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Micropropagation of Banana cv. BRS Terra-Anã: Evaluation of Genetic Stability Across Successive Subculture Cycles

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AUTHOR CONTRIBUTION

Alexssandro Rodrigues de Souza: Conceptualization, methodology, investigation, data curation, formal analysis, writing – original draft.

Alana Jeniffer Alves dos Santos; Gabrielle Balbo Crepaldi; Heitor Gomes Carvalho: Investigation and experimental assistance

Heitor Gomes Carvalho: English language revision.

Elyabe Monteiro de Matos: Flow cytometry analysis and writing – review & editing.

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MICROPROPAGATION OF BANANA cv. BRS TERRA-ANÃ: EVALUATION OF GENETIC STABILITY ACROSS SUCCESSIVE SUBCULTURE CYCLES

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ABSTRACT

Micropropagation is an essential technique for large-scale production of banana with phytosanitary quality and genetic uniformity. The cultivar BRS Terra-Anã, a short-stature plantain (2.83 m) developed by Embrapa, exhibits resistance to Yellow Sigatoka and *Fusarium* wilt and is recommended for cultivation in Mato Grosso and Vale do Ribeira (SP), Brazil. This study aimed to evaluate the efficiency of different benzyladenine (BA) concentrations on in vitro multiplication of shoot tips of cv. BRS Terra-Anã and to monitor genetic stability over eight successive subcultures. Shoot tips were established on MS medium supplemented with BA and subcultured every 30 days for 240 days. Multiplication rate was assessed by the average number of shoots per explant. Genetic stability was monitored by flow cytometry for ploidy level determination and by stomatal morphometric analysis on adaxial and abaxial leaf surfaces. The results demonstrated that 1.0 mg L^{-1} BA was the most efficient concentration, balancing high productivity and economic viability. Cytometric analysis revealed that most plants maintained the characteristic triploid pattern (3x); however, mixoploidy was detected in some individuals subjected to prolonged subculture cycles, indicating that prolonged BA exposure may induce mitotic instability. Mixoploid plants showed approximately 15% reduction in stomatal density on the abaxial surface and increased guard cell dimensions, confirming the relationship between ploidy level and stomatal characteristics. It is recommended to perform 3 to 4 subcultures to optimize productivity without compromising genetic stability. The protocol provides a reliable basis for plantlet production laboratories, enabling efficient and economically viable large-scale multiplication of cv. BRS Terra-Anã.

Keywords: Benzyladenine; Flow cytometry; *Musa spp*.; Shoot tips; Somaclonal variation; Stomata.

INTRODUCTION

The banana plant (*Musa spp*.) has high socioeconomic and alimentary importance, especially in tropical and subtropical regions. In Brazil, production reached approximately 7.04 million tons in 2024, with an estimated value of R$ 16.06 billion, highlighting the relevance of this crop to the national agricultural economy (IBGE 2024). In this context, the cultivar BRS Terra-Anã, released by the Brazilian Agricultural Research Corporation (Embrapa) in 2022, represents a strategic advance for the plantain segment (*Musa* AAB), standing out for its reduced size, greater ease of management, and resistance to yellow Sigatoka and *Fusarium* wilt race 1 (Embrapa 2022).

The commercial consolidation of this cultivar depends on the availability of seedlings with high phytosanitary quality and genetic fidelity. Conventional propagation via rhizomes presents limitations regarding multiplication rate and the risk of dissemination of systemic pathogens. In this context, plant tissue culture constitutes an important biotechnological tool, with micropropagation being the most widely used technique for large-scale clonal production of *Musa spp*. (Erol et al. 2023).

However, the efficiency and safety of the micropropagation process are directly associated with the management of growth regulators. Benzyladenine (BA), a cytokinin widely used in the multiplication phase, promotes the breaking of apical dominance and stimulates the proliferation of axillary buds. Despite its effectiveness in inducing multiple shoots, high concentrations and successive subculture cycles may increase the frequency of somaclonal variation, compromising the genetic uniformity of regenerated plants (Shirani Bidabadi et al. 2010; Venkatachalam et al. 2007).

Somaclonal variation may manifest through changes in nuclear DNA content, including events of aneuploidy and polyploidy, with potential impacts on agronomic performance and phenotypic stability of seedlings (Mohan Jain and Swennen 2004). Thus, monitoring genetic stability through flow cytometry has been widely used as a rapid and reliable tool to assess clonal fidelity in micropropagated plants (Erol et al. 2023). Additionally, stomatal morphometric parameters may act as complementary indicators of ploidy alterations.

Although there are established protocols for micropropagation of *Musa spp*., specific information on the morphogenic response and genetic stability of the BRS Terra-Anã cultivar under different BA concentrations over multiple subculture cycles is still scarce. The lack of such data limits the definition of strategies that reconcile high multiplication rates with the maintenance of genomic integrity.

Therefore, the present study aimed to: (I) evaluate the efficiency of different BA concentrations in the in vitro multiplication of cv. BRS Terra-Anã; (II) determine the multiplication rate over eight consecutive subculture cycles; (III) monitor genetic stability by flow cytometry; and (IV) correlate changes in nuclear DNA content with stomatal morphometric parameters.

MATERIAL AND METHODS

Plant material

Banana explants of the cv. BRS Terra-Anã (*Musa spp*., AAB genome) were used as plant material, obtained from mother plants maintained in the germplasm collection of EMPAER – Tangará da Serra – MT. The mother plants were previously identified and characterized regarding phytosanitary condition and morphological conformity with the cultivar standard.

For in vitro establishment, rhizomes from healthy adult plants were collected, from which shoot apices (sword suckers) approximately 15 to 30 cm in length were excised after removal of the external leaf sheaths. Alternatively, explants from plants already established in vitro, maintained in multiplication medium, were used.

Disinfestation and in vitro establishment

Rhizomes containing the shoot apices were initially washed under running water to remove soil residues and organic matter. Subsequently, they were subjected to surface disinfestation in a laminar flow chamber.

The procedure consisted of immersing the material in a 2% (v/v) neutral detergent solution for 10 minutes under agitation, followed by three rinses in sterile distilled water. Then, the explants were immersed in a 60% calcium hypochlorite [$Ca(ClO)_2$] solution for 20 minutes, and subsequently rinsed again three times in sterile distilled water. Next, immersion in 70% (v/v) ethanol solution for 5 minutes was performed, completing the process with three rinses in sterile distilled water.

After disinfestation, the shoot apices were isolated using a sterile scalpel and forceps, reduced to approximately 4 cm in height, and individually inoculated into 250 mL glass flasks containing 80 mL of culture medium (Fig. 1).



Fig. 1 Stages of apex preparation. (A) Sword sucker-type shoots. Bar: 10 cm. (B) Apex reduction after washing. (C) Reduced apices during the disinfestation process. Bar: 5 cm. (D) Reduced apex inoculated in MS medium. Bar: 4 cm.

The culture medium contained MS basal salts (Calsson Labs, Smithfield, UT, USA) (Murashige; Skoog, 1962) and White (1943) vitamins, supplemented with 30 g L^{-1} sucrose, 100 mg L^{-1} inositol (Acumedia®; Neogen, Lansing, MI, USA), and solidified with 2.8 g L^{-1} Phytagel (Sigma Aldrich®). The culture medium was supplemented with plant growth regulators, such as benzyladenine (Sigma Aldrich®), at concentrations of 1.0, 2.0, and 3.0 mg L^{-1}, with pH adjusted to 5.7 ± 0.1 prior to autoclaving. The medium was sterilized at 121 °C (1 atm) for 15 minutes, after which the explants were cultured.

Cultures were transferred to a growth room with a 16-hour photoperiod and irradiance of 36 µmol m^{-2} s^{-1} (Daylight Special Light, 20 W, Osram, Barueri, Brazil), at 25 ± 2 ºC.

In vitro multiplication using subculture cycles

After in vitro establishment (approximately 30 days of cultivation), explants that showed shoot formation were subcultured into culture medium. Subcultures were performed every 30 days over a total period of eight subcultures (240 days) to evaluate the multiplication rate and the stability of the morphogenetic response over time.

In the first subculture, the apices were inoculated whole, without sectioning. From the second, third, and fourth subcultures onward, the explants were longitudinally sectioned into four parts to increase the multiplication rate.

The variable analyzed was the mean number of shoots per explant, used as an indicator of the efficiency of in vitro multiplication over the subcultures. At each subculture, shoots were individualized and transferred to new 250 mL glass flasks containing 80 mL of culture medium.

Evaluation of genetic stability in each multiplication cycle

At each subculture cycle, shoots approximately 10 cm in length were isolated and inoculated into culture medium in the absence of BA for complete development. Three expanded leaves of cv. BRS Terra-Anã were collected from each treatment per cycle and isolated to evaluate genetic stability through estimation of nuclear DNA content.

Nuclei suspensions were prepared by chopping young leaves with a disposable steel blade (Galbraith et al. 1983) in WPB isolation buffer (Loureiro et al. 2007). The resulting suspension was aspirated through two layers of gauze using a plastic pipette and filtered through a 30 µm Nylon CellTrics® membrane to remove cell and tissue fragments. Subsequently, 25 µL (1 mg mL^{-1}) of propidium iodide and 5 µL (1 mg mL^{-1}) of RNase were added for DNA staining.

After 30 minutes in a light-protected environment, the samples were analyzed in a flow cytometer. Three replicates were performed, and at least 10,000 nuclei were analyzed for fluorescence emission quantification using a CytoFLEX flow cytometer (Beckman Coulter, CA, USA). As an internal standard, the species *Pisum sativum* with DNA content 2C = 9.09 pg was used (Dolezel et al. 1998). Histograms were generated and analyzed using CytExpert 2.0.1 software, and DNA content (pg) was calculated according to Dolezel and Bartos (2005) and Dolezel et al. (2007).

2C DNA content (pg):

$$\frac{\text{Mean G1 peak of } \textit{Musa spp}\text{. cv. BRS Terra-Anã} \times 9.09}{\text{Mean G1 peak of } \textit{Pisum sativum}}$$

Cytogenetic analysis

The chromosome number of regenerated plants of cv. BRS Terra-Anã was evaluated through DNA content analysis, allowing the identification of plants with ploidy corresponding to the cultivar.

Root tips approximately 1 to 2 cm in length were collected from confirmed mixoploid plants from cycles 4, 5, and 6, and from triploid plants (control), in the morning period. Subsequently, samples were pretreated with 0.003 M 8-hydroxyquinoline (HQ) solution for 7 hours at 4 °C.

After pretreatment, the material was fixed in methanol solution (methyl alcohol: acetic acid, 3:1 v/v) for 24 hours and stored under refrigeration until slide preparation. The roots were then washed in distilled water for 15 minutes and subjected to hydrolysis in 1 M HCl at 60 °C for 20 minutes.

Next, the meristematic apices were squashed between slide and coverslip for cell dispersion, followed by the freeze-crack technique in liquid nitrogen (−196 °C) (Conger; Fairchild, 1953), and air-dried. The slides were stained with 5% Giemsa for 10 minutes and subsequently observed under a light microscope.

Chromosome counting was performed in individualized cells, preferably at metaphase. The chromosome number was determined based on direct counting of visible chromosomes, and representative images were selected for documentation and plate preparation.

Plant acclimatization

Plants with developed roots and shoots, approximately 10 cm in height, were removed from the flasks and washed under running water to remove the culture medium. Then, they were submerged in water and maintained for a period of 24 hours, after which they were planted in plastic cups (11 cm × 8 cm) with holes, containing Carolina Soil® substrate (Fig. 2)

After 5 days maintained under controlled conditions (25 ± 2 ºC), the plants were transferred to a greenhouse, where they remained under cultivation and continuous monitoring (Fig. 2).

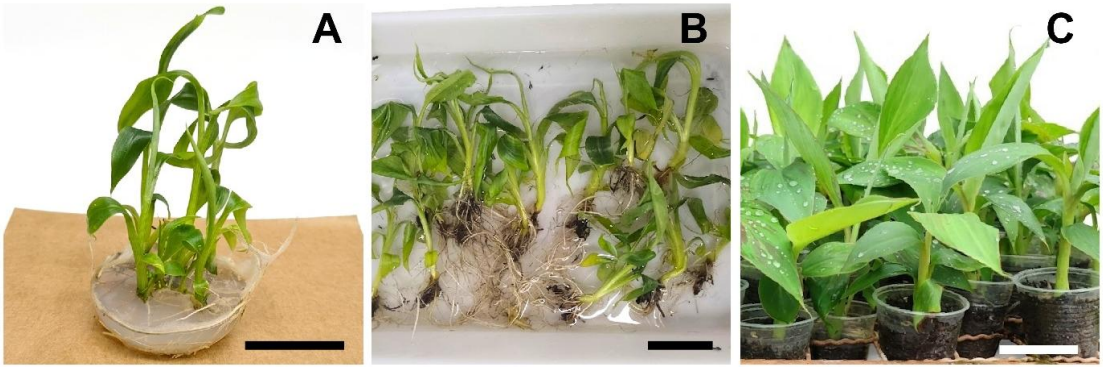


Fig. 2 Stages of acclimatization. (A) Shoots of cv. BRS Terra-Anã removed from the flask for standardized selection; (B) Plants submerged in water in a tray; (C) Plants in substrate under greenhouse conditions. Bar: 6 cm.

Variant plants and control individuals were maintained under cultivation in order to monitor vegetative development and evaluate morphophysiological stability during the ex vitro growth phase. As some individuals reached approximately 20 cm in height, showing adequate size and good vegetative vigor, they were distributed to smallholder farmers as a form of extension and technology transfer, while part of the material was kept under cultivation for continuity of experimental evaluations.

Stomatal characterization

The analyzed leaves were collected from plants previously acclimatized in a greenhouse under a controlled irrigation system. Triploid (2n = 3x) and mixoploid (2n = 3x/6x) individuals were sampled, previously confirmed for ploidy level.

Epidermal impressions were obtained from the median regions of the adaxial and abaxial surfaces of the leaf blade. For impression preparation, a cyanoacrylate-based instant adhesive (Super Bonder®) was used. A drop of adhesive was placed on a glass slide and immediately pressed against the selected leaf surface for approximately 1 minute. After complete removal of the leaf, the material was air-dried for 5 minutes before observation under a light microscope.

For stomatal density determination, three leaves per ploidy level were analyzed, selecting five fields of view per leaf surface in the median and central regions of the blade, totaling 15 observations per ploidy level. Counts were performed under a light microscope with a 10× objective.

Measurement of stomatal length and width, as well as guard cell dimensions, was performed under a light microscope with a 40× objective. Three leaves per ploidy level were evaluated, with five measurements per leaf, totaling 15 measurements per ploidy level. Biometric analyses were conducted using ImageJ software (National Institutes of Health, USA).

Experimental design

The experiment was conducted in a completely randomized design (CRD), consisting of four treatments corresponding to BA concentrations (1.0, 2.0, and 3.0 mg L^{-1}) and a control treatment (absence of BA). Each

treatment consisted of 13 replicates, with each replicate represented by one flask containing four explants, totaling 52 experimental units and 208 explants.

Experimental data were subjected to analysis of variance (ANOVA) and to generalized linear mixed models (GLMM) and Zero-Inflated Negative Binomial (ZINB) models, appropriate for count data with high overdispersion and excess zeros. The ZINB model allowed distinguishing structural zeros from sampling zeros, providing more robust estimates by considering the fixed effects of treatments and time, and the random effect of replicates. When significance was detected, means were compared using Tukey's test at a 5% probability level.

Analyses were performed using RStudio® software (Posit Software, PBC, 2024).

RESULTS

Consecutive cycles of in vitro regeneration of *Musa spp.* cv. BRS Terra-Anã from shoot apices are genetically stable

Shoot apices of cv. BRS Terra-Anã cultured in the presence of different concentrations of benzyladenine (BA) showed significant variations in shoot production over eight consecutive regeneration cycles. Initially, swelling of the basal region of the apices was observed with evident cell proliferation, and after 11 days of cultivation, small shoots became visible (Fig. 3).

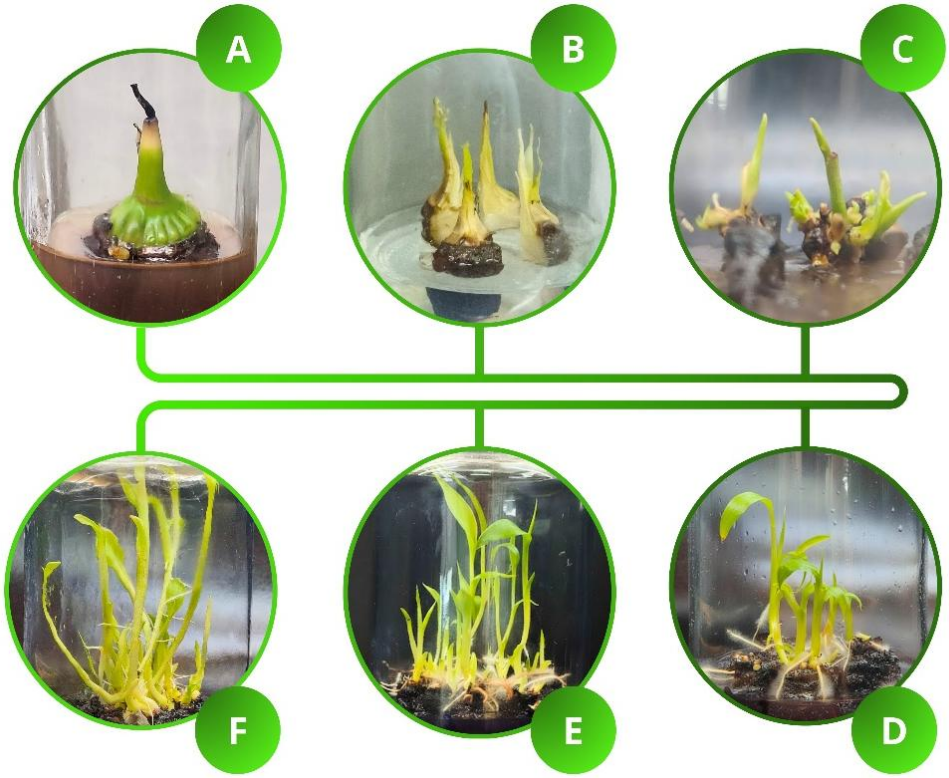


Fig. 3 Sequence of in vitro multiplication of *Musa spp*. cv. BRS Terra-Anã explants cultured in MS medium supplemented with BA. (A–F) Successive stages of development: (A) shoot apex established in MS culture medium; (B) onset of shoot emergence; (C) formation of multiple adventitious shoots; (D) shoot elongation and emergence of the first leaf primordia; (E) elongated shoots at different developmental stages; (F) plantlets with fully developed shoot and root systems.

Effect of BA concentrations on multiplication rate

Comparative analysis of means by Tukey's test ($p < 0.05$) revealed that the concentration of 1.0 mg L^{-1} BA was the most effective, producing an average of 1.20 shoots per explant over the eight cycles. This result was statistically superior to all other treatments tested (Fig. 4). The concentration of 3.0 mg L^{-1} also showed satisfactory

performance, with an average of 1.02 shoots per explant, being significantly superior to the control group (0.37 shoots per explant) and the treatment with 2.0 mg L^{-1} (0.38 shoots per explant), which did not differ statistically from each other.

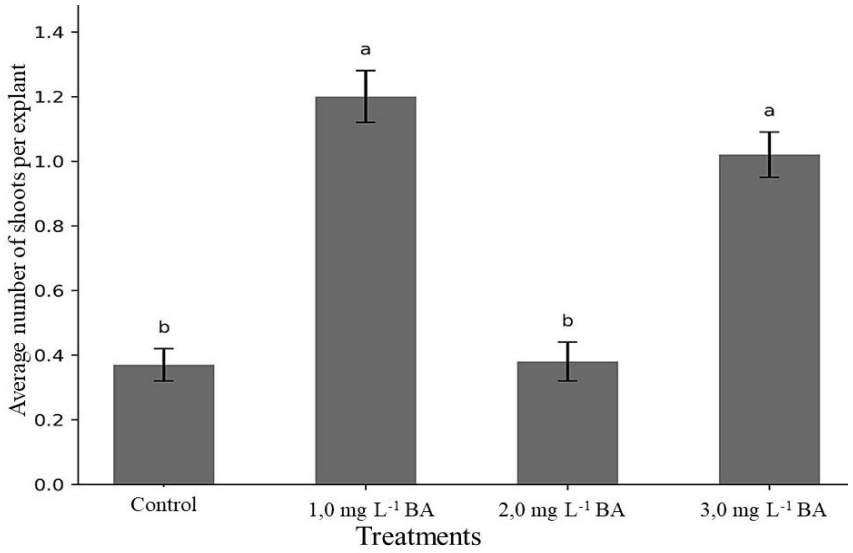


Fig. 4 Mean number of shoots per explant of *Musa spp*. cv. BRS Terra-Anã at different BA concentrations over eight consecutive in vitro multiplication cycles. Error bars indicate standard error. Letters indicate statistical differences among treatments per cycle according to Tukey's test at 5%.

Temporal dynamics of shoot production over cycles

In the first subculture cycle, in which explants derived from shoot apices were not sectioned, no shoot formation was observed. From the second subculture cycle onward, shoot proliferation began in all treatments, demonstrating that explant sectioning is a critical factor for the induction of adventitious organogenesis in *Musa spp*. (Fig. 5).

In cycle 2, treatments containing 1.0, 2.0, and 3.0 mg L^{-1} BA produced 140, 78, and 106 shoots, respectively, while the control treatment produced 68 shoots. In cycles 3 and 4, a significant increase in shoot number was observed in all treatments, with a production peak in cycle 4. The concentrations of 1.0 and 3.0 mg L^{-1} stood out, producing 150 and 126 shoots in cycle 3, and 186 and 194 shoots in cycle 4, respectively. The control treatment showed an intermediate response in cycle 4, with 106 shoots, while the concentration of 2.0 mg L^{-1} BA resulted in lower shoot production, with 82 shoots (Fig. 5).

From the fifth cultivation cycle onward, a marked reduction in shoot number was observed in all treatments (Fig. 5). The reduction was more evident in the treatment containing 2.0 mg L^{-1} BA, which showed only 13 shoots in cycle 5 and a complete absence of shoot formation in subsequent cycles.

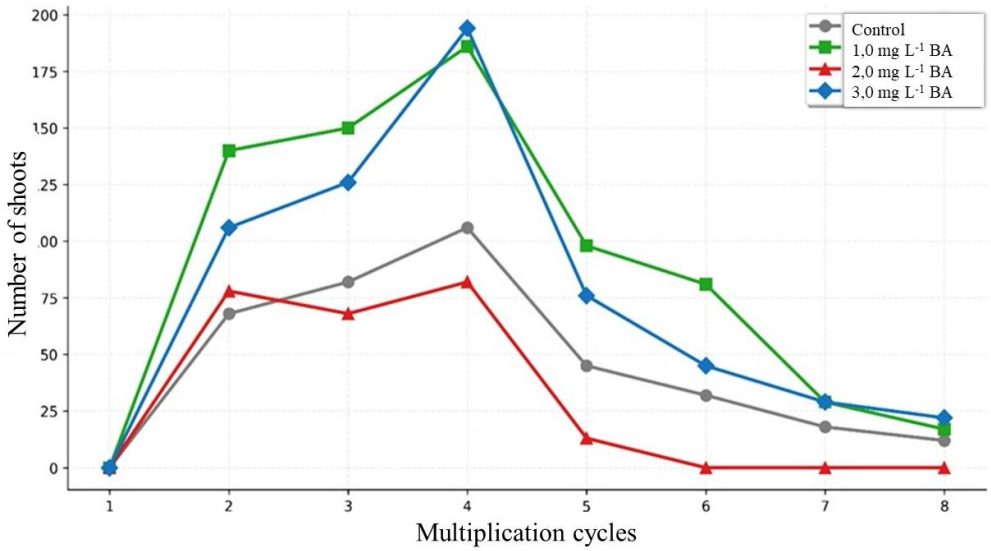


Fig. 5 Evolution of shoot production over eight consecutive in vitro multiplication cycles of *Musa spp*. cv. BRS Terra-Anã at different BA concentrations. Data represent the total number of shoots produced in each cycle for each treatment.

In contrast, the concentrations of 1.0 and 3.0 mg L^{-1} BA maintained shoot production up to cycle 8, although with a progressive reduction in relative production after the peak observed in cycle 4. In subsequent cycles, a gradual decline in regenerative capacity was observed, resulting in residual shoot production in the final multiplication cycles (Fig. 6).

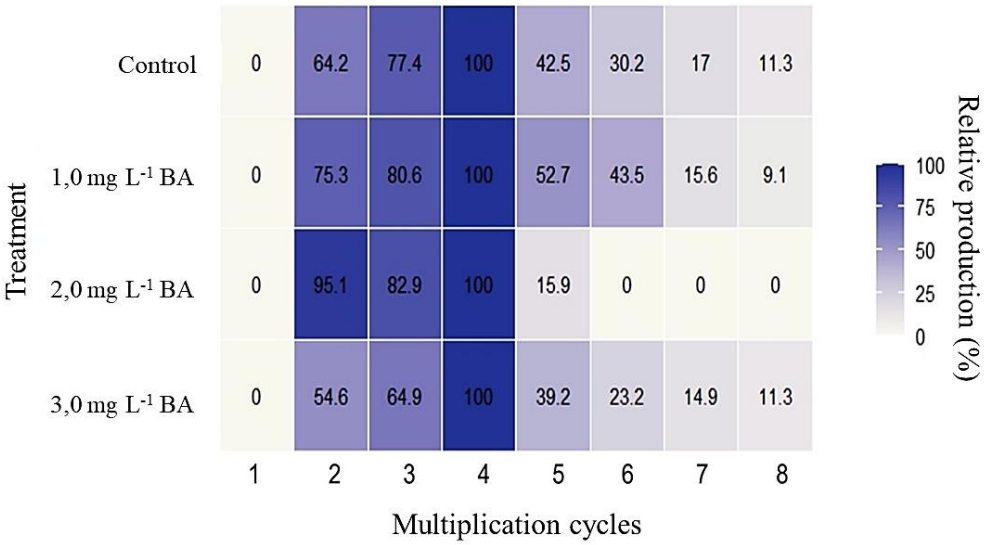


Fig. 6 Heatmap of relative shoot production (%) per cycle and treatment in *Musa spp*. cv. BRS Terra-Anã. Values were normalized relative to the maximum value observed in each treatment, allowing visualization of the dynamics of regenerative capacity over subcultures.

Accumulated production and multiplication efficiency

Quantification of accumulated production over the eight cycles confirms the pattern observed in the temporal analysis (Fig. 7). The treatment with 1.0 mg L^{-1} BA showed the highest total production, reaching 666 shoots, followed by 3.0 mg L^{-1} BA with 598 shoots. The control treatment produced 363 shoots, while 2.0 mg L^{-1} BA resulted in only 241 shoots over the entire experimental period.

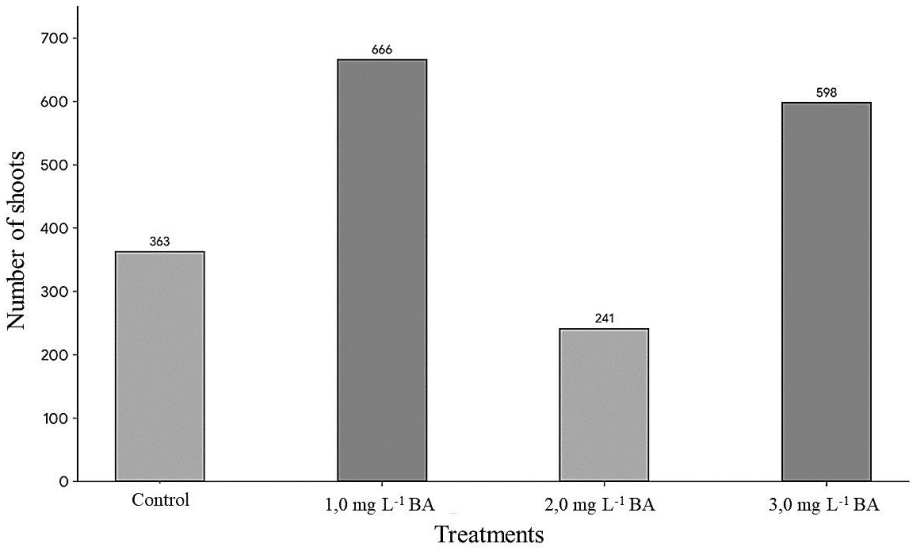


Fig. 7 Accumulated shoot production over eight in vitro multiplication cycles of *Musa spp*. cv. BRS Terra-Anã at different BA concentrations. Values represent the total number of shoots produced in each treatment throughout the experimental period.

Implications for subculture management and risk of somaclonal variation

The marked decline in regenerative capacity observed from the fifth cycle onward raises important questions regarding the ideal number of consecutive subcultures in commercial micropropagation protocols.

In the present study, the maintenance of regenerative capacity up to the eighth cycle in treatments with 1.0 and 3.0 mg L^{-1} BA, although with decreasing productivity, suggests that cv. BRS Terra-Anã presents relative genetic stability under these conditions. However, considering the production peak in cycle 4 and the subsequent decline, it is recommended that commercial protocols limit the number of consecutive subcultures to 3–4 cycles, periodically establishing new cultures from shoot apices of certified mother plants.

Successive cycles of in vitro regeneration of *Musa spp*. cv. BRS Terra-Anã show reduced genetic instability

The genomic stability of *Musa spp*. cv. BRS Terra-Anã plants regenerated after eight cycles of in vitro multiplication showed reduced genetic instability, as demonstrated by the evaluation of nuclear DNA content estimated via flow cytometry.

Fluorescence histograms revealed, in most samples, a unimodal peak of fluorescence intensity, consistent with the cytometric profile expected for triploid cv. BRS Terra-Anã plants (2n = 3x = 33 chromosomes). This observation confirms the preservation of genetic stability throughout successive in vitro proliferation cycles, with no evidence of significant aneuploidy or polyploidy (Fig. 8). Among the 72 evaluated plants, four (5.56%) showed variations in ploidy pattern and were classified as somaclonal variants.

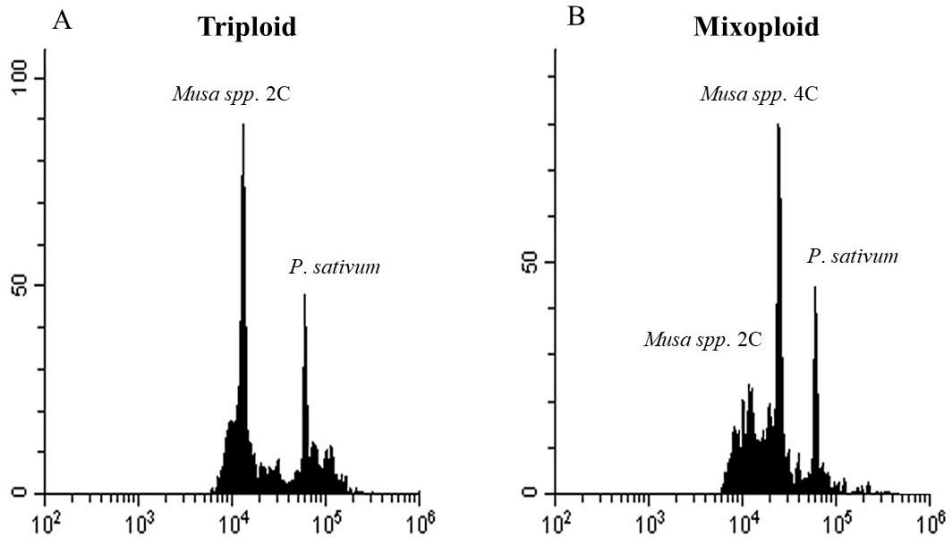


Fig. 8 Flow cytometry histograms comparing triploid and mixoploid plants of *Musa spp*. cv. BRS Terra-Anã. (A) Triploid plant (3x) with a single peak at 2C; (B) Mixoploid plant (3x/6x) showing two distinct peaks corresponding to triploid (2C) and hexaploid (4C) cell populations. The reference standard Pisum sativum is indicated in both histograms.

However, in a subset of samples, two distinct fluorescence peaks were detected, indicative of mixoploidy, characterized by the coexistence of triploid and hexaploid cell populations (Fig. 8B). This alteration in nuclear DNA content was more prevalent in treatments exposed to 2.0 mg L^{-1} BA, while the control and other concentrations predominantly exhibited the triploid pattern.

The relative DNA content in triploid samples ranged from 12.6 to 15.4 pg, with a coefficient of variation of 3.67%, consistent with the standard genotype values. In contrast, mixoploid samples showed ranges from 26.8 to 29.5 pg and a coefficient of variation of 6.86%, reflecting the proportion of hexaploid cells (2n = 6x = 66 chromosomes) (Fig. 9).

Peak	Sample	DNA Content (pg)	CV (%)
1	Triploid	12,6	3.67%
2	Mixoploids	26,8	6.86%
3	*P. sativum*	9.09	6.22%

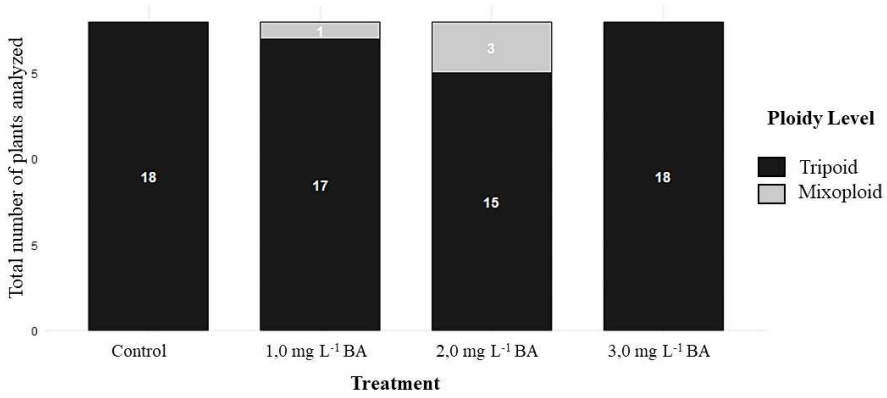


Fig. 9 Ploidy level based on nuclear DNA analysis in samples of *Musa spp*. cv. BRS Terra-Anã. Total number of plants analyzed by flow cytometry.

Triploid plants (2n = 3x) showed 33 chromosomes (Fig. 10A), while mixoploid plants showed cells (2n = 3x or 6x) with 40 chromosomes (Fig. 10B).

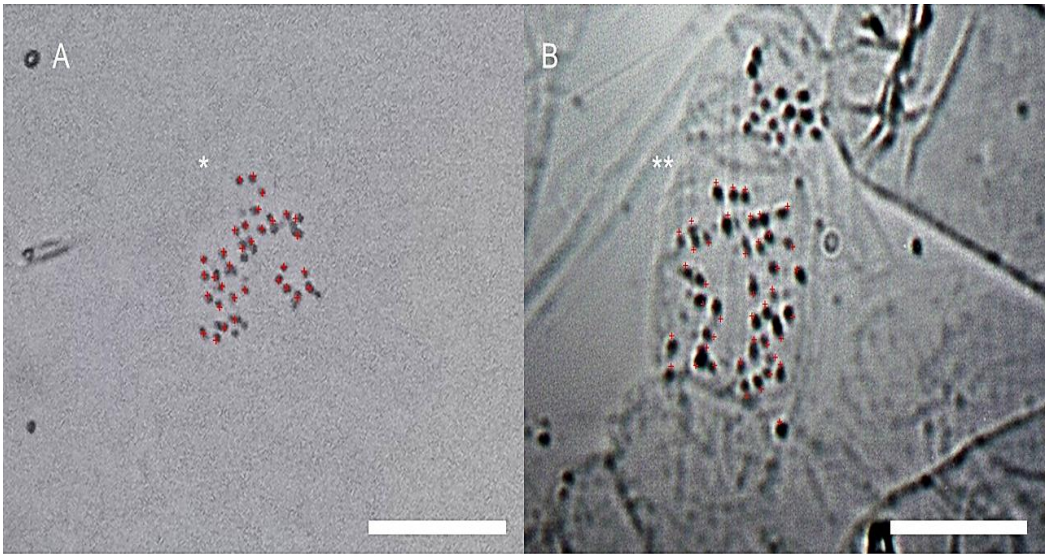


Fig. 10 Chromosomal arrangement in metaphases of *Musa spp*. cv. BRS Terra-Anã. (A)* triploid individual (2n = 3x = 33) and (B)** mixoploid individuals showing ploidy variation between 2n = 3x = 33 and 2n = 6x = 66. Bar: 40 µm.

Acclimatization and stomatal characterization

After acclimatization in substrate at each cycle with confirmed ploidy level, and maintenance under controlled greenhouse conditions, plants of cv. BRS Terra-Anã showed uniform growth, as expected for plants derived from a micropropagation system (Fig. 11).

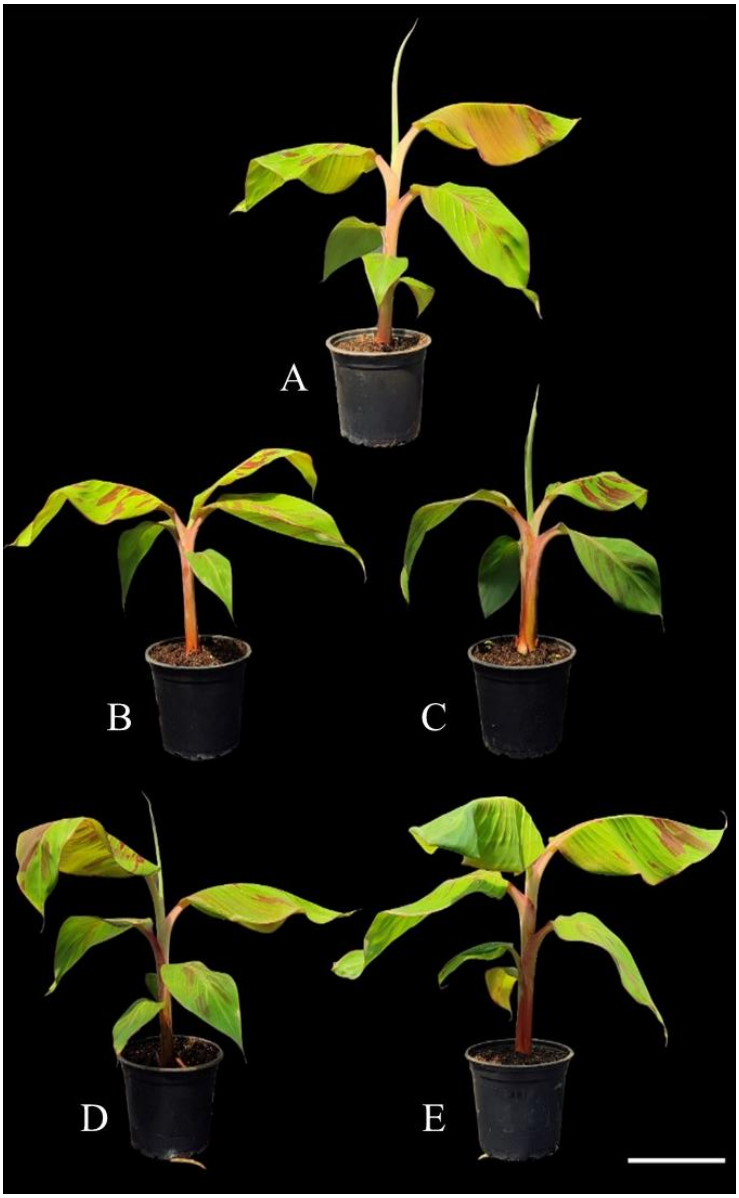


Fig. 11 Acclimatized plants of *Musa spp*. cv. BRS Terra-Anã, illustrating triploid and mixoploid individuals derived from different in vitro regeneration cycles. (A) Triploid plant (control); (B) mixoploid from cycle 4; (C) mixoploid from cycle 5; (D–E) mixoploids from cycle 6, maintained in a greenhouse. Bar: 15 cm.

Despite homogeneous vegetative development, differences in stomatal morphology were observed between triploid and mixoploid plants grown ex vitro, especially regarding stomatal density and width, as evidenced both by images (Fig. 12 A–B) and quantitative analyses. The highest stomatal density was observed in triploid plants on the abaxial surface, with a mean of 100.2 stomata mm^{-2}, followed by mixoploid plants on the abaxial surface, with 85.4 stomata mm^{-2}. Lower values were recorded on the adaxial surface, with 29.8 stomata mm^{-2} in triploid plants and 19.8 stomata mm^{-2} in mixoploid plants, with all comparisons being statistically different (Fig. 12C).

Regarding stomatal morphometry, stomatal width differed significantly between ploidy levels and leaf surfaces (Fig. 12D), being greater in mixoploid plants on the abaxial surface (54.2 µm) and lower in triploid plants on the adaxial surface (46.9 µm), while mixoploid plants on the adaxial surface and triploid plants on the abaxial surface showed intermediate values (50 and 46 µm, respectively).

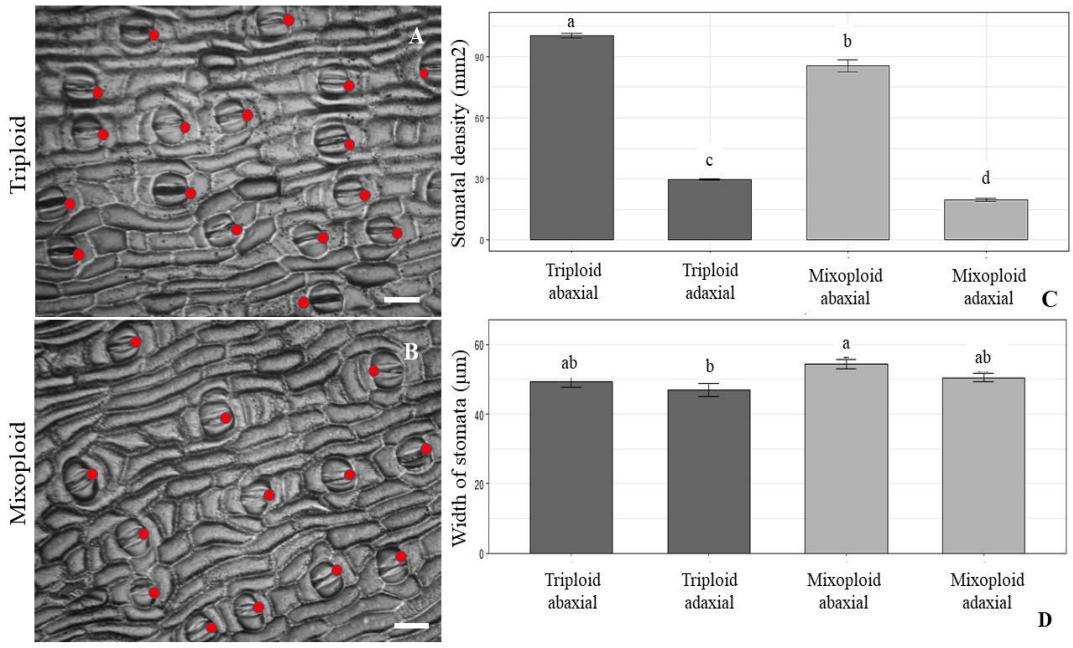


Fig. 12 Stomatal characterization of triploid and mixoploid plants of *Musa spp*. cv. BRS Terra-Anã grown in a greenhouse. (A) Triploid leaf epidermis, abaxial surface. (B) Mixoploid leaf epidermis, abaxial surface. Bars: 40 µm. (C) Stomatal density (mm²). (D) Stomatal width (µm). Bars indicate standard error; Red point indicate stomata; Different letters within each variable differ according to Tukey's test at 5%.

In contrast, stomatal length did not show significant differences among groups, ranging from 46.1 to 49.0 µm. There was no significant effect of treatments on stomatal area, stomatal length, or guard cell dimensions. Stomatal area ranged from 1884 to 2038 µm², stomatal length from 46.1 to 49.0 µm, and guard cells showed length between 38.5 and 40.0 µm and width between 17.8 and 19.8 µm.

Analyses regarding stomatal density and morphometry between triploid and mixoploid plants of cv. BRS Terra-Anã were limited to stomatal density and stomatal width.

DISCUSSION

Explant cutting is a fundamental step in the in vitro culture system, aiming to break the dominance of the apical meristem of the explant, a process directly reflected in plant physiology. It interrupts the dominance and activity of auxins present in the apex, allowing cytokinins to act directly on the proliferation of lateral buds and subsequently adventitious shoots (Sosnowski; Truba and Vasileva 2023; Suman 2017).

Variations in growth regulator concentrations showed significant responses in the number of shoots produced, with emphasis on the concentration of 1.0 mg L^{-1} BA, which promoted the highest multiplication rate over the eight subcultures. In a study conducted by Hasmayaputra, Asnawati and Arifin (2024), culture medium supplemented with 1.5 mg L^{-1} BA yielded an average of 12.0 shoots of *Musa spp*. Cavendish, highlighting the absence of 1.0 mg L^{-1} BA among the treatments. Tirkey (2021), in turn, used a combination of 1.0 mg L^{-1} BA with 50 mg L^{-1} adenine sulfate, resulting in an average of 5.5 shoots per explant of *Rauvolfia serpentina* (L.). However, it is observed that the concentration of 1.0 mg L^{-1} BA shows high responsiveness for shoot induction and multiplication in different cultivars of *Musa spp*. and in other species, demonstrating its strong regenerative potential. The accumulated multiplication rate obtained at this concentration (666 shoots from the initial number of explants) demonstrates the technical and economic feasibility of the protocol for commercial seedling production. Considering that each regenerated shoot can be used as a new source of explants in successive

subcultures, the exponential multiplication capacity of the system becomes substantial, reinforcing its applicability in large-scale micropropagation programs.

The concentration of 3.0 mg L^{-1} BA did not differ statistically from 1.0 mg L^{-1}, as also demonstrated in studies by Naitchede et al. (2025) and Nugrahani et al. (2024). However, from an economic and practical perspective, the concentration of 1.0 mg L^{-1} BA is more feasible and recommended for *Musa spp*. cv. BRS Terra-Anã, as it provides a higher rate of shoot proliferation and regeneration with lower cytokinin usage. This greater resilience suggests that these concentrations provide a more adequate hormonal balance for maintaining regenerative capacity over multiple subcultures.

The differences observed among studies also reflect the influence of genotype, as *Musa spp*. cultivars exhibit variable sensitivity to cytokinins. In addition, factors such as culture medium composition, addition of growth regulators, and the physiological age of explants contribute to these experimental variations (Šimoníková et al. 2022; Martanti et al. 2022).

The successive subculture technique constitutes a fundamental step in micropropagation, as it allows the serial multiplication of shoots from an initial explant (Chukwu et al. 2025; Mendonça et al. 2020), maintaining plant material metabolically active and suitable for new cultivation cycles (Jhurree-Dussoruth 2022). The production peak observed in the fourth cycle may be associated with the establishment of a physiological balance between the proliferative competence of explants and the physicochemical conditions of the culture medium, favoring maximum expression of morphogenic potential.

However, as observed, under in vitro culture conditions, especially under prolonged exposure to high cytokinin concentrations and successive subculture cycles, this hormonal stimulus may artificially intensify cell division, favoring the occurrence of failures in coordination between nuclear replication and cytokinesis (Yang et al. 2021). As a consequence, cytogenetic alterations may arise, such as endoreduplication, inducing somaclonal, epigenetic, and physiological variation, affecting genetic stability and, in some cases, the morphology of regenerated plants (Oliveira et al. 2022; Krishna et al. 2016).

Although often associated with morphological abnormalities and reduced vigor, somaclonal variation also constitutes an important source of genetic variability and may be exploited as a tool for plant breeding (Das et al. 2025). When controlled and properly characterized, this instability may contribute to obtaining cultivars with greater resistance to biotic and abiotic stresses and improved agronomic performance (Deepthi 2018; Nwauzoma; Jaja 2013). In this context, the formation of mixoploid individuals from triploid plants, observed in subcultures supplemented with BA, indicates the occurrence of cytogenetic instability induced by in vitro conditions, such as exposure to multiple subculture cycles, prolonged cytokinin exposure, and accumulated physiological stress such as oxidation (Sianipar et al. 2024; Ermini et al. 2021).

Flow cytometry constituted a fundamental step for estimating nuclear DNA content of the evaluated plants, being one of the fastest, most precise, and most sensitive methodologies for detecting variations in ploidy level and possible structural genomic alterations (Ho; Eng and Ling 2024).

The quantification of nuclei for DNA estimation was performed based on the relative fluorescence intensity of stained nuclei, using *Pisum sativum* as an internal reference standard. Histogram analysis revealed that most individuals presented a main peak compatible with the expected triploid level, with consistent and well-defined values, indicating cytogenetic stability. However, in certain samples, the presence of two distinct peaks in the histogram was observed, corresponding to triploid and near-hexaploid levels within the same individual,

characterizing the occurrence of mixoploidy and evidencing the coexistence of cell populations with different ploidy levels. These results demonstrate the efficiency of flow cytometry as a tool for monitoring genetic stability.

After preliminary screening by flow cytometry, variant and control individuals were subjected to cytogenetic confirmation through direct chromosome counting at metaphase using root meristems (Souza et al. 2023; Guimarães et al. 2009). Analyses confirmed that most cells presented 2n = 3x = 33 chromosomes, consistent with the expected triploid pattern for the Terra subgroup (AAB) (Dehury; Dehery and Das 2021; Nwauzoma et al. 2002). However, in certain individuals, the coexistence of cells with 33 and 66 chromosomes (2n = 6x) was observed, characterizing mixoploidy.

The occurrence of mixoploids suggests duplication events possibly associated with failures in chromosome segregation or the occurrence of endoreduplication. The incidence of these alterations was predominantly observed in individuals subjected to successive subculture cycles under BA exposure, indicating that prolonged cytokinin stimulus may act as an inducing factor of mitotic instability (Ferreira et al. 2023).

Although most plants maintained the characteristic triploid condition, the detection of mixoploidy demonstrates that the micropropagation protocol of cv. BRS Terra-Anã, under successive subculture cycles and prolonged BA exposure, may trigger alterations in ploidy level.

The occurrence of stomata on both adaxial and abaxial surfaces confirms the amphistomatic pattern, with predominance on the abaxial surface, as reported for different banana cultivars (Morais et al. 2023; Nyainleta; Pesik and Hiariej 2022; Costa et al. 2009). This anatomical arrangement is associated with optimization of gas exchange and reduction of water loss under edaphoclimatic conditions.

Comparison between ploidy levels revealed that mixoploid individuals showed a consistent reduction in stomatal density, accompanied by an increase in guard cell dimensions. The approximate 15% decrease in stomatal density on the abaxial surface suggests that changes in nuclear content directly interfere with the organization of the leaf epidermis. This pattern is consistent with the relationship described between ploidy and stomatal density, in which increased nuclear DNA promotes cell expansion, resulting in larger and fewer stomata (Vandenhout et al. 1995).

In general, the results indicate that ploidy in cv. BRS Terra-Anã mainly influences stomatal density, while characteristics related to stomatal morphometry (length, area, and guard cell dimensions) remain relatively stable among the evaluated groups.

The results demonstrate that micropropagation protocols must be adjusted according to the experimental system, with the choice of cytokinin, its concentration, and the number of cycles being decisive for efficiency and seedling quality. For the use of shoot apices, the concentration of 1.0 mg L^{-1} BA stood out by combining high multiplication efficiency and economic viability, since increasing concentrations did not provide greater productivity and increased physiological risks such as somaclonal variation. Furthermore, conducting the protocol between three and five subculture cycles constitutes a technically safe interval to maintain high productivity without significantly increasing the risk of somaclonal variation. Thus, the proposed protocol integrates technical efficiency, rational use of inputs, and control of genetic stability, presenting potential commercial applicability and direct contribution to the sustainable strengthening of banana production.

CONCLUSION

Among the evaluated benzyladenine (BA) concentrations, 1.0 mg L^{-1} stood out as the most responsive for in vitro multiplication of cv. BRS Terra-Anã, promoting a higher shoot proliferation rate combined with the

technical and economic viability of the protocol. The multiplication rate over eight consecutive subculture cycles showed variations among cycles, with a production peak observed between the third and fourth subcultures, indicating that limiting to three or four consecutive subcultures may optimize productivity while reducing prolonged exposure to the growth regulator and, consequently, the risk of cytogenetic alterations.

Monitoring genetic stability through flow cytometry revealed the occurrence of variations in ploidy level, including mixoploidy events, demonstrating that successive subcultures under continuous BA exposure may be associated with the induction of cytogenetic alterations. These findings reinforce the need for periodic control of genetic quality in commercial micropropagation programs.

Additionally, the correlation between ploidy and stomatal morphometric parameters confirmed the classical association between increased ploidy level and enlargement of stomatal dimensions, especially width, accompanied by a reduction in stomatal density. This relationship demonstrates that leaf anatomical analysis constitutes an efficient complementary tool for preliminary screening of cytogenetic variations detected by flow cytometry.

Thus, within the context of the adopted experimental conditions, an efficient micropropagation protocol is proposed for cv. BRS Terra-Anã, defining the most responsive BA concentration and the recommended number of subcultures, combined with the implementation of systematic cytogenetic monitoring to ensure the genetic quality of seedlings produced on a large scale.

DECLARATIONS

Conflict of interest The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript. There are no financial or personal relationships by the authors that could in-appropriately influence the work presented

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