



# Multiple shoot induction from embryonic axes of mature seeds supports a novel regeneration platform for biolistic DNA delivery in *Theobroma cacao* L.

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## Abstract

*Theobroma cacao* L. genetic transformation still relies predominantly on somatic embryogenesis-based systems, which are often genotype-dependent, labor-intensive, and associated with prolonged culture periods. In this study, we developed an alternative regeneration platform based on mature seed-derived embryonic axes and evaluated its suitability for biolistic DNA delivery. Embryonic axes from the cacao cultivars ‘TSH1188’ and ‘Parazinho’ were cultured on media supplemented with different cytokinins for multiple shoot induction. Among the plant growth regulators tested, 6-benzylaminopurine (BAP) was the most effective, promoting direct organogenesis from the apical dome and producing the highest shoot regeneration frequency and number of shoots per explant at 0.75 mg L<sup>-1</sup> for ‘TSH1188’ and 1.0 mg L<sup>-1</sup> for ‘Parazinho’. Sensitivity assays using antibiotics and herbicides identified genotype-dependent responses to kanamycin and hygromycin, whereas ammonium glufosinate, imazapyr, and glyphosate caused severe phytotoxic effects and completely inhibited shoot regeneration at relatively low concentrations. Hygromycin at 2.5 mg L<sup>-1</sup> (‘Parazinho’) and 5.0 mg L<sup>-1</sup> (‘TSH1188’) provided effective inhibition of shoot regeneration while maintaining the recovery of regenerating tissues, supporting its use in future selection protocols. For rooting, indole-3-acetic acid (IAA) at 0.5 mg L<sup>-1</sup> promoted the highest rooting efficiency, generating elongated roots and vigorous plantlets without intermediate callus formation. Preliminary biolistic assays using the pCAMBIA2301 vector resulted in localized transient GUS expression in the apical dome, demonstrating that this meristematic region is competent for exogenous DNA delivery. Collectively, these findings identify mature seed-derived embryonic axes as a promising explant for future stable genetic transformation and genome editing in cacao.

## Key message

A novel embryonic axis-based regeneration system enables rapid shoot recovery, rooting, and biolistic gene delivery in cacao, substantially shortening the timeline for plant transformation.

**Keywords** Bombardment · Cocoa · Genome editing · Rooting · Shoot regeneration · Transgenic plants

## Introduction

*Theobroma cacao* L. is one of the most economically important tropical crops worldwide, supporting the global chocolate industry and providing income for millions of smallholder farmers throughout tropical regions (Wickramasuriya and Dunwell 2018; ICCO 2024). Native to the Amazon Basin, cacao exhibits extensive genetic diversity distributed among wild and domesticated populations across South and Central America. This diversity has been extensively exploited in conservation programs

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and breeding initiatives aimed at improving productivity, bean quality, and resistance to biotic stresses (Cornejo et al. 2018; da Silva Guimarães et al. 2023; Colli-Silva et al. 2025). Recent genomic studies have further demonstrated that Amazonian germplasm harbors substantial intraspecific variation and represents a valuable reservoir of alleles for crop improvement (da Silva Guimarães et al. 2023; Colli-Silva et al. 2025).

Global cocoa production has exceeded five million tons in recent years, with approximately 70% originating from West African countries (FAOSTAT 2025; ICCO 2024). In Brazil, the states of Pará and Bahia remain the major production centers, where cacao is frequently cultivated in traditional agroforestry systems known as ‘cabruca’, characterized by cultivation beneath native forest canopies (Sambuichi et al. 2012). Beyond their economic importance, these systems contribute significantly to biodiversity conservation, ecosystem service maintenance, and carbon sequestration within remnants of the Atlantic Forest biome (Sambuichi et al. 2012).

Despite its economic and ecological relevance, cacao productivity remains severely constrained by several diseases, among which witches’ broom disease, caused by the hemibiotrophic fungus *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora, is one of the most destructive. The pathogen colonizes meristematic tissues and induces hypertrophy, hyperplasia, loss of apical dominance, and extensive tissue necrosis, resulting in severe impairment of both vegetative growth and reproductive development (Meinhardt et al. 2008; Pires et al. 2009; Santos et al. 2020). In Brazil, the introduction of witches’ broom disease into southern Bahia caused a dramatic collapse of regional cocoa production, generating long-lasting economic, social, and environmental consequences (Caldas and Perz 2013; Sousa Filho et al. 2021).

The development of efficient propagation and regeneration systems is essential for the long-term sustainability of cacao cultivation and for the implementation of modern crop improvement technologies. Although cacao is commonly propagated by seeds for the establishment of breeding populations and rootstocks, seed propagation does not provide a practical route for the rapid multiplication of selected materials or for the recovery of genetically engineered plants. Consequently, the development of reliable regeneration systems capable of producing whole plants from responsive explants has become a central requirement for functional genomics, genetic transformation, genome editing, and molecular breeding applications. Conventional vegetative propagation methods, including grafting and rooted cuttings, enable the multiplication of elite genotypes but remain constrained by low multiplication rates, labor-intensive management, and difficulties associated

with large-scale deployment (Schnell et al. 2005; Henao-Ramírez et al. 2021).

Plant tissue culture technologies have emerged as powerful tools for both clonal propagation and biotechnological applications in cacao. Among them, somatic embryogenesis has become the predominant regeneration system employed for micropropagation, germplasm conservation, and genetic transformation. Conventional protocols rely primarily on floral tissues, particularly petals and staminodes, for the induction of primary somatic embryos (Li et al. 1998; Garcia et al. 2018; Quinga et al. 2018; Guillou and Verdier 2022). Subsequent advances involving secondary somatic embryogenesis significantly enhanced embryo production by utilizing cotyledons derived from primary somatic embryos as explant sources (Florez et al. 2015; Guillou and Verdier 2022).

Although these developments have substantially expanded the biotechnological toolbox available for cacao, the establishment of efficient regeneration systems remains a major prerequisite for the successful application of genetic transformation and genome editing technologies. While somatic embryogenesis enables the clonal propagation of elite genotypes, its practical implementation is frequently constrained by strong genotype dependence, limited availability of responsive source tissues, prolonged culture periods, and relatively low embryo-to-plant conversion rates (Li et al. 1998; Maximova et al. 2002; Florez et al. 2015; Guillou and Verdier 2022). Moreover, the recovery of transformed plants through somatic embryogenesis involves multiple developmental stages, including embryo induction, maturation, germination, and plant conversion, which collectively increase production costs and extend regeneration timelines (Florez et al. 2015; Guillou and Verdier 2022). Long-term in vitro culture has also been associated with physiological and epigenetic alterations that may affect the developmental stability and quality of regenerated plants (Maximova et al. 2014; Adu-Gyamfi et al. 2016; Guillou et al. 2018; Fister et al. 2018; Garcia et al. 2022; Davis et al. 2026). Together, these limitations highlight regeneration efficiency as one of the principal bottlenecks restricting the broader implementation of transformation technologies in cacao.

In contrast, embryonic axes excised from mature seeds represent a readily available source of highly meristematic tissue with considerable regenerative potential. Direct shoot organogenesis from embryonic axes bypasses several developmental steps required for somatic embryogenesis, thereby enabling the rapid recovery of whole plants through multiple shoot induction. Importantly, the purpose of such a regeneration system is not clonal propagation, but rather the establishment of a highly responsive target tissue for DNA delivery, selection, and regeneration of transformed cells. This distinction is particularly relevant for biolistic

transformation and genome editing applications, where regeneration capacity frequently constitutes the primary factor limiting the recovery of transgenic or edited plants. Recent advances in plant biotechnology have emphasized the importance of developing highly regenerative explants to overcome transformation bottlenecks in recalcitrant species and to broaden the applicability of genetic engineering technologies (Maren et al. 2022).

The use of mature seed-derived embryonic axes also involves considerations that differ from those associated with somatic tissues obtained from clonally propagated donor plants. Because cacao seeds originate from sexual reproduction, individual embryonic axes may differ genetically from one another and from the maternal plant, particularly when seeds are produced through open pollination or crosses involving heterozygous parents (Cornejo et al. 2018; Colli-Silva et al. 2025). Such segregation may represent an additional source of biological variation in vitro responsiveness. This potential source of variation should therefore be considered when interpreting regeneration and transformation outcomes and when extending the protocol to additional genetic backgrounds. Nevertheless, zygotic embryonic axes offer important practical advantages as transformation targets, including minimal dependence on specialized donor tissues, the presence of pre-existing meristematic regions capable of rapid direct organogenesis, and the possibility of bypassing several developmental steps required for somatic embryogenesis, thereby accelerating recovery of transformed plants (Chen et al. 2022). Furthermore, recent advances in crop transformation have highlighted meristematic tissues as particularly attractive explants for overcoming regeneration bottlenecks and genotype dependence in recalcitrant species (Chen et al. 2022; Maren et al. 2022). Although zygotic embryonic axes are not intended for clonal propagation of elite genotypes, they constitute valuable target tissues for proof-of-concept transformation studies, genome-editing platforms, and the recovery of independently transformed seed-derived plants.

Successful organogenic regeneration depends on the coordinated interaction between plant growth regulators, cellular reprogramming processes, and developmental pathways associated with morphogenic competence. Recent studies have demonstrated that the acquisition of regenerative capacity involves extensive epigenetic remodeling and activation of developmental regulators controlling cellular totipotency and organ formation (Xu and Hu 2020; Long et al. 2022; Peng et al. 2025; Kowalik et al. 2026). Cytokinin such as 6-benzylaminopurine (BAP) and thidiazuron (TDZ) play central roles in shoot induction and meristem establishment, whereas auxins including indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthaleneacetic acid (NAA) are key regulators of adventitious root

formation (Da Costa et al. 2013; Bidabadi and Jain 2020). Consequently, optimization of regeneration, selection, and rooting stages remains critical for the development of rapid, efficient, and reproducible regeneration platforms in cacao.

Therefore, the establishment of a regeneration system based on multiple shoot induction from mature seed embryonic axes represents a promising alternative for cacao transformation studies. Rather than replacing somatic embryogenesis as a platform for clonal propagation, this approach complements existing regeneration systems by providing a rapid and efficient route for the recovery of genetically modified plants. The objective of this study was to establish an alternative and efficient protocol for in vitro regeneration, shoot selection, and rooting using embryonic axes isolated from mature seeds of two cacao genotypes as explant sources for multiple shoot induction, aiming at their application in biolistic-mediated DNA delivery in *Theobroma cacao* L.

## Materials and methods

### Plant material and explant preparation

Mature fruits of *Theobroma cacao* L. cvs. ‘TSH1188’ and ‘Parazinho’ (common cacao) were collected from plants grown in an experimental field at the Cocoa Research Center (CEPEC/CEPLAC), located in Ilhéus, Bahia, Brazil. Fruits were washed under running tap water with neutral detergent and subsequently surface-sterilized in a laminar flow cabinet by flaming after immersion in 100% ethanol. Under aseptic conditions, embryonic axes were excised and vertically inoculated following a basal cut, while preserving the circular shape of the explants to minimize mechanical injury during dissection and handling, particularly in the shoot apical meristem and surrounding tissues, thereby preserving explant structural integrity (Fig. S1).

### Culture media for shoot regeneration and selection

Explants were cultured on shoot induction medium consisting of MS basal salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 0.1 g L<sup>-1</sup> myo-inositol, 30 g L<sup>-1</sup> sucrose, and 2.5 g L<sup>-1</sup> Phytigel® (Sigma-Aldrich, USA). Different cytokinins were evaluated: zeatin (ZEA) at concentrations of 0.75, 1.0, and 1.5 mg L<sup>-1</sup> (MS1-MS3); 6-benzylaminopurine (BAP) at concentrations of 0.75, 1.0, and 1.5 mg L<sup>-1</sup> (MS4-MS6); and thidiazuron (TDZ) at concentrations of 0.1, 0.25, 0.5, and 0.75 mg L<sup>-1</sup> (MS7-MS10) (Table S1). Medium without plant growth regulators (MS11) was used as the control treatment. The pH of all media was adjusted to 5.7±0.1 prior to autoclaving at

121 °C for 20 min. Cultures were maintained in a growth chamber at 27±2 °C under a 16-h light/8-h dark photoperiod for 60 days. Evaluations were performed after 30 and 60 days of culture by recording callus formation and adventitious shoot induction using a stereomicroscope.

After identifying the most effective conditions for shoot regeneration, media containing 0.75 mg L<sup>-1</sup> BAP for ‘TSH1188’ and 1.0 mg L<sup>-1</sup> BAP for ‘Parazinho’ were selected for explant sensitivity assays to different selective agents. For these assays, culture media were supplemented with kanamycin (Sigma Chemical Co., USA) at 25 (MS12), 50 (MS13), 75 (MS14), and 100 mg L<sup>-1</sup> (MS15); hygromycin B (Sigma Chemical Co., USA) at 2.5 (MS16), 5.0 (MS17), 7.5 (MS18), and 10 mg L<sup>-1</sup> (MS19); ammonium glufosinate (Finale®, Bayer CropScience AG, Germany) at 0.2 (MS20), 0.4 (MS21), 0.6 (MS22), and 0.8 mg L<sup>-1</sup> (MS23); imazapyr (BioCarb Brasil) at 0.026 (MS24), 0.078 (MS25), 0.130 (MS26), and 0.156 mg L<sup>-1</sup> (MS27); and glyphosate (Insetimax Brasil) at 0.16 (MS28), 0.50 (MS29), 0.84 (MS30), and 1.0 mg L<sup>-1</sup> (MS31). Culture medium without selective agents was used as the control treatment (MS32), as described in Table S1.

### In vitro rooting

Based on the shoot regeneration experiments described above, ‘TSH1188’ genotype was selected for in vitro rooting due to its superior regenerative performance. Regenerated shoots of ‘TSH1188’ were excised near the shoot base and transferred to MS medium supplemented with different auxins:  $\alpha$ -naphthaleneacetic acid (NAA) at 0.25, 0.50, and 1.0 mg L<sup>-1</sup> (MS33–MS35); indole-3-butyric acid (IBA) at 0.25, 0.50, and 1.0 mg L<sup>-1</sup> (MS36–MS38); and indole-3-acetic acid (IAA) at 0.25, 0.50, and 1.0 mg L<sup>-1</sup> (MS39–MS41). Medium without plant growth regulators (MS42) was used as the control treatment, as described in Table S1. Cultures were maintained under the same environmental conditions described above.

### Biolistic-mediated genetic transformation

A transient transformation assay was designed as a proof-of-concept experiment to verify the suitability of mature seed-derived embryonic axes as targets for biolistic DNA delivery. Therefore, transformation efficiency was assessed qualitatively through transient GUS expression rather than by recovery of stable transgenic plants. ‘TSH1188’ was selected for biolistic transformation because its superior regenerative performance and agronomic relevance. Embryonic axes of ‘TSH1188’ were prepared as described above. To increase the exposure of meristematic cells to particle bombardment, the region corresponding to the

future primary leaves was removed, leaving the apical dome exposed. Explants were positioned on Petri dishes containing MS medium, with five embryonic axes per plate arranged in a circular pattern and oriented with the apical meristem facing upward (Fig. S1).

Particle bombardment was performed according to the protocol described by Rech et al. (2008), using tungsten microparticles coated with plasmid pCAMBIA2301 (Cambia, Australia), containing the selectable marker gene *nptII*, which confers kanamycin resistance, and the reporter gene *uidA*, both driven by the CaMV 35 S promoter. Microparticles were mixed with plasmid DNA, 2.5 M CaCl<sub>2</sub> and 0.1 M spermidine, washed twice with absolute ethanol, resuspended in ethanol and loaded onto the macrocarrier immediately before bombardment. Bombardment was performed using a Biolistic PDS-1000 Particle Delivery System (Bio-Rad) according to the instruction manual. Explants were bombarded using a rupture pressure of 1,200 psi, a target distance of 9 cm, a vacuum of 26 in Hg, and a single shot per plate. Following bombardment, explants were maintained under the growth conditions described previously for shoot induction. Explants were allowed to recover for 72 h before histochemical analysis. Non-bombarded embryonic axes were included as negative controls to verify the absence of endogenous GUS activity.

### Histochemical GUS assay

Transient expression of the *uidA* gene was evaluated three days after bombardment using a histochemical GUS assay as described by Jefferson (1987). Embryonic axes were incubated in a solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide), 100 mM sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, and 0.1% Triton X-100, followed by incubation for 5 h at 37 °C in the dark. Subsequently, chlorophyll was removed by repeated washing in 70% ethanol until blue staining became clearly visible. Explants were subsequently examined and photographed using a Leica® EZ4 stereomicroscope. Transient transformation efficiency was assessed qualitatively by the presence of blue staining in the apical meristematic region and surrounding tissues. Only discrete, localized blue foci associated with bombarded tissues were considered positive transformation events. Non-bombarded explants and explants incubated in staining solution without X-Gluc served as negative controls.

### Experimental design and statistical analysis

The shoot regeneration experiments were conducted in a completely randomized design arranged in an 11 × 2 factorial

scheme, corresponding to 11 treatments and two genotypes, with five replicates per treatment. Each replicate consisted of one Petri dish containing five embryonic axes. Callus frequency (total number of calli/total number of inoculated explants  $\cdot$  100), frequency of regeneration (total number of shoots/total number of inoculated explants  $\cdot$  100), and the mean number of shoots per explant (total number of shoots/total number of inoculated explants) were evaluated after 30 and 60 days of in vitro culture.

For the selective agent assays, a completely randomized design with five replicates per treatment was adopted, each replicate consisting of one Petri dish containing five embryonic axes. After 60 days of in vitro culture, callus formation and shoot regeneration responses were evaluated as a function of the concentrations of the selective agents tested.

The in vitro rooting experiments were conducted in a completely randomized design with five replicates per treatment, each replicate consisting of one Magenta® vessel containing four explants. Evaluations were performed after 30, 60, and 90 days of in vitro culture by recording the mean number of roots per explant.

For biolistic transformation, each experiment consisted of five independent biological replicates, each comprising of one Petri dish containing five embryonic axes.

The data were initially tested for normal distribution using the Shapiro-Wilk test, with a significance level of 5%. Once the hypothesis was confirmed, statistical differences were assessed based on analysis of variance (ANOVA) and the means were separated using the Scott-Knott or Student's *t* tests at the 5% probability level ( $P \leq 0.05$ ). All statistical analyses were conducted using Sisvar software version 5.6 (Ferreira 2011).

## Results

### Regeneration of embryonic axes of *Theobroma cacao* L.

The regeneration of embryonic axes from the cacao cvs. 'TSH1188' and 'Parazinho' was evaluated over 30 and 60 days of in vitro culture on media supplemented with different plant growth regulators. After 30 days of culture, both genotypes exhibited initial callus formation exclusively at the basal region of the embryonic axis, corresponding to the area in direct contact with the culture medium. At this stage, calli were predominantly compact and exhibited a whitish-yellow coloration. After 60 days, callus induction expanded to both the basal region and the apical dome of the embryonic axes. Calli remained compact and varied in color from white to pale yellow (Fig. 1A, B). In the control treatment, consisting of MS medium without plant growth regulators

(MS11), neither callus formation nor shoot induction was observed (Fig. 2). Instead, explants displayed normal seedling development, characterized by expanded green leaves and a well-developed root system, contrasting sharply with the morphogenic responses observed in explants cultured on media supplemented with plant growth regulators.

Significant differences were detected between 'TSH1188' and 'Parazinho' for both callus formation and shoot production. Overall, 'TSH1188' exhibited greater in vitro responsiveness, presenting higher mean values for the analyzed variables at 30 and 60 days of culture (Fig. 2). The genotype  $\times$  plant growth regulator interaction was also highly significant for callus frequency and mean number of shoots per explant, indicating that these morphogenic responses depended directly on the combination of genotype and the type and concentration of plant growth regulators employed. At 30 days of culture, callus formation was observed in all treatments supplemented with plant growth regulators, whereas no calli developed in the control (MS11) treatment (Fig. 2A). Among the culture media evaluated, those supplemented with zeatin at 0.75 mg L<sup>-1</sup> (MS1) and 1.5 mg L<sup>-1</sup> (MS3), as well as TDZ at 0.5 mg L<sup>-1</sup> (MS9), produced the highest levels of callogenesis in both genotypes (Fig. 2A). After 60 days of in vitro culture, significant differences among treatments were detected for callus formation. Media MS6 (1.5 mg L<sup>-1</sup> BAP) exhibited the lowest mean callogenesis values for both genotypes (Fig. 2B).

Regarding shoot induction after 30 days of culture, the lowest values were recorded in the plant growth regulator-free medium, confirming the requirement for hormonal supplementation to promote multiple shoot formation (Fig. 2C, E). Among the treatments, 'TSH1188' embryonic axes cultured on MS1 (0.75 mg L<sup>-1</sup> ZEA) and MS4 (0.75 mg L<sup>-1</sup> BAP) produced a significantly greater number of shoots than 'Parazinho' explants grown on the same media. Medium MS4, containing 0.75 mg L<sup>-1</sup> BAP, was particularly effective for 'TSH1188', yielding a significantly higher mean number of shoots than the other formulations. For 'Parazinho', although no significant differences were detected among BAP concentrations, the highest shoot induction rates were observed in media MS4, MS5, and MS6, containing 0.75, 1.0, and 1.5 mg L<sup>-1</sup> BAP, respectively (Fig. 2C, E).

After 60 days of culture, a general increase in shoot production was observed in both genotypes compared with the 30-day evaluation, indicating a cumulative effect of prolonged in vitro culture. Among the treatments, MS4 and MS5, containing 0.75 and 1.0 mg L<sup>-1</sup> BAP, respectively, produced the highest frequency of regeneration and mean numbers of shoots in 'TSH1188' and 'Parazinho', reaching a regeneration frequency of 48% and 68% and 0.40 and 0.38 shoots per explant, respectively (Fig. 2D, F). Extended culture duration significantly enhanced organogenic responses,

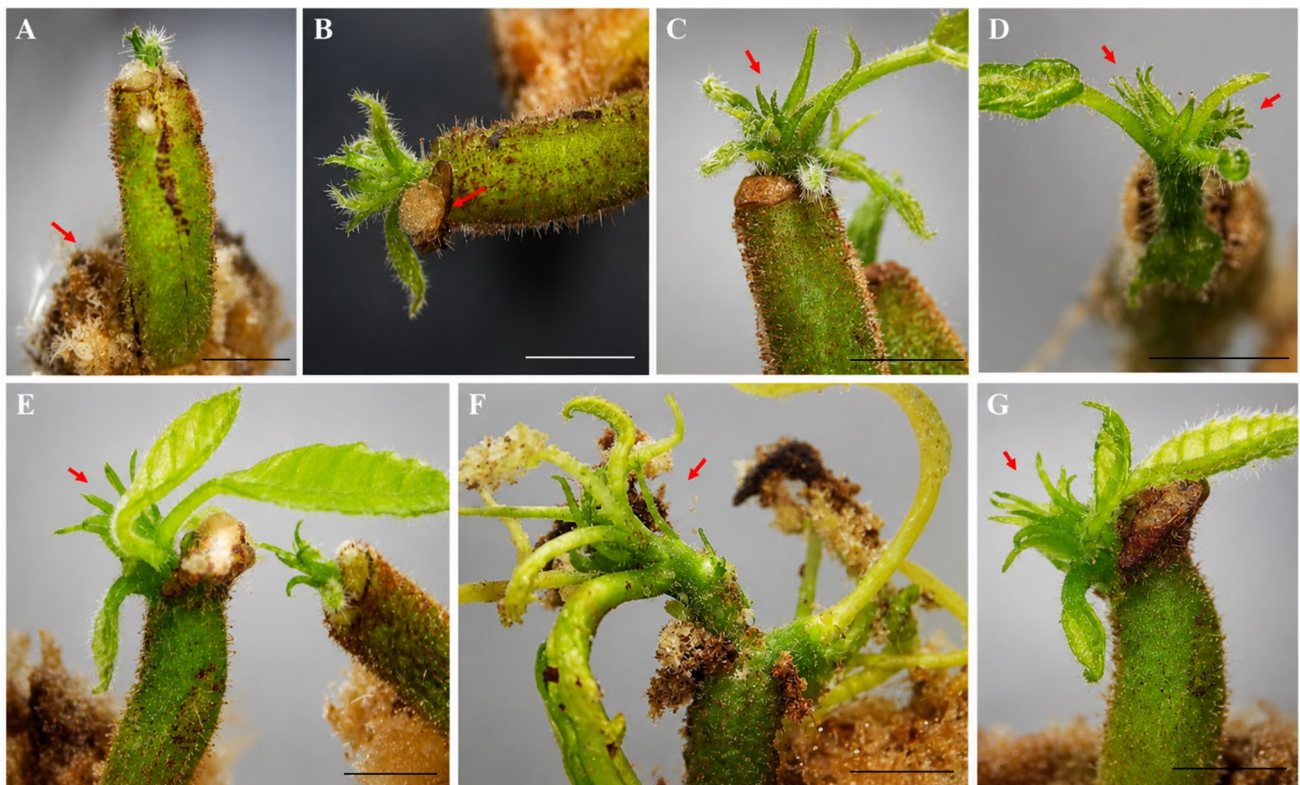


**Fig. 1** In vitro regeneration of embryonic axes of *Theobroma cacao* L. (A–B) Compact calli (red arrows) varying in color from white (A) to pale yellow (B) on the apical region and at the base of the mature seed-derived embryonic axis. (C–G) Embryonic axes exhibiting multiple shoot induction (red arrows) in the shoot region after 60 days of

in vitro culture. Images show the proliferation of adventitious shoot primordia around the apical region in embryonic axes of 'TSH1188' (C–D) and 'Parazinho' (E–G) cultured in MS medium supplemented with 0.75 mg L<sup>-1</sup> BAP (MS4) and 1.0 mg L<sup>-1</sup> BAP (MS5), respectively. Scale bar: 3 mm

particularly in media supplemented with BAP. In all treatments supplemented with plant growth regulators, shoot formation occurred exclusively in the aerial portion of the explants, and no organogenic structures were observed in the basal region, even in the presence of callus tissue (Fig. 1C–G). For both genotypes, shoot regeneration occurred through direct organogenesis.

Considering all variables evaluated, including mean callus frequency, frequency of regeneration, and number of shoots per explant, as well as genotype-specific responses, media MS4 and MS5, supplemented with 0.75 and 1.0 mg L<sup>-1</sup> BAP, respectively, were identified as the most effective treatments for in vitro multiple shoot induction in 'TSH1188' and 'Parazinho'. In addition to promoting superior morphogenic responses, these media required lower concentrations of plant growth regulators, thereby reducing costs and minimizing potential adverse effects associated with excessive cytokinin exposure.

### Selective medium for embryonic axes of *Theobroma cacao* L.

Embryonic axes of 'TSH1188' and 'Parazinho' cultured on the control medium (MS32), supplemented with the previously optimized BAP concentrations for each genotype and without the addition of antibiotics or herbicides, exhibited the highest levels of callus formation. Calli displayed a compact morphology, whitish-yellow coloration, and no signs of necrosis or tissue oxidation. In addition, embryonic axes maintained normal shoot development, characterized by expanded leaves and healthy tissues (Figs. 3A and 4A). This response pattern confirmed the healthy growth of explants under non-selective culture conditions.

The presence of kanamycin and hygromycin, regardless of the concentration tested, did not affect callus formation in embryonic axes of either genotype after 60 days of in vitro culture, as all explants produced calli (100% callus induction frequency) across all treatments (Fig. 5A, B), indicating a relatively low sensitivity of *T. cacao* embryonic tissues to these antibiotics with respect to callus formation. In contrast, genotype-dependent effects were observed in ammonium glufosinate medium. Embryonic axes of 'TSH1188'

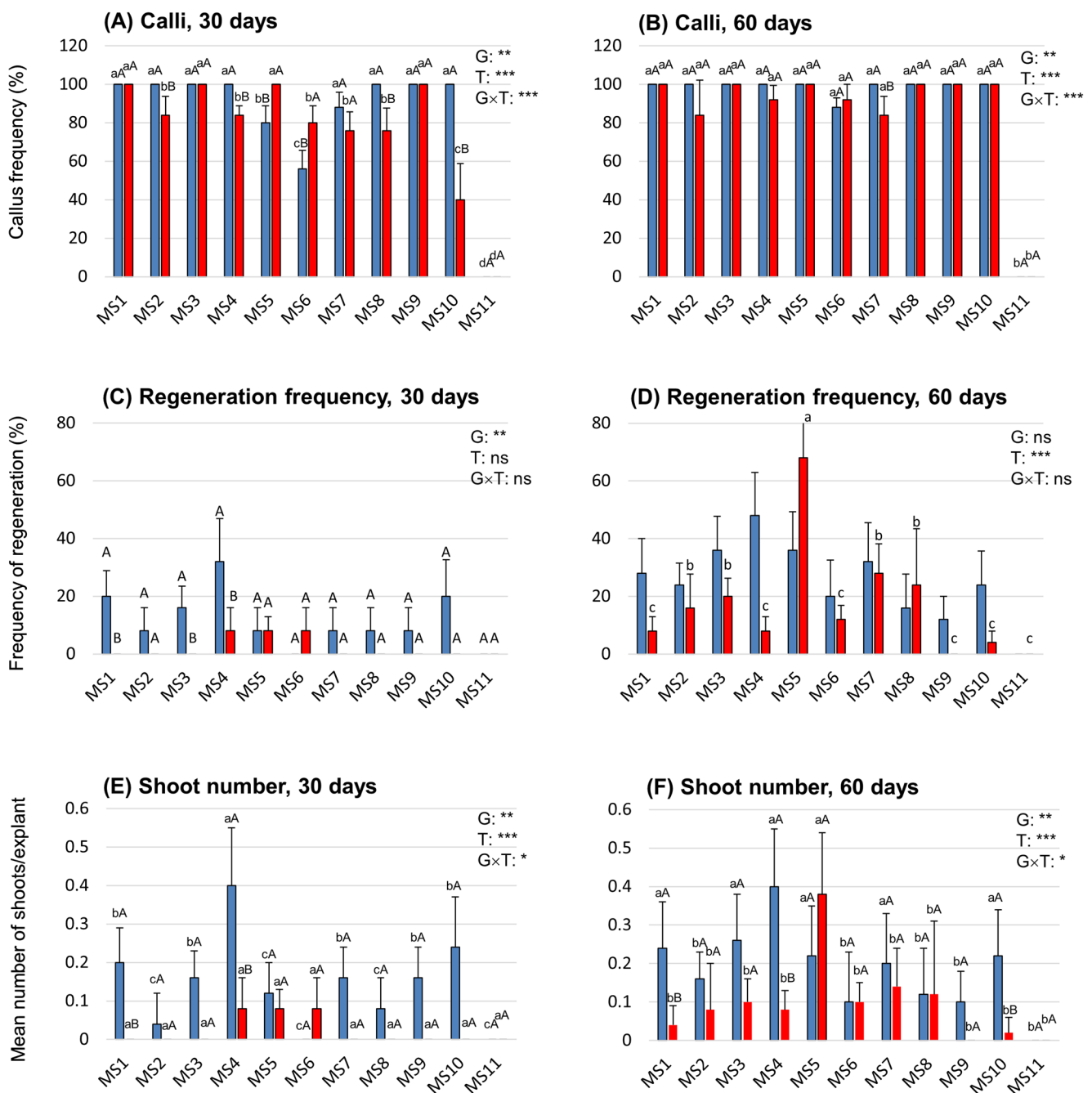


**Fig. 2** Callus frequency, frequency of regeneration, and mean number of shoots per explant in embryonic axes of ‘TSH1188’ and ‘Parazinho’ after 30 and 60 days of in vitro culture in response to different plant growth regulators. **(A–B)** Callus frequency after 30 and 60 days of in vitro culture. **(C–D)** Regeneration frequency after 30 and 60 days of in vitro culture. **(E–F)** Mean number of shoots per explant after 30 and 60 days of in vitro culture. MS1, MS2, and MS3: 0.75, 1.0, and 1.5 mg L<sup>-1</sup> zeatin (ZEA), respectively. MS4, MS5, and MS6: 0.75,

1.0, and 1.5 mg L<sup>-1</sup> 6-benzylaminopurine (BAP), respectively. MS7, MS8, MS9, and MS10: 0.1, 0.25, 0.5, and 0.75 mg L<sup>-1</sup> thidiazuron (TDZ), respectively. MS11: MS medium without plant growth regulators. Means followed by different letters, uppercase letters comparing genotypes and lowercase letters comparing treatments, differ significantly according to the Scott-Knott test ( $P \leq 0.05$ ). G: genotypes; T: treatment; G:T: interaction genotype · treatment. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

were more sensitive to the herbicide, whereas ‘Parazinho’ displayed greater tolerance (Fig. 5C). The lowest concentration tested (0.2 mg L<sup>-1</sup>; MS20) significantly affected callus formation in ‘TSH1188’, while higher concentrations

(MS21–MS23; 0.4–0.8 mg L<sup>-1</sup>) completely inhibited callus induction after 60 days of culture. In ‘Parazinho’, by contrast, the concentrations evaluated had no impact on callus induction.

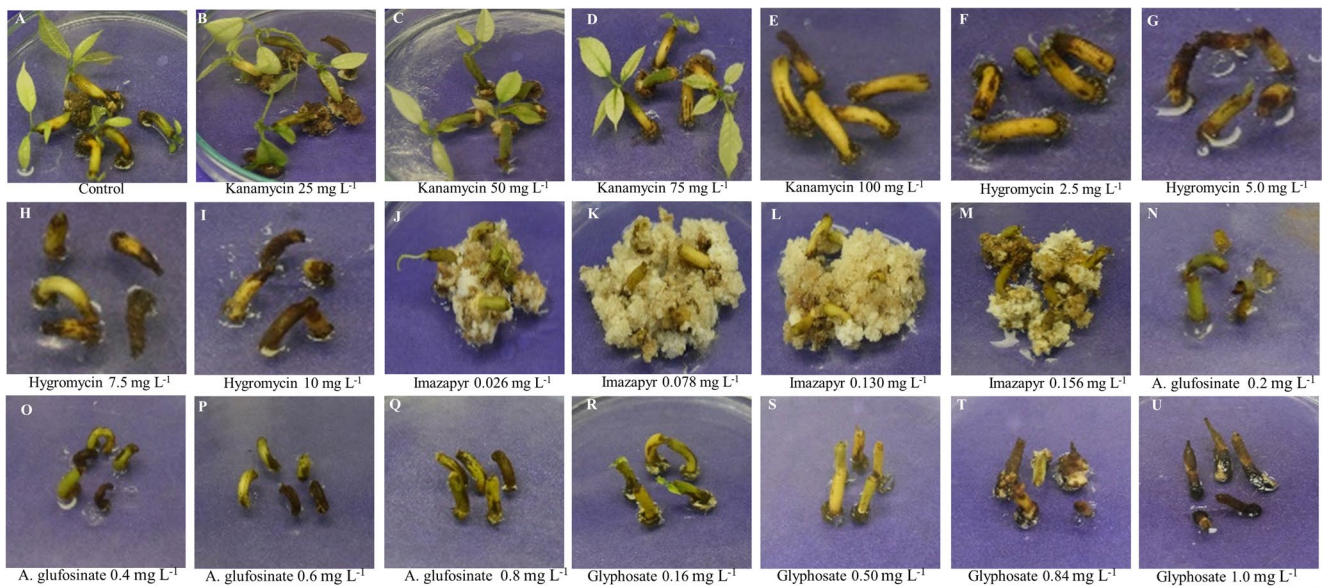


**Fig. 3** Representative photographs of 'TSH1188' embryonic axes cultured on selection media containing different concentrations of selectable agents, after 60 days of in vitro culture. **(A)** Control (culture medium without selection agents). **(B–E)** Kanamycin (25, 50, 75, and 100 mg L<sup>-1</sup>). **(F–I)** Hygromycin B (2.5, 5.0, 7.5, and 10 mg L<sup>-1</sup>). **(J–M)** Imazapyr (0.026, 0.078, 0.130, and 0.156 mg L<sup>-1</sup>). **(N–Q)** Ammonium glufosinate (0.2, 0.4, 0.6, and 0.8 mg L<sup>-1</sup>). **(R–U)** Glyphosate (0.16, 0.50, 0.84, and 1.0 mg L<sup>-1</sup>). Scale bar: 10 mm

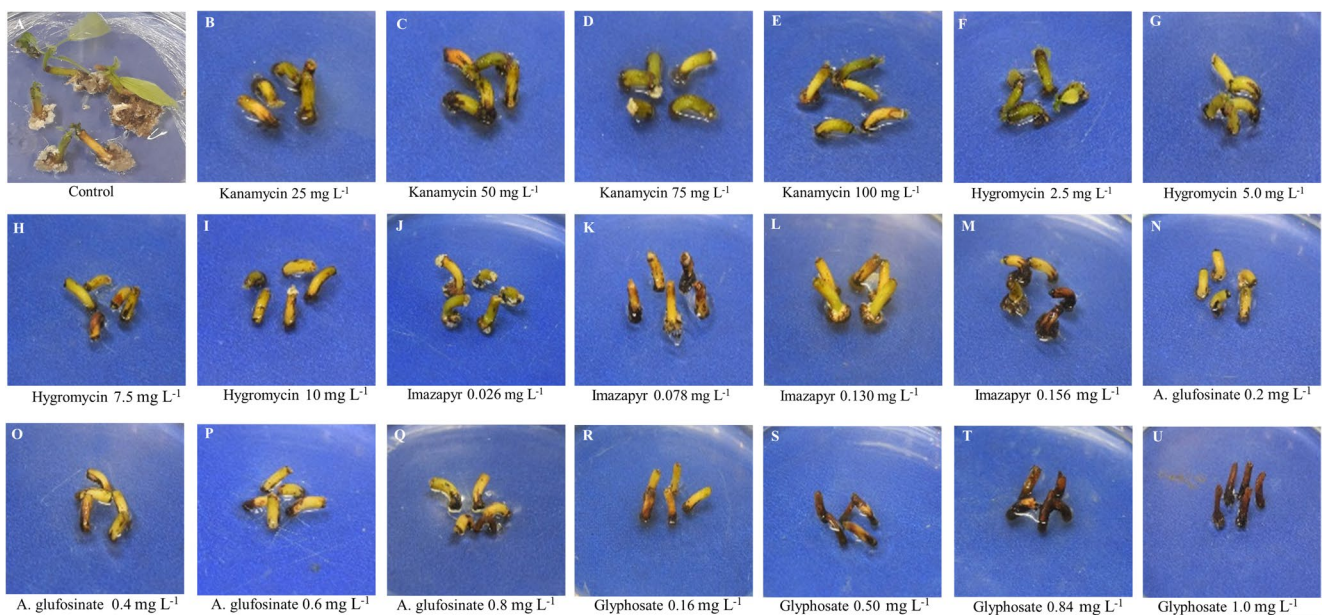


**Fig. 4** Representative photographs of 'Parazinho' embryonic axes cultured on selection media containing different concentrations of selectable agents, after 60 days of in vitro culture. **(A)** Control (culture medium without selection agents). **(B–E)** Kanamycin (25, 50, 75, and 100 mg L<sup>-1</sup>). **(F–I)** Hygromycin B (2.5, 5.0, 7.5, and 10 mg L<sup>-1</sup>). **(J–M)** Imazapyr (0.026, 0.078, 0.130, and 0.156 mg L<sup>-1</sup>). **(N–Q)** Ammonium glufosinate (0.2, 0.4, 0.6, and 0.8 mg L<sup>-1</sup>). **(R–U)** Glyphosate (0.16, 0.50, 0.84, and 1.0 mg L<sup>-1</sup>). Scale bar: 10 mm

Imazapyr reduced callus formation in both genotypes only at the highest concentration tested (Fig. 5D). Nevertheless, distinct morphological responses were observed between the genotypes irrespective of the herbicide concentration. In 'TSH1188', explants exhibited disorganized growth and excessive callus proliferation at the base of

the embryonic axis, producing friable whitish-yellow calli accompanied by tissue chlorosis (Fig. 3). In 'Parazinho', callus induction was more moderate and lacked the excessive proliferation observed in 'TSH1188' (Fig. 4).

Glyphosate exerted a strong inhibitory effect, particularly in 'Parazinho' (Fig. 5E). At concentrations of 0.16 mg L<sup>-1</sup>

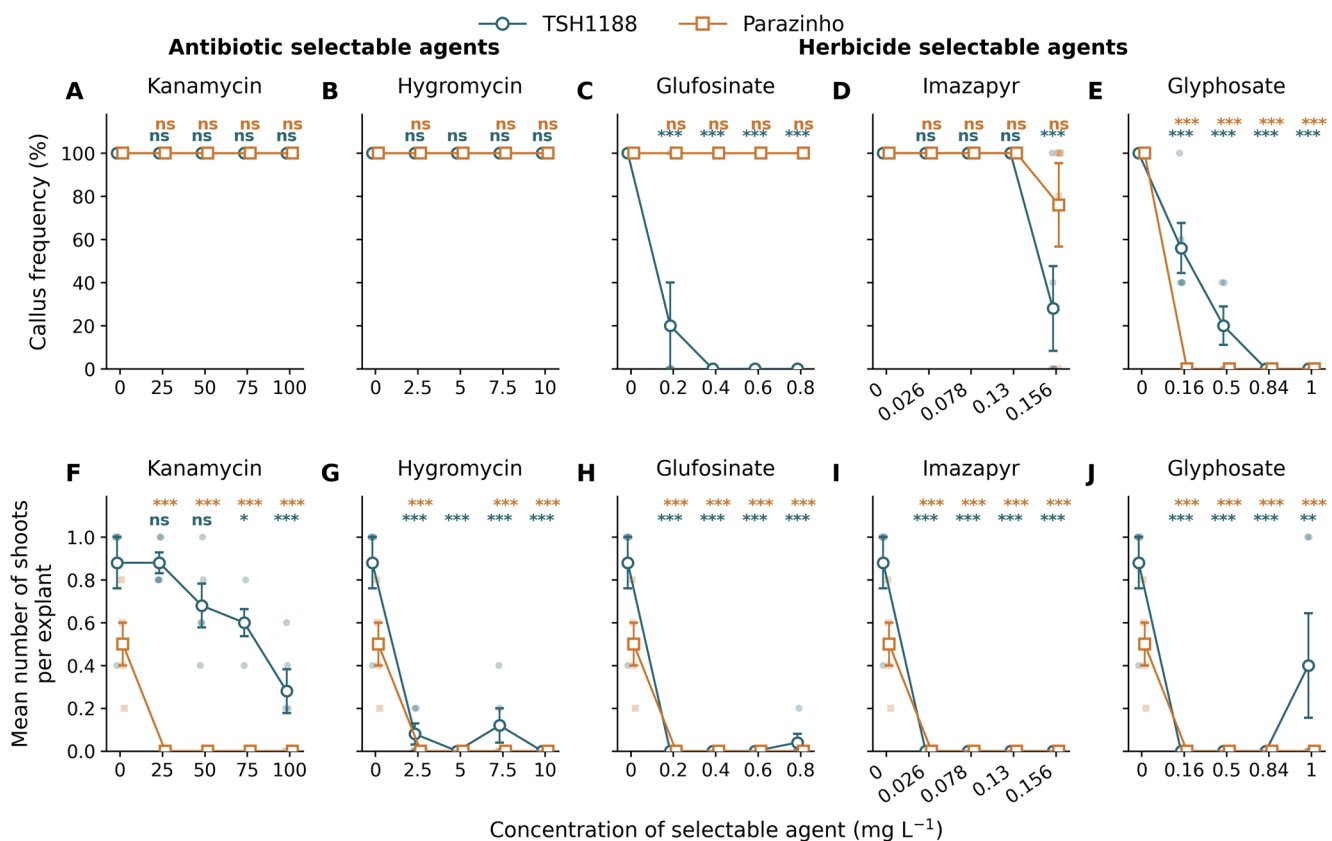


**Fig. 5** Effect of selectable agents on the callus frequency (A–E) and mean number of shoots per explant (F–J) in embryonic axes of ‘TSH1188’ and ‘Parazinho’ after 60 days of in vitro culture. (A, F) Kanamycin (25, 50, 75, and 100 mg L<sup>-1</sup>). (B, G) Hygromycin (2.5, 5.0, 7.5, and 10 mg L<sup>-1</sup>). (C, H) Ammonium glufosinate (0.2, 0.4, 0.6, and 0.8 mg L<sup>-1</sup>). (D, I) Imazapyr (0.026, 0.078, 0.130, and 0.156 mg L<sup>-1</sup>). (E, J) Glyphosate (0.16, 0.50, 0.84, and 1.0 mg L<sup>-1</sup>). Control:

MS medium supplemented with 0.75 mg L<sup>-1</sup> BAP for ‘TSH1188’ and 1.0 mg L<sup>-1</sup> BAP for ‘Parazinho’, without the addition of selectable agents. Symbols and lines indicate means  $\pm$  standard error (SE). Statistical significance between control and selectable agent treatments within each genotype was determined by the Student’s *t*-test, and is indicated as ns (not significant),  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), or  $P < 0.001$  (\*\*\*)

and above (MS29–MS31), callus formation was completely suppressed in this genotype after 60 days of culture, accompanied by progressive tissue browning and the complete absence of shoot development (Fig. 4). In ‘TSH1188’, callus formation was completely suppressed at concentrations of 0.84 mg L<sup>-1</sup> and above, and at the highest concentration tested (1.0 mg L<sup>-1</sup>) it was highly toxic, resulting in severe tissue oxidation followed by embryonic axis death (Fig. 3).

Considering the mean number of shoots, ‘Parazinho’ embryonic axes were more sensitive to the selectable agents, failing to regenerate shoots throughout the culture period, irrespective of the concentration tested (Fig. 5F–J). At the highest concentrations of the selectable agents, explants exhibited severe tissue chlorosis (Fig. 4). In ‘TSH1188’, kanamycin at concentrations ranging from 75 to 100 mg L<sup>-1</sup> (MS14 and MS15) and hygromycin from 2.5 to 10 mg L<sup>-1</sup> (MS16–MS19) significantly reduced (30 to 66% for kanamycin and 89 to 100% for hygromycin, respectively) shoot production compared with the control (MS32) treatment (Fig. 5F, G). The lower concentrations of kanamycin (25

and 50 mg L<sup>-1</sup>; MS12 and MS13) resulted in shoot production comparable to that of the control (Fig. 5F), indicating a threshold concentration below which shoot development was not inhibited. Finally, the herbicides ammonium glufosinate, imazapyr, and glyphosate strongly inhibited shoot regeneration in both genotypes at all concentrations evaluated (Fig. 5H–J). None of the herbicide-containing treatments supported shoot development, in sharp contrast to the vigorous aerial growth observed in the control medium (Figs. 3 and 4).

### In vitro rooting of adventitious shoots of *Theobroma cacao* L.

Root emergence was observed in all treatments after 30 days of in vitro culture (Table 1), indicating that adventitious shoots of ‘TSH1188’ were competent for root induction under all conditions tested. Although no significant differences were detected between the 60- and 90-day evaluation periods, the mean number of roots increased progressively

**Table 1** Mean number of roots per explant in embryonic axis-derived adventitious shoots of ‘TSH1188’ cultured on media supplemented with rooting-inducing agents at different concentrations (MS33-MS35: 0.25, 0.5, and 1.0 mg L<sup>-1</sup> naphthaleneacetic acid [NAA], respectively; MS36-MS38: 0.25, 0.5, and 1.0 mg L<sup>-1</sup> indole-3-butyric acid [IBA], respectively; MS39-MS41: 0.25, 0.5, and 1.0 mg L<sup>-1</sup> indole-3-acetic acid [IAA], respectively; and MS42: control MS medium without the addition of rooting inducers), after 30, 60, and 90 days of in vitro culture. Means followed by different letters, uppercase letters comparing days of in vitro culture and lowercase letters comparing treatments, differ significantly according to the Scott-Knott test ( $P \leq 0.05$ )

| Treatment | Auxin source and concentration | Number of roots per explant |                   |                   |
|-----------|--------------------------------|-----------------------------|-------------------|-------------------|
|           |                                | 30 days                     | 60 days           | 90 days           |
| MS33      | 0.25 mg L <sup>-1</sup> NAA    | 0.05 ± 0.05<br>aA           | 0.15 ±<br>0.10 aA | 0.35 ±<br>0.17 bA |
| MS34      | 0.5 mg L <sup>-1</sup> NAA     | 0.05 ± 0.05<br>aA           | 0.05 ±<br>0.05 aA | 0.05 ±<br>0.05 aA |
| MS35      | 1.0 mg L <sup>-1</sup> NAA     | 0.85 ± 0.36<br>aA           | 1.25 ±<br>0.45 aA | 1.70 ±<br>0.27 bA |
| MS36      | 0.25 mg L <sup>-1</sup> IBA    | 0.60 ± 0.23<br>aA           | 1.10 ±<br>0.57 aA | 1.10 ±<br>0.57 aA |
| MS37      | 0.5 mg L <sup>-1</sup> IBA     | 0.90 ± 0.49<br>aA           | 2.05 ±<br>0.75 bA | 2.10 ±<br>0.72 bA |
| MS38      | 1.0 mg L <sup>-1</sup> IBA     | 0.80 ± 0.24<br>aA           | 1.60 ±<br>0.57 bA | 1.75 ±<br>0.54 bA |
| MS39      | 0.25 mg L <sup>-1</sup> IAA    | 0.45 ± 0.28<br>aA           | 2.10 ±<br>0.58 bB | 2.95 ±<br>0.84 bB |
| MS40      | 0.5 mg L <sup>-1</sup> IAA     | 1.20 ± 0.59<br>aA           | 2.70 ±<br>0.44 bB | 2.75 ±<br>0.44 bB |
| MS41      | 1.0 mg L <sup>-1</sup> IAA     | 0.35 ± 0.15<br>aA           | 0.85 ±<br>0.38 aA | 0.90 ±<br>0.36 aA |
| MS42      | Control (no auxin)             | 1.30 ± 0.36<br>aA           | 1.70 ±<br>0.61 bA | 2.40 ±<br>0.66 bA |

over time, reaching the highest values after 90 days of culture (Table 1).

Rooting responses varied according to the auxin source and concentration. In the absence of auxins (MS42), explants developed roots (Fig. 6B); however, rooting efficiency was generally lower than that observed in media supplemented with indole-3-acetic acid (IAA) (Table 1; Fig. 6C). Treatments containing  $\alpha$ -naphthaleneacetic acid (NAA; MS33-MS35) produced lower rooting rates than the control, indicating limited effectiveness of this auxin for root induction in ‘TSH1188’. For indole-3-butyric acid (IBA; MS36-MS38), the intermediate concentration (0.5 mg L<sup>-1</sup>) consistently outperformed the lower and higher concentrations throughout the evaluation period (Table 1).

The most effective rooting responses were obtained on media supplemented with 0.25 and 0.5 mg L<sup>-1</sup> IAA (MS39 and MS40), particularly after 60 and 90 days of culture, significantly surpassing all other treatments (Table 1). Explants grown on these media developed vigorous shoots and well-developed root systems characterized by elongated, robust roots and the absence of tissue oxidation. In contrast, the highest IAA concentration (1.0 mg L<sup>-1</sup>) resulted in reduced

rooting performance and shorter roots, suggesting an inhibitory effect at this concentration (Fig. 6D).

Root formation occurred directly from the basal region of the explants in all treatments, with no intermediate callus formation (Fig. 6B-D), a desirable characteristic for subsequent acclimatization and ex vitro establishment. Among the auxins evaluated, 0.5 mg L<sup>-1</sup> IAA proved to be the most effective treatment, producing the highest mean number of roots after 90 days and generating morphologically normal root systems suitable for plant establishment.

### Biolistic transformation of embryonic axes from *Theobroma cacao* L.

Preliminary biolistic transformation assays performed with embryonic axes of ‘TSH1188’ demonstrated that this explant type is suitable for DNA delivery by particle bombardment, maintaining good structural integrity following the procedure (Fig. 7). The application of the previously established operational parameters, including rupture pressure, target distance, vacuum pressure, and number of bombardments, resulted in consistent responses compatible with efficient transformation protocols for woody plant species. Bombardment efficiency was evaluated by histochemical analysis of transient expression of the GUS reporter gene, performed three days after transformation. Embryonic axes exhibited intense and well-defined blue staining localized predominantly in the shoot apical region, indicating successful DNA delivery and transient transgene expression (Fig. 7). The staining pattern ranged from multiple independent transformation events distributed throughout the apical meristem (Fig. 7B) to localized expression in individual shoot primordia (Fig. 7C) and developing shoots (Fig. 7D), confirming successful DNA delivery into the tissues responsible for shoot regeneration. The spatial distribution of GUS staining suggests that the meristematic cells located within the apical dome are particularly receptive to particle bombardment and capable of sustaining detectable levels of transient gene expression. These findings demonstrate the feasibility of using mature seed-derived embryonic axes as target tissues for biolistic-mediated gene delivery in cacao and establish a foundation for the development of an alternative transformation platform based on multiple shoot regeneration.

### Discussion

The successful regeneration of *Theobroma cacao* L. from mature seed-derived embryonic axes demonstrates the high morphogenic competence of this explant type and highlights its potential as a target tissue for plant regeneration



**Fig. 6** Representative photographs of embryonic axis-derived adventitious shoots of ‘TSH1188’ cultured on rooting media supplemented with different concentrations of auxins (MS33–MS35: 0.25, 0.5, and 1.0 mg L<sup>-1</sup> NAA, respectively; MS36–MS38: 0.25, 0.5, and 1.0 mg L<sup>-1</sup> IBA, respectively; MS39–MS41: 0.25, 0.5, and 1.0 mg L<sup>-1</sup> IAA, respectively; and MS42: control MS medium without the addition of rooting-inducing agents). **(A)** Adventitious shoot regenerated directly from the apical region of the embryonic axis in MS medium supplemented

with 0.75 mg L<sup>-1</sup> BAP. The red bar indicates the excision site used for shoot removal prior to transfer to the different rooting media. **(B)** Shoot cultured on MS42 (control) after 60 days of in vitro culture. **(C)** Shoots cultured on rooting medium supplemented with 0.25 mg L<sup>-1</sup> IAA (MS39) after 60 days of in vitro culture. **(D)** Rooting response of adventitious shoots cultured on MS42 (control), MS33, MS34, MS35, MS36, MS37, MS38, MS39, MS40, and MS41, respectively, after 90 days of in vitro culture. Scale bar: 10 mm

and genetic transformation. The absence of callus and shoot formation in the regulator-free medium confirmed that exogenous plant growth regulators are essential for triggering morphogenic responses in cacao embryonic tissues. Plant regeneration in vitro is initiated by extensive cellular reprogramming involving hormonal signaling, chromatin remodeling, and activation of developmental pathways associated with cellular totipotency and organ formation (Pasternak and Steinmacher 2024; Lee et al. 2024). The induction of calli in both genotypes following cytokinin supplementation (Fig. 1A, B) suggests that endogenous auxin levels present in the embryonic axes were sufficient to establish the hormonal balance required for the acquisition of morphogenic competence.

Although callus formation was observed at both the basal region and apical dome of the embryonic axes, shoot regeneration occurred exclusively in the aerial meristematic region and proceeded predominantly through direct organogenesis (Fig. 1C–G). This response indicates that the apical dome possesses greater regenerative competence and responsiveness to cytokinin-mediated developmental signals than the basal tissues. Recent studies have demonstrated that meristematic regions maintain a transcriptional and epigenetic landscape that favors organogenic reprogramming, whereas non-meristematic tissues frequently require more extensive dedifferentiation before acquiring regenerative capacity (Xu and Hu 2020; Pasternak and Steinmacher 2024). The absence of shoot formation from callus tissues

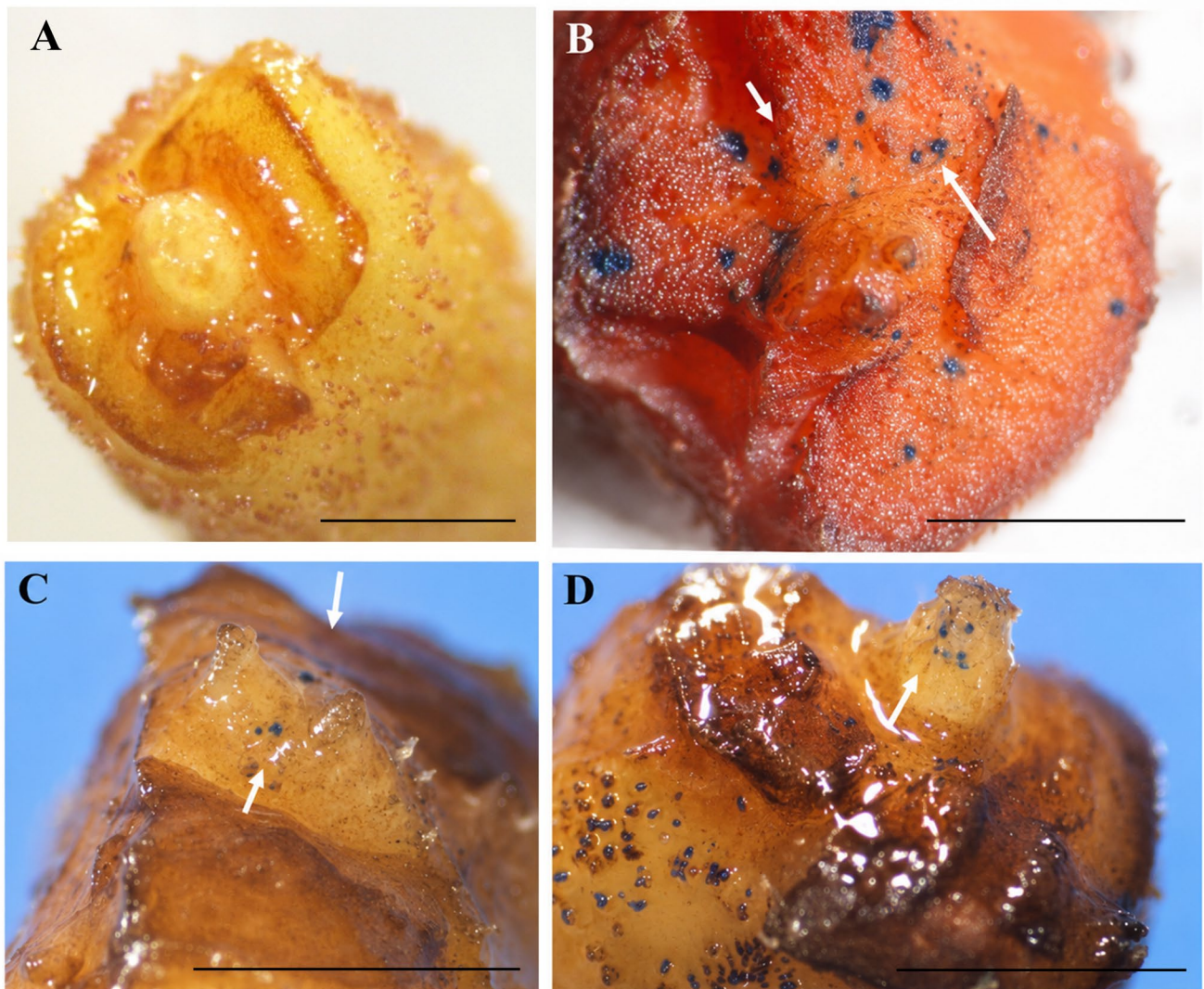


**Fig. 7** Biolistic-mediated genetic transformation of embryonic axes of *Theobroma cacao*. **(A)** Non-transformed control showing no GUS staining. **(B–D)** Representative patterns of transient *uidA* expression in embryonic axes of ‘TSH1188’ three days after particle bombardment with the pCAMBIA2301 vector. **(B)** Multiple independent GUS-

positive foci distributed within the shoot apical meristem region. **(C)** Localized GUS expression in an individual developing shoot primordium. **(D)** GUS expression associated with a developing shoot primordium emerging from the shoot apical region. White arrows indicate blue foci revealed by histochemical GUS assay. Scale bar: 1 mm

further suggests that callogenesis and organogenesis were not developmentally linked under the conditions employed, reinforcing the predominance of direct organogenesis in this regeneration system.

Among the cytokinins evaluated, BAP was consistently superior for multiple shoot induction in both genotypes (Fig. 2C–F). Similar responses have been reported in several woody species, in which BAP promotes efficient shoot proliferation while minimizing the excessive callogenesis and morphological abnormalities frequently associated with highly active synthetic cytokinins such as TDZ (Isah 2019; Bidabadi and Jain 2020). The superior performance of BAP may reflect its ability to promote sustained meristem activity while maintaining an appropriate balance between cell

proliferation and shoot differentiation. Importantly, the optimal concentrations identified in this study ( $0.75 \text{ mg L}^{-1}$  for ‘TSH1188’ and  $1.0 \text{ mg L}^{-1}$  for ‘Parazinho’) were relatively low, reducing both production costs and the risk of physiological disorders associated with prolonged cytokinin exposure.

Genotype-dependent responses were evident throughout the regeneration process. ‘TSH1188’ exhibited greater morphogenic responsiveness during the initial stages of culture (Fig. 2A, C, E), whereas ‘Parazinho’ required longer cultivation periods to achieve comparable shoot induction (Fig. 2B, D, F). Such differences are consistent with previous reports demonstrating strong genotype dependence in cacao tissue culture systems (Maximova et al. 2002; Tan

and Furtak 2003; Guillou and Verdier 2022). Variation in regenerative capacity has been associated with differences in endogenous hormonal composition, sensitivity to exogenous plant growth regulators, and genotype-specific regulation of developmental pathways controlling cell division and organogenesis (Fehér 2019; Rubio et al. 2025). These observations reinforce the importance of optimizing regeneration protocols for individual genetic backgrounds and highlight the need for explant systems capable of supporting transformation across diverse cacao germplasm.

The development of efficient selection systems is a critical prerequisite for successful genetic transformation. The results obtained in this study demonstrated that the response of cacao embryonic axes to selective agents was highly dependent on both genotype and the mode of action of the compound employed. Although kanamycin and hygromycin exerted limited effects on callus formation (Fig. 5A, B), both antibiotics significantly reduced shoot regeneration (Fig. 5F, G), indicating that organogenic development is more sensitive than callogenesis to selective pressure. Similar responses have been reported in other plant transformation systems, in which selective agents primarily affect shoot differentiation rather than the initial proliferation of undifferentiated tissues (Darbani et al. 2007; Tran and Sanan-Mishra 2015).

Among the antibiotic-based selection systems evaluated, hygromycin displayed the strongest inhibitory effect on shoot regeneration (Fig. 5G) and therefore appears to be the most promising selective agent for future transformation studies using embryonic axes. The hygromycin phosphotransferase (*hptII*)/hygromycin system has been widely adopted in plant biotechnology because of its high selection stringency and ability to efficiently eliminate non-transformed cells (Ortiz et al. 1996; Ahmad et al. 2013; Altpeter et al. 2016). In contrast, imazapyr, ammonium glufosinate, and glyphosate exhibited pronounced phytotoxic effects, completely suppressing shoot regeneration at relatively low concentrations (Fig. 5H–J). This response differs from observations reported in soybean embryonic axis-based transformation systems, where imazapyr has been successfully employed as a selective agent due to its preferential accumulation in apical meristematic tissues, enabling efficient selection of transformed cells without compromising embryonic axis viability (Aragão et al. 2000, 2002; Rech et al. 2008; Soto et al. 2017). Similarly, glyphosate has been proposed as a promising selective agent for meristem-based transformation systems because of its ability to translocate and accumulate in actively growing tissues (Mollenhauer et al. 1987; Araújo et al. 2005; Shaner 2009; Dill et al. 2010; Soto et al. 2017). However, the severe inhibition of shoot regeneration observed in cacao (Figs. 3 and 4) suggests that the meristematic cells of embryonic axes are highly

sensitive to these herbicides, even at low concentrations. Because the apical dome represents the primary site of organogenic competence and the target region for biolistic transformation, excessive herbicide toxicity likely compromises both cell survival and subsequent shoot development. These findings indicate that, despite their successful application in other species, herbicide-based selection systems may require substantial optimization for cacao and appear less suitable than antibiotic-based strategies for embryonic axis-mediated transformation.

Root induction represents another critical step for the recovery of complete plants. Among the auxins evaluated, IAA at 0.5 mg L<sup>-1</sup> produced the highest rooting efficiency and generated morphologically normal roots without callus formation (Table 1; Fig. 6). The superiority of IAA may be related to its rapid metabolism and dynamic regulation within plant tissues, allowing transient stimulation of root primordium formation while avoiding excessive callus proliferation (Ludwig-Müller 2011; Pacurar et al. 2014). Direct root formation is particularly advantageous because it generally results in improved vascular connectivity and greater plantlet stability during acclimatization. The production of elongated roots accompanied by vigorous shoot growth further indicates that this treatment promoted a favorable physiological balance between root and shoot development.

A major contribution of this study is the demonstration that mature seed-derived embryonic axes can serve as efficient target tissues for biolistic-mediated gene delivery in cacao. Removal of the leaf primordia exposed the apical dome and facilitated direct access of DNA-coated particles to the meristematic region, resulting in localized transient GUS expression (Fig. 7). The concentration of reporter gene activity within the apical dome suggests that these cells are particularly competent for DNA uptake and transgene expression. This finding is especially relevant because most transformation protocols currently available for cacao rely on *Agrobacterium tumefaciens*-mediated transformation of somatic embryo-derived tissues, particularly cotyledons obtained from primary or secondary somatic embryos (Maximova et al. 2003, 2006; Guillou and Verdier 2022). Although these systems have enabled the recovery of stable transgenic plants and constitute the current standard for cacao transformation, they remain highly genotype dependent and require multiple sequential developmental stages, including embryogenic tissue induction, embryo proliferation, maturation, germination, and plant conversion. In contrast, particle bombardment of mature seed-derived embryonic axes targets pre-existing meristematic tissues capable of direct organogenesis, thereby considerably simplifying the regeneration process. Rather than replacing established *Agrobacterium*-based protocols, the regeneration platform described here should be viewed as

a complementary strategy that may facilitate rapid evaluation of gene constructs, genome-editing reagents, and DNA delivery conditions before stable transformation systems are implemented.

The present study evaluated transient GUS expression as a proof-of-concept demonstration that mature seed-derived embryonic axes constitute competent targets for biolistic DNA delivery. Although transient reporter expression does not demonstrate stable genomic integration or transgene inheritance, it provides a rapid and widely accepted approach for validating DNA delivery into meristematic tissues and assessing the suitability of novel explants for transformation. The recovery of stable transgenic plants will require subsequent optimization of selection pressure, regeneration conditions, and molecular confirmation of transgene integration and inheritance across successive generations. Consequently, the regeneration system established here should be regarded as an important first step toward the development of an efficient stable transformation platform for cacao.

An additional aspect that merits consideration is the use of mature seed-derived zygotic embryos as transformation targets. Unlike somatic tissues obtained from clonally propagated donor plants, zygotic embryonic axes originate from sexual reproduction and therefore may exhibit genetic segregation among individual explants. Consequently, the proposed system is not intended as a platform for clonal propagation of elite genotypes, but rather as a highly regenerative target tissue for transformation, genome editing, and functional genomics studies. Once independent transformed events are recovered, they can subsequently be incorporated into breeding programs or used as donor material for secondary propagation systems. Future studies evaluating additional cacao genotypes will be important to determine the robustness and broader applicability of this regeneration platform across diverse genetic backgrounds.

Importantly, the significance of the regeneration system described here extends beyond its potential use for clonal propagation. In modern genetic transformation and genome-editing pipelines, particularly in recalcitrant species, the primary challenge is often the recovery of transformed events rather than the maintenance of clonal fidelity in the initial explant. Once an edited or transformed event is identified, it can subsequently be incorporated into breeding programs, self-pollinated, backcrossed, or used as a source material for secondary propagation systems. Consequently, the value of mature seed-derived embryonic axes lies in their ability to provide a rapid, accessible, and highly regenerative platform for DNA delivery, selection, and recovery of transformed plants. By reducing dependence on prolonged somatic embryogenesis cycles, this strategy has the

potential to accelerate functional genomics studies, genome editing, and molecular breeding applications in cacao.

Another important advantage of the regeneration system developed in the present study is the substantial reduction in the time required to obtain complete plantlets compared with conventional cacao regeneration protocols based on somatic embryogenesis. Most transformation and regeneration systems currently available for cacao rely on the induction, proliferation, maturation, and germination of somatic embryos, followed by their conversion into plantlets, a process that frequently requires several months and may extend to more than one year depending on the genotype and culture conditions (Maximova et al. 2002; Traore and Guiltinan 2006; Fister et al. 2018; Henao-Ramírez et al. 2021). In contrast, the use of embryonic axes isolated from mature zygotic seeds enabled direct shoot organogenesis within 30–60 days of culture, while rooted plantlets were obtained within approximately 90 days. Thus, the complete regeneration cycle from explant establishment to the production of rooted plants was achieved in approximately three months, representing a substantial reduction in the time required for plant recovery.

Beyond accelerating plant regeneration, the proposed system eliminates several labor-intensive stages associated with somatic embryogenesis, including embryogenic callus induction, secondary embryo proliferation, embryo maturation, and embryo-to-plant conversion. These stages are often characterized by low efficiencies, asynchronous development, and pronounced genotype dependence in cacao. The rapid regeneration response observed from embryonic axes therefore provides a practical advantage for biotechnological applications, particularly genetic transformation and genome editing, where the recovery of transformed tissues within a shorter timeframe is critical for increasing throughput and reducing production costs. Furthermore, the shorter in vitro culture period may reduce the accumulation of somaclonal variation frequently associated with prolonged tissue culture, thereby improving the genetic fidelity of regenerated plants. Nevertheless, because stable transformation efficiency was not evaluated in the present study, direct comparisons with previously published *Agrobacterium*- or biolistic-mediated cacao transformation protocols remain premature. Future studies focusing on stable transformation frequency, transgene inheritance, and genome-editing efficiency will be essential to fully establish the practical performance of this regeneration platform.

Collectively, the results demonstrate that mature seed-derived embryonic axes constitute a highly responsive explant source for regeneration, selection, rooting, and biolistic DNA delivery in *T. cacao*. By integrating rapid direct organogenesis with a simplified regeneration workflow, the proposed system complements existing somatic

embryogenesis-based transformation platforms and provides a promising foundation for future development of stable genetic transformation and genome-editing protocols in cacao.

## Conclusion

This study supports mature seed-derived embryonic axes as a novel and highly responsive explant source for the development of an integrated regeneration and DNA delivery platform in *T. cacao*. Cytokinin supplementation was essential for morphogenic induction, with BAP proving to be the most effective plant growth regulator for multiple shoot regeneration, yielding optimal responses at 0.75 mg L<sup>-1</sup> in ‘TSH1188’ and 1.0 mg L<sup>-1</sup> in ‘Parazinho’. Regeneration occurred predominantly through direct organogenesis restricted to the apical dome, highlighting the high meristematic competence of this region and its suitability for rapid and reproducible plant regeneration.

Among the selective agents evaluated, kanamycin and hygromycin were more suitable than the herbicide-based systems tested, which exhibited severe phytotoxic effects and completely suppressed shoot regeneration. Hygromycin showed the highest selection stringency while maintaining the capacity to recover regenerating tissues, making it a promising selective agent for future transformation protocols based on embryonic axes. For root induction, 0.5 mg L<sup>-1</sup> IAA promoted the formation of morphologically normal adventitious roots without intermediate callus formation, generating vigorous plantlets with characteristics favorable for subsequent acclimatization.

Importantly, transient GUS expression following particle bombardment demonstrated that the exposed apical dome of embryonic axes is highly receptive to DNA delivery, validating this explant as a suitable target tissue for biolistic-gene delivery. Although transient reporter expression does not demonstrate stable genetic transformation, these results provide proof of concept that mature seed-derived embryonic axes are competent recipients for exogenous DNA and represent a promising starting point for the development of stable transformation protocols in cacao. Unlike conventional cacao transformation systems, which rely on prolonged somatic embryogenesis cycles and floral-derived explants, the strategy described here provides a direct and simplified route for the recovery of regenerable tissues.

Collectively, these findings provide a basis for the use of mature seed-derived embryonic axes as a novel regeneration platform that integrates efficient shoot induction, selective-agent screening, rooting, and compatibility with biolistic DNA delivery into a single workflow. This approach substantially shortens the regeneration process compared with

conventional somatic embryogenesis-based systems and provides a practical foundation for future optimization of stable genetic transformation and genome-editing protocols in *T. cacao*. As such, it represents a valuable addition to the biotechnological toolkit available for functional genomics, molecular breeding, and crop improvement in cacao.

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**Author contributions** Conceptualization: SLSP, FCA, FJLA and MGCC; Methodology: SLSP, GMA, FJLA, and MGCC; Experiment and data analysis: SLSP, GMA, GCBS, and MGCC; Writing- original draft preparation: SLSP, GMA, and GCBS; Writing- review and editing: MGCC, FCA, and FJLA; Supervision: MGCC, FCA, and FJLA. All authors read and approved the manuscript for publication.

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**Data availability** All data generated or analyzed during this study are included in this published article and its supplementary information files.

## Ethics declarations

**Ethical approval** Not applicable.

**Competing interest** The authors declare no competing interests.

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