ASSESSORS' CONSOLIDATED REPORT ON MONSANTO PHILIPPINES' APPLICATION FOR DIRECT USE AS FOOD AND FEED, OR FOR PROCESSING OF ALFALFA J163

EXECUTIVE SUMMARY

On September 23, 2018, Monsanto Philippines Inc. submitted alfalfa J163 for direct use as food and feed, or for processing, as original application 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 assessors namely: Scientific and Technical Review Panel (STRP), BPI Plant Products Safety Services Division (BPI-PPSSD) and Bureau of Animal Industry- Biotech Team (BAI-BT), concurred that alfalfa J163 is as safe for human food and animal feed as its conventional counterpart.

The Department of Environment and Natural Resources – Biosafety Committee (DENR-BC), after a thorough scientific review and evaluation of the documents related to Environmental Risk along with the submitted sworn statement and accountability of the proponent, recommended the issuance of a biosafety permit for this regulated event provided the conditions set by DENR are complied.

Also, the Department of Health – Biosafety Committee (DOH-BC), after a thorough scientific review and evaluation of documents related to Environmental Health Impact, concluded that alfalfa J163 will not pose any significant risk to the health and environment and that any hazards could be managed by the measures set by the department. DOH-BC also recommended for the issuance of biosafety permit for alfalfa J163.

Furthermore, the Socio-economic, Ethical and Cultural (SEC) Considerations expert also recommended for the issuance of biosafety permit for this regulated article after assessing the socio-economic, social and ethical indicators for the adoption of Genetically Modified Organisms.

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, except for the SEC expert, the complete dossier submitted by Monsanto Philippines. The SEC expert, on the other hand, was provided with a questionnaire on socio-economic, ethical and cultural considerations that have been addressed by Monsanto in relation to their application.

Upon receipt of the individual reports from the assessors, the BPI Biotech staff prepared this consolidated risk assessment report for the information of the public.

STRP ASSESSMENT AND RECOMMENDATIONS

Based on the documents submitted by the applicant:

A. Host Organism

Alfalfa is mostly used as feed and it is also used for food as well. According to literature, it is a major source of protein to majority of domestic animals and is cultivated by more than 80 countries in an expand of 35 million hectares.

Food uses of alfalfa are very limited. It is consumed by humans in the form of compressed leaf material for dietary supplements and herbal teas and as alfalfa sprouts. It is not known to be allergenic. As animal feed, it is repared as dried alfalfa hay for ruminants, or as source of carotene especially for layer chickens. Alfalfa sprouts is a common source of fiber and carotene for humans

In addition, alfalfa contains lignin which was reported to elicit anti-nutritional property. J163 contains about 18 % lignin distributed through stems, leaves and other parts of the plant as reported in the evidences presented by the proponent

Searches on literature databases showed that alfalfa is not now nor has it been a substantial source of human food.

B. Transgenic Plant

Alfalfa J163 has been approved as food in the following countries: Australia/New Zealand, Canada, Japan, Korea, Mexico, and United States, and feed in the following countries: Canada, Japan, Korea, Singapore, Taiwan, and United States.

No change in consumption patterns by population subgroups is expected with the introduction of the novel food. This is due to its low consumption as food item. J163 is not materially different in composition, safety or nutrition from conventional alfalfa other than the introduction of the glyphosate-tolerance trait.

C. 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 has not been reported to pose a risk of allergenicity or pathogenicity to humans or animals.

J163 was developed through Agrobacterium-mediated transformation of alfalfa callus (from line R2336) using the double border, binary vector PV-MSHT4. This vector contains a region of DNA (T-DNA), which has the cp4 epsps expression cassette flanked by left and right border sequences. This sequence, of approximately 3.8 Kb, was transferred into the alfalfa genome by Agrobacterium tumefaciens during the transformation process. The cp4 epsps expression cassette contains the cp4 epsps coding sequence under the regulation of the 35S promoter, a heat shock protein intron (HSP70), a chloroplast transit peptide (CTP2) sequence and a E9 3' polyadenylation sequence.

E. Transformation System

The transformation method used was Agrobacterium – mediated transformation while the target of genetic modification is the genomic DNA found in the nucleus.

The experimental protocol was carried out with Agrobacterium-mediated transformation. The Agrobacterium strain 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 recipient for transformation was an alfalfa clone R2336, which

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. Selection for the Roundup Ready trait through the addition of glyphosate to the plant culture media was performed.

The double border, binary vector PV-MSHT4 was used. Plasmid vector PV-MSHT4 contains DNA sequences that are necessary for transfer of T-DNA into the plant cell. This sequence, of approximately 3.8 Kb, was transferred into the alfalfa genome by Agrobacterium tumefaciens during the transformation process.

All the genetic components of the construct as well as description and characterization of the coding and non -coding regions were thoroughly presented by the developer. Literature search prove the existence of these construct as well as its safe use in other plants.

F. Inserted DNA

J163 contains a single copy of T-DNA containing the cp4 epsps expression cassette that is stably integrated at a single insertion site and no detectable additional genetic elements. This was sufficiently demonstrated by Southern blot analysis and PCR and sequence analysis. No additional genetic elements, including backbone sequences from the transformation vector PV-MSHT4, linked or unlinked to the intact DNA insert, were detected in the genome of J163.

The integrity and order of genetic elements within the insertion site was demonstrated via Southern blot analysis. Alfalfa J163 and control (R2336) genomic DNAs were digested with Dra I and Mfe I. Both restriction endonucleases were each expected to cut twice at different sites within the T-DNA. When used together, the two restriction enzymes were expected to release three fragments of ~1.2 kb, ~2.3 kb, and 0.4 kb from an intact cp4 epsps gene cassette. Individual Southern blots were probed separately against the P-eFMV promoter region (Probe 1), the HSP70-ctp2-cp4 epsps coding region (Probe 2), and the E9 3' polyadenylation sequence (Probe 3). Separate hybridization of Probes 1, 2 and 3 against the Dra I + Mfe I digested J163 genomic DNA showed the expected fragment sizes of ~1.2 kb, ~2.3 kb, and 0.4 kb, respectively. No unexpected bands were detected indicating that alfalfa J163 does not contain any additional p-eFMV promoter, HSP70-ctp2-cp4 epsps, and E9 3' polyadenylation sequences. On the other hand, the appropriate control DNA (R2336) showed no hybridization signal in all blots

The potential for creating novel, chimeric ORFs was tested bioinformatically which showed that any putative polypeptides or proteins created from the ORFs in the insert or at the junction sequences did not show a sufficient degree of sequence similarity to known toxins and allergens. The transgene cp4 epsps has also been expressed in approved GM crops such as cotton, soybean, sugar beet, canola, and alfalfa events.

H. Genetic Stability

The stability of the T-DNA insert across multiple generations was assessed using Southern blot analysis. The results demonstrated that the J163 single integration locus was maintained through five generations of breeding; thus confirming the stability of the introduced trait. Segregation data were generated to assess the heritability and stability of the T-DNA present in J163. Chi square analysis, based on testing the observed segregation ratio to the expected segregation ratio according to Mendelian principles was performed over five generations to confirm the segregation and stability of T-DNA in J163.

The results support the conclusion that the cp4 epsps expression cassette in J163 resides at a single locus within the alfalfa genome and is inherited according to expected Mendelian inheritance

principles. The results are also consistent with molecular characterization data indicating a single insertion site for the cp4 epsps expression cassette.

I. Expressed Material

The level of expression of CP4 EPSP protein in forage obtained from Roundup Ready alfalfa populations containing the J163 event were obtained through Enzyme Linked Immunosorbent Assay (ELISA). Alfalfa populations were planted in the spring of 2001 at six field sites that represent geographies where alfalfa is typically grown in the United States. The CP4 EPSPS protein was determined at two different times during the growing season from two different years of forage growth (2001 and 2002). Forage was obtained from plants at the early to late bud stage which corresponds to the growth stage where alfalfa is usually harvested for maximum quality according to Marten et al. (1988). The mean level of CP4 EPSPS in J163 was 270 µg/g tissue fresh weights (TFW).

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, and is able to continuously synthesize aromatic amino acids even in the presence of glyphosate

J. Toxicological Assessment

Simulated gastric fluid (SGF) that contains a new formulation of pepsin was used to assess the susceptibility of the CP4 EPSPS protein to proteolytic digestion in vitro. SDS-PAGE, western blot analysis and EPSPS enzymatic activity assay were used to observe the digestibility of the E.coli – produced CP4 EPSPS. SDS-PAGE staining method showed that at least 98% of the mature CP4 EPSPS was digested in SGF within 15 seconds. No degenerative bands due to digestions were observed. Western blot analysis confirmed that greater than 95% of the mature CP4 EPSPS was digested in SGF within 15 seconds. Enzyme activity assay demonstrated that CP4 EPSPS activity was reduced by >90% within 15 seconds of incubation in SGF.

In additon, the effect of heat treatment on CP4 EPSPS protein was determined using a functional assay to assess the impact of temperature on activity and using SDS-PAGE to assess the impact of temperature on protein integrity. The analysis showed no significant change in band intensity of the CP4 EPSPS protein due heat treatment.

Furthermore, no biologically relevant structural similarities were observed between the CP4 EPSPS protein and pharmacologically active proteins (including protein toxins) that are known to cause adverse health effects in humans or animals, as shown by the bioinformatic analyses performed.

Meanwhile, an acute oral toxicity study was conducted with E. coli-produced CP4 EPSPS protein. The E. coli-produced CP4 EPSPS protein has been shown to be equivalent to the plant-produced CP4 EPSPS present in J163. The NOEL for oral toxicity of the CP4 EPSPS in mice was 572 mg/kg. No significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and the CP4 EPSPS-treated groups were observed.

The test protein was produced by Escherichia coli. Chemical and functional equivalence of the CP4 EPSPS produced by E. coli and the CP4 EPSPS produced in alfalfa J163 was demonstrated using five methods namely, N-terminal sequencing, MALDI-TOF MS analysis, Western blot analysis and immunoreactivity, SDS-PAGE, functional activity, and glycosylation analysis.

K. Allergenicity Assessment

A bioinformatic assessment of the CP4 EPSPS protein, using the allergen and public domain protein sequences databases, has been performed and demonstrates the absence of sequence similarity to proteins known to pose human health risks. No immunologically relevant sequences (eight contiguous amino acid identities) were detected when the amino acid sequence of the CP4 EPSPS protein was compared to the ALLERGEN3 sequence database.

In addition, the glycosylation analysis showed that the plant-produced CP4 EPSPS protein is not glycosylated and is equivalent to that of the E. coli -produced CP4 EPSPS protein. The apparent molecular weight of the plant-produced CP4 EPSPS protein was 43.3 kDa. Since this protein migrated with an identical molecular weight as that of the E. coli reference standard, it was concluded that this protein was the plant-produced CP4 EPSPS.

On the other hand, the overall mean CP4 EPSPS protein level in forage collected over two seasons is 270 g/g fresh weight, the CP4 EPSPS protein would represent approximately 0.52 percent of the total protein in J163. This calculation was based on total protein levels of 5.2 % for fresh alfalfa forage (Duke, 1981). Thus, the CP4 EPSPS protein represents a very small portion of the total protein in the forage of J163

L. Nutritional Data

The forage samples used in compositional analyses were taken from plants grown in 2001 at five replicated field sites across the alfalfa-producing regions of the United States (California, Illinois, New York, Washington, and Wisconsin). Plots were established using plants that were reared in a greenhouse and transplanted to the field. A randomized complete block design with four replicates per treatment was used at each location. The treatments were: a) non-transgenic conventional alfalfa; b) Roundup Ready J163 alfalfa were simplex (single copy, singe event); c) a synthetic population of Roundup Ready alfalfa (Syn 1) that contained a combination of J101 and J163, generated through conventional breeding (J101 x J163). Plots containing Roundup Ready alfalfa were treated with a Roundup agricultural herbicide at expected commercial treatment rates. Proximates include protein, fat, ash, and moisture. No significant difference was found in alfalfa J163, J101 x J163 and the conventional alfalfa.

Meanwhile, six statistically significant differences observed between the J163 and nontransgenic control: cystine, histidine, lysine, tyrosine, acid detergent fiber, and neutral detergent fiber. However, the mean values of all nutrients analyzed were within the 99% tolerance interval developed from the conventional alfalfa varieties grown at the same locations. Therefore, it is unlikely that these differences are biologically significant.

Furthermore, lignin levels were measured in alfalfa forage produced by alfalfa plants containing J163 and in control and conventional alfalfa varieties. The values were significantly greater in J163 (18 % increase) compared to the control, but were within the 99% tolerance interval established using data derived from the conventional varieties. Therefore, it is concluded that lignin level in Roundup Ready alfalfa is comparable to lignin levels in conventional alfalfa.

M. Recommendation

Find scientific evidence that the regulated article applied for human food and animal feed use is as safe as its conventional counterpart and shall not pose any significant risk to human and animal health

BPI-PPSSD and BAI ASSESSMENT AND RECOMMENDATION

Based on the assessments of BAI and BPI-PPSSD:

A. Toxicological Assessment

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.

In addition, E. coli-produced CP4 EPSPS protein behaves with a predictable tendency toward loss of functional activity at elevated temperature (55 °C and greater). SDS-PAGE was used to assess the impact of temperature on protein integrity.

Meanwhile, 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, and, the no observed effect level for oral toxicity of the CP4 EPSPS in mice was 572 mg/kg. No observable adverse effect on body weight cumulative body weight, or food consumption between control and CP4 EPSPS-treated groups was observed.

Lastly, E. coli-produced cp4 epsps was produced in an Escherichia coli expression system. The plant-produced CP4 EPSPS present in J163 shows equivalency to the E. coli-produced CP4 EPSPS protein.

B. Allergenicity Assessment

The results of bioinformatics analysis provided by the developer using the allergen and public domain protein sequences databases showed that CP4 EPSPS protein has no homology to any known allergens in the ALLERGEN3 sequence database.

The amino acid sequence, MALDI-TOF mass spectral analysis, and western blot analysis confirmed the equivalence in terms of physicochemical properties of J163- and E. coli- produced CP4 EPSPS protein. Glycosylation analysis showed CP4 EPSPS protein is not glycosylated, while the molecular weight analysis showed that the intact J163 produced CP4 EPSPS protein was calculated to be 43.6 kDa while E. coli-produced CP4 EPSPS protein was approximately 43.3 kDa.

C. Nutritional Data

Compositional analysis provided by the developer indicated no significant differences in the proximate levels for protein, fat, ash and moisture in J163 alfalfa and the non-transgenic alfalfa (McCann and Nemeth, 2003).

On the other hand, there are six (6) statistically differences observed between the J163 and non-transgenic control which are: cysteine, histidine, lysine, tyrosine, acid detergent fiber (ADF), and neutral detergent fiber (NDF). But these differences are unlikely to be biologically meaningful because the mean values of all analytes were within the 99% tolerance interval.

Meanwhile, there is 18% increase of lignin levels in J163 which is significantly greater than the control but within 99% tolerance interval established using data from conventional varieties. Hence, Roundup Ready alfalfa (J163) has comparable lignin levels with conventional alfalfa.

D. Recommendation

For J163 alfalfa, weight of evidences approach indicates the substantial equivalence of the single event in terms of nutritional composition and food safety, with the conventional alfalfa other than the tolerance to glyphosate-containing herbicides. After reviewing the provided material of Monsanto Philippines, Inc. and other literatures, it is therefore concluded that alfalfa J163 is as safe as its conventional counterpart.

DENR ASSESSMENT AND RECOMMENDATION

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 (J163), 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 pill 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).

Based on the evaluation and review of literatures cited, the DENR-BC considered the regulated article safe to the environment and biodiversity, and hereby submits the technical report relative to the application of Monsanto Philippines, Inc. for Biosafety Permit for direct use as food, feed, or for processing of Alfalfa J163.

DOH ASSESSMENT AND RECOMMENDATION

After a thorough review and evaluation of the documents provided by the proponent, Monsanto Philippines, Inc., through the Bureau of Plant Industry (BPI), in support of their application for approval for Direct Use as Food, Feed or for Processing (FFP) of Alfalfa J163, the DOH-BC:

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. 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 J163.

SEC ASSESSMENT AND RECOMMENDATIONS

The SEC expert has reviewed the SEC application of MONSANTO Philippines, Inc. on Event Roundup Ready Alfalfa J163 for direct use (feed, food, processing). The expert found the SEC application well documented in terms of the basic socio-economic date needed for the evaluation. Therefore, the SEC expert is favorably endorsing the SEC approval of Monsanto's Roundup Ready Alfalfa J163 for direct use.