

**CONSOLIDATED RISK ASSESSMENT REPORT OF MONSANTO'S ALFALFA J101
APPLICATION FOR DIRECT USE AS FOOD AND FEED OR FOR PROCESSING (FFP)**

EXECUTIVE SUMMARY

On September 10, 2018, Monsanto Philippines submitted alfalfa J101 application for direct use under the DOST-DA-DENR-DOH-DILG Joint Department Circular (JDC) No. 1 Series of 2016.

After reviewing the Risk Assessment Report and attachments submitted by the applicant, the STRP, BAI, and BPI-PPSSD found scientific evidence that alfalfa J101 is as safe as its conventional counterpart and shall not pose any significant risk to human and animal health.

The Department of Environment and Natural Resources – Biosafety Committee (DENR-BC), after a thorough scientific review and evaluation of the accomplished Project Description Report (PDR) and Environmental Risk Assessment (ERA) form along with the submitted sworn statement and accountability of the proponent, reported that the direct use of the regulated article will not cause any adverse effect on the environment (land and water) and biodiversity.

The DOH-BC, after a thorough scientific review and evaluation of documents related to Environmental Health Impact, found scientific evidence that the GM application will not cause significant adverse effects to human and animal health, is unlikely to result in allergenic reaction, and is as safe as food or feed derived from conventional varieties.

Furthermore, the Socio-economic, Ethical and Cultural (SEC) expert, after reviewing thoroughly the accomplished SEC questionnaire, also recommended for the issuance of biosafety permit.

BACKGROUND

In accordance with Article VII. Section 20 of the JDC, no regulated article, whether imported or developed domestically, shall be permitted for direct use as food and feed, or for processing, unless: (1) the Biosafety Permit for Direct Use has been issued by the BPI; (2) in the case of imported regulated article, the regulated article has been authorized for commercial distribution as food and feed in the country of origin; and (3) regardless of the intended use, the regulated article does not pose greater risks to biodiversity, human and animal health than its conventional counterpart.

The BPI Biotech Office provided the assessors the complete dossier submitted by Monsanto Philippines. Upon receipt of the individual reports from the assessors, the BPI Biotech Secretariat prepared this consolidated risk assessment report for the information of the public.

Information on Applied Event

Roundup Ready alfalfa J101 was developed using *Agrobacterium*-mediated transformation. Alfalfa J101 contains the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4 that encodes for the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein, which confers tolerance to glyphosate.

Countries Where Approvals Have Been Granted (for FFP; for Commercial Propagation)

Australia/ New Zealand (Food, 2007), Canada (Feed, Environment, 2005; Food, 2005), Japan (Food, 2005; Feed, 2006; Environment, 2006), Korea (Food, 2017; Feed, 2008), Mexico (Food, 2005), Philippines (Food, Feed & Processing, 2011), Singapore (Feed, 2012), Taiwan (Feed, 2018*), United States (Food and Feed, 2004; Environment, 2005).

Source: *Public Information Sheet of Alfalfa J101 for direct use as food and feed, or for processing*

STRP, BPI-PPSSD, BAI (Safety Assessment)

1. Host Organism (*Medicago sativa* L.)

Majority of alfalfa is grown and harvested for animal feed. Alfalfa is highly valued for animal feed because of its high protein content, high intake potential, and high digestibility. Alfalfa can provide the sole plant component in many livestock feeding programs when supplemented with the proper minerals. While alfalfa is primarily used as an animal feed, some food uses exist. For instance, alfalfa teas and alfalfa sprouts are occasionally consumed.

Lignin is considered as a key anti-quality/anti-nutritional factor associated with alfalfa. Lignin is a class of organic polymer that forms important structural materials particularly in the formation of cell walls in plants. Since it is cross-linked with other cell components, it minimizes the accessibility of cellulose and hemicellulose to microbial enzymes leading to a reduced digestibility of biomass. Furthermore, there are no toxicants or allergens found in alfalfa as evidenced by its long history of animal feed use.

2. Transgenic Plant

Alfalfa J101 has been reviewed and approved for food use in many countries including Australia (2007), Canada (2005), Japan (2005), Mexico (2005), New Zealand (2007), Philippines (2006), South Korea (2007) and United States of America (2004) while for feed use in Canada (2005), Japan (2006), Philippines (2006), Singapore (2014) South Korea (2008) and United States of America (2004) (ISAAA, 2018).

Alfalfa J101 was developed using *Agrobacterium*-mediated transformation. It contains the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4 that encodes for the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein, and confers tolerance to glyphosate, the active ingredient in Roundup® agricultural herbicides.

It was reported that the food uses of alfalfa are extremely minor. It is only consumed by humans as compressed leaf material for herbal teas and dietary supplements. Other historical or current food uses of alfalfa are extremely limited thus, consumption is not expected to change.

3. Donor Organism

The *cp4 epsps* gene is derived from the bacterium *Agrobacterium* sp. strain CP4, which is ubiquitous in the environment and has a well-established safety profile. *Agrobacterium* sp. strain CP4 is not known or reported to pose a risk of human allergenicity or pathogenicity to humans or animals.

The CP4 EPSPS protein represents less than 0.6% of the total protein in the forage. Bioinformatics analyses demonstrated that the CP4 EPSPS protein does not share structurally or immunologically relevant amino acid sequence similarities with known allergens. Thus, it is highly unlikely that the CP4 EPSPS protein contains immunologically cross-reactive allergenic epitopes.

4. Transformation System

Agrobacterium-mediated transformation was used to generate Alfalfa J101 and the genomic DNA was the target of genetic modification.

Agrobacterium-mediated transformation was carried out using a two-step procedure adapted from methods described by Walker and Sato (1981) and Austin et al. (1995). Plasmid PV-MSHT4 was used to generate Roundup Ready alfalfa event J101. *Agrobacterium*

tumefaciens binary strain (ABI) is an unregistered Monsanto proprietary *Agrobacterium* strain that contains the required transacting functional region, *trfA*, that in the presence of an introduced plasmid with the *ori-V* origin of replication allows plasmid replication and maintenance in *A. tumefaciens*. The ABI strain also contains additional genes that facilitate transfer of the T-DNA of interest, contained on plasmid PV-MSHT4, into the recipient plant. Plasmid PV-MSHT4 contains the *cp4 epsps* coding region under the control of a constitutive promoter. The recipient for transformation was an alfalfa clone R2336. Line R2336 was selected from an elite, high-yielding, fall-dormant FGI alfalfa breeding population using a tissue culture screen for callus formation and somatic embryo induction.

Each resultant callus from the *Agrobacterium*-mediated transformation was initially selected for the Roundup Ready trait through the addition of glyphosate to the plant culture media. Following somatic embryo induction, the glyphosate was removed and the embryos were allowed to develop. The resulting plantlets were transferred to soil pots as the T₀ generation. Rooted stem cuttings from the T₀ plants were selected for vegetative tolerance to glyphosate through application of Roundup Ultra herbicide. The subsequent F1 and MBC1 generations of Roundup Ready alfalfa plants were treated with Roundup Ultra herbicide at the two to three trifoliate stages. Roundup Ready alfalfa event J101 was determined to be hemizygous for the trait and displayed superior vegetative and reproductive tolerance in field studies with three sequential applications of Roundup Ultra herbicide. Introgression of Roundup Ready alfalfa event J101 into new alfalfa varieties was done using FGI's breeding process. (Source: Monsanto Petition to USDA. 2004. Petition for the Determination of Nonregulated Status: Roundup Ready® Alfalfa (*Medicago sativa* L.) Events J101 and J163. Monsanto Petition # 04-AL-116U. Monsanto Company. St. Louis, Missouri. Section III.A (Pages 28-30), Walker, K.A. and S.J. Sato. 1981. Morphogenesis in callus tissue of *Medicago sativa*: The role of ammonium ion in somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* 1:109-121, Austin, S., E.T. Bingham, D.E. Mathews, M.N. Shahan, J. Will and R.R. Burgess. 1995. Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing alpha-amylase and manganese-dependent lignin peroxidase. *Euphytica* 85:381-393.)

5. Inserted DNA

Using Southern blot analysis, PCR, and sequence analysis, it was confirmed that J101 contains a single copy of T-DNA containing the *cp4 epsps* expression cassette stably integrated at a single insertion site.

The integrity of the transformation cassette in J101 was determined by digesting test and control genomic DNA with the restriction endonuclease *Pst* I. Results show that: 1) probing the *Pst* I digested J101 genomic DNA with the P-eFMV genetic element (Probe 1) yielded the expected ~2.2 kb hybridization signal, 2) probing the *Pst* I digested J101 genomic DNA with the *HSP70-ctp2-cp4 epsps* genetic element (Probe 2) yielded two hybridization signals of ~1.2 kb and ~2.2 kb, and 3) probing the *Pst* I digested J101 genomic DNA with the E9 3' genetic element (Probe 3) yielded an ~1.2 kb hybridization signal.

No unexpected bands were detected, indicating that J101 does not contain any additional P-eFMV promoter element sequences, *HSP70-ctp2-cp4 epsps* coding region element sequences, and E9 3' polyadenylation sequence, other than that associated with the intact cassette.

6. Genetic Stability

The stability of the T-DNA insert across multiple generations was demonstrated by Southern blot analysis. The analysis demonstrated that the J101 single integration locus was maintained through five generations of breeding; thereby confirming the stability of the insert.

Furthermore, inheritance from five sexual generations was performed to determine the Mendelian heritability and stability of the inserted gene in J101 alfalfa (Monsanto, 2003). The result of analysis indicates that the Roundup Ready trait in J101 is inherited in one locus following Mendelian laws.

7. Expressed Material

The concentration of CP4 EPSPS protein in various plant tissues derived from J101 alfalfa were quantified by enzyme-linked immunosorbent assay (ELISA) (Monsanto, 2003). The mean level of CP4 EPSPS protein in J101 alfalfa forage is 276 µg/g fresh weight according to the 2001 study and 223 µg/g fresh weight according to the 2002 study.

The novel protein has no metabolic role. Glyphosate binds to the endogenous plant EPSPS enzyme and blocks the biosynthesis of 5-enolpyruvylshikimate-3-phosphate (EPSP) in conventional plants, thereby depriving plants of essential amino acids. In Roundup Ready plants, the presence of CP4 EPSPS reconstitutes the shikimic acid pathway thus, is able to continuously synthesize aromatic amino acids even in the presence of glyphosate.

8. Toxicological Assessment

The results of the SDS PAGE and western blot assays demonstrate that CP4 EPSPS protein is rapidly degraded in simulated gastric fluid containing pepsin within 15 seconds (Leach et al., 2002). The estimated T50 result for SGF is <15 seconds. Results from the digestibility experiments show that CP4 EPSPS protein will likely be digested in the typical mammalian gastric environment and it is highly unlikely to pose a safety concern to human and animal health.

Furthermore, at temperatures of 75 degree C and above, the CP4 EPSPS functional activity was below the limit of detection. SDS-PAGE results show that the heat-treated test substance after incubation for 15 or 30 minutes at all temperatures used had no effect on the band intensity of the CP4 EPSPS protein. Hence, the CP4 EPSPS protein is denatured at high temperatures.

Bioinformatics analyses using FASTA sequence alignment program and ALLPEPTIDES protein database provided by the developer indicated that CP4 EPSPS has no significant homology to any known toxin (Pearson and Lipman, 1988; Harrison et al., 1996).

Studies by Harrison, et al., (1996) showed that the CP4 EPSPS protein is not toxic and is equivalent to the CP4 EPSPS present in alfalfa Event J101. The proteins were administered by gavage to mice to assess acute toxicity. The No Effect Level (NOEL) for oral toxicity to mice was 572 mg/kg body weight which was the highest dose tested.

9. Allergenicity Assessment

Digestibility of J101 CP4 EPSPS in simulated gastric fluid (SGF, containing pepsin) was assessed using SDS-PAGE and western blot methods. T50 result for SGF is below 15 seconds. No protein bands due to degradation of the CP4 EPSPS protein were observed.

The CP4 EPSPS functional activity assay and SDS-PAGE analyses indicated loss of functional activity at elevated temperatures of 55 °C and greater due to protein denaturation. At 55 °C, the activity decreased to 70% of the control when treated for 15 minutes and to 25% of control activity when treated for 30 minutes (Hernan et al., 2011). The activity is significantly impacted by heat treatment.

No immunologically relevant sequences or eight contiguous amino acid identities were detected when compared to the ALLERGEN3 sequence database, thus, it does not share structurally relevant similarity with allergens or gliadins.

Based on the concentration of CP4 EPSPS protein in forage and % dry weight of total protein in J101, the percent of CP4 EPSPS protein in one gram of J101 forage is 0.49% of the total protein in J101 which represents a very small portion of total protein in J101 harvested forage (Duke et al., 1981). Hence, the margin exposure of humans or animals to risk of consuming high dosage of CP4 EPSPS is extremely high.

10. Nutritional Data

Compositional analysis indicated no significant differences in the proximate levels for protein, fat, ash and moisture in J101 alfalfa and the non-transgenic alfalfa (McCann and Nemeth, 2003).

Furthermore, 4 commercially available alfalfa varieties were grown under the same environmental condition. All test values of proximate were within the tolerance interval. Lastly, all test values of proximate were within the literature range or similar to literature values.

For the analysis of key nutrients, cysteine, glutamic acid and tyrosine were found to be statistically significant different between the J101 and non-transgenic control but mean values of all analytes were within the tolerance interval. Moreover, when compared with other commercially available alfalfa varieties grown under the same environmental conditions, results showed that test values of the key nutrients were found to be within the tolerance interval.

Lignin levels were also analyzed, the results showed that lignin levels were not statistically different between J101 and non-transgenic alfalfa, all test values were within the tolerance interval established from the commercial varieties, and within or similar to literature range.

11. Recommendation

BPI-PPSSD, BAI and STRPs find scientific evidence that the regulated article applied for direct use as food and feed or for processing is as safe as its conventional counterpart and shall not pose greater risk to human and animal health.

DENR Biosafety Committee (Environmental Safety)

After a comprehensive review and evaluation of the documents including the scientific evidence from references and literature submitted by Monsanto Philippines, Inc., on its application for Direct Use as FFP of Alfalfa J101, hereunder are the observations and appropriate actions:

1. The direct use of the regulated article whether for food, feed or for processing will not cause any significant adverse effect on the environment (land and water) and biodiversity. The transgenic crop will not increase its weediness potential in case the seeds spill out into the environment because the CP4 EPSPS protein product produced by the transgenic crop will degrade upon exposure to the natural environment and general conditions such as high temperatures (65°C and above), varying pH, enzyme digestion, etc. (Okunuki et al., 2002);
2. The donor organism for CP4 EPSPS protein, *Agrobacterium* sp. is ubiquitous to the environment and does not pose significant risk of pathogenicity to animals. Also, based on the bioinformatics analysis, CP4 EPSPS has no structural similarity to any putative toxins to mammals (Nida et al., 1996 and Fuchs et al., 1993);
3. Based on the reproductive biology of alfalfa, the alfalfa bloom can only be pollinated once by a single pollinating insect (primarily bees) because of its nonreversible "tripping" mechanism, which upon tripping, lodges the stigma into the groove of the standard petal preventing fertilization. Also, flowers do not shed its pollen grains to the wind (CFIA, 2012); and
4. The project description report (PDR) discusses the specified environmental management plan indicating the possible risk and harm to the environment and biodiversity as well as the mitigating measures and contingency plan. Furthermore, the chances of unintended release or planting of the regulated article is very minimal and will not cause any damaging and lasting effects because the receiving environment (areas near the port, roads, railways, etc.) is not conducive for plant growth. Also, alfalfa requires nutrients that are not commonly found in soil thus require human intervention for growth (Ottoman, 2010). Also, during ripening periods, rain causes poor seed quality and decrease in seed yield thus are suitable in Prairies where rainy season is unlikely (CFIA, 2012).

DOH Biosafety Committee (Environmental Health Safety)

The DOH-BC, after thorough review of the documents, find that the regulated article applied for Direct Use as Food, Feed or for Processing (FFP) is safe as its conventional counterpart and shall not pose any significant risk to human and animal health and environment.

The following are the observations and recommendations:

1. 1.Scientific pieces of evidence from Toxicity studies and references, find that the regulated article will not cause significant adverse health effects to human and animal health.
2. Dietary exposure to the regulated article is unlikely to result in allergic reaction.
3. The regulated article is as safe as food or feed derived from conventional alfalfa varieties.
4. The regulated article is not materially different in nutritional composition from that of the non-transgenic alfalfa or the conventional alfalfa.
5. It is suggested that the Bureau of Plant Industry (BPI) ensure that there shall be clear instructions that the product is only for the purpose of direct use for FFP and is not to be used as planting materials.

Based on the above considerations and with the submitted sworn statement and accountability of the proponent, we hereby submit our evaluation to BPI relative to the application of a Biosafety permit for Direct Use as Food, Feed, or for Processing (FFP) of Alfalfa J101.

SEC Expert (Socio-Economic Considerations)

Alfalfa is highly valued for animal feed because of its high protein content, high intake potential and digestibility. It can provide the sole plant component in many livestock feeding programs when supplemented with the proper minerals. In the Philippines importation of alfalfa meals and pellets for livestock purposes started in the early 1990s. It eventually reached its peak in 2008 - 2010 when almost 1M Kg of alfalfa meals and pellets were imported.

There is no local production of alfalfa hay for meals or pellets in the country and the Philippines relies heavily on importation as previously shown in the data provided for question no.1. In this regard, we do not believe this GM alfalfa product will drastically change current patterns of production, consumption/utilization and trade.

After a thorough and scientific review and evaluation of the documents provided by Monsanto Philippines Inc., relevant to alfalfa J101, the SEC Expert recommends the approval and issuance of biosafety permit for direct use as food and feed or for processing.