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SCIENTIFIC OPINION

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# Assessment of genetically modified maize DP202216 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2019-159)

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**Abstract**

Genetically modified maize DP202216 was developed to confer tolerance to glufosinate-ammonium-containing herbicides and to provide an opportunity for yield enhancement under field conditions. These properties were achieved by introducing the *mo-pat* and *zmm28* expression cassettes. The molecular characterisation data and bioinformatic analyses do not identify issues requiring food/feed safety assessment. None of the identified differences in the agronomic/phenotypic and compositional characteristics tested between maize DP202216 and its comparator needs further assessment, except for the levels of stearic acid (C18:0), which do not raise nutritional and safety concerns. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the PAT and ZMM28 proteins as expressed in maize DP202216, and finds no evidence that the genetic modification would change the overall allergenicity of maize DP202216. In the context of this application, the consumption of food and feed from maize DP202216 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize DP202216 is as safe as the comparator and non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary. In the case of accidental release of viable maize DP202216 grains into the environment, this would not raise environmental safety concerns. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of maize DP202216. The GMO Panel concludes that maize DP202216 is as safe as its comparator and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment.

**KEYWORDS**

DP202216, genetic engineering, GM, import and processing, maize (*Zea mays*), PAT, ZMM28

# CONTENTS

## SUMMARY

Following the submission of application EFSA-GMO-NL-2019-159 under Regulation (EC) No 1829/2003 from Pioneer Overseas Corporation (referred to hereafter as 'the applicant'), the Panel on genetically modified organisms of the European Food Safety Authority (referred to hereafter as 'GMO Panel') was asked to deliver a Scientific Opinion on the safety of genetically modified (GM) herbicide tolerant and potential yield enhanced maize (*Zea mays* L.) DP202216 according to Regulation (EU) No 503/2013. The scope of application EFSA-GMO-NL-2019-159 is for import, processing and food and feed uses within the European Union (EU) of maize DP202216 and does not include cultivation in the EU.

In this scientific opinion, the GMO Panel reports on the outcome of its risk assessment of maize DP202216 according to the scope of the application EFSA-GMO-NL-2019-159. The GMO Panel conducted the assessment of maize DP202216 in line with the principles described in Regulation (EU) No 503/2013 and its applicable guidelines for the risk assessment of GM plants. The molecular characterisation data establish that maize DP202216 contains a single insert, consisting of one copy of the *mo-pat* and *zmm28* expression cassettes. Bioinformatic analyses of the sequences encoding the newly expressed protein (NEP) and endogenous protein with altered expression (EPAE), and open reading frames (ORFs) present within the insert or spanning the junctions between the insert and genomic DNA, do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of the PAT and ZMM28 proteins is considered adequate. The protein characterisation data comparing the DP202216-produced PAT to those previously assessed in other maize events indicate that these proteins are equivalent. The protein characterisation data comparing the DP202216-produced ZMM28 to those in conventional maize varieties indicate that these proteins are identical.

Considering the selection of test materials, the field trial sites, the associated management practices and the agronomic–phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis.

None of the identified differences in the agronomic/phenotypic and compositional characteristics tested between maize DP202216 and its comparator needs further assessment, except for the levels of stearic acid (C18:0), which does not raise safety and nutritional concerns. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the PAT and ZMM28 proteins as expressed in maize DP202216, and finds no evidence that the genetic modification would change the overall allergenicity of maize DP202216. In the context of this application, the consumption of food and feed from maize DP202216 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize DP202216 is as safe as the comparator and non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

Considering the introduced traits, the outcome of the agronomic and phenotypic analysis and the routes and levels of exposure, maize DP202216 would not raise safety concerns in the case of accidental release of viable GM maize grains into the environment. The post-market environmental monitoring (PMEM) plan and reporting intervals are in line with the intended uses of maize DP202216.

Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issue pertaining to the intended uses of maize DP202216.

The GMO Panel concludes that maize DP202216 is as safe as its comparator and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment.

# 1 | INTRODUCTION

The scope of the application EFSA-GMO-NL-2019-159 is for food and feed uses, import and processing within the European Union (EU) of the genetically modified (GM) herbicide tolerant (HT) and potential yield enhancement (PYE) maize DP202216 and does not include cultivation in the EU.

## 1.1 | Background and Terms of Reference as provided by the requestor

On 3 July 2019, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands application EFSA-GMO-NL-2019-159 for authorisation of maize DP202216 (Unique Identifier DP-2Ø2216-6), submitted by Pioneer Overseas Corporation (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003.[1] Following receipt of application EFSA-GMO-NL-2019-159, EFSA informed EU Member States and the European Commission, and made the application available to them. Simultaneously, EFSA published the summary of the application.[2]

EFSA checked the application for compliance with the relevant requirements of Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013,[3] with the EFSA guidance documents, and, when needed, asked the applicant to supplement the initial application. On 23 November 2019, EFSA declared the application valid.

From the validity date, EFSA and its Panel on Genetically Modified Organisms (hereafter referred to as 'the GMO Panel') endeavoured to respect a time limit of six months to issue a scientific opinion on application EFSA-GMO-NL-2019-159. Such time limit was extended whenever EFSA and/or its GMO Panel requested supplementary information to the applicant. According to Regulation (EC) No 1829/2003, any supplementary information provided by the applicant during the risk assessment was made available to the EU Member States and to European Commission (for further details, see the section 'Documentation', below). In accordance with Regulation (EC) No 1829/2003, EFSA consulted the nominated risk assessment bodies of EU Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC.[4] The EU Member States had three months to make their opinion known on application EFSA-GMO-NL-2019-159 as of date of validity.

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

According to Articles 6 and 18 of Regulation (EC) No 1829/2003, EFSA and its GMO Panel were requested to carry out a scientific risk assessment of maize DP202216 in the context of its scope as defined in application EFSA-GMO-NL-2019-159.

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. In addition to the present scientific opinion, EFSA was also asked to report on the particulars listed under Articles 6(5) and 18(5) of Regulation (EC) No 1829/2003, but not to give an opinion on them because they pertain to risk management.[5]

# 2 | DATA AND METHODOLOGIES

## 2.1 | Data

The GMO Panel based its scientific risk assessment of maize DP202216 on the valid application EFSA-GMO-NL-2019-159, additional information provided by the applicant during the risk assessment, relevant scientific comments submitted by the Member States and relevant peer-reviewed scientific publications. As part of this comprehensive information package, the GMO Panel received additional unpublished studies submitted by the applicant in order to comply with the specific provisions of Regulation (EU) No 503/2013. A list of these additional unpublished studies is provided in Appendix A.

## 2.2 | Methodologies

The GMO Panel conducted its assessment in line with the principles described in Regulation (EU) No 503/2013, the applicable guidelines (i.e. EFSA GMO Panel, 2010a, 2011a, 2011b, 2015, 2017a; EFSA Scientific Committee, 2011) and explanatory notes and statements (i.e. EFSA, 2010a, 2014a, 2018, 2019a, 2019b; EFSA GMO Panel, 2010b, 2018a) for the risk assessment of GM plants.

[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]Available online: https://open.efsa.europa.eu/questions/EFSA-Q-2019-00419

[3]Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L157, 8.6.2013, p. 1–48.

[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.

[5]These particulars are available online at: https://open.efsa.europa.eu/study-inventory/EFSA-Q-2019-00419

For this application, in the context of the contracts OC/EFSA/GMO/2018/04, OC/EFSA/GMO/2018/02, OC/EFSA/GMO/2020/01, OC/EFSA/GMO/2021/06 and EOI/EFSA/SCIENCE/2020/01–CT02GMO, the contractors performed preparatory work for the evaluation of the applicant's literature search, methods applied for the statistical analysis, completeness and quality of DNA sequencing information, updated bioinformatic analyses and statistical analysis of the 90-day toxicity study on maize DP202216.

# 3 | ASSESSMENT

## 3.1 | Introduction

Maize DP202216 expresses the phosphinothricin acetyltransferase enzyme (PAT) (hereafter 'newly expressed protein' (NEP)), which confers tolerance to glufosinate-ammonium-containing herbicides.

In addition, maize DP202216 contains a transgene encoding the maize ZMM28 protein (hereafter 'endogenous protein with altered expression' (EPAE)), to extend and increase the expression of the endogenous protein, providing an opportunity for Yield Enhancement (YE) under field conditions (Wu et al., 2019). The ZMM28 protein is a MADS-box transcription factor (Castelán-Muñoz et al., 2019; Münster et al., 2002), involved in biochemical and physiological processes including photosynthesis, nitrogen assimilation and growth regulating hormone signalling, whose increased and extended expression can potentially lead to YE (Wu et al., 2019).

YE is driven by numerous biochemical and physiological processes and is highly dependent on the interaction between genotype, environment and management practices (Simmons et al., 2021). Therefore, an increased and extended expression of the ZMM28 protein may not lead to YE in all instances. This is evident from the extensive field evaluation conducted by Wu et al. (2019) where maize DP202216 was introgressed in different genetic backgrounds and tested in multiple years and locations, not always resulting into the expected YE.

The ZMM28 protein is expressed in different plant tissues of maize DP202216 throughout different stages of development, including edible parts of GM maize consumed as food and feed (e.g. grains and forage).

## 3.2 | Systematic literature review

The GMO Panel assessed the applicant's literature searches on maize DP202216, which included a scoping review, according to the guidelines given in EFSA (2010a, 2019). A systematic review as referred to in Regulation (EU) No 503/2013 has not been provided in support to the risk assessment of application EFSA-GMO-NL-2019-159. Based on the outcome of the scoping review, the GMO Panel agrees that there is limited value of undertaking a systematic review for maize DP202216 at present.

The GMO Panel considers the overall quality of the performed literature searches acceptable. The literature searches identified 11 relevant publications on maize DP202216 (Appendix B).

Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issues pertaining to the intended uses of maize DP202216.

## 3.3 | Molecular characterisation

### 3.3.1 | Transformation process and vector constructs [6]

Maize DP202216 was developed by *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*)-mediated transformation. Immature embryos of maize DP202216 were co-cultured with a disarmed *A. tumefaciens* strain LBA4404 containing the vector PHP40099. The plasmid PHP40099 used for the transformation contains two expression cassettes between the right and left border of the T-DNA, containing the following genetic elements:

- The *zmm28* expression cassette consists of the translation initiation factor *zm-gos2* promoter from *Zea mays*, the *ubi*ZM1 intron region from the *Zea mays* ubiquitin gene 1, the coding sequence of the *zmm28* gene from *Zea mays* and the *pin*II proteinase inhibitor II terminator region from *Solanum tuberosum*.
- The *mo-pat* expression cassette consists of the *ubi*ZM1 region, including the promoter, the 5′ untranslated, the intron sequences from the *Zea mays* ubiquitin gene 1, the maize codon-optimised *pat* sequence of the phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes*, and the *pin*II proteinase inhibitor II terminator region from *Solanum tuberosum*.

The vector backbone contained elements necessary for the maintenance and selection of the plasmid in bacteria.

[6]Dossier: Part II – Section 1.2.1.1 and 1.2.1.2.

### 3.3.2 | Transgene constructs in the GM plant[7]

Molecular characterisation of maize DP202216 was performed by Southern-by-Sequencing (SbS) to determine insert copy number and to confirm the absence of plasmid backbone elements, and by polymerase chain reaction (PCR) followed by Sanger sequencing to determine the size, organisation and sequence of the inserted T-DNA. The approach used is acceptable in terms of coverage and sensitivity. Overall, the quality of the sequencing methodology and data sets was assessed by the EFSA GMO Panel and is in compliance with the requirements listed in the EFSA Technical Note (2018a).

SbS indicated that maize DP202216 contains a single insert, consisting of a single copy of the T-DNA in the same configuration as in the PHP40099 transformation vector. SbS also indicated the absence of vector backbone sequences. The nucleotide sequence of the entire insert of maize DP202216 together with 1283 bp of the 5′ and 1372 bp of the 3′ flanking regions were determined. The insert of 7436 bp is identical to the T-DNA of PHP40099, except for the deletion of 22 bp of the right border region and 12 bp of the left border region.

The possible interruption of known endogenous maize genes by the insertion in maize DP202216 was evaluated by bioinformatics analyses of the pre-insertion locus and of the genomic sequences flanking the insert. The results of these analyses do not indicate the interruption of any known endogenous gene in maize DP202216.

The results of segregation (see Section 3.3.5) and bioinformatics analyses are compatible with a single insertion in the nuclear genome.

Bioinformatic analyses of the amino acid sequence of the NEP and EPAE reveal no significant similarities to toxins and allergens. In addition, bioinformatic analyses of the newly created open reading frames (ORFs) within the insert and spanning the junctions between the insert and genomic DNA indicated that three ORFs (DP-202216-6_189, DP-202216-6_260 and DP-202216-6_300) exceeded the allergenicity assessment threshold of 35% identity using an 80 amino acid sliding window approach. ORF DP-202216-6_189 is in the same orientation but in a different reading frame to the PAT ORF and does not contain any in-frame translational start codons (ATG). ORF DP-202216-6_260 is found within the transcriptional unit of PAT coding sequence, but in the reverse orientation, and lacks an upstream promoter and a start codon. ORF DP-202216-6_300 is found spanning part of the UbiZM1 intron, 5′ UTR and promoter sequence of PAT, but in the reverse orientation and does not contain any known promoter motifs upstream or a start codon. No significant similarities with toxins were identified for any ORF within the insert and spanning the junctions between the insert and genomic DNA. In conclusion, these analyses indicated that the expression of any ORF showing significant similarities to toxins or allergens in maize DP202216 is unlikely.

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis for maize DP202216 to microbial DNA. The likelihood and potential consequences of plant-to-microorganisms gene transfer are described in Section 3.6.1.2.1.

### 3.3.3 | Protein characterisation and equivalence[8]

Maize DP202216 expresses 2 proteins, ZMM28 and PAT.

The ZMM28 protein is a MADS-box transcription factor (Castelán-Muñoz et al., 2019; Münster et al., 2002; Pařenicová et al., 2003) which binds to specific DNA sequences termed the CArG-box as homo- or heterodimers, or even multimers to regulate expression of genes (Kaufmann et al., 2005; Smaczniak et al., 2012), involved in biochemical and physiological processes including photosynthesis, nitrogen assimilation and growth regulating hormone signalling, potentially leading to enhanced plant growth and increased grain yield (Wu et al., 2019).

The PAT protein is a phosphinothricin acetyltransferase enzyme which confers tolerance to the glufosinate-ammonium-containing herbicides (Hérouet et al., 2005; Krenchinski et al., 2018).

#### 3.3.3.1 | *ZMM28 protein characterisation and equivalence*

The deduced amino acid sequence as defined by the *zmm28* gene introduced in maize DP202216 is identical to the endogenous ZMM28 protein present in the GM plant and in most conventional maize varieties. The ZMM28 protein in DP202216 is the B73 isoform which was found also in 98% of the conventional maize considered in Anderson et al. (2019). Western blot analysis was employed to confirm the equivalence between endogenous and introduced ZMM28 in maize DP202216 with the ZMM28 protein present in near-isogenic control maize. These data showed that all the ZMM28 proteins migrated close to the expected molecular weight of ~28 kDa and were comparably immunoreactive to ZMM28 protein-specific antibodies. No ZMM28 protein was recombinantly produced in a heterologous expression system.

The protein characterisation data comparing the DP202216-produced ZMM28 to those in conventional maize varieties indicate that these proteins are identical.

[7]Dossier: Part II – Section 1.2.2.2 and additional information 16/1/2020, 25/2/2020, 5/6/2020, 17/8/2020, 23/4/2021, 27/8/2021, 27/3/2023.
[8]Dossier: Part II–Section 1.2.1.3 and additional information 16/1/2020.

#### 3.3.3.2 | *PAT protein characterisation and equivalence*

The PAT protein has been extensively characterised in previously authorised events assessed by the GMO Panel (e.g. EFSA GMO Panel, 2020 (AP142), 2018b (AP133), 2017b (AP92), and 2022 (AP161), 2023a (AP137), 2023b (AP140). Western blot analysis was employed to demonstrate the equivalence between PAT produced in maize DP202216 with plant-derived proteins in previously assessed maize events 1507 and 59122 as well as its corresponding *Escherichia coli*-produced PAT protein. These data showed that all the PAT proteins migrated close to the expected molecular weight of ~21 kDa and were comparably immunoreactive to PAT protein-specific antibodies.

The protein characterisation data comparing the DP202216-produced PAT to those previously assessed in other maize events indicate that these proteins are equivalent.

### 3.3.4 | Information on the expression of the insert [9]

Protein levels of ZMM28 and PAT were analysed by enzyme-linked immunosorbent assay (ELISA) and western blot in material harvested in a field trial across six locations in USA and Canada during the 2017 growing season. Samples analysed included leaves (V6, V9, R1, R4, R6), roots (V9, R1, R4, R6), whole plant (V9, R1, R6), forage (R4), grain (R6) and pollen (R1) from DP202216 plants treated and not treated with glufosinate. The mean values, standard deviations and ranges of protein expression levels in grains ($n = 24$), forage ($n = 24$) and pollen ($n = 24$) of the ZMM28 and PAT proteins used to estimate human and animal dietary exposure (see Section 3.5.5) are reported in Table 1. For ZMM28, protein levels were also measured in the control maize and for all tissues and conditions (Table 2). ZMM28 levels in leaf tissues are also reported in Table 2.

The data assessment, supported by a statistical analysis provided by the applicant, confirmed an increased and extended expression of ZMM28 in maize DP202216 in comparison with its control, in most of the stages and sites (Table 2).

**TABLE 1** Mean values, standard deviations and ranges of NEP and EPAE in grains (ng/mg dry weight [dw] and ng/mg fresh weight [fw]), forage and pollen (ng/mg dw) from maize DP202216 ($n = 24$).

| | **Glufosinate treatment** | | | |
|---|---|---|---|---|
| | **Not treated** | | **Treated** | |
| **Tissues** | **ng/mg dry weight (dw)** | **ng/mg fresh weight (fw)** | **ng/mg dry weight (dw)** | **ng/mg fresh weight (fw)** |
| Grain (R6) | | | | |
| ZMM28[d] | 0.012[a] ± 0.007[b] (< LOQ[e]-0.029)[c] | 0.0096 ± 0.0056 (< LOQ[e]-0.023) | 0.012 ± 0.0064 (< LOQ[e]-0.030) | 0.0096 ± 0.0051 (< LOQ[e]-0.024) |
| PAT | 16 ± 3.4 (8–22) | 13 ± 2.7 (6.4–18) | 16 ± 3.0 (12–23) | 13 ± 2.4 (9.6–18) |
| Forage (R4) | | | | |
| ZMM28[d] | 0.058 ± 0.024 (<LOQ[e]-0.14) | | 0.066 ± 0.024 (<LOQ[e]-0.12) | |
| PAT | 40 ± 10 (20–60) | | 38 ± 6.3 (28–55) | |
| Pollen (R1) | | | | |
| ZMM28[d] | < LOQ[e] (≤ LOQ) | | < LOQ[e] (< LOQ-0.029) | |
| PAT | 79 ± 10 (69–110) | | 77 ± 9.7 (57–100) | |

[a]Mean value.
[b]Standard deviation.
[c]Range.
[d]ZMM28 expression levels in maize DP202216 are the results of the combination of both endogenous and introduced proteins.
[e]A value equal to half of the limit of quantification (ZMM28 LOQ = 0.028 ng/mg dw in pollen, 0.036 ng/mg dw in forage and 0.0069 ng/mg dw in grain) was used to estimate the mean values when samples were reported as below LOQ.

**TABLE 2** Mean values, standard deviations and ranges of ZMM28 protein in grain, forage, pollen and in several developmental stages of leaf (ng/mg dw) from maize DP202216 and from non-GM maize control ($n = 24$).

| | **Glufosinate treatment** | | |
|---|---|---|---|
| | **Maize DP202216** | | |
| | **Not treated** | **Treated** | **Control** |
| | **ng/mg dry weight** | **ng/mg dry weight** | **ng/mg dry weight** |
| **ZMM28[d]** | | | |
| **Grain (R6)** | 0.012[a] ± 0.007[b] (< LOQ[e]-0.029)[c] | 0.012 ± 0.0064 (< LOQ[e]-0.030) | < LOQ[f] |
| **Forage (R4)** | 0.058 ± 0.024 (< LOQ[e]-0.14) | 0.066 ± 0.024 (< LOQ[e]-0.12) | < LOQ[e] (< LOQ[e]-0.068) |

[9]Dossier: Part II–Section 1.2.2.3 and additional information 16/1/2020, 5/6/2020, 14/1/2022 and 19/12/2022.

**TABLE 2** (Continued)

| | Glufosinate treatment | | |
|---|---|---|---|
| | Maize DP202216 | | |
| | Not treated | Treated | Control |
| | ng/mg dry weight | ng/mg dry weight | ng/mg dry weight |
| **Pollen (R1)** | < LOQ[e] (≤ LOQ) | < LOQ[e] (< LOQ-0.029) | < LOQ[f] |
| **Leaf (V6)** | 0.10 ± 0.11 (< LOQ[e]-0.38) | 0.11 ± 0.11 (< LOQ[e]-0.46) | 0.071 ± 0.093 (< LOQ[e]-0.32) |
| **Leaf (V9)** | 0.32 ± 0.21 (0.076–0.83) | 0.37 ± 0.17 (0.13–0.83) | 0.24 ± 0.15 (0.069–0.64) |
| **Leaf (R1)** | 0.37 ± 0.17 (0.097–0.76) | 0.44 ± 0.14 (0.20–0.66) | 0.25 ± 0.13 (< LOQ[e]-0.51) |
| **Leaf (R4)** | 0.14 ± 0.056 (< LOQ[e]-0.25) | 0.16 ± 0.063 (< LOQ[e]-0.28) | 0.091 ± 0.043 (< LOQ[e]-0.16) |
| **Leaf (R6)** | < LOQ[f] | < LOQ[f] | < LOQ[f] |

[a]Mean value.

[b]Standard deviation.

[c]Range.

[d]ZMM28 expression levels in maize DP202216 are the results of the combination of both endogenous and introduced proteins.

[e]A value equal to half of the limit of quantification (ZMM28 LOQ = 0.054 ng/mg dw in leaf, 0.028 ng/mg dw in pollen, 0.036 ng/mg dw in forage and 0.0069 ng/mg dw in grain) was used to estimate the mean values when samples were reported as below LOQ.

[f]All samples were below LOQ.

### 3.3.5 | Inheritance and stability of inserted DNA[10]

Genetic stability of maize DP202216 insert was assessed by Southern analysis of genomic DNA from five generations (T1, T2, BC1F1, BC3F3 and BC3F6). The restriction enzyme/probe combinations used were sufficient to conclude that all the plants tested retained the single copy of the insert and flanking regions, which were stably inherited in subsequent generations.

Phenotypic stability was assessed by segregation analysis of tolerance to glufosinate. The inheritance pattern was investigated by assessing the presence of *zmm28* and *pat* genes by quantitative polymerase chain reaction (qPCR) from five generations (T2, F1, BC1F1, BC3F3 and BC3F6). The results support the presence of a single insertion, segregating in a Mendelian fashion.

### 3.3.6 | Conclusion on molecular characterisation

The molecular characterisation data establish that maize DP202216 contains a single insert consisting of one copy of the *zmm28* and the *mo-pat* expression cassettes. Bioinformatics analyses of the sequences encoding the NEP and EPAE and other ORFs within the insert or spanning the junctions between the insert and genomic DNA do not give rise to safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of the ZMM28 and PAT proteins is considered adequate. The protein characterisation data comparing the DP202216-produced PAT to those previously assessed in other maize events indicate that these proteins are equivalent. The protein characterisation data comparing the DP202216-produced ZMM28 to those in conventional maize varieties indicate that these proteins are identical.

## 3.4 | Comparative analysis

### 3.4.1 | Overview of studies conducted for the comparative analysis

Application EFSA-GMO-NL-2019-159 presents data on agronomic and phenotypic characteristics as well as on forage and grain composition of maize DP202216 (Table 3).

[10]Dossier: Part II–Section 1.2.2.4 and additional information 25/2/2020.

**TABLE 3** Overview of the comparative analysis studies to characterise maize DP202216 in application EFSA-GMO-NL-2019-159.

| Study focus | Study details | Comparator | Non-GM reference varieties |
|---|---|---|---|
| Agronomic and phenotypic analysis | Field study, USA and Canada, 2017, 12 sites[a] | P0751 | Sixteen[b] |
| Compositional analysis | Field study, USA and Canada, 2017, 8 sites[a] | | |

[a]Eight field trials conducted in 2017 were used for both the compositional and the agronomic/phenotypic analysis: In USA, one site in Iowa, Illinois, Indiana, Missouri, Nebraska, Pennsylvania and Texas and in Canada one site in Ontario. Four field trials conducted in 2017 were used only for the agronomic/phenotypic analysis: two sites in Iowa, one sites in Illinois and in Kansas.

[b]Non-GM reference varieties used in the agronomic, phenotypic and compositional field trials, with their corresponding relative maturity indicated in brackets were 34N84(108), 35F38(103), 35P12(103), P0506(105), P0589(105), P0760(107), P0965(109), P0987(109), P0993(109), XL5140(105), XL5513(105), XL5828(110), XL5840(108), BK5883(108), XL5939(109) and BK6076(110).

As remarked in the introduction, YE is driven by numerous biochemical and physiological processes and is highly dependent on the interaction between genotype, environment and management practices (Simmons et al., 2021). Current knowledge precludes an advanced determination of whether the potential yield enhancement induced by the overexpression of ZMM28 will be phenotypically expressed in the selected field trials. For these reasons, the GMO Panel considered that it was not possible to rely on observed YE to assess the PYE in maize DP202216. However, protein levels were tested in six field trial sites used also for the compositional and agronomic/phenotypic analysis (Section 3.3.4; Table 3, footnote a). Therefore, the extended and increased expression of the ZMM28 protein in maize DP202216 (see Table 2) was used to assess the relevance of the field trials.

### 3.4.2 | Experimental field trial design and statistical analysis

The materials grown at each field trial site were maize DP202216 exposed to the intended glufosinate-containing herbicides (treated), maize DP202216 not exposed to the intended herbicide (untreated), the comparator P0751 and four of 16 commercial non-GM hybrid maize reference varieties (hereafter 'non-GM reference varieties').

The agronomic/phenotypic and compositional data were analysed as specified by EFSA GMO Panel (2010b, 2011a). This includes, for each of the two treatments of maize DP202216, the application of a difference test (between the GM maize and the comparator) and an equivalence test (between the GM maize and the set of non-GM reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[11]

### 3.4.3 | Suitability of selected test materials

*Selection of the GM maize line and comparator*

Maize DP202216 was obtained using the non-GM maize line PH14AW as recipient inbred line. Maize event DP202216 was then backcrossed and stabilised into the non-GM inbred line PHR1J.

For the field trial study, the transformed inbred line PHR1J was crossed with the non-GM inbred line PHW2Z to produce the GM hybrid used to conduct the agronomic and phenotypic and the compositional assessment.

The comparator selected in the field trials is the hybrid maize P0751 that was obtained by crossing the non-GM inbred lines PHR1J and PHW2Z. As documented by the pedigree, the GMO Panel considers the produced comparator suitable for the comparative analysis.

Maize DP202216 and its comparator, both with a comparative relative maturity (CRM) of 107, are appropriate for growing in a range of environments across United States and Canada.

*Selection of non-GM reference varieties*

The 16 non-GM reference varieties with a relative maturity ranging from 103 to 110 were selected by the applicant and at each field trial site four of them were tested (see Table 3). On the basis of the information provided on relative maturity classes and year of commercialisation, the GMO Panel considers the selected non-GM reference varieties appropriate for the comparative assessment.

*Seed production and quality*

Seeds of maize DP202216 and the comparator were produced, harvested and stored under similar conditions, before being sown in the field trials. The seed lots of maize DP202216 and the comparator were verified for their purity via event-specific polymerase chain reaction analysis. The germination of maize DP202216 of the comparator and of 12 non-GM reference

[11]In detail, the four outcomes are category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence).

varieties was tested following the ISTA (2014) test protocol. The GMO Panel considers that the starting seed used as test material in the agronomic, phenotypic and compositional studies was of adequate quality.

*Conclusion on suitability*

The GMO Panel is of the opinion that maize DP202216, its comparator and the non-GM reference varieties were selected according to the guidelines and are considered acceptable for assessment.

### 3.4.4 | Representativeness of the receiving environments

*Selection of field trial sites*

The selected field trial sites were located in commercial maize-growing regions of United States and Canada. The soil and climate characteristics of the selected fields[12] correspond to optimal, near optimal and suboptimal conditions for maize cultivation (Sys et al., 1993). The GMO Panel considers that the selected sites, including the subset chosen for the compositional analysis, reflect commercial maize-growing regions in which the test materials could be grown.

*Meteorological conditions*

Maximum and minimum mean temperatures and sum of precipitations were provided on a daily basis. No exceptional weather conditions were reported at any of the selected field trial sites. The GMO Panel considers that the meteorological data set falls within the range of climatic conditions normally occurring at these sites.

*Management practices*

The field trials included plots containing maize DP202216, plots with the comparator and plots with non-GM reference varieties, managed according to local agricultural practices. In addition, the field trials included plots containing maize DP202216 managed following the same agricultural practices, plus exposed to the glufosinate herbicide. Glufosinate-containing herbicide was applied at BBCH 14 growth stage. The GMO Panel considers that the management practices including sowing, harvesting and application of plant protection products were appropriate for the selected receiving environments.

*Conclusion on representativeness*

The GMO Panel concludes that the geographical locations, soil and climate characteristics, meteorological conditions and the management practices of the field trials are typical of the receiving environments where the test materials could be grown.

### 3.4.5 | Agronomic and phenotypic analysis

Thirteen agronomic and phenotypic endpoints[13] plus information on abiotic stressors, disease incidence and arthropod damage were collected from the field trials (Table 3). The endpoints ear count and lodging were not analysed as described in Section 3.4.2 because of insufficient variability in the data.

The outcome of the analysis for the remaining 11 endpoints was as follows:

- For maize DP202216 (not treated with the intended herbicide), statistically significant differences with the comparator were identified for two endpoints (final population and yield), which fell under equivalence category I.
- For maize DP202216 (treated with the intended herbicide), statistically significant differences with the comparator were identified for two endpoints (plant height and yield), which fell under equivalence category I.

A statistically significant decrease in yield with respect to the comparator was observed for maize DP202216 both untreated and when treated with the intended herbicide (4% and 5% decrease, respectively). The GMO Panel considered the results for protein levels, which were tested in six of the field trial sites used for the compositional and agronomic/phenotypic analysis (Section 3.2.4). The data assessment revealed the presence of increased and extended expression of ZMM28 across most stages and sites, a result which was supported by a statistical analysis provided by the applicant (Section 3.3.4). The GMO Panel considered this as an indication that the introduced ZMM28 gene has been expressed in the plant in most

[12]Soil types of the field trials were clay loam, silty clay loam, loam, silt loam and silty clay; soil organic carbon ranged from 1.0% to 2.7%; soil pH ranged from 5.2 to 7.1. Average temperatures and sum of precipitations during the usual crop growing season ranged, respectively, from 16.0°C to 24.3°C and from 368 to 709 mm.

[13]Early stand count, days to flowering, plant lodging, plant height, final stand count, days to maturity, ear count, harvest grain moisture, 100 kernel weight, yield, kernel rows per ear, kernels per row and kernels per ear.

sites and has the potential to affect crop development, even if it was not translated into the expected YE.[14] Although uncertainty remains as to how the plant will perform when yield increase would be observed, the GMO Panel considered that the field trials and the comparative analysis can be used for the risk assessment of maize DP202216.

The impact of ZMM28 on key metabolic pathways is discussed in Section 3.4.6.

### 3.4.6 | Compositional analysis

Maize forage and grains harvested from the field trials (Table 3) were analysed for 80 different constituents (10 in forage and 70 in grains), including the key constituents recommended by the Organisation for Economic Co-operation and Development (OECD) (OECD, 2002). Twelve grain constituents having more than 50% of the observations below the limit of quantification were excluded from the statistical analysis.[15]

The test of difference and the test of equivalence could be applied to 68 constituents (10 in forage[16] and 58 in grains[17]), with the following results (Table 4):

- For maize DP202216 (not treated), statistically significant differences with the comparator for nine constituents in grains. All these constituents fell under equivalence category I or II.
- For maize DP202216 (treated), statistically significant differences were identified for nine constituents in grains. All these constituents fell under equivalence category I or II, except for stearic acid (C18:0) which fell under equivalence category IV (Table 4).

**TABLE 4** Summary of the outcome of the comparative analysis in grain and forage from maize DP202216.

| | | Test of difference[a] | | | |
|---|---|---|---|---|---|
| | | Not treated[c] | | Treated[c] | |
| | | Not different | Significantly different | Not different | Significantly different |
| **Test of equivalence**[b] | Category I/II | 54 | 9[d] | 53 | 8[d] |
| | Category III/IV | 1[e] | – | 2[e] | 1[f] |
| | Not categorised | 4[g] | – | 4[g] | – |
| | Total endpoints | 68 | | 68 | |

[a]Comparison between the GM maize and the comparator.

[b]Four different outcomes: category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence). Not categorised means that the test of equivalence was not applied because of the lack of variation among the non-GM reference varieties.

[c]Treated/not treated with glufosinate ammonium.

[d]Endpoints with significant differences between the GM maize and the comparator and falling in equivalence category I–II. For forage, none. For grains, not treated only: arginine, glycine, serine, threonine and methionine. Treated only: crude fat, carbohydrates, potassium and inositol. Both treated and not treated: crude protein, iron, thiamine and pantothenic acid.

[e]Endpoints with no significant differences between the GM maize and the comparator and falling in equivalence category III/IV. In forage, not treated only: none. Treated only: moisture. In grain, not treated only: stearic acid (C18:0). Treated only: α-linolenic acid (C18:3).

[f]Levels of stearic acid (C18:0) in grain (treated only) were significantly different between the GM maize and the comparator and fell under equivalence category IV. Quantitative results are reported in Table 5.

[g]Endpoints that were not categorised for equivalence and for which no significant differences were identified between the GM maize and the comparator. In forage, both treated and not treated: crude fibre. In grains, both treated and not treated: cystine, sodium and pyridoxine.

The GMO Panel assessed all the significant differences between maize DP202216 and its comparator, taking into account the potential impact on plant metabolism and the natural variability observed for the set of non-GM reference varieties. Quantitative results for the endpoint showing a significant difference between maize DP202216 (treated) and its comparator and falling under equivalence category IV are given in Table 5.

[14]Additional information received on 19/12/2022.

[15]These were lauric acid (C12:0), myristic acid (C14:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), eicosadienoic acid (C20:2), behenic acid (C22:0), lignoceric acid (C24:0), riboflavin, β-tocopherol, δ-tocopherol and furfural.

[16]Moisture, crude protein, crude fat, crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), ash, carbohydrates, calcium, phosphorus.

[17]Moisture, crude protein, crude fat, crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), total dietary fibre, ash, carbohydrates, alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), alpha-linolenic acid (C18:3), arachidic acid (C20:0), erucic acid (C22:1), lignoceric acid (C24:0), β-carotene, thiamine, niacin, pantothenic acid, pyridoxine, folic acid, α-tocopherol, γ-tocopherol, total tocopherols, *p*-coumaric acid, inositol, ferulic acid, phytic acid, raffinose and trypsin inhibitor.

**TABLE 5** Quantitative results (estimated means and equivalence limits) for the compositional endpoint in maize DP202216 that is further assessed based on the results of the statistical analysis.

| | | Maize DP202216 | | | Non-GM reference varieties | |
|---|---|---|---|---|---|---|
| | Endpoint | Not treated[a] | Treated[a] | P0751 | Mean | Equivalence limits |
| **Grain** | Stearic acid (C18:0) (% FA) | 2.07 | 2.10* | 2.06 | 1.65 | 1.39–1.90 |

*Note*: For maize DP202216, significantly different values are marked with an asterisk. The outcome of the test of equivalence was category IV for both GM treatments (dark grey background).

Abbreviation: %FA, percentage total fatty acid.

[a]Treated/not treated with glufosinate ammonium.

Based on the submitted dossier, the increased and extended expression of ZMM28 in maize DP202216 results in maize plants with increased plant growth, photosynthesis capacity and nitrogen utilisation. Promoters of key photosynthetic pathway components were targeted by ZMM28, as were promoters of gibberellin and auxin receptor genes. The steady-state transcript levels of several genes involved in metabolic pathways were increased in V6 transgenic leaves. This was associated with phenotypic and biochemical evidence of increased photosynthesis in V6 leaves (Wu et al., 2019).

In order to investigate potential unintended effects of the ZMM28 transcription factor on the composition of maize DP202216, the GMO Panel asked the applicant to elaborate on additional endpoints posing potential food and feed safety concerns, such as levels of the auxins[18] indolylacetic acid and indolylbutyric acid and nitrate/nitrite.[19]

Regarding gibberellin and auxin receptor genes, the applicant noted that ZMM28 regulates expression of the genes *afb2* and *gid2*, which are receptors of auxin and gibberellin, respectively (Wu et al., 2019). The regulation of *afb2* and *gid2* by ZMM28 indicates that the auxin and gibberellin signalling pathways may be modulated, while the hormone biosynthesis pathways would not. The GMO Panel considered this information sufficient.

The applicant analysed nitrate and nitrite levels in grain and forage from a field trial study conducted at eight different sites in USA and Canada in 2021. The experimental design of the study was as specified by EFSA GMO Panel (2010b, 2011a). The materials grown at each site were maize DP202216 (treated), maize DP202216 (untreated), a near-isogenic control and four non-GM reference varieties (a total of 19 varieties across the sites). Nitrate and nitrite levels in grain and nitrite levels in forage were excluded from the statistical analysis because more than 50% of the values for the GM maize and the control were below the LOQ.[20] The statistical analysis (see Section 3.4.2) was performed only for nitrate levels in forage. A statistically significant difference was identified between maize DP202216 (treated) and the control; nitrate levels for both treatments fell under equivalence category I. Therefore, this difference does not need further assessment. The GMO Panel noted high variability in nitrate levels within each site, which is consistent with the variability observed for maize in the scientific literature (Maresma et al., 2019). The GMO Panel also noted that there were forage samples with high nitrate levels (up to 11,000 mg/kg dw); however, those high values do not result from the genetic modification as they were observed in both non-GM and GM materials.

### *Conclusions on the comparative analysis*

Considering the selection of test materials, the field trial sites and associated management practices, the agronomic–phenotypic characterisation as an indicator of the overall field trial quality and the extended and increased expression of the ZMM28 protein in maize DP202216 observed across the different receiving environments, the GMO Panel concludes that the field trials are acceptable for the comparative analysis.

Taking into account the natural variability observed for the set of non-GM reference varieties, the GMO Panel concludes that:

- None of the differences identified in agronomic and phenotypic characteristics between maize DP202216 and the comparator needs further assessment.
- None of the compositional differences identified between maize DP202216 and the comparator needs further assessment except for those in levels of stearic acid (C18:0), which are considered in Section 3.5.

## 3.5 | Food/feed safety assessment

Maize DP202216 expresses the PAT protein which confers tolerance to glufosinate-ammonium-containing herbicides, and carries a transgene encoding the ZMM28 protein to extend and increase the expression of the endogenous protein in different plant tissues of maize DP202216 throughout different stages of development, including edible parts of GM maize consumed as food and feed (e.g. grains and forage).

[18]Based on safety concerns raised by EFSA before (EFSA, 2010b, 2014b).

[19]Nitrate per se is relatively non-toxic, but its metabolites and reaction products, e.g. nitrite, have raised safety concerns (EFSA, 2008; EFSA CONTAM Panel, 2020).

[20]LLOQ for nitrite in forage and grain was 0.4 mg/kg dw; LLOQ for nitrate was 0.4 mg/kg dw in forage and 1 mg/kg dw in grain.

### 3.5.1 | Effects of processing

Maize DP202216 will undergo existing production processes used for conventional maize. No novel production process is envisaged. Based on the outcome of the comparative assessment, processing of the GM maize into food and feed products is not expected to result in products being different from those of conventional non-GM reference varieties.

### 3.5.2 | Stability of the newly expressed protein and the endogenous protein with altered expression

Protein stability is one of several relevant parameters to consider in the weight-of-evidence approach in protein safety assessment (EFSA GMO Panel, 2010c, 2011a, 2017a, 2021). The term protein stability encompasses several properties such as thermal stability, pH-dependent stability, proteolytic stability and physical stability (e.g. tendency to aggregate), among others (Li et al., 2019). It has been shown, for example, that when characteristics of known food allergens are examined, one of the most prominent traits attributed to food allergens is protein stability (Breiteneder & Mills, 2005; Costa et al., 2022; Foo & Mueller, 2021; Helm, 2001).

*Effect of temperature and pH on the newly expressed protein and the endogenous protein with altered expression*

The effects of temperature and pH on PAT protein have been previously evaluated by the GMO Panel (EFSA, 2005, 2007; EFSA GMO Panel, 2018c, 2024a, 2024b). In relation to the ZMM28 protein, the deduced amino acid sequence as defined by the *zmm28* gene introduced in maize DP202216 is identical to the endogenous ZMM28 protein present in the GM plant (see Section 3.3.3.1) and no studies were provided by the applicant. Furthermore, the applicant provided information supporting a history of safe use for consumption of the ZMM28 protein (see Sections 3.5.3.1, 3.5.5.1 and 3.5.5.2).

*In vitro protein degradation by proteolytic enzymes*

In vitro protein degradation studies on PAT protein have been previously evaluated by the EFSA GMO Panel (EFSA, 2005, 2007; EFSA GMO Panel, 2018c, 2024a, 2024b). In relation to the ZMM28 protein, no studies were provided by the applicant. The deduced amino acid sequence as defined by the *zmm28* gene introduced in maize DP202216 is identical to the endogenous ZMM28 protein present in the GM plant (see Section 3.3.3.1). Furthermore, the applicant provided information supporting a history of safe use for consumption of the ZMM28 protein (see Sections 3.5.3.1, 3.5.5.1 and 3.5.5.2).

### 3.5.3 | Toxicology

#### 3.5.3.1 | *Assessment of the newly expressed protein and the endogenous protein with altered expression*

a. PAT protein

The PAT protein was previously assessed by the GMO Panel in the context of other applications (EFSA, 2005, 2007; EFSA GMO Panel, 2018, 2024a, 2024b) and no safety concerns for humans and animals (i.e. farmed and companion animals) were identified. This protein has been characterised (Section 3.3.3). Updated bioinformatics analyses revealed no similarities of the PAT protein with known toxins (Section 3.3.2). The GMO Panel is not aware of any new information that would change previous conclusion on the safety of the PAT protein.

b. ZMM28 protein

The GMO Panel assessed the toxicological profile of the ZMM28 protein taking into account all the information relevant for its source, structure, function and bioinformatic analyses and history of safe use for consumption, which represent the main types of evidence supporting the safety of the ZMM28 protein.

*Molecular characterisation*

The protein characterisation of the ZMM28 protein encoded by the *zmm28* expression cassette in maize DP202216 provided by the applicant and assessed by the Panel is described in Section 3.3.3.1.

*Bioinformatics analysis*

Bioinformatics analyses of the amino acid sequence of the ZMM28 protein revealed no significant similarities to known toxins (Section 3.3.2).

*History of safe use for consumption as food/feed*

*a. Information on the source organism*

The ZMM28 gene source organism is *Zea mays*.

b. *Information on the structure, function and mode of action*

The ZMM28 protein encoded by the *zmm28* expression cassette in maize DP202216 is a transcription factor commonly found in conventional maize (Section 3.3.3).

*c. Information on identity/homology to other proteins in conventional sources*

The ZMM28 protein encoded by the *zmm28* expression cassette in maize DP202216 grains and forage is identical to the endogenous ZMM28 protein in its conventional maize forage (R4), in non-GM sweet corn grains (R3) (Anderson et al., 2019) and in other non-GM maize grains varieties (R4). [21] Details are presented in Section 3.5.5 to further consolidate the history of safe use for consumption of the ZMM28 protein in humans and animals considering the exposure to this protein through conventional food and feed products.

*d. Conclusions on ZMM28 protein*

Based on the available information, the GMO Panel did not identify indications that the ZMM28 protein raises food and feed safety concerns in humans and animals; therefore, no additional toxicological studies are needed to conclude on the safety of this protein. Humans and animals are naturally exposed to the ZMM28 protein through consumption of non-GM food and feed maize products, and no indications of potential toxicity of ZMM28 protein have been reported (Sections 3.2 and 3.5.3).

*Synergistic or antagonistic interactions among the newly expressed protein and the endogenous protein with altered expression*

The potential for a functional interaction among the PAT and ZMM28 proteins has been assessed with regard to human and animal health. Based on current scientific knowledge on the biological function of the two proteins (Table 6), no synergistic or antagonistic interactions between these two proteins which could raise safety concerns for food and feed from maize DP202216 are expected.

### 3.5.3.2 | *Testing of new constituents other than the newly expressed protein and the endogenous protein with altered expression*

No new constituents other than the NEP and EPAE have been identified in seed and forage from maize DP202216. Therefore, no further food/feed safety assessment of components other than the NEP and EPAE is required.

**TABLE 6** Intended effects of the PAT and ZMM28 proteins in maize DP202216.

| Protein | Intended effect in GM plant |
|---|---|
| PAT | The PAT protein confers tolerance to glufosinate-ammonium-containing herbicides acting by acetylation of glufosinate ammonium |
| ZMM28 | The ZMM28 protein is a transcription factor (Castelán-Muñoz et al., 2019, Münster et al., 2002), involved in biochemical and physiological processes including photosynthesis, nitrogen assimilation and growth regulating hormone signalling |

### 3.5.3.3 | *Information on altered levels of food and feed constituents*

Stearic acid (C18:0) in grains (treated with glufosinate ammonium) was significantly different in maize DP202216 when compared with its conventional counterpart and showed a lack of equivalence with the non-GM reference varieties (Section 3.4.6). No toxicological concern is identified regarding the observed difference in this compound. Further information on safety is provided in Section 3.5.6.

[21] Additional information received on 19/12/2022 and 3/5/2023.

#### 3.5.3.4 | *Testing of the whole genetically modified food and feed*[22]

Based on the outcome of molecular characterisation and comparative analysis assessment, no compositional modifications, or indication of possible unintended effects relevant to food and feed safety have been identified for maize DP202216. Therefore, animal feeding studies with food/feed derived from maize DP202216 are not considered necessary by the GMO Panel (EFSA GMO Panel, 2011a). In accordance with Regulation (EU) No 503/2013, the applicant provided a 90-day feeding study in rats receiving diets derived from maize DP202216 which was considered by the GMO Panel.

In this study, pair-housed Crl:CD (SD) rats (16 per sex per group; 2 rats per cage)[23] were allocated to six groups using a randomised complete block design with eight replications per sex. Groups were fed diets containing 50% of incorporation rate of grains either from maize DP202216 plants treated with the intended herbicide (test material, high dose), from the conventional counterpart (control material), or one of three non-transgenic commercial reference maize hybrids.[24] An additional group was fed diet containing 33% of incorporation rate of grains from maize DP202216 treated with the intended herbicide (test material, low dose) and 17% of incorporation rate of maize grain from the conventional counterpart.

The study was adapted from OECD test guideline 408 (1998), aligned with EFSA Scientific Committee guidance (EFSA Scientific Committee, 2011) and complied with the principles of good laboratory practice (GLP) with some minor deviations not impacting the results and interpretation of the study (i.e. test item stability, homogeneity and concentration), which are detailed below. Event-specific PCR analysis confirmed the presence of the event DP202216 in both the GM maize grains and diets and excluded the presence of the event in the respective controls. ELISA analyses also confirm the presence of event DP202216 (i.e. PAT concentration) in the GM maize grains and diets. Both GM and control maize grains and diets were analysed for nutrients, antinutrients and potential contaminants (e.g. selected heavy metals, mycotoxins and pesticides). Balanced diets were formulated based on the specifications for PMI Certified Rodent LabDiet® 5002. The stability of the test and control materials was not verified; however, in accordance to product expiration declared by the diet manufacturer, the constituents of the diets are considered stable for the duration of the treatment. The GMO Panel considered this justification acceptable. Diet preparation procedures and regular evaluations of the mixing methods guaranteed the homogeneity and the proper concentration of the test or control substances in them. The applicant provided information on concentration of PAT protein in the formulated test diets, further supporting the homogeneity of the formulations. Feed and water were provided ad libitum. In-life procedures and observations and terminal procedures were conducted in accordance to OECD TG 408 (1998).

An appropriate range of statistical tests were performed on the results of the study. Detailed description of the methodology and of statistically significant findings identified in rats given diets containing grains derived from maize DP202216 is reported in Appendix C.

There were no test diet-related incidents of mortality or clinical signs. One high-dose female showed signs of distress and was sacrificed on day 71; post-mortem investigations of this animal identified a multi-focal lymphoma which is an occasional spontaneous finding in SD rats and is considered unlikely to be related to treatment (Matsushima et al., 2010). No test diet-related adverse findings were identified in any of the investigated parameters. A small number of statistically significant findings were noted, but these were not considered adverse effects of treatment for one or more of the following reasons:

- were within the normal variation[25] for the parameter in rats of this age;
- were of small magnitude;
- were identified at only a small number of time intervals with no impact on the overall value;
- exhibited no consistent pattern with related parameters or end-points.

No gross pathology findings related to the administration of the test diet were observed at necropsy, and the microscopic examinations of a wide range of organs and tissues did not identify relevant differences in the incidence and severity of the histopathological findings related to the administration of the test diet compared to the control group.

The GMO Panel concludes that this study is in line with the requirements of Regulation (EU) No 503/2013 and that no treatment-related adverse effects were observed in rats after feeding diets including 50% defatted toasted meal from DP202216 maize for 90 days.

[22]Technical dossier, Part II, Section 1.4.4.; Additional information 19/11/2020 (AI7).

[23]On test day 66, female cagemates 661 and 662 (DP202216 High; block 6) were removed from their cage on the rack and moved to a new cage on a cart. Following the death of 662 on test day 71, female 661 was returned to its original position on the rack, without a cagemate for the remainder of the study.

[24]P0760, P0589, and XL5840.

[25]Although animals used in a toxicology study are of the same strain, from the same supplier and are closely matched for age and body weight at the start of the study, they exhibit a degree of variability in the parameters investigated during the study. This variability is evident even within control groups. To help reach a conclusion on whether a statistically significant finding in a test group is treatment-related account is taken of whether the result in the test group is outside the normal range for untreated animals of the same strain and age. To do this, a number of sources of information are considered, including the standardised effect size, the standard deviations and range of values within test and control groups in the study and, if applicable, data from other studies performed in the same test facility within a small timeframe and under almost identical conditions (Historic Control Data).

### 3.5.4 | Allergenicity

The strategies to assess the potential risk of allergenicity focus: (i) on the source of the recombinant protein; (ii) on the potential of the NEP and EPAE to induce sensitisation or to elicit allergic reactions in already sensitised persons; and (iii) on whether the transformation may have altered the allergenic properties of the modified plant. Furthermore, the assessment also takes into account potential adjuvant properties of the NEP and EPAE which is defined as the ability to enhance an allergic reaction.

#### 3.5.4.1 | *Assessment of the newly expressed protein and the endogenous protein with altered expression*

A weight-of-evidence approach was followed, taking into account all the information obtained on NEP and EPAE, as no single piece of information or experimental method yielded sufficient evidence to predict allergenicity (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a, 2017a; Regulation (EU) No 503/2013).

The *pat* and *zmm28* genes originate from *S. viridochromogenes* and *Zea mays*, respectively, which are not considered allergenic sources.

Updated bioinformatic analyses of the amino acid sequences of the PAT and ZMM28 proteins, using the criterion of 35% identity in a sliding window of 80 amino acids, revealed no relevant similarities to known allergens. The studies on protein stability of the PAT protein have been described in Section 3.5.2. The ZMM28 protein is identical to the endogenous protein in maize (see Section 3.3.3.1). The applicant provided information supporting a history of safe use for consumption of the ZMM28 protein in humans and animals with no indications of allergenicity (see Sections 3.5.3.1, 3.5.5.1 and 3.5.5.2). Therefore, no in vitro studies were provided by the applicant (see Section 3.4.2). Moreover, the GMO Panel did not find an indication that the proteins PAT and ZMM28 at the levels expressed in maize DP202216 might be adjuvants.

Furthermore, the applicant provided information on the safety of the PAT protein regarding its potential hazard to cause a coeliac disease response. For such assessment, the applicant followed the principles described in the EFSA GMO Panel guidance document (EFSA GMO Panel, 2017a). The assessment of the PAT protein revealed partial matches containing the Q/E-X1-P-X2 motif and required further investigations. Based on additional considerations on position and nature of amino acids flanking the ELPA[26] motif, such as the presence of two consecutive prolines and the charge and size of adjacent amino acids (EFSA GMO Panel, 2017a), the two relevant peptides containing the motif do not raise concern as they fail to mimic gluten sequences. Therefore, no indications of safety concerns were identified by the GMO Panel.

In the context of this application, the GMO Panel considers that there are no indications that the PAT and/or ZMM28 proteins in maize DP202216 may be allergenic.

#### 3.5.4.2 | *Assessment of allergenicity of the whole GM plant*

The GMO Panel regularly reviews the available publications on food allergy to maize. However, maize is not considered a common allergenic food[27] (OECD, 2002). Therefore, the GMO Panel does not request experimental data to analyse the allergen repertoire of GM maize.

In the context of this application and considering the data from the molecular characterisation, the compositional analysis and the assessment of the NEP and EPAE (see Sections 3.3, 3.4.6 and 3.5.3), the GMO Panel identifies no indications of a potentially increased allergenicity of food and feed derived from maize DP202216 with respect to that derived from the comparator.

### 3.5.5 | Dietary exposure assessment to new constituents

In line with Regulation (EU) No 503/2013, the applicant provided dietary exposure estimates to PAT and ZMM28 proteins in maize DP202216. Dietary exposure was estimated based on protein expression levels reported in this application for maize DP202216 treated with the intended herbicide, the current available consumption data and feed practices, the foods and feeds currently available in the market and the described processing conditions.

For the analysis of PAT and ZMM28 proteins in maize DP202216, samples were collected during the 2017 growing season at six sites in commercial maize-growing regions in USA and Canada. All analysed samples (grains, forage, pollen, etc.) were obtained from DP202216 maize treated with glufosinate. Table 1 in Section 3.3.4. shows the protein expression levels initially considered to estimate both human and animal dietary exposure. The expression of ZMM28 protein was also investigated in grains from six representative varieties of sweet corn at growth stage R3 as they are typically consumed as food.[28] In the six varieties of sweet corn, the predominant isoform of ZMM28 protein was B73, with the amino acid sequence of this

[26]E: Glutamic acid; L: Leucin; P: Proline; Y: Tyrosine.

[27]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 608/200.

[28]Study report PHI-R028-Y17, additional information: January 2020.

isoform being identical to that of the ZMM28 protein in DP202216 maize. Mean levels of ZMM28 protein across the six varieties of sweet corn ranged from 0.0017 to 0.019 ng/mg fw with the highest mean concentration reported in the variety 'Country Gentleman'.[29] The highest levels of ZMM28 protein were also found in the same sweet corn variety (0.033 ng/mg fw). Quantification of ZMM28 protein was carried out using a western blot method since ELISA showed matrix-related issues in grains. Western blot is considered as a semi-quantitative method which adds uncertainty to the reported values of ZMM28 protein in sweet corn.

#### 3.5.5.1 | *Human dietary exposure*

Human dietary exposure was estimated across different European countries on different population groups: young population (infants, toddlers, 'other children'), adolescents, adult population (adults, elderly and very elderly) and special populations (pregnant and lactating women). Since no specific consumption data were available on commodities containing, consisting of or obtained from maize DP202216 grains, a conservative scenario with 100% replacement of conventional maize by the GM maize was considered. Consumption figures for all relevant commodities (e.g. corn flakes, sweet corn, popcorn, etc.) were retrieved from the EFSA Comprehensive European Food Consumption Database (EFSA consumption database).[30] Corn oil was excluded from the assessment since no proteins are expected to be present in the oil. The applicant followed the methodology described by EFSA to estimate chronic and acute dietary exposure in the highly exposed population using summary statistics (EFSA, 2019). As indicated in the 2019 EFSA statement, the consumption data reported for processed commodities were converted into amounts of raw primary commodities before estimating dietary exposure, using the factors available in the EFSA website.[29]

*PAT protein*

For the highly exposed population, the highest estimated anticipated chronic dietary exposure to PAT protein following the consumption of maize DP202216 and derived commodities was 68.9 μg/kg bw per day, in infants. The highest anticipated acute dietary exposure, also in the highly exposed population, was identified in the age class 'Other children' with estimates of 155 μg/kg bw per day.

*ZMM28 protein*

For the highly exposed population, the highest anticipated estimated chronic dietary exposure to ZMM28 protein following the consumption of maize DP202216 and derived commodities was 0.055 μg/kg bw per day, in infants. The highest anticipated acute dietary exposure, also in the highly exposed population, was identified in the age class 'Other children' with estimates of 0.124 μg/kg bw per day.

A comparative dietary exposure assessment was conducted to assess whether the history of safe use for consumption of the ZMM28 protein via the consumption of conventional sweet corn covers the exposure to this protein through the consumption of DP202216 maize and derived commodities. Sweet corn is a commodity commonly consumed in Europe; among 'consumers only', the average long-term consumption goes from 2.1 to 54.1 g/day across different European countries, with high consumption (95th percentile) up to 116.7 g/day. Additionally, short-term (acute) consumption of sweet corn in 'consumers only' ranges from 8.6 to 160 g/day in average consumers while high consumers (95th percentile) can consume as much as 350 g/day.[31] Therefore, the European population is typically exposed to ZMM28 protein through the consumption of conventional sweet corn.

The applicant provided diverse dietary exposure estimations for ZMM28 protein making use of summary statistics of consumption from the EFSA consumption database. The maximum dietary exposure estimations to ZMM28 protein considering the consumption of conventional sweet corn alone were comparable to maximum estimates considering the consumption of conventional sweet corn together with DP202216 maize-derived commodities. This means that the intake of ZMM28 protein from DP202216 maize is covered by the history of safe use of ZMM28 protein.

Even though the applicant states that this event will not be commercialised in sweet corn or popcorn varieties, this scenario was also assessed. Since no hypothesis exists on a potential effect/interaction of the introduced *zmm28* gene on the expression of the endogenous ZMM28 gene, the increase in the ZMM28 levels in sweet corn varieties can be expected to be similar to that observed in DP202216 maize. This increase would have low impact on sweet corn varieties with relatively high levels of ZMM28 protein (e.g. the variety 'Country Gentleman') and, therefore, on the overall dietary exposure.

The history of safe use for consumption of the ZMM28 protein is further supported by the high similarity of ZMM28 proteins expressed in different foods (e.g. rice) with the ZMM28 protein in maize.

[29]Mean values are derived from quantified samples and samples with levels below the LOQ (0.0017 ng/mg fw) where half of the LOQ was used.

[30]https://www.efsa.europa.eu/en/food-consumption/comprehensive-database Date accessed: April 2019.

[31]https://www.efsa.europa.eu/en/food-consumption/comprehensive-database. Accessed December 2021.

*Ad hoc scenario on pollen supplements*

An ad hoc dietary exposure scenario was carried out for consumers of pollen supplements under the assumption that these supplements might be made of pollen from maize DP202216. PAT and ZMM28 levels in pollen as described in Table 1 (Section 3.3.4) were used to derive concentrations in pollen supplements considering around 6% moisture content. Consumption data on pollen supplements are available for few consumers across eight different European countries.[32] The low number of consumers available adds uncertainty to the exposure estimations which should be interpreted with care, and only allows the estimation of mean dietary exposure. The highest mean acute dietary exposure would be 53.7 and 0.010 μg/kg bw per day in the elderly population for PAT and ZMM28 proteins, respectively. Similarly, the highest mean chronic dietary exposure in consumers of pollen supplements would be 35.8 and 0.007 μg/kg bw per day also in the elderly population for PAT and ZMM28 proteins, respectively.

### 3.5.5.2 | *Animal dietary exposure*

Dietary exposure to PAT and ZMM28 proteins in maize DP202216 was estimated across different animal species, as below described, assuming the consumption of maize products commonly entering the feed supply chain (i.e. grain and forage). A conservative scenario with 100% replacement of conventional maize products by the maize DP202216 products was considered.

Mean levels (dry weight) of PAT and ZMM28 proteins in grains and forage from the maize DP202216 treated with the intended herbicide used for dietary exposure are listed in Table 1 in Section 3.3.4. Some of the grain and forage samples analysed in maize DP202216 for the presence of ZMM28 protein were below the limit of quantification (LOQ) (respectively, 0.0069 and 0.036 ng/mg dry weight); for the purpose of estimating dietary exposure, a value equal to half the LOQ value was assigned to those samples to calculate the mean.

The applicant estimated dietary exposure to PAT and ZMM28 proteins via the consumption of maize grains in chicken for fattening, laying hen, turkey for fattening, pig for fattening, sow lactating, cattle for fattening, dairy cow, sheep/goat, salmon, dog, cat and maize forage in laying hen, sow lactating, cattle for fattening and dairy cow, based on body weights and daily feed intakes as recommended by EFSA (EFSA FEEDAP Panel, 2017) and inclusion rates of maize grains and forage in diets, as recommended by OECD for the EU livestock population (OECD, 2013). Inclusion rates of maize grains in salmon diets (10%) were based on FAO recommendations,[33] while those in dogs and cats (45% and 25%) on additional information provided by the applicant.[34]

Estimated dietary exposures based on the consumption of maize grains and forage are reported in Appendix D.

The presence of the ZMM28 protein in non-GM maize feed products was taken into account to confirm the likely exposure in farmed and companion animals. The levels of the ZMM28 protein in non-GM maize R4 forage were quantifiable in 11 out of the 24 samples analysed in a field trial across six locations in USA and Canada during the 2017 growing season (Section 3.3.4). Furthermore, the presence of the ZMM28 protein was also reported in non-GM maize R4 grain (Wu et al., 2019, supplementary information) and levels were quantifiable in 9 out of the 12 samples analysed in a field trial across two locations in USA during the 2016 growing season.[35] However, the levels of the ZMM28 protein in non-GM R6 grains were not quantifiable in all 24 samples analysed in a field trial across six locations in USA and Canada during the 2017 growing season (Section 3.3.4).

Based on the information available, the GMO Panel confirms that farmed and companion animals, including herbivorous animals, e.g. ruminants and horses, but also gestating and lactating sow and laying hens (Ebertz et al., 2020; EFSA GMO Panel, 2023c; OECD, 2009, 2013; Steenfeldt et al., 2007), are naturally exposed to background levels of ZMM28 protein through consumption of rations containing maize R4 forage/silage.

The absence of ZMM28 protein reported in non-GM maize R6 grains does not allow the GMO Panel to conclude on a natural exposure to background levels of ZMM28 protein for those farmed and companion animals whose diets mainly consist of maize feed products and by-products based on R6 grains (e.g. fattening pig, broiler). The presence of ZMM28 in maize R4 grain cannot be used to support exposure to ZMM28 protein in these animals, since maize R4 grains and derived products are uncommon feed materials, even if on some occasions they can be used (Iverson & Thaler, 1996; OMAFRA, 2022; Patience et al., 2002).

The GMO Panel does not find indications of safety concern regarding the presence of ZMM28 protein in maize DP202216, being able to extrapolate the safe consumption to all farmed and companion animals, considering that: (i) information is available on natural exposure to ZMM28 protein for several animal species including herbivorous, birds and monogastric (mammals and humans) through consumption of maize R4 forage/silage and R3 sweet corn; (ii) the ZMM28 protein is identical to the endogenous protein; and (iii) no indications of potential toxicity of ZMM28 protein have been reported (see Sections 3.2 and 3.5.3).

[32] https://www.efsa.europa.eu/en/food-consumption/comprehensive-database. Accessed February 2021.

[33] https://www.fao.org/fishery/affris/species-profiles/atlantic-salmon/tables/en/–Atlantic salmon–table 3. Due to the absence of a corn grain inclusion values, the corn gluten meal value were used, resulting in a conservative estimate for salmon.

[34] Additional information: 26/4/2021.

[35] Additional information: 19/12/2022 and 12/6/2023.

### 3.5.6 | Nutritional assessment of endogenous constituents

The intended traits of maize DP202216 are enhanced grain yield potential and herbicide tolerance, with no intention to alter nutritional parameters. However, levels of stearic acid (C18:0) in grains from maize DP202216 (treated) were significantly different from its conventional counterpart and showed a lack of equivalence (both treated and not treated) with the set of non-GM reference varieties (Section 3.4.6) The biological relevance of stearic acid, the role of maize as contributor to its total intake and the magnitude and direction of the observed changes were considered during the nutritional assessment.

#### 3.5.6.1 | *Human nutrition*

Stearic acid is one the most commonly consumed saturated fatty acids together with palmitic acid (C16:0). Stearic acid is a minor fatty acid in maize oil representing approximately 2% of the total fatty acids; a barely 2% increase in the levels of stearic acid in grains from maize DP202216 (treated) was observed as compared to the levels in the conventional counterpart. After considering the extent of this increase and the limited role of maize and maize-based products as a source of stearic acid in the human diet, the GMO Panel concludes that the consumption of maize DP202216 and derived products does not raise any nutritional concern.

#### 3.5.6.2 | *Animal nutrition*

Stearic acid, a minor and not essential fatty acid for animals represents approximately 2% of the total fatty acids in maize grains and forage (lipids are below 4%). Fatty acid requirements in farmed and companion animals are mainly covered by other ingredients through the administration of balanced rations and diets formulations. After considering the extent of the increase (about 2%) in the levels of stearic acid in maize DP202216 (treated) compared to the levels in the conventional counterpart and the limited role of maize and maize-based products as a source of stearic acid in the animal diet, the GMO Panel concludes that the consumption of maize DP202216 and derived products does not raise any nutritional concern.

### 3.5.7 | Post-market monitoring of GM food and feed

Maize DP202216, as described in this application, does not raise any nutritional concern and is as safe as its conventional counterpart and the non-GM reference varieties tested. The GMO Panel concludes that, based on the information considered in its safety assessment, a post-market monitoring plan for food and feed is not necessary.

### 3.5.8 | Conclusion on the food and feed safety assessment

The newly expressed PAT protein and the endogenous ZMM28 protein with altered expression in maize DP202216 do not raise safety concerns for human and animal health. No interactions between the newly expressed PAT protein and the endogenous ZMM28 protein with altered expression relevant for food and feed safety were identified. Moreover, the GMO Panel did not identify indications of safety concerns regarding allergenicity or adjuvanticity related to the presence of the newly expressed PAT protein and the endogenous ZMM28 protein with altered expression. The GMO Panel found no evidence that the genetic modification impacts the overall safety of maize DP202216. Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the consumption of maize DP202216 does not represent any nutritional concern, in the context of the scope of this application. The GMO Panel concludes that maize DP202216, as described in this application, is as safe as the comparator and the non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

## 3.6 | Environmental risk assessment and monitoring plan[36]

### 3.6.1 | Environmental risk assessment

Considering the scope of application EFSA-GMO-NL-2019-159, which excludes cultivation, the environmental risk assessment (ERA) of maize DP202216 mainly takes into account: (1) the exposure of microorganisms to recombinant DNA in the gastrointestinal tract of animals fed with GM material and of microorganisms present in environments exposed to faecal material of these animals (manure and faeces); and (2) the accidental release into the environment of GM material, including viable maize DP202216 grains, during transportation and/or processing (EFSA GMO Panel, 2010a).

[36]Dossier: Parts 5 and 6; additional information 12/9/2022.

#### 3.6.1.1 | *Persistence and invasiveness of the GM plant*

Maize is highly domesticated, not winter hardy in colder regions of Europe, and generally unable to survive in the environment without appropriate management. Survival is limited mainly by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and cold climate conditions (OECD, 2003), even though occasional feral GM maize plants may occur outside cultivation areas in the EU (e.g. Pascher, 2016). Field observations indicate that maize grains may survive and overwinter in some EU regions, resulting in volunteers in subsequent crops (e.g. Gruber et al., 2008; Palaudelmàs et al., 2009; Pascher, 2016). However, maize volunteers have been shown to grow weakly and flower asynchronously with the maize crop (Palaudelmàs et al., 2009). Thus, the establishment and survival of feral and volunteer maize in the EU is currently limited and transient.

It is unlikely that the intended traits of maize DP202216 will provide a selective advantage to maize plants, except when they are exposed to glufosinate-containing herbicides. However, this fitness advantage will not allow the GM plant to overcome other biological and abiotic factors (described above) limiting plant's persistence and invasiveness. Due to the scope of the application, which excludes cultivation, the potential yield enhancement trait was not considered as a concern.

In conclusion, the GMO Panel considers it unlikely that maize DP202216 will differ from conventional maize hybrid varieties in its ability to survive until subsequent seasons, or to establish occasional feral plants under European environmental conditions in case of accidental release into the environment of viable maize DP202216 grains.

#### 3.6.1.2 | *Potential for gene transfer*

##### 3.6.1.2.1 | *Plant to microorganism gene transfer*

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

Genomic DNA can be a component of food and feed products derived from maize. It is well documented that such DNA becomes substantially degraded during processing and digestion in the human or animal gastrointestinal tract. However, bacteria in the digestive tract of humans and animals, and in other environments, may be exposed to fragments of DNA, including the recombinant fraction of such DNA.

Current scientific knowledge of recombination processes in bacteria suggests that horizontal transfer of non-mobile, chromosomally located DNA fragments between unrelated organisms (such as from plants to bacteria) are not likely to occur at detectable frequencies under natural conditions (for further details, see EFSA, 2009).

Homologous recombination is known to facilitate horizontal transfer of non-mobile, chromosomal DNA fragments to bacterial genomes. This requires the presence of at least two stretches of DNA sequences that are similar in the recombining DNA molecules. In the case of sequence identity with the transgene itself, recombination would result in gene replacement. In the case of identity with two or more regions flanking recombinant DNA, recombination could result in the insertion of additional DNA sequences in bacteria and thus confer the potential for new properties.

In addition to homology-based recombination processes, at a lower transformation rate, the non-homologous end joining and microhomology-mediated end joining are theoretically possible (EFSA, 2009; Hülter & Wackernagel, 2008). Independently of the transfer mechanism, the GMO Panel did not identify a selective advantage that a theoretical HGT would provide to bacterial recipients in the environment.

The bioinformatic analysis for event DP202216 revealed no homology with known DNA sequences from bacteria which would facilitate homologous recombination. The analysis confirmed that the genetic elements encoding for PAT protein were plant codon-optimised and did not provide sufficient sequence identity to bacterial DNA.

In summary, there is no indication for an increased likelihood of horizontal transfer of DNA from maize DP202216 to bacteria. Given the nature of the recombinant DNA, the GMO Panel identified no safety concern linked to an unlikely but theoretically possible HGT.

##### 3.6.1.2.2 | *Plant to plant gene transfer*

The potential for occasional feral maize DP202216 plants originating from grain 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 maize grains need to germinate and develop into plants in areas containing sympatric wild relatives and/or cultivated maize with synchronous flowering and environmental conditions favouring cross-pollination.

Maize is an annual predominantly cross-pollinating crop. Cross-fertilisation occurs mainly by wind (OECD, 2003). Vertical gene transfer from maize is limited to *Zea* species. Wild relatives of maize outside cultivation are not known/reported in Europe (Eastham & Sweet, 2002; EFSA, 2016, 2022; OECD, 2003; Trtikova et al., 2017). Therefore, potential vertical gene transfer is restricted to maize and weedy *Zea* species, such as teosintes, and/or maize-teosinte hybrids, occurring in cultivated areas (EFSA, 2016, 2022; Le Corre et al., 2020; Trtikova et al., 2017).

The potential of spilled maize grains to establish, grow and produce pollen is extremely low and transient (see Section 3.6.1.1). Therefore, the likelihood/frequency of cross-pollination between occasional feral GM maize plants resulting from grain spillage, and weedy or cultivated *Zea* plants is considered extremely low (EFSA, 2016, 2022). Even if cross-pollination would occur, the GMO Panel is of the opinion that environmental effects as a consequence of the spread of genes from occasional feral GM maize plants in Europe will not differ from that of conventional maize varieties for the reasons given in Section 3.6.1.1, even if exposed to the intended herbicide.

#### 3.6.1.3 | *Interactions of the GM plant with target organisms*

Taking the scope of application EFSA-GMO-NL-2019-159 (no cultivation) and the absence of target organisms into account, potential interactions of occasional feral maize DP202216 plants arising from grain import spills with target organisms are not considered a relevant issue.

#### 3.6.1.4 | *Interactions of the GM plant with non-target organisms*

Environmental exposure of non-target organisms to spilled GM maize material or occasional feral GM maize plants arising from spilled maize DP202216 seeds will be limited. Additionally, ingested proteins are typically degraded before entering the environment through faecal material of animals fed with GM maize (Harmon & Swanson, 2020; Miner-Williams et al., 2014; Mok & Urschel, 2020; Santos-Hernández et al., 2018; van Bruchem et al., 1985), and the data provided for the assessment of protein stability (see Section 3.5.2.) support that the NEPs will also be degraded. Given the limited environmental exposure, the GMO Panel considers that potential interactions of maize DP202216 with non-target organisms do not raise any environmental safety concern.

#### 3.6.1.5 | *Interactions with abiotic environment and biogeochemical cycles*

Environmental exposure to spilled GM maize material or occasional feral GM maize plants arising from spilled maize DP202216 seeds will be limited. Additionally, ingested proteins are typically degraded before entering the environment through faecal material of animals fed with GM maize (Harmon & Swanson, 2020; Miner-Williams et al., 2014; Mok & Urschel, 2020; Santos-Hernández et al., 2018; van Bruchem et al., 1985), and the data provided for the assessment of protein stability (see Section 3.5.2) support that the NEPs will also be degraded. Given the limited environmental exposure, the GMO Panel considers that potential interactions of maize DP202216 with non-target organisms do not raise any environmental safety concern.

### 3.6.2 | Post-market environmental monitoring

The objectives of a post-market environmental monitoring (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 rationale of the PMEM plan provided by the applicant (EFSA GMO Panel, 2011b).

As the ERA did not identify potential adverse environmental effects from maize DP202216, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for maize DP202216 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 GMOs on human health and the environment; (2) a coordinating system established by CropLife Europe 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 for the duration 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 maize DP202216. The GMO Panel agrees with the reporting intervals proposed by the applicant in its PMEM plan.

#### 3.6.2.1 | *Conclusion of the environmental risk assessment and monitoring plan*

The GMO Panel concludes that it is unlikely that maize DP202216 would differ from conventional maize varieties in its ability to persist under European environmental conditions. Considering the scope of application EFSA-GMO-NL-2019-159, interactions of occasional feral maize DP202216 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from maize DP202216 to bacteria does not indicate a safety concern. Therefore, considering the introduced traits, the outcome of the agronomic and phenotypic analysis and the routes and levels of exposure,

the GMO Panel concludes that maize DP202216 would not raise safety concerns in the event of accidental release of GM material, including viable GM maize grains, into the environment.

The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of maize DP202216.

## 4 | OVERALL CONCLUSIONS

The GMO Panel was asked to carry out a scientific assessment of maize DP202216 for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003. The molecular characterisation data establish that the GM maize DP202216 contains a single insert, consisting of one copy of the *mo-pat* and *zmm28* expression cassettes. The quality of the sequencing methodology and data sets was assessed by the EFSA GMO Panel and is in compliance to the requirements listed in the EFSA Technical Note, 2018a. Updated bioinformatic analyses of the sequences encoding the newly expressed proteins and other ORFs present within the insert or spanning the junctions between the insert and genomic DNA, do not raise any safety concerns. The stability of the inserted DNA and of the introduced traits is confirmed over several generations. The methodology used to quantify the levels of the PAT and ZMM28 proteins is considered adequate. The protein characterisation data comparing the DP202216-produced PAT to those previously assessed in other maize events indicate that these proteins are equivalent. The protein characterisation data comparing the DP202216-produced ZMM28 to those in conventional maize varieties indicate that these proteins are identical.

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic–phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis. None of the identified differences in the agronomic/phenotypic and compositional characteristics tested between maize DP202216 and its conventional counterpart needed further assessment, except for the levels of stearic acid (C18:0) which do not raise nutritional and safety concerns. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of PAT and ZMM28 proteins as expressed in maize DP202216. The GMO Panel finds no evidence that the genetic modification impacts the overall safety of maize DP202216. In the context of this application, the consumption of food and feed from maize DP202216 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize DP202216 is as safe as the conventional counterpart and non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary. The GMO Panel concludes that there is a very low likelihood of environmental effects resulting from the accidental release of viable grains from maize DP202216 into the environment. The PMEM plan and reporting intervals are in line with the intended uses of maize DP202216.

Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issues pertaining to the intended uses of maize DP202216.

In addition, the GMO Panel considered the additional unpublished study listed in Appendix A. This new information does not raise any concern for human and animal health and the environment.

In conclusion, the GMO Panel considers that maize DP202216, as described in this application, is as safe as its conventional counterpart and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment.

## 5 | DOCUMENTATION AS PROVIDED TO EFSA

- Letter from the Competent Authority of the Netherlands received on 3 July 2019 concerning a request for authorization of the placing on the market of genetically modified maize DP202216 submitted in accordance with Regulation (EC) No 1829/2003 by Pioneer Overseas Corporation (EFSA Ref. EFSA-GMO-NL-2019-159; EFSA-Q-2019-00419).
- The application was made valid on 23 September 2019.
- Additional information (1) was requested on 8 November 2019.
- Additional information (1) was received on 16 January 2020.
- Additional information (2) was requested on 23 December 2019.
- Additional information (2) was received on 25 February 2020.
- Additional information (3) was requested on 22 January 2020.
- Additional information (3) was received on 24 January 2020.
- Additional information (4) was requested on 13 February 2020.
- Additional information (4) was received on 26 June 2020.
- Additional information (5) was requested on 7 April 2020.
- Additional information (5) was received on 5 June 2020.
- Additional information (6) was requested on 7 August 2020.
- Additional information (6) was received on 17 August 2020.
- Additional information (7) was requested on 24 September 2020.
- Additional information (7) was received on 19 November 2020.
- Additional information (8) was requested on 10 October 2020.

- Additional information (8) was received on 11 November 2022.
- Additional information (9) was requested on 9 February 2021.
- Additional information (9) was received on 23 April 2021.
- Additional information (10) was requested on 7 June 2021.
- Additional information (10) was received on 27 August 2021.
- Additional information (11) was requested on 21 December 2021.
- Additional information (11) was received on 14 January 2022.
- Additional information (12) was requested on 16 September 2022.
- Additional information (12) was received on 19 December 2022.
- Additional information (13) was requested on 21 December 2022.
- Additional information (13) was received on 27 March 2023.
- Additional information (14) was requested on 16 February 2023.
- Additional information (14) was received on 3 May 2023 partial; 12 June 2023 complete.
- Additional information (15) was requested on 13 April 2023.
- Additional information (15) was received on 9 June 2023.
- Additional information (16) was requested on 17 July 2023.
- Additional information (16) was received on 7 November 2023.
- Supplementary information was provided on voluntary basis on 16 January 2020; 23 March 2021; 27 August 2021 and 19 December 2022.

## ABBREVIATIONS

| | |
|---|---|
| ATG | in-frame translational start codons |
| bw | body weight |
| CRM | comparative relative maturity |
| dw | dry weight |
| ELPA | Glutamic acid, Leucin, Proline and Tyrosine |
| EPAE | endogenous protein with altered expression |
| ERA | environmental risk assessment |
| FA | Fatty acid |
| fw | fresh weight |
| GLP | good laboratory practice |
| GMO | genetically modified organism |
| HGT | horizontal gene transfer |
| HR | homologous recombination |
| HT | herbicide tolerant |
| LOQ | limit of quantification |
| NEP | newly expressed protein |
| OECD | Organisation for Economic Co-operation and Development |
| ORFs | open reading frames |
| PAT | phosphinothricin acetyltransferase enzyme |
| PMEM | post-market environmental monitoring |
| PMI | phosphomannose isomerase |
| PYE | potential yield enhancement |
| qPCR | qualitative polymerase chain reaction |
| SbS | Southern-by-Sequencing |
| T-DNA | transfer-deoxyribonucleic acid |
| UTR | untranslated region |
| YE | Yield Enhancement |

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

## REQUESTOR

Competent Authority of The Netherlands

## QUESTION NUMBER

EFSA-Q-2019-00419

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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# APPENDIX A

## Additional studies

| | |
|---|---|
| PHI-2017-135 | Evaluation of germination and viability of maize lines containing event DP-2Ø2216-6 |
| PHI-2018-033[a] | Nutritional Equivalency Study of Maize Grain DP-2Ø2216-6 – Poultry Feeding Study |
| PHI-2018-038 DP202216[a] | 8-week channel catfish dietary tolerance study: catfish study still to be assessed as additional study |
| PHI-2018-084 DP202216 | Evaluation of Zea m 14 Concentrations in Grain from a Maize Line Containing DP-2Ø2216-6 Using Ultra Performance Liquid Chromatography–Tandem Mass Spectrometry (UPLC-MS/MS) |
| PHI-2017-042/700 | Evaluation of Seed Germination, Health, and Viability of DP-2Ø2216-6 Maize Seed Lots Identified for use in Regulatory Science Studies |

[a]The GMO Panel notes that the submitted study report contained limited details about the materials and methods used for the production of the test diets. As the study was not a requirement for the EU, clarification of the limitations was not sought.

# APPENDIX B

## List of relevant publications identified by the applicant through systematic literature searches (January 2009 to June 2023)

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# APPENDIX C

## Statistical analysis and statistically significant findings in in vivo toxicity and feeding studies

### C.1 | Statistical analysis of the 90-day study on DP202216 maize in rats

The following endpoints were statistically analysed: body weight, body weight gain, feed consumption and feed efficiency, forelimb and hindlimb grip strength, motor activity data, haematology values, coagulation values, clinical chemistry and urinalysis values, absolute and relative organ weights and functional observation battery data.
For all continuous endpoints, mean and standard deviation were provided for each dose group for each sex, variable and period or time interval. In the statistical analysis, rats consuming the low- and high-dose test diets were compared with those consuming the control diet. For continuous parameters, a linear mixed model was applied to data for individual animals for the two sexes combined (fixed effects: diet, sex and sex-by-diet interaction; random effects: block and cage). Test–control comparisons were done both across sexes and separately for males and females; in case a significant sex-by-diet interaction was identified, only the sex-specific results were considered for the assessment. The model was modified as needed for the analysis of sex-specific endpoints and cage-level data (food consumption and food efficiency). Ranges of variability for the parameters were calculated from data for the three reference groups and from historical control data. For each comparison, point estimates and 95% confidence intervals of the standardised effect size were reported to aid the assessment. With the exception of pH (which was analysed as a continuous endpoint), the data for discrete endpoints were not analysed statistically because of lack of variability. Missing data were considered by the Panel and found not to affect the results (Table C.1).

**TABLE C.1** Statistically significant findings in 90-day study on DP202216 maize in rats.

| Statistically significant parameter/endpoint | Finding | GMO panel interpretation |
|---|---|---|
| Body weight gain, food consumption, feed efficiency | Statistically significant increases and decreases at different time periods (±100%) | Sporadic changes at individual time points. No impact on body weight or body weight gain over the entire study period. Not an adverse effect of treatment |
| Forelimb grip strength | Statistically significant decrease in top dose animals (10%) | Low magnitude, within normal variation, evident pre-dosing and consistent with reference diet animals. Not an adverse effect of treatment |
| Red blood cell count, haemoglobin concentration and haematocrit | Statistically significant increase in low-dose animals (3%) | Low magnitude, not adverse in isolation, within normal variation not seen in high-dose animals and consistent with reference diet animals. Not an adverse effect of treatment |
| Sorbitol dehydrogenase activity | Statistically significant increase in low-dose males (100%) | Within normal variation, not evident in the high-dose males, no consistent pattern in other liver marker enzymes and no liver pathology findings. Not an adverse effect of treatment |
| BUN | Statistically significant decrease in low-dose females (15%) | Low magnitude, within normal variation (control value high, driven by one animal), not adverse in isolation. Not an adverse effect of treatment |
| Triglycerides | Statistically significant increase in high-dose males (25%) | Within normal variation, not present in females. Not an adverse effect of treatment |
| Inorganic phosphate | Statistically significant decrease in low-dose females (7%) | Within normal variation, not evident in the high-dose groups. Not an adverse effect of treatment |
| Potassium | Statistically significant increase in low-dose males (7%) | Low magnitude, not present in high-dose males. Not an adverse effect of treatment |
| Chloride | Statistically significant decrease in low-dose females | Low magnitude (< 1%), within normal variation not present in high-dose animals. Not an adverse effect of treatment |
| Heart, spleen and thymus weights (absolute and relative to brain weights) | Statistically significant increase in high-dose males (10%–25%) | Partially related to a higher body weight, not significant relative to body weight and with no associated pathological findings. Not an adverse effect of treatment |
| Adrenal weights (absolute and relative to brain and body weights) | Statistically significant increase in females (10%–20%) | Moderate magnitude, control values below reference group values (~ 5%–10%), not seen in males, no associated pathological findings. Not an adverse effect of treatment |
| Liver weight relative to body weight | Statistically significant increase in low-dose females (6%) and decrease in high-dose males (6%) | Low magnitude, not significant in high-dose females or low-dose males or both sexes combined; not significant for absolute weight or relative to brain weight. No associated clinical chemistry or pathological findings. Not an adverse effect of treatment |

# APPENDIX D

## Animal dietary exposure

**TABLE D.1** Dietary exposure to PAT and ZMM28 proteins protein in selected animals, based on the consumption of maize grain and forage.

| | Dietary exposure (mg/kg bw per day) | | | | | |
|---|---|---|---|---|---|---|
| | ZMM28 | | | PAT | | |
| | Grain (G) | Forage (F) | G + F | Grain (G) | Forage (F) | G + F |
| **Chicken for Fattening** | 0.00066 | NA | NA | 0.88 | NA | NA |
| **Laying hen** | 0.00045 | 0.00035 | 0.00080 | 0.59 | 0.20 | 0.79 |
| **Turkey for fattening** | 0.00035 | – | – | 0.47 | – | – |
| **Pig for fattening** | 0.00031 | – | – | 0.41 | – | – |
| **Sow lactating** | 0.00025 | 0.00040 | 0.00065 | 0.34 | 0.23 | 0.56 |
| **Cattle for fattening** | 0.00019 | 0.000106 | 0.00125[a] | 0.26 | 0.61 | 0.86[a] |
| **Dairy cow** | 0.00011 | 0.00123 | 0.00134 | 0.15 | 0.71 | 0.86 |
| **Sheep/goat** | 0.00007 | – | – | 0.10 | – | – |
| **Salmon** | 0.00002 | – | – | 0.03 | – | – |
| **Dog** | 0.00009 | – | – | 0.12 | – | – |
| **Cat** | 0.00006 | – | – | 0.08 | – | – |

Abbreviation: –, forage not included in the daily ration.

[a]For DP202216 maize grain+forage combination replacement scenario, the inclusion rate for cattle for fattening would be 160% of the diet; therefore, the exposure reported to each protein is an overestimation.