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Safety of genetically modified glyphosate-tolerant eucalyptus designed for integrated weed management

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Abstract: Background: Eucalyptus is the primary cultivated wood species in Brazil, covering 7.5 million hectares. Weed competition in eucalyptus plantations reduces yield and increases operational costs. FuturaGene/Suzano has developed genetically modified (GM) eucalyptus varieties with glyphosate herbicide tolerance (HT) as a modern tool for improving weed management practices in plantations. The first event received regulatory approval for commercial deployment in 2021. However, the introgression of a new GM trait into eucalyptus, a non-isogenic species, cannot be achieved through selfing or backcrossing. To overcome this limitation and expedite the introgression of HT into the breeding population, multiple GM events were generated, in various genetic backgrounds and genomic locations, enabling simultaneous crossing with numerous elite parents.

Objective: To characterize the newly developed HT GM eucalyptus events and assess their safety for the environment and wood production.

Methods: HT GM eucalyptus events were subjected to genome sequencing and glyphosate tolerance testing. Biosafety analyses and environmental impact assessments were conducted through field trials in various eucalyptus cultivation regions, comparing the HT GM eucalyptus with conventional clones.

Results: The new events proved highly tolerant to glyphosate and displayed different genomic insertion sites. No adverse effects on non-target organisms were observed, and there were no significant differences in the soil microbiota or decomposition profile.

Conclusions: The HT GM events have been proven to be safe, posing a low risk to the environment, humans, and animals. Consequently, these HT GM eucalyptus varieties can be confidently utilized for wood production.

Keywords: Herbicide Tolerance; Glyphosate; Biosafety; GM trees; GM Eucalyptus

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1. Introduction

Species of the genus *Eucalyptus* are the primary trees grown for wood production in Brazil, which is one of the countries with the largest non-native eucalyptus plantations (Seng Hua et al., 2022). Despite their small fraction in the world's forested areas (approximately 0.5% or around 22.57 million hectares), according to the Food and Agriculture Organization of the United Nations (2020), eucalyptus plantations account for approximately 10% of the current global demand for roundwood. As a result, eucalyptus is considered a vital species for future wood supply.

To address the increase in global roundwood demand, expected to reach four billion cubic meters in the next decade (Barua et al., 2014), significant advancements in sustainable production, trade and consumption of forest products are required worldwide. Eucalyptus plantation management faces ongoing challenges, including substantial yield limitations and increased operational costs due to significant weed competition, which is one of the major limiting factors in eucalyptus production (Osiecka, Minogue, 2013; Little et al., 2003). Studies conducted on eucalyptus plantations have revealed that weed competition can lead to higher yield loss, with reported percentages reaching up to 91% (Bacha et al., 2016; Vargas et al., 2018). In other crops, direct competition with weeds for crucial resources like water, light and minerals is responsible for an estimated 40% yield loss (Korav et al., 2018). Glyphosate serves as the primary herbicide employed for weed management in eucalyptus plantations. It is applied across the entire area prior to planting to control both weeds and regrowth from cut stumps. Additionally, post-planting applications are carried out within and between planted rows. These post-planting glyphosate applications usually involve manual techniques using backpack sprayers and specialized tractors with sprayer shields. These methods are costly and result in excessive chemical usage, primarily implemented to safeguard young eucalyptus plants from herbicide exposure. Preliminary estimative from Suzano S.A. shows that advanced over-the-top herbicide application technology, when coupled with tolerant trees, may reduce weed control costs by average of 36%, potentially leading to savings on labor, tools, and chemicals according to the company.

However, eucalyptus exhibits a high sensitivity to glyphosate (Cerveira Junior et al., 2020; Pereira et al., 2013) and even slight herbicide drift (2.4% of the recommended 1.8 Kg a.e/ha application dose) during application can cause significant damage to young eucalyptus plantlets, leading to a substantial decline in yield (Meloni, Martínez, 2021; Santos et al., 2015; Santos et al., 2019). To address this issue, Suzano and its subsidiary FuturaGene have developed genetically modified (GM) glyphosate-tolerant eucalyptus varieties. These GM eucalyptus events offer protection against yield losses caused by glyphosate, and enable more uniform, mechanized herbicide application using tractor-mounted horizontal sprayers. This reduces chemical waste and operational costs.

These GM eucalyptus events carry an *Agrobacterium tumefaciens* strain CP4 (CP4) gene, which encodes a glyphosate-tolerant 5-enolpyruvyl-shikimate-3-phosphate-synthase (EPSPS) enzyme. In contrast to the native glyphosate sensitive EPSPS, this CP4-EPSPS exhibits a reduced affinity for glyphosate but a higher affinity for phosphoenolpyruvate (PEP) (Sammons, Gaines, 2014). As a result, when glyphosate is applied and the native EPSPS is inhibited, CP4-EPSPS serves as an alternative pathway, enabling the conversion of shikimate-3-phosphate (S3P) and PEP to 5-enolpyruvylshikimate-3-phosphate (EPSP). It is important to note that both CP4-EPSPS (Organisation for Economic Co-operation and Development, 1999; Agriculture & Food Systems Institute, 2016) and the selectable marker neomycin phosphotransferase II (NPTII) (European Food Safety Authority, 2007) have been used in GM crop for over 25 years and deemed safe for human and animal consumption, as well as for environmental exposure. The first glyphosate-tolerant GM eucalyptus event, named 751K032, was subjected to rigorous biosafety analyses under laboratory, greenhouse and field conditions and was granted commercial approval by the National Technical Commission of Biosafety (CTNBio) of Brazil in 2021 (Avisar et al., 2023).

When compared to other crops, eucalyptus breeding presents unique challenges. One major challenge is that eucalyptus presents high inbreeding depression, which greatly diminishes its ability to self-pollinate and renders backcrossing nearly impossible (Hedrick et al., 2016). Additionally, eucalyptus is a semi-domesticated species with high genomic variability, resulting in a lack of genetic uniformity. Furthermore, the genetic map of eucalyptus remains largely unexplored, posing significant obstacles to breeding efforts (Bartholomé et al., 2015). Introduction of a new trait from a single glyphosate-tolerant GM event to the entire breeding population is impossible, due to the narrow genetic diversity. Furthermore, the selected GM event may not be the optimal parent for certain environmental conditions and the genomic DNA insertion site can sometimes be linked to or in close proximity to an undesirable genetic quantitative trait loci (QTLs) creating a linkage drag that can interfere with breeding efforts (Mumm, 2013; Peng et al., 2014). Such undesired QTLs can result in reduced yield and high sensitivity to environmental stress as well as susceptibility to diseases and pests. To overcome these limitations and expedite the integration of the desired trait into the breeding population, multiple glyphosate-tolerant GM events were created. These events were obtained on diverse genetic backgrounds and with distinct T-DNA genomic localizations, enabling simultaneous crosses with numerous families. This approach reduces the risk of linkage with hidden undesirable genetic loci (cryptic bad QTLs) and accelerates the introgression process, whilst maximizing genetic diversity.

This paper presents the selection and genomic characterization of four recently developed GM eucalyptus events: 955S019, 955S024, 751K022, and 955P082. These events were carefully selected based on having unique and diverse genetic backgrounds, high levels of glyphosate tolerance, and unique locations of the new genes in the genome, as described here. Additionally, we provide a thorough examination of the safety assessment studies carried out on event 955S019, evaluating its potential effects on humans, animals, the environment.

2. Material and Methods

2.1 DNA sequencing and insertion site detection

The cetyltrimethylammonium bromide (CTAB) protocol was used to extract genomic DNA from eucalyptus plants. Firstly, fresh leaf tissue (2g) was frozen in liquid nitrogen before being finely powdered. Next, an extraction buffer consisting of 15 ml was added, containing the following components: 2% CTAB, 100 mM Tris at pH 8, 1.5 M NaCl, 0.2 mM EDTA at pH 8, 1% β-mercaptoethanol, and 0.1% PVP. The resulting mixture was subjected to incubation at 65°C for 60 minutes, periodically swirling the contents, followed by cooling to room temperature. Subsequently, 15 ml of Chloroform Isoamylic Alcohol was added, thoroughly mixed, and centrifuged at 9,000 rpm for 15 minutes at 22 °C. The supernatant was carefully transferred to a new tube, and this step was repeated twice. Afterward, an equal volume of ice-cold Isopropyl alcohol was added, and the tube was incubated at -20 °C for 30 minutes. The mixture was then centrifuged at 14,000 rpm for 20 minutes at 4 °C. The resulting supernatant was discarded, and the pellet was treated with 500 µl of 70% ice-cold ethanol. This solution was centrifuged at 14,000 rpm for 2 minutes at 4 °C, and the ethanol was subsequently removed. The tubes were left open at room temperature to allow complete ethanol evaporation, after which the pellet was resuspended in 250 µl of RNAse (10 ng/µL; Sigma R6513) in TE buffer at a concentration of 20 ng/µl. The tube was maintained at 37 °C until complete dissolution of the pellet.

Genomic DNA (0.5 μg) was sequenced on the Illumina Hiseq2500 platform, utilizing a single individual lane that produced raw read data (150PE, 80 gigabytes). The Geneious Prime software version 11 (http://www.geneious.com) was utilized for read mapping. Two nearly identical T-DNA constructs were used in this study (Figure S01). The first construct (751) contains a single CP4-EPSPS expression cassette driven by the constitutive cauliflower mosaic virus 35S promoter. The second construct (955) consists of two CP4-EPSPS expression cassettes driven by both the constitutive cauliflower mosaic virus 35S promoter and the figwort mosaic virus segmented promoter. Both T-DNA constructs share an identical NPTII selectable marker cassette. Reads that successfully aligned to both the T-DNA and the genome DNA sequences were used to determine the specific location of the insert within the genome. A published eucalyptus genome BRASUZ 2.0 (Myburg et al., 2014) served as a reference for locating the insertion within the genome.

2.2 Glyphosate tolerance assay

The experiment was conducted in a polycarbonate greenhouse with a pad-fan cooling system. Temperatures ranged from 18-28 °C and relative humidity from 70–90%. Three wild type Eucalyptus urophylla hybrid clones and five glyphosate-tolerant GM events derived from those clones (see Table 1) were evaluated for tolerance to direct application of increasing doses of glyphosate herbicide (0, 0.45, 1.8 or 3.6 kg acid equivalent/ha).

120-day old plants were transplanted individually into 1.2 L pots with an organic media containing peat moss, vermiculite, limestone, gypsum and NPK fertilizer (in milligrams per liter: N-271, P-40, K-233, C-232, Mg-50, S-70, Micronutrients: Cu, Fe, Zn, Mo and Co – 13). Water and nutrients were supplied via drip irrigation (3.2 L/day). Glyphosate was applied at the aforementioned doses inside a spray booth using an automated spray bar with a 110° flat fan nozzle calibrated to deliver 200 L/ha.

The experiment used a randomized design with 4 replications (one plant per plot). Treatments were arranged in a double factorial with 5 doses and 8 genotypes. The herbicide was applied 7 days after transplanting. Aerial parts were oven-dried to constant weight at 60 °C for 24 hours 15 days after application and the biomass (g) was measured.

Analysis of variance was conducted and means separations within genotype and dose were determined by Tukey's test at 5% error probability.

2.3 Field design

Eucalyptus event 955S019 and the control clone FGN-S were planted across four sites in Brazil in a randomized complete block design: two sites in São Paulo (SP), one site in Bahia (BA), and one site in Maranhão (MA). At each site, there were five square plots containing 16 plants for each clone (955S019 and FGN-S). The five plots for each clone were randomly distributed among plots containing other unrelated clones. The plantings used 3-month old cuttings at all sites. The plantations were grown for at least 3 years at each site.

2.4 Degradability of event 955S019 tissues in the field

Screened nylon litter bags with a tight 2 mm weave and dimensions of 20 x 20 cm were prepared. Each bag was filled with 35g of plant biomass, comprising 5 g of branches and 30 g of leaves taken from samples of GM event 955S019 and non-GM commercial eucalyptus FGN-S. For each sample type, 5 replicate bags were prepared for each time point (day zero and day 270) resulting in 10 bags per sample. These litter bags were placed in experimental plots at four different field sites (section 2.3), so at each site there were 20 bags total - 10 bags for the GM event 955S019 sample and 10 bags for the non-GM eucalyptus FGN-S sample.

On both days 0 and 270, samples were subjected to analysis using the method and calculations outlined in Santos and Whitford's study from 1981 (Santos, Whitford, 1981). The samples were first dried for approximately 1 h, at a temperature of 60–70 °C, and the weight of the dry matter was recorded. Subsequently, the samples were incinerated in a muffle furnace at 700 °C to determine the content of ashes and organic matter. The loss of ashes and

Table 1. Information of Eucalyptus genotypes used in this study.

#	Name	Background	Type	Eucalyptus species	Source
1	FGN-K		Wild type	*E. urophylla* hybrid	Suzano S.A
2	FGN-S		Wild type	*E. urophylla* hybrid	Suzano S.A
3	FGN-P		Wild type	*E. urophylla* hybrid	Suzano S.A
4	751K022	FGN-K	GM Event	*E. urophylla* hybrid	FuturaGene Ltd.
5	751K032	FGN-K	GM Event	*E. urophylla* hybrid	FuturaGene Ltd.
6	955S019	FGN-S	GM Event	*E. urophylla* hybrid	FuturaGene Ltd.
7	955S024	FGN-S	GM Event	*E. urophylla* hybrid	FuturaGene Ltd.
8	955P082	FGN-P	GM Event	*E. urophylla* hybrid	FuturaGene Ltd.

organic matter was calculated by comparing the results to those obtained on day 0. Averages across all sites were calculated for the event 955S019 and the commercial reference eucalyptus FGN-S. Analysis of variance was conducted and means separations within treatment and weight were determined by Tukey's test at 5% error probability.

2.5 Arthropod collection and analysis

Over three years, twelve arthropod samplings were conducted at each of four eucalyptus farms. The 955S019 event plots were compared to the eucalyptus wild type control FGN-S plots. Four arthropod collection methods with five repeats each were applied in 16 random square plots:

1. Pitfall traps (10 cm diameter, 15 cm high, with insect preservative solution) were placed for 72 hours to count epigean species.
2. Yellow adhesive cards at treetop height were placed for 72 hours to count flying species.
3. Soil samples (10 per plot, 10 cm diameter, 5 cm depth) used a Berlese-Tüllgren funnel to extract organisms.
4. Litter samples (5 per plot, 25 cm^2) used the Winkler extractor method.

Organisms collected using the 5 methods were preserved in 70% ethanol and 5% glycerin solution for later analysis and classification. Taxa were classified by comparison with reference collections or consultation of specific literature.

The arthropod community was analyzed using the DivEs computer program (version 4.0) to determine the Shannon ecological diversity index (H'). The H' index indicates species diversity by considering richness (number of species) and abundance. The obtained H' results underwent analysis of variance and the means were compared using the Agricolae package (version 1.3.5) for R language (version 4.2.1) with the Tukey's Test at 5% significance.

2.6 Microbial community analysis

Soil samples were collected 30 months after planting using an auger to 15 cm depth. Samples were taken from locations cleared of plant material, sealed in plastic bags, and refrigerated at 4 °C for up to 72 hours before analysis.

To evaluate microbial density, soil samples were diluted in phosphate buffered saline, centrifuged, and plated on culture media. Bacteria were quantified by plating serial dilutions on TSB agar with carbendazim and incubating at 28 °C for 24 hours. Fungi were quantified on PDA agar with tetracycline, incubating at 28 °C for 96 hours. Microbial density was calculated as Log10 CFU/g soil.

For microbial diversity analysis, DNA was extracted from soil samples using a commercial kit. Bacterial 16S rRNA and fungal ITS regions were PCR amplified and sequenced. Bacterial 16S rRNA was amplified with the primers V3 and V4 (V3-341F: 5"-CC TAC GGG NGG CWG CAG–3' and V4-805 R: 5'-GAC TAC HVG GGT ATC TAA TCC– 3'). Fungal rDNA (gene 5.8S rRNA partial, ITS2 and gene 28S rRNA par- tial) were amplified with the forward primer ITS3 (5'-GCA TCG ATG AAG AAC GCA GC–3') and reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC–3"). Sequences were processed with QIIME software (Caporaso et al., 2010) using the SILVA database. Taxonomic assignments were made based on sequence similarity thresholds. Alpha diversity metrics including Chao1, Shannon, and Simpson indices were calculated. Principal coordinate analysis (PCoA) was utilized to compare groups of samples based on phylogenetic and count-based distance metrics (Podani, Miklós, 2002; Hammer et al., 2001).

3. Results and Discussion

3.1 Characterization and selection of glyphosate tolerance events

Glyphosate susceptible *urophylla* hybrid clones FGN-K, FGN-S, and FGN-P (Table 1 and Figure 1), underwent genetic modification using either 751 or 955 T-DNA constructs (Figure S01). The GM events 751K022, 751K032, 955S019, 955S024, and 955P082 that were selected had a single insertion site in their genomes, with no other foreign DNA present. All five events demonstrated tolerance to at least double the commercial dose (3.60 Kg a.e/ha) of glyphosate, rendering them resistant to accidental overspray or secondary drift if a higher-than-intended dose is applied in the field (see Figure 1). Fifteen days after glyphosate application, the aerial biomass data (Figure 1A) showed that each engineered eucalyptus event gained biomass similarly across all applied doses compared to the wild type clones FGN-K, FGN-S and FGN-P, which displayed sensitivity to glyphosate and impaired biomass accumulation. The biomass of the engineered eucalyptus events was also similar to or higher (for event 955P082) than that of the corresponding wild type clones without glyphosate application. This demonstrates that glyphosate application did not impact the growth of the engineered eucalyptus events. The greenhouse results were consistent with data from field trial studies (Suzano A.S unpublished data; Porto et al., in preparation).

DNA sequencing on these events identified the specific insertion sites on the chromosome and nucleotide levels. The insertion site of the 751K022 event was identified on chromosome 05 at position 52,792,218 nt. The insertion site of the 751K032 event was found on chromosome 10 at position 23,009,898 nt. The insertion site of the 955S019 event was located on chromosome 03 at position 55,945,341 nt. The insertion site of the 955024 event was detected on chromosome 11 at position 28,078,899 nt. Finally, the insertion site of the 955082 event was identified on chromosome 06 at position

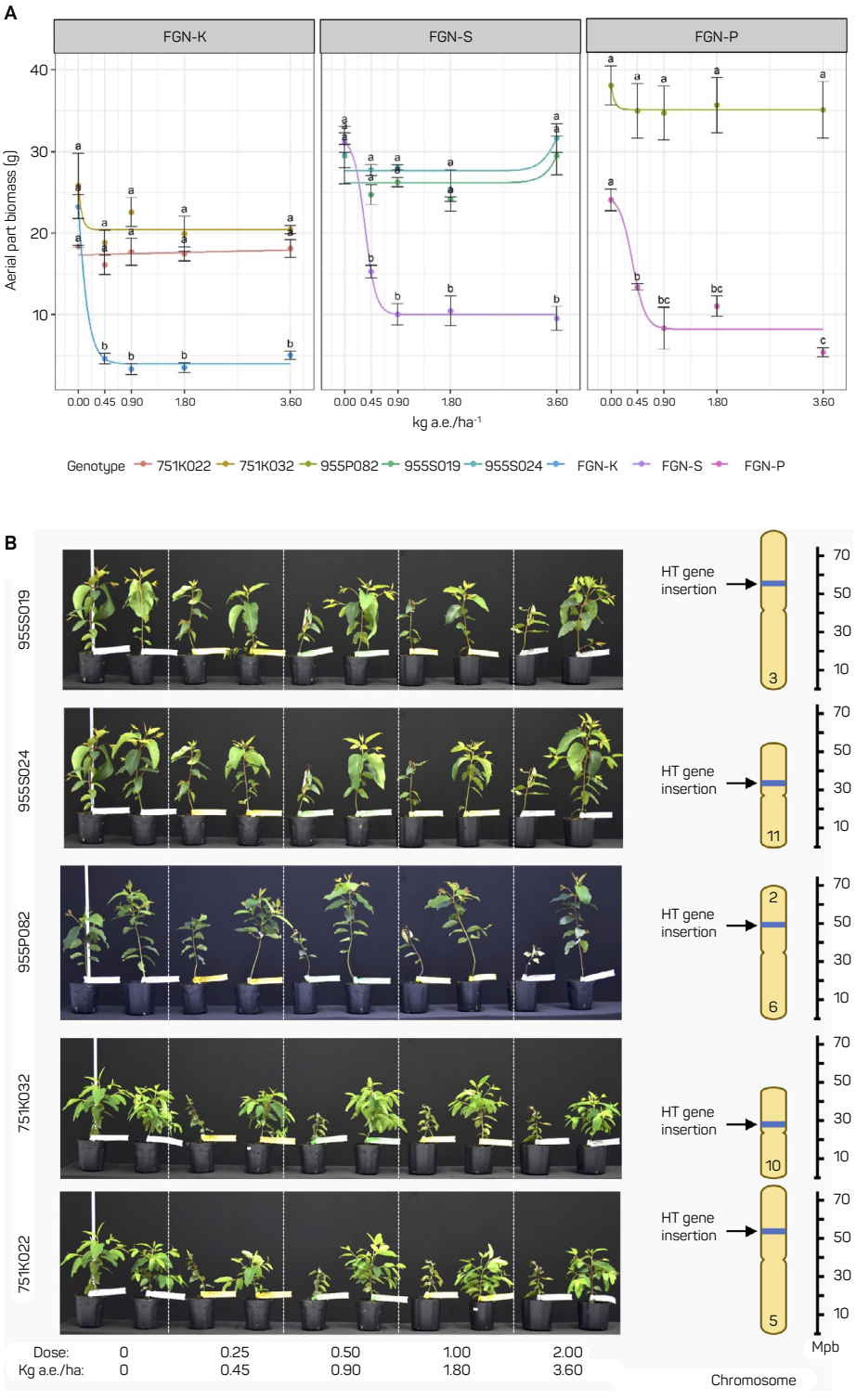


Figure 1 - Glyphosate tolerance and genomic insertion sites. Five glyphosate-tolerant genetic modification (GM) events were subjected to different doses of glyphosate, equivalent to 0, 0.25, 0.5, 1.0, and 2.0 times the recommended application dose. These doses correspond to 0, 0.45, 0.90, 1.80, and 3.60 Kg a.e/ha. A. Aerial part biomass 15 days post glyphosate application. The data for each GM event was presented alongside the data for its matched wild type clone. The biomass of the sprayed events was either similar to or greater than that of their corresponding unsprayed wild type clones. This indicates that applying glyphosate at twice the recommended dose did not inhibit the growth of the selected events. B. Left: Corresponding wild type (wt) FGN-K, FGN-S, and FGN-P. Right: GM events. All of the selected events exhibited high tolerance to at least double the recommended dose, whereas the corresponding wt plants showed signs of death. The sites where the herbicide tolerance (HT) genes were inserted in the genome are indicated by the chromosome number and a line

41,371,983 nt, and it was found to be fused with a fragment of chromosome 2 that had been translocated to position 53,849,328 nt (see Figure 1B).

Glyphosate-tolerant events have the potential to play a crucial role in enhancing the sustainability of eucalyptus plantations in Brazil. They can offer a significant opportunity to reduce glyphosate usage in the field (per area), as shown in other HT GM crops (Brookes, Barfoot, 2020) by implementing unified mechanical spraying techniques instead of manual and protected spraying that increase chemical waste. Additionally, these events can potentially boost yield by safeguarding the plants against any glyphosate herbicide-related damage. However, relying solely on glyphosate-tolerant eucalyptus is insufficient, as weeds resistant to glyphosate, whether naturally occurring, evolved, or migrated from nearby fields, can still compete and thrive in plantations. While planting glyphosate-tolerant trees has the potential to reduce glyphosate usage and selection pressure, this strategy must be complemented with additional weeding techniques. These could include using alternative herbicide modes of action, manual weeding, and potentially developing GM eucalyptus with tolerance to multiple herbicides, similar to new GM crops being engineered worldwide (Shyam et al., 2021).

The development of multiple highly glyphosate-tolerant events as presented here, can expedite the integration of the herbicide-tolerance gene into the eucalyptus breeding population. Eucalyptus trees possess distinctive characteristics that impose unique breeding strategies to overcome challenges, such as limited self-fertilization and constrained backcrossing possibilities (Hedrick et al., 2016). By employing diverse genetic backgrounds and conducting simultaneous crossings with different families, the HT gene can be efficiently disseminated. Furthermore, the presence of the HT gene in multiple genomic locations can mitigate the risk of linkage with undesirable QTLs, including those associated with subpar wood quality, disease susceptibility, and sensitivity to environmental stresses such as drought. Instances of undesired linkage between the HT gene and a negative QTL can be eliminated from the breeding program, while parallel crossings involving other events can continue without disruption.

3.2 Safety Assessments

In the following sections, we present the environmental safety analyses conducted for event 955S019. The *in-vitro* safety study that examined allergenicity, toxicity, digestibility, and heat lability of the NPTII and CP4-EPSPS proteins was described previously (Avisar et al., 2023; Fuchs et al., 1993; Harrison et al., 1996). Additionally, bee safety studies were conducted, as outlined by Santos et al. (2023). These studies collectively demonstrated that there are no safety risks associated with the NPTII and CP4-EPSPS proteins to humans, animals, or the environment.

The other events 751K022, 955S024, and 955P082 are subjected to a simplified assessment process based on the comprehensive assessment process made for events 955S019 and 751K032. The CTNBio allows for a simplified risk assessment process, without redundant safety analyses, if a new GM event possesses a DNA construct that is identical or similar to a previously approved reference GMO of the same species (Normative Resolution no. 32 of June 15, 2021 - Anexo IV – Part A[1]).

3.2.1 *Degradability of event 955S019 tissues in the field*

Degradability assays conducted over 270 days evaluated the degradation of branch and leaf tissues over time. These assays revealed no significant differences in degradation outcomes between the 955S019 event and the FGN-S wild-type control bags across all parameters under field conditions in various biomes (Figure 2). Each farm is situated in a unique biome characterized by diverse precipitation levels, soil composition, humidity, and temperatures. These variations can influence the degradation rate observed on

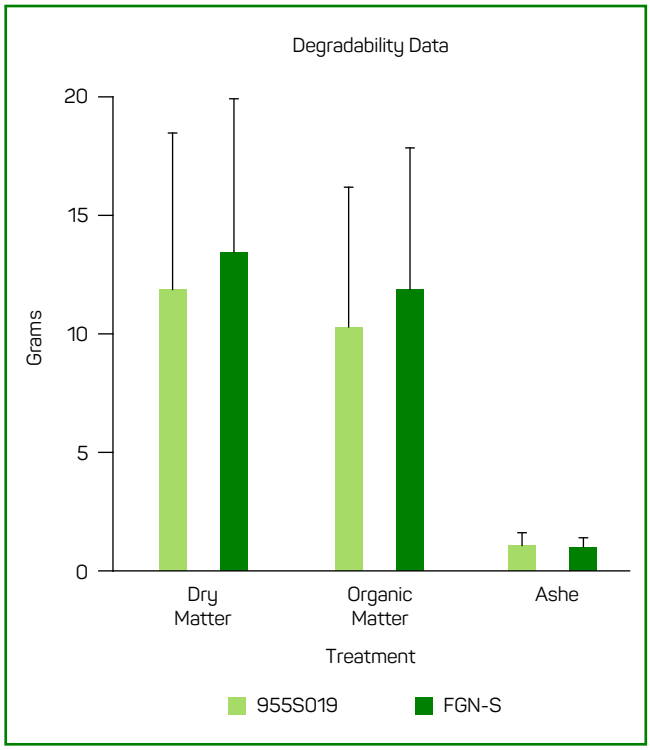


Figure 2 - Similar degradability rate results of 955S019 event and wt FGN-S leaves and branches over 270 days in field soil at four sites in Brazil. Dry matter weight and the organic matter and ashes weight loss averages of all four farms are presented. There were no significant variations in the degradability of the 955S019 event compared to wt FGN-S, as determined by Tukey's test at a significance level of 5%

[1] https://www.in.gov.br/en/web/dou/-/resolucao-normativa-n-32-de-15-de-junho-de-2021-326241632

each farm (Ribeiro et al., 2018) and probably contribute to the relatively high standard deviations.

3.2.2 *Effect of eucalyptus 955S019 event on soil microbial communities and arthropod diversity*

Arthropods and soil microbes play crucial roles in maintaining ecological balance, particularly in agricultural fields and plantations (Paoletti, 1999) (Zhang et al., 2023). Due to their susceptibility to changes in crop growth and silviculture practices, they serve as valuable bioindicators of specific ecological scenarios in the field. Within this context, the present study conducted on four farms aimed to investigate the effects of the GM 955S019 event on arthropod and soil microbial populations in comparison to the non-modified wt clone FGN-S. Thirty months after planting, no significant differences were found in the density of bacteria and fungi ($p>0.05$) in plots of event 955S019 and wt FGN-S (Figure 3). The PCoA (Figure 3 upper panel) indicated no correlation between the cultivation of the GM event 955S019 and the structure of the soil

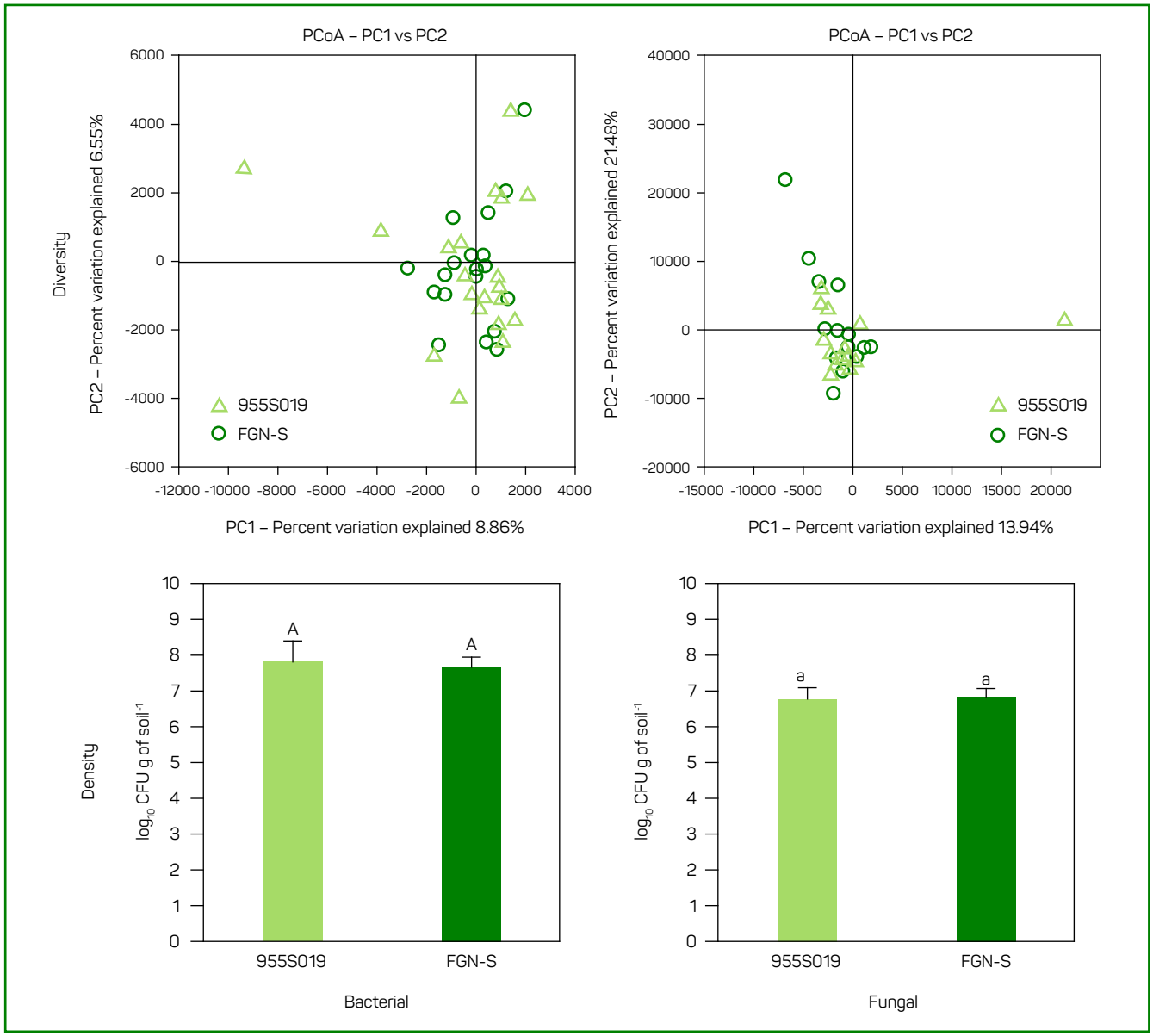


Figure 3 - Comparison of microbial composition of 955S019 and FGN-S soil samples. Soil samples were obtained from both 955S019 and the corresponding wt clone FGN-S plots, 30 months after planting. To evaluate the diversity of microbial populations based on 16S rRNA sequencing, Principal Coordinate Analysis (PCoA) was conducted using the "alpha diversity" tool from the QIIME pipeline, as shown in the upper panel. Additionally, colony-forming unit analyses were performed on five biological replicates to assess microbial densities, as depicted in the lower panel. Both analyses found no significant differences between the 955S019 and wt FGN-S samples, indicating similarity in microbial population diversity and densities (Tukey's test at a significance level of 5%)

microbial community when compared to wt FGN-S. Overall, cultivation of event 955S019 did not have a significant impact on the soil microbial community in comparison to the non-modified wt FGN-S eucalyptus genotype (see supplemental material for microbial diversity revealed through metataxonomic sequencing).

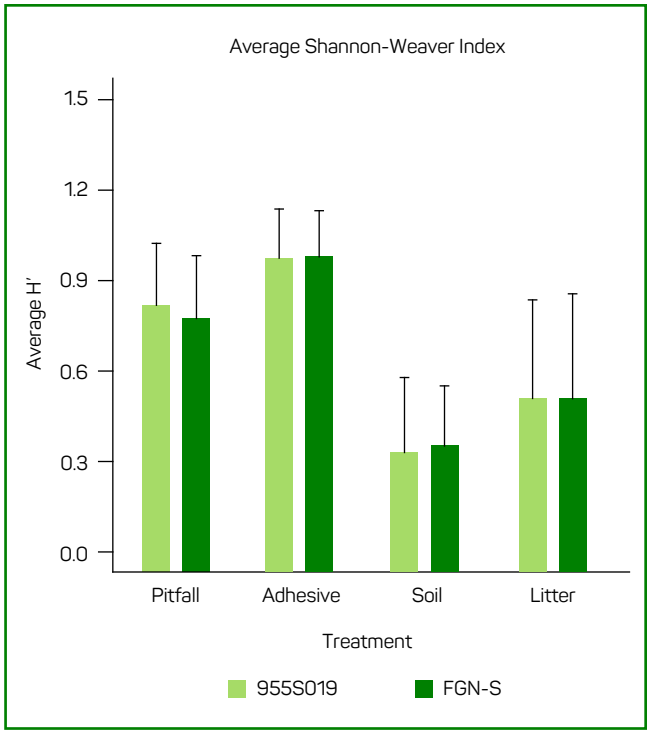
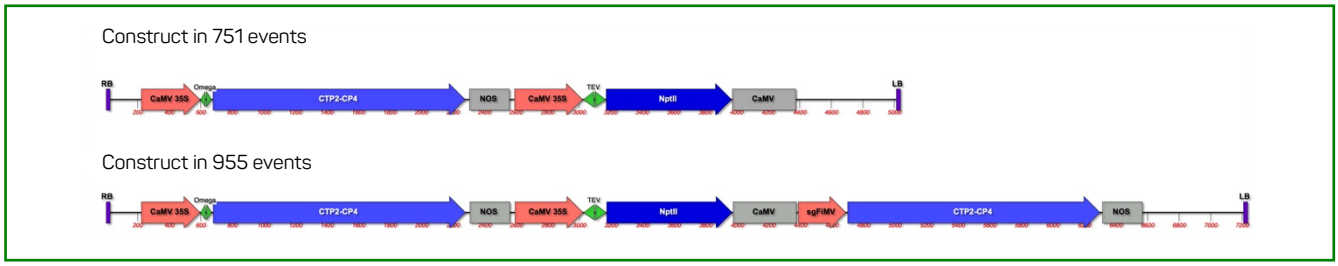


Figure 4 - Arthropod richness and abundance of 955S019 vs. wt clone FGN-S soil samples. Specimen were collected from 955S019 and wt FGN-S plots using the pitfalls, adhesive traps, soil sampling, and litter sampling methods, in four farms over a three-year period. The collected samples were analyzed in the laboratory and the Shannon ecological diversity index (H') was used to assess the richness and abundance of arthropod species in the 955S019 and wt FGN-S plots.Variance analysis was performed, and means were compared using the Tukey's Test, at a significance level of 5%, with the Agricolae package (version 1.3.5) for the R language (version 4.2.1). No significant differences were found between the two types of plots across all farms and collection methods

Furthermore, the Shannon ecological diversity index analysis, conducted across all farms and using various arthropod collection methods (Figure 4), revealed no significant differences between the event 955S019 plots and the wt FGN-S plots. In addition, introduction of eucalyptus event 955S019 did not have a significant impact on the arthropod populations in any of the tested regions in Brazil; the populations exhibited similar parameters whether they were sampled from within event 955S019 plots or nearby FGN-S clone plots. Taken together, the three-year study found no significant differences in the populations of arthropods and soil microbes between the 955S019 and FGN-S plots. These findings suggest that the introduction of the GM event does not cause any ecological changes and can be considered environmentally friendly, posing a low risk of environmental harm.

4. Conclusions

After evaluating the biosafety of event 955S019, it can be concluded that this GM eucalyptus variant is suitable for the production of wood and fiber. The present work together with previous findings suggest that the event poses no risks to humans, animals, or the environment, and its safety is comparable to that of the conventional clone FGN-S. The five distinct glyphosate-tolerability events described here, featuring diverse genetic backgrounds and genomic localizations, will accelerate incorporation of the HT gene into the eucalyptus breeding population, and promote sustainability within the eucalyptus plantation sector. Planting glyphosate-tolerant eucalyptus is the first step in improving weed control practices in eucalyptus plantations. However, this needs to be followed by developing trees with traits that confer tolerance to both glyphosate and additional herbicides. Pursuing multi-herbicide tolerance will enable more diverse, integrated approaches for sustainable weed control.



Supplementary Figure S1. T-DNA construct maps. Two nearly identical constructs were employed to produce transgenic eucalyptus events with glyphosate tolerance. The first construct (751) contains a single CP4-EPSPS expression cassette driven by the constitutive cauliflower mosaic virus 35S promoter. The second construct (955) consists of two CP4-EPSPS expression cassettes driven by both the constitutive cauliflower mosaic virus 35S promoter and the figwort mosaic virus segmented promoter. Both T-DNA constructs share an identical NPTII selectable marker cassette

Author contributions

All authors read and agreed to the published version of the manuscript. DA, RNG, and ACP: conceptualization of the manuscript and development of the methodology. JMWG, SL, ACMP, AAS, TRD, and CSR: data collection and curation. AAS, ACMP, TBD, MPG, TRD, SL, CSR, and AM: data analysis. DA, RNG, and ACP: data interpretation. DA, RNG, and ACP: funding acquisition and resources. TBD, MPG, and AM: project administration. DA and ACP: supervision. DA: writing the original draft of the manuscript. DA, AAS, RNG, TRD, and ACP: writing, review and editing.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript. The authors are employed by FuturaGene and Suzano, where they are involved in the development of genetically modified eucalyptus.

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