TESTBIOTECH Background 21 - 09 - 2016

Testbiotech comment on EFSA´s Scientific Opinion on an application (EFSA-GMO-NL-2007-47) for the placing on the market of the herbicide-tolerant, high-oleic acid, genetically engineered soybean 305423 x 40-3-2 for food and feed uses, import and processing under Regulation (EC) No. 1829/2003

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Introduction

Of the parental plants, soybean 305423 was genetically engineered with the intention of changing the oil composition in the plants: The content of monounsaturated fatty acids (MUFA) in the soybean is increased, while the production of polyunsaturated fatty acids (PUFA) is suppressed.

Further, soybean 305423 is engineered to be resistant to acetolactate synthase (ALS)-inhibiting herbicides, which include herbicides of the imidazolinone, sulfonylurea, triazolopyrimidine, pyrimidinyl(thio)benzoate and the sulfonylaminocarbonyltriazolinone chemical families. The metabolic pathway introduced for providing resistance to ALS inhibitor herbicides is suspected of being the reason for an additional unintentional change in oil composition in the plants, causing a lower content in odd chain fatty acids.

In addition, soybean 305423 inherits a promoter and a terminator derived from soybean Kunitz trypsin inhibitor gene 3 (KTi3). This gene unintentionally interacts with the activity of the plant's own trypsin inhibitor gene, causing a lower content of trypsin inhibitors in the plants.

As a result, soybean 305423 shows a wide range of intended and unintended changes in its composition and has to be regarded as being substantially different from its conventional counterparts used as comparators.

The other parental plant, soybean 40-3-2 incorporates the epsps (5-enolpyruvylshikimate-3-phosphate synthase) gene to make it resistant to spraying with glyphosate. This soybean is also known to show unintended effects, due to flaws in the inserted DNA construct.

The two above-mentioned soybean plants were combined by crossing to create a so-called stacked event that is resistant to two herbicides and altered in oil composition. However, crossing also leads to unintended effects being combined in the resulting plants. In addition, the residues from spraying with the complementary herbicides can accumulate in the parts of the plants used for food and feed.

Molecular characterisation

Both parental soybeans were produced by particle bombardment. This method is known to have a major impact on plant DNA (see, for example, Makarevitch et al., 2003).

In soybean 305423, molecular characterisation revealed multiple rearrangements, and several complete and truncated copies of gene constructs were detected. Soybean 305423, for example,

contains in total, eight copies of the KTi3 promoter, seven copies of the gm-fad2-1 gene fragment and five copies of the KTi3 terminator. Gene products such as RNA produced from these additional and unintended copies can render various biological effects. One unintended effect - in regard to the KTi3 gene - is evident from the data provided on the plant's composition, which show a reduction in the concentration of the plant´s own trypsin inhibitor protein. It is likely that this effect results from RNAi. The molecular characterisation has also revealed that one of the investigated plants showed signs of genetic instability.

In soybean 40-3-2, Rang et al (2005) showed occurrence of unintended open reading frames, due to the non-functioning of the nos-stop codon, causing the occurrence of additional RNA in the plants. Even though no fusion proteins were identified the plants produce additional RNA that needs further assessment. For example, if small double stranded RNA is produced it could be transmitted as a biologically active compound at the stage of consumption. However, no detailed investigations were performed to assess these unintended gene products in detail.

In general, beyond that, RNAi effects are highly relevant for the risk assessment of these genetically engineered soybeans: RNAi is used to achieve the intended changes in the oil quality of soybean 305423. Therefore, the assessment of the biological effects of intended and unintended miRNA produced in the plants should have been a priority. However, no investigations were requested to assess the newly produced miRNA in detail.

In 2012, it was reported for the first time that miRNA produced by plants can enter the blood of mammals (including humans) at the stage of consumption (Zhang et al, 2012). These findings were called into question by several experts. However, looking at more recent publications one has to assume that the plant miRNA can indeed enter the blood, organs and urine of mammals after ingestion (Beatty et al., 2015; Yang et al., 2015; Liang et al., 2015; Hirschi et al, 2015). Certainly, the amount being taken up and the biological impact depend on factors that need further research.

This uptake of small RNAs via ingestion is relevant for risk assessment. There is evidence that small RNAs taken up from the intestine do indeed interfere with gene regulation in humans and animals. For example, it was found that miRNA transferred via milk shows biological activity (Baier et al., 2014; see also: Lukasik & Zielenkiewicz, 2014)). Small RNAs produced by plants are able to interfere with the immune system in humans and animals (Zhou et al., 2015; Cavalieri et al., 2015).

Therefore, EFSA should have requested data on the emergence of new variations, combinations and concentrations of small, biologically active RNA in the parental plants as well as in the stacked event.

Furthermore, both the expression of the enzymes that confer herbicide resistance and the concentration of small biologically active RNA molecules should have been tested under a wide range of defined environmental conditions, taking into account stressful conditions that, for example, emerge under ongoing climate change. It is known that under stress conditions, genetically engineered plants can show reactions that are not obvious under normal agricultural conditions, and these can be very different from those of conventionally bred plants (see, for example, Gertz et al., 1999). Environmental stress can also cause unexpected patterns of expression of the newly introduced DNA (Trtikova et al., 2015).

Finally, since the KTi3 gene inserted into the plants unintentionally renders biological effects (lower content of the trypsin inhibitor protein in the plants), the expression rate of the additional KTi3 gene

and its specific gene products should also have been investigated in detail.

To summarise, the inserted DNA, its truncated sequences, rearrangements and open reading frames can interfere with gene regulation in the plants and cause a broad range of unintended effects. Apparently such effects occur, since the plants show a lower content in trypsin inhibitor and a reduction in odd chain fatty acids. Metabolic and genomic screening would be required to assess the real magnitude of these effects and to exclude other effects. In these investigations, the plants should also be subjected to defined environmental stress factors. But no such investigations were requested by EFSA.

Comparative analysis (for compositional analysis and agronomic traits and the phenotype)
Despite field trials only being conducted in the USA and Canada and only for one year, significant differences were found for several compounds and agronomic characteristics. These differences should have been investigated over more than one year and under a broad range of environmental conditions, including defined biotic and abiotic stressors.

Some of the significant changes observed, such as lower content of trypsin inhibitor and odd chain fatty acids, are apparently caused by unintended effects due to the insertion of the additional DNA.

As a result, the soybean shows a wide range of intended and unintended changes in its composition and agronomic characteristics, and therefore has to be regarded as substantially different from its conventional counterparts used as comparators. As the EFSA Guidance Document from 2006 shows, this requires more in-depth investigation of the whole food and feed, regardless of whether the observed effects are known to be detrimental to human or animal health:

> *"If the composition of the GM plant is modified substantially, or if there are any indications for the potential occurrence of unintended effects, based on the preceding molecular, compositional or phenotypic analysis, not only new constituents, but also the whole GM food/feed should be tested. In such a case, the testing programme should include at least a 90-day toxicity study in rodents".*

However, EFSA did not request any further investigation such as testing of the whole food and feed.

Toxicology
The applicant provided a 90-day feeding study of insufficient quality and so it was rejected by EFSA. However, EFSA should have consequently requested a new study, due to the many intended and unintended effects observed in the composition of the plants.

It should also be taken into account that the feeding studies with the parental plant 305423 suffer from major deficiencies. Furthermore, according to Magaña-Gómez et al. (2009), a number of the studies with soybean 40-3-2 revealed signs of possible health effects.

Thus, investigations with the whole food and feed are definitely needed for the risk assessment of the combination of the two soybeans. This was also noted by experts from Member States (EFSA, 2016 b) such as the German authority, the Federal Office of Consumer Protection and Food Safety (BVL).
However, EFSA failed to ensure that necessary data were provided.

Also relevant in this context, but omitted in the risk assessment of the GMO Panel, is the potential toxicity caused by the residues from spraying with the complementary herbicides. Due to the specific agricultural practices that go along with the cultivation of these herbicide resistant plants,

there are, for example, specific patterns of applications, exposure, occurrence of specific metabolites and emergence of combinatorial effects that require special attention. For example, commercial large-scale cultivation of these plants results in a strong selective pressure on weeds to develop resistance to these herbicides (Sammons & Gaines, 2014), this can lead to increasing amounts of sprayed herbicides and subsequently of residues in the harvest. Further, herbicide-tolerant plants are meant to survive the application of the complementary herbicide while most other plants will die after short time. Thus, for example, residues of glyphosate, its metabolites and additives to the formulated product might accumulate and interact in the plants. As a publication by Kleter et al. (2011) shows, using herbicides to spray genetically engineered herbicide-resistant plants does indeed lead to patterns of residues and exposure that are not taken into account in regular pesticide registration:

> *"1. GM herbicide-resistant crops can change the way that herbicides can be used on these crops, for example: (a) post-emergent over-the-top applications (i.e. on the crop itself) instead of directed sprays, avoiding herbicide contact with the crop; or (b) pre-emergent and pre-harvest applications made to the conventional crop and not, or in different quantities, to the GM crop. 2. The residue profile of the applied pesticide may have been altered on the basis of the nature of the modification. 3. The overall pattern of pesticides applied to the particular crop may have been altered, leading to different exposure to pesticide residues overall."*

According to a reasoned legal opinion drawn up by Kraemer (2012), residues from spraying with complementary herbicides have to be taken into account in the risk assessment of genetically engineered plants from a regulatory point of view:

> *"It is the objective of Directive 2001/18 to avoid any adverse effect of the genetically modified plant on human health. The provisions of the Directive on the environmental risk assessment are very broad and try to cover - in the abstract, it is true – all possible cases, where direct or indirect, immediate, delayed or unforeseen adverse effects might occur. Then, it is only logical that, when genetically modified plants which are tolerant to certain herbicides, are exposed to pesticide or herbicide treatment, the effects of such treatment on the plant – and later on human or animal health – must be examined during the environmental risk assessment."*

Following on from this, that the applicants have to provide a comprehensive environmental risk assessment of the genetically engineered plants, which includes all and potential adverse effects on the environment as well as on human and animal health. This requirement includes long-term potential and accumulative effects and also all other harmful effects on human or animal health which are, in one way or another, related to the genetically modified plant, such as residues from spraying with complementary herbicides.

This is also in accordance with pesticide regulation, which requires specific risk assessment of imported plants if the usage of pesticides is different in the exporting countries compared to the one in the EU: Recital 26 of Regulation 396/2005 requires Maximum Residues Levels (MRLs) are set for food and feed produced outside the Community if produced by different agricultural practices as regards the use of plant protection products. Article 14 of Regulation 396/2005 requires that the presence of pesticide residues arising from sources other than current plant protection uses and their known cumulative and synergistic effects are determined. Further, Article 29 of Regulation 1107/2009 states that active substances and synergists have to be approved, and the maximum residue levels for each specific agricultural products have to be determined.

In any case, both the EU pesticide regulation and the GMO regulation require a high level of protection for health and the environment. Thus, in regard to herbicide-resistant plants, specific assessment of residues from spraying with complementary herbicides must be considered to be a prerequisite for granting authorisation. In addition, cumulative effects have to be investigated if a plants contains or produces other compounds of potential toxicity.

A basic prerequisite for risk assessment in this context is the availability of valid and reliable data on residue loads from spraying with herbicides. This is especially relevant in the case of glyphosate: A study published in 2015 (IARC) found that glyphosate is probably carcinogenic. While carcinogenicity of the active ingredient remains a matter of debate (EFSA 2015 a), there is a scientific consensus that additives and their mixtures used in commercial formulations for spraying glyphosate can show a much higher toxicity than the active ingredient alone (Mesnage et al., 2015). The amount of these residues depends on the specific agronomic management used in the cultivation of the herbicide resistant plants. Data from some publications (Bøhn et al., 2014, Cuhra, 2015) show, a considerable amount of residues from spraying can be expected in genetically engineered soybeans resistant to glyphosate formulations. In general, the level of residues is likely to increase due to increasing problems with herbicide resistant weeds (Benbrook, 2016)

However, as the EFSA Pesticide Panel stated (EFSA 2015 b), safety of residues from spraying glyphosate formulations could not be concluded on the data provided so far. Thus, EFSA was unable to deliver a conclusive risk assessment on the actual risks of residues from spraying with glyphosate and the various glyphosate formulations.

Furthermore, there is no comprehensive risk assessment of residues from spraying ALS inhibitors as complementary herbicides on genetically engineered soybeans. On the opposite, major data gaps were identified by the Pesticide Panel of EFSA (EFSA, 2015c) in the case of thifensulfuron, which is one of the active ingredients that act as an ALS inhibitor:

> *“Data gaps were identified in the residue section. Pending the ability of a sufficient evaluation of consumer exposure and/or further information on the toxicological profile for specific plant and livestock metabolites, the consumer risk assessment can not be finalised for the representative uses."*
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> *“In the area of mammalian toxicology and non-dietary exposure, data gaps were identified to define the toxicological profile of some metabolites and impurities. The equivalence of the different sources produced by Cheminova and Rotam to the agreed technical specification by DuPont (that was supported by the toxicological studies) should be re-assessed leading to a data gap. The potential endocrine disruption of thifensulfuron-methyl was identified as an issue that could not be finalised and a critical area of concern.”*

As a result, risk assessment of the genetically engineered soybeans cannot be concluded.

In this context, EFSA´s risk assessment omitted further relevant health risks: There is a considerable amount of literature indicating that glyphosate formulations can act as so-called endocrine disruptors (see, for example, Thongprakaisang et al., 2013; Caglar and Kolankaya, 2008; de Liz Oliveira Cavalli et al., 2013; Omran et al., 2013). Since soybeans also produce a number of plant estrogens (de Lemos, 2001), there might be some synergistic or additive interaction with the residues from spraying with glyphosate formulations. However, the impact of the soybeans on the hormone system of mammals was not investigated.

Allergenicity
It is known that toxicants, if applied together with the allergens, can have adjuvant effects, triggering a stronger immune reaction to the proteins. This is a specific risk that needs to be addressed in the context of residues from spraying with the complementary herbicides.

Furthermore, soybeans are known to have a substantial variation in their natural concentrations, depending on specific varieties and on interaction with the environment. The applicant failed to show that the level of endogenic allergens in specific varieties and/ or under specific environmental conditions is not increased. For this purpose, further crossing with other varieties should have been performed as well as subjecting the soybeans to suitable tests including biotic and abiotic stressors.

The applicant provided data on testing with blood samples stemming from a small group of people known to be sensitive to soybean allergens to find out if they had a changed reaction to the genetically engineered soybeans. However, the number of samples used for testing was too small to get reliable results. Furthermore, no analysis was undertaken of the risks for individuals with an impaired immune system such as the elderly or infants, as requested by the EFSA guidance (EFSA, 2010).

Nutritional assessment
In the process of risk assessment, potential hazards need to be identified first, before the level of exposure is taken into account. However, EFSA appears to be setting the decisive steps in risk assessment aside: Based on data for average exposure of consumers, it is concluded that no further risk assessment is needed.

The average exposure is far from being reliable: Only the anticipated average but not maximum intake of soybean food in Europe was estimated. In reality, habits regarding the consumption of soybean products can vary greatly over time, in different regions, subpopulations and individuals.

Thus, the applicant has to show that all relevant food products are safe, for all kind of diets as well as accumulated and long-term effects. However, there are no data on the safety of products that are processed, such as soybean milk and baby food. Without such data, no conclusion can be drawn upon food safety. Data on the nutrient (and anti-nutrient) composition of all the foods within the scope of the application (salad dressings, margarines, cooking oils, salty snacks, tofu, soymilk etc.) must be provided by the applicant, including data on secondary products such as soy lecithin.

Since the soybeans are intentionally and unintentionally are changed in their oil composition, there are many open questions regarding the potential health effects of the products derived from the soybeans. For example, a higher concentration of MUFA as well as a lower content in odd chain fatty acids are under discussion regarding negative health effects (Jenkins, et al., 2015, Chua et al., 2013).

Long-term feeding studies including various concentrations of the relevant fatty acids would be needed, to conclude on the safety of the products derived from the soybeans. However, from an ethical point of view there are considerable doubts about whether the potential benefits of these soybeans would justify such trials.

Monitoring
As a legal dossier compiled by Professor Ludwig Kraemer (Kraemer, 2012) shows, EU regulations require the monitoring of effects on health at the stage of consumption in cases where there are uncertainties. Thus, for example, there must be a requirement for the monitoring of health effects that takes residues from spraying with herbicides into account.

In this case, case specific monitoring would be needed to investigate negative health impacts from residues of spraying as well as effects stemming from the intended and unintended changes in the plants´ composition. Further, any spillage of the kernels has to be closely monitored, since the data on agronomic characteristics show significant changes in the performance of the plants.

Conclusions and recommendations
Based on the data presented and assessed, the risk assessment cannot be concluded. Consequently, the application should be rejected.

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