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Ministério da Ciência e Tecnologia

GOVERNO FEDERAL BRASIL PAÍS RICO É PAÍS SEM POBREZA

Ministry of Science, Technology and Innovation – MCTI

National Biosafety Technical Commission – CTNBio

comissão técnica nacional CTNBio de biossegurança

Executive Secretary

Opinion by Rapporteur – Commercial Release

TECHNICAL OPINION

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Address:	Rua Domingos Jorge, 1100, Prédio 9701, Térreo, 0477-900 São Paulo, SP.
CIBio Chairman:	Denis Lima
Title:	"Commercial Release of genetically modified soybean named 'FG72' soybeans, tolerant to herbicides based on glyphosate and

isoxaflutole."

GMO Description: Soybean, event FG72, tolerant to glyphosate and isoxaflutole herbicides (IFT).

CTNBio, following examination of request for a technical opinion on commercial release of genetically modified soybeans event FG72, concluded for its **GRANTING** on the terms of this Technical Opinion.

Bayer S.A. requested CTNBio to prepare an opinion on biosafety of genetically modified soybean tolerant to herbicides based on glyphosate and isoxaflutole, event FG72, for the purpose of its release in the environment, marketing, consumption and any other activities related to the GMO and progenies thereto. Tolerance to herbicides was reached by introduction of two genes: one modified version of the maize (*Zea mays*) gene *epsps* that grants tolerance to glyphosate; and one also modified version of bacterium *Pseudomonas fluorescens* gene *hppd* isolate *A32* that grants tolerance to herbicide isoxaflutole (IFT). The coding sequence of maize *epsps* gene, coding for the native enzyme EPSPS (5-enolpyruvylshikimate 3-phosphate synthase), was isolated from the maize genome and changed in two positions through site directed mutation, originating mutated gene *2mepsps*, which codifies the double-mutant enzyme 2mEPSPS. The presence of enzyme 2mEPSPS grants soybean FG72 tolerance to glyphosate herbicide. The coding sequence of gene *hppd* of bacterium *Pseudomonas fluorescens* isolate A32, which codes for enzyme HPPD (p-hydroxyphenylpyruvate dioxygenase), was also altered in one position through site directed mutation, originating mutated gene *hppd*, coding for enzyme HPPD. The presence of enzyme HPPD grants soybeans FG72 tolerance to herbicide isoxaflutole. Analysis of the event's risk assessment was conducted according to Article 3 of Ruling Resolution no. 5/2008. Information

previously submitted included the molecular characterization of the event; assessment of proteins expression; compositional analysis of different plant tissues; and the result of experiments for the event agronomic and phenotypic assessment. CTNBio studied the molecular characterization (PCR and Southern Blot); composition of kernels; expression of proteins; tolerance to herbicides; toxicity; allergenicity; carcinogenicity; and no evidence was found of additional risks contrasted to conventional soybeans regarding aspects of human and animal health and effects to the environment.

CTNBio reviewed reports submitted by applicant as well as independent scientific literature. Scientific studies conducted to assess biosafety and agronomic and phenotypic characteristics as part of the risk assessment of this GMO included the representative regions for soybean farming within the Brazilian territory.

TECHNICAL OPINION

I. GMO Identification.

GMO Designation:	Soybeans event FG72
Species:	Glycine max
Inserted Characteristics:	Tolerance to glyphosate and isoxaflutole herbicides.
Proposed Use:	Release to the environment; marketing; consumption and any other activities related to the GMO and progenies derived therefrom.
Characteristics introduction method:	Introduction in soybean variety JACK through microparticle bombardment method (biolistics).

Expressed Proteins: HPPD and EPSPS.

II. General Information

Genetically modified soybeans event FG22, ("FG72 soybean" hereinafter), an GMO classified as Risk I Class, shows tolerance to glyphosate and isoxaflutole herbicides, granted by introduction of two genes: one modified version of gene *epsps* of maize (*Zea mays*), which grants tolerance to glyphosate herbicide and one also modified version of gene *hppd* from bacterium *Pseudomonas fluorescens*, isolate *A32*, granting tolerance to isoxaflutole herbicide (IFT).

The coding sequence of maize gene *epsps*, coding for the native enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), was isolated from the maize genome and altered in two loci through site directed mutation, originating mutated gene 2*mepsps*, which codes for double-mutant enzyme 2mEPSPS. Presence of enzyme 2mEPSPS grants FG72 soybean tolerance to glyphosate.

The coding sequence of gene *hppd* of bacterium *Pseudomonas fluorescens* isolate *A32* - Genebank A69533 (MCKELLAR, 1982), which codes for enzyme HPPD (p-hydroxyphenylpyruvate dioxygenase), was also altered in one locus through site directed mutation, originating the mutated gene *HPPD W336* (BOUDEC *et al., 2001*), which codes for enzyme HPPD W336. The presence of enzyme HPPD W336 grants FG72 soybean tolerance to isoxaflutole (SAILLAND *et al.,* 2001).

Genes 2*mepsps* and *HPPD W336*, present in one expression cassette, were introduced in JACK soybeans plants by the method of micro-particles bombardment (biolistics). After selection in a medium containing the herbicide isoxaflutole, the cells were submitted to multiplication, regeneration to embryos and budding in absence of the selective agent. Regenerated seedlings

were transferred to a greenhouse and submitted to a further selection, this time with the glyphosate herbicide (pulverization). Surviving plants were cultivated up to flowering and production of seeds.

FG72 soybean was obtained by integrating the linear DNA filament isolated from plasmid vector pSF10 containing genes 2*mepsps* and *HPPD W336* expression cassettes. Psf10 was constructed by inserting genes 2*mepsps* and *HPPD W336* in the cloning plasmid PBR322 (BOLIVAR *et al.* 1977). The fragment of interest, of 7.3 kb, containing the expression cassettes of genes 2*mepsps* and *HPPD W336*, was freed from the plasmid by cleavage with restriction enzyme SA1I and purified by HPLC, and then used to transform the JACK variety soybean by biolistics. Therefore, the vector sequence was not introduced in the GMO.

The expression cassette of gene 2*mepsps* contains the modified coding sequence of gene *epsps* of maize (*Zea mays*) controlled by the following regulating sequences: 1) promoter region Ph4a748At of Histone 4 gene from *Arabidopsis thaliana* (CHABOUTE *et al.*, 1987); 2) intron *1 h3At*, the first intron of gene II of Histone H3.III variant of *Arabidopsis thaliana* (CHAUBET *et al.,* 1992); 3) optimized transit peptide TPotp C, containing gene sequences of maize (*Zea mays*) and of sunflower (*Helianthis annuus*) RuBisCO small subunits, directing the modified protein to plastids, where the native protein EPSPS is located (LEBRUN *et al.,* 1996). Specific peptidases are responsible for the cleavage and degradation of the peptide after transport (RICHTER & LAMPPA, 1999); and 4) the polyadenylation signal sequence 3'histonAt of gene histone H4 of *Arabidopsis thaliana* (CHABOUTE *et. Al.,* 1987).

Gene *HPPD W336* expression cassette contains the modified coding sequence of gene *hppd* of *Pseudomonas fluorescens* controlled by the following regulatory sequences: 1) one variant of promoter Ph4a478 of *Arabidopsis thaliana* that contains one internal duplication, namely

Ph4a748 ABBC, and the leading sequence etch (coming from tobacco), which operates as an enhancer; 2) the optimized transit peptide TPotp C that contains gene sequences of maize (*Zea mays*) RuBisCO small subunit and of sunflower (*Helianthus annuus*) directing the protein to the plastids where the native protein EPSPS is located (LEBRUN *et al.,* 1996); 3) the termination region 3'nos of nopaline synthase of *Agrobacterium tumefaciens*, responsible for the polyadelynation signal.

Southern Blot analyses of FG72 soybean genomic DNA and of JACK variety of control plants digested with 10 different restriction enzymes, using as probe the different regions of the cassette used for the transformation, showed that FG72 soybean contains two T-DNA complete copies in the head-to-tail orientation, flanked by two partial sequences of 3'histonAt (polyadenylation signal) in portion 5' in the head-to-head orientation. This means that two "in tandem" copies of the 2*mepsps* and *HPPD W336* expression genes were inserted in the FG72 soybean. Southern Blot analyses failed to reveal in Event FG72 the presence of any sequence of plasmid vector pSF10 wherefrom the transformation cassette was obtained. Therefore the antibiotic-resistant gene present in the vector has not been transferred to FG72 soybean.

Sequencing of the inserted region inserted in FG72 soybean and of flanking regions 3' and 5' showed that: 1) insertion of transgene DNA failed to create any new open reading frame (ORF) in the soybeans genome that could originate a protein with biologic activity and failed to interrupt any ORF of the soybeans genome at the insertion locus. 2) occurrence of translocation of 4 small fragments of soybean DNA during the insertion of the cassette in the receiving conventional soybeans, which created 3 interruption points in the FG72 soybean event sequence, but failed to cause any interruption in endogen genes existing in the conventional soybean.

Data obtained in assessments conducted in FG72 soybean progenies after auto-pollination and crossing with conventional materials displayed a segregation pattern that shows insertion in only one mendelian inheritance locus.

Genetic stability refers to keeping, in successive generations, a change introduced in the genome. To assess this characteristics in event FG72 soybean, Southern Blot was used from the genomic DNA isolated from leaves of different individual plants of three generations (T2,T7 and T9), from three different genetic backgrounds and cultivation in four different locations in the United States. Isolated DNA was digested with restriction enzyme HindIII, that provides a single pattern for event FG72 soybean, and the results of hybridization in Southern Blot, using two different probes, which confirmed the presence of the expected fragments in all DNAs supplied from transgene samples and in the positive control, yet not in the negative control (summarized in Table 19, on page 87). This is an indication that the change introduced was stable along generations, in different genetic backgrounds and different environments, suggesting that the insertion is integrated to the FG72 soybean genome in a stable way.

Phenotypic stability refers to maintaining the expression of characteristics introduced along generations and is assessed by analyses to determine the mendelian inheritance which, in this case, is the tolerance to glyphosate and isoxaflutole herbicides. Samples from 901 F2 plants, coming from the crossing of sixth generation plants (T6) crossing with conventional lineages of soybeans to initiate the introgression of the selectivity characteristics to herbicides in commercial germplasm, were analyzed by PCR in order to identify the level of zygosity of the event. The segregation pattern observed is stable along several generations.

III. Aspects Related to Human and Animal Health

Soybean (*Glycine max*) is a leguminous plant that is present in the food chain for over 4,000

years and is part of Asian diet for thousands of years. Soybean is currently cultivated and marketed in over 35 countries, being the following countries the largest producers: United States (33.3%), Brazil (30,3%), Argentina (17,8%), China (3,6%) and India (3,6%) (USDA, 2015). The first to process soybean were the Chinese. Soybean grain was crushed to obtain oil that was used as food, and its flour initially employed as fertilizer and animal feeding and, later, in human food (TEIXEIRA, *et al.*, 2009). Soybean became known in the West only in the 19th century, planted in the United States in 1804 and in Brazil, in 1882. Soybean is the main source of raw material for extraction of edible plant oil and production of high protein content soybean meal, used in human food and animal ration, respectively (SEDIYAMA *et al.,* 2009). Soybean is currently used as edible oil, animal ration, food component for humans, in addition to flour, soap, cosmetics, resins, solvents and biodiesel. Before its consumption, soybean shall undergo thermal processing because of its anti-nutrients such as stachyose, raffinose, phytic acid, trypsin inhibitor and lectine.

Soybean is held as a complete source of protein to the human species, since it contains adequate amounts of all amino acids that are essential to humans, that is to say, those amino acids that the human body cannot synthesize and that shall therefore be obtained from food. Essays have suggested that a diet rich in soybean may reduce the risk of some types of cancer, such as lung cancer (SCHABATH *et al.*, 2005).

Being soybeans a beneficial food, with a history of safe use in human and animal feeding, the question to be answered is whether FG72 soybean is as safe as conventional soybean, namely, whether it fails to add any risk to human and animal health in comparison with other varieties of the grain already in use. To answer this question, several studies were conducted by applicant to assess Event FG72 safety for use as food, including analysis of the inserted DNA,

proteins expressed and the derived product in animal nutrition. The studies, following internationally accepted and recognized methodologies, would brace submissions to GMO regulatory bodies of different countries, including the Brazilian CTNBio.

FG72 soybean expresses a modified version of protein EPSPS of maize (*Zea mays*), which grants tolerance to herbicide glyphosate (protein 2mEPSPS) and an also modified version of protein HPPD of bacterium *Pseudomonas fluorescens* isolate *A32*, which grants tolerance to herbicide isoxaflutole (protein HPPD W336).

The coding sequence of maize protein *epsps* was changed in two positions through site directed mutation. Changes led to the change of amino acids in positions 102 (substitution of threonine by isoleucine and 106 (substitution of proline by serine) of native protein EPSPS, originating the double mutant 2*mepsps* (LEBRUN *et al.,* 1997), which codifies protein 2mEPSPS. Native protein EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) is one enzyme that is part of the shikimate pathway, used by bacteria, fungi and plants for the biosynthesis of aromatic amino acids phenylalanine, tyrosine and tryptophan. Its inhibition by glyphosate leads the plant to death. Double-mutant enzyme 2mEPSPS displays diminished bonding to glyphosate, enabling plants containing gene 2*mepsps* and expressing this mutated enzyme to be tolerant to glyphosate. Although presenting diminished affinity to glyphosate, the mutated enzyme shows sufficient enzymatic activity on other natural substrate shikimate-3-phosphate and phosphoenolpyruvate, which enables normal growth of the plant, even in the presence of this herbicide. The only difference between the native protein EPSPS of maize and protein 2mEPSPS expressed in FG72 soybean is the change of amino acids in positions 102 and 106 of their sequences, which contain a total of 445 amino acids. This means that the sequence

identity among them is 99.6%. Therefore, this is a protein found in maize, with a long history of use in human and animal food.

The equivalence of 2mEPSPS produced in *Escherichia coli* and in event FG72 soybean was shown through SDS-PAGE, Western Blot, N-terminal sequencing, enzymatic activity and mass spectrometer (MARTONE, 2009). Analyses by SDS-PAGE and Western blot showed that molecular weight and mobility of both proteins are the same. Western blot also showed that immunoreactivity of both proteins is equal. Mass spectrometer, used in fragments resulting from digestion with trypsin of both 2mEPSPS (produced in *Escherichia coli* and expressed in FG72 soybean) indicated that 98% of the 2mEPSPS produced in *Escherichia coli* was covered, while the protein expressed in Event FG72 soybean had its sequence covered in 71%. N-terminal sequencing showed that both 2m3EPSPS expressed in Event FG72 soybean and produced in *Escherichia coli* do not present N-terminal methionine. This loss of methionine is not uncommon, given that post-traductional modifications are frequent in eukaryotic and prokaryotic organisms (YANG *et al.,* 2014).

Coding sequence of *hppd* gene of bacterium *Pseudomonas fluorescens* isolate A32 was changed by one position through site directed mutation, giving origin to mutated gene *HPPD W336*. Amino acid tryptophan (W) on position 336 was substituted by one glycine (G) giving origin to mutant gene *HPPD W336* (BOUDEC *et al.,* 2001). Gene *hppd* codes for protein HPPD (p-hydroxyphenylpyruvate dioxygenase), which is a key enzyme in catabolizing tyrosyne in microorganisms, plants and mammals, responsible for transformation of p-hydroxyphenylpyruvate into homogentisic acid (HGA). In plants, HPPD has a relevant role, since the product of its reaction is also the aromatic precursor for the synthesis of all plastoquinones and tocopherols, essential elements for the transportation chain of

photosynthetic electrons and antioxidant systems, respectively (MORAN, 2005). The inhibition of native HPPD by herbicide isoxaflutole results in destabilizing photosynthesis, with the consequent foliage whitening and plant death. Mutated enzyme HPPD W336, coded by mutant gene *HPPD* W336, has diminished affinity with isoxaflutole (SAILLAND *et al.*, 2001) enabling survival of plants that have expressed it when treated with this herbicide. The only difference between the native plant of *Pseudomonas fluorescens*, which contains 358 amino acids, and the mutated protein HPPD W336 expressed in FG72 soybean is the change of amino acid tryptophan (W) to glycine (G) in position 336 of the sequence, that is to say, the two proteins are 99.7% identical. It shall be noted that *Pseudomonas fluorescens* from where gene *hppd* was isolated is a gram-positive non-pathogenic bacterium, ubiquitous in nature and found in water, soils and plant rhizosphere.

Protein HPPD W336 expressed in FG72 soybean corresponds to the amino acid sequence coded by the introduced gene *HPPD W336*. Western Blot indicated that the immunoreactivity of the two proteins is the same. Mass spectrometry conducted in fragments resulting from the digestion of the two proteins with trypsin indicated that 95.2% of the HPPD W336 sequence expressed in *Escherichia coli* was covered, while the protein expressed in Event FG72 soybean had its sequence covered in 70.1%.

Expression of Event FG72 soybean proteins 2mEPSPS and HPPD W336 was determined both for field cultivated plants and in different tissues along different phases of FG72 soybean in greenhouse.

Expression of proteins 2mEPSPS and HPPD W336 in grains of soybean Event FG72 cultivated in the field was determined by applicant and presented in Annex 1 of the Biosafety Report (POE, 2009), in a study conducted in 10 locations of the United States. Delineation used was that of

random blocks, with three portions with conventional soybean (Jack), 3 with commercial soybean varieties (Stine 2686-6, Stine 2788 and Stine 23000-0 and 6 portions with soybean Event FG72 soybean. Portions with Event FG72 were subdivided into 2 systems (with and without application of herbicides) and 3 repetitions and, in the whole, there were 12 portions in each of the 10 locations. Plants were cultivated under typical cultivation conditions and cultural traits and cultivation practices were identical for portions containing GMO and conventional soybean (isoline), except for application of the herbicides glyphosate and isoxaflutole in the portions with the GM soybean. Applications with isoxaflutole (70g a.i./ha) and glyphosate (1060 g a.i./ha) were made in phase V4-V5. Samples of grains were collected in two central tracks of portions, processed to extract proteins and subsequent dosage of proteins 2mEPSPS and HPPD W336 through the ELISA method.

Results indicate that the content of 2mEPSPS in grains is 1.5µg/g of fresh matter (FM) for samples under application of conventional herbicide and 1.3µg/g of FM for samples under application regime of glyphosate + isoxaflutole, representing 0.0004% and 0.0003% of total protein contained in grain, respectively. Average contents of HPPD W336 in grain were 0.94 µ/g of FM for samples under application of conventional herbicide and 0.89µg/g of FM for samples under the regime of glyphosate + isoxaflutole, representing 0.00024% and 0.00023% of total protein contained in grain, respectively.

Contents of HPPD W336 and 2mEPSPS were determined by applicant in different tissues (leaves, stalk, root and kernels) and along different growing phases of FG72 soybean (V4, V6 and V8) as described in the study submitted in Annex 2 of the Biosafety Report (HABEX & DEBAVEYE, 2009). Plants of FG72 soybean and of the non-modified variety Jack were cultivated in greenhouses of the Bayer facilities in Astene, Belgium, and 10 samples of each tissue were

collected. Samples of leaves were collected in phases V4, V6 and V8; samples of roots and stalk were collected in phases V4 and V8 and samples of kernels were collected for analysis, where the expression of target-proteins was quantified by the ELISA method. The results (Tables 13 and 14) show that proteins HPPD W336 and 2mEPSPS were detected in plants of FG72 soybean and all tissues and all growth phases. The strongest expression (µg/g FM) for the two proteins was in foliar tissues (569 to 668 for 2mEPSPS and 27.2 to 38.4 for HPPD W336), while the weakest expression were detected in kernels (2.62 for 2mEPSPS and 1,41 for HPPD W336).

Genetically modified plants that expressed genes 2*mepsps* and *HPPD W336* should be likely to be identified by swabbing the leaves or spraying the plants with the herbicides, since they are selective. Plants that maintained full growth (no phytotoxicity) with both herbicides indicate the presence of the insert. This is a general method. A more specific manner is the use of molecular detection methods, such as PCR (Polymerase Chain Reaction) or Southern Blot. Applicant developed a detection methodology by quantitative PCR in real time (Real Time PCR). Since the technique is highly specific and has high sensitivity detection, it shall be used in well-known laboratories, accredited by the competent monitoring office, as well as applying a recognized PCR methodology, validated by other institutions. Applicant proposes to make available the reference materials (seeds), initiators and probes in addition to specific methodology to laboratories interested in validating the PCR detection procedure. Therefore, the event may be immediately and specifically detected and identified in the environment.

IV. Risk Assessment for Human and Animal Health

Regarding the studies of compositional analyses initially submitted by applicant, such compositional analysis had been conducted in samples originated from cultivars in 10 different locations within the United States. CTNBio requested this analysis to be also conducted in

samples coming from Brazil and that the results were compared. The studies were conducted by applicant with samples coming from three field essays that were selected in the 2012-2013 crops in Taquaravi, State of São Paulo; Poxoréu, State of Mato Grosso; and Água Santa, State of Rio Grande do Sul. The analyses of samples produced in Brazil were conducted by *Laboratório TECAM*, São Paulo, SP (analytical phase of the study and determination of centesimal composition and fibers, minerals, Vitamin A, Vitamin B1, Vitamin B2, folic acid, tryptophan, phytic acid, stachyose, raphynose and fat acids), by *Centro de Ciências e Qualidade de Alimentos, The Food Technology Institute – CCQA/ITAL* (determination of lecithin, total isoflavones and tocopherols) and by *LABTEC – Laboratório de Análises Químicas,* located in Hortolândia, State of São Paulo (determination of total amino acids, except tryptophan).

Components selected for nutritional composition analyses represent important basic nutrients of soybean, such as centesimal composition (including fibers), micronutrients, such as minerals and vitamins, isoflavones, anti-nutrients such as raphynose, stachyose, phytic acid, trypsin inhibitors, total amino acids, and fat acids.

- **Centesimal Composition**: Centesimal composition analyses of soybean seeds, both for treated and untreated event FG72 soybean (application of herbicides IFT and glyphosate) and for its conventional isoline, failed to display difference between them. Averages remained within the range of values calculated from three commercial varieties of soybean and of reference intervals widely known and reported in the specialized literature for this culture.
- **Minerals and Vitamins**: Amounts of minerals and vitamins found in seeds of event FG72 soybean, either treated or not with herbicides, and its conventional isoline were equivalent between genotypes of the three commercial varieties and within reference

intervals described in the literature for this culture.

- **Isoflavones and Anti-nutrients**: Analyses of FG72 soybean seeds, either treated or not with herbicides and seeds of its conventional isoline showed that the amounts of isoflavones and non-nutrients failed to display significant differences between the treatments, and all amounts were within the range of values described by the literature for soybean seeds. When such analyses were conducted in toasted and untoasted soybean meal, the content of anti-nutrients in genetically modified and non-genetically modified samples was no different.
- **Amino acids:** The amount of amino acids found in soybean seeds and toasted and untoasted soybean meal coming from FG72 soybean, either treated or untreated with herbicides and from seeds of its conventional isoline failed to show significant differences both between the treatments and the ranges of reference reported in the literature.
- **Fat Acids**: The fat acid profile in samples of genetically modified seed and oils (both plants treated and untreated with herbicides) and non-genetically modified ones, were very similar. There were only small differences for oleic acid (C18:1) and linoleic acid (C18:2); however, the values remained between their respective ranges of reference.
- **Phospholipids**: The profile of phospholipids found in genetically modified soybean (either treated of untreated with herbicides) and in its non-genetically modified isoline is similar to each other and comparable to the levels found in conventional soybeans.

Therefore, these results taken as a whole, indicate that FG72 soybean displays substantial

equivalence to the non-genetically modified soybean. The principle of substantial equivalence, which is accepted by the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO), is a concept that has contributed towards construction of a robust framework to analyze alimentary safety of new food, including those coming from transgenic plants (FAO/WHO, 2000).

Regarding nutritional assessment and animal performance, studies were conducted in boiler chicken lineage ROSS 308 (*Gallus gallus domesticus*) using two-day birds, in a total of 420 individuals randomly selected (210 male and 210 female). In an experimental delineation, the birds were also randomly selected for the three treatment groups, with 140 birds in each such groups, accommodated in 14 replicates (seven male cages and seven female cages). The three treatment consisted of ration containing: (A) toasted soymeal seeds from soybeans conventional isoline; (B) toasted soymeal from FG72 soybean event; and (C) toasted soymeal seeds from commercial non-GM soybeans. Birds were fed for 6 weeks (42 days) and health effects, mortality, body weight, alimentary conversion efficiency, carcasses, muscles (breast, thigh, foot, wing) and abdominal fat weight were assessed (STAFFORD, 2009).

The results failed to identify any change related to consumption of FG72 soybean, that is to say, no adverse effect was recorded. For using animals that are very sensible to environmental and nutritional changes, the experiment also evidences that genes 2*mepsps* and *HPPD W336* do not produce toxins or other metabolites that may be hazardous to human or animal health.

Regarding digestibility of proteins 2mEPSPS and HPPD W336 expressed by FG72 soybean, it shall be emphasized that one of the physicochemical characteristics of allergenic proteins is resistance to proteolytic hydrolysis (AALBERSE, 2000) VERHOECK *et al.*, 2015). Therefore, resistance to digestibility of a protein has been used as a measure of its allergenic potential.

Results obtained by applicant in gastric system and intestine simulation essays conducted according to the methodology recommended by ILSI (International Life Science Institute) (THOMAS *et al.*, 2004) show that protein 2mEPSPS is rapidly degraded (<30 seconds) in a medium with pH 1.2 in the presence of pepsin, or in a medium with pH 7.5 in the presence of pancreatin (HEROUET-GUICHENEY *et al.*, 2009). Similarly, protein HPPD W336 was promptly degraded in a gastric simulation medium (pH 1.2 in a medium with enzyme pepsin) in thirty seconds of incubation. In the presence of pancreatin and pH 7.5 (simulating the intestine system), total degradation of HPPD W336 took place in less than 0.5 minutes. The results indicated that the two proteins are promptly digested when exposed to media containing digestive enzymes and under pH and temperature similar to the ones found in the gastrointestinal systems of humans, failing therefore to display allergenic characteristics.

Acute toxicity of proteins 2mEPSPS and HPPD W336 were assessed for 14 days in OF1 mice through administration of highly concentrated proteins by oral (2000 mg/kg of the target protein) and intravenous routes (up to 10 mg/kg). Clinical signs or individuals were accompanied on a daily basis with the purpose of recording the onset, severity, reversibility and duration of such symptoms. However, no change or mortality was found along the period of assessment. Weight gain in animals treated with the target proteins was assessed on a weekly basis and contrasted with that of animals treated with BSA (control), and no significant difference was found. At the end of the experiment, the animals were necropsied and macro- and microscopic assessments were conducted in internal organs and cavities. Results showed that the proteins 2mEPSPS and HPPD W336 failed to display any toxic effect on individuals submitted to oral ingestion or intravenous administration of these polypeptides in high concentration, indicating the safety of these compounds for the organisms.

In silico studies were conducted to asses, in public databases, similarity of proteins HPPD W336 and 2mEPSPS against sequences of allergens and toxins. No homology of epitopes or putative sites of n-glycosylation was found between the target-sequences and those sequences present in different publicly assessed databanks and knowingly being allergenic or toxic.

Summarizing, results indicate that proteins 2mEPSPS and HPPD W336 expressed in FG72 soybean have no potential adverse effect on human and animal health.

V - Assessment of Risk to the Environment

Soybean (*Glycine max*) is the currently world leading oilseed culture in production and consumption (WILCOX, 2004). Soybeans culture has its center of origin in the eastern region of China (BORÉM, 1999) and is recognized as one of the first plants that were cultivated. The species *Glycine max* belongs to the subgenus Soja, which includes *Glycine soja* and *Glycine gracilis*. *Glycine soja* is a feral species that grows naturally in fields, roadsides, and ravines, but only in some Asian countries. Cytological, morphologic and molecular analyses suggest that *Glycine soja* is an ancestor species of *Glycine max*. Feral species are endemic in China, Korea, Japan, Taiwan and the former Soviet Union. *Glycine gracilis* occurs only in northeastern China and has a morphology described as intermediary between *Glycine max* and *Glycine soja* (SKVORTZOV, 1927). *Glycine gracilis* is held to be a pest or semi-feral species of *Glycine max*, featuring intermediary phenotypic characteristics between *Glycine max* and *Glycinia soja* (OECD, 2000), or may be presented as a hybrid of *Glycine max* and *Glycinia soja* (OECD 2000). Besides the subgenus *Soja* there are 12 perennial species belonging to the subgenus *Glycine*. These species originated in Australia, South Pacific Islands, China, Papua New Guinea, Philippines and Taiwan. Attempts to hybridization between annual and perennial species failed to succeed in producing new individuals (HYMOWITZ, 1970).

Evolution of soybean started with the appearance of plants formed by natural mating between two species of feral soybean that were domesticated and improved by Chinese scientists. After domesticated, soybean was introduced in other Western regions and countries, such as Manchuria, Korea, Japan, Soviet Union and Southeastern Asian countries.

Soybean currently cultivated is typically herbaceous, annual and short days, with characteristics quite distinct from the ancestor that originated it, which were creeping and climbing species (SEDIYAMA *et al.*, 2005). Feral species that belonged to the genus *Glycine* may cross with cultivated soybean. However, according to VERNETTI (1983) and BORÉM (1999), there is no native, sylvan or feral species in Brazil that may intercross with *Glycine max*. The surveys conducted by HYMOWITZ (1970) and SINGH and HYMOWITZ (1999) prove that no selvatic species of soybean occur naturally in Brazil.

Regarding the history of cultivation and use of a parental organism in terms of safety for the environment, and human and animal consumption, informing on the likelihood of introgressive hybridization with species sexually compatible and on the possible selective advantage of the transgene it is important to stress that soybean (*Glycine max*) is a leguminous plant that is in the alimentary chain for over 4000 years and has been an essential part of the Asian diet for thousands of years. Currently cultivated soybean is quite different from its originating ancestors: creeping plants that developed in the eastern coast of Asia, mainly along the Yellow River in China. Its evolution started with the appearance of plants coming from natural crossings between two species of feral soybean that were domesticated and improved by scientists of ancient China. Its importance in the alimentary diet in the ancient Chinese civilization was so large that soybean, jointly with wheat, rice, rye and millet were held to be sacred grains, with right to ritualistic ceremonies at seeding and harvesting (EMBRAPA, 2004).

Soybean is currently cultivated in over 35 countries. The first to process soybean were the Chinese. Grains were crushed to obtain oil, which was used as food, and the soymeal initially used as fertilizer and food to the animals and, later used in human feeding (TEIXEIRA *et al*., 2009). Soybean was introduced to the West only in the twentieth century, arriving to the United States of America in 1804 and to Brazil in 1882. Soybean was the main source of raw material for extracting edible plant oil and of production of high protein bran used as human food and animal ration, respectively (SEDIYAMA *et al*., 2009). The production of grain is directed to different uses, such as oils (used in margarines, salads, cooking), alimentary products such as tofu, soy sauce, soy milk, soy meat (texturized protein) among others. Besides, soybean is used as alimentary supplement in animal rations. Industrial use of soybeans is related to a range of products, since production of antibodies to the use in soaps and disinfectants. (OECD, 2000).

Soybean arrived to Brazil, coming from the United States, in 1882. The first studies involving assessments of cultivar introduced from that country were conducted by Gustavo Dutra, professor of the State of Bahia School of Agronomy. In 1900 and 1901, the Agronomic Institute of Campinas (IAC), State of São Paulo, promoted the first distribution of soybean seeds for farmers of that State and, simultaneously, the first record of soybean farming was recorded in the State of Rio Grande do Sul (EMBRAPA, 2004). Currently, Brazil is the second larger world producer, with a planted area of 32.50 million hectares and a production of 97 million tons of the grain in the 2015/2016 crop (USDA, 2015).

The crossing of a sylvan species of soybean with a cultivated soybean species may lead to the creation of interspecific hybrid which, when successively recrossed with the cultivated species and followed by selection, originate descendants similar to the cultivated parental, though

enriched by some genes derived from the sylvan species. Through this process, named introgressive hybridization, many cultivated plants developed to be intensely cultivated by the human species (AZEVEDO *et al.*, 2000).

Cultivated soybean (*Glycine max*) crossed naturally with the sylvan species *Glycine soja*, although this has been recorded naturally only in China, Japan, Taiwan and Russia, and is not found in the Brazilian environment. Therefore, the likely transfer of the selectivity characteristics to glyphosate and isoxaflutole herbicides (introgression of genes 2*mepsps* and *hppd W336*, respectively) to their parents or other species through genic flow in Brazil is close to null.

Regarding analysis of the transgene selective advantage, it important to stress that, for being a domesticated species, soybean depends highly on the human species for its survival and scientific reasons would not exist to forecast the survival of soybean plants outside farming environments. Besides, in the absence of a selective pressure (use of the herbicide), expression of the inserted gene does not grant the plant any adaptive advantage.

The probability that soybean Event FG72 would present any selective advantage is very low, since genes 2*mepsps* and *hppd W336* are solely related with selectiveness to the glyphosate and isoxaflutole herbicides. The study carried out in Brazil by applicant (MORAIS, 2011a - Annex 5) showed that the plants of FG72 soybean keep being sensitive to the active ingredients Paraquat and Ammonium Gluphosinate, the same as any other soybean conventional variety.

Data presented by applicant related to phenotypic assessments resulting from essays carried out in Brazil (INOUE, 2011) and the United States of America ((KOWITE,2009), indicate that no characteristic was recorded between GM FG72 soybean and non-GM soybean that could

indicate any interference in of genes in the species adaptability. Therefore, the genetic change failed to ascribe any selective advantage to FG72 soybean as against conventional soybean.

Regarding symbiont organisms, soybeans is one of the cultures that fix N_2 existing in the atmosphere due to the symbiotic association of its roots with bacteria of the Rhyzobium genus. Inoculation of seeds with bacteria of the genus Rhyzobium is one of the practices adopted. The association may also occur with some species of this bacterium naturally present in soils.

Therefore, Rhysobium may be held as a relevant symbiont organism to act as an indicator to show whether the genetic modification introduced by FG72 soybean resulted in a product offering greater environment risk than the commercial varieties of the plant. To answer this question, applicant presented studies carried out in releases planned in the 2010-2011 harvests (Cascavel, State of Paraná; Palotina, State of Pará; Capão do Leão, State of Rio Grande do Sul, and the 2012-2013 harvests (Paulínea, State of São Paulo; Trindade, State of Goiás; Poxoréu, State of Mato Grosso; and Água Santa, State of Rio Grande do Sul) with the purpose of assessing the association ability of Rhyzobium with plants of Event FG72 soybean, as against its Jack conventional lineage (MORAIS, 2011b, MORAIS e ARAÚJO, 2014).

Results obtained show that the genetic change failed to affect the ability of symbiotic association between *Bradyrhisobium japonicum* (lineages SEMIA 5079 and SEMIA 5080) and soybean plants, with no impact on nitrogen fixation.

Regarding the entomofauna, data collected in the 2010-2011 crop indicate that there was not significant modification in the population of natural enemies inhabiting the plots, while the pests recorded did not present any differential preference either variety.

In what regards the GMO reproduction and propagation structures dispersion ability beyond

the cultivation areas and the mechanisms for their dispersion in air, water and soil, supplying information on the plant's pollen viability and indicating the potentially pollinizing and their geographic distribution in Brazil, it is worth stressing that soybean is an essentially autogamous plant, with perfect flowers, with both male and female organs protected within the corolla, which favors autopollination (AHRENT and CAVINESS, 1994; MORSE and CARTTER, 1937; RUBIS, 1970), being commercially propagated through seeds.

Insects, such as *Trigona spinipes* Fabricius (*Hymenopera*: *Apidae*) and, mainly, *Aphis mellifera* (*Hymenoptera: Apidae*), are able to transport the pollen and provide for pollination of flowers from different plants (ABUD *et al.,* 2007). However, the soybean flower is little attractive to bees, which are the most efficient pollinizers for soybean (THOMAS, 1989), notwithstanding the natural soybean mating rate rarely reaching figures higher than one per cent for any variety (ABUD *et al.*; NELSON and BERNARD, 1984). Studies conducted in greenhouse indicate that there is no difference between these parameters between FG72 and the non-GM soybean.

Regarding the likelihood of long term reproduction structures formation in the parental organism, genes 2*mepsps* and *hppd W336* are specific in attributing selectivity to herbicides glyphosate and isoxaflutole (IFT) and fails do display any relation with the formation of reproductive structures.

Post-harvesting monitoring assessments conducted in field essays in Brazil showed that FG72 soybean does not persist in the environment and failed to change into a more aggressive and invasive species.

Soybean is a cultivated plant that underwent a domestication process and reproduces almost exclusively by inbreeding, which limits the transfer of the inserted gene to other plants.

Besides, soybean is not original in Brazil. Therefore, there are no plants kin of the species in our environment, which eliminates the likelihood of transfer, in our conditions, of the introduced gene (AZEVEDO *et al.,* 2000).

There is a discrete fear that genes inserted in genetically modified plants may be transferred to other species and cause damages, especially to soil microorganisms and microorganisms of the digestive tract of humans and animals (DROGE *et al*., 1998). However, several studies conducted to this purpose were unable to show the occurrence of transference, in normal conditions of genes through horizontal flow between GM plants and bacteria (BERTOLLA *and* SIMONET, 1999; GEBHARD *and* SMALLA, 1999; NELSEN *et al.,* (1998). Essays conducted under environment conditions with non-sterile soil failed to record occurrence of horizontal transfer between plants and microorganisms, suggesting that soil conduction in itself inhibits the transformation (THOMSON, 2001). A study with soil bacteria in an area that had been cultivated for ten years with Bt maize evidenced that maize transgenic plants do not increase the likelihood of acquisition of resistance to antibiotics by soil bacteria (GMO Safety, 2008).

Nevertheless, if we consider the remote hypothesis of transfer of cassettes containing genes 2*mepsps* and *hppdPfw336* to some soil organism, the result would be an organism that presents just tolerance to herbicides glyphosate and/or isoxaflutole. This characteristics does not imply any adaptive advantage to microorganisms and fails to make possible for the organisms a better reproduction ability and stability in the environment. Therefore, it could not cause significant damage to the ecosystem.

Regarding possible negative and positive impacts to target and non-target organisms that could take place with the release of the GMO into the environment, the listing of assessed species, the reasons for the choice and the techniques used to show the impacts, it comes to

surface that the use of FG72 soybean has the purpose of incrementing the management of pest plants in post-emergence by using more than one herbicide featuring large range of action. This characteristics of tolerance to herbicides described for Event FG72 does not result in tolerance to any biotic element of the environment, and therefore there is no target-organism for the technology.

In field studies carried out in Brazil, United States of America, Canada and Argentina since 2002, the applicant reports that no adverse or unexpected effect was recorded as a result of cultivation of FG72 soybean.

In 2007, a revision of works published up to the one regarding experimentation in areas of research and commercial farming at global level, focusing on genetically modified cultures already passed for commercial use and having relevant importance for agriculture in Central and Western Europe (such as maize, canola and soybean) and more, considering the main characteristics of tolerance to herbicides and resistance to insects, showed that the data then available failed to show any scientific evidence that cultivation of GM assessed had caused damages to the environment (SANVIDO, *et al.*, 2007). The conclusion holds to date.

The assessment of changes in the ability of the plant to add or remove soil substances as a result of the introduction of new characteristics, describing possible physical and chemical changes of the soil and contamination of surrounding water bodies resulting from interactions with the GMO, contrasted with conventional systems, according to data the only difference between genetically modified soybean, event FG72 and conventional soybean varieties are proteins 2mEPSPS and HPPD W336 expressed by FG72 soybean.

Event FG72 is substantially equivalent to conventional soybean and no difference was recorded of phenotypic or agronomic difference in field tests carried out in Brazil and the United States

between FG72 and conventional soybean. Each essay was conducted under the same environment conditions, including soil fertility, an indication that Event FG72 soybean has no differenced ability to use or remove components of soils.

Biodegradability of FG72 soybean refers to its susceptibility in suffering the action of soil microorganisms, depending such action on: (i) physical characteristics of the plant; (ii) chemical characteristics, and (iii) availability and activity of microorganisms. The expression of proteins 2mEPSPS and HPPD W336 is not expected to change either physical or chemical characteristics of FG72 soybean, or capable to change the biodegradability of the plant by leading to the production of composts displaying any toxicity to microorganisms, preventing or extending the term for degradation or to differentiated expression of some molecule that could make decomposition by microorganisms difficult.

Introduction of genes 2*mepsps* and *hppdPfW336* in Event FG72 had the specific purpose of making the Event specifically selective to group G and group F herbicides, glyphosate and isoxaflutole, respectively. FG72 soybean remains sensitive to registered herbicides to the control of pest plants both during soybean cultivation and in rotation of soybean with other cultures. Voluntary soybean plants may be treated with pre-emergence or post-emergence herbicides including 2,4D, atrazine, ammonium gluphosinate, mesotrione, acetochlor and dicamba. It must be stressed that integrated management, with rotation of herbicides, is the best way to reduce the resistance of weed to herbicides (BOEBOOM and OWEN, 2006).

FG72 soybean had been already PASSED in nine countries (Australia, Canada, South Korea, United States of America, Japan, Malaysia, Mexico, New Zealand and Taiwan) either for human food or animal ration.

Other cultivars exist in Brazil of genetically modified soybean with the same characteristic of

tolerance to herbicides (glyphosate, imidazolinones) that are commercially planted in millions of hectares and, up to now, the technologies proved to be efficient, with no reference to proven adverse effects when compared to cultivation of the conventional varieties.

Regarding Changes in the survival ability of the GMO in environments different from those occupied by the parental, caused by new characteristics introduced, it shall be stressed that the characteristics introduced in FG72 soybean is the selectiveness to herbicides glyphosate and isoxaflutole as a result of the expressed proteins 2mEPSPS and HPPD W336. Given the specificity of such enzymes, the long history of use of genetically modified soybean in the country and the similarity of the phenotypic/agronomic characteristics of FG72 soybean as against conventional varieties, there is no record of any fact that may corroborate the hypothesis of changing the survival ability of Event FG72 in environments different from the agricultural ecosystem.

SANVIDO *et al.* (2007), revising the literature resulting from 10 years of research in experimental and commercial cultivation areas of genetically modified cultures report that there is no evidence that an extensive cultivation of genetically modified canola with selectivity to herbicides in the west of Canada had resulted in increased numbers of voluntary canola due to its characteristics of tolerance to herbicides. Similarly, there is no evidence that the genetic modification for such characteristics had increased the potential invasiveness of genetically modified canola in natural environments.

In an extensive review of scientific publication on analysis of genetically modified products use risk, LEMAUX (2008, 2009) concluded that though no human activity is able to reach a 100 safety level, genetically modified cultivars and their products commercially available are as safe as those generated from conventional methods.

VI- Opinion

Whereas

1) Soybean species *Glycine max* is in the food chain for over 4,000 years with no record of damage to humans, animals and the environment;

2) Over the relevant millennia, soybean failed to present characteristics of being a pest plant;

3) There is not in Brazil sylvan species with which soybeans may intercross;

4) Genes inserted in one single locus of the plant genome, the characteristics of such genes showed to be stable along generations and the segregation is mendelian;

5) Studies conducted in Brazil, Canada and United States of America showed that FG72 soybean is not different from the conventional variety in agronomic, morphologic, reproductive characteristics, in survival characteristics and the form of dissemination of plants, in the response to may pathogens and pests, except for the characteristics of tolerance to herbicides glyphosate and isoxaflutole, granted by the presence and expression of genes *epsps* of maize and *hppd* of bacterium *Pseudomonas fluorescens*, respectively;

6) Chemical composition analysis, conducted in planned releases conducted in different locations in Brazil and the United States of America, present similar results and show that FG72 is substantially equivalent to conventional soybean;

7) Studies of nutritional assessment and animal performance conducted in broiler chicken fail to reveal any change related to the consumption of FG72 soybean when compared to conventional soybean and any adverse effect to the birds;

8) Proteins 2mEPSPS and HPPD W336 produced by the genetically modified soybean failed to present any toxic or allergenic effect;

9) The change introduced failed to interfere in the ability to symbiotic association of genetically modified plants with *Bradyrhizobium japonicum* with no impact, therefore, in nitrogen fixation;

10) The event represents one additional available for farmers to manage populations of weed in soybean cultures;

11) Biosafety analyses of FG72 soybean already conducted by regulatory agencies of countries where the event had been analyzed and passed;

12) Information currently available in the scientific literature;

One may conclude that FG72 soybean is as safe as its conventional equivalent. Therefore, CTNBio opinion is favorable to the **granting** of the request for commercial release of this Event.

VII - Restrictions to the use of the GMO and derivatives thereto

As established by Article 1 of Law no. 11460, of March 21, 2007, "*research and cultivation of genetically modified organisms in indigenous lands and areas of conservation units are forbidden*".

There is no difference in agronomic performance of transgenic and conventional plants, as well as there is substantial equivalence between these transgenic and conventional plants. Therefore, the information suggests that transgenic plants are not fundamentally different from the genotypes of unmodified soybean, except for the tolerance to herbicides glyphosate and isoxaflutole. Besides, there is no evidence of adverse allergic reactions resulting from the use of FG72 soybean, there are no restrictions to the use of such Event or derivatives thereto,

either in human food or in animal rations.

Soybean is not native in Brazil and there is not in the country any native species, either sylvan or feral, that may intercross with *Glycine max*. Therefore, there are no additional risks to the environment with the cultivation of FG72 soybean, except for those risks already caused to the different varieties of conventional soybean in use across the country.

VIII – Considerations on particulars of different regions of the Country (subsidies to monitoring agencies)

As established by Article 1 of Law no. 11460, of March 21, 2007 "*research and cultivation of genetically modified organisms in indigenous lands and areas of conservation units are forbidden*". In Brazil there are no kin species of soybean in natural distribution. The genetic modification inserted does not alter the botanical characteristics of the plant, so that soybean behaves as its conventional counterpart would in cultivation conditions, except for the inserted characteristics.

IX – Conclusion

For the foregoing and considering the internationally accepted criteria in the process of risk analyses of genetically modified raw-materials, one may conclude that FG72 soybean is as safe as its conventional equivalents. Regarding the competences attributed to it by Article 14 of Law no. 11105/05, CTNBio reached the opinion that the request meets the rules and legislations in effect aimed at guaranteeing environment biosafety, agriculture, human and animal health and concluded that Event FG72 is substantially equivalent to conventional soybean and that its consumption is safe for human and animal health. As regards the environment, CTNBio concluded that FG72 soybean is not a potential cause of significant degradation of the

environment, keeping with the biota a relation identical to that of the conventional soybean.

CTNBio considers that the activity is not a potential cause of significant degradation of the environment and harm to human and animal health. Restrictions to the use of the GMO analyzed and its derivatives are conditioned to the provisions of Law no. 11460, of March 21, 2007.

CTNBio analysis took into consideration the opinions issued by the Commission members; documents supplied to the CTNBio Executive Secretary by the applicant; results from planned releases into the environment and related texts. Also, consideration was given and consultations made to studies and scientific independent publications of the applicant and prepared by third persons, as well as the analyses already carried out in other countries by the respective agencies of genetically modified organisms regulation.

X – Monitoring

Regarding the plan for post-commercial release, CTNBio determines that instructions and technical actions of monitoring appearing in Ruling Resolution no. 09, of December 02, 2011, shall be followed.

Therefore, according to Article 3 of Ruling Resolution 09, “applicant shall submit the post-commercial monitoring plan, or request exemption thereof, within 30 (thirty) days from publication of the granting of the request for GMO commercial release, according to CTNBio risk assessment, as well as the opinion containing in CTNBio technical decision.”

The monitoring plan submitted in the Technical Opinion is generic and does not meet the provisions of Ruling Resolution no. 09. Therefore, applicant shall submit a new monitoring plan within the term stipulated by Ruling Resolution no. 09, of December 02, 2011. This plan shall

necessarily include the size of the sample, regions to be covered, frequency of information collection and remaining actions, and the methodology to be used.

XI – Dissenting Votes:

The following were dissenting votes:

- Paulo Kageyama, Representative of the Ministry of Agrarian Development.

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Date: 10/08/2015

Edivaldo Domingues Velini

CTNBio President

Advisor: Gutemberg D. Sousa.

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In Witness Whereof, I have hereunto set my hand and seal in this City of Brasília, Federal District, Brazil, this Thursday, April 07, 2016.

Fees according to
Official Gazette of 04/15/2011

Page 73

Marco Antônio Rochadel

Public Translator