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Testbiotech comment on the Scientific Opinion on an application by Dow Agrosciences LLC (EFSA-GMO-NL-2009-68) for placing on the market of cotton 281-24-236 x 3006-210-23 x MON 88913 for food and feed uses, import and processing under Regulation (EC) No 1829/2003

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Introduction
Cotton 281-24-236 x 3006-210-23 x MON 88913 was developed to confer resistance to lepidopteran target pests and tolerance to glyphosate-based herbicides. Resistance to lepidopteran target pests is achieved by the expression of Cry1Ac and Cry1F. Tolerance to glyphosate is achieved by expression of CP4 EPSPS. In addition, this cotton also expresses the PAT protein, which confers tolerance to glufosinate ammonium-based herbicides.
EFSA had previously issued opinions on the single events (281-24-236 x 3006-210-23 9 counts as a single event).

Molecular characterisation
According to EFSA (2016), the molecular data "establish that the events stacked in cotton 281-24-236 x 3006-210-23 x MON 88913 have retained their integrity. Protein expression analyses showed that the levels of the newly expressed proteins are similar in the three-event stack and the GM parental lines."

However, no analysis was performed with the stacked event to prove that there was no interaction between genes and partial gene sequences.

Further, no assessment was made (neither for the parental plants nor the stacked event) on the various forms of interfering RNAs that are likely to emerge from both the intended and unintended insertion sites. Some of these interfering RNAs, for example, might occur in the form of miRNA that can be taken up from the gut at the stage of consumption without losing biological activity (Zhang et al., 2012).

There is an ongoing debate about the results of this study. While some researchers reported data consistent with the original study (Wang et al., 2012; Beatty et al., 2014), others have failed to replicate the results. But the research group reporting the initial finding has responded to some of these reports (Chen et al., 2013), and has published additional work detailing the detection of other plant-derived miRNAs in humans and mice after feeding, with biological activity (Zhou et al., 2014; Liang et al., 2015). Furthermore, RNAi effects might also impact the metabolism in the plant.

As Trtikova et al. (2015) show, gene regulation in the plants might be affected by stressors occurring under ongoing climate change. This might also affect food quality or food safety. Thus, expression of intended or unintended proteins stemming from the additional DNA as well as occurrence of other new biologically active compounds such as interfering RNA should have been investigated under various defined stress conditions. However, no such data are available, either for the parental plants or the stacked events.

Comparative analysis (for compositional analysis and agronomic traits and the phenotype)

Field trials were conducted during the 2005 growing season in five locations in the USA. The number of locations is not in line with current EFSA guidance.

According to EFSA, statistically significant differences regarding phenotype were observed between three-event stack cotton compared to its conventional counterpart for fibre micronaire and fibre elongation, which might be related to the interruption of the gibberellin-20-oxidase gene.

According to Member States experts, statistically significant differences in the composition of the genetically engineered plants were also found for several compounds such as
• protein
• carbohydrates
• calcium
• glutamic acid
• valine
• vitamins E and A
• sterculic- and malvelic acids.

However, no further investigations were deemed necessary by the GMO Panel and all differences were declared irrelevant, even using non-scientific ad hoc assumptions. For example, a significant reduction in sterculic acid was considered irrelevant, just because this compound is seen as an anti-nutrient.

Overall, further investigations taking defined stress conditions into account to examine genetic stability should be carried out to assess the composition of the plants. Moreover, these investigations should take into account conditions which, for example, can occur under ongoing climate change.

Toxicology

The GMO Panel notes that the free gossypol content in cotton 281-24-236 x 3006-210-23 x MON 88913 and its conventional counterpart is higher than the limits set in Directive 2002/32 EC22 (5,000 mg/kg as fed) on undesirable substances in feed materials.

It is known that the content of gossypol in cotton seeds is affected by the genetic background of the plant variety as well as by environmental factors such as climate, soil type, and fertilisation. It is readily absorbed from the gastrointestinal tract, and is highly protein-bound to amino acids, especially lysine, and to dietary iron. The precise mechanism of action is not known, but gossypol renders many amino acids unavailable. Gossypol also affects enzymatic reactions critical for many biological processes, including the ability of cells to respond to oxidative stress and inhibition of oxygen release from haemoglobin. All animals are susceptible, with monogastrics, preruminants, immature ruminants, and poultry appearing to be affected most frequently. Toxic effects usually only occur after long-term exposure to gossypol, often after weeks or months. Signs of toxicity may relate to effects on the cardiac, hepatic, renal, reproductive, or other systems. (see, for example: www.merckmanuals.com/vet/toxicology/gossypol_poisoning/overview_of_gossypol_poisoning.html)

But although a significantly higher level of gossypol was found in the plants, no detailed assessment of risks to health was carried out. Instead, EFSA concluded that because of general EU regulations limiting the maximum content of free gossypol in feed, the elevated content of gossypol was not a safety concern. Further, some preparations used for human food consumption are not supposed to contain free gossypol. Thus, it appears that EFSA is unable to exclude toxic effects in farm animals

(and humans?) when they are fed with stacked events, and is simply relying on EU controls and inspections of animal feed (which are not normally very frequent).

Such a weighing up of risk management measures has nothing to do with the scientific risk assessment of genetically engineered plants. EFSA should have requested a detailed investigation of the underlying mechanisms that cause the higher level of gossypol in the stacked event, in addition to a lot more data on the real content of gossypol under various defined environmental conditions, and after crossing with a large number of other varieties.

Despite this fact, not a single feeding study with the whole food and feed was requested by EFSA to explore potential health effects and no nutritional study or toxicological study was conducted.

Further, possible effects due to the introduction of the four transgenic traits into the cotton genome and due to residues of the complementary herbicides, their metabolites or interactions with the Cry toxins were not assessed. Also in this regard, existing evidence – largely ignored in the EFSA opinion - shows that indeed more investigations would be needed to conclude risk assessment on this stacked genetically engineered plant:

- Hilbeck & Otto (2015) give an overview on open questions regarding the mode of action of Bt toxins, synergistic and additional effects. Specific synergistic effects were shown by Bergamasco et al. (2013). Further, the mode of action of Vip3A is not well characterised and should therefore have been much more thoroughly assessed in the stacked event and in the parental plants.
- Hilbeck & Otto (2015) show that there is not just one mode of action that has to be taken into account. Thus, the EFSA panel cannot set aside potential combinatorial effects as being relevant only for insects, just because mammals are supposed to lack relevant receptors (EFSA, 2016). As Rubio-Infante & Moreno-Fierros (2015) show, negative health effects of Bt toxins on mammals cannot simply be excluded, the proteins cannot be regarded as harmless for mammals.
- Further effects on the immune system that are known to be relevant in the context of Bt toxins are not dependent on a specific mode of action but on dosage effects. This is relevant in this context, since the stack shows a higher overall concentration of Bt toxins than the parental plants.
- It is also known, that degradation of Bt toxins under artificial digestion tests are not reliable when it comes to persistence of Bt toxins in the gut. For example, if fed with soybeans, degradation of the Bt toxins can be delayed substantially by plant enzymes, enhancing toxicity significantly (Pardo-López et al., 2009).
- Residues from spraying with glyphosate are thought to have carcinogenic effects (IARC, 2015). Negative effects from residues might be enhanced by combinatorial effects with Bt toxins. The existence of combinatorial effects between glyphosate and different Cry toxins was recently shown by Bøhn et al. (2016) in *Daphnia magna*.
- Regarding glyphosate, the EU Commission (2016) recently requested EFSA to assess the effects of glyphosate residues in feed on animal health. The outcome of this assessment should be taken into account by the GMO Panel before premature conclusions not based on data are reached.
- The investigations should not only cover direct effects on health but also indirect effects via changes in the microbiological composition in the gut (see, for example, Shehata, et al., 2012).

Thus, whatever the case, potential combinatorial health effects need to be assessed in detail before any conclusion can be drawn on food safety.

Allergenicity

According to EFSA, no concerns on allergenicity were identified for Cry toxins produced by parental lines, and no new information on allergenicity of these proteins that might change the previous conclusions of the GMO Panel has become available.

No tests were conducted to substantiate these claims.

The “weight of evidence” approach as applied by the EFSA is inadequate, since it is largely based on methods such as the pepsin test that is known to be unreliable. Further, the EFSA approach does not take potential adjuvant / synergistic effects that may emerge in stacked events into account. No non-IGE-mediated immune reactions were assessed, although these effects must be considered relevant (Mills et al., 2013).
Furthermore, EFSA (2010) requests detailed investigations into allergenic risks for infants and individuals with impaired digestive functions. “The specific risk of potential allergenicity of GM products in infants as well as individuals with impaired digestive functions (e.g. elderly people, or individuals on antacid medications) should be considered, taking into account the different digestive physiology and sensitivity towards allergens in this subpopulation.” However, these specific risks were left aside during EFSA risk assessment.

Environmental risk assessment

As the comments from experts from Member States show, some plant species in Europe can cross with cotton. Apart from this, cotton is grown in several regions. Spillage from cotton seeds is likely to occur and concerns were raised by experts from EU Member States that transgenes might be distributed in the environment. However, EFSA considers the risks for the uncontrolled spread of the transgenes to be low. In doing so, EFSA has ignored data from Mexico (Wegier et al., 2012) showing that it is difficult to predict the distribution of transgenic cotton in the environment once spillage occurs. Thus, the risk for contamination and uncontrolled spread of the transgenes seems to be much more relevant than assumed by EFSA.

Monitoring

According to EFSA, post-market monitoring of food/feed derived from cotton 281-24-236 x 3006-210-23 x MON88913 is not necessary, given the absence of safety concerns.

However, 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. This is especially relevant in this case, because of the elevated level of gossypol that has been found, and because a specific pattern of residues from spraying with herbicides can be expected in the plants. Directive 2001/18 and Regulation 1829/2003 both require that potential adverse effects on human health from genetically modified plants are monitored during the use and consumption stage. Therefore, the EFSA opinion that monitoring the effects on health is unnecessary contradicts current EU regulations.

Conclusions and recommendations
EFSA risk assessment is based to a large extent on assumptions instead of valid data. Therefore, risks cannot be assessed properly and market authorisation for import and usage in food and feed cannot be given.

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