

TESTBIOTECH Background 20 - 2 -2019

Testbiotech comment on EFSA's assessment of genetically engineered soybean SYTH0H2 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2012-111) by Syngenta

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Introduction

Soybean SYTH0H2 contains genes conferring resistance to two groups of herbicides:

- pat - for tolerance to the herbicide glufosinate
- avhppd-03 - for tolerance to mesotrione and other HPPD inhibitors (such as isoxaflutole).

Implementing Regulation 503/2013 was not applied in this case because the application was submitted in 2012, one year before the Implementing Regulation came into force. Therefore, EFSA assessed the application under its old guidance documents which are now seven (!) years out of date.

Soybean (*Glycine max*) was genetically modified to express an hppd gene derived from oat (*Avena sativa*), designated avhppd-03. This gene encodes a modified p-hydroxyphenylpyruvate dioxygenase isozyme (AvHPPD-03) that differs from the native HPPD isozyme from *A. sativa* by one amino acid. The avhppd-03 gene is linked to a constitutive promoter that expresses avhppd-03 at a higher level than that of soybean native hppd gene (see EFSA, 2020a and 2020b).

Tolerance to glufosinate ammonium herbicides is accomplished by the expression of a pat gene derived from the soil microorganism *S. viridochromogenes*, strain Tü494. The PAT protein inactivates the herbicide glufosinate ammonium, which is an inhibitor of glutamine synthetase, an enzyme in the nitrogen assimilation pathway (see EFSA, 2020a and 2020b).

If the soybean would be allowed for import, it is not unlikely the products a unique toxic mix of residues from herbicides that are carcinogenic, endocrine disruptive and show reproductive toxicity - with no testing of combinatorial effects at the stage of consumption being carried out. Furthermore, it is likely that the genetic engineering caused the plants composition to change unintentionally. The data presented are insufficient to demonstrate safety.

1. Molecular characterisation

It is known that 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 Argentina. No systematic comparison in gene expression was made between those plants (from US field trials) treated with the complementary herbicide and those plants which were not, although the data presented (from Argentina) seem to

indicate that the application of the trait-specific herbicides may lead to an increase in the expression of the respective proteins AvHPPD-03 and PAT (see EFSA, 2020a and 2020b).

There is a relatively high number of different genetic elements inserted into the plants: to facilitate the expression of the additional and modified HPPD enzymes, the company used four different sequence(s) in SYHT0H2 soybean as well as four promoter/enhancer elements (derived from different species). In addition, the construct as inserted has four copies of the pat gene along with further promoters and stop codons. In addition, further unintended fragments, truncations and insertions were observed (see EFSA, 2020a and 2020b).

According to EFSA (2020a), the process of genetic engineering led to several open reading frames (ORFs) that give rise to biologically active molecules:

“Bioinformatic analyses of the sequences encoding the newly expressed proteins and other ORFs present within the insert or spanning the junctions between the insert and genomic DNA indicate a ~ 30% sequence identity of AvHPPD-03 to some proteins of bacterial origin annotated as haemolysins. [...]

*In addition, an eight amino acid exact match between an ORF and a putative serine carboxylpeptidase from *Triticum aestivum* was identified. This ORF is found within the transcriptional unit of the AvHPPD-03 coding sequence but in a reverse orientation and does not contain any in-frame translational start codons (ATG).”*

Given the high number of genetic elements used in the transformation (to facilitate a high level of tolerance to the complementary herbicides) as well as the unintentionally inserted fragments, much more data and much greater scrutiny is needed for the molecular characterisation of the soybeans. For example, other gene products (besides proteins), 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.

Furthermore, as also shown in the compositional analysis, the change in the metabolic pathway involving HPPD should have been assessed much more thoroughly. Plants utilise the HPPD enzyme to produce the cofactors plastoquinone and tocopherol which are essential for the plant to survive (EFSA, 2020b). Interfering with this metabolism can have various impacts on biological characteristics, such as growth, stress resistance and fitness of the plants. There are several cases of genetically engineered plants showing, for example, unintentionally enhanced fitness that can be influenced by environmental factors (for overview, see Bauer-Panskus et al., 2020). Therefore, the material derived from the plants should have been assessed by using ‘omics-techniques’ to investigate changes in gene activity of the transgene and the plant genome, as well as changes in metabolic pathways and the emergence of unintended biologically active gene products.

Furthermore, the plants should have been subjected to a much broader range of defined environmental conditions and stressors (which, for example, have to be expected under ongoing climate change) to gather reliable data on gene expression and functional genetic stability. The generation of these data should have taken all relevant patterns of herbicide applications and the application of all relevant complementary herbicides, such as isoxaflutole, into account.

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

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 Brazil, Argentina, Paraguay or Uruguay.

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.

Regarding agronomic parameters, only six agronomic/phenotypic endpoints were submitted for statistical analysis; two in the group where the complementary herbicide was applied were considered to be significantly different.

The compositional analysis showed statistically significant differences with the conventional counterpart in about half of the analysed compounds (treated as well as not treated with mesotrione and glufosinate ammonium) (EFSA, 2020a):

- *“For soybean SYHT0H2 (not treated), the test of difference identified statistically significant differences with the conventional counterpart for 32 endpoints (one in forage and 31 in seeds). All these endpoints fell under equivalence category I/II, except for the levels of α -tocopherol and γ -tocopherol that fell under equivalence category III/IV.*
- *For soybean SYHT0H2 (treated), the test of difference identified statistically significant differences with the conventional counterpart for 27 constituents (two in forage and 25 in seeds). All these endpoints fell under equivalence category I/II, except for the levels of γ -tocopherol that fell under equivalence category III.*

To assess these compositional differences, the metabolic pathways in which HPPD is involved, should have been taken into account in more detail. Plants utilise this enzyme to produce the cofactors plastoquinone and tocopherol, which are essential for the plant to survive (EFSA, 2020b). Interfering with the metabolism in the plants can have various impacts on their biological characteristics, such as growth, stress resistance and fitness. There are several cases of genetically engineered plants, showing, for example, unintentionally enhanced fitness which can be influenced by environmental factors (for overview, see Bauer-Panskus et al., 2020 in print). The only test carried out to find changes in the fitness of the plants is a germination test under controlled temperatures, which is not sufficient to assess unintended changes in plant biology. Stress tests under defined environmental conditions with the whole plants should have been carried out, also 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 soybean. This is especially relevant in this case because of the extremely high expression levels of the additionally produced enzymes compared to wild-type cereals (EFSA, 2020a). These data would need to take into account other patterns of herbicide applications, and the application of other active complementary herbicides, such as isoxaflutole, which are also likely to be applied to the plants.

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. Furthermore, the plant material should have been assessed in more detail by using omics techniques to investigate changes in plant composition and agronomic characteristics.

However, instead of assessing the overall pattern of changes 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 concerning the overall safety of the whole food and feed.

In addition, the quality of the comparative analysis suffers from the fact that soybean SYTH0H2 was not assessed under the more stringent guidance of the Commission Implementing Regulation 503/2013, which came into force in 2013. Instead, as the application for soybean SYTH0H2 was submitted in 2012, EFSA assessed the application under its old guidance documents which are now already seven (!) years out of date. This is not acceptable.

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

3. Toxicology

Significant changes in plant composition were identified in more than half of the compared parameters. In addition, feeding studies carried out with the isolated HPDD protein showed many significant effects. Amongst other effects, statistically significant effects included increased movement activities in all male groups exposed to the HPPD protein. Likewise, the body temperature in the male 10 mg group was statistically significantly higher when compared to the control. The latter was correlated with statistically significant lower weights of brain, liver, and spleen (EFSA, 2020b). In the light of these findings, the absence of a subchronic feeding trial was also criticised by several member states (EFSA, 2020b).

Furthermore, there are specific health risks resulting from the intended use of the GE soybeans that are engineered to be resistant to herbicides such as glufosinate, mesotrione and isoxaflutole.

As the EFSA peer review shows (EFSA, 2016a), mesotrione and its metabolite AMBA is associated with risks such as endocrine disruption. Further, the peer review identified a large number of data and knowledge gaps in regard to genotoxicity and other human and animal health aspects. As the summary states:

“Regarding the mammalian toxicology area, a number of data gaps were identified. The toxicological relevance of individual impurities present in the technical specification in comparison with the toxicity profile of mesotrione needs to be addressed. Interspecies comparative in vitro metabolism should be conducted to identify at least potentially unique human metabolites to mesotrione. As the genotoxic potential of metabolite AMBA could not be ruled out due to positive results obtained in an in vitro cytogenetic assay, and no in vivo genotoxicity testing was performed, a critical area of concern has been identified regarding consumer risk assessment; repeated dose toxicity would also have to be addressed for this

metabolite. Mesotrione is proposed to be classified as Repr. 2 for development by the peer review (in contrast with the harmonised classification according to CLP Regulation) and adverse effects were observed on endocrine organs. Therefore, according to the interim provisions of Annex II, point 3.6.5 of Regulation (EC) No 1107/2009 concerning human health, mesotrione may be considered to have endocrine disrupting properties. As no study is available to investigate a potential ED mode of action, a general data gap has been identified such as level 2 and 3 indicated in the OECD Conceptual Framework to address this issue; this was identified as another critical area of concern. The consumer dietary risk assessment could not be finalised with regard to products of animal origin considering the requested clarification of the genotoxic potential and the toxicological profile of AMBA. Furthermore, the consumer risk assessment from consumption of drinking water could not be finalised whilst the nature of residues in drinking water following water treatment had not been addressed.”

Regarding endocrine effects, the peer review (EFSA, 2016a) further states:

“With regards to the assessment of endocrine disruptive properties of mesotrione, the substance is proposed to be classified as Repr. 2 for development and adverse effects were observed on endocrine organs: increased testes and epididymides weights, and thyroid adenomas in female rats. Therefore, according to the interim provisions of Annex II, point 3.6.5 of Regulation (EC) No 1107/2009 concerning human health, mesotrione may be considered to have endocrine disrupting properties.”

The EFSA review (EFSA, 2016a) concludes:

“The consumer dietary risk assessment could however not be finalised with regard to products of animal origin as the genotoxic potential of AMBA in vivo could not be ruled out due to positive results obtained in an in vitro cytogenetic assay.”

Furthermore, EFSA presented its peer review of pesticide risk assessment of the active substance isoxaflutole in 2016, which clearly shows major data deficiencies in regard to the safety of GE soybeans treated with the herbicide (EFSA, 2016b):

- Carcinogenicity and developmental toxicity were confirmed for the active substance.
- Three different metabolites of isoxaflutole were found in soybean seeds, most of them at higher levels compared to other usages.
- Risk assessment of the residues in food and feed derived from genetically engineered soybeans could not be concluded and no MRL could be determined due to a lack of data.
- Further data gaps concern the method of determining residues in food and feed of plant origin.

Since no MRL could be set for the residues of isoxaflutole applied to the genetically engineered soybeans as the complementary herbicide, products containing such residues cannot be allowed on the EU market.

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>).

In summary, the GE soybean meant for import, are not unlikely to contain a toxic mix of chemicals which are carcinogenic, endocrine disruptive and show reproductive toxicity, 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), combinatorial effects (or potential mixed toxicity) emerging from simultaneous exposure to a fixed combination of potential stressors emerging from GE plants at the stage of consumption, need to be assessed in far more detail. Consequently, the GE soybeans should be tested following the ‘whole mixture’ approach, considering them as “*insufficiently chemically defined to apply a component-based approach*” (EFSA, 2019).

Currently, the most appropriate method to test these substances is 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.

However, soybean SYTH0H2 was not assessed under the Commission Implementing Regulation 503/2013, which came into force in 2013 and requests mandatory feeding studies. Instead, as the application for soybean SYTH0H2 was submitted in 2012, EFSA simply assessed the application under its old guidance documents which are now already seven (!) years out of date. This is not acceptable.

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

4. Allergenicity

Due to the data provided and assessed by EFSA, the real allergenic, or adjuvant, or immunogenic potential of the soybean cannot be assessed.

Interfering with plant metabolic pathways with the involvement of HPPD can have various impacts on the biological characteristics of the plants, including elevating the content of allergenic proteins. The changes in the tocopherol content of the plants strongly indicates that the additionally produced enzymes do indeed unintentionally interfere with plant metabolism.

Furthermore, changes in the composition of the relevant 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, taking into account all relevant agronomic practices. Furthermore, the plant material should have been assessed in more detail by using omics techniques to investigate changes in plant composition and targeted measuring of allergenic proteins. The

inclusion of allergens in the compositional analysis is mandatory, as is the case for applications subject to Regulation 503/2013, which should have been applied. However, the Implementing Regulation 503/2013 was not applied in this case because the application was submitted in 2012, one year before the Implementing Regulation came into force. Therefore, EFSA assessed the application under its old guidance documents which are now already seven (!) years out of date.

Overall, the assessment of risk to the immune system carried out by EFSA is not acceptable.

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. However, as our analysis shows, safety of the products derived from the GE soybeans was not shown, while on the other hand, there are substantial indications that consumption of the soybeans may provoke adverse health effects. Therefore, risk assessment is not conclusive and no permission for entering the EU market can be issued.

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