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Submission on the Assessment Report for

Application A1063 – Food derived from herbicide tolerant soybean line MON 87708 by Monsanto Europe S.A.

Submitted to Food Standards Australia/New Zealand (FSANZ)
by

Submitter: Centre for Integrated Research in Biosafety and GenØk – Centre for Biosafety
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Introduction

This submission from the Centre for Integrated Research in Biosafety (INBI) is in response to an invitation from Food Standards Australia/New Zealand (FSANZ) to comment on application A1063. A1063 is an application to amend the Australia New Zealand Food Standards Code to allow foods derived from corn line MON 87708 into the human food supply. In this case, dicamba mono-oxygenase (DMO) from *Stenotrophomonas maltophilia* is produced in soybean to confer tolerance to the herbicide dicamba (3,6-dichloro-2-methoxybenzoic acid). Dicamba increases the growth rate of treated plants. If the growth rate outgrows the nutrient supply, the plants die off. DMO is part of a protein complex converting dicamba into 3,6-dichlorosalicylic acid (DCSA), which does not exhibit herbicidal activity.

Our submissions were built in large part using the Biosafety Assessment Tool (https://bat.genok.org/bat/) produced by the University of Canterbury and GenØk – Centre for Biosafety. It is a resource registered with the Biosafety Information Resource Centre of the Biosafety Clearing House (ID 48544). The BAT is a free-to-the-public resource for hazard identification and risk assessment of genetically modified organisms.

Our submission is based on the Executive Summary of Application A1063 - Food derived from herbicide tolerant soybean line MON 87708 Assessment Report and its Supporting Document 1, prepared by FSANZ, as well as the studies supplied by the Applicant.

We conclude that the preferred action is Option 1: reject, unless further information from both FSANZ and the Applicant can address the questions raised in this submission.

Recommendations

Based on our findings, we propose a number of specific recommendations, summarized here and detailed in the critique below.

FSANZ is encouraged to request the following:

1. FSANZ should report how it determined that the activity of DMO in processed soy at/below the detection limit was not biologically relevant.

2. The Applicant should provide data of the effects of MON 87708 through inhalation studies using animal models of acute respiratory syndrome, compared with inhalation of the proper conventional comparator. This should include an appropriately sensitive analysis for signs of immuno-modulation, including allergenicity, and toxicity.

3. The Applicant should supply additional evidence about the substrate specificity of DMO by testing substances more relevant to the safety assessment, using the *in planta* produced DMO proteins.

4. We recommend that FSANZ requests data from proper immuno-modulatory and allergenicity testing of MON 87708 including tests from diet and inhalation exposures.

5. FSANZ should review the data from the feeding trial conducted by the Applicant.

6. In accordance with Codex Alimentarius, the Applicant should report detection limits for all methods.

7. The Applicant should comply with FSANZ guidelines and provide evidence that all isoforms of the newly expressed proteins are not post-translationally modified. Additionally, *in planta*-produced isoforms should be evaluated using antibodies capable of detecting all occurring isoforms.

8. The Applicant should provide data to substantiate claims of specificity of DMO, either by using the *in planta*-produced DMO protein or by demonstrating equivalence between the test protein and the *in planta*-produced form.

9. Given the deletion and insertions reported after integration of the recombinant DNA using the techniques of modern biotechnology, the Applicant should provide a survey of the actual RNAs produced or absent at the integration junctions and in the DNA surrounding the insert, preferably using high throughput transcriptome sequencing techniques.

Overall recommendation

Based on our detailed assessment, we find that the informational, empirical and deductive deficiencies identified in the dossier do not provide sufficient evidence to the regulator to conclude that MON 87708 treated with dicamba under normal commercial production conditions will be as safe as or safer than alternatives already available. A new application or reapplication should only be reconsidered with the delivery of the information requests recommended here, including any additional information deemed significant by FSANZ.

About the event

The product MON 87708, developed using the techniques of modern biotechnology by Monsanto Europe S.A., is intended to be tolerant to the herbicide dicamba (3,6-dichloro-2-methoxybenzoic acid). This is achieved by expression of *dmo*, coding for dicamba oxygenase.

1. Misuse of terms

The Applicant incorrectly states the trait is **biotechnology-derived**, as in:

> "*Monsanto Company has developed biotechnology-derived soybean MON 87708 that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide*" (p. 17 of the main application).

FSANZ also refers the event being a product of biotechnology in its assessment report:

"The affected parties may include the following:

- *Consumers of soybean-containing food products, particularly those concerned about the use of biotechnology to generate new crop varieties"*

(p. 9 of the assessment report).

According to internationally agreed definitions, the trait in MON 87708 is derived from **modern biotechnology** (CBD, 2003). Unlike biotechnology-derived traits as defined by the Convention on Biodiversity, the techniques and products of modern biotechnology have no

history of safe use, and are therefore subject to special regulation. While this may seem to be merely semantics, there are in fact important legal, scientific and socio-economic ramifications specific to this difference. Therefore, the erroneous use of terms should be corrected whenever possible.

2. *Stenothrophomonas maltophilia*, the source of the *dmo* gene, is a known human pathogen

The Applicant uses the fact that *Stenotrophomonas maltophilia* is ubiquitous in the environment to support its claim of safety for using DMO sourced from this organism For example:

"The safety of the donor organism, S. maltophilia*, [is] based on its ubiquitous presence in the environment, presence in healthy individuals, and the incidental presence in foods without any adverse safety reports"* (p. 18 of the main application).

"*[...] infections in humans caused by* S. maltophilia *are extremely uncommon (Cunha, 2010). Similar to the indigenous bacteria of the gastrointestinal tract,* S. maltophilia *can be an opportunistic pathogen (Berg, 1996)*" (p. 19 of the main application).

FSANZ acknowledges the fact that *S. maltophilia* can cause "*a wide range of sometimes fatal infections in immuno-compromised hospital patients*" (p. 5 of SD1), but no mention is made about the high mortality of those with infections, and the increase of reported cases in recent years.

S. maltophilia is the second most common non-fermentative gram-negative bacillus isolated from clinical specimens, although until recently was considered an unusual organism to isolate in the diagnostic microbiology laboratory. The frequency of infections related to *S. maltophilia* has tripled in the last decade both in the USA and France (Denton and Kerr, 1998). The main reason for the high numbers of fatalities seems connected to multiple antibiotic resistances in *S. maltophilia*.

Therefore, we argue that a history of successful suppression of *S. maltophilia* is not reason to discount the potential of this organism to cause disease. A full evaluation of how *S. maltophilia* causes diseases, and any known or plausible contribution to pathogenicity by DMO, should be provided by the Applicant or the regulator to justify any decision to appove MON 87708.

3. Exposure of humans and animals to DMO

The technical dossier claims that "*MON 87708 DMO would not be consumed as an active protein in food products*" (p. 113 of the main application) because no protein activity was detected after 15 min at 55°C. However, the Applicant's assay is only able to detect "<25% activity" with a detection limit of 4.4 nmol DSCA/min/mg DMO enzyme. Further, there is no rationale provided to assume that there is a relationship between amount of DMO protein and its potential to cause harm. Only properly designed feeding studies using processed soy and based on actual consumption patterns in the target population could substantiate this point.

Recommendation: FSANZ should report how it determined that the activity of DMO in processed soy at/below the detection limit was not biologically relevant.

While soy is predominantly consumed after some processing, there are many stages in the production chain that would create potential exposures before processing, particulary through inhalation. The identified use of MON 87708 as a highly processed product, involves milling the grain to soy flour. Humans may more likely have direct, non-dietary exposure to soy flour than through dietary exposure, yet the Applicant did not take this into account.

Inhalation provides possible direct lung cell exposure to any soy flour, including MON 87708. Moreover, inhalation sensitisation to allergens can bemore important than dietary sensitisation:

> "*[I]t has to be considered that transgenic plants may be used in industrial processing; hence other exposure routes and sensitization scenarios might become important. For example, manufacturing large amounts of transgenic soy containing a food allergen may induce respiratory sensitization due to the generation of allergen-containing dust*" (Spok et al., 2005).

Therefore it is essential for safety testing to ensure that MON 87708 is as safe as conventional soy in its raw state using proper inhalation studies.

The Applicant should provide data of the effects of MON 87708 through inhalation studies using animal models of acute respiratory syndrome, compared with inhalation of the proper conventional comparator. This should include an appropriately sensitive analysis for signs of immuno-modulation, including allergenicity, and toxicity.

4. Substrate specificity testing

The substrate specificity test included in the present dossier is insufficient. First, none of the tested substances, except for o-Anisic acid, have a methylated group in the ortho- position. No substance was tested that contains one or two halogen substitutions in the ring, and there is no test of other ring structures besides the benzene ring.

Second, the DMO protein actually used in the specificity assays does not have the same amino acid sequence as DMO and DMO+27 produced in MON 87708. Rather, it is identical to the protein ("wild type" or WT) as produced by *S. maltophilia* with an additional N-terminal His-tag (Fig 21 and p. 77 of the main application). The WT and DMO from MON 87708 differ in two positions: the latter contains an additional alanine at position 2 added for cloning purposes, and a Trp112Cys substitution was reported. No experimental evidence for the bio-equivalence of these different versions was presented by the Applicant. In addition, the Applicant only reports testing o-Asinic acid for DMO conversion of endogenous substrates (p. 78 of the main application).

Recommendation: The Applicant should supply additional evidence about the substrate specificity of DMO by testing substances more relevant to the safety assessment, using the *in planta* produced DMO proteins.

5. Phenotypic and agronomic data was collected in only one season

To evaluate phenotypic and agronomic traits of MON 87708, the Applicant conducted field tests in eight field sites in the USA during the 2008 growing season. While this may be

consistent with the latest guidelines by FSANZ, we believe that more than one trial year is necessary to capture changes resulting from annual variations in biotic and abiotic stressors.

6. Comparisons using immune sera from subjects sensitized to conventional soy are not appropriate for detecting immune responses that may be unique to MON 87708

In section 7.9.2, "Assessment of allergenicity of the whole GM plant or crop" a quantitative ELISA assessment of human IgE binding to MON 87708 soybean, control and reference soybean extracts was performed. The Applicant submitted the results of an allergenicity test in which the sera from 13 "soybean allergic patients" and 5 sera from non allergic control individuals were incubated with protein extracts prepared from the MON 87708 seed, control soybean, and 17 commercial soybean varieties and then was analyzed by enzyme linked immunosorbent assay (ELISA). Focusing on the similarity of reaction profiles, the Applicant concluded that, based on the levels of endogenous soybean allergens:

> *"... MON 87708 and products derived from MON 87708 do not pose an increased endogenous allergenicity concern to humans over currently consumed soybean foods" (p. 118 of the main application).*

Based on our understanding of the experimental design, the study used sera from people sensitized to conventional soybean, not soybeans expressing DMO. These individuals would not have mounted an immune reaction to an unknown allergen unique to dicamba tolerant soybean MON 87708. Therefore the study only provides baseline data about the generic allergenicity of soybeans; it is not capable of distinguishing the allergenic potential of MON 87708 from conventional soybean for people never exposed to MON 87708. We fail to understand the relevance of this study for demonstrating the safety of MON 87708. Moreover, the study was limited to 13 soy-sensitive individuals with unknown histories of sensitisation. People could be exposed to MON 87708 both in the diet and through inhalation of flour. Therefore, the study should include an assessment of the allergenic potential of MON 87708 through both dietary and inhalation sensitisation. Especially given the statistically significant differences in the detected levels of two anti-nutrients (trypsin inhibitors and genistein) between MON 87708 and the comparator, a more thorough investigation should be carried out.

Recommendation: We recommend that FSANZ requests data from proper immunostimulation and allergenicity testing of MON 87708 including tests from diet and inhalation exposures.

7. Absence of feeding trial data

According to the FSANZ Application Handbook Section 3.5.1

> "C. The application must contain […]
>
> 2. Data from an animal feeding study, **if available"**
>
> (emphasis added to p. 88 FSANZ, 2011).

The Applicant conducted a 90 day feeding study in rats with processed soybean meal from MON 87708 (report number MSL0022868), which was supplied to the European Food Safety Agency (EFSA). Clearly, the data is available and should therefore be evaluated by FSANZ before making a decision on the safety of MON 87708.

Recommendation: FSANZ should review the data from the feeding trial conducted by the Applicant.

8. Protein characterisation

There are several shortcomings in the protein characterisation section of Application A1063.

First, the antigen used to raise anti-DMO antibodies, and the antibodies themselves utilized in the immunoreactivity assays, lack description. It is not clear what the origin of the protein was that was used to raise the antibodies in the first place, or how the antibodies were purified from serum (e. g. which antigens were used to purify by immunoaffinity chromatography). Post-translational modifications vary by species, tissue and time of development and epitopes can be masked or altered by post-translational modifications (Kuester et al. 2001; Petersen et al., 2009). Therefore, raising antibodies against the *E. coli*-produced form will obviously bias all subsequent equivalence testing against the detection of potential novel *in planta*-produced isoforms. It is impossible to say, using the evidence provided, that the polyclonal antibodies would in fact detect all isoforms of the recombinant proteins that might be produced *in planta*, were they present in the sample. A precautionary approach should conclude that the Applicant has profiled only a subset of epitopes on the unglycosylated isoform of the recombinant protein.

Second, many of the experiments lack a description of detection limits. This includes the immunoblot analysis, MALDI-TOF MS and the glycosylation analysis. Codex Alimentarius states that:

> *"[...] The sensitivity of all analytical methods should be documented"*
>
> (§20 of Codex, 2003).

Recommendation: In accordance with Codex Alimentarius, the Applicant should report detection limits for all methods.

Third, the Applicant's means of determining glycosylation status of DMO via hybridisation of glycoproteins to probes is not the ideal method for sensitive detection of protein glycosylation. A more complete profile is possible using oligosaccharide mapping, liquid chromatography, and mass spectrometry (Werner et al, 2007).

Determining only the glycosylation status of the proteins does not satisfy FSANZ guidelines:

> Section 3.5.1
> *"The characeriaztion of novel proteins or other novel substances*
> *This part includes all of the following:*
> *[...]*
> *(e) information on whether the newly-expressed protein has undergone* ***any*** *unexpected post-translational modification in the new host"*
>
> (emphasis added to p. 86 of FSANZ Application Handbook 2011).

Indeed, the Applicant identified an unexpected DMO variant, DMO+27, which is not a product of glycosylation:

"It was anticipated that during translocation into chloroplasts the [chloroplast transit peptide] and the additional 27 amino acids would be fully cleaved resulting in the appropriate amino terminus for mature DMO. However, analysis of leaf and mature seed tissue by western blot shows the presence of two bands […]. One band corresponds to the mature DMO protein (referred as to DMO), whereas the second band is DMO plus 27 amino acids originating from the pea Rubisco small subunit on its N-terminus" (p 12 of Wang et al., 2010).

Recommendation: The Applicant should comply with FSANZ guidelines and provide evidence that all isoforms of the newly expressed proteins are not post-translationally modified. Additionally, *in planta*-produced isoforms should be evaluated using antibodies capable of detecting all occurring isoforms.

9. Equivalence of expressed DMO proteins

The Applicant states that:

"The differences in the amino acid sequence between the wild-type DMO protein and MON 87708 DMO protein and the MON 87708 DMO+27 protein are not expected to have an effect on structure, activity, or specificity because they are sterically distant from the catalytic site" (p. 74 of the main application).

However, there is no experimental evidence to support these claims, because the experiments conducted to determine the crystal structure and the specificity did not use the actual DMO isolated from MON 87708 (Fig 21, p. 75):

- The crystal structure was resolved using a version containing the alanine at position 2 but the wild-type (WT) tryptophan at position 112. This makes it impossible to determine if the substitution in MON 87708 DMO changed the tertiary structure of the protein;

- The specificity was determined using a N-terminally His-tagged WT protein.

No experiments were reported that would establish equivalence between these different proteins, or differences between DMO and DMO+27 produced by MON 87708. Changes of single amino acids can significantly alter the characteristics of proteins μ(e.g., Doyle and Amasino, 2009, Hanzawa et al., 2005, Zubieta et al., 2008)§, a fact that underpins the field of directed evolution μ(reviewed in Bloom and Arnold, 2009, Tracewell and Arnold, 2009)§. One of the characteristics that can be changed is immunogenicity. For example, several groups reported significant decreases of IgE binding to a major peanut allergen after mutating single nucleotides μ(King et al., 2005, Ramos et al., 2009)§. Even more surprising, in some cases even a synonymous (i.e., differences in the nucleotide sequence of a gene that do not alter the resulting amino acid sequence) coding change can alter the characteristics of a transcript or protein, or levels of expression (Parmley and Hurst, 2007). A single nucleotide polymorphism that results in a synonymous change can change the substrate specificity of the resulting protein, potentially by affecting its folding patterns during translation (Kimchi-Sarfaty et al., 2007). Furthermore, sequence identical proteins with differing tertiary structures can turn benign proteins into toxins μ(Bucciantini et al., 2002, Ellis and Pinheiro, 2002, Ross and Poirier, 2005)§ or agents that cause pathogenesis as demonstrated for the Prp proteins causing Creutzfeld-Jacob disease and mad cow disease (Caughey and Baron, 2006).

In MON 87708, we find additional amino acids (alanine in position 2 and 27 amino acids in DMO+27), and a substitution of the original tryptophan in position 112 (with a large rigid aromatic ring in the side chain) by a cysteine (containing a sulf hydryl (SH) group). Both additions and substitutions may well result in changes in the folding of the protein and thus its activity.

It is only through proper scientific testing that FSANZ can rule out unintended or unanticipated effects.

Recommendation: The Applicant should provide data to substantiate claims of specificity, either by using the *in planta*-produced proteins or by demonstrating equivalence between the test protein and the *in planta*-produced form.

10. Detection of absence of backbone vector DNA/unintended transgenes in event MON 87708

'Backbone' transfers are common when introducing recombinant DNA using the Ti plasmid system found in *Agrobacterium*. Historical data underestimates the number of backbone transfers because:

> *"Usually, transfer of only the non-T-DNA sequences to the plant would remain undetected because: (1) there is no selection for the transfer of such sequences; and (2) scientists generally have not looked for the transfer of these sequences"* (Kononov et al., 1997).

The amount of DNA that can transfer can be many times the length of the T-DNA region, and short backbone sequences can transfer and be difficult to detect:

> *"extremely long regions of DNA (greater than 200 kbp) can transfer to and integrate into the genome of plants. […]*
> *In many instances, vector 'backbone' regions of a binary vector are smaller than what is conventionally termed the 'T-DNA' region"* (Kononov et al., 1997).

The Applicant used Southern blotting to raise confidence in the conclusion that there were no insertions of unintended material.

To test for the presence of backbone sequences in event MON 87708, the Applicant used probes ranging from 171 to 1700 bp, covering the whole backbone sequence with the exception of the LB and RB sequence of DNAII (which are identical to DNAI sequences and would therefore produce bands in the Southern blots). No bands were detected in the Southern blots with probes covering the backbone sequence. Controls spiked with 0.1 and 1 genome equivalent of the backbone DNA added to genomic DNA from the conventional comparator did result in the expected bands.

However, the Applicant failed to account for potential inserts that are only partial, either smaller than the probes or with rearrangements, both of which could prevent binding of the probe and therefore detection of rDNA integrated elsewhere in the genome. No detection limits for these potential targets were given. This leads us to conclude that there is not enough evidence to support the Applicant's claim.

11. Organisation and sequence adjacent to the introduced DNA in MON 87708

The Applicant sequenced about 1-1.2 kb of the chromosomal DNA on either side of the inserted T-DNA. The resulting sequence was compared to that of the comparator A3525. A deletion of 899 bp (RB) and insertions of 128 (RB) and 35 bp (LB) were reported (Song et al., 2011).

In accordance with FSANZ guidelines, the Applicant reports bioinformatics analysis of the inserted DNA (the translated sequence) and the junctions between recombinant and endogenous sequences to determine if any new substances might be expressed as a result of the insertion.

However, the analysis carried out by the Applicant does not survey for either disrupted endogenous ORFs or regulatory sequences that may have been disrupted or altered in a way that will affect gene expression in the host plant. Codex takes this into account when requesting that

> *"In addition, information should be provided: […]*
> *E) to indicate whether there is any evidence to suggest that one or several genes in the host plant has been affected by the transformation process*" (§ 33 Codex 2003a).

In addition to the direct disruption of genes or regulatory sequences at the insertion site, *Agrobacterium*-mediated transformation frequently leads to deletions and rearrangements in chromosomal sequences around the insertion site (Latham et al., 2006 and references therein), as detected in MON 87708 as well. Zolla et al (2008) conclude that:

> "*[I]t is also evident that the insertion of a single gene does not result in a unique newly expressed protein, but rather in many differently expressed genes with respect to the control. This could be due to the fact that, when the transgene enters the nucleus, many genetic loci are randomly affected by the insertion procedure*" (p. 1854 Zolla et al., 2008).

Thus, in addition to new junctions caused by insertions of recombinant DNA and thus possible novel RNAs in the transcriptome and proteins in the proteome, there may be a loss of endogenous RNAs and proteins that have no apparent effect on agronomic qualities but may have an effect on the expression or accumulation of toxins or anti-nutrients. The bioinformatic analysis provided by the Applicant does not substitute for a survey of actual RNAs produced at the junctions or for a survey of deleted RNAs.

Recommendation: Given the deletion and insertions reported after integration of the transgenic DNA into the host genome, the Applicant should provide a survey of the actual RNAs produced or absent at the integration junctions and in the DNA surrounding the insert, preferably using high throughput transcriptome sequencing techniques (Heinemann et al., 2011).

Conclusion

Available information for risk assessment evaluation

FSANZ has for the most part relied only upon the Applicant's own submitted information for its evaluation. While the directly relevant scientific literature is very limited in some cases, we have tried to extract relevant indirect information from the peer-reviewed literature to constructively contribute to FSANZ's ongoing evaluation.

All product-related safety testing should have an independent and unbiased character. This goes both for the production of data for risk assessment, and for the evaluation of those data. The lack of compelling or complete scientific information to support the claims of the Applicant highlights the need for independent evaluation of safety studies and molecular information provided, including the raw data produced by the Applicant. We therefore request that FSANZ exercise a higher level of transparency in how it decides what information is acceptable for inclusion in its decision-making processes. This would extend to any information provided by the Applicant used to justify confidentiality claims on any scientific data. We encourage the regulator to insist on a higher level of transparency and accessibility to all scientific data (including raw data) to ensure the scientific validity of the information presented.

Overall recommendation

Based on our detailed assessment, we find that the informational, empirical and deductive deficiencies identified in the dossier do not provide sufficient evidence to the regulator to conclude that MON 87708 treated with dicamba under normal commercial production conditions will be as safe as or safer than alternatives already available. A new application or reapplication should only be reconsidered with the delivery of the information requests recommended here, including any additional information deemed significant by FSANZ.

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