GenØk - Centre for Biosafety

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Assessment of the technical dossier submitted under EFSA/GMO/NL/2011/93 for approval of transgenic soya event MON 87708 by Monsanto Europe S.A.

Submitted to

Direktoratet for Naturforvaltning

by

Centre for Biosafety – GenØk
and
Center for Integrated Research in Biosafety
July 2011

KONKLUSJON PÅ NORSK

Vi trekker frem flere begrepsmessige, empiriske og informatoriske mangler i dossieret som ikke gir grunnlag for en konklusjon om sikker bruk, samfunnsnytten og bidrag til bærekraftighet av MON 87708 . Søker har ikke inkludert noe av den informasjonen omkring samfunnsnytten og bærekraftighet til MON 87708 som kreves i den norske genteknologiloven (Appendix 4) for godkjenning i Norge.

Basert på våre funn foreslår vi en rekke konkrete anbefalinger som vi poengterer i vårt høringssvar, og som vi har oppsummert her

Direktoratet for Naturforvaltning bes vurdere følgende

1. Fra søker må det kreves en "post-release" plan for å forsikre tilsynsmyndighetene om:
    a. mengde dicamba som er planlagt brukt: dvs tilsiktet mengde og maksimums mengde per sesong pr lokalitet;
    b. mulighet søker eller "adoptant" av dicamba-tolerant soya har for å detektere dicamba-tolerant ugress med en sensitivitet som er god nok til at dette kan kontrolleres uten å øke mengde dicamba eller andre typer herbicide.
2. Søker bør gi informasjon om:
    a. tilsiktet og mulig maksimums mengde dicamba rester på dicamba tolerant plantemateriale på ulike stadier i produksjonskjeden;
    b. tilsiktet og mulig maksimums mengde dicamba metabolitter på dicamba tolerant plantemateriale på ulike stadier i produksjonskjeden;
    c. effekt av dicamba på ikke-målorganismer (mikroorganismer), inkludert de som potensielt kan selektere for kryss-resistens ved og i klinisk eller veterinær bruk av antibiotika og ved de mulige maksimums frekvenser og doser som er tenkt brukt;
    d. effekter på nitrogen-fikserende mikroorganismer ved påtenkt/tilsiktet og mulige maksimums mengder av dicamba.
3. Søker bør fremskaffe bevis for substrat-spesifisitet av DMO ved å teste forbindelser som er mer relevante iht bedømmelsen av sikkerhet, og ved å bruke "in-planta" produsert DMO protein/enzym.
4. Søker bør bli påkrevd å forelegge data fra utprøvinger i praksis som dekker mer enn èn felt-sesong og som tar i betraktning hensiktsmessig eksponering i de varierende betingelsene som er i naturen (Codex, 2003).
5. For å kunne karakterisere risiko av MON87708 brukt i norsk mat er det helt nødvendig med informasjon om hvordan en eksponering av produktet kan tenkes å foregå, i hvilken mengde og i hvilken form dette kan skje. Her har søker ikke gitt tilstrekkelig informasjon, som medfører at risiko ikke kan vurderes på en god måte.
6. Søker bør bli påkrevd å undersøke forskjellen i de ulike sammensetningene av DMO som kan tilskrives til behandlingen med dicamba eller relevans dette

eventuelt kan ha for risikovurderingen.

7. Direktoratet for Naturforvaltning bør kreve data fra en egnet immunstimulering og allergitesting av MON87708, inkludert tester fra diett og eksponering via inhalasjon.
8. Søker må oppgi konsentrasjon av DMO i fòret som er brukt i fòrings-forsøkene ved oppstart og avslutning av forsøkene.
9. Søker bør fremskaffe data fra fòring med MON 87708 som har vokst under relevante betingelser, som ved tilstedværelse av dicamba.
10. Søker bør fremskaffe bevis for at effekten av MON 87708 på milt parametrene i rotte fòrings forsøket virkelig var tilfeldige, eller eksperimentelt bestemme årsaken til variasjonen i størrelsen av milt hos hunn-rotter fòret med 15 % MON 87708.
11. Søker bør fremskaffe bevis for at antistoffer brukt i karakteriseringen av DMO proteinet er i stand til å detektere alle hoved isoformer produsert "in-planta".
12. Søker bør angi deteksjonsgrense for alle metoder som er brukt.
13. Søker bør følge retningslinjene fra EFSA og Codex og skaffe bevis for at alle isoformer av nylig uttrykte proteiner ikke er post-translasjonelt modifisert.
14. Søker bør fremskaffe data som støtter påstanden om spesifisitet, enten ved å bruke "in-planta" produserte proteiner eller ved å demonstrere ekvivalens mellom test protein og "in-planta" produsert form av proteinet.
15. På grunnlag av delesjoner og insersjoner som er rapportert etter integrering av transgent DNA til verts-genom, burde søker fremlegge en oversikt over de reelle RNAer som blir dannet eller som er borte ved integrasjons punkter og i DNA som omgir insertet, helst ved å bruke "high throughput transcriptome sequencing" teknikker (Heinemann et al., 2011).
16. I henhold til genteknologiloven Vedlegg 4 del V "*Samfunnsmessige fordeler og ulemper* " ligger det til grunn at samfunnsmessig nytte skal tillegges vekt. Det er imidlertid høyst tvilsomt om de endringene i fettsyreprofilene til MON87705 forårsaket av genmodifisering, faktisk er etterspurt eller nødvendige i den norske dietten. Dette må vies ytterlig oppmerksomhet.

Hovedkonklusjon og anbefalinger

Vi har i vår gjennomgang funnet flere svakheter av begrepsmessig art, mangel på informasjon, feilaktige konklusjoner og mangelfulle empiriske data som hver for seg og til sammen ikke støtter søkers påstand om sikker bruk av MON 87708 soya. Søker har ikke fremskaffet noe av den informasjonen som er nødvendig for å kunne vurdere samfunnsnytte og bærekraftighet, noe som er påkrevd i den norske genteknologiloven for godkjenning i Norge. Disse manglene gjør at vi mener at denne søknaden er ufullstendig i sin nåværende form. Vi anbefaler derfor å avslå søknaden samt at en ny søknad bare bør vurderes om søker har adressert de mangler vi har belyst.

SUMMARY OF THE ASSESSMENT OF THE TECHNICAL DOSSIER RELATED TO EFSA/GMO/NL/2011/93

As a designated National Competence Center for Biosafety, our mission at GenØk in advice giving is to provide independent, holistic and useful analysis of technical and scientific information/reasoning in order to assist authorities in the safety evaluation of biotechnologies proposed for use in the public sphere.

The following information is respectfully submitted for consideration in the evaluation of product safety and corresponding impact assessment of event MON 87708, setting out the risk of adverse effects on the environment and health, including other consequences of proposed release under the pertinent Norwegian regulations.

This submission is structured to address specific provisions for an impact assessment required under the Norwegian Gene Technology Act of April 1993, focusing on the requirements in Appendix 2 - Principles for environmental risk assessment pursuant to sections 13-16 of the regulations, and Appendix 4 - Evaluation of ethical considerations, sustainability and benefit to society, cf section 17 of the "Regulations relating to impact assessment pursuant to the Gene Technology Act" of December 2005, pursuant to section 11 cf section 8. The information presented here may be applicable to more than one provision in different appendices. We focused our critique to address the information needs under the relevant provisions that relate to our particular area of competence in biotechnology assessment as comprehensively as possible. Lack of commentary on our part towards any information under consideration should not be interpreted as specific endorsement of that information.

This submission was 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. This is a free-to-the-public resource for hazard identification and risk assessment of genetically modified organisms.

All page numbers not directly referenced refer to the document Part 1 of the technical dossier "Application for authorization to place on the market MON 87708 soybean in the European Union, according to Regulation (EC) No. 1829/2003 on genetically modified food and feed", submitted by the Applicant.

Key findings

After a detailed analysis of many of the portions of the dossier on MON 87708 submitted by the Applicant, we outline a number of informational, methodological and conceptual weaknesses that do not justify the Applicant's conclusion of safety, based on the given data. Our input focuses on a critique of the Applicant's dossier and covers three broad issues:

1. Flawed assumptions, reasoning, or interpretations by the Applicant

2. Missing, incomplete or inadequate information to support scientifically sound claims of safety

3. Missing information in relation to requirements under the Norwegian Gene Technology Act

Within we suggest appropriate action to address the specific deficiencies where possible, and conclude our assessment with a summary recommendation.

Lastly, Codex Alimentarius guidelines allow Norway to ask for specific data of the type we identify and recommend obtaining below. Norway therefore may request such information without concern of a challenge from the World Trade Organisation.

Recommendations

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

The Direktoratet for naturforvaltning is encouraged to request the following:

1. The Applicant should be required to provide a post-release plan that provides certainty to the regulator on:
    a. intended and maximum levels of dicamba applications per season per locality;

    b. ability of the Applicant or adopters of dicamba-tolerant soybeans to detect the emergence of dicamba-tolerant weeds with a sensitivity that would allow them to be controlled without resort to higher levels of dicamba application or alternative herbicides.

2. The Applicant should provide information on

    c. intended and possible maximum dicamba residues on dicamba-tolerant plant materials at various stages in the production chain;

    d. intended and possible maximum dicamba metabolite residues on dicamba-tolerant plant materials at various stages in the production chain;

    e. non-target effects on microorganisms including those that could select for cross-resistance to clinical or veterinary antibiotics at possible maximum frequencies and doses of application;

    f. effects on nitrogen-fixing microorganisms at both intended and possible maximum dicamba application levels.

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

4. The Applicant should be required to submit data from field trials covering more than one field season in order to allow adequate exposure to the variety of conditions met in nature (Codex, 2003).

5. The Applicant should the clarify functional status of the transgenic protein after processing with properly designed experiments, and further test the effects of MON 87708 inhalation in animals that are used as models of acute respiratory syndrome, compared with inhalation of the proper conventional comparator. This should include an analysis of allergenicity and toxicity.

6. The Applicant should be requested to investigate the differences in composition that may be directly attributed to the treatment with dicamba and the relevance of these for the risk assessment.

7. The Direktoratet for naturforvaltning should request data from proper immunostimulation and allergenicity testing of MON 87708 including tests from diet and inhalation exposures.

8. The Applicant should report the DMO concentration of feed used in the feeding trials at the beginning and the end of the studies.

9. The Applicant should provide feeding data obtained with MON 87708 that has been grown under the relevant agronomic conditions, i.e. in the presence of dicamba.

10. The Applicant should provide evidence that the effect of MON 87708 on spleen parameters in the rat feeding study was indeed incidental or experimentally determine the cause for the variation in spleen size of female rats fed with 15% MON 87708.

11. The Applicant should provide evidence that the antibodies used in the protein characterization would detect all novel in-planta produced isoforms.

12. The Applicant should report detection limits for all methods.

13. The Applicant should comply with EFSA and Codex guidelines and provide evidence that all isoforms of the newly expressed proteins are not post-translationally modified.

14. 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.

15. 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).

16. The Applicant should submit required information on the social utility of MON 87708 and its contribution to sustainable development, in accordance with the Norwegian Gene Technology Act

Overall recommendation

Based on our detailed assessment, we find that the informational, empirical and deductive deficiencies identified in the dossier do not support claims of safe use, social utility and contribution to sustainable development of MON 87708. **Critically, the Applicant has not included any of the required information to assess social utility and sustainability as required in Appendix 4 of the Norwegian Gene Technology Act, which would be necessary for consideration of approval in Norway.** Hence at minimum, the dossier is deficient in information required under Norwegian law. 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 the Norwegian authorities.

Therefore, in our assessment of MON 87708, we conclude that based on the available data, including the safety data supplied by the Applicant, the Applicant has not substantiated claims of safety satisfactorily or provide the required information under Norwegian law to warrant approval in Norway at this time.

ASSESSMENT OF THE TECHNICAL DOSSIER RELATED TO EFSA/GMO/NL/2011/93

About the event

The transgenic soy MON 87708, developed by Monsanto Europe S.A., has been genetically engineered to be tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide. The event also expresses PMI (phosphomannose isomerase), which was used as a selectable marker.

1. Missing, incomplete or inadequate information to support the Applicants claims

1.1 Misuse of terms

The Applicant incorrectly states that 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).

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 a quibble about words, there are in fact important legal, scientific and socio-economic ramifications specific to this difference and not all regulatory authorities in the world may be equally primed to these differences. Therefore, it is important to state our objection to this practice of claiming these products as products of biotechnology rather than as products of modern biotechnology.

1.2 *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. On p. 32., the Applicant states that "there is no evidence of human or animal pathogenicity for any of the donor organisms of the coding and non-coding DNA sequences present in MON 87708." Elsewhere, the dossier makes reference to how uncommon *S. maltophilia* infections are in humans. However, 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*. Particularly noteworthy is the resistance to drugs of the β-lactam class.

1.3 Exposure of humans and animals to DMO

The technical dossier claims that the consumption of DMO protein expression in soy is extremely low due to its low concentration in the total diet of animals and humans (p. 180). This is an erroneous statement. There is no absolute relationship between the level or protein expression 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 clarify this point.

Additionally, the Applicant assumes that "*DMO is likely to denature during soybean toasting and processing* (p 184)". No data is presented to support this statement.

Further, the Applicant has only considered dietary exposure pathways in its assessment of possible adverse effects from MON 87708. Inhalation exposure can be expected to be a significant pathway for many people, and a more direct cause of potential adverse effects. 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. Edible soybean flour production was estimated at 2 million tons by 1992, up from only 60,000 tons in 1960 (Berk, 1992). It is used in baking, cereals and pasta. It has important uses in replacing wheat flours especially for those with coeliac disease (Berk, 1992).

Inhalation provides possible direct lung cell exposure to any soy flour, including MON87708. Moreover, inhalation sensitization to allergens can be more important than dietary sensitization:

> "*[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).

We recommend that the Direktoratet for naturforvaltning request information from the Applicant the functional status of the transgenic protein after processing and also on the effects of MON 87708 inhalation in animals that are used as models of acute respiratory syndrome, compared with inhalation of the proper conventional comparator. This should include an analysis of allergenicity and toxicity.

2. Improper assumptions and/or unsupported reasoning by the Applicant related to assessment needs

2.1 Effect of dicamba on agricultural sustainability

The Applicant should be required to provide a post-release plan that provides certainty to the regulator on:

1. intended and maximum levels of dicamba applications per season per locality;
2. ability of the Applicant or adopters of dicamba-tolerant soybeans to detect the emergence of dicamba-tolerant weeds with a sensitivity that would allow them to be controlled without resort to higher levels of dicamba application or alternative herbicides.

Failure to meet these post-release monitoring requirements is expected to undermine the use of this or similar herbicides in sustainable conventional integrated pest management systems and undermine the economic viability of poor and subsistence farmers especially those in developing countries.

Rationale:

Dicamba is presumed to act as a plant growth hormone. When the herbicide reaches an effective concentration, plants are stimulated to grow without reference to their nutrient limitations and subsequently die. It is likely that the incorporation of dicamba tolerance on a scale necessary to compensate for the loss of glyphosate tolerance as a specific weed control strategy in soybeans will result in the same herbicide "treadmill" that is rapidly senescing glyphosate as a commercial option (Binimelis et al., 2009). Indeed, dicamba tolerance in wild plants has been reported (Cranston et al., 2001, Jasieniuk et al., 1995). As with glyphosate, weed control using dicamba and dicamba-tolerant crops will involve multiple applications during the growing season at ever higher doses as the agroecosystem becomes more welcoming to weeds less susceptible to dicamba, or traditionally susceptible but newly arising resistant variants of current weeds.

The Applicant has previously denied what was long predicted by the scientific community, and that is that overuse of glyphosate-based herbicides, namely Roundup, in the way special to Roundup Ready crops, would result in weed resistance[1]. Although the Applicant now recognises that the pattern of use special to glyphosate tolerant crops did eventually result in glyphosate-tolerant weeds (e.g. Monsanto, 2008), it has made no attempt to address this fundamental problem of this form of pest management. It is important to note that prior to the

[1] "Monsanto, which once argued that resistance would not become a major problem, now cautions against exaggerating its impact. 'It's a serious issue, but it's manageable,' said Rick Cole, who manages weed resistance issues in the United States for the company." http://www.nytimes.com/2010/05/04/business/energy-environment/04weed.html

introduction of GM glyphosate-tolerant crops, the long-term use of glyphosate as an effective herbicide was not threatened (Heinemann, 2009, Heinemann and Kurenbach, 2008, Young, 2006). And the loss of glyphosate to farmers, especially poor farmers in developing countries, undermines locally sustainable agriculture under an integrated pest management system (Heinemann, 2009).

The socio-economic ramifications of imprudent use of dicamba are important, and ever more so given the rapid loss of glyphosate effectiveness. Together with the Applicant's failure to achieve a managed use of glyphosate during a time when it arguably had exclusive proprietary control of both the herbicide and the associated GM germplasm, it is now incumbent upon governments committed to the call for sustainable agriculture and the use of agriculture as a vehicle to achieve the Millenium Assessment Goals (IAASTD, 2009) to require Applicants of these technology products to ensure a safe use plan for products considered safe in a pre-market assessment.

2.2 Unintended effects of dicamba on the microbial ecosystem

The Applicant should provide information on:

1. intended and possible maximum dicamba residues on dicamba-tolerant plant materials at various stages in the production chain;
2. intended and possible maximum dicamba metabolite residues on dicamba-tolerant plant materials at various stages in the production chain;
3. non-target effects on microorganisms including those that could select for cross-resistance to clinical or veterinary antibiotics at possible maximum frequencies and doses of application;
4. effects on nitrogen-fixing microorganisms at both intended and possible maximum dicamba application levels.

Failure to do so could result in a regulatory decision with unacceptably high levels of uncertainty for unintended ecosystem effects from farm to fork.

Rationale:

Dicamba and its normal metabolites (e.g. 3,6-dichlorosalicylic acid which is similar to 3,5-dichlorosalicylic acid) have structural similarity to classes of salicylic acid-based compounds with antimicrobial activity (Gershon and Parmegiani, 1962). There is very little information about the antimicrobial activities, if any, of dicamba metabolites.

> "*Even though some soil bacteria are able to tolerate or degrade some pesticides by using them as their sole carbon or nitrogen source, bacteriostatic and lethal effects can also occur*" (p. 780 Drouin et al., 2010).

However, it is known that salicylic acid-based compounds with antimicrobial activities can create a selection for bacteria likely to be resistant to antibiotics (Heinemann et al., 2000). As bacteria throughout the production chain, from soil through to processing and on to the gut of

consumers and wild and domestic animals, will be exposed to intended higher levels of dicamba and its metabolites, the effects on microorganisms should be determined before approval is granted.

Although dicamba is presumed to act as a plant growth hormone, it is a genotoxin and a potential carcinogen (Knopper and Lean, 2004, Kovalchuk and Kovalchuk, 2008). Thus, the herbicide has the potential to select for a variety of novel phenotypes in microbes and in plants, as well as to accelerate the evolution of resistance. Other antibiotics with DNA damaging activites, e.g. bleomycin, have been known to select for resistance and resistance has been beneficial to potential pathogens even in the absence of the antibiotic (Heinemann et al., 2000).

Information of this kind should be required for:

- dicamba;
- 3,6-dichlorosalicylic acid;
- 6-dichlorosalicylic acid; and
- 5-hydroxy-2-methoxy-3,6-dichlorobenzoic acid (Casida and Lykken, 1969).

The unintended antimicrobial activities may also have an adverse effect on soil productivity. Of special significance would be an effect on nitrogen fixation, since soybeans are used as an important source of fixed nitrogen in mixed cropping agroecosystems.

> "*The effect of pesticides on rhizobia and their symbiosis with legume, will vary according to the rhizobial species, the rhizobial strains within a given species, the type of pesticide involved, and the pesticide concentration*" (p. 780 Drouin et al., 2010).

Reductions in fixation would have to be supplemented using fertilizers produced at high fossil fuel costs.

Holst et al. (1982) found that lower levels (0.1-1 ppm) of dicamba stimulated growth of *Anabaena azollae*, the nitrogen-fixing symbiont of *Azolla mexicana*, but higher concentrations inhibited growth. Concentrations of 1-10 ppm inhibited nitrogen fixation and reduced chlorophyll levels (Holst et al., 1982).

Reported effects of dicamba on *Rhizobium* and *Bradyrhizobium* have been concentration and strain-dependent. Two studies reported strains that were inhibited by dicamba. 5% and 3% of *Rhizobium* and *Bradyrhizobium* strains, respectively, surveyed by Drouin et al. (2010) were inhibited by 450 µg of dicamba. While reassuring that so few responded to dicamba, and then only at concentrations that would be relevant to seed treatment rather than current soil application concentrations, this study did not examine susceptibility in the field under field conditions, leaving some uncertainty as to actual environmental impact of dicamba use. More importantly, given the mode of action of dicamba, current application concentrations may not be predictive of future concentrations and therefore the effects on these symbionts. Finally, again it should be noted that even in this limited survey there were strain-specific differences

in susceptibility to dicamba and thus any environmental risk assessment should be conducted on local soil and nodule isolates.

Nitrogen-fixing bacteria of four different genera were isolated from soil that originated from a single soybean farm in Argentina (Zabaloy and Gómez, 2005). Of the 76 strains isolated, only 1 (a strain of *Bradyrhizobium*) demonstrated sensitivity to dicamba. Again, this study is reassuring in that a minority of strains surveyed appeared susceptible to dicamba. However, it is concerning that a general prediction about dicamba's effects on important soil microorganisms cannot be reached, and emphases the need for agroecosystem-specific sampling and large surveys. Moreover, this study did not measure sub-lethal effects on nodule formation and fixation, which are important variables for any comprehensive assessment on soil microorganisms.

2.3 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. The rationale for using the substances described in the dossier is missing.

Second, the DMO protein actually used in the specificity assays does not have the same amino acid sequence as DMO and DMO+27 expressed in MON 87708. Rather, it is identical to the wildtype-DMO from *S. maltophilia* with an additional N-terminal His-tag (Fig 24, p. 192). 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. The Applicant only reports testing o-Asinic acid for DMO conversion of endogenous substrates (p. 193).

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

2.4 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 2009 growing season. While this may be consistent with the latest guidelines by EFSA, which state that

> "*[t]he trials may be conducted in a single year, or spread over multiple years"* (p. 14 EFSA 2011),

Codex Alimentarius allows Norway to request field testing in several relevant locations and over several seasons:

> *1"[…] trials should be conducted over a sufficient number of generations to allow adequate exposure to the variety of conditions met in nature." (§ 45 of Codex, 2003a)*

Recommendation: We recommend that the Direktoratet for naturforvaltning requires the Applicant to submit data from field trials covering more than one field season in order to allow adequate exposure to the variety of conditions met in nature (Codex, 2003a).

2.5 Comparison of nutrients and anti-nutrients for dicamba treated and untreated MON 87708 in field trials.

According to the Applicant,

> "*[c]ompositional analyses were conducted in the US in 2009 to assess whether the nutrient and anti-nutrient levels in the seed and forage derived from MON 87708 are comparable to those in the conventional soybean control, A3525, which has background genetics similar to MON 87708, but does not possess the introduced gene (dmo)*" (p. 104).

Of the 84 components measured from seeds of dicamba treated and untreated MON 87708, 40 (47%) were statistically significantly different in the combined site analysis. However, there were seven statistically significant differences in the nutrient and anti-nutrient content of MON 87708 (when compared to the isogenic comparator A3525) that occurred only in either the treated or the untreated seed. Three only appear in the untreated plots (levels of isoleucine, valine and trypsin inhibitors), while four differences are only significant in the dicamba treated plots (levels of 18:2 and 18:3 linoleic acid and genistein). The latter may be caused by the dicamba treatment itself. Since treatment with dicamba is the practice that will in reality be used when growing MON 87708, differences to the plant metabolism occurring during this treatment should be further investigated. The low number of observed differences makes follow up experiments feasible.

Recommendation: We recommend that the Direktoratet for naturforvaltning requests the Applicant to investigate the differences in composition that may be directly attributed to the treatment with dicamba and the relevance of these for the risk assessment.

2.6 Comparisons using immune sera from subjects sensitized to conventional soy are not capable of detecting immune responses 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 were performed. The Applicant submitted the results of an allergenicity test in which the sera from "soybean allergic patients" was 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 does not pose an increased endogenous allergenicity concern to humans over currently consumed soybean foods." (p. 236).

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 sensitization. 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 sensitization. Especially given the statistically significant differences in the detected levels of two anti-nutrients (trypsin inhibitors and genistein; see below) between MON 87708 and the comparator, a more thorough investigation should be carried out.

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

2.7 90-days rat feeding trial

The Applicant reports data from a 90 days feeding trial on rats. Here, several issues are highlighted that call into question the Applicant's conclusion of safety of MON 87708.

First, the Applicant does not report the concentration of DMO in the animal feed after processing. This measurement should be performed from randomly selected samples at the beginning and the end of the trial to determine how storage of the food is affecting the concentration of the protein and its breakdown products and therefore the concentration the test animals are actually consuming. This is relevant since the feed has been shipped from the producer at ambient temperature.

Recommendation: The Applicant should report the DMO concentration of feed used in the feeding trials at the beginning and the end of the studies.

Second, the test material used as feed did not contain any residues of dicamba[2]. However, since dicamba-treated soy will be the product humans and animals will be exposed to, it should be taken into account in feeding trials, which are performed to provide evidence of the safety of MON 87708.

[2] According to Report MSL0022868, Appendix C, p. 1348, dicamba is not one of the organo chlorinated substances present in the diet.

Recommendation: The Applicant should provide feeding data obtained with MON 87708 that has been grown under the relevant agronomic conditions, i.e. in the presence of dicamba.

Third, the Applicant reports statistically significant differences in spleen weight in the females 15% group (absolute spleen weight, spleen weight relative to final body weight, spleen weight relative to brain weight). These values were outside the control range and outside historical control ranges. The Applicant argues a lack of dose-response relationship and the absence of any histological changes and concludes that

> *"[c]ollectively, these observations suggest an incidental variation unrelated to test substance administration".*

However, no follow up data was presented to substantiate this assumption.

Recommendation: The Applicant should provide evidence that the effect of MON 87708 on spleen parameters in the rat feeding study was indeed incidental or experimentally determine the cause for the variation in spleen size of female rats fed with 15% MON 87708.

2.8 Protein characterization

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 by post-translational modifications (Kuester et al. 2001). 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.

Recommendation: The Applicant should provide evidence that the antibodies used in the protein characterization would detect all novel in-planta produced isoforms.

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

Recommendation: The Applicant should report detection limits for all methods.

Third, the Applicant's means of determining glycosylation status of DMO via hybridization 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 EFSA or Codex guidelines:

> *"To demonstrate the safety of newly expressed proteins, the Applicant should provide: a) molecular and biochemical characterisation of the newly expressed protein, including the amino acid sequence, molecular weight,* post-translational modifications *and a description of the function…."* (emphasis added to p. 23 of EFSA, 2011)

and

> *"33. In addition, information should be provided:*
> *[…]*
> *B) to demonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its* post-translational modification *or affect sites critical for its structure or function;`[…]" (emphasis added to p. 6 of Codex, 2003)*

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 EFSA and Codex guidelines and provide evidence that all isoforms of the newly expressed proteins are not post-translationally modified.

2.9 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. 189).

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 24, p. 192):

- 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. Indeed, changes of single amino acids can drastically alter the characteristics of proteins (e. g. Doyle and Amasino, 2009, Hanzawa et al., 2005, Zubieta et al., 2008), a fact that underpins the field of directed evolution (reviewed in e. g. 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 (Glaspole et al., 2005, King et al., 2005, Ramos et al., 2009). Even more surprising, in some cases not even an amino acid change is necessary to alter the characteristics of a protein! Kimchi-Sarfaty et al. demonstrated that even synonymous single nucleotide polymorphisms (i.e. differences in the nucleotide sequence of a gene that do not alter the resulting amino acid sequence) can change the substrate specificity of the resulting protein, potentially by affecting its folding patterns during translation (Kimchi-Sarfaty et al., 2007). Changes in the tertiary structure alone can turn benign proteins into toxins (Bucciantini et al., 2002, Ellis and Pinheiro, 2002, Ross and Poirier, 2005), 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 cystein (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.

2.10 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 that "*MON 87708 contains no detectable backbone elements from the transformation vector PV-GMHT4355*" (p. 24 of Song et al., 2011).

2.11 Organization 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).

The resulting sequence was then submitted to BLASTn and BLASTx analyses to determine

> *"if any endogenous ORFs were disrupted by the insertion of the T-DNA present in MON 87708 or whether ORFs from the soybean genome are present in the flanking genomic DNA adjacent to the T-DNA after transformation. […] The results […]*

> *provided no evidence that genes at or in the flanking genomic DNA of the MON 87708 insertion site were disrupted by the insertion of the T-DNA sequence"* (p. 7 Tu, 2011b).

However, the analysis carried out by the Applicant does not survey for 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). 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).

3. Missing information in relation to requirements under the Norwegian Gene Technology Act

3.1. Social utility and sustainability aspects

In addition to the EU regulatory framework for GMO assessment, an impact assessment in Norway follows the Norwegian Gene Technology Act. In accordance with the aim of the Norwegian Gene Technology Act, production and use of the GMO shall take place in an

ethically and socially justifiable way, under the principle of sustainable development. This is further elaborated in section 10 of the Act (approval), where it is stated that

> *"significant emphasis shall also be placed on whether the deliberate release represent a benefit to the community and a contribution to sustainable development"*.

These issues are further detailed in the regulation on consequence assessment section 17 and its annex 4. The Applicant has not provided relevant information that allows an evaluation of the issues laid down in the aim of the Act, regarding ethical values, social justification of the GMO within a sustainable development. Given this lack of necessary information for such an evaluation, the Applicant has not demonstrated a benefit to the community and a contribution to sustainable development from the use of MON 87708. The Applicant should thereby provide the necessary data in order to conduct a thorough assessment on these issues, or the application should be refused.

It is also important to evaluate whether alternative options, (e. g. the parental non-GM version of this MON 87708 has achieved the same outcomes in a safer and ethically justified way.

Further, the Norwegian Gene Technology Act, with its clauses on societal utility and sustainable development, comes into play with a view also to health and environmental effects in other countries, such as where GMOs are grown. For instance, it is difficult to extrapolate on hazards or risks taken from data generated under different ecological, biological, and genetic contexts as regional growing environments, scales of farm fields, crop management practices, genetic background, interactions between cultivated crops, and surrounding biodiversity are all likely to affect the outcomes. Hence it cannot be expected that the same effects will apply between different environments and across continents.

Recommendation: The Applicant should submit required information on the social utility of MON 87708 and its contribution to sustainable development, in accordance with the Norwegian Gene Technology Act.

Conclusion

Available information for risk assessment evaluation

This evaluation is for the most part based on the Applicant's own submitted information. The directly relevant scientific literature is very limited in some cases, yet we have tried to extract relevant indirect information from the peer-reviewed literature.

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 mechanisms become elucidated that would allow any scientific information used in pursuit of regulatory approval to be transparent. This would include any information provided

by the Applicant used to justify confidentiality claims on any scientific data. We encourage the authorities to insist on this level of transparency and accessibility to all scientific data (including raw data) to ensure the scientific validity of the information presented.

Overall recommendation

Above we highlight a number of conceptual, empirical and informational deficiencies in the dossier that do not justify a conclusion of safe use, social utility and contribution to sustainable development of **MON 87708**. Critically, the Applicant has not included any of the required information to assess social utility and sustainability as required in Appendix 4 of the Norwegian Gene Technology Act, which would be necessary for consideration of approval in Norway. Taken together, these deficiencies fail to address the necessary safety regulations under Norwegian Law, and thus the application is incomplete and should not be approved. 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 the Norwegian authorities.

Therefore, in our assessment of MON 87708 we conclude that based on the available data, including the safety data supplied, the Applicant has not substantiated claims of safety satisfactorily to warrant approval in Norway at this time.

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