

TESTBIOTECH Background 16 - 9 - 2021

Testbiotech comment on Scientific Opinion on the assessment of genetically engineered cotton GHB811 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-ES-2018-154) by BASF

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Introduction

The GMO panel assessed the cotton event GHB811. The cotton is engineered to be resistant to glyphosate and a group of herbicides known as HPPD inhibitors, such as isoxaflutole, mesotrionine and tembotrionine. Implementing Regulation 503/2013 was applied in this case.

Molecular characterisation and gene expression

Annex II of Implementing Regulation 503/2013 requests that

“Protein expression data, including the raw data, obtained from field trials and related to the conditions in which the crop is grown (in regard to the newly expressed proteins).” (Scientific requirements 1.2.2.3)

“In the case of herbicide tolerant genetically modified plants and in order to assess whether the expected agricultural practices influence the expression of the studied endpoints, three test materials shall be compared: the genetically modified plant exposed to the intended herbicide; the conventional counterpart treated with conventional herbicide management regimes; and the genetically modified plant treated with the same conventional herbicide management regimes.” (Scientific requirements 1.3.1)

“The different sites selected for the field trials shall reflect the different meteorological and agronomic conditions under which the crop is to be grown; the choice shall be explicitly justified. The choice of non-genetically modified reference varieties shall be appropriate for the chosen sites and shall be justified explicitly.” (Scientific requirements 1.3.2.1)

Open reading frames and gene insertion

The genetic engineering process led to the emergence of many new open reading frames in the genome of the cotton. 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 proteins that may emerge from these DNA sequences would raise no safety concerns. Other gene products, such as ncsRNA 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.

Impact of environmental factors, agricultural practice and genetic backgrounds

The data presented by BASF do not meet the requirements of Implementing Regulation 503/2013: (1) the field trials were not conducted in all relevant regions where the GE cotton may be cultivated, and no specific extreme weather conditions were taken into account; (2) the field trials did not take all relevant agricultural management practices into account; (3) not all relevant genetic backgrounds were taken into account.

Data on environmental factors, stress conditions and their impact on gene expression

Data was only presented from field trials carried out at three sites in the US for just one year. Environmental stress can cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). However, the data from the field trials do not allow conclusions to be drawn on how gene expression will, for example, be affected by climate stress due to drought, watering or high temperatures. Therefore, to assess gene expression, the plants should have been grown in different environmental conditions and exposed to defined environmental stress conditions.

In conclusion, the cotton plants tested in field trials may not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact of environmental factors or stress conditions on gene expression as requested by the EU Regulation 503/2013.

Data on herbicide application rates and their impact on gene expression

Of the relevant groups of HPPD inhibitors (one group consists of benzoylisoxazoles, i.e. bleaching herbicides such as isoxaflutole, the other of β -triketones such as mesotrione), only one active substance (isoxaflutole) was tested in field trials. Other HPPD inhibitors that might be used in the cultivation of cotton were ignored. Isoxaflutole was only sprayed at the pre-emergence stage (see also comments made by experts from Member States; EFSA, 2021b) but not onto the plants as described by Foster (2021). Furthermore, isoxaflutole was not mixed with other herbicides in the tank as described by Foster (2021) and suggested in BASF patent applications (see, for example, WO2020025367).

As shown by Foster and detailed in BASF patent applications, the responses of the transgenic cotton to the application of the complementary herbicide depends on the way it is applied. EFSA did not take this issue into account. It signifies new evidence that the data generated by BASF and assessed by EFSA do not sufficiently recognise the expected agricultural practices which may influence the expression of the studied endpoints.

EFSA should have requested the applicant to submit data from field trials that included all the relevant agricultural practices, all active ingredients, all dosages and all combinations of the complementary herbicides that might be used in agricultural practice of the cotton producing countries. Without these data, no reliable conclusion can be drawn as requested in Implementing Regulation 503/2013 (in particular for herbicide tolerant GE plants) to assess whether anticipated agricultural practices influence the expression of the studied endpoints. This also includes the application of glyphosate, which was only used in a single application at low dosage (1067 to 1222 g ai/ha, see comments made by experts from Member States; EFSA, 2021b).

Consequently, the cotton plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition or biological characteristics of the plant as requested in EU Regulation 503/2013.

Impact of genetic backgrounds on gene expression

It is known that the genomic background of the subspecies / varieties can influence both the expression of the inserted genes and plant metabolism. However, the data on gene expression were confined to the *Gossypium hirsutum* species, although it is stated that the application also covers insertion of the GHB811 event into the species of *G. barbadense*. Therefore, EFSA should also have requested additional data from transgenic *G. barbadense* (see also statement of experts from Member States).

However, EFSA has not taken these issues into consideration. Consequently, the cotton plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are therefore insufficient to conclude on the impact of the genetic backgrounds on gene expression as requested in EU Regulation 503/2013.

Comparative assessment of plant composition and agronomic and phenotypic characteristics

Implementing Regulation 503/2013 requests:

“In the case of herbicide tolerant genetically modified plants and in order to assess whether the expected agricultural practices influence the expression of the studied endpoints, three test materials shall be compared: the genetically modified plant exposed to the intended herbicide; the conventional counterpart treated with conventional herbicide management regimes; and the genetically modified plant treated with the same conventional herbicide management regimes.”

“The different sites selected for the field trials shall reflect the different meteorological and agronomic conditions under which the crop is to be grown; the choice shall be explicitly justified. The choice of non-genetically modified reference varieties shall be appropriate for the chosen sites and shall be justified explicitly.”

The data presented by BASF do not meet the requirements of Implementing Regulation 503/2013: (1) the field trials were not conducted in all relevant regions where the cotton will be cultivated, and no defined extreme weather conditions were taken into account; (2) the field trials did not take all relevant agricultural management practices into account; (3) not all relevant genetic backgrounds were taken into account.

Data on environmental factors and stress conditions - and their impact on plant composition and phenotype

Field trials to assess plant composition as well as agronomic and phenotypic characteristics of the cotton were only conducted in the US (each site only for one year). Some extreme weather conditions were reported from the field trials. These, however, remain arbitrary and not well defined and do not allow any conclusions to be drawn on how gene expression will be affected by more severe climate stress due to drought, watering or high temperatures. In order to assess gene expression, the plants should have been grown in various environmental conditions and exposed to well defined environmental stress conditions. This requirement is especially relevant in this case,

since it is known that the additional epsps genes may show pleiotropic effects, also affecting seed dormancy, growth and stress responses of the plants (see for example Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; Beres et al., 2018, Beres, 2019).

However, no experiments were requested to show to which extent specific environmental conditions influence plant composition and agronomic characteristics. Hence, no data were made available as requested in Implementing regulation 503/2013 to assess whether the expected environmental conditions under which the plants are likely to be cultivated will influence the expression of the studied endpoints.

Data on herbicide application rates and their impact on plant composition as well as agronomic and phenotypic characteristics

Due to the mode of action of the active ingredients in the complementary herbicides, it is plausible that complementary herbicide applications will cause stress responses in the plants, and thus impact gene expression and plant composition. These effects may vary with the amount of herbicide sprayed onto the crop and the various active ingredients which can be used. This is especially relevant for isoxaflutole, which causes specific plant responses if applied on top of the plants (post-emergence; see Foster, 2021). Therefore, it has to be assumed that the differences in complementary herbicide applications will not only lead to a differing burden of residues in the harvest, but will impact the composition of the plants and agronomic characteristics. This assumption is supported by a higher number of significant differences in plant composition in plants sprayed with the complementary herbicide compared to those not treated with isoxaflutol.

Of the relevant groups of HPPD inhibitors (one group is known as benzoylisoxazoles, i.e. bleaching herbicides such as isoxaflutole, the other as β -triketones such as mesotrionine), only one active substance (isoxaflutole) was tested in field trials. Other HPPD inhibitors that might be used in the cultivation of cotton were ignored. Isoxaflutole was only sprayed pre-emergence (see also comments made by experts from Member States; EFSA, 2021b) but not on top of the plants as described by Foster (2021). Furthermore, isoxaflutole was not mixed with other herbicides in the tank as described by Foster (2021) and set out in BASF patent applications (see, for example, WO2020025367). As shown by Foster and set out in the BASF patent applications, the response of the transgenic cotton to the complementary herbicide application depends on how it is applied. EFSA did not take this issue into account. This is new evidence that the data generated by BASF and assessed by EFSA do not represent the expected agricultural practices which may influence the data on the studied endpoints.

EFSA should have requested the applicant to submit data from field trials on all the relevant active ingredients used in agricultural practice, including all dosages and combinations of the complementary herbicides which might be used in agricultural practice in cotton producing countries. Without these data, no reliable conclusions can be drawn as requested in Implementing Regulation 503/2013 (in particular for herbicide tolerant GE plants) to assess whether anticipated agricultural practices influence the expression of the studied endpoints. This also includes the application of glyphosate, which was only used in a single application at low dosage (1067 to 1222 g ai/ha, see comments made by experts from Member States; EFSA, 2021b).

Consequently, the cotton plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition or biological characteristics of the plant as requested in EU Regulation 503/2013.

Impact of genetic backgrounds on plant composition as well as on agronomic and phenotypic characteristics

It is known that the genomic background of the subspecies / variety can influence the expression of the inserted genes and plant metabolism. It is known that the genomic background of the subspecies / varieties can influence both the expression of the inserted genes and plant metabolism. However, the data on plant composition and agronomic and phenotypic characteristics were confined to the *Gossypium hirsutum* species, although it is stated that the application also covers insertion of the GHB811 event into the species of *G. barbadense*. Therefore, EFSA should also have requested additional data from transgenic *G. barbadense* (see statement made by experts from Member States).

However, EFSA has not yet taken these issues into consideration. Consequently, the cotton plants tested in the field trials do not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact of the genetic backgrounds on plant composition or agronomic and phenotypic characteristics as requested in EU Regulation 503/2013.

Data from compositional analysis show the need for further investigations

Forty-five agronomic and phenotypic endpoints were collected from the field trials. Of those, 14 endpoints (including information on biotic and abiotic stressors) were measured on a categorical scale and analysed with the Cochran–Mantel–Haenszel (CMH) test. For the disease stressor rating, the CMH test identified statistically significant differences between both treatments of cotton GHB811 and the comparator; the mean values for cotton GHB811 were within the range of the non-GM reference varieties, however the comparison with this non-isogenic varieties causes larger uncertainties.

In addition, for % lint and lint length, highly significant statistically differences were identified between cotton GHB811 and the conventional counterpart treated with and without the complementary herbicides (endpoints fell under equivalence category III/IV).

The outcome of the field trials clearly shows the need for more detailed data on agronomic and phenotypic endpoints, which need to be generated from a wider range of clearly defined stress factors, including all relevant agricultural practices and genetic backgrounds. This requirement is especially relevant in this case, since it is known that the additional epsps genes may show pleiotropic effects, also affecting seed dormancy, growth and stress responses of the plants (see for example Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; Beres et al., 2018, Beres, 2019).

Fuzzy seeds of cotton GHB811 harvested from the field trials (Table 2) were analysed for 73 constituents. The statistical analysis was applied to a total of 56 constituents. For cotton GHB811 not treated with the intended herbicide, significant differences between the GE cotton and the comparator were found for 11 endpoints, for GHB811 treated with the intended herbicide, significant differences between the GE cotton and the comparator were found for 18 endpoints, with one endpoint (dihydrosterculic acid) being highly significant (equivalence category IV).

As shown, the data showed a lower number of significant findings in plant composition and phenotypic characteristics when the plants were not sprayed with the complementary herbicides. This indicates that application of the complementary herbicide might have impacted metabolic pathways. This should have been investigated in more detail, also taking defined environmental

biotic and abiotic stressors into account, including all relevant genetic backgrounds and various herbicide application regimes, such as those used by Foster (2021).

More detailed analysis would have been necessary to investigate changes in plant composition and phenotype, and also to investigate potential unintended changes in metabolic pathways and the emergence of unintended biologically active gene products.

Conclusion on the comparative assessment of plant composition as well as on phenotypic and agronomic characteristics

The data provided by the applicant and accepted by EFSA are insufficient to conclude on the impact of environmental factors, herbicide applications and genetic backgrounds on gene expression, plant metabolism, plant composition, or on agronomic and phenotypic characteristics.

To gather reliable data on compositional analysis and agronomic characteristics, the plants should have been subjected to a much broader range of defined environmental conditions and stressors. Furthermore, EFSA should have requested the applicant to submit data from field trials which reflect current agricultural practices, including all relevant complementary herbicides and all relevant genetic backgrounds.

However, only samples from field sites located in the US were used to generate the data, and the impact of environmental factors and agricultural practices were not assessed in detail. Herbicide applications in the field trials did not represent all the relevant agricultural practices. Only *G. hirsutum* was used for generating the data.

Consequently, the data presented by the applicant and accepted by EFSA are insufficient to conclude on the impact of environmental factors, herbicide applications or different genetic backgrounds on plant composition and agronomic characteristics.

Based on the available data, no final conclusions can be drawn on the safety of the plants. Therefore, the data neither fulfill the requirements of Implementing Regulation 503/2013 nor Regulation 1829/2003. This is also underlined by several statements made by experts from Member States (EFSA, 2021b).

In summary, the cotton plants tested in the field trials do not sufficiently represent the products intended for import.

Toxicity

Implementing Regulation 503/2013 requests:

“Toxicological assessment shall be performed in order to:

(a) demonstrate that the intended effect(s) of the genetic modification has no adverse effects on human and animal health;

(b) demonstrate that unintended effect(s) of the genetic modification(s) identified or assumed to have occurred based on the preceding comparative molecular, compositional or phenotypic analyses, have no adverse effects on human and animal health;”

“In accordance with the requirements of Articles 4 and 16 of Regulation (EC) No 1829/2003, the applicant shall ensure that the final risk characterisation clearly demonstrates that:

(a) the genetically modified food and feed has no adverse effects on human and animal health;”

Effects of residues from spraying with complementary herbicide specific to GE plants and their mixed toxicity

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 management practices in the cultivation of the herbicide-resistant plants, there are, for example, specific patterns of spraying, exposure, occurrence of specific metabolites and emergence of combinatorial effects that require special attention.

Isoxaflutole is classified as a “suspected human carcinogen”. In this case, specific residues from applications of isoxaflutole have to be expected (EFSA, 2016). Safety of the products cannot be demonstrated as long as the toxicity of these residues and their impact as co-stressors are not fully investigated.

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 a prerequisite for granting authorisation.

EU legal provisions such as Regulation 1829/2003 (and Implementing Regulation 503/2013) state that “*any risks which they present for human and animal health and, as the case may be, for the environment*” have to be avoided. Therefore, potential adverse effects resulting from combinatorial exposure of various potential stressors need to be tested for mixed toxicity (EFSA, 2019b).

HPPD enzymes are not only found in plants but in almost all living organisms, including microorganisms, where they are involved in the tyrosine degradation pathway (Moran, 2005). The *hppd* gene coding the targeted enzyme has been described in about 2000 bacterial species (Thiour-Mauprivez et al., 2020).

Therefore, the potential impact on the gut microbiome from chronic exposure to food and feed derived from GE plants resistant to HPPD inhibitors, should be considered a relevant issue for the risk assessment of the GE cotton since they may trigger significant changes in intestinal bacteria (see also Testbiotech, 2021).

The same is true for the resistance to glyphosate: glyphosate is known to cause shifts in the microbial composition and associated microbiomes of plants and animals, thus leading to a specific situation in regard to chronic exposure from food consumption. Glyphosate has indeed been shown to cause shifts not only in soil organisms (van Bruggen et al., 2018) but also in the composition of the intestinal flora of cattle (Reuter et al., 2007), poultry (Shehata et al., 2013; Ruuskanen et al., 2020) and rodents (Mao et al., 2018; Mesnage et al., 2021; Tang et al., 2020) as well as honey bees (Motta et al., 2020) and daphnia (Suppa et al., 2020). Therefore, antibiotic effects caused by chronic exposure to food and feed derived from glyphosate-resistant GE plants, including this GE cotton, are not unlikely to trigger significant changes in intestinal bacteria (see also Testbiotech, 2021).

In general, the microbiome can be seen as a common network of life, encompassing and closely interacting with plants, animals and humans. Microbial networks are thought to have co-evolved with their hosts and have developed a mutualistic relationship that benefits both the host and microorganisms. They act at the interphase and communicate between the organisms and their wider environment while at the same time being part of an organism’s closer environment. Microbiomes are considered to be vital for the health of higher organisms, i.e. human, animal and plants.

Just recently, a document published by EFSA (EFSA, 2020) called attention to the role of the microbiome in environmental risk assessment and food and feed safety. In regard to food and feed safety, EFSA (2020) considers microbiomes to be highly relevant to the health status of their hosts. Therefore, it is desirable to understand the importance of their role in risk assessment. EFSA expects that gut microbiome research (not only in the case of GE plants) will play a relevant role in regulatory science with potential implications for future risk assessments and predictive risk models. As EFSA states: *“considering that the gut microbiome is a biological component directly and indirectly involved in the metabolism of food/feed components and chemicals and in the protection of the host against adverse environmental exposure, it would be useful to establish criteria on how to evaluate the potential adverse impacts of perturbators on this defensive barrier, and consequently, on human/animal health.”*

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

These findings are highly relevant for the risk assessment of the GE cotton, which inherits combinations of herbicide resistance to glyphosate and HPPD inhibitors. Therefore, the plants may be expected to carry a higher burden and a specific pattern of the residues from spraying with the complementary herbicides in comparison to conventional plants. These residues may cause a perturbation of the gut microbiome. Further, it has to be considered a plausible hypothesis that the effects on the microbiome can trigger effects on the immune system. This hypothesis needs to be tested before any conclusion can be drawn on the health safety of food and feed.

However, no attempts have been made to integrate the microbiome into the risk assessment of food and feed derived from the GE cotton. This is in direct contradiction to Regulation 1829/2003 which requests *“genetically modified food and feed should only be authorised for placing on the Community market after a scientific evaluation of the highest possible standard, to be undertaken under the responsibility of the European Food Safety Authority (Authority), of any risks which they present for human and animal health and, as the case may be, for the environment.”* (Recital 9).

In conclusion, the EFSA opinion on the application for authorisation of the GE cotton (EFSA, 2021a) cannot be said to fulfill assessment requirements of EU GMO regulation.

Results from the subchronic feeding study

In light of the analysis provided above, we believe that a relevant hypothesis should be tested to assess immune system responses after chronic exposure from consumption of whole food and feed. However, this hypothesis is not covered by the design of the feeding study.

In conclusion, the subchronic feeding study does not appear to be adequate to demonstrate the safety of food and feed derived from the GE cotton.

Conclusions on toxicity

The safety of products derived from the GE cotton at the stage of consumption was not sufficiently demonstrated.

Others

It is known that GE cotton has been spreading within populations of wild cotton species in Mexico (Wegier et al., 2011). Resulting offspring are often transgenic and, consequently, produce insecticides or are resistant to the herbicide, glyphosate. A recently published paper (Vázquez-Barrios et al., 2021) has shown that there are disturbances in the interactions between the transgenic offspring and their environment. This finding has serious implications for the protection of wild cotton species because Mexico is one of the centres of origin for cotton.

Cotton plants naturally produce a kind of nectar on their surface. If plants are attacked by pest insects, they produce higher amount of the nectar. This attracts predatory ants which help to reduce the pest insects. The new study shows that the amount of nectar is significantly changed in the hybrid transgenic offspring.

Both types of transgenic offspring were examined: those that produce insecticides (Bt toxins) and those resistant to glyphosate. There were significant differences in comparison to the wild cotton plants: in the wild species, the amount of nectar and the composition of the ant population was changed after infestation with pest insects. However, these reactive changes were absent in the transgenic plants: instead, the amount of nectar was permanently reduced in the herbicide-resistant cotton and in the Bt plants it was permanently enhanced. Consequently, there were also changes in the ant populations: in general, there were more ants in wild cotton plants. There were also differences in the types of ant species: there was a reduction in ant species that are especially useful in combating pest insects in cotton with herbicide resistance, whereas it was higher in the Bt plants. Ants are important as a defence against pest insects and in the distribution of wild cotton seeds; therefore, the disturbance in the interactions between the cotton plants and their environment can have significant long-term impacts. The higher nectar production may enable the Bt cotton plants to become invasive. It could mean that wild cotton species may be replaced by transgenic plants, which would be a disaster for the centre of biodiversity of cotton species.

On the other hand, the herbicide-resistant cotton seems to show reduced fitness: in these plants, there was an increase in pest insect damage. However, findings from other researchers indicate that additional EPSPS enzymes produced in the transgenic plants to make them resistant to glyphosate, also unintentionally enhance plant growth and number of seeds (see for example Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; Beres et al., 2018, Beres, 2019). Consequently, the herbicide-resistant GE plants could acquire the characteristics of invasive plants. The researchers emphasised that the transgenes are currently spreading rapidly in natural populations, and some offspring even inherit combinations of several transgenes. Against this backdrop, recommended measures must be urgently taken to protect the centres of biodiversity much more efficiently.

In this context, as also pointed out by experts from Member States (EFSA, 2021b), the EU should consider the consequences of cultivation in the exporting countries, such as spread in the environment, adverse agronomic consequences in both the producing countries and third countries.

The European Union has ratified the Convention on Biological Diversity, therefore both the exporting and importing countries have international responsibilities regarding biological diversity.

Consequently, the import of GE cotton cannot be allowed if its cultivation may contribute to further damage in the centres of origin for wild cotton plants.

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