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CNPJ: 18.459.628/0001-15
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Previous extract: 2459/2010, published in the Federal Official Gazette of 07.26.2010.
Meeting: 140th Regular Meeting held on 02.17.2011.
Matter: Commercial release of GMO
Decision: GRANTED.

CTNBio, following examination of the application for commercial release of insect resistant and ammonium glyphosate herbicide tolerant genetically modified cotton T304-40 x GHB119, styled TwinLink cotton, decided to GRANT the request for commercial release on the terms of this technical opinion.
Bayer S.A., holder of Biosafety Quality Certificate Nº CQB 05/96, requested CTNBio a Technical Opinion related to biosafety of genetically modified cotton T304-40 x GHB119, styled TwinLink cotton, with the purpose of free use in the environment, trade and industrial use and any other use or activity related to this event and byproducts thereof.
Event T304-40 x GHB119 results from crossing, through classic genetic improvement, of genetically modified T304-40 and GHB119 cotton parents. Parental events were obtained through transformation mediated by A. tumefasciens and molecular characterization by Southern Blot confirmed both presence and structure of the inserts. Genotypic stability was confirmed for parental events and the stacked event through analysis of multiple generations, different germoplasms and cultivation locations. Stacked event T304-40 x GHB119 inherited from its parental T304-40 genes cry1Ab and bar and from its parental GHB119, genes cry2Ae and bar. Genes cry1Ab and cry2Ae are derived from Bacillus thuringiensis and codify proteins Cry1Ab and Cry2Ae, respectively, responsible for granting resistance to insects. It is known that the action mechanism of Cry proteins is mediated by specific receptors located at the middle intestine of susceptible insects. Association of Cry proteins to these receptors leads to the formation of pores that cause the insect death. Gene bar is derived from Streptomyces hygroscopicus and codes the PAT protein (phosphinothricin-N-acetyltransferase), responsible for granting tolerance to the herbicide ammonium glyphosate . Tolerance to the herbicide is granted by “acetylation”, catalyzed by PAT, of synthetic phosphinothricin (styled ammonium gluphosinate) producing the inactive compound N acetyl phosphinothricin, which is later metabolized in plant cells. Expression levels of proteins Cry1Ab, Cry2Ae and PAT in leaves, flower-buds and kernels of TwinLink cotton and its byproducts, such as husk, cakes and oil. Besides, the identity confirmation of target proteins was conducted by mass spectrometry (MS). The results were compared with the adequate controls and all results obtained point towards stability of constructs and adequate expression of the target-proteins and, in some byproducts, the amount of proteins is below detection level (oil and cotton cake). Results of studies of centesimal composition, mineral contents, E vitamin, antinutrients, gossypol, amino acids and fat acids, were used to compare the genetically transformed event TwinLink with conventional cotton. The majority of the components examined did not show differences between the genotypes and, in cases where the change took place, it remained between the range described in the literature and therefore does not represent impact of nutritional relevance. There was no performance difference between birds treated with cakes of genetically modified cotton (coming from event T304-40, GHB119 and T304-40 x GHB119) and cakes of conventional corn. No adverse effect was recorded in acute toxicity studies by oral ingestion of proteins Cry1Ab and Cry2Ae in mice. Essays in simulated gastric and intestinal fluids showed that proteins Cry1Ab and Cry2Ae are completely degraded, so that no cumulative effects, or persistence of such proteins in the organism, are expected. In silico analysis showed that there is no homology between heterolog proteins Cry1Ab and Cry2Ae with allergens and know toxic proteins. The studies indicate that Events T304-40 and GHB119, as well as their combination in TwinLink cotton, are substantially equivalent to other cotton varieties, and also indicate that the DNA inserted and the expressed proteins Cry1Ab, Cry2Ae and PAT fail to pose significant risk to human/animal health comparatively with the use of conventional cotton and its byproducts in food. Environment security aspects were analyzed in a series of studies conducted both in Brazil and abroad. Experiments conducted in regions featuring favorable edaphoclimatic conditions, which are representative for the culture of cotton in Brazil, assessed reproduction, survival, and agronomic aspects comparing lineages of genetically modified cotton and the conventional variety. The results recorded a regular occurrence of phenologic phases along the cycle and similarity of growth, development and phenotypic characters between genetically modified lineages and the isogenic non-modified cultivar, and therefore no additional characteristic was recorded that could regulate the survival of genetically modified cotton in the environment, discarding the possibility of occurrence of epistatic and pleiotropic effects of the genes introduced. Analyses of development and vegetative growth and reproduction during the cycle showed that the genetically modified genotypes T304-40, GHB119 and T304-40 x GHB119 are as sensitive as conventional corn when submitted to biotic stresses, that is to say, no competitive advantage of such genetically modified genotypes is observed when they are exposed to environmentally adverse conditions. Furthermore, TwinLink cotton displayed the same resprouting behavior, so that the data taken together demonstrate that there are no evidences relating the expression of genes bar, cry1Ab and cry2Ae with appearance of characteristics that may lead TwinLink cotton to be more invasive or display higher ability to survive as against conventional genotypes. The likelihood of a gene flow between TwinLink

cotton and Brazilian feral cotton plants is remote due to the isolated spatial distribution envisaged for commercial tillage in areas recognizedly exempt from feral types. Even assuming that the gene flow does occurs, the adaptive advantage represented by tolerance to the herbicide and resistance to insects shall be null, since feral cotton plants are cultivated in restricted areas, manually weeded and featuring low infestation by pests. In Brazilian conditions, cotton cultivation regions do not display any pest plant that may be sexually compatible with the cultivated species of cotton, so that the likelihood that transgenes are transferred from TwinLink cotton to plant pests making them more invasive is remote. Field assessments showed that events T304-40, GHB119 and T304-40 x GHB119 fail to have any activity on non-target organisms and natural enemies, while showing, on the other hand, efficient and specific control on target lepidopteran pests. In addition, purified proteins Cry1Ab and Cry2Ae were included in the diet of selected non-target organisms and no effect was recorded on their survival, development or reproductive ability. Degradability analyses of proteins Cry1Ab and Cry2Ae in the soil evidenced that, though in different times, both came to undetectable values, which represents zero risk for the soil microbiological and chemical processes. Further, there was no change in biodegradability of plant tissues of genetically modified TwinLink cotton as against the conventional isoline. Additional assessments, such as soil respirometry, Carbon and Nitrogen biomass, protozoa and flagellate, even though those parameters were more influenced by environmental changes than by genetically modified or conventional genotypes. Several events that express protein Cry1Ab are already approved to be commercially used in many countries around the world, being five corn events approved in Brazil. The same can be said of the protein PAT, expressed in eleven events (including corn, soybeans and cotton), already approved for commercial use in Brazil. Besides the data supplied by applicant, CTNBio examined the independent scientific literature to analyze safety and occurrence of some unexpected effect generated by the crossing between events T304-40 and GHB119 to the generation of TwinLink cotton event T304-40 x GHB119. For the foregoing, one may conclude that cultivation and consumption of TwinLink cotton event T304-40 x GHB119 is not a potential cause of significant degradation of the environment, or risks to human and animal health. For this reason, there are no restrictions to the use of this cotton and its byproducts. CTNBio determines that post-release monitoring be conducted in commercial, and not in experimental cultivation. Areas selected for monitoring shall not be isolated from other areas, possess margins or any other situation extraneous from the commercial standard. Monitoring shall be conducted in a comparative model between the conventional cultivation system and the GMO corn cultivation system, and the data gathering conducted by sampling. Monitoring shall be conducted in biomes that represent the main areas of GMO cultivation and, whenever possible, include different types of farmers. Monitoring shall be carried out for at least five years. The reports submitted shall include detailed information on all activities conducted in pre-sowing and sowing, their carrying out, reporting on activities conducted in monitoring areas during the cultivation cycle, on harvesting activities and climate conditions. Follow up reports shall be prepared on eventual harm to human and animal health, through official adverse effect notification systems, such as SINEPS (Health Products Related Adverse Events Notification System), regulated by Anvisa. The analytical methods, the results achieved and their interpretation shall be developed according to principles of independence and transparency, except for aspects of commercial secrecy previously justified and accepted as such. Regarding the bar gene, granting resistance to the herbicide, the following shall be monitored: nutritional state and sanity of GM plants; chemical and physical features of the soil in what relates to fertility and other basic pedologic characteristics; soil fauna dispersion bank; community of invading plants; development of resistance to the herbicide in invading plants; residues of herbicide in the soil, kernels and aerial portion of the plant; and gene flow. Regarding genes cry1Ab and cry2Ae, granting resistance do insects, the following shall be monitored: impact on target and non-target insects; impact on soil indicator invertebrates not belonging to the Insecta Class; residues of insecticide proteins in organic matter in decomposition, in the soil and watercourses near the monitoring area; development of resistance among target insects and gene flow of the two inserted genes.

TECHNICAL OPINION

I. Identification of the GMO

Name of GMO: T304-40 x GHB119 coton, styled TwinLink cotton

Applicant: Bayer S.A.

Species: Gossypium hirsutum L.

Inserted Characteristics: Tolerance to the herbicide ammonium glyphosate and resistance to insects

Method of insertion: T304-40 x GHB119 cotton, classified as Risk Class I, was developed through classical genetic improvement, by sexual crossing between genetically modified lineages containing event T304-40 and event GHB119.

Proposed use: Free use in the environment, registration, human and animal consumption, commerce and industrial use and any other use or activity related to TwinLink cotton and its byproducts.

II. General Information

Cotton belongs to genus Gossypium, Tribe Gossypiae, Family Malvaceae, order Malvales(1, 2). The genus is divided into four sub-genera (Gossypium, Sturtia, Houzingenia and Karpas) that, in turn, are subdivided into nine sections and several sub-sections(3). Genus Gossipyum currently encompasses 52 species distributed in Asia, Africa, Australia and America, of which only 4 yield commercial fiber and are cultivated. In general, species considered as feral species do not display fibers or, when presenting them, the fibers have no torsions and therefore are not spinnable, have short length and low resistance, which makes their industrial use unfeasible. Among commercial species, Gossypium arboretum L. is cultivated

in India and Gossypium herbaceum L. which was more important in the past, is currently planted just in some dry regions of Africa and Asia. About 90% of the world production of cotton comes from to Gossypium hirsutum and 8% from Gossypium barbadense(4). Out of the species producing commercial fiber, G. hirsutum and G. barbadense are tetraploid, coming from the New World. G. herbaceum and G. arboreum are diploid plants of the Old World(5).
Brazil is the center of G. mustelinum origin and an important center of diversity for G. barbadense and G. hirsutum r. maria galante, all tetraploid plants. None of them is held as a pest plant in neither agricultural nor natural environments.
Species G. barbadense as its domestication center at the North of Peru and South of Ecuador(6). It was introduced by pre-Columbian peoples and its fiber was used in production of some textile crafts by some indigenous tribes before the Portuguese arrived(7). Its use as a textile plant spread among colonizers, yet it became decadent with dissemination of two exotic races of G. hirsutum. G. barbadense cannot be found in natural environments and is maintained basically as a backyard plant. It is widely distributed and is present almost all over the country and its in situ conservation is directly connected to maintenance of traditional use as a medicinal plant(8).
According to Freire and collaborators(9), G. mustelinum, the only feral species to be genuinely Brazilian, was never improved and commercially exploited, in spite of the evident introgression of alleles of G. hirsutum in its genome(10). Its center of origin is the Brazilian Northeastern Region, where some populations can be found in Caiacó, RN, Macurerê, BA, and Caraíba, BA, municipalities that do not figure as cotton producers(10). Two issues endanger the in situ maintenance of G. mustelinum. First, and more important, is the destruction of riparian forests in rivers and non-perennial rivulets, the habitat of the species. Second, the extensive cattle raising common in the region, especially of caprine cattle. The animals feed on cotton sprouts, leaves, fruits, seeds and stem husk, damaging the plant development and, in some cases, killing adult plants. Renewal of populations is also jeopardized, since feeding on young individuals causes destruction of part of the plants(8).
Gossypium hirsutum is represented in two Brazilian biotypes. The latifolium race, also known as Upland cotton is the main cotton farmed in the country. The maria galante race, also known as “Mocó” or “arboreal” cotton was very cultivated in the Brazilian Northeast up to the end of the eighties, when several problems caused sudden interruption in cultivation(12). The race originates in the Antilles and the way by which it was introduced in Brazil remains unknown. There are hypotheses of the plant being brought by Netherlanders or Africans in colonial times(7).
Cotton is an agricultural product held as basic and greatly important to Brazil for its complex process of production/industry and high use of manpower. According to ABRAPA (Brazilian Association of Cotton Producers)(13), Brazil farmed 1.07 million hectares in the 2008/2009 harvest, of which 300 thousand hectares were planted with genetically modified cotton(14).
Use of cotton in the food chain is limited to animal (cotton meal, cotton cake) and human (oil) feeding. After the fiber has been separation of, the cotton main product is, in importance, edible oil. In extracting the oil, the main primary byproducts obtained are: linter, husk, and kernel; the second, whole flour, raw oil, cake and bran; and the tertiary, refined oil, cotton waste, and defatted flour(15). Processing the kernel yields on average 16% of oil 45% of bran, 9% of short fibers and 26% of other products(16).
III. Description of GMO and Proteins Expressed
T304-40 x GHB119 or TwinLink cotton is the result of a crossing, through classical genetic improvement, of the genetically modified parental T304-40 and GHB119, resulting in a lineage that expresses genes cry1Ab, cry2Ae and bar and, consequently, protein crystals Cry1Ab, Cry2Ae and PAT enzyme. Proteins Cry1Ab and Cry2Ae come from a soil bacterium Bacillus thuringiensis (Bt), feature biological action on certain insects, causing the plants derived from TwinLink cotton to express the characteristics of self-defense against certain lepidopteran pests, such as cotton leafworm (Alabama argilacea), cotton earworm (Helicoverpa zea), tobacco budworm (Heliothis virescens), fall armyworm (Spodoptera spp), pink bollworm (Pectinophora gossypiella) and soybean looper (Pseudoplusia includens). Protein PAT grants tolerance to the herbicide ammonium gluphosinate, enabling it to be used in doses of agronomic utility in controlling pest plants in post-emergence.
Parental T304-40 and GHB119 were obtained by inserting genes of interest and regulators in the genome of Coker 315 and Coker 312 cotton, respectively. Event T304-40 has gene cry1Ab that grants resistance to insects, and gene bar that grants tolerance to the herbicide ammonium gluphosinate. Event GHB119 has gene cry2Ae that confers resistance to insects and gene bar that confers tolerance to the herbicide ammonium gluphosinate. The genetic modification of Events T304-40 and GHB119 uses a system mediated by Agrobacterium tumefasciens(17, 18) for insertion of gene constructs.
Bacillus thuringiensis is the main biological control agent currently in use, responsible for about 2% of the insecticide world market(19). Its entomopathogenic activity relates to the production of crystals featuring insecticide action that may be formed by one or more than one proteins coded by genes cry and known as δ-endotoxines or crystal proteins. Cry insecticide proteins are extremely selective for target insects of the Lepidoptera Order(20, 21, 22, 23, 24) that have, in their intestine, specific receptors for this protein. Mammals and other non-target organisms (including other arthropods, pollinators and natural enemies of target-pests) fail to possess such bonding sites and therefore are not affected by the Bt protein(25, 26, 27, 28). Δ-endotoxine Cry1Ab coded by gene cry1Ab in Event T304-40 corresponds to one fragment of Cry1Ab produced by the bacterium Bacillus thuringiensis subspecies Berliner 1715, preceded, in its sequence, by one methionine and one alanine. Plants containing gene cry1Ab produce a protein with 617

amino acids, with a molecular weight about 69 kDa, equivalent to the original Bacillus thuringiensis protein after cleavages by trypsin and with the exception that the C-terminus is truncated and the presence of one alanine in the N-terminator.
The first step on the action mode of takes place after ingestion of the crystals, and consists on solubilization of protoxines by the insect alkaline pH middle intestine. Activation of the protoxines occurs later through action of proteases. In the case of Cry1, the toxic fragment with molecular weight from 65 to 55 kDa is generated after removal of about 500 amino acids from the carboxi-terminus and 38 amino acids from the amino-terminus portion(29).After activated, the toxins pass through the pores of the peritrophic membrane and interact with the epithelial cells of the insect, which contains receptors(30, 31). According to the model described by Gill and others(32), the δ-endotoxine irreversibly bond to the receptors and after the protein conformational change, its toxic domain is inserted in the plasmatic membrane. At this point, oligomerization, pore formation and cell lyse take place. The lyse leads to rupture of the middle intestine integrity and the insect dies by inanition or septicemia(33, 34).
Gene cry2Ae, present in event GHB119, was isolated from Bacillus thuringiensis subspecies Dakota whose C/G ration nucletid sequence has been modified to better expression in plant cells. Protein Cry2Ae has 86% identity with Cry2Ab. Protein Cry2Ae contains 631 amino acids and its molecular weight is about 71 kDa. This δ-endotoxine forms cubic crystals and is also associated to toxicity to lepidopterans through the same mechanism involving solubilization, hydrolysis specific-site bonding on epithelial cells, pore formation and cell lyse displayed by protein Cry1Ab.
The bar gene was obtained from part of the Streptomyces hygroscopicus bacterium genome. Genus Streptomyces (Actinomyces), is one of the most well characterized of the Streptomyces family due to its wide distribution in nature, especially in soils(35). Gene bar expression product, which is present in events T304-40 and GHB119, is enzyme PAT (phosphinothricin-N-acetyltransferase), a molecule comprising 183 amino acids with a molecular weight of about 22 kDa. PAT is highly specific, catalyzing the reaction of phosphinothricin “acetylation” that produces compound N-acetyl phosphinothricin (inactive), later metabolized in the plant cells. In plant cells, there is no known substrate for this enzyme, except in cases when an herbicide derived from phosphinothricin is applied to the plants. Synthetic phosphinothricin is also known as ammonium gluphosinate and its herbicide action takes place through inhibition of glutamine synthase, which leads to accumulation of ammonium levels to levels that are toxic to cells causing the plant to desiccate(36, 37). Expression of PAT in genetically modified plants grants tolerance to this herbicide, for promoting its inactivation(35, 38).
Molecular characterization of events T304-40 and GHB119 was made through Southern blot analyses in genomic DNA samples, using as hybridation probes several components of the expression cassette used in the transformation of each such events. Hybridation results analyzed for event T304-40 showed that it has an almost complete copy of T-DNA, bordered by an incomplete inverted copy of cassette cry1Ab and an additional of the terminator. In case of event GHB119, results show that it consists of one full copy of T-DNA, corresponding to the original sequence delineated in the vector.
Presence of the insert and genotypic stability of events T304-40 and GHB119 was confirmed by Southern blot analyses along different generations, germoplasms and even in different environments. Analyses also showed the genetic stability of inserts (related to events T304-40 and GHB119) in the stacked event T304-40 x GHB119.
Different events expressing protein Cry1Ab have already been approved for use in commercial scale in several countries around the world. In Brazil, corn events approved include MON810; Bt11; Bt11 x GA21; MON810 X NK603; and Bt11 x MIR 162 x GA21. The same with protein PAT, which in Brazil appears in events such as corn T25; cotton LLCotton25; Soybean A2704-12; Soybean A5547-127; Widestrike Cotton; Herculex Corn; Bt11 Corn; Bt11 x GA21 Corn; TC1507 x NK603 Corn; Bt11 x MIR162 x GA21 Corn; MON89034 x TC1507 x NK603 Corn; all of them already approved by CTNBio for commercial release.
IV. Aspects Related to Human and Animal Health
Quantification analyses of proteins Cry1Ab, Cry2Ae and PAT proteins using ELISA methodology (Enzyme Linked Immuno Sorbent Assay) showed their low levels of expression in cored seeds derived from TwinLink cotton plants with and without application of the herbicide ammonium gluphosinate. In the samples the average concentration of protein Crya1b was, in fresh matter, 1.4µg/g and in dry matter, 1.6µg/g, 10.7 and 163, respectively. Quantification of the proteins in byproducts (linter, husk, linter free seeds, cake, roast cake, raw oil, refined/deodorized oil) of TwinLink cotton sprayed twice with ammonium gluphosinate showed that Cry1Ab protein is not detected or quantified in samples of oil, fiber and husk. It was not possible to measure the expression of Cry2Ae in samples of oil, fiber and cotton cake after roasting. Similarly, it was not possible to detect PAT protein in samples of oil. Expression of proteins Cry1Ab, Cry2Ae and PAT in other tissues, such as leaves, flower buds and linter free seeds was carried out with TwinLink cotton and showed that leaves concentrate the higher content of the proteins.
To assess possible nutritional differences in genetically modified cotton when compared with conventional cotton, several parameters were analyzed. TwinLink cotton , with and without application of ammonium gluphosinate, was compared to the unmodified isoline, Coker 315, regarding centesimal composition (humidity, fatty acid, ashes, carbohydrates, ADF fiber and NDF fiber), fibers, micronutrients (minerals and E vitamin), total antinutrients, gossypol, cyclopropenoids, phytic acid, total amino acids and fatty acids. The comparison was carried out using cored seeds (kernels + linter) coming from seven field experiments established in 2007 in the United States Southeastern Region. Besides linter free seeds, compositional analyses were carried out in byproducts, such as linter, husk, linter free seeds, cakes (raw and roasted)

and oil (raw and refined/deodorized), coming from seeds produced in a field essay conducted in Levelland, Texas, in 2007, with application of the herbicide.
In most of components examined in cored seeds, no significant difference was identified between genetically modified and conventional cotton. In cases in which changes were noticed (magnesium, cystine, and some fatty acids), these are not likely to represent any nutritional impact, since the average measured values are within the range described by the literature for the species. The same with assessments on byproducts (fiber, linter, husk, linter free seeds, cakes and oil), where little statistical differences were recorded between the genotypes, though without displaying average values exceeding the amplitude described in the literature and therefore failing to represent impact of nutritional relevance.
Part of the biosafety studies were conducted with proteins Cry1Ab and Cry2Ae produced in E. coli to enable collection of sufficient volume of material, since their expression in plant tissues is deemed as being low. Protein Cry1Ab, isolated and purified from leaf tissues of TwinLink cotton and event T304-40, as well as the protein produced by E. coli, were denatured and analyzed by SDS-PAGE, showing that such proteins display the same mobility, molecular weight and immunoactivity (verified by Western blot). Mass spectrometric analysis revealed that the sequence of protein Cry1Ab expressed in TwinLink cotton is 93% equivalent to the one of the E. coli protein, and that the sequence of the N-terminus region was not changed. The same analyzes were conducted with protein Cry2Ae (obtained from E. coli and purified from TwinLink cotton and DHB119 cotton leaves), and the results evidenced that molecular weight, mobility and immunoreactivity of the protein isolated from E. coli are similar to that of protein Cry2Ae isolated from the leaves of TwinLink and GHB199 cotton. Mass spectrometer studies conducted for protein Cry2Ae showed that the protein expressed in TwinLink cotton is 70% equivalent to that isolated from E. coli and the analysis of the N-terminus sequence indicated that the protein expressed in TwinLink cotton failed to display any change.
Performance of birds was assessed, treated with cotton cake coming from events T304-40, GHB119 and TwinLink contrasted with that of the conventional isoline and with one commercial cotton variety(39, 40, 41). This studies used chicken (Gallus gallus domesticus) ROSS #308 during their growth phase, in which the animals are very sensitive during their first 40 days of life and increase their weight up to 45 times(40). Three studies were conducted in 42 days, when three types of balanced diet was offered ad libitum to the birds. In one of the studies, the birds were fed with a diet containing 10% of cotton roast cake prepared with conventional cotton (commercial variety), conventional corn (Coker isoline) or genetically modified T304-40 cotton. In other study, 5% of cotton roast cake was added to the birds' diet prepared from conventional cotton (commercial variety), conventional cotton (Coker isoline) or genetically modified GHB119 cotton. In the third study the diet contained 10% of cotton roast cake prepared from conventional cotton (commercial variety), conventional cotton (Coker isoline) or genetically modified TwinLink cotton. During the study, health and survival conditions of the animals were assessed together with weekly food consumption, weight gain, alimentary conversion, live weight (weekly), carcass weigh, abdominal fat, among others. Assessment results showed no significant statistical variation among the groups and treatments, and that the birds consuming the diet containing events T304-40, GHB119 and TwinLink indicated sanitary and growth characteristics similar to those individuals treated with a diet containing conventional cotton. Therefore, it is not likely that changes in performance occur in animals fed with products/by products of TwinLink cotton and its parental T304-40 and GHB119.
Simulated essays of gastric and intestinal system were conducted to assess degradability of Cry proteins. In order to assess degradability of Cry1Ab and Cry2Ae proteins in human gastric juice, a simulated gastric fluid (SGF), according to the methodology described by Thomas et al.(43), was used. The results showed that proteins Cry1Ab and Cry2Ae were rapidly and fully degraded under the essay conditions. The peroxidase control protein was rapidly digested, while ovalbumin, slowly digested, as expected(44, 45). In order to assess degradability of the proteins in a system simulating the human intestinal fluid (SIF), an in vitro essay was conducted following the protocol established by ILSI (International Life Science Institute). In this essay, the Cry proteins were partially degraded after 60 minutes or incubation at 37oC. The data indicate the intrinsic safety of the proteins for use in human and animal food due to its rapid degradation, which minimizes the likelihood of the proteins to be integer and active in the digestive tract, as well as due to low exposure, since they were not detected (or detected in very low values) in quantification analyses conducted in the byproducts.
To strengthen the alimentary safety of Cry proteins, oral acute toxicity essays were conducted in OF1 mice. In the studies, protein Cry1Ab or protein Cry2Ae(46) were administered through the use of probes and in two doses of 1000 mg/kg of live weight, totaling 2000mg/kg of each tested protein, with an interval of 4 hours between doses. Clinical signs were assessed on a daily basis between the first and the 15th day, with the purpose of recording the beginning, severity, reversibility and duration of the signs. Besides, the cages were inspected every day to check occurrence of blood and alteration of the feces, as well as check the presence of dead animals. No change or mortality was recorded in the studies. At the end of the 15 days of the study, the individuals were sacrificed and undergone necropsy for macroscopic analysis, and no change in the organs studied was recorded.
In silico surveys were conducted to identify all known allergens displaying over 35% of identity with proteins Cry1Ab and Cry2Ae. For the purpose, the full amino acid sequence of the proteins were compared to sequences of known and putative allergens deposited in the databank AllergenOnline. Searches for epitopes were conducted using the CGC (Genetic Computer Group) algorithm FindPatterns. For searches of homology with toxic compounds, BLASTP (Protein-protein Basic Local Alignment Search

Tool) was used. No identity was found between blocks of 8 amino acids (epitopes) in the sequence of proteins Cry1Ab and Cry2Ae and the recognizedly allergenic compounds. Comparing the sequence as a whole, no relevant similarity was found between proteins Cry1Ab and Cry2Ae and the proteins listed in the AllergenOnline databank. Similarly, proteins Cry1Ab and Cry2Ae failed to show homology with toxins, except with other Cry proteins of different organisms.

Degradability of the PAT protein in SIF and SGF environments, acute oral toxicity essays and bioinformatics studies to assess similarity of allergenic and toxic compounds were already conducted and reported for events T25, LLCotton25, A2704, and A5547-127, which are approved for commercial use in Brazil and several other countries. By the same token, there is a publication in a scientific article describing all studies related to safety of this protein, including degradation in simulated gastric and intestinal fluids(47).

The above studies indicate that events T304-40 and GHB119, as well as their combination (TwinLink cotton) are substantially equivalent to other cotton varieties, as well as that the inserted DNA or the expressed proteins (Cry1Ab, Cry2Ae and PAT) do not pose any significant risk to human/animal health comparatively with the use of conventional cotton and its byproducts in food.

V. Environmental and Agronomic Aspects

Studies conducted in Brazil with events T304-40, GHB119 and TwinLink cotton cultivated in regions that are currently traditional for cotton farming (States of Mato Grosso and Bahia) assessed parameters related to growth, development and phenotypic characters contrasting genetically modified varieties (GMV) with isogenic non modified cultivars(48). The results showed regular occurrence of phonologic phases along the cycle and similarity of growth and development and of phenotypic characters between the GMV and the non modified isogenic cultivar, indicating that the attributes granting resistance to some lepidopteran pests and tolerance to the herbicide ammonium gluphosinate are not likely to be related to any change in the cotton metabolic pathways, morphologic aspects or phenology missing, therefore, any additional characteristics that may regulate the survival of genetically modified events into the environment, discarding the possibility of pleiotropic or epistatic events of the genes introduced.

Besides, no evidence was found of attributes that may lead the genetically modified lineages to display greater reproductive ability than the conventional cultivar, such as: beginning and duration of the flowering and fructification (productive potential and prolificity), leaf architecture and density (photosynthetic ability) and growth habit (tendency to sprout again at the end of the cycle).

Without distinction, plants derived from events TG304-40 and GHB119, plants derived from the combination T304-40 x GHB119 and plants of the conventional cultivar were affected by absence of pest control, which negatively affected the culture growth and development and, consequently, its productive efficiency(48). The results show that genetically modified and conventional genotypes were evenly sensitive when submitted to biotic stresses, and consequently that there is no competitive advantage of genetically modified plants when submitted to environmental adverse conditions. No evidence was additionally found of change in survival and invading ability of genetically modified plants, since the number of fruits per plant, production of seed cotton and vigor of new sprouting had similar values to the ones recorded for the conventional genotype. Therefore, there is no scientific evidence that may relate expression of genes bar, cry1Ab and cry2Ae to irruption of characteristics that could lead events T304040, GHB119, or TwinLink cotton (T304-40 x GHB119) to be more invasive or more able to survive than the conventional genotypes in non-farming ecosystems(48), and therefore these events of genetically modified cotton keep relying on human intervention in controlling pest plants for the good development of the culture.

According to Freire(11), the likelihood of gene flow between GM cotton and feral cotton is remote due to the isolation of the spatial distribution foreseen for commercial cultivars (distributed in high-end tillage of the Cerrado) in areas recognizedly distant from the centers of origin and distribution of feral species. In case the transfer does occur, the adaptive advantage represented by the tolerance to the herbicide and resistance to the lepidopteran will be null, since these cotton plants are cultivated in small areas, manually weeded and featuring low infestation by pests. Besides, in Brazilian conditions, cotton planting regions fail to present any pest plant that is sexually compatible with the cultivated Gossypium species, and therefore the likelihood that the modified genes are transferred from the TwinLink cotton to pest plants making them more invasive, is remote.

The study by Degrande(49) had the purpose of assessing the impact of genetically modified cotton lineages T304-40 x GHB119 (TwinLink cotton), GHB119 and T304-40, on bioecological aspects of target and non-target phytophagous Arthropods and natural enemies in two regions of cotton farming in the Brazilian Cerrado (Santo Antonio do Leste/MT and Riachão das Neves/BA) under two conditions of chemical pest control. With this purpose, the incidence intensity was assessed in all occurring species and population dynamics of more relevant species, and parameters were studied related to certain types of injuries caused by some species of pests and the productivity of lineages tested was appraised. In the first study, TwinLink cotton was evaluated against its conventional lineage, in tillage with no application of insecticides to control lepidopterans (condition 1) and in tillage with systematic application of insecticide for controlling all pest species (condition 20. In the second study, TwinLink cotton was compared with its isolated events T304-40 and GHB119 and with the conventional isoline, also under cultivation with no application of insecticides (condition 1) and with total control of insects (condition 2).

The results of this study(49) indicated that genetically modified TwinLink cotton was highly efficient in controlling lepidopteran pests Alabama argilacea, Heliothis virescens, Pseudoplusia includes, Spodoptera

eridania, Spodoptera frugiperda and Spodoptera cosmioides, showing the efficiency of the technology. It was also demonstrated that protein Cry1Ab expressed in lineage T304-40, protein Cr2Ae expressed in event GHB119, and both proteins expressed in event T304-40 x GHB119, or TwinLink cotton, failed to show any direct effect on non-target pests A. gossypii, B tabaci, A. grandis, Liriomiza sp., T. urticae and P. latus. Besides, no direct effect of the proteins was identified on natural enemies C. sanguine, E. connexa, H convergens, Chrysoperia sp., D. luteipes, P. nigrispinus, M. confuse, Calososoma sp., Syrphidae, Euplectrus sp., Tachinidae, Trichogramma ssp., and N. rileyi. Lineages of genetically modified cotton T304-40, GHB119, and T304-40 x GHB119, or TwinLink cotton, by suppressing the lepidopteran populations are able to reduce important enemy populations in biologic control of lepidopterans, such as N. rileyi, E. connexa, D. luteipes, Aranae and Tachinidae. Differences found for these species of natural enemies may be associated to lower availability of preys on genetically modified lineages studied, and not to the direct action of the proteins expressed by genetically modified lineages, since for the majority of natural enemy species no significant differences were recorded for the number of individuals between genetically modified lineages and the conventional control, and that the lepidopteran populations were efficiently controlled by the TwinLink cotton technology.

Proteins Cry1Ab and Cry2Ae are both very specific for lepidopteran insects. Even though there is no evidence of toxicity on other non-target species, a range of organisms was selected (Apis mellifera, Coleomegilla maculata, Chrysoperla rufilabris, Folsomia candida, Elsenia fetida and Daphnia magna) with higher probability of exposure to GM cotton and its parts in order to assess its sensitivity to the Cry proteins produced by TwinLink cotton. For most of the species identified, the test was conducted with purified protein expressed in E. coli. This way, Cry1Ab and Cry2Ae could be incorporated to diets appropriate to each tested organism and, besides, in concentrations above those found in environmental conditions. The results showed that addition of the proteins to the diet or non-target species failed to cause significant change in survival rates, development or reproductive ability.

Degradability of Cry proteins expressed in TwinLink cotton was also analyzed to confirm whether or not the structural stability of such components is maintained and, consequently, maintained their biological activity. To this end, a study(49) was conducted where genetically modified plants of TwinLink cotton and conventional plants were cultivated in vases containing the soil of the Santo Antonio do Leste/MT region, which is important to the cotton production region in Brazil. The plants were cultivated in a greenhouse. The cultivation in vase was maintained until the first flowering bud, when they were removed, weighted, hacked and incorporated to the soil of their respective vases, in a way to add the same amount to each vase. The proteins were extracted from the soil according to a method described by Murase et al.(51). After isolation and confirmed the presence of Bt protein in the soil, the amount of proteins Cry1Ab and Cry2Ae were determined. Results showed that the quantities of protein Cry1Ab were below detection levels of ELISA, meaning that they are insignificant quantities that may remain free in the environment without posing risk to communities of soil microorganisms and macrofauna. Degradability of protein Cry2Ae recorded a behavior different from that of Cry1Ab. While Cry1Ab was not detected, Cry2Ae was detected in increasing levels from 30 days of incubation, reaching the top level in 45 days. Subsequently the concentration decreased to values close to zero. The results show that the two proteins Cry1Ab and Cry2Ae when produced by the plant feature a different behavior in the soil during degradation of the plant tissues. However, both reach non-detectable values in different times, which represents no risk to the soil microbiological and biochemical processes. The study also assessed different parameters of the soil microbiota in samples collected in different areas planted with TwinLink cotton and conventional cotton using chemical control with insecticides. The results showed that none of the parameters assessed, such as population of protozoa, mychorrizal fungi, soil respiration rate and carbon and nitrogen biomass were negatively impacted by the presence of plant tissues containing proteins Cry. Biodegradability of TwinLink cotton plants was also assessed against the conventional lineage. To this end, a greenhouse experiment was conducted where plants were maintained in vases containing soil coming from the traditional cotton-growing region (Santo Antonio do Leste/MT). The plants were cultivated to the first flowering bud, then removed from the vases, separated into leaves, branches and foots. One gram of each plant part was weighted to pack them in nylon mesh bags and buried into the soil. Each treatment had five repetitions for each part of the plant. After thirty days maintained under greenhouse conditions, the bags were removed and weighted and again buried. After sixty days buried into the soil, the bags were again removed and the non-degraded biomass was weighted, dried and the dry matter remaining in the two assessments was estimated. Results obtained showed that root biodegradability, as well as that of the aerial part, were equal when the GM variety was compared to the conventional isoline. The GM plant leaves displayed greater degradability when contrasted to the isogenic non-Bt, which does not represent change in the product safety to the environment or, specifically, to the soil biota.

Available data indicate that plants derived from TwinLink cotton do not show characteristics that could grant undesired selective advantage to the genetically modified cotton or atypic behavior to the species. Assessments on non-target orbanisms and natural enemies showed that the events so tested are safe and act specifically on the lepidopteran target pests. During the post-harvest monitoring, conducted in experimental areas, presence of residual (spontaneous) cotton plants from seeds and new sprouting was assessed and showed to be equally and easily eliminated by usual practices, indicating that the GMO does not remain in the agricultural environment without human intervention, in a different form, nor displays characteristics that could make it more aggressive or invader of the ecosystem than its conventional parental.

VI. Restrictions to the use of the GMO and its derivatives

Pursuant to Article 1 of Law no. 11,460, of March 21, 2007, “research and cultivation of genetically modified organisms are forbidden in indigenous and conservation unit areas, except in Environment Protection Areas” as well as in municipalities mentioned in the Annex of the Ministry of Agriculture – MAPA Directive 21, of 01.16.2006.

Studies submitted by Applicant showed that there were no significant differences between genetically modified cotton and its conventional isoline regarding agronomic characteristics, reproduction, dissemination and survival ability. All evidence submitted in the process and bibliographic references verify the transgenic variety level of risk as equivalent to non-transgenic varieties vis-à-vis the soil microbiota, as well as other plants and human and animal health. Therefore, cultivation and consumption of TwinLink cotton are not potential causes of significant degradation to the environment or risk to human and animal life. For the foregoing, there are not restrictions to the use of this cotton and its byproducts, except in locations mentioned by Law nº 11,460, of March 21, 2007 and in the Annex to the Ministry of Agriculture – MAPA Directive nº 21, of 01.16.2006.

After 17(52) years of use in different countries, no problem has been detected for human and animal health or to the environment that could be ascribed to transgenic cotton. It shall be stressed that lack of negative effects resulting from farming of transgenic cotton plants does not mean that such results may not occur in the future. Zero risk and absolute safety do not exist in the biologic world, although there is a host of reliable scientific information and a safe use history of transgenic varieties in agriculture.

VII. Considerations on particulars of different regions of the country (subsidies to monitoring agencies)

Pursuant to Article 1 of Law no. 11,460, of March 21, 2007, “research and cultivation of genetically modified organisms are forbidden in indigenous and conservation unit areas, except in Environment Protection Areas” as well as in municipalities mentioned in the Annex of the Ministry of Agriculture – MAPA Directive 21, of 01.16.2006.

VIII. Conclusion

Considering that the cotton (Gossypium hirsutum) variety TwinLink, event T304-40 x GHB119 belongs to the most well-characterized species with a solid safety history for human consumption and that genes cry1Ab, cry2Ae and bar introduced in this variety code proteins that are ubiquitous in nature.

Considering that the construct of this genotype occurred through classical genetic improvement that resulting in the insertion of a stable and functional copy of genes cry1Ab, cry2Ae and bar that granted the plant tolerance to the herbicide ammonium gluphosinate and resistance to insects.

Considering that centesimal composition data failed to point out significant differences between genetically modified varieties and conventional varieties, suggesting nutritional equivalence between them.

Whereas:

1. Event T304-40 x GHB119 was very well characterized at the molecular level and verified the integrity of genic constructs inherited from the respective parental during the process of classical genetic improvement;
2. Proteins Cry1Ab and Cry2Ae belong to the family of Cry proteins derived from Bacillus thuringiensis, an organism that is being commercially used for over forty years in producing microbial formulations for controlling insects;
3. PAT protein, granting tolerance to the herbicide ammonium gluphosinate is expressed in several events of different agricultural cultures already approved for commercial use in different countries around the world;
4. During agronomic assessments conducted with cotton T304-40 x GHB119 there was no record of any other characteristics, but the expected ones, being expressed, discarding the possibility of pleiotropic and epistatic effects from the genes introduced.
5. Birds treated with cotton cake containing the TwinLink event, or its parental varieties separately, failed to show any variation in sanitary and growth characteristics when compared to the conventional cake.
6. The rapid degradation of proteins in gastric and intestinal system simulations demonstrated its safety for use in human and animal food;
7. Essays of oral acute toxicity showed no abnormality or significant clinical signs in mice after administration of proteins Cry1Ab and Cry2Ae;
8. In silico analyzes showed no similarity between Cry proteins and allergens or known toxins;
9. Agronomic assessments revealed that there are no changes in parameters growth, development, reproducing ability and phenotypic characters between plants of genetically modified cotton and the conventional cultivar;
10. Genetically modified plants showed to be equally sensitive to biotic stress and therefore there is no evidence of change in their survival invading ability;
11. No direct effect was recorded on non-target pests and natural enemies derived from cultivation of genetically modified cotton events;
12. No toxicity was recorded of Cry proteins on non-target species selected after incorporation of the purified proteins to the diet;
13. Degradability analyzes of Cry proteins showed that both reach undetectable values, though in different times, posing no risk to the soil microbiological and biochemical processes;
14. Several parameters related to soil microbiota, such as population of protozoa, mycorrhizal fungi, carbon and nitrogen biomass and soil respiration were not affected by the presence of plant tissues from genetically modified TwinLink cotton;

15. Biodegradability of roots and aerial parts of TwinLink cotton was the same when compared to the traditional lineage. Leaves of TwinLink cotton displayed higher degradability, which fails to represent change in its safety to the soil biota;
16. Internationally accepted criteria in analyzing risk of genetically modified raw materials(53)
One may conclude that TwinLink cotton is as safe as its conventional equivalent.
Under Article 14 of Law nº 11,105/2005, CTNBio considered that the request meets the applicable rules and legislation in effect aiming at securing environment, agriculture, human and animal health biosafety, and concluded that TwinLink cotton is substantially equivalent to conventional cotton and that is consumption is safe for human and animal health. Regarding the environment, CTNBio concluded that TwinLink cotton is not a potential cause to significant degradation of the environment, keeping with the biota a relation identical to that of conventional cotton.
As far as post-commercial release is concerned, CTNBio determines that the instructions are to be met and the following technical monitoring actions carried out:
(I) Instructions:
(a) Monitoring shall be carried out in commercially tilled lands and not in experimental cultures. Areas selected for monitoring shall not be isolated from remaining areas, have borders or any other situation extraneous to the commercial standard;
(b) Monitoring shall be carried out in a model comparative between the conventional cultivation system and the GMO cultivation system, and data collection conducted by sampling;
(c) Monitoring shall occur in biomass representative of main GMO farming areas and, whenever possible, include different types of producers;
(d) Monitoring shall be conducted for a period of at least five years;
(e) In every monitoring event, Applicant shall detail the data on activities carried out in pre-sowing and sowing, its execution, reporting activities conducted in monitored areas during the culture cycle, on harvesting activities and climate conditions;
(f) Monitoring shall include eventual harm to human and animal health through official adverse effect notification systems, such as SINEPS (Health Products Related Adverse Events Notification System), regulated by ANVISA;
(g) Analytical methods, results obtained and their interpretation shall be developed according to principles of independence and transparency, protecting aspects of commercial secrecy previously justified and defined as such;
(h) CTNBio reserves the right to review this Opinion at any time on technical and scientific grounds.
(II) Technical monitoring actions to be carried out:
1 - Regarding gene bar that grants resistance to the herbicide, the following shall be monitored:
(a) Nutritional and sanity conditions of GMO plants;
(b) Chemical and physical soil aspects related to fertility and other basic pedologic characteristics;
(c) Soil microbial diversity;
(d) Soil dispersion bank;
(e) Community of invading plants;
(f) Resistance to the herbicide developed by invading plants;
(g) Herbicide residues in soil, kernels and aerial part of the plant;
(h) Gene flow;
2 - Regarding genes cry1Ab and cry2Ae, which grant resistance to insects, the following shall be monitored:
(a) Impact on target and non-target insects;
(b) Impact on soil invertebrate indicators that do not belong to the Insecta Class;
(c) Insecticide protein residues in decomposing organic matter, soil and watercourses near to the monitoring area;
(d) Development of resistance among target insects;
(e) Gene flow of the two genes inserted.
CTNBio assessment considered the opinions issued by Commission members, documents submitted by Applicant to the Office of CTNBio Executive Secretary and results of planned releases into the environment. Applicant's independent studies and scientific publications, carried out by third parties, were additionally considered.
IX. Bibliography
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Dr. Edilson Paiva
CTNBio President

Technical Advisor: Thais Haline Vaz
Dissenting Vote
CTNBio Members Doctor Paulo Yoshio Kageyama and Doctor Solange Teles da Silva, both of the Plant Area Permanent Sector Subcommission and Doctor José Maria Ferraz, of the Environment Area Permanent Sector Subcommission voted against the commercial release of TwinLink cotton.