



NATIONAL TECHNICAL COMMISSION OF BIOSECURITY
TECHNICAL OPINION No. 180/2023/SEI-CTNBio - Members
TECHNICAL OPINION 8393/2023

Process: 01245.022752/2022-35

Applicant: SUZANO SA

Subject: Commercial release of genetically modified Eucalyptus
1521K059 and its derivatives

CQB: 325/11

President of CIBio: Ana Cristina Pinheiro

Risk Class: 1

Meeting: 259th. Ordinary Meeting held on 03/02/2023

Decision: DEFERRED

Technical Basis

The applicant requests CTNBio to issue a technical decision for the commercial release of eucalyptus FGN-Ø6K59-4, called 1521K059, pursuant to CTNBio Normative Resolution No. of 2005 and Law 11,105, of March 24, 2005.

Eucalyptus 1521K059 carries genes cry2Aa, cry1Ab and cry1Bb, which encode proteins derived from the bacteria *Bacillus thuringiensis*, conferring resistance to insect pests of the order Lepidoptera, specifically of the Geometridae family, such as *Thyrinteina arnobia*, considered one of the main pests of eucalyptus in the Brazil. The data presented by the applicant come from 9 trials, each in different locations in the national territory, which provided the evidence for the present request.

The applicant informs that eucalyptus 1521K059 will be cultivated and processed in the same way as its conventional clone FGN-K, since both are

equivalent in silvicultural characteristics and chemical composition, differing only in terms of their insecticidal characteristics on certain orders or species of insect pests, Genetic Transformation Method

Eucalyptus 1521K059 was produced by the method of genetic transformation mediated by *Rhizobium radiobacter* (also recognized as *Agrobacterium tumefaciens*) using the plasmid pBI121. The vector contains the expression cassettes for genes cry2Aa, cry1Ab and cry1Bb, which encode proteins from *B. thuringiensis*, in addition to the expression of the nptII gene, which encodes neomycin phosphotransferase type II (NPTII), derived from *Escherichia coli*. The construct present in eucalyptus event 1521K059 has a copy of the cry2Aa gene regulated by the 35S promoter of the Cauliflower mosaic virus (CaMV) and by the NOS terminator of *A. tumefaciens*; a copy of the cry1Ab gene regulated by the 35S promoter and NOS terminator from *A. tumefaciens*; one copy of the cry1Bb gene, regulated by the Rubisco promoter and terminator (pRBS), and one copy of the nptII gene, which is controlled by the 35S and the CaMV polyA terminator.

The cry genes inserted in eucalyptus 1521K059 encode proteins Cry2Aa, Cry1Ab and Cry1Bb, of approximately 70.86 kDa (633 amino acids), 69.76 kDa (622 amino acids) and 74.06 kDa (655 amino acids), respectively, which confer resistance to *T. arnobia*.

The characterization of the T-DNA inserted in eucalyptus 1521K059 was carried out by means of DNA sequencing analyzes (New Generation Sequencing - NGS). The results showed that eucalyptus 1521K059 contains a T-DNA insertion in the genome, with a functional copy of the genes cry2Aa, cry1Ab, cry1Bb and nptII. Sequencing analyzes confirmed the absence of unwanted DNA sequences in the eucalyptus genome, such as fragments of the bacterial plasmid. Insert segregation studies in progenies obtained through controlled crosses involving eucalyptus 1521K059 and non-transformed conventional matrices confirmed the Mendelian segregation for T-DNA.

Proteins Cry2Aa, Cry1Ab, Cry1Bb and NPTII were biochemically characterized and quantified by means of ELISA analysis (Enzyme Linked Immunosorbent Assay). Protein expression was evaluated in young leaves, mature leaves, branches, roots and in pollen grains.

The pBI121 binary vector was used to transform eucalyptus cells to obtain event 1521K059. This vector was originally developed with the purpose of being a general purpose vector for transforming plants with variations of constructs using the coding region of the *E. coli* β -glucuronidase (GUS) gene, for use in expression analyses.

Its external region to the T-DNA (of 8565 pb) was constructed based on the Bin 19 vector. The plasmid received the insert of genes cry2Aa, cry1Ab, cry1Bb and nptII together with their regulatory elements, constructed by Suzano SA (FuturaGene – Division of Biotechnology). Plasmid pBI121 is a T-DNA plasmid vector, which contains *E. coli* and *A. tumefaciens* bacteria in its host spectrum. Its total original size, with the expression cassette of the nptII and gus genes, is 14,758 bp, and its final size, after replacing these cassettes by the expression cassettes of the genes cry2Aa, cry1Ab, cry1Bb and nptII, is 21,501 bp

Human health risk assessment

Potential toxic or allergenic effects of proteins Cry2Aa, Cry1Ab, Cry1Bb and NPTII were analyzed using bioinformatics analysis, not indicating significant homologies with known allergens or toxins. All four (4) proteins were thermolabile and rapidly hydrolyzed in simulated gastric and intestinal fluids. The results of the overall safety assessment of proteins Cry2Aa, Cry1Ab, Cry1Bb and NPTII do not indicate possible allergenic or toxic effects.

Leaf chemical composition studies were carried out comparing the genetically modified event 1521K059 with the respective conventional clone, in addition to comparing it to other conventional clones used as commercial references. Total protein, ether extract, carbohydrates, fixed mineral residue, energy value, minerals, fibers and moisture were quantified. The results proved that eucalyptus 1521K059 does not differ from conventional eucalyptus in its chemical composition, except for the presence and expression of genes cry2Aa, cry1Ab and cry1Bb, which confer resistance to insect pests of the Geometridae family, such as *T. arnobia*, and the gene nptII, which confers tolerance to the antibiotic kanamycin, acting as a selection marker.

The T-DNA of the pBI121 vector contains, after modifications by cloning, 4 (four) expression cassettes, for the genes cry2Aa, cry1Ab, cry1Bb and nptII. The first cassette in the T-DNA contains the coding sequence of the cry2Aa gene regulated by the CaMV 35S promoter fused to the intron of the *Eucalyptus* spp Elongation Factor. (EF1) (Eucgr.J01112) and terminator NOS. The second cassette in the T-DNA contains the coding sequence of the nptII gene optimized for eucalyptus under the control of the 35S promoter linked to the Tobacco etch virus (TEV) enhancer sequence and the CaMV terminator at the end 3'. Subsequently, the cry1Bb gene sequence was added under the control of the Rubisco promoter and terminator from *Eucalyptus* spp. (RbcS; Eucgr.J01502.2). Lastly is the cry1Ab gene expression cassette

regulated by the 35S promoter, fused by the Omega enhancer, and by the NOS terminator.

The expression cassette of the neomycin phosphotransferase II (nptII) gene, controlled by the NOS promoter and terminator of the original plasmid pBI121, was replaced by the synthetic gene (Genewiz) containing the coding sequence (CDS) of the nptII gene optimized for expression in *Eucalyptus* spp., under the control of the Cauliflower mosaic virus (CaMV) 35S promoter, fused to the Tobacco etch virus (TEV) enhancer sequence and the CaMV terminator at the 3' end. This cassette was cloned using BstZ17I/PmeI-HindIII restriction enzymes.

The beta-glucuronidase (GUS) cassette was removed using EcoRI restriction enzyme.

The cry2Aa synthetic DNA expression cassette (GENEWIZ) driven by the CaMV 35S promoter fused to the *Eucalyptus* Elongation Factor (EF1) intron (Eucgr.J01112) and NOS terminator, was cloned before the nptII cassette into modified pBI121 using the FseI restriction enzyme site.

A second synthetic DNA expression cassette (Genewiz) of the cry1Ab gene controlled by the CaMV 35S promoter, fused by the 5' untranslated region (5'UTR) of Tobacco mosaic virus (TMV) and NOS terminator, was cloned posterior to the cassette of nptII expression using the EcoRI restriction enzyme site.

A third synthetic DNA expression cassette (Genewiz) of the cry1Bb gene, controlled by the *Eucalyptus* RuBisCO small subunit promoter (RbcS; Eucgr.J01502.2), fused to the 5' untranslated region (5'UTR) of RbcS and terminated with the 3' UTR region and the RbcS terminator. This sequence was cloned after the expression cassette sequence of the cry1Ab gene using the AsiSI restriction enzyme site.

All cry gene coding sequences were optimized for *Eucalyptus* spp. The general plasmid was named as FGN#1521

The nucleotide sequence of the FGN#1521 binary vector was verified by restriction enzyme analysis and Next Generation Sequencing (NGS).

The binary vector, plasmid FGN#1521, was extracted from *E. coli* from a liquid culture grown overnight (37°C, 200 RPM, LB + Km 50 µg/mL) using the QIAGEN Plasmid PlusMidi Kit (cat#A12943).

About 1.5 μ g of plasmid DNA was digested by the following restriction enzymes: FseI (NEB R0588S) 4 units, AscI (NEB R0558S) 20 units, NsiI (Thermo ER0731) 20 units, MfeI (NEB R0589S) 20 units, XmaI (NEB R0180S) 20 units and SphI (NEB R0182S) 20 units. Each reaction was supplemented with 3 μ L of the x10 reaction buffer and water was used to bring the final volume to 30 μ L. The reaction was incubated at 37 °C for 2 hours with half the unit amount of enzymes. Then, a second amount of enzyme unit was added to the reaction and incubated at 37°C for an additional 2 hours. Subsequently, the reaction was inactivated by heating at 65°C for 20 minutes. The total volume was loaded onto a 1.2% TBE agarose gel, separated for 2.5 hours at 150 V and incubated in EtBr for 3 hours.

NGS analysis was performed and genomic DNA was isolated and quantified by spectrophotometer. To build the library, approximately 100 ng Genomic DNA was broken into fragments approximately 250 bp in length, isolated with the Nextflex Rapid XP DNA-Seq kit from the Illumina Platform (Perkin Elmer), as described by the manufacturer. The library concentration was measured by Qubit and the size was checked by TapeStation. The library was then loaded onto Illumina's Miseq equipment and sequenced using the Miseq V2 kit (500 cycles) to generate 2x250 paired-end reads and concluded that clean reads were aligned against the FGN#1521 vector sequence using the Geneious program version 11.

The expression product of cry2Aa, cry1Ab, cry1Bb and nptII genes was evaluated by immunoenzymatic test ELISA (Enzyme-Linked Immunosorbent Assay), to detect and quantify the concentration of proteins Cry2Aa, Cry1Ab, Cry1Bb and NPTII in different tissues of eucalyptus 1521K059 and in different stages of crop development.

Environmental Risk Assessment

The significance of the segregation pattern was evaluated by chi-square analysis (χ^2) in F1 progenies of eucalyptus 1521K059 (used as female parent), produced by means of controlled pollinations, using pollen from conventional male parents (FGN-L, FGN-P and FGN-AL). The seeds from the crossings were used for the production of seedlings, through sowing in plastic tubes containing organic substrate. Leaf tissue samples were collected from seedlings produced from seeds of the three crosses (1521K059 x FGN-L, 1521K059 x FGN-P and 1521K059 x FGN-AL) and sent for analysis in the molecular biology laboratory. The result of the analyzes of the data of the progenies obtained

crossings such as eucalyptus 1521K059, indicate that the observed frequencies of plants with genes cry2Aa, cry1Ab, cry1Bb and nptII follow the ratio of 1:1, indicating Mendelian segregation of T-DNA in the 3 (three) evaluated progenies

No pleiotropic or epistatic effects were observed in eucalyptus 1521K059 plants resulting from the presence of the cry2Aa, cry1Ab, cry1Bb and nptII genes, whether in phenotypic, agronomic, morphological, reproductive and compositional characteristics in containment experiments, carried out since their acquisition in a greenhouse, and in the field, carried out under Planned Environmental Releases (LPMAs),

No significant differences were observed in the phenotypic characteristics studied between GM eucalyptus 1521K059 plants when compared to conventional eucalyptus plants, at various stages of tree development, except for their ability to present resistance to caterpillars of the order Lepidoptera, through the introduction of genes cry2Aa, cry1Ab, cry1Bb and nptII in the plant through Agrobacterium-mediated transformation.

The applicant carried out tests to evaluate the emergence of eucalyptus seedlings under field conditions in two locations where eucalyptus is cultivated. Eucalyptus 1521K059 did not differ statistically from its corresponding conventional control FGN-K in germination percentage; After 60 days of sowing in the field, no new germination occurred until the end of the test at 90 DAI (days after installation).

The results of the phenotypic evaluations indicate that the eucalyptus 1521K059 does not have characteristics that could confer a risk of significant impact on the environment or of becoming invasive (with an increase in its adaptability or invasiveness), when compared to the conventional eucalyptus clone FGN-K. Data from ecological interactions also indicate that eucalyptus 1521K059 does not confer any greater susceptibility or tolerance to disease. The compositional data also support the conclusion about the chemical equivalence of eucalyptus 1521K059 and conventional eucalyptus FGN-K.

Cry2Aa protein toxicity tests were performed on non-target organisms associated with GM eucalyptus forestry, including pollinators such as bees (*Apis mellifera*), soil organisms such as earthworms (*Eisenia fetida*) and springtails (*Folsomia candida*), and microcrustaceans such as the *Daphnia magna*. The NOECs from these studies were compared to the EECs environmental exposure estimates of the eucalyptus Cry2Aa protein,

aiming to determine a safe environmental exposure margin of the protein to non-target organisms.

Data on chemical and physical characteristics of soils analyzed in experimental plots of the genetically modified eucalyptus 1521K059, its conventional clone FGN-K, and two commercial references FGN-T and FGN

P, in 4 locations, at pre-planting (baseline) and 24 months after planting, allow us to conclude:

Due to the uniformity of the chemical and physical characteristics of the soil before planting, it was demonstrated that the soil conditions in the experimental area were adequate to allow a comparative analysis of the effect of different eucalyptus clones on the chemical and physical properties of the soils where they were cultivated.

Eucalyptus 1521K059 did not change the chemical and physical characteristics of the soil that could differentiate it from its conventional FGN-K control.

The genetically modified eucalyptus 1521K059 (treatment 2) and its non-genetically modified control, Isolinha FGN-K (treatment 3), were similar in the period of material degradation in the evaluated variables, and presented, in most cases, similarity with the loss of matter of conventional commercial references.

The greatest decomposition of the biomass of the materials of the 4 clones occurred between 120 and 180 days of deposition of the samples in the soil.

There was a difference in biomass decomposition depending on the evaluated location. These differences in biomass decomposition between sites are expected because specific edaphoclimatic conditions are present among the evaluated agroecosystems.

Final Opinion

The applicant presented the information requested by RN nº 32/2021. There was no identification of non-negligible risks in the risk assessment conducted. Therefore, and in accordance with the provisions of RN 32/2021, it did not present a post-commercial release monitoring plan (page 461).

Within the powers of art. 14 of Law 11.105/05, as well as the internationally accepted criteria for assessing the safety of genetically modified foods and raw materials, it is considered that the biosafety data of event 1521K059 meet the standards and relevant legislation that aim to guarantee the biosafety of the environment environment, agriculture,

human and animal health. Thus, once the conditions described in the process and in this technical opinion are met, this activity does not potentially cause significant degradation of the environment or human health.

Data: 02/03/2023

Paulo Augusto V. Barroso
President of CTNBio