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Testbiotech comment on EFSA's assessment on application for authorisation of genetically modified of insect-resistant and herbicide-tolerant soybean DAS-81419-2 x DAS-44406-6 (EFSA-GMO-NL-2016-132) for food and feed uses, import and processing submitted in accordance with Regulation (EC) No 1829/2003 by Dow Agrosciences LCC

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Introduction

Stacked soybean DAS-81419-2 x DAS-44406-6 contains genes conferring resistance to three groups of herbicides:

- glyphosate (2mEPSPS protein),
- 2,4-dichlorophenoxyacetic acid (2,4-D) and other related phenoxy herbicides (AAD–12 protein),
- glufosinate ammonium (PAT protein).

Further, the plants have resistance to lepidopteran pests through the expression of Bt toxins, Cry1F and Cry1Ac.

Implementing Regulation 503/2003 was applied in this case. In its opinion, EFSA "*does not identify any safety issues pertaining to the intended uses of soybean DAS-81419-2 x DAS-44406-6"* (EFSA, 2020a).

However, according to Testbiotech, the data presented are insufficient to demonstrate safety.

1. Molecular characterisation

It is known that environmental stress can cause unexpected patterns of expression in newly introduced DNA (see, for example, Trtikova et al., 2015). More specifically, Fang et al. (2018) show 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. Further, according to Member States' experts, the genetic stability of the insert was only shown in a very low number of plants (3 single individual plants) which is insufficient for reliable results (EFSA, 2020b).

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 also have been tested in the largest soybean producing countries in South America.

A high number of open reading frames (ORFs) is reported in Member States comments (see EFSA, 2020b). Uncertainties remain about biologically active substances arising from the method of genetic engineering and newly introduced gene constructs, such as non-coding small RNAs.

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

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, the data presented do not represent the conditions in which the plants will be grown as the field trials were not conducted in all the relevant regions, and no extreme weather conditions were taken into account. Furthermore, it is not clear from the EFSA opinion whether the field trials actually represent current agricultural management practices.

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; Lohn et. al., 2020; de Campos et al., 2020).

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 plant 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."*

Field trials for the compositional and agronomic assessment of the stacked soybeans were only conducted in the US for one year, but not in other relevant soybean production areas such as Brazil, Argentina, Paraguay or Uruguay - and even for the US, it is disputed if the field trial locations are representative. According to Member States' experts (EFSA, 2020b):

> *"Soybean is for example also grown in the South and South East of the US and not only in Midwest and East of the US, where the trial sites were located. Therefore, the presented data*

are considered insufficient to establish that the trials are representative for the whole range of possible agronomic and environmental conditions, under which soybean is produced in North America."

It is not acceptable that EFSA failed to require further studies, e.g. field trials lasting for 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. Nevertheless, regarding agronomic parameters, multiple significant differences were detected in GE soybean plants whether or not they were treated with the intended herbicides (EFSA, 2020a):

- *"For soybean DAS-81419–2 x DAS-44406-6 (not treated with the intended herbicides), the test of difference identified statistically significant differences with the conventional counterpart for early stand count, days to maturity, plant height, 100-seed weight, yield and lodging. Of those endpoints, 100-seed weight fell under equivalence category IV while the other endpoints fell under equivalence category I or II.*
- *For soybean DAS-81419-2 x DAS-44406-6 (treated with the intended herbicides), the test of difference identified statistically significant differences with the conventional counterpart for days to 50% flowering, days to maturity, plant height, 100-seed weight and lodging. Of those endpoints, 100-seed weight fell under equivalence category IV while the other endpoints fell under equivalence category I."*

Further, the compositional analysis also showed statistically significant differences to the conventional counterpart in many analysed compounds (treated and not treated with glyphosate, 2,4-D and glufosinate ammonium) (EFSA, 2020a).

- *"For soybean DAS–81419–2 x DAS–44406–6 not treated with the intended herbicides, statistically significant differences with the conventional counterpart were identified for 32 endpoints (all in seeds). For two of them (acid detergent fibre (ADF) and phosphatidylinositol), the test of equivalence was not applied because the variability among the reference varieties was estimated to be zero, while lectin activity fell under equivalence category IV (Table 6). The other 29 endpoints fell under equivalence category I or II.*
- *For soybean DAS–81419–2 x DAS–44406–6 treated with the intended herbicides, statistically significant differences with the conventional counterpart were identified for 39 endpoints (34 in seeds and 5 in forage). The test of equivalence was not applied to four of the forage endpoints, while lectin activity and glutamic acid levels in seed fell under equivalence category III and IV, respectively (Table 6). The other 33 endpoints fell under equivalence category I or II."*

There are several cases where genetically engineered plants show, for example, unintentionally enhanced fitness which can be influenced by environmental factors (for overview, see Bauer-Panskus et al., 2020). More specifically, Fang et al. (2018) showed that stress responses can lead to unexpected changes in plant metabolism inheriting additional EPSPS enzymes.

Stress tests under a broad range of defined environmental conditions should have been carried out, including taking pollen viability and seed dormancy into account.

Whatever the case, much more data would be needed to develop a sufficiently defined hypothesis for risk assessment in regard to phenotypical characteristics and compositional analysis of the soybeans. This is especially relevant in this case because of the extremely high expression levels of the additionally produced proteins compared to wild-type cereals (EFSA, 2020a).

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 (see Miyazaki et al., 2019).

Therefore, EFSA should have requested further tests to be carried out under exposure to a wider range of environmental conditions, which should also have taken all relevant agronomic practices into account. The plant material should, in addition, have been assessed in more detail by using omics techniques to investigate changes in plant composition and agronomic characteristics.

Compositional analysis should also include measuring the herbicide residues and metabolite levels. This is requested by several Competent Authorities. For example, according to Austrian experts (EFSA, 2020b):

> *"We consider that the scope of the comparative analysis concerning food and feed risk assessment is too narrow with a view to the characteristics of GM soybean DAS-81419-2 x DAS-44406-6 and that the presence of residual levels of herbicides as well as residual metabolites of the complementary herbicides in GM soybean seed material should be determined."*

However, instead of assessing the overall patterns of change in plant components in greater detail, as well as 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 on the overall safety of the whole food and feed.

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

3. 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;"*

<u>Feeding studies</u>
Significant changes in plant composition were identified in many parameters; these should have triggered a request for a 90-day subchronic study in rats. However, according to EFSA, this was not considered necessary.

Instead of testing the stacked soybean, EFSA asked the applicant to provide a study in which groups of rats were given diets containing DAS-81419-2 or DAS-44406-6 soybean. According to EFSA, this study found *"that no treatment-related adverse effects were observed in rats after feeding diets including soybean DAS-81419-2 or soybean DAS-44406-6 (up to 30% defatted toasted meal, 2% hulls and 2.7% oil) for 90 days."*

Interestingly, the applicant even conducted a study with the stacked soybean for the authorisation process. However, this study was not accepted by EFSA, mainly because the percentage of GE soybean in the diet in the high dose group was considered too low. Nevertheless, this study yielded interesting results which should have been scrutinised in a second, better planned study. Whereas the outcome of this study is not reported in the EFSA opinion, comments from Member States point to the fact that many significant effects were found when feeding rats with the stacked soybean. One Competent Authority lists these significant effects (EFSA, 2020b), which should have prompted more detailed investigations:

> *"- Kidney weight males (Dunnett's Test) - Relative kidney weights males - Albumin males (Dunnett's Test) - Glucose males (Dunnett's Test) - Potassium males (Dunnett's Test) - Red Blood cell Count males (Dunnett's Test) - Haemoglobin males (Dunnett's Test) - Haematocrit males (Dunnett's Test) - Relative heart weight females (Dunnett's Test) - Relative liver weight females - Relative spleen weight females (Dunnett's Test) - Urea nitrogen females (Dunnett's Test) - Glucose females (Dunnett's Test) - Relative adrenal weight sex*dose (Dunnett's Test) - Relative kidney weight sex*dose (Dunnett's Test) - Relative kidney weight males - Relative liver weight sex*dose (Dunnett's Test) - Relative liver weight females - Reticulocytes sex*dose (Dunnett's Test)"*

Herbicides

Furthermore, there are specific health risks resulting from the intended use of the GE soybeans engineered to be resistant to herbicides, such as glyphosate, glufosinate or 2,4-D.

The residues from spraying were considered to be outside the remit of the GMO Panel. However, without a 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 the herbicide-resistant plants, there are, e.g. specific patterns of application, 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 differs 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 in genetically engineered plants resistant to this herbicide. Further, a recent review comes to the conclusion that *"literature on the potential effects of glyphosate on livestock is very scarce and mainly reporting in vitro studies; hence, a solid basis of in vivo studies with livestock in physiological and productive phases, particularly sensitive to disorders in mineral status and in the gut microbiota, is needed"* (Sørensen et al., 2020).

In addition, glufosinate is classified as showing reproductive toxicity and there are indications of additive or synergistic effects of the residues from spraying (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN).

Further, recent research regarding 2,4-D seems to indicate that health risks may be underestimated (de Azevedo Mello et al., 2020).

Mixtures

In summary, the GE soybeans intended for import are not unlikely to contain a toxic mix of chemicals without any testing of combinatorial effects at the stage of consumption being requested. In addition, 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 can be triggered by the process of genetic engineering as well as by interactions with the complementary herbicides.

Therefore, as shown in a recent report (Then et al., 2020), a far more detailed assessment is needed of combinatorial effects (or potential mixed toxicity) arising from simultaneous exposure to a fixed combination of potential stressors from GE plants at the stage of consumption. Consequently, the GE soybeans should be tested following the 'whole mixture' approach, which considers them to be "*insufficiently chemically defined to apply a component-based approach*" (EFSA, 2019).

Currently, the most appropriate method to test these substances are life-time feeding studies with whole plant materials. To generate reliable data for products that are used daily in the food chain, the feeding studies will need to be long-term and include several generations.

In addition, *in vitro* testing systems and testing systems using non-vertebrates might also be applied to reduce the overall number of animals needed for feeding studies.

The material derived from the plants should be assessed in regard to organ toxicity, immune system responses and reproductive toxicity, also taking combinatorial effects with other plant components into account.

Bt toxins and protease inhibitors

Selectivity and efficacy of Bt toxins as produced in GE plants can be influenced by many co-factors (see, for example, Then, 2010; Hilbeck & Otto, 2015). One crucial impact factor are protease inhibitors (PI), which delay the degradation of Bt proteins and thereby enhance their toxicity (see Pardo-López et al., 2009).

Already in 1990, Monsanto showed that maize, cotton and soybeans produce protease inhibitors (PI) which considerably enhance the toxicity of Bt proteins in plants. In the presence of PIs, Bt toxin will degrade much more slowly than in isolation. This results in a much higher toxicity of the Bt toxin (if it is taken up together with the plant tissue) compared to the isolated toxin (MacIntosh et al., 1990; Zhao et al., 1999; Zhang et al., 2000; Gujar et al., 2004; Zhu et al., 2007; Pardo-López et al., 2009; Ma et al., 2013; Mesén-Porras et al., 2020). The effects described indicate, for example, a 20-fold higher toxicity of Bt proteins if produced in the plants and taken up with PIs (MacIntosh et al., 1990).

Therefore, any risk assessment which does not take a combination of plant material with the Bt toxin into account is not reliable and systematically underestimates the risks.

It is known from scientific publications that co-factors which enhance the toxicity of the Bt proteins can also impact their selectivity (for overview see Then, 2010): if synergistic or additive effects occur that increase efficacy of the Bt toxin, its selectivity may be decreased and a wider range of non-target organisms may become susceptible. In addition, there has never been any systematic

research into these combinatorial effects. There are just a few publications available which indicate the effects of protease inhibitors combined with Bt toxins on non-target insects (Babendreier et al., 2005; Liu et al., 2005a; Liu et al., 2005b; Han et al., 2010).

The synergistic effects described by MacIntosh et al. (1990), Zhao et al. (1999), Zhang et al. (2000) Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Ma et al. (2013), Mesén-Porras et al. (2020) causing higher toxicity of the Bt toxins are also relevant to the risk assessment of food and feed safety: the combination with protease inhibitors is likely to be associated with a delay in the degradation of the Bt toxins after consumption. This delay in degradation extends the exposure of the intestinal immune system to Bt toxins and may trigger or enhance health impacts, such as chronic inflammation and allergies.

Overall, the toxicological assessment carried out by EFSA is not acceptable.

4. Allergenicity

The synergistic effects described by MacIntosh et al. (1990), Zhao et al. (1999), Zhang et al. (2000) Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Ma et al. (2013), Mesén-Porras et al. (2020) causing higher toxicity of the Bt toxins are also relevant to risk assessment in regard to the immune system: the combination with protease inhibitors is likely to be associated with a delay in the degradation of the Bt toxins after consumption. This delay in degradation extends the exposure of the intestinal immune system to Bt toxins and may trigger or enhance health impacts, such as chronic inflammation and allergies (see also Then & Bauer-Panskus, 2017)

EFSA does not mention that Cry1Ac is thought to be allergenic (Santos-Vigil et al., 2018; see also: www.testbiotech.org/en/press-release/can-bt-toxins-cause-allergies). In the published reports and also in references made by EFSA (2020a), there is a general lack of empirical data. Consequently, EFSA can only conclude on an absence of evidence, but not on evidence of safety for the immune system.

None of the reports mention, discuss or assess the potential enhancement of toxic or immunogenic effects caused by interaction with plant components such as PI. Furthermore, EFSA (2020a) does not address non IGE-immune reactions.

Although lectins are known immunogens (Parenti et al., 2019), the highly significant increase in the concentration of lectins was not investigated as a risk for the immune system if taken up together with higher concentration of Cry toxins present in the stacked event.

Furthermore, Parenti et al. (2019) state that "*one of the most important drivers of immune response is the gut microbiota and other microbial constituent of the human body which are able to regulate host-pathogen balance and to produce systemic pro-inflammatory stimuli. The lifelong antigenic load represented by foods and bacteria/bacterial products leads to a profound remodeling of the gut microbiota and these changes are emerging as a driving force of the functional homeostasis of the immune system. As a matter of fact, a perturbation of the gut microbiota homeostasis due to irregular lifestyles, stress and age may lead to gut microbiota dysbiosis. This condition may predispose the host to metabolic disorders and inflammation*."

However, potential changes in the microbiota were not taken into account by EFSA (2020a) even though this was also mentioned by experts of Member States (EFSA, 2020b).

In conclusion, the safety of the GE soybeans in regard to potential impacts on the immune system was not demonstrated.

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 from 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, the impact on the environment from organic waste material, by-products, sewage, or faeces containing GE products during or after the production process, should be part of the monitoring process; both during and after human or animal consumption.

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. However, the applicant only presents four studies from the past 14 years deemed to be important for risk assessment of the stacked soybean, all of them conducted by Dow. Clearly, this form of literature review cannot be taken seriously and should have been rejected by EFSA.
Further, monitoring data should be provided on imports of parental plants into the EU.

5. Conclusions and recommendations

Regulation 1829/2003 (Recital 9) states that *"…any risks which they present for human and animal health and, as the case may be, for the environment…"* have to be avoided. Our analysis shows that the safety of the products derived from the GE soybeans could not be demonstrated. There are however substantial indications that the consumption of the soybeans may provoke adverse health effects. Therefore, the risk assessment is not conclusive and approval for the EU market cannot be granted.

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