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RESEARCH

Clonal propagation of segregating genotypes through somatic embryogenesis from zygotic embryos in an interspecific oil palm backcross

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Abstract

Oil palm breeding programs are constrained by the reliance on sexual propagation, in which each seedling represents a unique genotype. This intrinsic genetic variability prevents the replicated evaluation of segregating genotypes across genetically comparable individuals and increases the risk of false-positive selection driven by environmental variance. In this study, we investigated whether somatic embryogenesis from zygotic embryos of an interspecific oil palm backcross (*Elaeis oleifera* × *Elaeis guineensis*) x *E. guineensis* could enable the production of multiple plants from a single zygotic embryo, allowing the evaluation of segregating genotypes across genetically similar individuals while maintaining genetic stability. A total of 198 zygotic embryos obtained from mature seeds of a single backcross genotype (SQ 150) were used as explants for somatic embryogenesis induction and plant regeneration. Molecular analyses were performed on 36 regenerated plants derived from three independent donor embryos using ISSR and methylation-sensitive AFLP (MSAP) markers. Differences among classes were assessed using a chi-square (χ^2) test and considered significant at $p<0.05$. ISSR analysis revealed high genetic similarity (97.5–100%) among regenerants derived from the same donor embryo, indicating strong intra-line genetic stability. In contrast, comparisons among regenerants originating from different donor embryos showed approximately 80% similarity, reflecting the expected genetic variability among zygotic embryos. MSAP analysis identified 357 loci, of which 69% were sensitive to DNA methylation. Epigenetic variation was mainly associated with fragment gain events (58%), indicating a predominance of genomic hypomethylation during regeneration. These results demonstrate that somatic embryogenesis from zygotic embryos can generate genetically consistent regenerants within embryogenic lines while maintaining epigenetic plasticity. This approach enables the production of multiple plants from a single zygotic embryo and supports the evaluation of segregating genotypes across genetically similar individuals, potentially improving selection accuracy in oil palm breeding programs.

Key message

Somatic embryogenesis from zygotic embryos of an interspecific oil palm backcross produces genetically consistent regenerants within embryo-derived lines, enabling clonal propagation of segregating genotypes and supporting the evaluation these genotypes across genetically similar individuals in breeding programs.

Keywords DNA methylation · Genetic fidelity · MSAP · Plant breeding · Somaclonal variation · Tissue culture

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Introduction

Somatic embryogenesis is a developmental process involving extensive cellular reprogramming, in which differentiated somatic cells reacquire totipotency and regenerate complete plants. This phenomenon represents an important model system for studying plant developmental plasticity and has become a valuable tool for clonal propagation, germplasm conservation and plant breeding. However, the dedifferentiation and redifferentiation processes involved in somatic embryogenesis may induce genetic and epigenetic alterations that can affect the stability and performance of regenerated plants (Lei et al. 2006; Gantait et al. 2015; Hussain and Nisar 2020; Majumder et al. 2025), as widely reported in studies demonstrating that tissue culture conditions act as abiotic stress factors capable of promoting DNA methylation and demethylation events associated with somaclonal variation (Orłowska 2021), which comprises a wide range of genetic and epigenetic alterations, including sequence polymorphisms, structural variations, and epigenetic modifications that may either contribute to breeding or compromise clonal fidelity (Wang et al. 2022; Das et al. 2025).

Oil palm is one of the most important oil-producing crops worldwide, with yields that may reach 5–7 tons of oil per hectare per year, making it the most productive source of vegetable oil currently cultivated (Feroldi et al. 2014; Murphy et al. 2021). Despite this high productivity, global palm oil production remains largely concentrated in Southeast Asia, particularly in Malaysia and Indonesia (Forster et al. 2018), whereas Brazil contributes only a small fraction to global production, accounting for less than 1% of total world output based on recent estimates (USDA 2024).

The American oil palm (*Elaeis oleifera*) and its hybrids with African oil palm (*Elaeis guineensis*) have been associated with resistance to Fatal Yellowing (FY), an important disease affecting oil palm cultivation in South and Central America (Barcelos et al. 2002; Bittencourt et al. 2021). Consequently, oil palm improvement strategies have focused on interspecific hybridization between *E. oleifera* and *E. guineensis* to combine complementary traits, including slow vertical growth, disease resistance, and improved oil quality from *E. oleifera* with the higher productivity and shorter reproductive cycle of *E. guineensis* (Rios et al. 2012). Backcrossing has been widely employed to progressively recover desirable agronomic traits while maintaining disease resistance (Bespalhok et al. 2011; Cunha et al. 2014).

Despite the importance of these breeding strategies, oil palm propagation relies almost exclusively on sexual reproduction. As a consequence, each seedling represents a unique genotype generated through recombination, resulting in high genetic variability among individuals and limiting the possibility of evaluation of segregating genotypes across genetically comparable individuals. This limitation may reduce the accuracy of phenotypic selection during early stages of breeding programs. In addition, oil palm seeds frequently exhibit slow and irregular germination due to dormancy, further complicating large-scale propagation and evaluation of breeding populations (Gomes et al. 2017). In this context, plant tissue culture techniques, particularly somatic embryogenesis, represent an important alternative for the clonal propagation of elite genotypes (Gomes Jr et al. 2014). In oil palm, somatic embryogenesis is widely used for clonal propagation; however, the occurrence of genetic and epigenetic variation associated with this process has been reported in more recent studies (Gomes et al. 2024). Beyond its role in large-scale propagation, somatic embryogenesis may also allow the production of multiple plants from a single zygotic embryo, enabling the generation of genetically similar individuals sharing essentially the same genetic background. Such an approach could facilitate the evaluation of segregating genotypes and potentially improve the reliability of phenotypic selection in oil palm breeding programs.

However, the extensive cellular reprogramming associated with somatic embryogenesis raises concerns regarding the occurrence of genetic and epigenetic alterations during plant regeneration. For this reason, assessing the genetic fidelity and epigenetic stability of regenerated plants is essential to ensure the reliability of this propagation strategy. Molecular markers provide powerful tools for detecting both genetic polymorphisms and epigenetic modifications associated with in vitro culture processes (Lei et al. 2006; Gantait et al. 2015; Hussain and Nisar 2020). Among these markers, ISSR (Inter-Simple Sequence Repeats) and AFLP (Amplified Fragment Length Polymorphism) are widely used due to their high reproducibility and ability to detect polymorphism across the genome (Kumar et al. 2009; Akçali Giachino et al. 2020). In addition, methylation-sensitive AFLP (MSAP) allows the detection of DNA methylation changes, providing insights into epigenetic variation associated with plant regeneration (Lei et al. 2006). MSAP is a widely used and cost-effective technique for detecting genome-wide DNA methylation changes, particularly in species with complex or uncharacterized genomes (Hermawaty et al. 2024).

Although somatic embryogenesis has been extensively applied in oil palm, previous studies have primarily focused on the clonal propagation of elite genotypes derived from somatic tissues, with emphasis on large-scale multiplication and genetic fidelity assessment. However, the potential of somatic embryogenesis from zygotic embryos to clonally propagate individual segregating genotypes remains largely unexplored. In this context, the present study aimed

to investigate the genetic and epigenetic stability of plants regenerated through somatic embryogenesis from zygotic embryos of an interspecific oil palm backcross (*Elaeis oleifera* × *Elaeis guineensis*). Using ISSR and methylation-sensitive AFLP (MSAP) markers, we evaluated the extent of molecular variation among regenerants derived from different donor embryos and within individual embryogenic lines. By assessing the similarity among plants originating from the same zygotic embryo, we also evaluated the potential of this approach to support genotype-level assessment in oil palm breeding programs.

Materials and methods

Somatic embryogenesis induction in an interspecific oil palm backcross

For the induction of somatic embryogenesis, zygotic embryos obtained from mature seeds of an interspecific oil palm backcross (*Elaeis oleifera* × *Elaeis guineensis*) x *E. guineensis*, genotype SQ 150, were used. The backcross was generated from (*Elaeis oleifera* (RU 3724 D) x *Elaeis guineensis* (LM 2032 T)) x *Elaeis guineensis* (RU 2681 P). The seeds were provided by the Oil Palm Genetic Improvement Program conducted by Embrapa Western Amazon (Rio Urubu Experimental Station), located in Rio Preto da Eva, Amazonas, Brazil (2°35′ S, 59°28′ W; 200 m asl).

Initially, the seed endocarp was removed using a mechanical lathe to obtain the kernels. In a laminar flow cabinet, the kernels were disinfected by immersion in 70% ethanol for 3 min, followed by immersion for 40 min in commercial sodium hypochlorite solution (2–2.5% active chlorine) supplemented with one drop of Tween-20 (Sigma, St. Louis, MO, USA) per 100 mL of solution. Subsequently, three rinses with autoclaved distilled water were performed to remove residual disinfectants. To facilitate embryo excision, the seeds were immersed in sterile water for 24 h prior to embryo excision. After excision under aseptic conditions, embryos were rehydrated in sterile water for 1–2 h prior to inoculating into sterile culture medium, as described in the following section.

A total of 198 zygotic embryos from the SQ 150 genotype were used. Approximately ten embryos were placed in each Petri dish (15 × 90 mm), where each embryo was considered an independent experimental unit. The process was evaluated at two time points: five months and 36 months after culture initiation. The methodology followed that described by Balzon et al. (2013), with minor modifications. All culture media were solidified with 2.5 g L^{-1} Phytagel (Sigma®), and the pH was adjusted to 5.8 ± 0.1 prior to the addition of the gelling agent. Media were sterilized by autoclaving at 121 °C for 20 min.

Induction of embryogenic calluses

Callus induction was performed in Petri dishes (15 × 90 mm) containing 25 mL of modified MS medium (Murashige and Skoog 1962) prepared from MS basal salts (4.4 g L^{-1}; Sigma-Aldrich, St. Louis, MO, USA), supplemented with 20 g L^{-1} sucrose, 0.5 g L^{-1} L-glutamine and 2.5 g L^{-1} activated charcoal. The auxin 4-amino-3,5,6-trichloropicolinic acid (picloram) was added at a concentration of 450 µM, according to the methodology proposed by Scherwinski-Pereira et al. (2010), and all media were prepared and sterilized as described below.

During the callus induction phase, cultures were maintained in darkness in a growth chamber at 25 ± 2 °C and subcultured every four weeks onto fresh medium of the same composition. Cultures remained under these conditions for approximately 150 days before transfer to the callus multiplication medium. During this stage, evaluations of primary callus formation and embryogenic callus differentiation were performed every 30 days for up to 150 days of culture.

Multiplication and maintenance of calluses

For callus multiplication, cultures were transferred after approximately 150 days of induction to modified MS medium supplemented with 20 g L^{-1} sucrose, 0.5 g L^{-1} glutamine, 0.5 g L^{-1} hydrolyzed casein, 10 µM 2-isopentenyladenine (2iP) and 40 µM picloram, without activated charcoal. Embryogenic calluses were visually selected and distributed among Petri dishes in approximately uniform amounts, without precise standardization of callus mass. Cultures were maintained in darkness at 25 ± 2 °C and subcultured every four weeks on medium of the same composition until they reached morphological conditions suitable for transfer to the differentiation medium (approximately three months).

Differentiation of somatic embryos

For somatic embryo differentiation, embryogenic calluses were cultured on modified MS medium supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} glutamine, 12.3 µM 2iP and 0.54 µM naphthaleneacetic acid (NAA), without activated charcoal.

Cultures were maintained in the growth chamber under the same temperature conditions described above and in darkness until the regeneration stage of differentiated somatic embryos.

Somatic embryo regeneration

For plant regeneration, calluses containing differentiated somatic embryos were transferred to test tubes (25 × 150 mm) containing 10 mL of modified MS medium with half-strength salts and vitamins, supplemented with 20 g L^{-1} sucrose and 1.5 g L^{-1} activated charcoal, without plant growth regulators. At this stage, cultures were maintained at 25 ± 2 °C under a 16-h photoperiod with light intensity of approximately 100 µmol m^{-2} s^{-1} provided by LED lamps (Philips® TLED, 20 W, 220–240 V, 50/60 Hz). Cultures remained under these conditions until they were suitable for rooting.

After 36 months of culture, the number of somatic embryos formed per embryogenic callus and the number of regenerated plants were evaluated. Somatic embryo production was classified as follows: class 0 = no somatic embryos produced; class 1 = 1–3 embryos per explant; class 2 = 4–10 embryos per explant; class 3 = > 10 embryos per explant; and class X = explant death or contamination. The number of regenerated plants was recorded individually.

In vitro rooting of regenerated plants

Following regeneration, plantlets lacking roots were individualized and subsequently subjected to in vitro rooting according to the methodology described by Gomes et al. (2016). Plantlets were cultured in test tubes (25 × 150 mm) containing 10 mL of MS medium supplemented with 30 g L^{-1} sucrose and 53.7 µM indole-3-butyric acid (IBA). Cultures were maintained in a growth chamber at 25 ± 2 °C under a 16-h photoperiod with light intensity of 100 µmol m^{-2} s^{-1} until the emergence of at least two roots, after which plantlets were acclimatized in a greenhouse.

Statistical analysis

Statistical analyses were performed using the R statistical software (V. 3.3.0.; R Core Team, 2026). A chi-square (χ^2) test was applied to evaluate differences among classes of somatic embryo production. The null hypothesis (H_0) assumed homogeneous proportions among classes 1, 2 and 3, while the alternative hypothesis (H_a) assumed that at least one class differed significantly. Differences were considered significant at $p < 0.05$.

Molecular analysis of regenerated plants from an interspecific oil palm backcross

For molecular analyses, young leaves were collected from plants regenerated through somatic embryogenesis from three independent donor zygotic embryos, identified as matrices 1, 2 and 3. Donor zygotic embryos were not selected based on somatic embryo production classes, but rather from responsive explants that successfully produced embryogenic callus and regenerated plants. For each donor embryo, 12 regenerated plants of the interspecific backcross SQ 150 were sampled, totaling 36 individuals. Each plant was considered an independent biological replicate ($n = 12$ per donor embryo; total $n = 36$).

Genomic DNA was extracted from plant material using a CTAB-based method (Doyle and Doyle 1990). DNA concentration was estimated using a spectrophotometer and its integrity verified by 1% agarose gel electrophoresis stained with ethidium bromide. DNA samples were stored at -20 °C until use. Working solutions were prepared at concentrations of 3 and 20 ng μL^{-1} for subsequent PCR amplifications.

ISSR analysis

For the assessment of genetic fidelity, 29 ISSR primers were screened based on the number of amplified bands and the quality of the generated fragments (Table 1). ISSR reactions were performed in a final volume of 13 µL containing 1x PCR buffer, 1.3 µL of 2.5 µg μL^{-1} BSA, 0.25 mM each dNTP, 1.3 U Taq DNA polymerase, 3 µL of primer (1.2 mM), and 9 ng genomic DNA.

PCR amplifications comprised initial denaturation at 94 °C for 5 min, then 35 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 1 min; followed by a final extension at 72 °C for 7 min. Amplified fragments, together with a 1-kb DNA ladder (Invitrogen), were separated by electrophoresis on 1.5% agarose gels stained with ethidium bromide. The resulting banding patterns were visualized under UV light and recorded using a gel documentation system.

For ISSR data analysis, fragments of the same molecular weight were considered a locus and scored as binary data, with "1" indicating presence and "0" indicating absence of the band. The proportion of polymorphic fragments was used to estimate the level of genomic alteration associated with in vitro culture.

A fragment was considered polymorphic when it was present in at least one individual and absent in others, or absent in at least one individual and present in others. To increase the reliability of the results, ISSR reactions corresponding to loci that showed putative somaclonal variation, as well as weakly amplified bands, were repeated three times. Genetic similarity among isolates was estimated using the Jaccard coefficient (Sneath and Sokal 1962) in NTSYSpc (v. 2.1; Rohlf 2000). The dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA).

Table 1 List of the 29 primers used in the genetic fidelity analysis of oil palm plants produced via somatic embryogenesis using ISSR markers and their respective sequences

Primer	Sequence (5' −3')	Primer	Sequence (5' −3')
2zm	CACACACACACACAGG*	52zm	GGCACCACACACACACACACA
3zm	CACACACACACACACAAT	53zm	CGCAACACACACACACACACA
4zm	CACACACACACACACAAC	54zm	GGCTACACACACACACACACA
6zm	CACACACACACACACAGC	55zm	CCTCCACACACACACACACA
7zm	CACACACACACACAATCT	56zm	GCTACCACACACACACACACA
8zm	CACACACACACACAATCC	57zm	CGTCCACACACACACACACA
11zm	TGTCACACACACACAC	58zm	CGAACCACACACACACACACA
12zm	GGTCACACACACACAC	59zm	GGCCAGCTGCTGCTGCTGCTGCT
15zm	GTGCACACACACACAC	60zm	GCCACGCTGCTGCTGCTGCTGCT
16zm	CGGCACACACACACAC	5pv	ACTGACTGACTGRG
17zm	CAGCTCTCTCTCTCTC	9ps	ACACACACACACACACYA
18zm	GTGCTCTCTCTCTCTC	10ps	AGAGAGAGAGAGAGAGYT
32zm	AGCAGCAGCAGC	11ps	GTGTGTGTGTGTGTGTYC
39zm	AGCAGCAGCAGCAC	12ps	CACACACACACACA
51zm	GCACCCACACACACACACACACA		

Table 2 Sequences of MSAP adapters and primers used in the analysis of epigenetic fidelity of oil palm clones produced through somatic embryogenesis

Name	Enzyme	Type	Sequence (5'–3')
EcoA+	EcoRI	Adapter (+)	CTCGTAGACTGCGTACC
EcoA-	EcoRI	Adapter (-)	AATTGGTACGCAGTCTAC
Mse/HpaA+	MspI/HpaII	Adapter (+)	GACGATGAGTCTAGAA
Mse/HpaA-	MspI/HpaII	Adapter (-)	CGTTCTAGACTCATC
Eco+A	EcoRI	Primer+1	GACTGCGTACCAATTCA
MH+T	MspI/HpaII	Primer+1	GATGAGTCTAGAACGGT
Eco+AAG	EcoRI	Primer+3	*GACTGCGTACCAATTCAAG
Eco+AGG	EcoRI	Primer+3	*GACTGCGTACCAATTCAGG
Eco+ACG	EcoRI	Primer+3	*GACTGCGTACCAATTCACG
MH+TAA	MspI/HpaII	Primer+3	GATGAGTCTAGAACGGTAA
MH+TCC	MspI/HpaII	Primer+3	GATGAGTCTAGAACGGTCC
MH+TAG	MspI/HpaII	Primer+3	GATGAGTCTAGAACGGTAC
MH+TAC	MspI/HpaII	Primer+3	GATGAGTCTAGAACGGTAC
MH+TGA	MspI/HpaII	Primer+3	GATGAGTCTAGAACGGTGA
MH+TGG	MspI/HpaII	Primer+3	GATGAGTCTAGAACGGTGG
MH+TTA	MspI/HpaII	Primer+3	GATGAGTCTAGAACGGTTA

*Primers marked with VIC® fluorochrome

MSAP analysis

Epigenetic variation was assessed using a methylation-sensitive amplified polymorphism (MSAP) approach (Xiong et al. 1999). Initially, 100 ng of genomic DNA were digested using parallel reactions comprising enzyme EcoRI in combination with one or other of the differentially methylation-sensitive frequent-cutting isoschizomers HpaII or MspI. Restriction digestion and AFLP adapter ligation were performed simultaneously in a final volume of 20 µL containing 1X ligase buffer (Promega), 50 mM NaCl, 0.05 µg/µl bovine serum albumin, one unit T4 DNA ligase (Promega), 5 pmol EcoRI adapter, 50 pmol MspI/HpaII adapter, five units EcoRI (EcoRI-HF high fidelity, NEB), five units of either MspI or HpaII and 100 ng genomic DNA (Table 2). The reaction mixture was incubated at 37 °C for 2 h, followed by 1 h at 17 °C. Subsequently, 80 µL of ultrapure water was added to the digestion-ligation product.

Pre-selective amplification was performed using primers containing one additional selective nucleotide, with base A for the EcoRI primer and base T for the HpaII/MspI primer (Table 2). Reactions were carried out in a final volume of 20 µL containing 1 M betaine, 1X PCR buffer with 2 mM Mg^{2+}, 0.25 mM dNTPs, 1 U Taq DNA polymerase, 0.5 µM Eco+A primer, 0.5 µL MH+T primer, and 2 µL of the diluted adapter-ligase DNA. The isoschizomers HpaII and MspI recognize the same restriction site (5′-CCGG-3′), but differ in their sensitivity to cytosine methylation. MspI cleaves the site when the internal cytosine is methylated (5′-CCmGG-3′), but does not cut when the external

cytosine is methylated (5′-mCCGG-3′). In contrast, HpaII cleaves when the external cytosine is hemi-methylated but is blocked when the internal cytosine is methylated (Francischini et al. 2017).

Pre-selective PCR was carried out using the following program: 72 °C for 2 min; 20 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 2 min; followed by 72 °C for 2 min and 60 °C for 30 min. After pre-selective amplification, 80 µL of ultrapure water was added to dilute the reaction products. Selective amplification was then carried out using the following EcoRI/HpaII-MspI primer combinations with three selective bases: AAG-TAA, AAG-TCC, AAG-TAG, AAG-TAC, AAG-TGA, ACG-TGG, and AGG-TTA (Table 2). Each selective amplification reaction was performed in a final volume of 10 µL containing 1X PCR buffer with 2 mM Mg^{2+}, 1 U Taq DNA polymerase, 0.15 µM of Eco + 3 primer labeled with VIC® fluorochrome, 0.15 µM MH + 3 primer, 0.2 mM dNTPs, and 2 µL of diluted pre-amplified DNA. Selective PCR consisted of an initial denaturation at 94 °C for 2 min, followed by 10 cycles of 94 °C for 30 s, 66 °C for 30 s with a decrease of 1 °C per cycle, and 72 °C for 1 min; then 25 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; followed by a final extension at 72 °C for 3 min and 60 °C for 30 min.

After amplification, 1 µL of each selectively amplified sample was mixed with 9.5 µL of Hi-Di formamide (Applied Biosystem) and 0.4 µL GeneScan™ 600 LIZ™ dye size standard v2.0 (Applied Biosystems), containing 36 fragments between 20 and 600 bp. The mixture was denatured at 95 °C for 5 min, and fragments were separated by capillary electrophoresis using an ABI 3730 Genetic Analyzer (Applied Biosystems).

Analysis of MSAP data

MSAP profiles were initially inspected using Genographer 2.1 to evaluate amplification quality. Electrophoretic data was converted to sized fragment length Peaks using Peak Scanner 2 (Applied Biosystems). Data was then filtered, scored, and converted to a binary matrix using the R CRAN RawGeno AFLP package (v.2.0–1; Arrigo et al. 2009), when "1" indicated presence and "0" absence of a fragment. Only fragments ranging from 50 to 550 bp were considered, and to improve scoring consistency, automatic peak calling was refined by manual inspection.

To evaluate epigenetic relationships among regenerated individuals, binary presence/absence data was further processed using the R CRAN package MSAP (v. 1.1.9; Pérez-Figueroa 2013). Data were subjected to principal coordinate analysis (PCoA) and analysis of molecular variance (AMOVA; Excoffier et al. 1992). The proportion of methylation-sensitive and methylation-insensitive loci was also determined, and the number of HpaII- and MspI-associated fragments lost or gained among regenerated individuals quantified. As described for ISSR analysis, a fragment was considered polymorphic when it was present in at least one individual and absent in others, or absent in at least one individual and present in others.

Results and discussion

Somatic embryogenesis of an interspecific oil palm backcross

Of the 198 zygotic embryos of genotype SQ 150 inoculated for callus induction, 161 (81.3%) showed swelling after 15 days of culture, indicating the onset of callogenic response. After 30 days in induction medium, these responsive embryos had developed primary callus, whereas 37 embryos (18.7%) remained unresponsive. At this stage, however, no embryogenic callus formation was observed (Fig. 1).

Initial development progressed steadily over time, with 87.5% of the explants forming primary callus after 60 days, of which approximately 56.5% exhibited embryogenic characteristics. At 90 days, 93.1% of the responsive zygotic embryos had developed primary callus, and 59.0% of them subsequently differentiated into embryogenic calluses. By 120 days, more than 98% of the explants had formed primary callus, reaching 100% at 150 days of culture. At this stage, more than 78% of the primary calluses had differentiated into embryogenic calluses, with no substantial changes thereafter.

Such high callogenesis efficiency is consistent with the broad variability reported for palms, which is strongly influenced by genotype, explant type, developmental stage, and culture conditions, particularly the type and concentration of auxins used for induction. Studies using zygotic embryos across Arecaceae report primary callus formation ranging from approximately 20% to nearly 100% under optimized conditions (Balzon et al. 2013; Campos et al. 2020; Saleh et al. 2024).

Within this context, oil palm exhibits a similar pattern, in which callus formation from zygotic embryos may exceed 90% under favorable conditions, although strong genotypic variation has been documented (Silva et al. 2012; Balzon et al. 2013). Accordingly, the near-complete callogenesis observed herein represents a high-response scenario within the upper range reported for the species.

In contrast, explant origin markedly affects morphogenic competence. Somatic embryogenesis from immature leaf explants, which are widely used in oil palm propagation, typically show much lower responsiveness, with callus

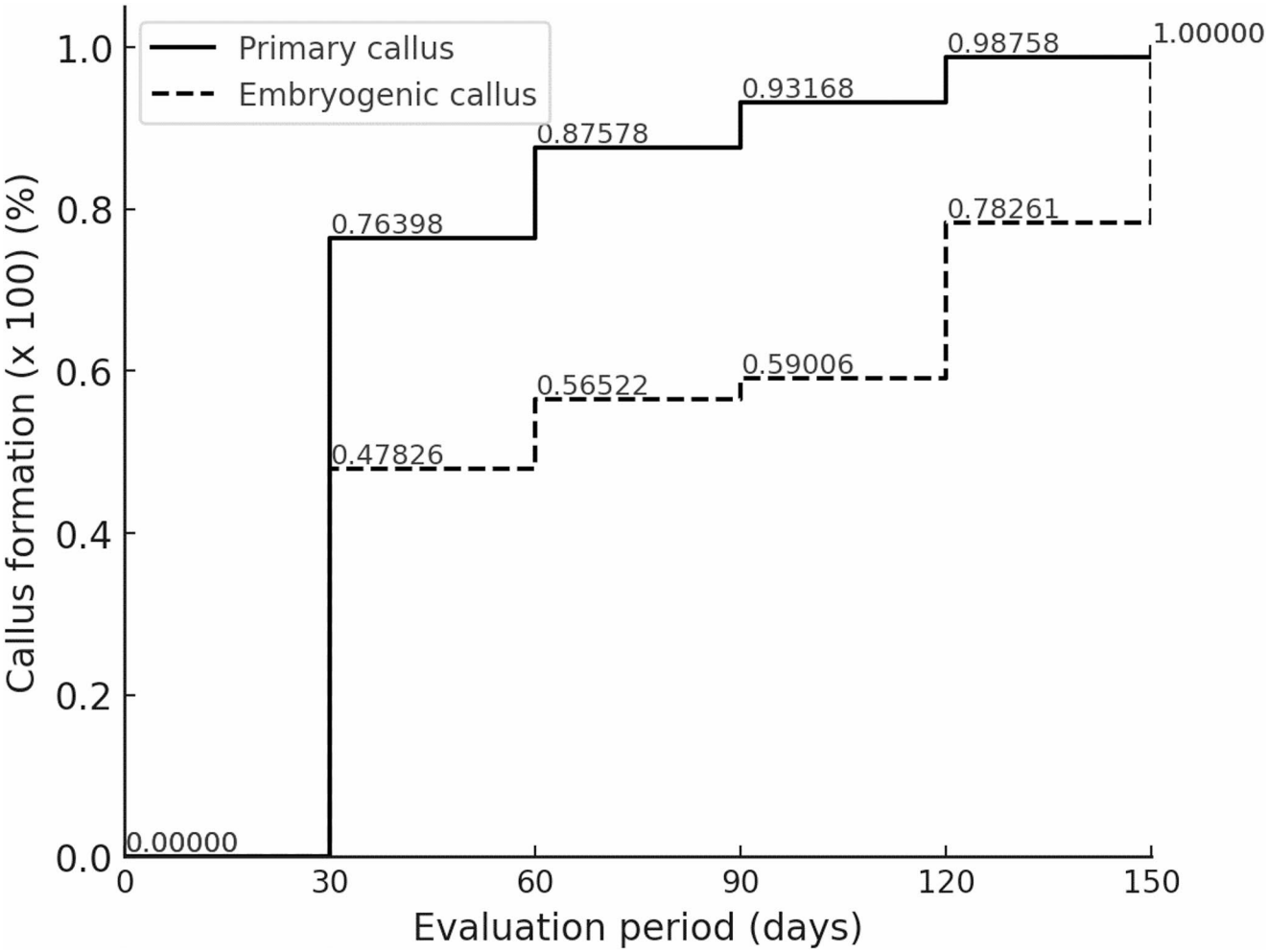


Fig. 1 Kaplan–Meier curve showing the formation of primary and embryogenic callus from 161 zygotic embryos responsive to callus induction. Evaluations were performed at 0, 30, 60, 90, 120, and 150 days of culture in the induction medium

formation rates around 10–15% even under optimized conditions (Silva-Cardoso et al. 2022; Gomes et al. 2024).

While primary callus formation may reach high frequencies in zygotic embryo-based systems, the subsequent transition to embryogenic callus remains the critical limiting step. This phase is widely recognized as the main bottleneck of indirect somatic embryogenesis, with reported frequencies often below 50%, although higher values may be achieved under optimized conditions (Silva et al. 2012; Campos et al. 2020; Ferreira et al. 2022). In this regard, the embryogenic differentiation rates observed here (> 78%) indicate a highly efficient system, comparable to the best-performing protocols reported for palms.

The progressive increase in responsiveness observed over time (Fig. 1) further suggests a strong effect of prolonged exposure to picloram, ultimately reaching full induction efficiency. The effectiveness of picloram for inducing callogenesis and somatic embryogenesis has been reported for *Elaeis guineensis* (Balzon et al. 2013) and other palm species, including *Acrocomia aculeata* (Granja et al. 2018), *Bactris gasipaes* (Steinmacher et al. 2007), *Calamus merrillii* and *C. subinermis* (Goh et al. 2001), and *Euterpe oleracea* (Scherwinski-Pereira et al. 2012).

These results indicate that the high morphogenic competence observed in this study is likely associated with the characteristics of the zygotic embryo explant and the sustained inductive effect of picloram, which promotes cellular dedifferentiation and reprogramming required for the establishment of embryogenic competence. Following the induction phase described above, calluses were transferred to multiplication medium for at least four months, followed by three months in differentiation medium and subsequent transfer to regeneration medium. Plant regeneration was evaluated 36 months after culture initiation.

Analysis of somatic embryo production per explant (Table 3) showed that 59 explants (36.6%) did not produce somatic embryos, whereas 55 explants (34.2%) produced between 1 and 3 embryos, 29 explants (18.0%) produced

Table 3 Absolute and relative frequency of the 161 SQ 150 genotype explants that formed somatic embryos

Class	Number of somatic embryos per explant	Explants (*n*)	Frequency (%)
Class 0	0	59	36.6
Class 1	1–3	55	34.2
Class 2	4–10	29	18.0
Class 3	>10	17	10.6
Total	—	161	100

Table 4 Observed and expected frequencies of somatic embryo production per explant in the SQ150 genotype used for χ^2 analysis

Class	Somatic embryos per explant	Observed frequency (*n*)	Expected frequency (*n*)
Class 1	1–3	55	33.7
Class 2	4–10	29	33.7
Class 3	>10	17	33.7
Total	—	101	101

Expected frequencies were calculated assuming equal distribution among embryo-producing classes (101/3 = 33.7)

Table 5 Chi-square contributions of each class of somatic embryo production per explant in genotype SQ150

Class	Somatic embryos per explant	χ^2 contribution
Class 1	1–3	13.518
Class 2	4–10	0.647
Class 3	>10	8.251
Total χ^2	—	22.416

χ^2 test assuming equal expected frequencies among embryo-producing classes; df = 2; $p = 1.357 \times 10^{-5}$

between 4 and 10 embryos, and 17 explants (10.6%) produced more than 10 embryos per explant. This variability is consistent with the observations of Quiroz-Figueroa et al. (2006), who emphasized that not all cells exposed to inductive conditions express totipotency. Such differences may reflect variation in cellular competence, differential sensitivity to growth regulators, or the availability of regulatory factors required for the transition to the embryogenic state (Guzzo et al. 1994). Accordingly, the predominance of explants producing relatively few somatic embryos suggests that embryogenic competence may be unevenly distributed within the induced callus tissues. This interpretation is consistent with the concept that somatic embryos often originate from small clusters of competent cells rather than from the entire callus mass (Yeung 1995).

Overall, the number of somatic embryos obtained for genotype SQ 150 fell within the range expected for oil palm (Table 4). To verify whether embryo production differed significantly among the defined classes (1–3, 4–10, and >10 embryos per explant), a chi-square test was performed. The null hypothesis assumed homogeneous proportions among classes, whereas the alternative hypothesis assumed that at least one class differed significantly (Table 5). The test yielded $\chi^2 = 22.416$ and $p = 1.357 \times 10^{-5}$, indicating significant differences among classes and rejection of the null hypothesis. These results confirm that embryo production is not uniformly distributed among explants, with a clear predominance of low-output classes, whereas only a small proportion of explants exhibited high embryogenic performance. Despite this variability, the system proved capable of producing a substantial number of regenerants, yielding an average of 88 regenerated plants for the SQ 150 genotype, corresponding to approximately 55% of the responsive explants. The morphological sequence of somatic embryogenesis from zygotic embryos of the interspecific oil palm backcross is illustrated in Fig. 2. All regenerated plants successfully established during greenhouse acclimatization and exhibited no visible morphological abnormalities. Plants showed uniform growth patterns, with consistent leaf morphology and overall architecture among individuals derived from the same embryogenic line.

Beyond its propagation value, this pattern of embryogenic response also has relevant implications for breeding. Since each zygotic embryo corresponds to a distinct segregating genotype, the ability to regenerate multiple plants from a single embryo creates the possibility of generating genetically similar individuals with essentially the same genetic background. This may help reduce false-positive selection during phenotypic evaluation by allowing genotype-level assessment rather than single-plant evaluation, an aspect of particular relevance for perennial crops such as oil palm.

Genetic fidelity assessed by ISSR markers

Figure 3 shows the UPGMA dendrogram generated to assess the genetic relationships among plants regenerated through somatic embryogenesis from zygotic embryos of the interspecific oil palm backcross SQ 150. ISSR analysis was able to clearly differentiate each of the three donor zygotic embryos used as the explant source. However, no detectable genetic variation was observed among plants regenerated from donor embryos 1 and 3 (Fig. 3), which is consistent with the expectation that regenerants derived from a single zygotic embryo represent genetically coherent lineages, reflecting the propagation of a segregating genotype rather than independent somatic events.

In contrast to donor embryos 1 and 3, significant polymorphism was detected among regenerants derived from donor embryo 2, particularly with primers 32 zm and 59 zm (Fig. 4A and B), reaching 2.5% dissimilarity (Fig. 3). In some contexts, such polymorphism may represent exploitable variability for breeding purposes (Naz et al. 2016; Bahmankar et al. 2017). However, in the context of clonal

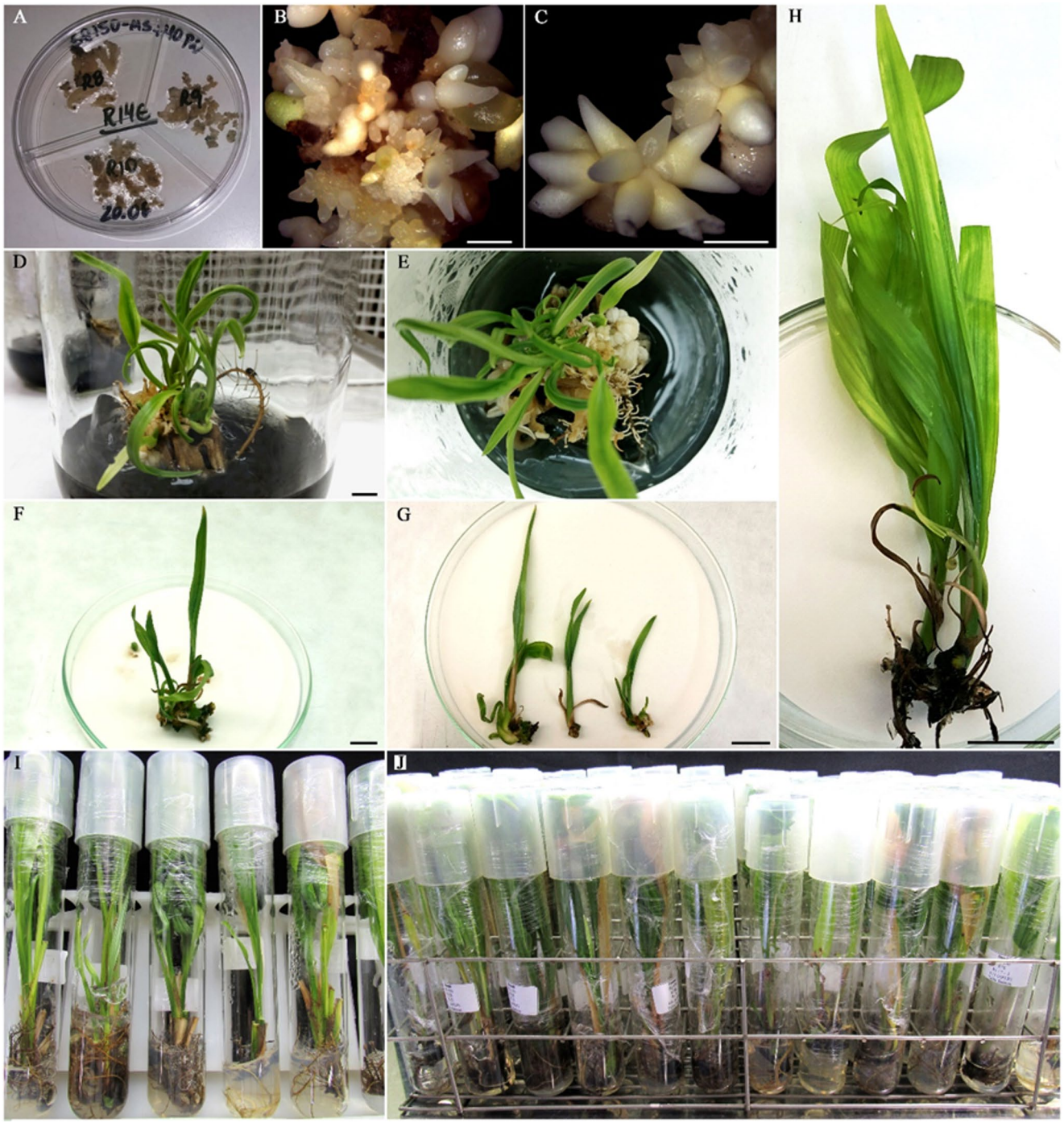


Fig. 2 Morphological aspects of somatic embryogenesis in an interspecific oil palm backcross (genotype SQ 150). (**A**) Callus multiplication. (**B**) Callus with somatic embryos at different developmental stages. (**C**) Torpedo-stage somatic embryos. (**D**, **E**) Cluster of rooted plantlets. (**F**) Regenerated plantlets ready for individualization. (**G**) Individualized plantlets. (**H**) Plantlets with developed roots used for molecular analyses. (**I**, **J**) Regenerated plants. Bars: (**B**, **C**) 2 mm; (**D**, **G**, **H**) 1 cm; (**F**) 0.5 cm

propagation and germplasm conservation, genetic variation among regenerants is generally considered undesirable because it is indicative of somaclonal variation (Krishna et al. 2016; Naz et al. 2016). Recent genomic-scale evidence indicates that somatic embryogenesis-based systems can exhibit markedly increased mutation rates and structural genomic variation, including chromosomal duplications and large-scale rearrangement (Davis et al. 2026). In this context, the low level of polymorphism detected within most embryo-derived lines in the present study contrasts with

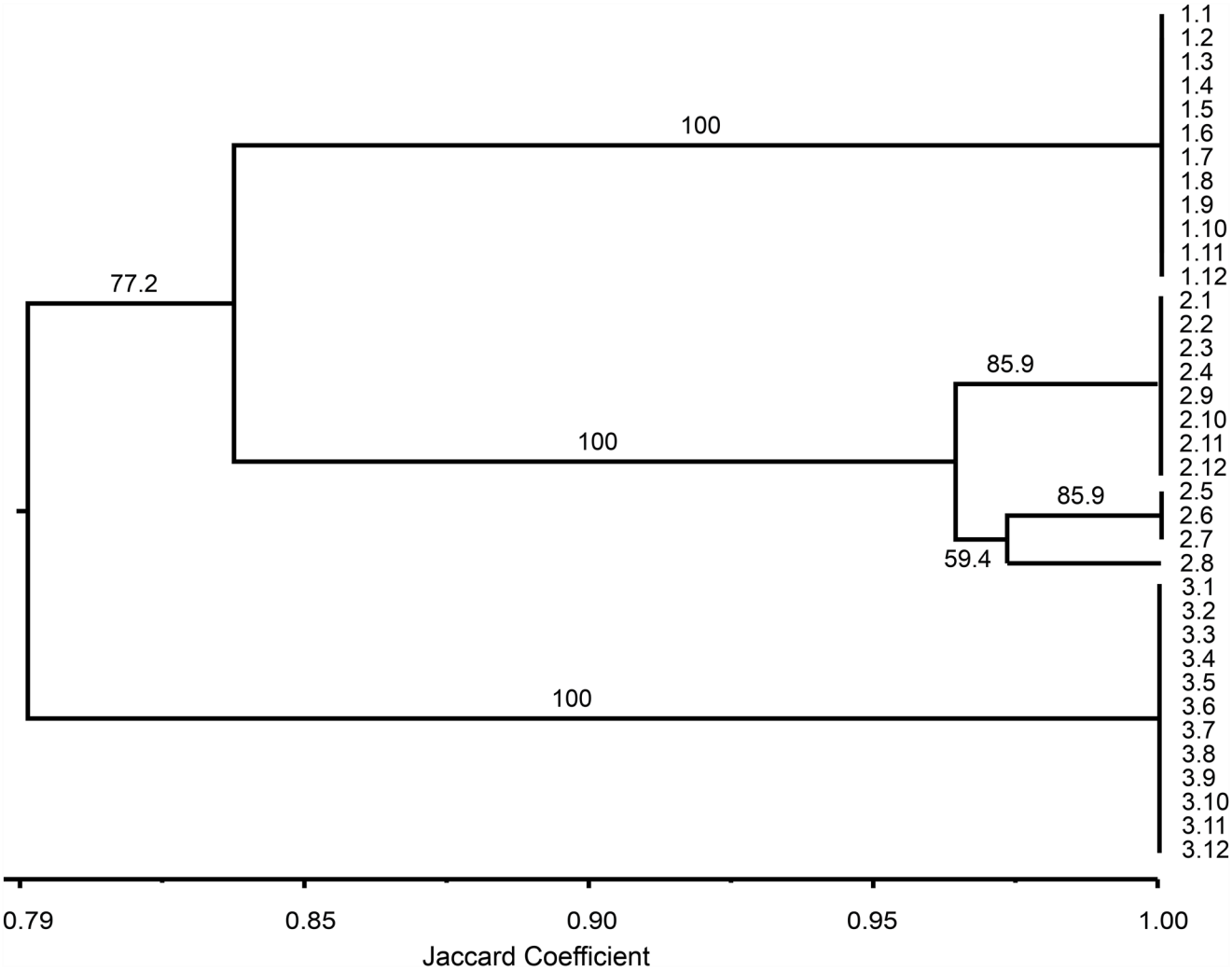


Fig. 3 UPGMA dendrogram showing the genetic relationship among plants regenerated via somatic embryogenesis from zygotic embryos of an interspecific oil palm backcross (genotype SQ 150). Labels 1.1–1.12 correspond to plants regenerated from donor embryo 1; 2.1–2.12 correspond to plants regenerated from donor embryo 2; and 3.1–3.12 correspond to plants regenerated from donor embryo 3

scenarios of extensive genome instability reported under prolonged or highly proliferative in vitro conditions, suggesting that such effects were limited under the conditions employed in the present study.

This level of genetic homogeneity supports the view that, within a given embryogenic line, regenerated plants are genetically consistent enough to be considered clonally derived and highly similar individuals for practical purposes. In contrast, regenerants derived from different donor embryos showed approximately 80% similarity (Fig. 3), which reflects the expected genetic divergence among distinct zygotic embryos produced by sexual reproduction.

This distinction is critical because it separates culture-induced variation from inherent genetic variability associated with the zygotic origin of the explants. Therefore, the lower similarity observed among donor embryos should be interpreted as a consequence of genetic segregation rather than somaclonal variation.

From a practical perspective, somatic embryogenesis from zygotic embryos may provide an effective approach to clonally propagate segregating genotypes and thus support the evaluation of individual genotypes across genetically similar individuals in breeding programs. The low level of polymorphism detected within donor-derived lines may be associated with the stressful conditions inherent to in vitro culture, which can promote genetic instability in regenerants (Modgil et al. 2005). According to Cullis (1999), genetic changes may arise as adaptive responses to stress. However, the limited polymorphism observed here suggests that, under the conditions employed, the mutagenic effects of tissue culture were relatively constrained, particularly when compared to highly proliferative systems or long-term maintained cultures. Similar ISSR-based detection of

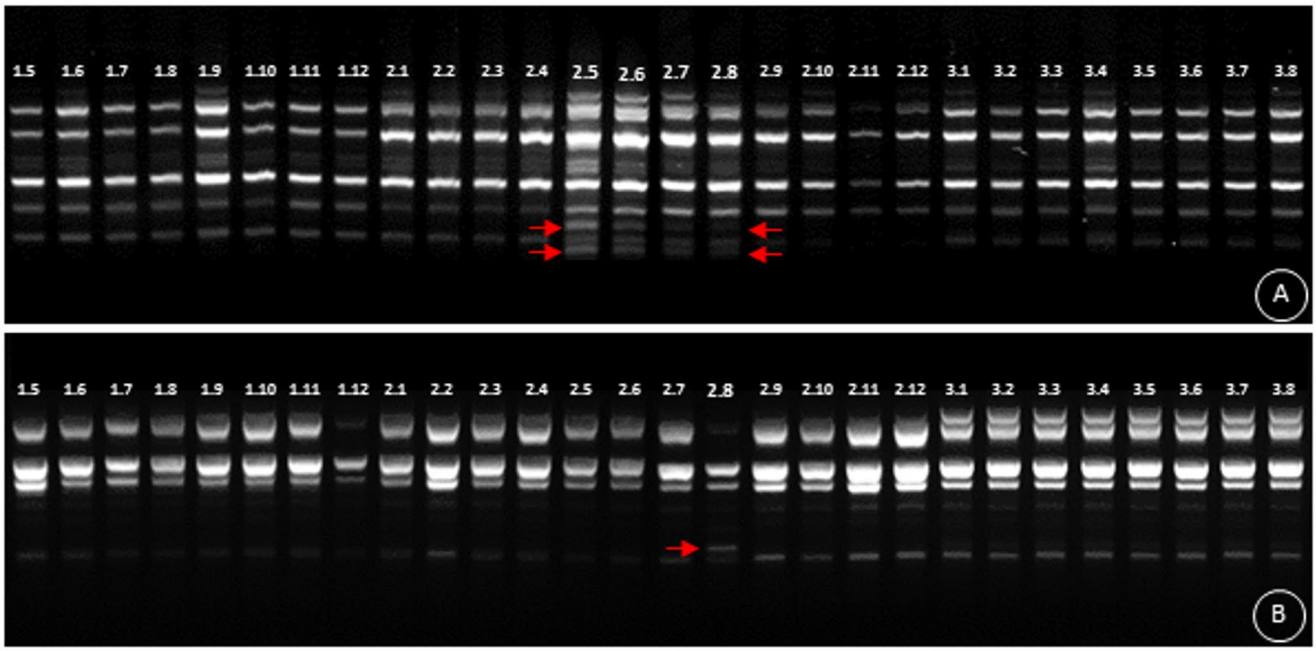


Fig. 4 Genetic fidelity analysis using ISSR markers in plants regenerated by somatic embryogenesis from zygotic embryos of an interspecific oil palm backcross (genotype SQ 150). (**A**) Primer 32 zm. (**B**) Primer 59 zm. Red arrows indicate somaclonal variation

Table 6 Summary of methylation-sensitive AFLP (MSAP) loci detected in regenerated plants of an interspecific oil palm backcross

Number of individuals	Total amplified loci	Methylation-sensitive loci	Methylation-free loci
36	357	245 (69%)	112 (31%)

Table 7 Analysis of molecular variance (AMOVA) of the loci submitted or not to the methylation process

Source of variation	df	Sum of squares	Variance component	Percentage of variation
Among groups (embryos)	2	125.8	0.79	3.86
Within groups (regenerants)	32	570.4	19.68	96.14
Total	34	696.1	20.47	100

AMOVA based on methylation-sensitive loci (MSL). Significance tested by permutation ($p < 0.0001$)

tissue culture-induced polymorphism has been reported in several species, including *Calliandra tweedii* (Heikrujam et al. 2014), *Ficus carica* (El-Dessoky et al. 2016), *Ananas comosus* (Kohpaii et al. 2017), *Saccharum officinarum* (Thorat et al. 2017), and *Vitellaria paradoxa* (Attikora et al. 2025). Taken together, the very high similarity observed within most embryo-derived lines in the present study indicates that somatic embryogenesis can maintain substantial genetic fidelity in this oil palm backcross.

Epigenetic variation assessed by MSAP markers

A total of 357 loci were scored in the 36 regenerated plants, of which 245 (approximately 69%) were sensitive to methylation (Table 6). This relatively high proportion of methylation-sensitive loci, together with the low level of genetic variation detected by ISSR, is consistent with the notion that epigenetic changes are often more frequent than genetic alterations in plants regenerated through tissue culture (Miguel and Marum 2011). A possible inverse relationship between the extent of genetic and epigenetic variation has also been suggested by Linacero et al. (2011). However, more recent evidence indicates that genetic and epigenetic changes are not necessarily inversely related but may coexist and be mechanistically interconnected (Bednarek and Orłowska 2020).

In line with this, AMOVA of the methylation-sensitive loci indicated that most of the epigenetic variation was distributed within donor embryo derived regenerant groups rather than among the three donor embryos. Specifically, 96.14% of the total variation occurred within donor embryo-derived groups, whereas only 3.86% was detected among donor embryos (Table 7). This pattern indicates that epigenetic modifications arising during somatic embryogenesis were largely independent of the donor embryo of origin. Consistent with these results, principal coordinate analysis (PCoA) based on the 245 methylation-sensitive loci revealed detectable epigenetic dissimilarity among regenerated plants, although the overall magnitude of this variation remained relatively limited (Fig. 5). Plants derived

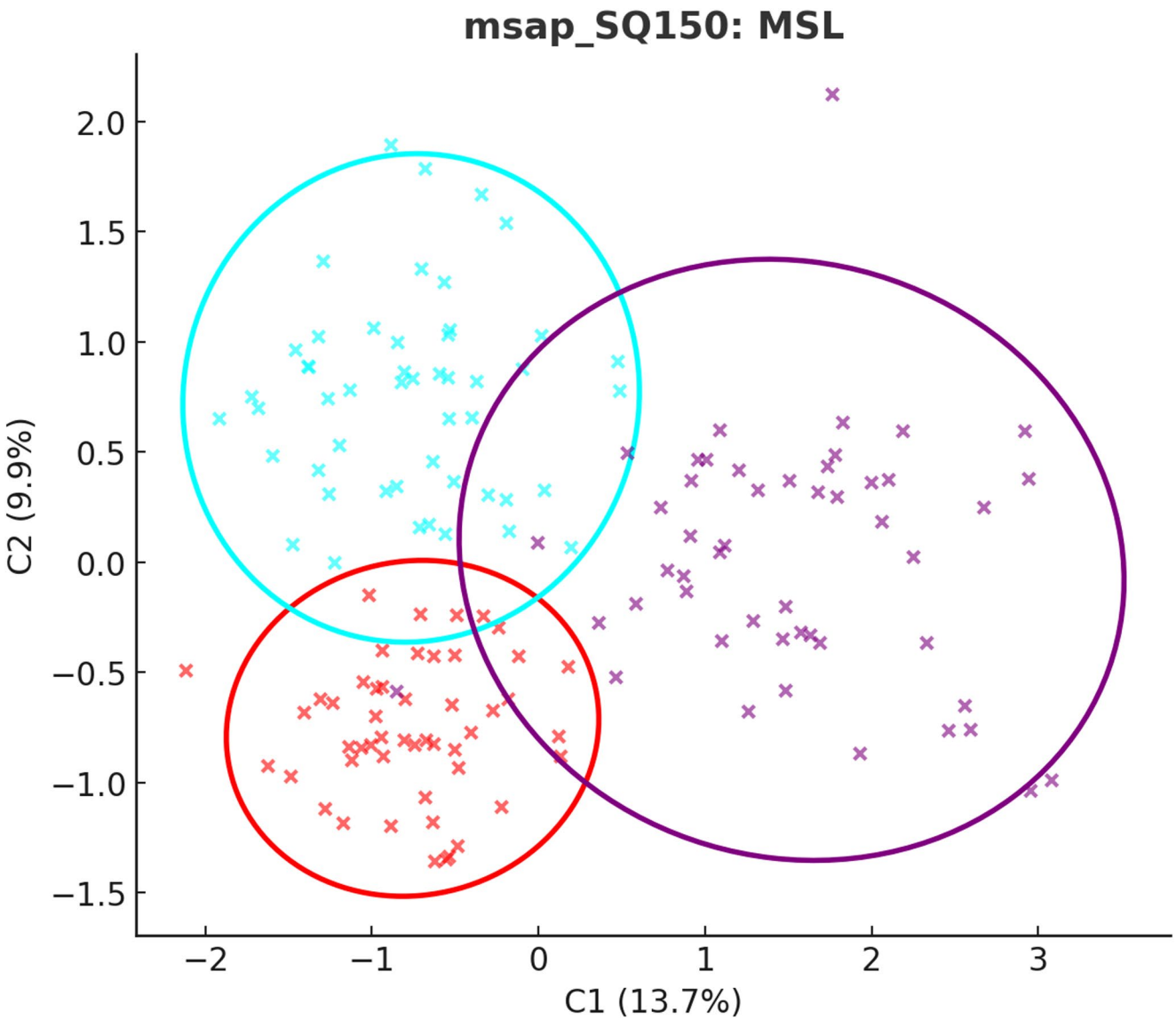


Fig. 5 Principal coordinates analysis (PCoA) based on methylation-sensitive loci for regenerated plants obtained by somatic embryogenesis from zygotic embryos of an interspecific oil palm backcross (genotype SQ 150). Axes C1 and C2 indicate the percentage of explained variance. Each point represents an individual plant. Ellipses represent the dispersion of individuals within each donor-derived group

from donor embryo 1 exhibited the lowest dispersion in the ordination space, indicating greater epigenetic similarity within this group, whereas regenerants originating from donor embryo 3 showed comparatively higher levels of epigenetic variation relative to those derived from donor embryos 1 and 2.

The epigenetic profiles observed here are consistent with the role of DNA methylation dynamics during somatic embryogenesis. Recent experimental evidence indicates that DNA methylation changes are characteristic of the transition of somatic cells to an embryogenic state and may regulate chromatin accessibility and the activation of totipotency-related genes during somatic embryogenesis (Peng et al. 2025). In addition, transient DNA demethylation has been shown to promote somatic embryogenesis induction and proembryogenic mass proliferation, whereas sustained demethylation may impair subsequent embryo differentiation (Carneros et al. 2024). In oil palm, Silva-Cardoso et al. (2022) demonstrated that embryogenic competence is associated with DNA hypomethylation during the induction phase, coinciding with the reactivation of procambial cells and the onset of callus formation, while non-responsive genotypes maintained higher methylation levels throughout culture. These findings also support the interpretation that stress conditions associated with in vitro culture may induce genome-wide methylation changes, which in some cases have been associated with genomic instability through mechanisms such as transposable element activation

(Bednarek and Orłowska 2020; Das et al. 2025). Together, these results indicate that this pattern of hypomethylation among individual regenerants observed in the present study may reflect molecular processes associated with cellular reprogramming that are typically linked to the acquisition of embryogenic competence.

These findings agree with previous reports showing that in vitro culture frequently induces epigenetic remodeling (Zhang et al. 2009; Miguel and Marum 2011; Linacero et al. 2011; De-la-Peña et al. 2012; Us-Camas et al. 2014). Such epigenetic changes are often associated with stress imposed by in vitro conditions and with the dedifferentiation and cellular reprogramming processes required for somatic embryo formation (Koukalova et al. 2005; De-la-Peña et al. 2015; Liu et al. 2017). Therefore, the methylation changes detected here are likely part of the broader developmental plasticity associated with the embryogenic transition rather than merely indicators of instability.

Approximately 58% of the epigenetic alterations detected in regenerated plants were associated with fragment gain, indicating a predominance of genomic hypomethylation, whereas fragment loss events, interpreted here as hypermethylation, accounted for about 42% (Fig. 6). Similar patterns, with a predominance of hypomethylation, have been reported for *Humulus lupulus* (Peredo et al. 2009), *Freesia hybrida* (Gao et al. 2010), maize (Kaeppler and Phillips 1993), rice (Stroud et al. 2013), and sugarcane regenerants obtained through somatic embryogenesis (Francischini et al. 2017). These observations support the idea that loss of methylation is a common feature of tissue culture-induced epigenetic remodeling.

The prolonged culture period adopted in the present study (up to 36 months), including repeated subculturing and an extended callus phase, may have contributed to the predominance of hypomethylation (58%) observed in the regenerated plants. Long-term in vitro culture has been associated with cumulative epigenetic changes, including DNA demethylation, driven by sustained stress and repeated subculturing (Miguel and Marum 2011; Bednarek and Orłowska 2020; Ghosh et al. 2021), particularly in systems involving prolonged dedifferentiation phases such as callus cultures (Das et al. 2025). In this context, the observed hypomethylation may reflect both developmental reprogramming and the temporal accumulation of epigenetic changes during extended culture. From a developmental perspective, hypomethylation has often been linked to the activation of gene expression programs required for cell fate transition and embryo development. DNA methylation changes are considered an integral component of somatic embryogenesis, contributing to the regulation of genes involved in dedifferentiation, embryogenic competence, and embryo maturation (De-la-Peña et al. 2015; Kumar and

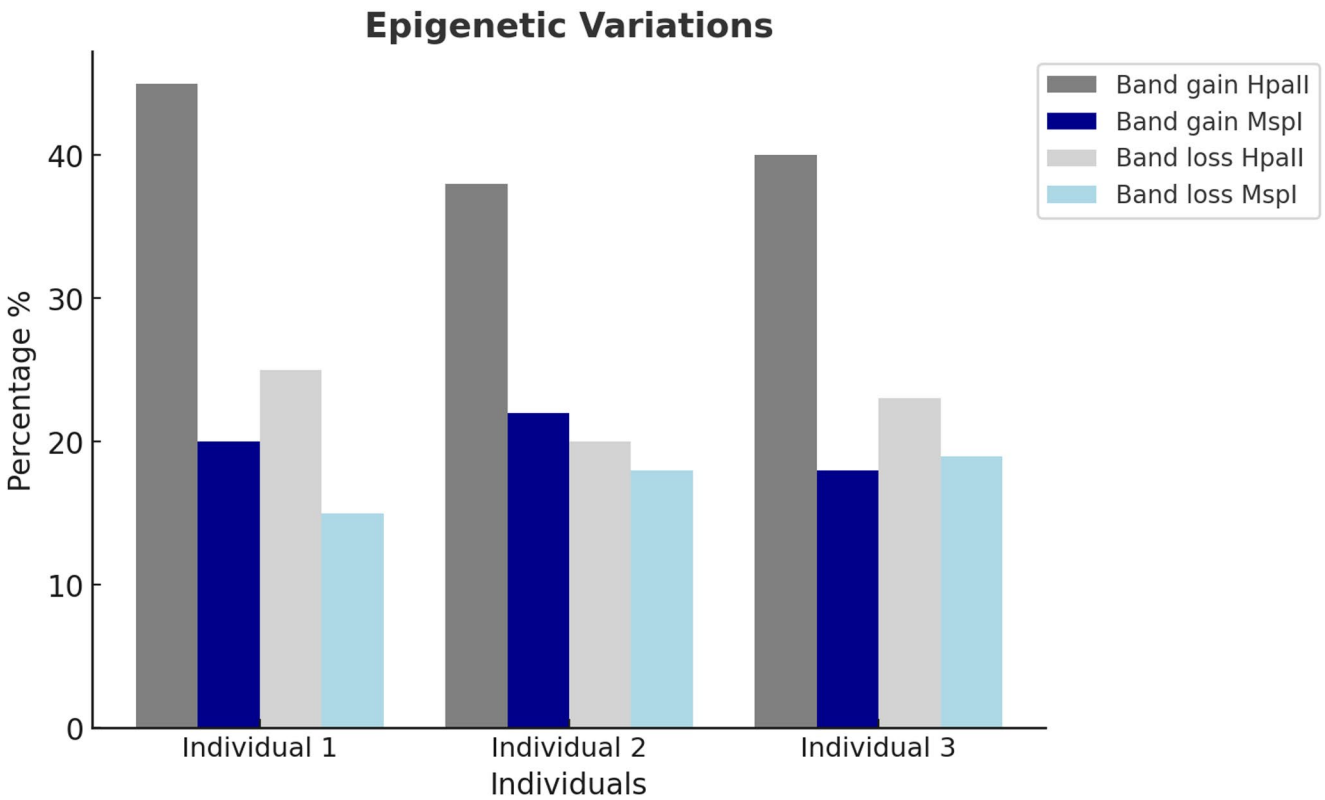


Fig. 6 Epigenetic variation detected by MSAP markers in plants regenerated by somatic embryogenesis from zygotic embryos (donor embryos 1, 2, and 3) of an interspecific oil palm backcross

Van Staden 2017). Thus, the predominance of hypomethylation detected here may reflect the molecular reprogramming required for embryo induction and plant regeneration.

In oil palm, epigenetic instability is particularly relevant because altered methylation patterns have been associated with the “mantled” phenotype, a well-known floral abnormality observed in some clones derived from tissue culture (Jaligot et al. 2000, 2004; Ong-Abdullah et al. 2015). "Mantled" abnormalities are characterized by feminization of male floral structures and severe reproductive impairment, often leading to reduced fruit set or fruit abortion (Corley et al. 1986; Ong-Abdullah et al. 2015). For this reason, the detection of methylation changes in regenerants always deserves careful consideration. However, the presence of epigenetic changes does not necessarily imply abnormal development. Francischini et al. (2017) showed that apparently normal in vitro-derived plants can still exhibit detectable methylation changes relative to their donor source. Similarly, Matthes et al. (2001) and Lei et al. (2006), working with oil palm, reported that tissue culture-induced methylation polymorphisms were not always consistently associated with abnormal phenotypes. Taken together, these observations indicate that epigenetic remodeling is a common component of the regeneration process, but only part of this variation is likely to have phenotypic consequences. This is consistent with the absence of visible morphological abnormalities observed during greenhouse acclimatization, suggesting that the epigenetic variation detected at the molecular level did not translate into evident phenotypic instability at this stage.

From a practical perspective, the association between DNA hypomethylation and the “mantled” phenotype raises important considerations regarding the long-term risk of somaclonal variation in oil palm. Although the epigenetic variation detected in the present study were not directly linked to phenotypic abnormalities, the extended culture period and the predominance of hypomethylation observed among regenerants suggest that latent epigenetic alterations may occur and potentially be expressed under specific developmental or environmental conditions. In this context, the relatively moderate level of epigenetic variation observed here, together with the high genetic fidelity within embryo-derived lines, indicates that the potential risk of somaclonal variation may be limited under the conditions employed. However, long-term field evaluation and reproductive assessment remain essential to determine whether these epigenetic modifications are stable, reversible, or associated with phenotypic instability.

In the present study, the combination of high intra-line genetic similarity and detectable epigenetic variation suggests that somatic embryogenesis from zygotic embryos can produce regenerants that are genetically stable enough for clonal propagation purposes while still undergoing the epigenomic adjustments typically associated with cellular reprogramming. This balance between stability and epigenetic plasticity reinforces the potential of this system for applications in in vitro breeding.

The number of donor embryos and regenerated plants analyzed in this study was limited; therefore, the observed patterns reflect the conditions and material evaluated. Further studies including a larger number of embryogenic lines and regenerants, as well as an analysis of methylation patterns over time, would be useful to assess the broader applicability of these findings.

Morphoanatomical analyses

Although embryogenic structures were already visible approximately 2 months after induction and torpedo-stage embryos were observed after about 32 weeks of culture, morphoanatomical analyses were performed at the same evaluation stage used for regenerated plants, namely 36 months after culture initiation. For these analyses, clusters containing callus and torpedo-stage somatic embryos were used (Fig. 7A).

Histological sections revealed that torpedo-stage somatic embryos exhibited a well-defined protoderm composed of a uniseriate layer of tightly connected tabular cells with prominent nuclei (Fig. 7B, C). Similar to zygotic embryos of the species, these somatic embryos displayed distinct distal and proximal regions (Fig. 7B), as well as a fundamental meristem predominantly formed by rounded, isodiametric cells. Distinct procambial strands were also observed (Fig. 7C), together with a clearly defined embryonic axis in the proximal region, where the plumule was evident (Fig. 7D). These anatomical characteristics, combined with the absence of vascular connection between the somatic embryos and the original explant, confirm that regeneration occurred through somatic embryogenesis rather than organogenesis.

In addition, in the peripheral region of the somatic embryos, proembryos surrounded by polysaccharide mucilage were observed, indicating the occurrence of secondary embryogenesis, possibly of unicellular origin (Konar et al. 1972; Verdeil et al. 2001). The presence of mucilage surrounding embryogenic structures has been associated with embryogenic competence and with the capacity for continued development in vitro (Goh et al. 2001).

These observations are consistent with previous descriptions of somatic embryogenesis in oil palm. Scherwinski-Pereira et al. (2010) described similar morphohistological characteristics in torpedo-stage embryos of *E. guineensis*, including the presence of procambial strands formed by elongated cells with dense cytoplasm and prominent nuclei. Comparable anatomical features were also reported by

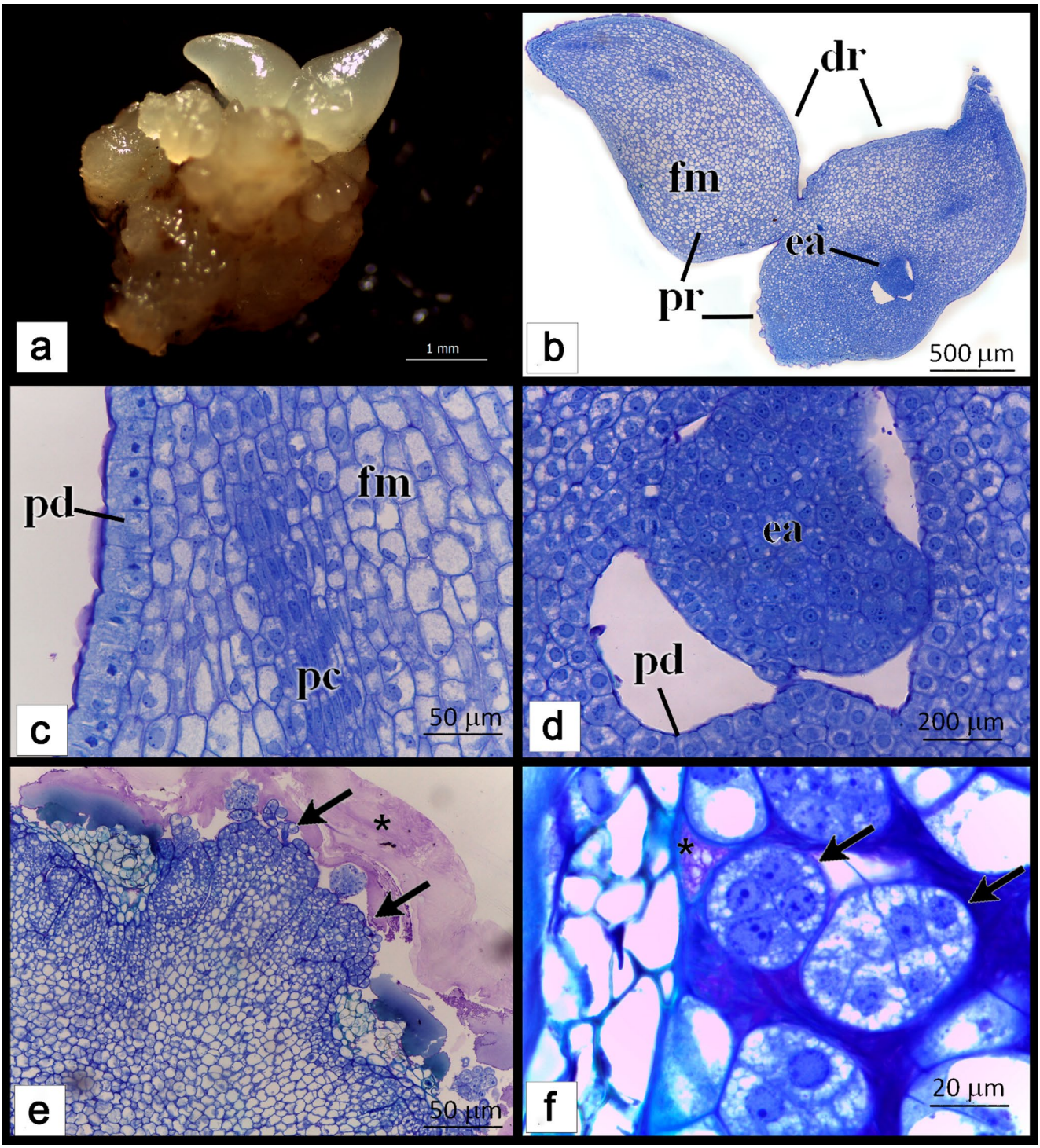


Fig. 7 Morphoanatomical characterization of oil palm somatic embryogenesis. (**A**) Torpedo-stage somatic embryos. (**B**) Longitudinal section of a torpedo-stage embryo showing a well-defined embryonic axis. (**C**) Detail of the protoderm, procambium, and fundamental meristem. (**D**) Embryonic axis with a clearly defined plumule. (**E**) Proembryos developing in the peripheral region of somatic embryos (arrows), surrounded by polysaccharide mucilage. (**F**) *Detail of proembryos showing nuclei and nucleoli within cells surrounded by polysaccharide mucilage*. Abbreviations: ea, embryonic axis; dr, distal region; pr, proximal region; fm, fundamental meristem; pc, procambium; pd, protoderm

Gomes et al. (2017) for an interspecific oil palm hybrid. More recently, Gomes et al. (2024) described the structural organization of somatic embryos, including the establishment of protoderm, fundamental meristem, and procambial differentiation at advanced developmental stages. Therefore, the morphoanatomical evidence obtained here corroborates the embryogenic nature of the regenerants and supports the interpretation that the system followed the typical developmental route of palm somatic embryogenesis.

Conclusions

The present study demonstrates that somatic embryogenesis from zygotic embryos of an interspecific oil palm backcross can generate regenerants with high genetic similarity within individual embryo-derived lines, while maintaining epigenetic plasticity associated with developmental reprogramming. ISSR analysis indicated strong intra-line genetic stability, whereas MSAP profiling revealed detectable differences in DNA methylation patterns, suggesting that epigenetic variation, rather than genetic mutation, may represent an important source of molecular variability in this system. Importantly, these epigenetic variations do not necessarily imply phenotypic abnormality.

From an applied perspective, the ability to obtain multiple genetically consistent regenerants from a single zygotic embryo supports the potential use of somatic embryogenesis as a strategy for clonal propagation of segregating genotypes in oil palm. This approach may contribute to the evaluation of individual genotypes across genetically similar individuals, improving selection accuracy in breeding programs. However, the genetic variation detected in a small number of regenerants highlights the importance of molecular screening and stability assessment before large-scale application in breeding and clonal propagation programs. Further studies under ex vitro and field conditions are required to determine the stability and potential phenotypic effects of the epigenetic variation observed.

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Data availability Data will be made available on request.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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