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Testbiotech comment on EFSA's assessment of genetically engineered maize Bt11 x MIR162 x MIR604 x 1507 x 5307 x GA21 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2011-103) by Syngenta

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Introduction

The GMO panel assessed the six-event stacked maize Bt11 x MIR162 x MIR604 x 1507 x 5307 x GA21, which is derived from crossing genetically engineered maize events and produced by Syngenta (EFSA, 2019a). The maize contains genes conferring resistance to two herbicides and produces five insecticidal proteins.

- Bt11 expressing Cry1Ab insecticidal protein and PAT protein for tolerance to glufosinate-containing herbicides;
- MIR162 expressing Vip3Aa20 insecticidal protein and phosphomannose isomerase (PMI) protein (used as a selectable marker);
- MIR604 expressing Cry3A (mCry3A) insecticidal protein and phosphomannose isomerase (PMI) protein (used as a selectable marker);
- 1507 expressing the Cry1F insecticidal protein and phosphinothricin acetyl transferase (PAT) protein for tolerance to glufosinate-containing herbicides;
- maize 5307 expressing eCry3.1Ab insecticidal protein and and phosphomannose isomerase (PMI) protein (used as a selectable marker);
- GA21 expressing mEPSPS protein for tolerance to glyphosate-containing herbicides.

Consequently, the stacked maize produces five insecticidal toxins; Cry1Ab, Vip3Aa20 and Cry1F that target L*epidoptera* insects, and mCry3A, eCry3.1Ab (both synthetic, not corresponding to natural templates) that target C*oleoptera*. The maize is also resistant to two groups of complementary herbicides (glyphosate and glufosinate). Even though Implementing Regulation 503/2003 has been in force since 2014, EFSA has not applied it in this case.

1. Molecular characterisation

The process of genetic engineering involved several deletions and insertions in the parental maize 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 miRNA 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.

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 reactions 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. It is unclear, to which extent specific environmental conditions will influence the overall concentration of the enzymes in the plants. 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.

The data on the expression rate of the insecticidal proteins were generated without applying the complementary herbicides. The differences in the expression of the insecticidal proteins in comparison to the parental plants and their subcombinations were not investigated in detail. The methods used for measuring the content of the insecticidal proteins were not evaluated by testing in other laboratories. Therefore, genetic stability and integrity of the expression rate of the additional proteins was not demonstrated.

Due to increased weed pressure, it has to be expected that these plants can and will be exposed to high and also repeated dosages of glyphosate alone and/or in combination with glufosinate. 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.

Industry suggests dosages of up to 1,5 kg a.i./ha of glufosinate and 3,6 l/ha of glyphosate for herbicide resistant maize. EFSA should have requested that Syngenta submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also 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 transgenes, as well as the natural genome of the plants.

2. Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)
Field trials for compositional and agronomic assessment of the stacked maize were conducted in the US for only one year (2012) and not in other relevant maize production areas, such as Brazil or Argentina.

Only data from a low number of agronomic parameters (9) were subjected to statistical analysis, 4 or even 6 (treated with the complementary herbicides) of these were found to be statistically different with some falling within categories III and IV, indicating major differences. Thus, there were many significant differences even in this small data set, and therefore EFSA should have requested more data, for example, from exposure to higher dosages of the complementary herbicides.

Compositional analysis revealed many (and major) statistically significant differences:

- Statistically significant differences between the six-event stack maize (untreated) and the non-GM comparator were identified for 25 out of 64 endpoints, with several endpoints in category III / IV.
- Statistically significant differences between the six-event stacked maize (treated with complementary herbicides) and the non-GM comparator were identified for 24 of 64 endpoints, with several endpoints in category III / IV, indicating major differences.

The most relevant differences identified concern ash, b-carotene, folic acid, ferulic acid, potassium, zinc, arachidic acid (C20:0) and methionine.

Despite the high number of significant differences, EFSA did not request any further tests (toxicological data, repeated spraying with higher herbicide dosages or exposure to a wider range of environmental conditions). Instead of assessing the overall pattern of changes in plant components as well as their causes and possible impacts, EFSA only assessed each of the compounds in isolation (!). This approach turns the comparative approach into a trivial concept of only assessing bits and pieces and ignores questions concerning the overall safety of the whole food and feed.

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

Due to high weed pressure in many maize growing regions, it has to be expected that these plants can and will be exposed to higher amounts, and also repeated dosages of the herbicides. Industry suggests dosages on herbicide resistant maize up to 1,5 kg a.i./ha glufosinate and 3,6 l/ha glyphosate. 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 risk assessment. EFSA should have requested that Syngenta submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying with each active ingredient in isolation as well as in combination. In addition, more varieties carrying the transgenes should have been included in the field trials to see how the gene constructs interact with the genetic background of the plants. The material derived from those plants should have been assessed by using omics techniques to investigate changes in plant composition or agronomic characteristics.

The need for further analysis is also underlined by research indicating that the stacking of transgenic inserts in genetically engineered maize can induce changes in the overall proteome, impacting the associated endogenous metabolic pathways (Agapito-Tenfen et al., 2014).
Based on the available data, no final conclusions can be drawn on the safety of the plants.

Toxicology
Despite many highly significant changes in the composition of the plants and agronomic characteristics, no testing of the whole plant (feeding study) was requested. 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 investigation of their potential health impacts. In addition, as mentioned, a higher number of applications of the complementary herbicide is not likely to just 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 due to interaction with the additionally inserted gene constructs.

a) Regarding Bt toxins

Furthermore, the stacked maize differs from the parental lines in regard to the overall amount of toxin produced, which is much higher than in the parental lines. In processed products, such as maize gluten, the toxins can even show a much higher concentration. These higher concentrations are relevant for the assessment of overall toxicology as well as for the immune system; nevertheless, there were no empirical investigations.

In regard to toxicology and potential synergistic or other combinatorial effects, negative impacts of Bt toxins on human and animal health cannot be excluded a priori. Bt toxins have several modes of action and are altered in their biological quality; they are therefore not identical to their natural templates (Hilbeck & Otto, 2015; Latham et al., 2017). These facts were completely ignored by EFSA.

Despite what is claimed by EFSA, not all modes of action are dependent on the specific mechanisms that only occur in the target insect species. Only very few Bt toxins (especially Cry1Ab, for overview see, for example, Then, 2010) were investigated in more detail in regard to their exact mode of action, and there is no data on the Bt toxins produced in the maize. Further, no data were presented to show that the toxins produced in the plants are only activated and effective in insects. On the other hand, several publications exist showing the effects of Bt toxins in mammals: some Cry toxins are known to bind to epithelial cells in the intestine of mice (Vázquez-Padrón et al., 1999, Vásquez-Padrón et al., 2000). As far as potential effects on health are concerned, Thomas and Ellar (1983), Shimada et al. (2003) Huffmann et al. (2004), Ito et al. (2004), Mesnage et al. (2012) and Bondzio et al. (2013) show that Cry proteins could potentially have an impact on the health of mammals. De Souza Freire et al. (2014) and Mezzomo et al. (2014) confirm hematotoxicity of several Cry toxins, including those being used in genetically engineered plants, such as Cry 1Ab and Cry1Ac. These effects seem to occur with high concentrations and tend to become stronger after several days. Such observations make the need for studies on effects after long-term exposure to various dosages clear, including in combination with material that was sprayed with the complementary herbicides. In this context, it is important that the stacked maize is also resistant to the herbicides glyphosate and glufosinate, which should be seen as potential co-stressors (see also Then & Bauer-Panskus, 2017).

Moreover, it is evident that Bt toxins can survive digestion to a much higher degree than has been assumed by EFSA: Chowdhury et al., (2003) as well as Walsh et al. (2011) have found that Cry1A proteins can frequently and successfully still be found in the colon of pigs at the end of digestion when they were fed with Bt maize. The Cry1A proteins can show much higher stability at least in monogastric species than predicted by current in vitro digestion experiments. This shows that Bt toxins are not degraded quickly in the gut and can persist in larger amounts until digestion is completed, and there is enough time for interaction between various food compounds. Consequently, there is substantiated concern that especially the stacked event can trigger immune system responses and have adverse health effects.

b) Regarding herbicide residues

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, for example, 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 the usage of pesticides is different in the exporting countries compared to the usage in the EU. In this regard, it should be taken into account that EFSA (2018) explicitly stated that no conclusion can be derived on the safety of residues from spraying with glyphosate occurring in genetically engineered plants resistant to this herbicide.

Consequently, EFSA should have requested that Syngenta 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 and the Bt toxins into account.

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.

c) Regarding effects on intestine microbiome

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; see also comments from Member States; EFSA 2019b). 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 (Bremmer & Leist, 1997).

In general, antibiotic effects and other adverse health effects might occur from exposure to a diet containing these plants that were not assessed under pesticide regulation. These adverse effects on health might be triggered by the residues from spraying with the complementary herbicide (see also van Bruggen et al., 2017). Further attention should be paid to the specific toxicity of the metabolites of the pesticide active ingredients that might occur specifically in the stacked event.

Whatever the case, both EU pesticide regulation and 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. The urgency for performing more investigations with whole food & feed is supported by the results of a feeding study (Zdziarski et al., 2018) with a similar stacked maize indicating significant health effects. Despite all these open questions regarding potential health impacts, we are not aware of a single sub-chronic or chronic feeding study carried out with whole food and feed derived from the six-event stacked maize.

Consequently, EFSAs opinion does not account for health effects arising from the complexity of the

stacked maize, which simultaneously bring multiple potential stressors into our food chains. The combinatorial effects (or potential mixed toxicity) arising from simultaneous exposure to a fixed combination of potential stressors emerging from the genetically engineered maize at the stage of consumption have to be assessed in much more detail. More specifically, food and feed derived from these plants, including specific products such as gluten, should have been tested by following a whole mixture approach considering them "*insufficiently chemically defined to apply a component-based approach*" (EFSA, 2019c). Similarly, the plants could be considered equivalent to UVCB substances (substances of unknown or variable composition, complex reaction products or biological materials) as defined under Regulation (EC) No 1907/2006 (REACH). Therefore, the food and feed analysed should be relevant to the product to be consumed, including the residues from spraying with complementary herbicides (with dosages taking the real conditions of agricultural practice into account). To generate reliable data for daily food products in the food chain, the feeding studies should be long-term and include several generations.

In conclusion, the EFSA opinion on the application for authorisation of the stacked maize (EFSA, 2019a) cannot be said to fulfil the requirements for assessment of potential synergistic or antagonistic effects resulting from the combination of transformation events in regard to toxicology and allergenicity. The hypothesis which should have been used as a starting point is that there will be synergistic effects between the various Bt toxins and between the various Bt toxins and other stressors, such as residues from spraying. Therefore, the effects of the Bt toxins in regard to mammalian cell systems and intestinal microbiomes should have been tested in combination with other stressors. Furthermore, combinatorial (adjuvant) effects triggered by Bt toxins occurring in high concentrations in the stacked maize and especially in gluten prepared from the maize, have to be tested in interaction with known allergens, such as the one occurring in soybeans. For this purpose, EFSA should have requested that Syngenta submit data from field trials with the highest dosage of glyphosate 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 and the Bt toxins into account.

As a result, the toxicological assessment carried out by EFSA is not acceptable, since safety of the products applied for import was not shown.

Allergenicity

According to Santos-Vigil et al. (2018), the Bt toxin Cry1Ac can act as an allergen if ingested. This publication is highly relevant since high concentrations of the Bt toxins can be expected, especially in products such as maize gluten.

The EU Commission initially noted that the Santos-Vigil et al (2018) publication was relevant for the risk assessment of genetically engineered plants producing Bt toxins, and therefore requested the European Food Safety Authority (EFSA) for an assessment. However, EFSA (EFSA, 2018b) came to the conclusion that the Santos-Vigil et al. (2018) publication does not provide any new information and suffers from methodological flaws. This EFSA opinion, however, is based on a rather biased interpretation of existing publications, and it does not provide any evidence that the Santos-Vigil (2018) findings are invalid or irrelevant (Moreno-Fierros et al., 2018).

In conclusion, the EFSA assessment of the stacked maize cannot be said to fulfil the requirements for assessing allergenicity of the source of the transgene. The Santos-Vigil et al. (2018) publication has to be considered to be both valid and not properly assessed by EFSA (Moreno-Fierros et al.,

2018). In awareness of the high concentrations of Bt toxins produced in the stacked maize and products derived thereof, EFSA should have started with the hypothesis that the consumption of products derived from the maize can trigger allergic reactions – and should therefore have requested empirical investigations.

Furthermore, there are several studies indicating that immune responses such as adjuvanticity in mammals are triggered by Bt toxins and have to be considered in this context (for review also see Rubio-Infante et al., 2016).

In its risk assessment, EFSA did not consider that under real conditions and contrary to what is suggested by the findings of in-vitro studies, Bt toxins will not degrade quickly in the gut but are likely to occur in substantial concentrations in the large intestine and faeces (Chowdhury et al., 2003; Walsh et al., 2011).

In regard to the degradation of the Bt toxins during ingestion, there is specific cause for concern that the maize or gluten is likely to be fed together with soybeans that naturally produce enzymes, which can substantially delay the degradation of Bt toxins in the gut (Pardo-López et al., 2009). In addition, soybeans are known to produce many food allergens. Therefore, the immune responses caused by the allergens in the soybeans might be considerably enhanced by the adjuvant effects of the Bt toxins. Such effects are likely to lead to detrimental effects on health.

Furthermore, it also has to be taken into account that so far only very few Bt toxins produced in genetically engineered plants have been investigated in regard to their potential impact on the immune system. As yet, only two Bt toxins (Cry1Ac and Cry1Ab) have been tested for their possible effects on the immune system; none of the toxins produced in the maize were investigated in this regard in any empirical research. This is a major flaw, since two of the toxins produced in the plants to not have a natural template.

The effects caused by a combination of these toxins also remain untested. The need for more detailed investigations in regard to potential immunogenic effects is also underlined in the minority opinion in another EFSA opinion (Annex II of EFSA, 2018c).

In conclusion, the EFSA assessment of the stacked maize cannot be said to fulfill the requirements for assessing risks to the immune system. Safety of the products applied for import was not shown.

Others

We support several statements made by experts of Member States requesting a much more detailed design for post-marketing monitoring (EFSA, 2019b).

Besides the methods of detection, other methods for quantifying exposure to Bt toxins need to be made publicly available in order to facilitate monitoring. Food and feed producers, farmers as well as experts dealing with environmental exposure (for example, via waste material, spillage and manure) have to be able to gather independent information on their exposure to the toxins via independent laboratories. As yet, these methods are regarded as confidential business information and are not made available upon request by EFSA. Thus, the Commission should ensure that the relevant data are both publicly available and also reliable.

As existing evidence shows (Székács et al., 2011; Shu et al., 2018), the methods need to be carefully evaluated to ensure that the results are reliable, comparable and reproducible. Therefore,

fully evaluated methods have to be published that allow the Bt concentration in the maize to be measured by independent scientists as is the case for other plant protection compounds used in food and feed production. This is necessary to make sure that the environment as well as humans and animals coming into contact with the material (for example, via dust, consumption or manure) are not exposed to higher quantities of Bt toxins than described in the application.

Environmental risk assessment

Syngenta completely ignored the appearance of teosinte in Spain and France (see Testbiotech, 2016; Trtikova et al, 2017). In its assessment of the volunteer potential, the information provided by Syngenta is largely outdated. As Pascher et al (2016) show, the volunteer potential of maize is higher than assumed by Syngenta. Further, in awareness of the findings of Fang et al. (2018), the glyphosate-resistant maize needs to be examined in detail regarding next generation effects, volunteer potential (persistence) and gene flow. There are substantial reasons for following a hypothesis that the maize can show higher fitness compared to conventional maize.

In its opinion, EFSA (2019a) was aware of the occurrence of teosinte in the EU and tried to assess the risks of gene flow. However, EFSA (2019a) is wrong for several reasons:

- Without more data on the teosinte species growing in the EU, the likelihood of gene flow from the maize to teosinte cannot be assessed (Trtikova et al, 2017). The same is true for gene flow from teosinte to genetically engineered plants.
- Furthermore, the characteristics of potential hybrids and next generations have to be investigated and cannot be predicted simply from the data of the original event. It is well known that there can be next generation effects and interference from genetic background that cannot be predicted from the assessment of the original event. This issue is relevant for gene flow from maize to as well from teosinte to maize.
- Finally, it is well established under EU regulation that it is the applicant who has to present data sufficient to show that the respective event is safe before the application can be considered to be valid (see Kraemer, 2016). Thus, an application with incorrect or missing information on crucial aspects of environmental risk assessment cannot be accepted as a starting point for EFSA risk assessment.

EFSA should have requested data from the applicant to show that no adverse effects can occur through gene flow from the maize to teosinte and / or from teosinte to the maize volunteers. In the absence of such data, the risk assessment and the authorisation have to be regarded as not valid.

Without detailed consideration of the hazards associated with the potential gene flow from maize to teosinte and from teosinte to maize, no conclusion can be drawn on the environmental risks of spillage from the stacked maize.

Consequently, environmental risk assessment carried out by EFSA is not conclusive.

Conclusions and recommendations

The EFSA risk assessment cannot be accepted since it is not conclusive and safety of the products applied for import was not demonstrated.

References

Agapito-Tenfen S.Z., Vilperte V., Benevenuto R.F., Rover C.M., Traavik T.I., Nodari R.O. (2014) Effect of stacking insecticidal cry and herbicide tolerance epsps transgenes on transgenic maize proteome. BMC plant biology 14: 346.

Ahmad, I., Malloch D. (1995) Interaction of soil microflora with the bioherbicide phosphinothricin. Agriculture, Ecosystems and Environment 54(3): 165-174.

Bondzio, A., Lodemann, U., Weise, C., Einspanier, R. (2013) Cry1Ab treatment has no effects on viability of cultured porcine intestinal cells, but triggers hsp70 expression. Plos One, 8(7): e67079. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0067079

Bremmer, J.N. & Leist, K.-H. (1997) Disodium-N-acetyl-L-glufosinate; AE F099730 – Hazard evaluation of Lglufosinate produced intestinally from N-acetyl-L-glufosinate. Hoechst Schering AgrEvo GmbH, Safety Evaluation Frankfurt. TOX97/014. A58659. Unpublished.

Chowdhury, E. H., Kuribara, H., Hino, A., Sultana, P., Mikami, O., Shimada, N., ... & Nakajima, Y. (2003) Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. Journal of Animal Science, 81(10): 2546-2551. https://academic.oup.com/jas/article-abstract/81/10/2546/4789819

de Souza Freire, I., Miranda-Vilela, A.L., Pereira Barbosa, L.C., Soares Martins, E., Gomes Monnerat, R., Koppe Grisolia, C. (2014) Evaluation of Cytotoxicity, Genotoxicity and Hematotoxicity of the Recombinant Spore-Crystal Complexes Cry1Ia, Cry10Aa and Cry1Ba6 from Bacillus thuringiensis in Swiss Mice. Toxins, 6: 2872-2885. https://www.mdpi.com/2072-6651/6/10/2872/htm

EFSA (2018a) Reasoned Opinion on the review of the existing maximum residue levels for glyphosate according to Article 12 of Regulation (EC) No 396/2005. EFSA Journal 2018;16(5):5263, 230 pp. https://doi.org/10.2903/j.efsa.2018.5263)

EFSA (2018b) Relevance of new scientific information (Santos-Vigil et al., 2018) in relation to the risk assessment of genetically modified crops with Cry1Ac. EFSA supporting publication 2018:EN-1504. 13 pp. doi:10.2903/sp.efsa.2018.EN-1504. https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/sp.efsa.2019.EN-1504

EFSA (2018c) Scientific opinion on the assessment of genetically modified maize Bt11 x MIR162 x 1507 x GA21 and three subcombinations independently of their origin, for food and feed uses under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2010-86). EFSA Journal 2018;16(7):5309, 35 pp. https://doi.org/10.2903/j.efsa.2018.5309

EFSA (2019a) Scientific Opinion on assessment of genetically modified maize Bt11 x MIR162 x MIR604 x 1507 9 5307 x GA21 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2011-103). EFSA Journal 2019;17(4):5635, 36 pp. https://doi.org/10.2903/j.efsa.2019.5635

EFSA (2019b) Scientific Opinion on assessment of genetically modified maize Bt11 x MIR162 x MIR604 x 1507 9 5307 x GA21 and subcombinations, for food and feed uses, under Regulation

(EC) No 1829/2003 (application EFSA-GMO-DE-2011-103). Comments from the Member States, derived from Register of EFSA http://registerofquestions.efsa.europa.eu/roqFrontend/login?0

EFSA (2019c) Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. EFSA Journal 2019;17(3):5634, 77 pp. https://doi.org/10.2903/j.efsa.2019.5634

Fang, J., Nan, P., Gu, Z., Ge, X., Feng, Y.-Q., Lu, B.-R. (2018) Overexpressing Exogenous 5-Enolpyruvylshikimate-3-Phosphate Synthase (EPSPS) Genes Increases Fecundity and Auxin Content of Transgenic Arabidopsis Plants. Frontiers in Plant Sciences, 9: 233. https://doi.org/10.3389/fpls.2018.00233

Hilbeck, A. & Otto, M. (2015) Specificity and combinatorial effects of Bacillus thuringiensis Cry toxins in the context of GMO risk assessment. Frontiers Environmental Science, 3: 71.

Hsiao, C-L., Young, C-C., Wang, C-Y.W (2007) Screening and identification of glufosinate-degrading bacteria from glufosinate-treated soils. Weed Science, 55(6): 631-637.

Huffmann, D.L., Abrami, L., Sasik, R., Corbeil, J., van der Goot, G., Aroian, R.V. (2004) Mitogenactivated protein kinase pathways defend against bacterial pore-forming toxins. Proceedings of the National Academy of Sciences, 101(30): 10995-11000. http://www.pnas.org/content/101/30/10995.short

Ito, A., Sasaguri, Y., Kitada, S., Kusaka, Y., Kuwano, K., Masutomi, K., Mizuki, E., Akao, T., Ohba, M. (2004) Bacillus thuringiensis crystal protein with selective cytocidal action on human cells. Journal of Biological Chemistry, 279: 21282-21286. http://www.jbc.org/content/279/20/21282.short

Kleter, G.A., Unsworth, J.B., Harris, C.A. (2011) The impact of altered herbicide residues in transgenic herbicide-resistant crops on standard setting for herbicide residues. Pest Managment Science, 67(10): 1193-1210. https://onlinelibrary.wiley.com/doi/abs/10.1002/ps.2128

Kopcáková, A., Legáth, J., Pristaš, P., Javorský, P. (2015) Already a short-term soils exposure to the field-rate glufosinate concentration significantly influences soil bacterial communities. Soil and Water Research 10(4): 271-277.

Kraemer, L. (2016) Teosinte plants in the European environment and its implication for market authorisation of genetically engineered maize Legal analysis commissioned by Testbiotech, http://www.testbiotech.org/node/1773

Latham, J.R., Love, M., Hilbeck, A. (2017) The distinct properties of natural and GM cry insecticidal proteins. Biotechnol Genet Eng Rev 33(1): 62-96.

Mao, Q., Manservisi, F., Panzacchi, S., Mandrioli, D., Menghetti, I., Vornoli, A., Bua, L., Falcioni, L., Lesseur, C., Chen, J., Belpoggi, F., Hu, J. (2018) The Ramazzini Institute 13-week pilot study on glyphosate and Roundup administered at human-equivalent dose to Sprague Dawley rats: effects on the microbiome. Environmental Health, 17: 50. https://doi.org/10.1186/s12940-018-0394-x

Mesnage, R., Clair, E., Gress, S., Then, C., Székács, A., Séralini, G.-E. (2012) Cytotoxicity on human cells of Cry1Ab and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide. Journal of Applied Toxicology, 33(7): 695–699.

https://onlinelibrary.wiley.com/doi/abs/10.1002/jat.2712

Mezzomo, B.P., Miranda-Vilela, A.L., de Souza Freire, I., Pereira Barbosa, L.C., Portilho, F.A., Marques Lacava, Z.G., Koppe Grisolia, C. (2013) Hematotoxicity of Bacillus thuringiensis as Spore-crystal Strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss Albino Mice. J Hematol Thromb Dis, 1:1.

Moreno-Fierros, L., Santos-Vigil, K., Ilhicatzi-Alvarado, D. (2018) Response to assessment of the Relevance of new scientific information (Santos-Vigil et al., 2018) in relation to the risk assessment of genetically modified crops with Cry1Ac of European Food Safety Authority (EFSA). www.testbiotech.org/node/2304

Pampulha, M.E., Ferreira, M.A.S.S., Oliveira, A. (2007) Effects of a phosphinothricin based herbicide on selected groups of soil microorganisms. J Basic Microbiol 47(4): 325-331.

Pardo-López L, Muñoz-Garay C, Porta H, Rodríguez-Almazán C, Soberón, M, Bravo A (2009) Strategies to improve the insecticidal activity of Cry toxins from Bacillus thuringiensis. Peptides 30(3): 589–595. https://www.sciencedirect.com/science/article/pii/S0196978108003264

Pascher, K. (2016) Spread of volunteer and feral maize plants in Central Europe: recent data from Austria. Environmental Sciences Europe, 28(1): 30. https://link.springer.com/article/10.1186/s12302-016-0098-1

Reuter, T., Alexander, T.W., Martinez, T.F., McAllister, T.A. (2007) The effect of glyphosate on digestion and horizontal gene transfer during in vitro ruminal fermentation of genetically modified canola. J Sci Food Agric 87: 2837-2843. https://onlinelibrary.wiley.com/doi/abs/10.1002/jsfa.3038

Rubio Infante, N., & Moreno-Fierros, L. (2016) An overview of the safety and biological effects of Bacillus thuringiensis Cry toxins in mammals. Journal of Applied Toxicology, 36(5): 630-648. http://onlinelibrary.wiley.com/doi/10.1002/jat.3252/full

Santos-Vigil, K.I., Ilhuicatzi-Alvarado, D., García-Hernández, A.L., Herrera-García, J.S., Moreno-Fierros, L. (2018) Study of the allergenic potential of Bacillus thuringiensis Cry1Ac toxin following intra-gastric administration in a murine model of food-allergy. International immunopharmacology, 61: 185–196. https://www.sciencedirect.com/science/article/pii/S1567576918302467

Shehata, A.A., Schrödl, W., Aldin, A.A., Hafez, H.M., Krüger, M. (2012) The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. Curr Microbiol 6(4): 350-358. https://link.springer.com/article/10.1007/s00284-012-0277-2

Shimada, N., Kim, Y.S., Miyamoto, K., Yoshioka, M., Murata, H. (2003) Effects of Bacillus thuringiensis Cry1Ab toxin on mammalian cells. J Vet Med Sci, 65: 187-191. https://www.jstage.jst.go.jp/article/jvms/65/2/65_2_187/_article/-char/ja/

Shu, Y., Romeis, J., Meissle, M. (2018) No interactions of stacked Bt maize with the non-target aphid Rhopalosiphum padi and the spider mite Tetranychus urticae. Frontiers in Plant Science, 9: 39. https://www.frontiersin.org/articles/10.3389/fpls.2018.00039

Székács, A., Weiss, G., Quist, D., Takács, E., Darvas, B., Meier, M., Swain, T., Hilbeck, A. (2011) Interlaboratory comparison of Cry1Ab toxin quantification in MON 810 maize by ezyme-

immunoassay. Food and Agricultural Immunology, 23(2): 99-121. www.tandfonline.com/doi/abs/10.1080/09540105.2011.604773

Testbiotech (2016) Cultivation of genetically engineered maize: Risks not under control - Overview: Why the EU should not allow the cultivation of transgenic maize engineered to produce insecticidal toxins. Testbiotech Background, 24 - 11 – 2016. https://www.testbiotech.org/node/1759

Then, C. (2010) Risk assessment of toxins derived from Bacillus thuringiensis: synergism, efficacy, and selectivity. Environmental Science and Pollution Research, 17(3): 791-797. https://link.springer.com/article/10.1007/s11356-009-0208-3

Then, C., & Bauer-Panskus, A. (2017) Possible health impacts of Bt toxins and residues from spraying with complementary herbicides in genetically engineered soybeans and risk assessment as performed by the European Food Safety Authority EFSA. Environmental Sciences Europe, 29(1): 1. https://enveurope.springeropen.com/articles/10.1186/s12302-016-0099-0

Thomas, W.E. & Ellar, D.J. (1983) Bacillus thuringiensis var israelensis crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. Journal of Cell Science, 60(1): 181–197. http://jcs.biologists.org/content/60/1/181.short

Trtikova, M., Lohn, A., Binimelis, R., Chapela, I., Oehen, B., Zemp, N., Widmer, A., Hilbeck, A. (2017) Teosinte in Europe – Searching for the Origin of a Novel Weed. Scientific Reports, 7: 1560. https://www.nature.com/articles/s41598-017-01478-w

Trtikova, M., Wikmark, O.G., Zemp, N., Widmer, A., Hilbeck, A. (2015) Transgene expression and Bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions. PloS one, 10(4): e0123011. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0123011

Van Bruggen, A.H.C., He, M.M., Shin, K., Mai, V., Jeong, K. C., Finckh, M.R., Morris, J.G. (2018) Environmental and health effects of the herbicide glyphosate. Science of The Total Environment, 616: 255-268. https://www.sciencedirect.com/science/article/pii/S0048969717330279

Vásquez-Padrón. R.I., Gonzáles-Cabrera. J., Garcia-Tovar. C., Neri-Bazan. L., Lopéz-Revilla. R., Hernández. M., Morena-Fierros. L., de la Riva, G.A. (2000) Cry1Ac Protoxin from Bacillus thuringiensis sp. kurstaki HD73 binds to surface proteins in the mouse small intestine. Biochem and Biophys Research Comm, 271: 54-58. www.sciencedirect.com/science/article/pii/S0006291X00925841

Vázquez-Padrón, R.I., Moreno-Fierros, L., Neri-Bazán, L., de la Riva, G.A., López-Revilla, R. (1999) Intragastric and intraperitoneal administration of Cry1Ac protoxin from Bacillus thuringiensis induces systemic and mucosal antibody responses in mice. Life Sciences, 64(21): 1897-1912. www.academia.edu/download/43177637/Intragastric_and_intraperitoneal_adminis20160228-1573-204m62.pdf

Walsh, M. C., Buzoianu, S. G., Gardiner, G. E., Rea, M. C., Gelencsér, E., Jánosi, A., ... & Lawlor, P. G. (2011) Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. PLoS One, 6(11): e27177. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0027177

Walsh, M. C., Buzoianu, S. G., Gardiner, G. E., Rea, M. C., Gelencsér, E., Jánosi, A., ... & Lawlor, P. G. (2011) Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. PLoS One, 6(11): e27177. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0027177

Zdziarski, I.M., Carman, J.A. and Edwards, J.W. (2018) Histopathological investigation of the stomach of rats fed a 60% genetically modified corn diet. Food and Nutrition Sciences, 9: 763-796. https://doi.org/10.4236/fns.2018.96058