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# Assessment of genetically modified cotton GHB614 × LLCotton25 × MON 15985 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2011-94)

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## Abstract

The three-event stack cotton GHB614 × LLCotton25 × MON 15985 was produced by conventional crossing to combine three single cotton events, GHB614, LLCotton25 and MON 15985. The EFSA GMO Panel previously assessed the three single events and did not identify safety concerns. No new data on the single events that could lead to modification of the original conclusions on their safety were identified. Based on the molecular, agronomic, phenotypic and compositional characteristics, the combination of the single events and of the newly expressed proteins in the three-event stack cotton did not give rise to food and feed safety or nutritional issues. Food and feed derived from cotton GHB614 × LLCotton25 × MON 15985 are expected to have the same nutritional impact as those derived from the non-GM comparator. In the case of accidental release of viable GHB614 × LLCotton25 × MON 15985 cottonseeds into the environment, this three-event stack cotton would not raise environmental safety concerns. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of cotton GHB614 × LLCotton25 × MON 15985. In conclusion, the GMO Panel considers that cotton GHB614 × LLCotton25 × MON 15985, as described in this application, is as safe as the non-GM comparator with respect to potential effects on human and animal health and the environment.



**Keywords:** GMO, cotton GHB614 × LLCotton25 × MON 15985, Regulation (EC) 1829/2003, Cry1Ac, Cry2Ab2, GUS, NPTII, 2mEPSPS, PAT

**Requestor:** Competent Authority of the Netherlands

**Question number:** EFSA-Q-2011-00134

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## Summary

Following the submission of an application (EFSA-GMO-NL-2011-94) under Regulation (EC) No 1829/2003 from Bayer CropScience AG, the Panel on Genetically Modified Organisms of the European Food Safety Authority (GMO Panel) was asked to deliver a scientific opinion on the safety of insect-resistant and herbicide-tolerant genetically modified (GM) cotton GHB614 × LLCotton25 × MON 15985 (Unique Identifier BCS-GHØØ2-5 × ACS-GHØØ1-3 × MON-15985-7). The scope of the application EFSA-GMO-NL-2011-94 is for import, processing, and food and feed uses of cotton GHB614 × LLCotton25 × MON 15985 within the European Union (EU) but excludes cultivation in the EU.

In delivering its scientific opinion, the GMO Panel considered the data available on the single events, the information presented in application EFSA-GMO-NL-2011-94, additional information provided by the applicant, the scientific comments submitted by the Member States and relevant scientific publications. The three-event stack cotton GHB614 × LLCotton25 × MON 15985 was produced by conventional crossing to combine three single cotton events: GHB614, expressing the 2mEPSPS protein for tolerance to glyphosate-based herbicides; LLCotton25, expressing the PAT protein for tolerance to glufosinate-ammonium-based herbicides; and MON 15985, expressing the Cry1Ac and Cry2Ab2 proteins which confer resistance to certain lepidopteran pest and the NPTII and GUS proteins which were used as selectable markers.

The GMO Panel evaluated cotton GHB614 × LLCotton25 × MON 15985 with reference to the scope and appropriate principles described in its guidelines for the risk assessment of GM plants and derived food and feed, the environmental risk assessment of GM plants and the post-market environmental monitoring of GM plants. The GMO Panel Guidance Documents establish the principle that where all single events have been assessed, the risk assessment of stacked events should focus mainly on issues related to (a) stability of the inserts, (b) expression of the introduced genes and their products and (c) potential synergistic or antagonistic effects resulting from the combination of the events.

For application EFSA-GMO-NL-2011-94, the previous assessment of the three single events (GHB614, LLCotton25 and MON 15985) provided the basis to evaluate the three-event stack cotton. Cotton GHB614, LLCotton25 and MON 15985 were previously assessed by the GMO Panel and no safety concerns were identified. No safety issue was identified by updated bioinformatics analyses nor reported by the applicant for any the three single cotton events since the publication of the respective scientific opinions. Consequently, the GMO Panel considers that its previous conclusions on the safety of the single cotton events remain valid.

The risk assessment of the three-event stack cotton included the molecular characterisation of the inserted DNA and analysis of protein expression. An evaluation of the comparative analysis of compositional and agronomic/phenotypic characteristics was undertaken, and the safety of the newly expressed proteins and the whole food/feed was evaluated with respect to nutritional characteristics and potential toxicity and allergenicity. An evaluation of environmental impacts and post-market environmental monitoring plans was also carried out.

The molecular data establish that the events stacked in cotton GHB614 × LLCotton25 × MON 15985 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 single events. No indications of interactions that may affect the integrity of the events and the levels of the newly expressed proteins in this three-event stack cotton were identified.

The comparative analysis of cottonseed composition and agronomic/phenotypic characteristics identified no differences between cotton GHB614 × LLCotton25 × MON 15985 and the non-GM comparator that required further assessment for food/feed safety or environmental impact, except for increased levels of gossypol, dihydrosterculic acid and α-tocopherol in GM cottonseed.

Based on the molecular, agronomic, phenotypic and compositional characteristics, the combination of cotton events GHB614, LLCotton25 and × MON 15985 in the three-event stack cotton did not give rise to issues regarding food and feed safety and nutrition. The combination of the newly expressed proteins in the three-event stack cotton did not raise concerns for human and animal health. The toxicological and nutritional assessment identified no concern related to the increased levels of gossypol, dihydrosterculic acid and α-tocopherol. The nutritional impact of food and feed derived from cotton GHB614 × LLCotton25 × MON 15985 is not expected to differ from that of food and feed derived from the non-GM comparator.

Considering the combined events, the outcome of the comparative analysis, the routes of exposure and limited exposure levels, the GMO Panel concludes that this three-event stack cotton would not raise safety concerns in the case of accidental release of viable GM cottonseeds into the environment.

In conclusion, the GMO Panel considers that the information available for cotton GHB614 × LLCotton25 × MON 15985 addresses the scientific comments raised by Member States and that cotton GHB614 × LLCotton25 × MON 15985, as described in this application, is as safe as the non-GM comparator with respect to potential effects on human and animal health and the environment in the context of the scope of this application.

Given that no safety concerns were identified on food/feed derived from cotton GHB614 × LLCotton25 × MON 15985, the GMO Panel considers that post-market monitoring of these products is not necessary. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of cotton GHB614 × LLCotton25 × MON 15985.

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# 1. Introduction

## 1.1. Background

On 18 February 2011, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands application EFSA-GMO-NL-2011-94, for authorisation of genetically modified (GM) cotton GHB614 × LLCotton25 × MON 15985 for food and feed uses, import and processing submitted by Bayer CropScience AG within the framework of Regulation (EC) No 1829/2003 on genetically modified food and feed.[1,2]

After receiving the application EFSA-GMO-NL-2011-94 and in accordance with Articles 5(2)(b) and 17 (2)(b) of the Regulation (EC) No 1829/2003, EFSA informed the Member States and the European Commission, and made the summary of the application publicly available on the EFSA website.[3] EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of the Regulation (EC) No 1829/2003. On 16 June 2015, EFSA received additional information requested under the completeness check (requested on 20 April 2011). On 15 July 2015, EFSA declared the application as valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

EFSA made the valid application available to the Member States and the European Commission, and consulted nominated risk assessment bodies of Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC[4] following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1829/2003, to request their scientific opinion. Member State had three months after the opening of the Member State commenting period (until 4 July 2016) to make their opinion known.

The GMO Panel carried out a scientific risk assessment of the GM cotton GHB614 × LLCotton25 × MON 15985. On 24 July 2015, 7 April 2016, 12 May 2016, 29 September 2016 and 17 November 2017, the GMO Panel requested additional information from the applicant. The applicant provided the requested information on 22 October 2015, 6 July 2016, 16 August, 3 November 2017 and 2 February 2018, respectively. On 20 February 2018, the applicant provided supplementary information spontaneously. After receipt and assessment of the full data package, the GMO Panel finalised its risk assessment of cotton GHB614 × LLCotton25 × MON 15985.

In giving its scientific opinion to the European Commission, the Member States and the applicant, and in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003, EFSA has endeavoured to respect a time limit of 6 months from the acknowledgement of the valid application. As additional information was requested by the GMO Panel, the time limit of 6 months was extended accordingly, in line with Articles 6(1), 6(2), 18(1), and 18(2) of Regulation (EC) No 1829/2003.

According to Regulation (EC) No 1829/2003, this scientific opinion is to be seen as the report requested under Articles 6(6) and 18(6) of that Regulation and thus will be part of the EFSA overall opinion in accordance with Articles 6(5) and 18(5).

## 1.2. Terms of Reference as provided by the requestor

The GMO Panel was requested to carry out a scientific assessment of cotton GHB614 × LLCotton25 × MON 15985 (*Gossypium hirsutum*) for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003.

Where applicable, any conditions or restrictions which should be imposed on the placing on the market and/or specific conditions or restrictions for use and handling, including post-market monitoring requirements based on the outcome of the risk assessment and, in the case of GMOs or food/feed containing or consisting of GMOs, conditions for the protection of particular ecosystems/environments and/or geographical areas should be indicated in accordance with Articles 6(5)(e) and 18(5)(e) of Regulation (EC) No 1829/2003.

[1] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23.

[2] The original submission also included the two-event stack cotton LLCotton25 × MON15985. However, the two-event stack was subsequently removed by the applicant and is not included in the scope of this application.

[3] Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2011-00134

[4] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 12.3.2001, p. 1–38.

The GMO Panel was not requested to give an opinion on the information required under Annex II to the Cartagena Protocol. Furthermore, the GMO Panel did not consider proposals for labelling and methods of detection (including sampling and the identification of the specific transformation event in the food/feed and/or food/feed produced from it), which are matters related to risk management.

# 2. Data and methodologies

## 2.1. Data

In delivering its scientific opinion, the GMO Panel took into account application EFSA-GMO-NL-2011-94, additional information provided by the applicant, scientific comments submitted by the Member States and relevant scientific publications.

## 2.2. Methodologies

The GMO Panel carried out a scientific risk assessment of cotton GHB614 × LLCotton25 × MON 15985 for food and feed uses, in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. The GMO Panel took into account the appropriate principles described in its guidelines for the risk assessment of GM plants and derived food and feed (EFSA, 2006a, 2007; EFSA GMO Panel, 2011a), the environmental risk assessment (ERA) of GM plants (EFSA GMO Panel, 2010), and the post-market environmental monitoring (PMEM) of GM plants (EFSA GMO Panel, 2011b).

The EFSA guidance applicable to this application establishes that 'Where all single events have been assessed, the risk assessment of stacked events should focus mainly on issues related to (a) stability, (b) expression of the events and (c) potential interactions between the events' (EFSA, 2006a, 2007). Additional information received after May 2011 was assessed in accordance with 2011 guidance (EFSA GMO Panel, 2011a).

The comments raised by Member States are addressed in Annex G of EFSA's overall opinion and were taken into consideration during the scientific risk assessment.

# 3. Assessment

## 3.1. Introduction

Cotton GHB614 × LLCotton25 × MON 15985 was developed by conventional crossing of single lines GHB614 (expressing 2mEPSPS protein), LLCotton25 (expressing PAT protein) and MON 15985 (expressing Cry1Ac, Cry2Ab2, NPTII and GUS proteins) to confer resistance to certain lepidopteran pests and tolerance to glyphosate- and glufosinate-ammonium-based herbicides. Resistance to certain lepidopteran pests, such as the cotton bollworm larvae (*Helicoverpa zea*), tobacco budworm larvae (*Heliothis virescens*), pink bollworm (*Pectinophora gossypiella*) and beet armyworm larvae (*Spodoptera exigua*), is provided by the expression of the Cry1Ac and Cry2Ab2 proteins, which have an insecticidal effect on larvae of certain lepidopteran species. Tolerance to glyphosate is achieved by the expression of the 2m*epsps* gene (modified 5-enolpyruvylshikimate 3-phosphate synthase gene), and tolerance to glufosinate-ammonium-based herbicides is achieved by the expression of phosphinothricin acetyltranferase (PAT), an enzyme that acetylates L-glufosinate ammonium.

All three single events were assessed previously (see Table 1) and no concerns for human and animal health or environmental safety were identified.

**Table 1:** Single cotton events already assessed by the GMO Panel

| Event | Application | EFSA Scientific Opinion |
|---|---|---|
| GHB614 | EFSA-GMO-NL-2008-51 | EFSA (2009a) |
| LLCotton25 | EFSA-GMO-NL-2005-13 | EFSA (2006b) |
| MON 15985 | EFSA-GMO-UK-2008-57<br>EFSA-GMO-RX-MON15985 | EFSA GMO Panel (2014a) |

## 3.2. Updated information on single events

Since the publication of the scientific opinions on the single cotton events (see Table 1), no safety issue pertaining to any of the three single events has been reported by the applicant.

Updated bioinformatic analyses for events GHB614, LLCotton25[5] and MON 15985 confirmed that no known endogenous genes were disrupted by any of the inserts.[6]

Updated bioinformatic analyses of the amino acid sequence of the newly expressed Cry1Ac, Cry2Ab2, 2mEPSPS, NPTII, GUS and PAT proteins confirmed previous results indicating no significant similarities to known toxins and allergens.[6,7] Updated bioinformatics analyses of the newly created open reading frames (ORFs) within the inserts, or spanning the junctions between the inserts to identify ORFs with significant similarity to toxins or allergens not previously assessed, revealed that for event MON 15985 a single ORF exceeded the allergenicity assessment threshold of 35% identity using an 80 amino acid sliding window approach. This ORF is found within the transcriptional unit of the Cry2Ab2 coding sequence driven by the cauliflower mosaic virus 35S promoter, but it is in a different reading frame to the Cry2Ab2 ORF and does not contain any in-frame translational start codons (ATG). In conclusion, these analyses indicated that the expression of an ORF showing significant similarities to toxins or allergens for any of the events in cotton GHB614 × LLCotton25 × MON 15985 is highly unlikely.[6,7,8]

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis for events GHB614, LLCotton25 and MON 15985 to microbial DNA.[6] The likelihood and potential consequences of plant-to-bacteria gene transfer are described in Section 3.6.

Based on the above information, the GMO Panel considers that its previous conclusions on the safety of the single cotton events remain valid.

## 3.3. Molecular characterisation

Possible interactions that would affect the integrity of the events, the expression levels of the newly expressed proteins or the biological function conferred by the individual inserts are considered.

### 3.3.1. Genetic elements and biological functions of the inserts[9]

Cotton events GHB614, LLCotton25 and MON 15985 were combined by conventional crossing to produce the three-event stack cotton GHB614 × LLCotton25 × MON 15985. The structure of the inserts introduced into the three-event stack cotton is described in detail in the respective EFSA scientific opinions (Table 1) and no new genetic modifications were involved. Genetic elements in the expression cassettes of the single events are summarised in Table 2.

Intended effects of the inserts in cotton GHB614 × LLCotton25 × MON 15985 are summarised in Table 3.

Based on the known biological function of the newly expressed proteins (Table 3), the only foreseen interactions at the biological level are between the two Cry proteins in susceptible insects.

[5] The LLCotton25 event sequence used to perform the bioinformatic analyses was the sequence submitted in the original application EFSA-GMO-NL-2005-13 but corrected for sequencing errors (http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2017-00803).
[6] Additional information: 22/10/2015 and 2/2/2018.
[7] Additional information: 6/7/2016.
[8] Additional information: 20/2/2018.
[9] Dossier: Part I – Section C3.

**Table 2:** Genetic elements in the expression cassettes of the events stacked in cotton GHB614 × LLCotton25 × MON 15985

| Event | Promoter | 5′ UTR | Transit peptide | Coding region | Terminator |
|---|---|---|---|---|---|
| GHB614 | *Ph4a748At* (*Arabidopsis thaliana*)* | – | TPotpC (*Zea mays* and *Helianthus annuus*) | 2me*psps* (*Z. mays*) | *3′histon At* (*A. thaliana*) |
| LLCotton25 | P35S3 (CaMV) | – | No | *bar* (*Streptomyces hygroscopicus*) | *3′ nos* (*Agrobacterium tumefaciens*) |
| MON 15985 | e35S (CaMV) | – | No | *cry1Ac* (*Bacillus thuringiensis*) | *7S 3′* (*Glycine max*) |
| | 35S (CaMV) | – | No | *nptII* (Tn*7*) | *3′ nos* (*A. tumefaciens*) |
| | e35S (CaMV) | – | No | *uidA* (*Escherichia coli*) | *3′ nos* (*A. tumefaciens*) |
| | e35S (CaMV) | *hsp70* (*Petunia*) | CTP2 (*A. thaliana*) | *cry2Ab2* (*B. thuringiensis*) | *3′ nos* (*A. tumefaciens*) |

UTR: untranslated region; CaMV: cauliflower mosaic virus.
*: Source of genetic material.

Cotton event MON 15985 also contains a gene coding for an aminoglycoside adenylyltransferase (AAD) enzyme, which confers resistance to spectinomycin and streptomycin. This gene was only used as a selectable marker during the development of MON 15985. This gene is under the control of a prokaryotic promoter and it is therefore not expressed in parental line cotton MON 15985 (EFSA GMO Panel, 2011c, 2014a).

**Table 3:** Characteristics and intended effects of the events stacked in cotton GHB614 × LLCotton25 × MON 15985

| Event | Protein | Donor organism and biological function | Intended effects in GM plant |
|---|---|---|---|
| GHB614 | 2mEPSPS | Based on a gene from *Zea mays*. 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Lebrun et al., 2003) | The amino acid sequence of the maize EPSPS enzyme was modified by two substitutions to render it tolerant to glyphosate. Expression of 2mEPSPS confers tolerance to glyphosate-based herbicides |
| LLCotton25 | PAT | Based on a gene from *Streptomyces hygroscopicus*. Encoded by the *bar* gene, phosphinothricin-acetyl-transferase (PAT) confers resistance to the antibiotic bialaphos (Thompson et al., 1987) | The *bar* gene inserted in cotton LLcotton25 differs from the native by two N-terminal codons. Expression of PAT in cotton LLCotton25 confers tolerance to glufosinate-ammonium-based herbicides |

| Event | Protein | Donor organism and biological function | Intended effects in GM plant |
|---|---|---|---|
| MON 15985 | Cry1Ac | Based on genes from *Bacillus thuringiensis* subsp. *kurstaki* HD-73. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (*cry*) genes (Donovan et al., 1992) | Cotton MON 15985 expresses a synthetic *cry1Ac* gene which was modified to enhance its expression in plants. Cry1Ac is 99.4% identical to its native (differs by 7 amino acids). This protein is toxic to certain lepidopteran larvae feeding on cotton |
| | AAD | Bacterial gene comprising its own regulatory elements and coding for an aminoglycoside-modifying enzyme, 3′(9)-*O*-nucleotidyltransferase from the transposon Tn7 under a prokaryotic promoter (Flung et al., 1985) | The expression of the *aad* gene provides resistance to spectinomycin and streptomycin and was used as a selectable marker for plasmid maintenance during product development |
| | NPTII | Based on a gene from bacterial transposon Tn5. Neomycin phosphotransferase II (NPTII) inactivates by phosphorylation a range of antibiotics, including kanamycin and neomycin (Fraley et al., 1983; EFSA, 2009b) | The *nptII* gene inserted in cotton MON 15985 also contains 153 bp of the gene encoding the bleomycin binding protein. Expression of the *nptII* gene allowed selection of transformed plant cells with kanamycin in the development of MON 531, the parental line of MON 15985 |
| | GUS | Based on a gene from *Escherichia coli* strain K12. Encoded by the *uidA* gene, β-D-glucuronidase (GUS) catalyses the hydrolysis of a range of β-glucuronides (Gilissen et al., 1998) | Expression of the GUS protein in cotton MON 15985 was used as a histochemical marker during product development |
| | Cry2Ab2 | Based on genes from *B. thuringiensis* subsp. *kurstaki*. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (*cry*) genes (Widner and Whiteley, 1990) | The amino acid sequence of the Cry2Ab2 protein expressed in MON 15985 is 88% identical to the Cry2Aa protein produced by the *B. thuringiensis* subsp. *kurstaki*. Cotton 15985 expresses the synthetic Cry2Ab2, which is toxic to certain lepidopteran larvae feeding on cotton |

### 3.3.2. Integrity of the events in the three-event stack[10]

The genetic stability of the inserted DNA over multiple generations in the single cotton events GHB614, LLCotton25 and MON 15985 was demonstrated previously (see Table 1). Integrity of these events in cotton GHB614 × LLCotton25 × MON 15985 was demonstrated by Southern analyses.

### 3.3.3. Information on the expression of the inserts[7,11]

Cry1Ac, Cry2Ab2, 2mEPSPS, PAT, NPTII and GUS protein levels were analysed by enzyme-linked immunosorbent assay (ELISA) in material harvested from replicated field trials at three locations in the USA in 2014. Samples analysed included leaf (4–6 leaf, square initiation and 2 weeks after flowering), root (square initiation), pollen (flowering) and fuzzy seed (maturity) both those treated and not treated with glyphosate and glufosinate. Since cottonseeds are the main raw commodities used for food and feed purposes, protein levels in cottonseeds from GHB614 × LLCotton25 × MON 15985 (the highest mean values, regardless the treatment) are summarised in Table 4.

[10] Dossier: Part I – Section D2; additional information: 16/8/2016 and 3/11/2017.
[11] Dossier: Part I – Section D3.

**Table 4:** Highest mean values, standard deviation and ranges of protein levels (μg/g dry weight) in seed (n = 15) from cotton GHB614 × LLCotton25 × MON 15985 treated with glyphosate and glufosinate

| Tissue/ developmental stage | Cry1Ac | Cry2Ab2 | 2mEPSPS | PAT | GUS | NPTII |
|---|---|---|---|---|---|---|
| **Seed/ maturity** | 11.35[(a)] ± 1.13[(b)] (9.92–13.22)[(c)] | 171.21 ± 24.03 (136.07–215.60) | 105.70 ± 12.79 (81.48–127.63) | 161.02 ± 19.35 (122.48–193.10) | 32.06 ± 4.20 (21.77–39.52) | 6.60 ± 0.91 (5.19–8.34) |

Cry: crystal protein; EPSPS: 5-enolpyruvyl-shikimate-3-phosphate synthase; PAT: phosphinothricin acetyltransferase; GUS: β-D-glucuronidase; NPTII: neomycin phosphotransferase II.
(a): Mean.
(b): Standard deviation.
(c): Range.

In order to assess changes in protein expression levels which may result from potential interactions between the events, protein levels were determined for the three-event cotton stack and the corresponding single events in different parts of the plant.

The levels of all the newly expressed proteins in the three-event cotton stack and the corresponding singles were similar in all tissues (Appendix A). Therefore, there is no indication of an interaction that may affect the levels of the newly expressed proteins in this stack.

#### 3.3.4. Conclusion

The molecular data establish that the events stacked in cotton GHB614 × LLCotton25 × MON 15985 have retained their integrity. Protein expression analyses showed that the levels of the newly expressed proteins are similar in the three-event stack and in the single events. Therefore, there is no indication of an interaction that may affect the integrity of the events and the levels of the newly expressed proteins in this stack.

Based on the known biological function of the newly expressed proteins (Table 3), the only foreseen interactions at the biological level are between the two Cry proteins, which will be dealt with in Sections 3.5 and 3.6.

### 3.4. Comparative analysis[12]

#### 3.4.1. Choice of comparator and production of material for the comparative assessment[13]

Application EFSA-GMO-NL-2011-94 includes data on agronomic and phenotypic characteristics of cotton and compositional data on ginned cottonseeds (fuzzy seeds). The data were collected from field trials performed in the USA. The field trials included the three-event stack cotton GHB614 × LLCotton25 × MON 15985, the non-GM comparator FiberMax958 (FM958) and the three single events GHB614, LLCotton25 and MON 15985. The non-GM comparator FM958 had a similar genetic background to the three-event stack and was considered an appropriate comparator by the GMO Panel.

The field trials were conducted during the 2008 growing season in seven locations in the southern-eastern USA[14] in typical cotton-growing regions.[15] At each site, a randomised complete block design was used, with three replications of the test materials. All the materials received the same (site-specific) maintenance treatments to ensure good plant health conditions; in what follows, 'untreated' is used to refer to materials treated only with conventional herbicides. The test materials were: cotton GHB614 × LLCotton25 × MON 15985 (untreated), cotton GHB614 × LLCotton25 × MON 15985 (treated with the target herbicides[16]), the non-GM comparator FM958 (untreated) and the three single

[12] Dossier: Part I – Section D7.1.
[13] Dossier: Part I – Section D7.2.
[14] Tift, GA; Jackson, AR; Crittenden, AR; Tate, MS; St. Landry, LA; Wharton, TX; and Hockle, TX.
[15] Dossier: Part I – Section D7.1 (study DQ08B001).
[16] The target herbicides were: a combination of glyphosate-based and glufosinate ammonium-based herbicides for the three-event stack cotton GHB614 × LLCotton25 × MON 15985; glyphosate-based herbicides for the single event GHB614; and glufosinate ammonium-based herbicides for the single event LLCotton25.

events: GHB614 (untreated), GHB614 (treated with the target herbicides), LLCotton25 (untreated), LLCotton25 (treated with the target herbicides) and MON 15985 (untreated).

The GMO Panel took into account the full data set provided, including the results for the three single events. In the following sections, only the results for the three-event stack and the non-GM comparator are discussed.

### 3.4.2. Agronomic and phenotypic analysis[17]

#### 3.4.2.1. Agronomic and phenotypic characteristics tested under field conditions

In total, 29 agronomic and phenotypic endpoints were measured, related to plant growth and morphology at different life stages, reproduction, agricultural productivity, fibre properties and disease susceptibility.[18]

In order to test for differences between the three-event stack cotton and the non-GM comparator, an analysis of variance (ANOVA) was applied across the field trial sites.[19]

Significant differences were observed between cotton GHB614 × LLCotton25 × MON 15985 (untreated) and the non-GM comparator for the following endpoints: boll type and percentage lint, all the fibre properties, number of bolls at first position and parameters related to lepidopteran susceptibility (Table 5).

Cotton GHB614 × LLCotton25 × MON 15985 (treated with the target herbicides) showed statistically significant differences from the non-GM comparator for the following endpoints: percentage open bolls, boll size, micronaire, fibre length uniformity, fibre strength, fibre elongation, number of bolls at first position, total number of bolls and parameters related to lepidopteran susceptibility (Table 5).

The significant differences are further assessed for their potential environmental impact in Section 3.6.

**Table 5:** Agronomic and phenotypic endpoints for which statistically significant differences were observed between cotton GHB614 × LLCotton25 × MON 15985 (treated or not treated with the intended herbicides) and the non-GM comparator FM958

| Endpoint | Comparator FM958 | Cotton GHB614 × LLCotton25 × MON 15985 | |
|---|---|---|---|
| | | Untreated[(a)] | Treated[(b)] |
| Boll type (rating 0–9) | 5.5 | 5.7* | 5.6 |
| % open bolls | 49.5 | 46.4 | 46.2* |
| % lint | 40.5 | 39.2* | 39.9 |
| Boll size (g) | 5.77 | 5.70 | 5.52* |
| Micronaire (mic units) | 4.20 | 3.90* | 3.80* |
| Fibre length (inches) [(c)] | 1.19 | 1.17* | 1.17 |
| Fibre length uniformity (%) | 85.3 | 84.0* | 84.4* |
| Fibre strength (g/tex) | 33.4 | 31.9* | 31.6* |
| Fibre elongation (%) | 4.60 | 4.90* | 4.90* |
| Number of bolls at first position (N/plant) | 4.70 | 5.60* | 5.70* |
| Total number of bolls (N/plant) | 10.60 | 11.50 | 12.30* |
| Larvae in squares | 2.2 | 0.4* | 0.2* |
| Damaged squares | 7.2 | 0.8* | 0.3* |
| Larvae in flowers | 2.8 | 1.2* | 1.1* |
| Damaged flowers | 3.6 | 1.2* | 0.1* |

[17] Dossier: Part I – Section D7.4.

[18] Agronomic parameters: plant stand, strain uniformity, lodging, number of days to first flower, number of days to first open boll, boll type, % open bolls, yield, % lint, no. of seeds per boll, boll size and seed index. Fibre properties: length, length uniformity, strength, micronaire and elongation. Plant mapping: plant height, number of nodes, first position bolls and total number of bolls. Pest reaction: larvae in squares, damage square, larvae in flowers, damaged flowers, larvae in bolls and damaged bolls.

[19] A linear mixed model was used, where genotype (each combination of material and herbicide treatment) was the fixed effect, and the random effects were location, block-within-location and location-by-genotype.

| Endpoint | Comparator FM958 | Cotton GHB614 × LLCotton25 × MON 15985 | |
|---|---|---|---|
| | | Untreated[(a)] | Treated[(b)] |
| Larvae in bolls | 1.6 | 0.0* | 0.0* |
| Damaged bolls | 3.4 | 0.2* | 0.1* |

The values shown are estimated means. Significantly different entries for the GM cotton are marked with an asterisk.
(a): Cotton GHB614 × LLCotton25 × MON 15985 treated only with conventional herbicides.
(b): Cotton GHB614 × LLCotton25 × MON 15985 treated with glyphosate- and glufosinate-ammonium-based herbicides.
(c): The p-value for the untreated test material was: 0.034; the p-value for the treated material was: 0.059.

#### 3.4.2.2. Agronomic and phenotypic characteristics tested under controlled conditions

*Pollen characteristics*[20]

Pollen germinability and viability for cotton GHB614 × LLCotton25 × MON 15985, the non-GM comparator (FM958) and the three single cotton events were measured according to Barrow (1981). The pollen was obtained from plants grown under greenhouse conditions in 2009. The applicant observed no statistically significant differences between cotton GHB614 × LLCotton25 × MON 15985, the non-GM comparator and the single cotton events for pollen germinability and viability.

While Barrow's test provides an indication of germination capacity, it does not measure directly pollen germinability and viability (Barrow, 1981; Burke et al., 2004). Therefore, the data on pollen viability supplied by the applicant in support of the comparative assessment were not considered suitable by the GMO Panel. Given that the genetic modification of cotton GHB614 × LLCotton25 × MON 15985 is not designed to target specifically pollen germinability and viability, and that the scope of application EFSA-GMO-NL-2011-94 excludes cultivation, the GMO Panel considers that data on pollen germinability and viability are not required for the risk assessment of cotton GHB614 × LLCotton25 × MON 15985.

### 3.4.3. Compositional analysis[21]

Comparative compositional analysis was performed on fuzzy cottonseed samples obtained from cotton GHB614 × LLCotton25 × MON 15985 and the non-GM comparator FM958 grown in the USA field trials in 2008.

Samples were analysed for 66 compositional parameters, selected in line with OECD recommendations (OECD, 2009). Eighteen fatty acids had most of the sample values below the limit of quantification (LOQ) and were not analysed statistically.[22] The data for the remaining 48 compounds[23] were analysed with ANOVA, in order to test for differences between the three-event stack cotton and the non-GM comparator.[16]

Significant differences in fuzzy cottonseed (with maintenance management treatment only; 'Untreated', Table 6) were identified for 10 endpoints. Significant differences in fuzzy cottonseed (treated with the target herbicides; 'Treated', Table 6) were identified for 14 endpoints.[24]

[20] Dossier: Part I – Sections D4 and D7.4 (report DQ09Q003).
[21] Dossier: Part I – Section D7.3.
[22] The fatty acids were: caprylic (C8:0), capric (C10:0), lauric (C12:0), myristoleic (C14:1), pentadecanoic (C15:0), pentadecenoic (C15:1), heptadecenoic (C17:1), γ-linolenic (C18:3), octadecatetraenoic (C18:4), eicosenoic (C20:1), eicosadienoic (C20:2), eicosatrienoic (C20:3), arachidonic (C20:4), eicosapentaenoic (C20:5), erucic (C22:1), docosapentaenoic (C22:5) and docosahexaenoic (C22:6).
[23] Ash, total fat, moisture, total protein, carbohydrate, acid detergent fibre (ADF), neutral detergent fibre (NDF), calcium, iron, magnesium, phosphorus, potassium, zinc, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine, tryptophan, myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), behenic acid (C22:0), lignoceric acid (C24:0), α-tocopherol, sterculic acid, malvalic acid, dihydrosterculic acid, phytic acid, free gossypol and total gossypol.
[24] A significant difference between the GM cotton and the non-GM comparator was also identified for lignoceric acid (C24:0). However, lignoceric acid (C24:0) had many analytical results below the LOQ (50 out of 126 samples) and the statistical results for this compound were not considered by the EFSA GMO Panel.

**Table 6:** Compositional endpoints in fuzzy cottonseeds (from USA 2008 field trials) for which statistically significant differences were identified between the three-event stack cotton (treated or not treated with the intended herbicides) and the non-GM comparator

| Component | Comparator FM958 | Cotton GHB614 × LLCotton25 × MON 15985 | |
|---|---|---|---|
| | | Untreated[(a)] | Treated[(b)] |
| Total fat (% DM) | 20.0 | 19.1* | 19.0* |
| Carbohydrate (% DM) | 55.1 | 56.1* | 55.9 |
| Calcium (% DM) | 0.131 | 0.153* | 0.155* |
| Phosphorus (% DM) | 0.63 | 0.60* | 0.60* |
| Magnesium (% DM) | 0.37 | 0.36* | 0.36* |
| Iron (mg/kg) | 54.1 | 47.0* | 47.5* |
| α-Tocopherol (mg/kg) | 109.8 | 130.2* | 134.1* |
| Myristic acid (C14:0) (% FA) | 0.77 | 0.76 | 0.68* |
| Palmitoleic acid (C16:1) (% FA) | 0.55 | 0.53 | 0.50* |
| Linoleic acid (C18:2) (% FA) | 54.47 | 54.07 | 56.22* |
| Arachidic acid (C20:0) (% FA) | 0.30 | 0.30 | 0.28* |
| Behenic acid (C22:0) (% FA) | 0.19 | 0.19 | 0.18* |
| Free gossypol[(c)] (mg/kg DM) | 5,400 | 6,500* | 6,400* |
| Total gossypol (mg/kg DM) | 6,000 | 7,200* | 7,200* |
| Dihydrosterculic acid (% FA) | 0.25 | 0.27* | 0.26* |

DM: dry matter; FA: total fatty acids.
The values shown are estimated means. Significantly different entries for the GM cotton are marked with an asterisk.
(a): Cotton GHB614 × LLCotton25 × MON 15985 treated only with conventional herbicides.
(b): Cotton GHB614 × LLCotton25 × MON 15985 treated with glyphosate- and glufosinate-ammonium-based herbicides.
(c): The EFSA GMO Panel noted that the free gossypol content in raw cottonseeds of cotton GHB614 × LLCotton25 × MON 15985 and its comparator was higher than the limits set in Directive 2002/32 EC (5,000 mg/kg as fed) on undesirable substances in feed materials.

#### 3.4.4. Conclusion

The GMO Panel concluded that the differences identified in the agronomic and phenotypic characteristics between cotton GHB614 × LLCotton25 × MON 15985 and the non-GM comparator do not require further assessment regarding food and feed safety. The differences are further assessed for their potential environmental impact in Section 3.6.

The GMO Panel assessed all significant compositional differences between cotton GHB614 × LLCotton25 × MON 15985 and the non-GM comparator, taking into account the potential impact on plant metabolism and the variability reported in the OECD consensus document (OECD, 2009) and published literature (e.g. Berberich et al., 1996). The GMO Panel did not identify any need for further food/feed safety assessment except for gossypol, dihydrosterculic acid and α-tocopherol (see Section 3.5).

### 3.5. Food and feed safety assessment

#### 3.5.1. Effect of processing[25]

Cotton GHB614 × LLCotton25 × MON 15985 will undergo existing production processes used for conventional cotton. No novel production process is envisaged.

#### 3.5.2. Toxicology[26]

##### 3.5.2.1. Toxicological assessment of newly expressed proteins

Six proteins (PAT, 2mEPSPS, GUS, NPTII, Cry1Ac and Cry2Ab2) are newly expressed in various tissues of cotton GHB614 × LLCotton25 × MON 15985. The GMO Panel has previously assessed these proteins individually in the context of the single events (Table 2), and no safety concerns were identified for humans and animals (Section 3.2).

[25] Dossier: Part I – Section D7.6.
[26] Dossier: Part I – Section D7.8.

The potential for a functional interaction between the proteins newly expressed in cotton GHB614 × LLCotton25 × MON 15985 was assessed with regard to human and animal health. The four enzymatic proteins (PAT, 2mEPSPS, GUS and NPTII) catalyse distinct biochemical reactions and act on unrelated substrates. The two insecticidal proteins (Cry1Ac and Cry2Ab2) act through cellular receptors found in target insect species and it is reported that the gastrointestinal tract of mammals, including humans, lacks receptors with high affinity specific to Cry proteins (Hammond et al., 2013; Koch et al., 2015).

On the basis of the known biological function of the newly expressed proteins (Table 3), there is currently no expectation for possible interactions relevant to the food and feed safety assessment of the three-event stack cotton. Similar conclusions on possible interactions between PAT and 2mEPSPS were drawn by the GMO Panel on the two-event stack cotton GHB614 × LLCotton25 (EFSA-GMO-NL-2010-77; EFSA GMO Panel, 2014b).

The GMO Panel concludes that there are no safety concerns to human and animal health related to the newly expressed proteins PAT, 2mEPSPS, GUS, NPTII, Cry1Ac and Cry2Ab2 in cotton GHB614 × LLCotton25 × MON 15985.

#### 3.5.2.2. Toxicological assessment of components other than newly expressed proteins

This section focuses on the safety assessment of the higher levels of gossypol, dihydrosterculic acid and α-tocopherol observed in cotton GHB614 × LLCotton25 × MON 15985 when compared to the non-GM comparator (see Section 3.4.3).

Gossypol is a terpenoid phytoalexin with toxic effects mainly on the male genital system of humans and animals (EFSA, 2008; OECD, 2009). The EFSA Panel on Contaminants in the Food Chain described gossypol as undesirable substance in animal feed (EFSA, 2008). Current EU feed legislation has introduced limits to the maximum content of free gossypol for feed materials and complete feeds.[19] These limits are not allowed to be exceeded in order to protect target species and consumers of animal products derived from animals fed gossypol-containing feed. Direct human consumption of cottonseed products is predominantly via the refined oil which is essentially free from gossypol (0–0.09% DW total gossypol, OECD, 2009). An alternative source of consumption of cottonseed products may arise from the use of flour prepared from seeds. However, the source of such flour is cottonseeds from gland-free varieties, which do not produce gossypol (EFSA, 2008). Having considered the information on gossypol toxicity in animals and humans, the effect of processing on the gossypol content of cottonseed feed materials and the use of certain cottonseed materials as food or feed, the GMO Panel concludes that the higher content of gossypol in cottonseed of GHB614 × LLCotton25 × MON 15985 (both treated and untreated with the intended herbicides) as compared to the non-GM comparator is of no safety concern for animals and humans in practice because: (i) the maximum content of free gossypol in feed is regulated by European legislation[19]; and (ii) bleached and refined cottonseed oil as well as flour produced from cottonseed, which may be directly consumed by humans, are essentially free from gossypol.

Dihydrosterculic acid belongs to the category of cyclopropenoid fatty acids (CPFA) described as antinutrients in cottonseeds (OECD, 2009). CPFA inhibit the desaturation of saturated fatty acids and have been associated with detrimental effects in animals (OECD, 2009). The minimal increase in the level of dihydrosterculic acid in cotton GHB614 × LLCotton25 × MON 15985 as compared to the non-GM comparator is considered of no toxicological concern by the GMO Panel.

The toxicity of α-tocopherol has been investigated in animals and in clinical studies, with clotting time prolongation identified as the main critical adverse effect (no-observed-adverse-effect-level (NOAEL): 125 mg/kg body weight (bw) per day in the rat). EFSA (EFSA NDA Panel, 2015) set a tolerable upper intake level (UL) for humans at 300 mg α-tocopherol/day for adults, pregnant and lactating women; and, for children, 100 mg α-tocopherol equivalent (TE)/day (1–3 years) to 260 mg α-TE/day (15–17 years). The minimal increase in α-tocopherol observed in cotton GHB614 × LLCotton25 × MON 15985 as compared to the non-GM comparator is considered of no toxicological concern by the GMO Panel.

The GMO Panel concludes that there are no toxicological concerns as regards the changes in the levels of gossypol, dihydrosterculic acid and α-tocopherol observed in GHB614 × LLCotton25 × MON 15985 as compared to the non-GM comparator.

### 3.5.3. Testing of the whole genetically modified food and feed

No compositional modifications relevant for safety or nutrition are expected in food and feed derived from cotton GHB614 × LLCotton25 × MON 15985 (Sections 3.4, 3.5.2 and 3.5.5). There was no indication of interactions relevant for food/feed safety (Sections 3.3 and 3.4). Therefore, animal feeding studies were not considered necessary (EFSA, 2006a; EFSA GMO Panel, 2011a).

However, a 42-day broiler study[27] with animals fed diets including toasted cottonseed meals from cotton GHB614 × LLCotton25 × MON 15985 was provided and evaluated by the GMO Panel.

A total of 420 (210/sex) one-day old chicken broilers (Ross 308) were randomly allocated to three dietary groups with 140 broilers per treatment (7 pens/treatment per gender, 10 birds/pen) and fed balanced diets containing up to 10% toasted cottonseed meals from cotton GHB614 × LLCotton25 × MON 15985,[28] the non-GM comparator (FM958) or a non-GM commercial cotton variety (FM966). Diets (as crumble pellets or pellets) and water were offered *ad libitum*. Overall mortality was high (approximately 10%) with no statistically significant difference between the GM group and the two non-GM groups, in either male or female chickens. During the study, a total of 118 out of 420 birds (almost 30%) exhibited clinical signs unrelated to treatment. There were no statistically significant differences between treatment groups in male or female body weight by period or total body weight gain; in weekly mean feed consumption, total mean feed consumption or feed conversion ratio; in chilled carcass, fat pad, leg, thigh, wing or breast weights. A subset of 126 birds processed for carcass and tissue weights were also examined for gross pathology at study termination and only four birds[29] exhibited abnormal conditions, considered unrelated to treatment.

The GMO Panel is able to draw only limited conclusions from this study, because of the high mortality and the number of clinical signs observed. However, the measured performance endpoints were similar in groups fed balanced diets containing 10% of GM and non-GM cottonseed meals.

### 3.5.4. Allergenicity

For allergenicity assessment, a weight-of-evidence approach is followed, taking into account all information on the newly expressed proteins as no single piece of information or experimental method yields sufficient evidence to predict allergenicity (EFSA, 2006a; Codex Alimentarius, 2009; EFSA GMO Panel, 2011a). In addition, when known functional aspects of the newly expressed protein or structural similarity to known adjuvants may indicate an adjuvant activity, the possible role of these proteins as adjuvants is considered. When newly expressed proteins with a potential adjuvant activity are expressed together, possible interactions increasing adjuvanticity and impacting the allergenicity of the GM crop are assessed.

#### 3.5.4.1. Assessment of allergenicity of the newly expressed proteins[30]

The GMO Panel has previously evaluated the safety of the PAT/*bar*, 2mEPSPS, GUS, NPTII, Cry1Ac and Cry2Ab2 proteins, and no concerns for allergenicity were identified (Table 1). No new information on the allergenicity of these proteins that might change the previous conclusions of the GMO Panel in the context of the GM events assessed has become available. Based on current knowledge, and as none of the newly expressed proteins showed allergenicity, no reasons of concern regarding the simultaneous presence of these newly expressed proteins in the three-event stack cotton affecting their allergenicity were identified.

For adjuvanticity, proteins derived from *Bacillus thuringiensis* (Bt proteins) have been suggested to possess adjuvant activity, based on animal studies on Cry1Ac when applied at relatively high doses (e.g. Vazquez et al., 1999). The GMO Panel has previously evaluated the safety of the Cry1Ac and Cry2Ab2 proteins in the context of applications EFSA-GMO-UK-2008-57 and EFSA-GMO-RX-MON15985 and no concerns on adjuvanticity were identified (EFSA GMO Panel, 2014a). The levels of Bt proteins in the three-event stack cotton are similar to those in the respective single events (Section 3.3.2). From the limited experimental evidence available, the GMO Panel did not find indications that the simultaneous presence of the Bt proteins at the levels expressed in this three-event stack cotton might act as adjuvants with the potential to enhance a specific immunoglobulin E (IgE) response and to favour the development of an allergic reaction.

[27] Dossier: Part I – Section D7.8 (study 13798.4125).
[28] Sprayed once with glufosinate-ammonium-based herbicides and twice with glyphosate.
[29] Two in the non-GM commercial variety group, one in the non-GM comparator group and one in the GM group.
[30] Dossier: Part I – Section D7.9; additional information: 3/11/2017.

From a broader perspective, the effects on the immune system of Cry proteins, in particular Cry1Ac and Cry1Ab, have been investigated (e.g. Guerrero et al., 2004; Adel-Patient et al., 2011) and the GMO Panel identified no indications of safety concern in the context of the applications assessed (EFSA GMO Panel, 2012, 2016a,b). A recent study by Torres-Martínez et al. (2016) involving the Cry1Ac protein was assessed by the GMO Panel.[31] No reasons for concern were identified in the context of this application.

#### 3.5.4.2. Assessment of allergenicity of the whole GM plant[32]

The GMO Panel regularly reviews the available publications on food allergy to cottonseed-derived products. However, to date, cotton has not been considered a common allergenic food[33] (OECD, 2009). Therefore, the GMO Panel did not request experimental data to analyse the allergen repertoire of GM cotton.

#### 3.5.4.3. Conclusion

In the context of this application, and considering the data from the molecular characterisation, the compositional analysis and the assessment of the newly expressed proteins (see Sections 3.3, 3.4.3 and 3.5.2), the GMO Panel found no reasons of concern regarding the allergenicity of food and feed derived from cotton GHB614 × LLCotton25 × MON 15985 with respect to that of food and feed derived from the non-GM comparator.

### 3.5.5. Nutritional assessment of GM food/feed

The intended traits of cotton GHB614 × LLCotton25 × MON 15985 are herbicide tolerance and insect resistance, with no intention to alter the nutritional parameters. However, gossypol, dihydrosterculic acid and α-tocopherol levels were significantly different from those in the non-GM comparator (see Section 3.4). The biological role of these compounds, their levels in cottonseed and the magnitude and direction of the observed changes were considered during the nutritional assessment.

#### 3.5.5.1. Human nutrition

Oil is the most important cottonseed-derived product used for human consumption. In Europe, the consumption of cottonseed oil as such is relatively limited as reflected by the lack of consumption data reported in the EFSA Comprehensive Food Consumption Database (EFSA, 2011). However, cottonseed oil can also be used as an ingredient in the production of a wide variety of food products such as dressings, mayonnaise, fine bakery wares, chocolate spreads and chips.

Dihydrosterculic acid (cyclopropaneoctanoic acid, 8-(2-octylcyclopropyl)octanoic acid) is one of a group of cyclopropane fatty acids (CPA) that together with cyclopropene fatty acids (CPE) occur infrequently in most plants. Dihydrosterculic acid makes up 0.2–0.4% of total fatty acids in cottonseed (Xiao-Hong et al., 2011). CPAs are derived from unsaturated fatty acids, e.g. oleic acid, by cyclopropanation. No information is available about effects in humans. A very small increase (~5%) was observed in the GM cotton as compared to the non-GM comparator. However, CPAs will be destroyed during refining and hydrogenation of vegetable oils; therefore, changes in levels of dihydrosterculic acid

[31] Torres-Martínez et al. (2016) studied the macrophage activation and the signal transduction pathways by Cry1Ac proteins using a specific cell line to elucidate the molecular basis underlying its immunostimulatory mechanisms. The authors concluded that the Cry1Ac protein tested induced macrophage activation and overproduction of proinflammatory cytokines in the specific cellular system used. The information collected in this model system was considered as a first step which should be further studied using other cell types of the intestinal mucosa for instance, where relevant exposure to the tested protein could occur. Additional aspects to investigate included: relevance of the dose, nature of the protein tested (toxin vs protoxin, crystalline vs soluble), immunisation route and target cells. The authors highlighted the need for investigating such aspects in future studies to better understand: (i) how Cry1Ac protein is recognised by mammalian cells; and (ii) other signalling pathways that might be implicated in the effects induced by this protein, including the cytokine profile. The GMO Panel agrees with the authors that, to elucidate these aspects, additional studies on the immunogenicity of Cry1Ac proteins would be useful. In the context of the risk assessment of GM plants containing Cry1Ac protein assessed by the GMO Panel, the study by Torres-Martínez et al. (2016) does not put forward new elements that would invalidate the previous conclusions made by the GMO Panel (EFSA GMO Panel, 2014a).

[32] Dossier: Part I – Section D7.9.

[33] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

in cotton GHB614 × LLCotton25 × MON 15985 are considered to be of no concern as regards human nutrition.

As already commented in the toxicology section (Section 3.5.2), bleached and refined cottonseed oil does not contain detectable levels of free gossypol; a similar situation occurs for flours produced from cottonseed. Therefore, the changes in levels of gossypol observed in cotton GHB614 × LLCotton25 × MON 15985 are considered to be of no concern as regards human nutrition.

α-Tocopherol (vitamin E) is an important micronutrient and antioxidant that prolongs the shelf life of the oil and food products containing the oil (OECD, 2009). Considering the relatively minor consumption of cottonseed oil and its negligible contribution to the total intake of vitamin E (EFSA NDA Panel, 2015), the observed increase of α-tocopherol in cotton GHB614 × LLCotton25 × MON 15985 is considered to be of no concern as regards human nutrition.

#### 3.5.5.2 Animal Nutrition

Cotton can be fed to animals, mainly as cottonseed cake/meal or as full fat cottonseeds especially in ruminants. Current EU feed legislation has introduced limits to the maximum contents of free gossypol for feed materials and complete feeds.[34] The GMO Panel considers that the changes observed in the concentration of α-tocopherol (vitamin E) in GHB614 × LLCotton25 × MON 15985 cottonseeds are not of concern for animal nutrition, since animal complete feeds are balanced with vitamins. Based on the current knowledge available on the metabolic activity of dihydrosterculic acid on animals (Phelps et al., 1965; Page et al., 1997), the minimal increase (~ 5%) observed in GHB614 × LLCotton25 × MON 15985 cottonseeds is not expected to raise health concerns.

### 3.5.6. Conclusion

In the context of this application, the GMO Panel considers that the newly expressed proteins do not raise safety concerns for human and animal health in cotton GHB614 × LLCotton25 × MON 15985. No adverse effects for humans and animals resulting from interactions between these proteins are expected based on their known mode of action. The GMO Panel did not identify safety concerns regarding allergenicity or adjuvanticity resulting from interactions between the newly expressed proteins or regarding the overall allergenicity of the three-event stack cotton. The GMO Panel considered that the compositional differences observed between cotton GHB614 × LLCotton25 × MON 15985 and the non-GM comparator do not raise toxicological concerns. Likewise, the nutritional impact of food and feed derived from cotton GHB614 × LLCotton25 × MON 15985 is not expected to differ from that of food and feed derived from the non-GM comparator.

## 3.6. Environmental risk assessment[35]

Considering the scope of application EFSA-GMO-NL-2011-94, which excludes cultivation, the ERA of cotton GHB614 × LLCotton25 × MON 15985 mainly takes into account: (1) the exposure of bacteria to recombinant DNA in the gastrointestinal tract of animals fed GM material and bacteria present in environments exposed to faecal material of these animals (manure and faeces); and (2) the accidental release into the environment of viable GHB614 × LLCotton25 × MON 15985 cottonseeds during transportation and/or processing (EFSA GMO Panel, 2010).

### 3.6.1. Persistence and invasiveness of the GM plant[36]

In southern Europe, *Gossypium herbaceum* and *G. hirsutum* have been grown since the 19th century, and led to transient or locally naturalised cotton plants in the same area (Davis, 1967; Tutin et al., 1992; Sarno et al., 1993; Celesti-Grapow et al., 2010). However, survival of cottonseeds outside cultivation areas in Europe is limited due to the absence of a seed dormancy phase. Even if seeds from spillage germinate, the resulting cotton plants are unlikely to survive because of factors such as cold climatic conditions, susceptibility to diseases and low competitiveness (Eastick and Hearnden, 2006). For example, after the end of cotton cultivation in Italy in 1950s, no feral cotton was reported in southern Italy, except in some restricted areas (Sarno et al., 1993; Celesti-Grapow et al., 2010). Also,

[34] Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed.
[35] Dossier: Part I – Sections D8–10 and Annex 2.
[36] Dossier: Part I – Sections D7.1, 7.2, 7.4, 9.1, 9.2 and study 050006.03.

in other cotton-growing regions such as in Australia, surveys showed that feral GM cotton established infrequently along transportation routes and mostly as transient populations (Addison et al., 2007).

It is unlikely that the intended traits of cotton GHB614 × LLCotton25 × MON 15985 will provide a selective advantage to cotton plants, except when they are exposed to glyphosate- and/or glufosinate-ammonium-containing herbicides or infested by insect pests that are susceptible to the Cry1Ac and/or Cry2Ab2 proteins.

The GMO Panel considers that the fitness advantage provided by the intended traits, and the observed differences in percentage open bolls, boll size, micronaire, fibre length uniformity, fibre strength, fibre elongation, number of bolls at first position, total number of bolls and parameters related to lepidopteran susceptibility (see Section 3.4.2) will not allow the GM plant to overcome other biological and abiotic factors (described above) limiting plant's persistence and invasiveness. Therefore, the presence of the intended traits and other observed differences will not affect the persistence and invasiveness of the GM plant.

In conclusion, the GMO Panel considers that it is very unlikely that cotton GHB614 × LLCotton25 × MON 15985 will differ from conventional cotton varieties in its ability to survive until subsequent seasons, or to establish feral plants under European environmental conditions in case of accidental release into the environment of viable GHB614 × LLCotton25 × MON 15985 cottonseeds.

### 3.6.2. Effects of gene transfer[37]

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through HGT of DNA or through vertical gene flow via cross-pollination from feral plants originating from spilled seeds.

#### 3.6.2.1. Plant-to-microorganism gene transfer

The potential for HGT of the recombinant DNA of the single events has already been assessed in previous opinions (see Table 1 and EFSA GMO Panel, 2011c). For cotton events GHB614 and LLCotton25, the GMO Panel concluded that there is no increased likelihood for HGT; for cotton event MON 15985, the GMO Panel concluded that there is increased likelihood of transfer of the antibiotic resistance gene *nptII*, but that MON 15985 material is highly unlikely to contribute to the environmental prevalence of this gene. No adverse effects on human and animal health and the environment are expected (EFSA, 2006b, 2009a; EFSA GMO Panel, 2014a). New bioinformatic data[38] confirmed the previous conclusions.

Synergistic effects of the recombinant genes, for instance due to combinations of recombinogenic sequences, which would cause an increase in the likelihood for HGT or a selective advantage were not identified.

Therefore, the GMO Panel concludes that the unlikely, but theoretically possible, horizontal transfer of recombinant genes from this three-event stack cotton to bacteria does not raise any environmental safety concern.

#### 3.6.2.2. Plant-to-plant gene transfer

The potential for occasional feral cotton GHB614 × LLCotton25 × MON 15985 plants originating from cottonseed import spills to transfer recombinant DNA to sexually compatible plants and the environmental consequences of this transfer were considered.

For plant-to-plant gene transfer to occur, imported GM cottonseeds need to germinate and develop into plants in areas containing sympatric wild relatives and/or cultivated cotton with synchronous flowering and environmental conditions favouring cross-pollination.

Cotton is predominantly an annual self-pollinating crop, although cross-pollination can occur at low frequencies in the presence of insect pollinators (such as wild bees, honeybees, bumblebees) (OECD, 2008). For cotton, no wild relatives have been reported in Europe; therefore, any vertical gene transfer is limited to *G. hirsutum* and *G. herbaceum* cotton plants. However, gene transfer to *G. herbaceum* is considered unlikely due to the difference in ploidy level.

The potential of spilled cottonseeds to establish, grow and produce pollen is extremely low and transient (see Section 3.6.1). The likelihood/frequency of cross-pollination between occasional feral GM cotton plants resulting from seed spillage and weedy or cultivated *Gossypium* plants is considered

[37] Dossier: Part I – Section D 9.3.
[38] Additional information: 2/2/2018.

extremely low. Even if cross-pollination would occur, the GMO Panel is of the opinion that the likelihood of environmental effects as a consequence of the spread of genes from occasional feral GM cotton plants in Europe will not differ from that of conventional cotton varieties for the reasons given in Section 3.6.1, even in the case of treatment with the intended herbicides.

### 3.6.3. Interactions of the GM plant with target organisms[39]

Taking the scope of application EFSA-GMO-NL-2011-94 into account, potential interactions with the target organisms of occasional feral cotton GHB614 × LLCotton25 × MON 15985 plants arising from seed import spills are not considered by the GMO Panel to raise any relevant environmental concern.

### 3.6.4. Interactions of the GM plant with non-target organisms[40]

Given that environmental exposure of non-target organisms to spilled GM cottonseeds or occasional feral GM cotton plants arising from spilled GM cottonseeds is limited and because most proteins are degraded before entering the environment through faecal material of animals fed GM cotton, potential interactions of the GM plant with non-target organisms are not considered by the GMO Panel to raise any relevant environmental concern. Interactions that may occur between the Cry proteins (as mentioned in Section 3.3) will not alter this conclusion.

### 3.6.5. Interactions with the abiotic environment and biogeochemical cycles[41]

Considering the scope of application EFSA-GMO-NL-2011-94, and the low level of exposure to the environment, potential interactions with the abiotic environment and biogeochemical cycles were not considered a relevant issue by the GMO Panel.

Given that environmental exposure to spilled seeds or occasional feral cotton GHB614 × LLCotton25 × MON 15985 plants arising from seed import spills is limited and because most proteins are degraded before entering the environment through faecal material of animals fed GM cotton, potential interactions with the abiotic environment and biogeochemical cycles are not considered a relevant issue by the GMO Panel.

### 3.6.6. Conclusion

The GMO Panel concludes that it is unlikely that cotton GHB614 × LLCotton25 × MON 15985 would differ from conventional cotton varieties in its ability to persist under European environmental conditions. Considering the scope of application EFSA-GMO-NL-2011-94, interactions of occasional feral cotton GHB614 × LLCotton25 × MON 15985 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from cotton GHB614 × LLCotton25 × MON 15985 to bacteria does not indicate a safety concern. Therefore, considering the combined traits and their interactions, the outcome of the comparative analysis, the routes and levels of exposure, the GMO Panel concludes that cotton GHB614 × LLCotton25 × MON 15985 would not raise safety concerns in the event of accidental release of viable GM cottonseeds into the environment.

## 3.7. Post-market monitoring

### 3.7.1. Post-market monitoring of GM food/feed

No compositional changes relevant for food/feed safety or nutrition were identified in cotton GHB614 × LLCotton25 × MON 15985 when compared with the non-GM comparator. The GMO Panel therefore considers cotton GHB614 × LLCotton25 × MON 15985 to be as safe as the non-GM comparator and that post-market monitoring (EFSA, 2006a; EFSA GMO Panel, 2011a) of the food/feed derived from cotton GHB614 × LLCotton25 × MON 15985 is not necessary.

[39] Dossier: Part I – Section D9.4.
[40] Dossier: Part I – Section D9.5.
[41] Dossier: Part I – Section D9.8.

### 3.7.2. Post-market environmental monitoring[42]

The objectives of a PMEM plan, according to Annex VII of Directive 2001/18/EC, are: (1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the ERA are correct; and (2) to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment that were not anticipated in the ERA.

Monitoring is related to risk management, and thus a final adoption of the PMEM plan falls outside the mandate of EFSA. However, the GMO Panel gives its opinion on the scientific methodology of the PMEM plan provided by the applicant (EFSA GMO Panel, 2011b).

As the ERA did not identify potential adverse environmental effects from cotton GHB614 × LLCotton25 × MON 15985, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for cotton GHB614 × LLCotton25 × MON 15985 includes (1) the description of a monitoring approach involving operators (federations involved in import and processing) reporting to the applicant, via a centralised system, any observed adverse effect(s) of the GMOs on human health and the environment; (2) a coordinating system established by EuropaBio for the collection of information recorded by the various operators; and (3) the review of relevant scientific publications retrieved from literature searches (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis and a final report at the end of the authorisation period.

The GMO Panel considers that the scope of the PMEM plan provided by the applicant is consistent with the intended uses of cotton GHB614 × LLCotton25 × MON 15985. The GMO Panel agrees with the reporting intervals proposed by the applicant in its PMEM plan.

## 4. Overall conclusions

The GMO Panel was asked to carry out a scientific assessment of cotton GHB614 × LLCotton25 × MON 15985 for import, processing, and food and feed uses in accordance with Regulation (EC) No 1829/2003.

No new data on the single cotton events GHB614, LLCotton25 and MON 15985 that would lead to a modification of the original conclusions on their safety were identified. Based on the molecular, agronomic/phenotypic and compositional characteristics, the combination of cotton events GHB614, LLCotton25 and MON 15985 in the three-event stack cotton did not give rise to issues regarding food and feed safety. The newly expressed proteins in the three-event stack cotton did not raise concerns for human and animal health. Food and feed from cotton GHB614 × LLCotton25 × MON 15985 are expected to have the same nutritional impact as those derived from the non-GM comparator.

The GMO Panel concluded that there is a very low likelihood of environmental effects resulting from the accidental release of viable seeds from cotton GHB614 × LLCotton25 × MON 15985 into the environment.

No scientific information that could change the conclusions on this three-event stack was retrieved in a literature search covering the period since the time of validity of the application.[43]

The GMO Panel concludes that cotton GHB614 × LLCotton25 × MON 15985, as described in this application, is as safe as the non-GM comparator with respect to potential effects on human and animal health and the environment.

The GMO Panel considers that post-market monitoring of food/feed derived from cotton GHB614 × LLCotton25 × MON 15985 is not necessary, given that no safety concerns were identified. The PMEM plan and reporting intervals are in line with the intended uses of cotton GHB614 × LLCotton25 × MON 15985.

## Documentation provided to EFSA

1) Letter from the Competent Authority of the Netherlands received on 18 February 2011 concerning a request for placing on the market of genetically modified cotton GHB614 × LLCotton25 × MON 15985 submitted by Bayer CropScience in accordance with Regulation (EC) No 1829/2003 (application reference EFSA-GMO-NL-2011-94).
2) Acknowledgement letter dated 14 March 2011 from EFSA to the Competent Authority of The Netherlands.

[42] Dossier Part I – Section D11 and Annex 3.
[43] Additional information: 3/11/2017.

3) Letter from EFSA to applicant dated 20 April 2011 requesting additional information under completeness check.
4) Letter from applicant to EFSA received on 31 May 2011 providing a timeline for submission of responses.
5) Letter from applicant to EFSA received on 27 January 2012 extending the timeline for submission of responses.
6) Letter from applicant to EFSA received on 2 April 2013 extending the timeline for submission of responses.
7) Letter from applicant to EFSA received on 3 March 2014 extending the timeline for submission of responses.
8) Letter from applicant to EFSA received on 16 June 2015 providing additional information
9) Letter from EFSA to applicant dated **15 July 2015** delivering the 'Statement of Validity' of application EFSA-GMO-NL-2011-94 for placing on the market of genetically modified cotton GHB614 × LLCotton25 × MON 15985 submitted by Bayer CropScience in accordance with Regulation (EC) No 1829/2003.
10) Letter from EURL-JRC to EFSA dated 16 October 2015 requesting EFSA to stop the clock.
11) Letter from EFSA to applicant dated 24 July 2015 requesting additional information and stopping the clock.
12) Letter from applicant to EFSA received on 22 October 2015 providing additional information.
13) Letter EFSA to applicant dated 22 February 2016 re-starting the clock.
14) Letter from EFSA to applicant dated 7 April 2016 requesting additional information and stopping the clock.
15) Letter from EFSA to applicant dated 12 May 2016 requesting additional information and maintaining the clock stopped.
16) Letter from applicant to EFSA received on 6 July 2016 providing additional information.
17) Letter from applicant to EFSA received on 16 August 2016 providing additional information.
18) Letter from applicant to EFSA dated 16 August 2016 re-starting the clock.
19) Letter from EFSA to applicant dated 29 September 2016 providing additional information.
20) E-mail from applicant to EFSA received on 9 November 2016 requesting an extension of deadline for the info requested by EFSA on 29 September 2016.
21) Letter from EFSA to applicant dated 3 November 2017 providing additional information.
22) E-mail from EFSA to applicant dated 11 November 2017 re-starting the clock.
23) Letter from EFSA to applicant dated 11 November 2017 stopping the clock.
24) Letter from applicant to EFSA received on 2 February 2017 providing additional information.
25) E-mail from EFSA to applicant dated 7 November 2018 re-starting the clock from 2 February 2017.
26) Letter from applicant to EFSA received on 20 February 2018 providing complementary information.
27) E-mail from EFSA to applicant dated 23 February 2018 re-starting the clock on 20 February 2018.

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## Abbreviations

| | |
|---|---|
| AA | amino acid |
| AAD | aminoglycoside adenylyltransferase |
| ADF | acid detergent fibre |
| ANOVA | analysis of variance |
| bp | base pair |
| bw | body weight |
| CaMV | cauliflower mosaic virus |
| CPA | cyclopropane fatty acids |
| CPE | cyclopropene fatty acids |
| CPFA | cyclopropenoid fatty acids |
| Cry | crystal protein |
| DM | dry matter |
| ELISA | enzyme-linked immunosorbent assay |
| EPSPS | 5-enolpyruvyl-shikimate-3-phosphate synthase |
| ERA | environmental risk assessment |
| FA | total fatty acids |
| FM | FiberMax |
| GM | genetically modified |
| GMO | genetically modified organism |
| GMO Panel | EFSA Panel on Genetically Modified Organisms |
| GUS | β-D-glucuronidase |
| HGT | horizontal gene transfer |
| HR | homologous recombination |
| IgE | immunoglobulin E |
| LOD | limit of detection |
| LLOQ | lower limit of quantification |
| LOQ | limit of quantification |
| NDF | neutral detergent fibre |
| NOAEL | no-observed-adverse-effect-level |
| NPTII | neomycin phosphotransferase II |
| OECD | Organisation for Economic Co-operation and Development |
| ORF | open reading frame |
| PAT | phosphinothricin acetyltransferase |
| PMEM | post-market environmental monitoring |
| TE | tocopherol equivalent |
| UL | tolerable upper intake level |
| UTR | untranslated region |

## Appendix A – Protein expression data

**Table A.1:** Means, standard deviation and ranges of protein levels (μg/g dry weight) from cotton GHB614 × LLCotton25 × MON 15985 (treated with glyphosate and glufosinate), GHB614 (treated with glyphosate), LLCotton25 (treated with glufosinate), and MON 15985 (not treated), from field trials performed in USA in 2014[a]

| | **GHB614 × LLCotton25 × MON 15985** | **GHB614** | **LLCotton25** | **MON 15985** |
|---|---|---|---|---|
| **Cry1Ac** | | | | |
| Leaf (4–6 leaf) | 32.22[b] ± 9.85[c] (21.19–54.12)[d] | | | 31.90 ± 8.38 (15.72–47.80) |
| Leaf (square initiation) | 31.00 ± 15.85 (14.79–64.51) | | | 27.59 ± 8.66 (16.79–45.74) |
| Leaf (2 weeks after first flower) | 25.37 ± 7.20 (14.93–34.45) | | | 22.55 ± 6.35 (16.16–40.31) |
| Root (square initiation) | 4.04 ± 0.64 (< LLOQ–5.01) | | | 5.25 ± 1.64 (< LLOQ–7.84) |
| Pollen (flowering)[e] | 0.21 ± 0.05 (0.15–0.34) | | | 0.18 ± 0.02 (0.14–0.21) |
| Fuzzy seed (maturity) | 11.35 ± 1.13 (9.92–13.22) | | | 10.14 ± 1.50 (7.13–11.96) |
| **Cry2Ab2** | **GHB614 × LLCotton25 × MON 15985** | **GHB614** | **LLCotton25** | **MON 15985** |
| Leaf (4–6 leaf) | 167.35 ± 36.91 (121.95–255.62) | | | 139.60 ± 20.86 (103.94–175.15) |
| Leaf (square initiation) | 156.29 ± 32.36 (111.96–203.41) | | | 182.70 ± 20.21 (140.73–218.23) |
| Leaf (2 weeks after first flower) | 180.57 ± 26.88 (140.52–232.73) | | | 189.77 ± 16.09 (164.39–221.56) |
| Root (square initiation) | 78.71 ± 15.84 (47.63–101.15) | | | 84.39 ± 13.06 (62.19–98.64) |
| Pollen (flowering) | 0.90 ± 0.75 < LLOQ–2.88 | | | 0.80 ± 0.51 (< LLOQ–2.27) |
| Fuzzy seed (maturity) | 171.21 ± 24.03 (136.07–215.60) | | | 152.77 ± 18.24 (122.26–181.50) |
| **2mEPSPS** | **GHB614 × LLCotton25 × MON 15985** | **GHB614** | **LLCotton25** | **MON 15985** |
| Leaf (4–6 leaf) | 279.67 ± 125.83 (104.87–453.86) | 303.66±78.10 (207.34–470.87) | | |
| Leaf (square initiation) | 507.11 ± 188.84 (234.08–891.96) | 567.24 ± 171.45 (272.34–827.28) | | |
| Leaf (2 weeks after first flower) | 410.80 ± 42.95 (310.88–495.99) | 359.55 ± 79.40 (227.92–478.53) | | |
| Root (square initiation) | 86.62 ± 27.25 (44.05–127.19) | 91.96 ± 21.52 (58.55–125.74) | | |
| Pollen (flowering) | 11.91 ± 3.03 (6.56–17.48) | 13.18 ± 5.90 (7.85–30.01) | | |
| Fuzzy seed (maturity) | 105.70 ± 12.79 (81.48–127.63) | 117.59 ± 12.24 (98.99–133.69) | | |

| PAT | GHB614 × LLCotton25 × MON 15985 | GHB614 | LLCotton25 | MON 15985 |
|---|---|---|---|---|
| Leaf (4–6 leaf) | 519.38 ± 158.43 (261.28–1029.36) | | 461.68 ± 49.44 (401.12–608.19) | |
| Leaf (square initiation) | 540.34 ± 66.92 (422.13–636.93) | | 418.55 ± 43.17 (365.42–502.18) | |
| Leaf (2 weeks after first flower) | 464.49 ± 43.19 (391.05–543.93) | | 416.55 ± 57.51 (355.89–539.01) | |
| Root (square initiation) | 144.67 ± 43.15 (73.83–194.07) | | 153.11 ± 20.87 (115.69–183.99) | |
| Pollen (flowering) | 1.49 ± 1.06 (0.09–3.81) | | 0.74 ± 0.66 (0.08–2.24) | |
| Fuzzy seed (maturity) | 161.02 ± 19.35 (122.48–193.10) | | 152.43 ± 19.53 (104.93–172.19) | |
| **GUS** | **GHB614 × LLCotton25 × MON 15985** | **GHB614** | **LLCotton25** | **MON 15985** |
| Leaf (4–6 leaf) | 180.76 ± 48.27 (112.71–254.48) | | | 205.32 ± 19.53 (162.90–232.64) |
| Leaf (square initiation) | 247.33 ± 35.71 (201.32–294.17) | | | 245.48 ± 38.46 (179.05–308.26) |
| Leaf (2 weeks after first flower) | 174.58 ± 37.33 (119.31–223.39) | | | 147.11 ± 41.61 (87.40–210.30) |
| Root (square initiation) | 65.23 ± 35.17 (18.24–122.00) | | | 58.04 ± 13.88 (33.29–78.46) |
| Pollen (flowering) | 1.56 ± 0.24 (1.17–2.13) | | | 1.42 ± 0.24 (< LLOQ–1.98) |
| Fuzzy seed (maturity) | 32.06 ± 4.20 (21.77–39.52) | | | 29.36 ± 3.35 (20.81–34.06) |
| **NPTII** | **GHB614 × LLCotton25 × MON 15985** | **GHB614** | **LLCotton25** | **MON 15985** |
| Leaf (4–6 leaf) | 16.09 ± 2.85 (10.86–22.99) | | | 16.32 ± 1.48 (13.71–18.66) |
| Leaf (square initiation) | 15.24 ± 2.53 (11.35–18.99) | | | 13.79 ± 2.07 (10.65–17.77) |
| Leaf (2 weeks after first flower) | 18.55 ± 1.33 (16.29–20.76) | | | 15.76 ± 1.26 (13.37–17.88) |
| Root (square initiation) | 3.88 ± 0.62 (2.42–4.77) | | | 3.57 ± 0.47 (3.10–4.41) |
| Pollen (flowering) | < LOD (NA) | | | < LOD (NA) |
| Fuzzy seed (maturity) | 6.60 ± 0.91 (5.19–8.34) | | | 6.05 ± 0.62 (4.89–7.52) |

Cry: crystal protein; EPSPS: 5-enolpyruvyl-shikimate-3-phosphate synthase; PAT: phosphinothricin acetyltransferase; GUS: β-D-glucuronidase; NPTII: neomycin phosphotransferase II; LOD: limit of detection; LLOQ: lower limit of quantification; NA: not applicable.

(a): Number of samples is n = 15 except for: Cry1Ac in root (n = 6 and n = 10 of GHB614 × LLCotton25 × MON 15985 and MON 15985, respectively); Cry2Ab2 in pollen (n = 12 of GHB614 × LLCotton25 × MON 15985 and MON 15985); GUS in pollen (n = 14 of MON 15985).

(b): Mean.

(c): Standard deviation.

(d): Range.

(e): Reported values for pollen are derived from fresh weight (FW) tissue.