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Testbiotech comment on EFSA's assessment of genetically engineered soybean MON 87708 x MON 89788 x A5547-127, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2016-135) by Bayer / Monsanto

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Introduction

The EFSA GMO panel assessed the triple-stacked soybean MON 87708 x MON 89788 x A5547-127 derived from crossing genetically engineered soybean events. This soybean had undergone previous assessment (EFSA, 2019a). The soybean contains genes conferring resistance to three herbicides:

- MON 89788 expressing CP4 EPSPS protein for tolerance to glyphosate-containing herbicides;
- MON 87708 expressing dicamba mono-oxygenase (DMO), for tolerance to the herbicide dicamba;
- A5547-127 expressing PAT protein, for tolerance to the herbicide glufosinate.

Consequently, the stacked GE soybean is resistant to three groups of complementary herbicides (glyphosate, glufosinate and dicamba). These herbicides can be applied in combination or individually. Implementing Regulation 503/2003 was applied in this case.

1. Molecular characterisation

The process of genetic engineering involved several deletions and insertions in the parental soybean plants. In order to assess the sequences encoding the newly expressed proteins or any other open reading frames (ORFs) present within the insert and spanning the junction sites, it was assumed that the proteins that might emerge from these DNA sequences would raise no safety issues; therefore, no detailed investigations were carried out in this regard. Furthermore, other gene products, such as dsRNA 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.

Therefore, EFSA should have requested much more detailed investigation into potential biologically active gene products and changes in metabolic pathways.

In regard to 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 (in regard to the newly expressed proteins)."*

However, there are three reasons why the data presented do not represent the conditions in which the plants will be grown: (1.1) the field trials were not conducted in all relevant regions where the

soybeans will be cultivated, and no extreme weather conditions were taken into account; (1.2) the field trials did not take into account current agricultural management practices; (1.3.) only one transgenic variety was included in the field trials.

1.1.
Environmental stress can cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). More specifically, Fang et al. (2018) showed that stress responses can lead to unexpected changes in plant metabolism inheriting additional EPSPS enzymes. However, the expression of the additional enzymes was only measured under field conditions in the US for one year. 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. Whatever the case, they should have been tested in large soybean producing countries in South America.

1.2.
Due to increased weed pressure, it has to be expected that these plants will be exposed to high and also repeated dosages of glyphosate alone and / or in combination with the other complementary herbicides. Higher applications of 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 ignored in the EFSA risk assessment.

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

1.3.
It is known that the genomic background of the variety can influence the expression of the inserted genes (see, for example, Trtikova et al., 2015). Therefore, EFSA, should have requested additional data from several varieties, including those cultivated in South America.

The material derived from the plants should have been assessed by using omics techniques to investigate changes in the gene activity of the transgene and the plants genome, as well as changes in metabolic pathways and the emergence of unintended biological active gene products. Such in-depth investigations should not depend on findings indicating potential adverse effects, they should always be necessary to come to sufficiently robust conclusions to inform the next steps in risk assessment.

2. Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)

Implementing Regulation 503/2013 requests:

> *"In the case of herbicide tolerant genetically modified plants and in order to assess whether the expected agricultural practices influence the expression of the studied endpoints, three test materials shall be compared: the genetically modified plant exposed to the intended herbicide; the conventional counterpart treated with conventional herbicide management regimes; and the genetically modified plant treated with the same conventional herbicide management regimes."*

> *"The different sites selected for the field trials shall reflect the different meteorological and agronomic conditions under which the crop is to be grown; the choice shall be explicitly justified. The choice of non-genetically modified reference varieties shall be appropriate for the chosen sites and shall be justified explicitly."*

However, the data that were presented do not represent anticipated agricultural practices, or the different meteorological and agronomic conditions where the crop is to be grown. The following three reasons can be given: (2.1) the field trials were not conducted in all relevant regions where the soybeans will be cultivated, and no extreme weather conditions were taken into account; (2.2) the field trials did not take current agricultural management practices into account; (2.3) only one transgenic variety was included in the field trials.

2.1.
Field trials for the compositional and agronomic assessment of the stacked soybeans were conducted in the US for only one year, but not in other relevant soybean production areas such as Brazil, Argentina, Paraguay or Uruguay. As stated in the EFSA opinion (2019a), "No exceptional weather conditions were reported at any of the selected field trial sites."

It is not acceptable that EFSA failed to require further studies e.g.

- No field trials were conducted that lasted more than one season. Thus, based on current data, it is hardly possible to assess site-specific effects.
- Further, no data were generated representing more extreme environmental conditions, such as those caused by climate change.

More specifically, Fang et al (2018) showed that stress responses can lead to unexpected changes in plant metabolism inheriting additional EPSPS enzymes. However, no experiments were requested to show to which extent specific environmental conditions will influence plant composition or agronomic characteristics. Whatever the case, the plants should have been subjected to a much broader range of defined environmental conditions and stressors to gather reliable data.

2.2.
Due to high weed pressure in many soybean growing regions, it has to be expected that these plants will be exposed to higher amounts and repeated dosages of the herbicides. It has to be taken into account that the herbicides can be sprayed in combination or individually at high dosages and repeatedly. These agricultural practices have to be taken into account to assess whether the expected agricultural practices will influence the expression of the studied endpoints. However, this requirement was mostly ignored by EFSA and the applicant: the herbicides were only sprayed in combination, each just one time, at early stage of vegetation and comparably low dosages.

Available publications show much that the complementary herbicides get sprayed at much higher dosages and repeatedly on the GE syobeans: on its product label Monsanto recommends about 7 kg (a.i.)/ha is sprayed (Monsanto, 2017), with up to three applications during cultivation. Official figures from the USDA data base show that up to 6-7 kg (a.i.)/ha of glyphosate can be expected in soybean cultivation, including pre- and post-emergence applications (USDA, 2019). The same data base (USDA 2019) also shows that spraying with dicamba reached up to 3,5 kg (a.i.)/ha in soybean cultivation, and glufosinate was around 660 g (a.i.)/ha. In its patent application concerning the "*cropping systems for managing weeds*", Monsanto recommends spraying up to 8 kg (a.i.)/ha onto HT soybeans, in addition 2,2kg (a.i.)/ ha Dicamba and 907 g (a.i.)/ha glufosinate. Data from South America show that even higher amounts are possible (Avila-Vazquez et al., 2018).

From the data that is available, it has to be assumed that the specific patterns of complementary herbicide applications 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 in the EFSA risk assessment.

It is known that soybeans contain many biologically active substances e.g. estrogens, allergens and anti-nutritional compounds, which may interact with trait-related characteristics and act as stressors. Changes in the composition of these components may not only be triggered by the process of genetic engineering, but also by interactions with the complementary herbicides. For example, Zobiole et al. (2012) and also Bøhn et al. (2014) found that glyphosate application can cause significant changes in soybean plant constituents. More specifically, Zobiole et al. (2012) applied glyphosate at three different dosages (800, 1200 and 2400 g/ha), which resulted in dose-correlated changes in plant agronomic performance and plant composition.

It also should be taken into account that a mixture of all the complementary herbicides will not always be used in the fields where the soybeans are cultivated; in some cases, just one of them will be used. This might lead to an increase in dosages of the respective complementary herbicides. The choice of herbicide will depend on the price of the herbicide formulations, the respective weed problem and regional agricultural practices. For example, it can be expected that in Argentina, Brazil and the US, there will be different prices, different herbicide formulations and varying regimes of herbicide applications under which the maize is cultivated. None of these specific agronomic practices were considered in the design of the field trials or in EFSA risk assessment.

EFSA should have requested the company to submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, including repeated spraying with each active ingredient individually as well as in combination.

2.3.
It is known that the genomic background of the variety can influence the expression of the inserted genes (see, for example, Trtikova et al., 2015). Therefore, EFSA should have requested additional data from several varieties, including those cultivated in South America, to see how the gene constructs interact with the genetic background of the plants.

2.4.
Only data from a low number of agronomic parameters (8), were subjected to statistical analysis in accordance with EFSA guidance; 5 of these were found to be statistically and significantly different in plants sprayed with the complementary herbicides (!). Against the backdrop of many significant differences even in this small data set, EFSA should have requested much more data (see also above).

Compositional analysis of 53 endpoints in the grains revealed many (and partly major) statistically significant differences: 28 endpoints were statistically and significantly different (!), with 2 indicating major differences between the transgenic stack and its comparator.

Therefore, EFSA should have requested further tests (toxicological data, repeated spraying with higher herbicide dosages or exposure to a wider range of environmental conditions). Furthermore, the plant material should have been assessed in more detail by using omics techniques to investigate changes in plant composition and agronomic characteristics.

But instead of assessing in more detail the overall pattern of changes in plant components, their

causes and possible impacts, EFSA only assessed the observed changes in isolation. This approach turns the comparative approach into a trivial concept of assessing bits and pieces, and ignores questions concerning the overall safety of the whole food and feed.

More in-depth investigations should not depend on findings indicating adverse effects, they should always be necessary to come to sufficiently robust conclusions to inform the next steps in risk assessment.

It has to be assumed that this event is essentially different from its comparator in regard to composition as well as biological characteristics. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects and their clear significance has to be taken as a starting point for much more detailed investigations.

Based on the available data, no final conclusions can be drawn on the safety of the plants. The data do not fulfill the requirements of Implementing Regulation 503/2013.

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;"*

There were many significant changes in plant composition and agronomic characteristics, but testing of the whole stacked plant (feeding study) was not requested. It has to be assumed that this event is essentially different from its comparator in regard to several compositions and biological characteristics. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects should have been considered as a starting point for much more detailed investigation of their potential health impacts.

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 specific agricultural practices in the cultivation of these herbicide-resistant plants, there are, e.g. specific patterns of applications, exposure, occurrence of specific metabolites and emergence of combinatorial effects that require special attention (see also Kleter et al., 2011).

More detailed assessment is also in accordance with pesticide regulation that requires specific risk assessment of imported plants if pesticide usage in the exporting countries is different compared to EU usage. In this regard, it should be taken into account that EFSA (2018) explicitly stated that no conclusion can be drawn on the safety of residues from spraying with glyphosate occurring in

genetically engineered plants resistant to this herbicide. In addition, glufosinate is classified as showing reproductive toxicity (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN) and there are indications of additive or synergistic effects of the residues from spraying (Reuter, 2015). EFSA should have at least requested data on the combined toxicity of the residues from spraying with the complementary herbicides.

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 tallowamine 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. (…)." (https://www.testbiotech.org/content/eu-commission-request-consider-impact-glyphosate-residues-feed-animal-health-february-2016)*

Consequently, EFSA should have requested the company to submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, including repeated spraying. The material derived from those plants should have been assessed in regard to organ toxicity, immune system responses and reproductive toxicity, also taking combinatorial effects with other plant components into account.

It is known that soybeans contain many biologically active substances e.g. estrogens, allergens and anti-nutritional compounds, which may interact with trait-related characteristics and act as stressors. Changes in the composition of these components cannot only be triggered by the process of genetic engineering but also by interactions with the complementary herbicides. For example, Zobiole et al. (2012) and also Bøhn et al. (2014) found that glyphosate application can cause significant changes in soybean plant constituents. More specifically, Zobiole et al. (2012) applied glyphosate at three different dosages (800, 1200 and 2400 g/ha) which resulted in dose-correlated changes in plant agronomic performance and plant composition.

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), poultry (Shehata et al., 2013) and rodents (Mao et al., 2018). Such effects might be also be caused by the residues from spraying with glufosinate since glufosinate interferes with bacterial growth and, in certain circumstances, acts as an antimicrobial agent causing shifts in bacterial community structures (Ahmad and Malloch 1995; Hsiao et al. 2007; Pampulha et al. 2007; Kopcáková et al. 2015). In general, antibiotic effects and other adverse health effects might occur from exposure to a diet containing these plants which were not assessed under pesticide regulation. 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 that most of the conversion was caused by bacteria in the colon and rectum, although toxicity findings indicate partial bioavailability.
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). Furthermore, attention should be paid to the specific toxicity of the metabolites in the active ingredients of the pesticide that might occur specifically in the stacked event.

Whatever the 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.

EU legal provisions such as Regulation 1829/2003 (as well as Implementing Regulation 503/2013) state that "*any risks which they present for human and animal health and, as the case may be, for the environment*" have to be avoided. Therefore, potential adverse effects that result from combinatorial exposure of various potential stressors need specification, and their assessment needs to be prioritised. We conclude that the health risk assessment as currently performed by EFSA for the stacked soybean is unacceptable. We propose that these plants are tested following the whole mixture approach, considering them to be "*insufficiently chemically defined to apply a component-based approach"* (EFSA, 2019b).

Despite all these open questions regarding potential health impacts, we are not aware of a single sub-chronic or chronic feeding study performed with whole food and feed derived from the stacked maize. This observation is supported by a literature review carried out by the applicant that did not yield any peer-reviewed publication.

In conclusion, the EFSA opinion on the application for authorisation of the stacked soybean (EFSA, 2019a) cannot be said to fulfil assessment requirements of potential synergistic or antagonistic effects resulting from the combination of the transformation events in regard to toxicology.

For this purpose, EFSA should have requested Monsanto to submit data from field trials with the highest dosage of complementary herbicides that can be tolerated by the plants, including repeated spraying. The material derived from those plants should have been assessed in regard to organ toxicity, immune responses and reproductive toxicity, also taking combinatorial effects with other plants components into account.

Further, EFSA should have rejected the feeding study carried out with parental plant MON89788, which used material that was stored for up to 27 months (!). It cannot be assumed that the identity of this material was sufficiently preserved over such a long period of time.

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

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;"*

For this purpose, EFSA should have requested Monsanto to submit data from field trials with the highest dosage of complementary herbicides that can be tolerated by the plants, including repeated spraying. The material derived from those plants should have been assessed in regard to organ toxicity, immune responses and reproductive toxicity, also taking combinatorial effects with other plants components into account.

Others

For monitoring and methods to identify the specific event, Implementing Regulation 503/2013 requests:

> *"The method(s) shall be specific to the transformation event (hereafter referred to as 'event-specific') and thus shall only be functional with the genetically modified organism or genetically modified based product considered and shall not be functional if applied to other transformation events already authorised; otherwise the method cannot be applied for unequivocal detection/identification/quantification. This shall be demonstrated with a selection of non-target transgenic authorised transformation events and conventional counterparts. This testing shall include closely related transformation events."*

However, no such method for identification was made available. Based on the information that is available, it will not be possible to distinguish the stacked event from a mixture of single parental events or stacked events that overlap with the actual stack.

If approval for import is given, the applicant has to ensure that post-market monitoring (PMM) is developed to collect reliable information on the detection of indications showing whether any (adverse) effects on health may be related to 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 was transferred to;
- iv) the amount of the GE products used on farms for feed;
- 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 (see also comments from experts of Member States, EFSA, 2019c).

Finally, in regard to the literature research, we do not agree with the way it was carried out. The review should take into account all publications on the parental plants and provide all relevant information regarding gene expression, findings from field trials and feeding studies. Further, monitoring data should be provided on imports of parental plants into the EU.

Conclusions and recommendations

The EFSA risk assessment cannot be accepted.

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