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Research Article

Somatic embryogenesis and plant regeneration from leaf callus with genetic stability validation using SCoT markers in *Paramignya trimera*, a medicinal plant native to Vietnam

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Abstract

Xao tam phan (*Paramignya trimera* (Oliv.) Guillaum) is a medicinal plant native to Vietnam, that is renowned for its therapeutic properties, particularly for the treatment of various ailments, including cancer. This study investigated *in vitro* propagation of *P. trimera* through somatic embryogenesis and shoot organogenesis using leaf callus. Various culture media, plant growth regulators, malt extract, and carbon sources were evaluated to optimize callus induction and somatic embryo formation from leaf explants. DNA barcoding confirmed 96.96% to 100% homology with *P. trimera* specimens from Khanh Hoa province, Vietnam. The highest callus formation rate, reaching 100%, was observed in one-year-old explants cultured in Woody Plant Medium (WPM) supplemented with 2.0 mg L^{-1} naphthaleneacetic acid (NAA) and 0.2 mg L^{-1} benzylaminopurine (BAP) in the dark for over six weeks. In WPM supplemented with 30 g L^{-1} sucrose, 4.0 mg L^{-1} BAP, and 500 mg L^{-1} malt extract, globular stage embryos developed into embryoids and shoots and buds clumped at 10 and 18 weeks, respectively. Shoot organogenesis was observed in WPM supplemented with 30 g L^{-1} sucrose and 0.07 mg L^{-1} thidiazuron (TDZ) after 18 weeks of culture. Genetic fidelity assessments using 12 SCoT markers indicated that *in vitro* plantlets were homologous to the mother plant. This study provides a viable method for the conservation and sustainable cultivation of Xao tam phan, ensuring a stable supply of this valuable medicinal resource.

Keywords: genetic stability; *in vitro* propagation; *Paramignya trimera*; shoot organogenesis; somatic embryogenesis

Introduction

Paramignya trimera, commonly known as "Xao tam phan", is a perennial woody vine native to Southeast Asia, especially Vietnam (POWO, 2023). This species belongs to the Rutaceae family, which is recognized for

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its economic and medicinal significance, although it has not been studied as extensively as its well-known relatives, such as *Citrus* fruits. In Vietnamese traditional medicine, "Xao tam phan" is celebrated for its health benefits, particularly in cancer treatment, thanks to its rich array of bioactive compounds including flavonoids, alkaloids, and terpenoids (Le *et al.*, 2021; Nguyen *et al.*, 2023). These compounds have shown promising anti-cancer properties in preliminary screenings, effectively inhibiting the growth of specific cancer cell lines and underscoring the plant's potential as a natural cancer therapy (Tran Le *et al.*, 2023). This highlights the critical need for conservation, given the risks posed by medicinal overexploitation.

The increasing demand for "Xao tam phan" has led to intensive harvesting practices that, along with habitat loss, have placed this species at risk in some regions. Consequently, there is an urgent need to develop effective propagation methods to ensure sustainable cultivation and maintain a steady supply of this medicinal plant. Traditional propagation techniques such as seed germination and vegetative cutting face challenges such as low germination rates and slow growth, which are further complicated by genetic diversity and environmental factors (Tran and Le, 2017). In response, recent research has shifted toward *in vitro* propagation techniques to cultivate uniform plants essential for medicinal use however success is still limited (Phi *et al.*, 2017; Tran *et al.*, 2017; Nguyen *et al.*, 2020). To date, somatic embryogenesis has shown particular promise for its ability to rapidly multiply plants and generate true to true types. This technique involves reverting plant cells or tissues to an embryonic state from which they can develop into completely, genetically identical plants.

In plant regeneration, organogenesis and somatic embryogenesis are favored for *in vitro* plant generation. However, long-term culture in media enriched with various plant growth regulators can lead to somaclonal variations (Kairuz *et al.*, 2021), necessitating measures to preserve the genetic stability of the regenerated plants (Faisal *et al.*, 2014). Techniques such as flow cytometry and DNA markers (AFLP, RAPD, ISSR, and SSR), increasingly employed in studies (Ajithan *et al.*, 2019), along with SCoT markers, which are instrumental in verifying the genomic polymorphism of microplants and ensuring the fidelity of plantlets to the mother plant.

This study aims to present comprehensive research on the identification, callus formation, somatic embryo regeneration, shoot multiplication, and assessment of the genetic stability of *Paramignya trimera* plantlets. Through a detailed examination of DNA barcodes, somatic embryogenesis, shoot multiplication from callus derived from leaf samples, and genetic uniformity assessment using SCoT molecular markers, we sought to establish a reliable and efficient method for the conservation and sustainable cultivation of this medicinally valuable species.

Materials and Methods

The study was conducted at the Plant Integrative Biology Laboratory (PIB), Faculty of Biological Sciences, Nong Lam University – Ho Chi Minh City, Vietnam, from January 2021 to December 2023.

Species identification based on DNA barcodes

Young leaf tissue was used for DNA extraction using the GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific). The purity and concentration of the extracted DNA samples were verified using a spectrometer (BioDrop, UK) and electrophoresed on 1% agarose gel. PCR reactions to amplify 4 DNA barcodes (*rbc*L, *mat*K, *trn*H-*psb*A, and ITS1) using Thermo Scientific 2X PCR Master Mix were performed with 0.2 µM of each primer (ITS-p5: CCT TAT CAY TTA GAG GAA GGA G and ITS-p4: CCG CTT AKT GAT ATG CTT AAA) (Cheng *et al.*, 2016) (*rbc*L: *rbc*LaF: GAC AAC TGT GTG GAC CGA TG và *rbc*LaR: CCA CCG CGA AGA CAT TCA TA; *mat*K (3FKIMf: CGT ACA GTA CTT TTG TGT TTA CGA G và 1RKIMr: ACC CAG TCC ATC TGG AAA TCT TGG TTC) (Kress *et al.*, 2007); (*trn*H-*psb*A: CGCGCATGGTGGATTCACAATCC and GTTATGCATGAACGTAATGCTC) (Kress *et al.*,

2005). The thermal conditions consist of 94 °C for 5 min; 35 cycles of 94 °C for 30 s, Ta °C for 30 s, 72 °C for 1 min, and 7 min at 72 °C for elongation. The products were electrophoresis on agarose gels 1% and observed based on a 1 kb ladder (Thermo Scientific). The amplified products were sequenced using the Sanger method in both directions (1st BASE, Malaysia). The obtained nucleotide sequences of both DNA strands were aligned with forward and reverse sequences to ensure accuracy. Sequence correction was performed using a BioEdit Sequence Alignment Editor (Hall, 1999) and Chromas 2.6.6 (Technelysium Pty Ltd). The assembled sequences were aligned with the sequences available in the NCBI GenBank for species identification.

In vitro somatic embryogenesis and shoot organogenesis

Plant materials and surface sterilization

Young, healthy second and third leaves from the tops of one-year-old and four-year-old plants were used as explants. Initially, the leaves were washed under running water, then soaked in a 5% Javel solution (v/v) for 10 min, and again rinsed under running water for 20 min. Subsequently, the leaves were sterilized using 70% ethanol for 60 s and a 10% calcium hypochlorite solution (v/v) for 15 min, followed by five rinses with sterile distilled water.

Culture media and conditions

MS (Murashige and Skoog, 1962), MT (Murashige and Tucker, 1969), and WPM (Wood Plant Media, Lloyd and McCown, 1980) media, supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, were used as basal salts in the experiments. Two types of carbon sources including sucrose, maltose and malt extract (ME), were added to the medium at varying concentrations depending on the experiment. Plant growth regulators such as α-naphthaleneacetic acid (NAA), benzyl adenine (BAP), gibberellic acid 3 (GA3), and thidiazuron (TDZ) were added either individually or in combination, at various concentrations as needed. The pH of the medium was adjusted to 5.7 with HCl or NaOH before sterilization at 121 °C for 20 min. All cultures were placed in a growth chamber, illuminated for 16 hours per day with a light intensity of 100 µmol $m^{-2}s^{-1}$ PPFD, and maintained at 25 ± 2 °C with a relative humidity of 65-70%.

Callus induction

The second and third leaves from shoots of both one-year-old and four-year-old plants were sterilized and transversely cut into thin slices approximately 2 mm thick. The samples were inoculated in 250 ml flasks containing either WPM or MT basal salts, each supplemented with 0.2 mg L^{-1} BAP and varying concentrations of NAA (0, 1, 2, 3, and 4 mg L^{-1}) according to the treatment required. The callogenesis rate was recorded biweekly. Three replicates were conducted for each treatment, each consisting of five flasks with five explants per flask. The morphological characteristics of the callus were noted after eight weeks of culture.

Somatic embryo induction

For embryo induction, embryogenic calli were transferred on MS, MT, and WPM media containing BAP (1-5 mg L^{-1}), GA3 (1; 1.5 mg L^{-1}), carbon sources including sucrose (0; 30; 50 g L^{-1}) and maltose (30 g L^{-1}); malt extract (500 or 1000 mg L^{-1}) depending treatments. All treatments were repeated thrice, and each replicate comprised 5 flasks inoculated with 5 explants per flask. Embryogenesis rate (%) and embryo number were recorded every two weeks of culture.

Shoot organogenesis from callus

For shoot formation, embryogenic calli were transferred to WPM medium adding 30 g L^{-1} sucrose and TDZ (0; 0.01; 0.02; 0.03; 0.04; 0.07; 0.1; 0.3; 0.7; 1 mg L^{-1}) depending treatments. All treatments were repeated thrice, and each replicate comprised 5 flasks inoculated with 5 explants per flask. The embryogenesis rate (%) and embryo number were noted after 4, 8, weeks of culture. Shoot formation was observed after 18 and 22 weeks of culture.

Histology

The embryogenic callus was cut into thin slices with a razor blade, treated with 5% (v/v) Javel for 10 min, and washed with distilled water 5 times. The slices were treated with acetic acid 1% for 2 min and subsequently rinsed with distilled water 5 times. Fine slides were placed on glass slides and stained with carmine red for 4 min. The prepared slides were visualized and photographed under a microscope (Olympus).

Statistical analysis

All *in vitro* culture experiments were arranged in a completely randomized design (CRD), and analysis of variance (ANOVA) was performed using XLStat (Addinsoft, 2019), and the results were read based on the mean value using Duncan's multiple range test (if any). The data were converted to ensure a normal distribution before statistical analysis (if necessary).

Assessment of genetic fidelity

Total genomic DNA of 9 randomly selected *in vitro* plantlets and mother plants was extracted according to the description above. Leaf samples from two seeded plants and *Citrus maxima* were used as control. The purity and quantity of the extracted DNA were measured using BioDrop and electrophoresis on 0.8% agarose gel. SCoT-PCR was conducted using high-quality DNA samples and 12 SCoT primers (Collard and Mackill, 2009) for genetic fidelity analysis. All reactions were carried out with 50 ng of DNA template using the Thermo Scientific 2X PCR Master Mix with 50 pmol of primer. The thermal program includes 94 °C for 5 min, 40 cycles of 94 °C for 30 s, Ta °C for 45 s, 72 °C for 1 min, and 72 °C for 10 min (Applied Biosystem). SCoT-PCR amplification was performed twice. PCR products were examined by electrophoresis on 1.5% agarose gel with 0.5x TBE buffer, and GelRed dye. The size of DNA fragments was estimated based on a DNA ladder (Thermo Scientific). The DNA fragments of each SCoT primer were photographed and scored using ImageJ software. The genetic distance of samples was analysed using the UPGMA method of NTSYS tool (Rohlf, 2000).

Results

Species identification based on DNA barcodes

The sequencing results showed the sizes of products 391 bp (*rbc*L, AN: OR438074), 842 bp (*mat*K, AN: OR421491), 707 bp (ITS1, AN: OP763653), and 403 bp (*trn*H-*psb*A, AN: OR438075). BLAST results showed that the coverage and similarity between the study and available sequences of the *Paramignya* genus in the *rbc*L gene were 97%-100% and 97.1%-98.4%, respectively; in the *mat*K gene were 95%-98% and 97.2%-97.9%. The ITS1 region was similar to *Paramignya trimera* at 92.5%-99.8%. The *trn*H-*psb*A region was firstly sequenced and submitted on NCBI. The phylogenetic analysis confirmed that the studied sample belonged to *Paramignya trimera* (data not shown).

Impact of explant types, basal salts, and plant-growth regulator concentrations on callogenesis from leaf samples

The experiment assessed the impact of tree age, basal salts, and concentrations of NAA and BAP on callogenesis in leaf explants over an eight-week culture period. Statistical analysis confirmed that the rate of callogenesis was influenced by all three factors: explant types, basal salts, and PGR concentrations, with a significant p-value of 0.013.

Results shown in Table 1 indicated that tree age significantly affected the callogenesis process; one-year-old explants demonstrated a 2.87-fold increase in responsiveness to PGR treatments compared to four-year-old explants. The callogenesis rate on the WPM medium was 1.5 times higher than on the MT medium.

Table 1. The impact of tree age, basal salts, and growth regulators on callogenesis from leaf explants after 8 weeks of culture

Explant type	Basal salts	PGRs (mg L^{-1})		Callogenesis rate (%)			Morphogenesis (8 weeks)
		NAA	BAP	4 weeks	6 weeks	8 weeks	
One- old year	MT	0	0	0.0 ± 0.0 d	0.0 ± 0.0 f	0.0 ± 0.0 g	No callus; some explants yellowing
		1	0.2	62.5 ± 4.2 abc	62.5 ± 4.2 bc	66.7 ± 0.0 b	Large, dark yellow calluses with small, compact nodules and a few white, friable calluses on the upper surface.
		2	0.2	50.0 ± 8.3 abcd	50.0 ± 8.3 cd	50.0 ± 8.3 bcd	Large dark yellow calluses with small, compact nodules and few white, friable calluses on the upper surface.
		3	0.2	45.8 ± 4.2 abcd	50.0 ± 8.3 cd	50.0 ± 8.3 bcd	Small calluses, some dark yellow and nodular with smooth, compact surfaces; others are white and friable.
		4	0.2	20.8 ± 12.5 cd	45.8 ± 4.2 cde	45.8 ± 4.2 bcde	Small calluses, some dark yellow and nodular with smooth, compact surfaces; others are white and friable.
	WPM	0	0	0.0 ± 0.0 d	0.0 ± 0.0 f	0.0 ± 0.0 g	No callus; some explants yellowing
		1	0.2	83.3 ± 16.7 ab	95.8 ± 4.2 ab	95.8 ± 4.2 a	Small light-yellow calluses with smooth, compact nodular clusters; some upper surface ones are white and friable
		2	0.2	95.8 ± 4.2 a	100 ± 0.0 a	100 ± 0.0 a	Large light-yellow calluses with smooth, compact nodular clusters; some upper surface ones are white and friable
		3	0.2	33.3 ± 16.7 bcd	50 ± 25 cd	66.7 ± 8.3 b	Small calluses: some compact and light yellow, others white and friable
		4	0.2	45.8 ± 20.8 abcd	62.5 ± 12.5 bc	62.5 ± 12.5 bc	Small calluses: some compact and light yellow, others white and friable
Four-old year	MT	0	0	0.0 ± 0.0 d	0.0 ± 0.0 f	0.0 ± 0.0 g	No callus; some explants yellowing
		1	0.2	0.0 ± 0.0 d	8.3 ± 0.0 ef	12.5 ± 4.2 fg	Very small, white, friable calluses form on leaf veins
		2	0.2	12.5 ± 12.5 cd	25.0 ± 8.3 cdef	29.2 ± 4.2 def	Small, white, friable calluses
		3	0.2	12.5 ± 12.5 cd	20.8 ± 4.2 cdef	20.8 ± 4.2 ef	Small, white, friable calluses
		4	0.2	4.15 ± 4.2 cd	4.15 ± 4.2 f	16.7 ± 0.0 fg	Small, white, friable calluses
	WPM	0	0	0.0 ± 0.0 d	0.0 ± 0.0 f	0.0 ± 0.0 g	No callus; some explants yellowing
		1	0.2	0.0 ± 0.0 d	8.3 ± 0.0 ef	20.8 ± 4.2 ef	Small calluses: white and friable on the surface, pale yellow, nodular, and compact underneath.
		2	0.2	12.5 ± 12.5 cd	25 ± 8.3 cdef	37.5 ± 4.2 bcdef	Small calluses: white and friable on the surface, pale yellow, nodular, and compact underneath.
		3	0.2	8.3 ± 8.3 cd	33.3 ± 0.0 cdef	33.3 ± 0.0 cdef	Small, white, friable calluses
		4	0.2	4.15 ± 4.2 cd	12.5 ± 4.2 def	16.7 ± 0.0 fg	Small, white, friable calluses

Values (mean ± SE) with the same letter are not significantly different ($p < 0.05$), Duncan's test

In media free of PGRs, callogenesis was absent across all age groups and basal salts, often accompanied by explant yellowing. With the addition of PGRs, four-year-old explants generally yielded small, white, friable calluses under similar PGR concentrations, indicating a diminished regenerative capacity with increased tree age (Figure 2 a, a'). Conversely, one-year-old explants exhibited a marked increase in callogenesis rates, most effectively at 2 mg L^{-1} NAA and 0.2 mg L^{-1} BA, producing large, dark yellow calluses with nodular structures and occasional friable areas or globular structures (Figure 1, 2 b, c). These cells, capable of generating embryos, were predominantly found in experimental setups using one-year-old leaf explants cultured on WPM medium supplemented with 1-2 mg L^{-1} NAA and 0.2 mg L^{-1} BAP.

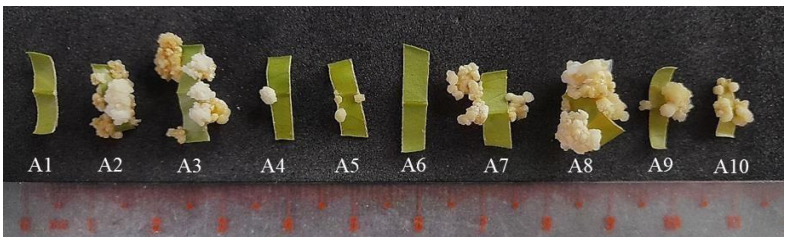

Figure 1. Callus formation from one-year-old leaf explants on basal salts complemented with different concentrations of NAA and BAP after 8 weeks of culture. (A1 – A5) MT basal salt; (A6-A10) WPM basal salt. (A1, A6) non PGRs, (A2, A7) 0.2 mg L^{-1} BAP + 1 mg L^{-1} NAA, (A3, A8) 0.2 mg L^{-1} BAP + 2 mg L^{-1} NAA, (A4, A9) 0.2 mg L^{-1} BAP + 3 mg L^{-1} NAA, (A5, A10) 0.2 mg L^{-1} BAP + 4 mg L^{-1} NAA

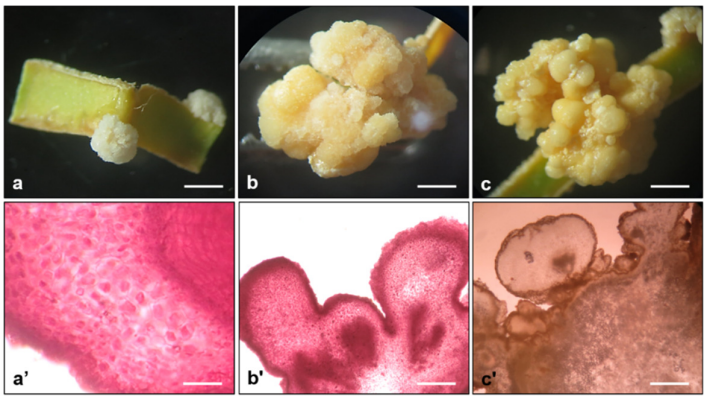

Figure 2. Various types of callus and histological section
(a, a') small, friable callus; (b, b') proliferated callus with granular-like structures; (c, c') globular callus. Bar = 5 mm (a-c), 1 mm (a'-c')

Effect of basal salts, plant growth regulators, carbon sources, and malt extract on somatic embryogenesis

Effect of basal salts, malt extract, and PGRs on embryogenesis

The results presented in Table 2 indicate that WPM medium, supplemented with 4 mg L^{-1} BAP, significantly enhanced embryogenesis rates, achieving an 83.8% rate after six weeks of culture. This treatment promoted the development of green globular structures by the tenth week. In contrast, MT yielded moderate embryogenesis rates; further increases in BAP concentration beyond 3 mg L^{-1} did not yield significant improvements. MS medium proved less effective for supporting embryogenesis. Cultures in MS medium experienced growth arrest and necrosis after ten weeks. By the end of the culture period, all samples in the MS medium had ceased growth and exhibited signs of senescence, with no further development of embryonic shapes observed.

Table 2. Impact of basal salts, malt extract, and BAP on embryogenesis rate

Basal salt	ME (mg L^{-1})	BAP (mg L^{-1})	Embryogenesis rate (%)		Morphogenesis	
			4 weeks	6 weeks	6 weeks	10 weeks
MS	500	3	22.5 ± 0.27 e	38.8 ± 1.3 c	Globular structure, white	Brownish with necrotic zones
MS	500	4	32.5 ± 0.95 c	55.0 ± 5.0 bc	Globular structure, white	
MT	500	3	28.8 ± 0.99 d	67.5 ± 7.5 b	Globular structure, yellow	Growth arrest
MT	500	4	45.0 ± 0.88 b	67.5 ± 7.5 b	Globular structure, green	
WPM	500	3	45.0 ± 0.88 b	67.5 ± 7.5 b	Globular structure, white	
WPM	500	4	61.5 ± 1.03 a	83.8 ± 3.8 a	Globular structure, green	

Values (mean ± SE) with the same letter are not significantly different ($p < 0.05$), Duncan's test.

The result presented in Table 3 showed the effect of basal salts and combinations of BAP and GA3 on the proliferation of embryonic callus at eight weeks post-induction. In WPM, the highest rate of embryogenesis was recorded at 60%, achieved with 0.2 mg L^{-1} BAP and 1.0 mg L^{-1} GA3. At this concentration, the callus predominantly exhibited a green coloration by the fourth week. However, by the eighth week, the majority of callus samples across all treatments had turned brownish, with some exhibiting necrosis. In contrast, the MT medium presented a lower embryogenic rate, with a maximum of 53.33% observed using 0.2 mg L^{-1} BAP and 1.5 mg L^{-1} GA3. This treatment yielded initially green and later yellow globular callus. The statistical analysis indicates significant variability in the response to different PGR treatments, suggesting a nuanced relationship between hormone concentrations and embryogenesis.

Table 3. Impact of basal salts, BAP and GA3 on proliferation of embryonic callus at 8 weeks of induction

Basal salt	PGRs (mg L^{-1})		Embryogenic rate (%)		Morphogenesis	
	BAP	GA3	4 weeks	8 weeks	4 weeks	8 weeks
WPM	0	1.0	16.67 ± 2.74 c	33.33 ± 2.87 cd	Globular structures, white or yellow	Brownish
	0	1.5	23.33 ± 3.75 bc	40.00 ± 3.69 c	Globular structures, yellow	Brownish and necrosis
	0.2	1.0	43.33 ± 2.71 a	60.00 ± 5.87 a	Globular structures, green	Brownish
	0.2	1.5	40.00 ± 2.93 a	56.66 ± 1.41 a	Globular structures, green	Brownish
MT	0	1.0	6.67 ± 1.64 d	23.33 ± 1.80 d	Globular structures, green	Brownish
	0	1.5	20.00 ± 2.42 bc	36.66 ± 2.80 c	Globular structures, white or green	Brownish
	0.2	1.0	26.67 ± 2.22 b	43.33 ± 2.45 bc	Globular structures, green	Globular structures, yellow
	0.2	1.5	36.67 ± 3.81 a	53.33 ± 7.64 ab	Globular structures, green	Globular structures, yellow

Values (mean ± SE) with the same letter are not significantly different ($p < 0.05$), Duncan's test.

Effect of sucrose and BAP on somatic embryogenesis

The results presented in Table 4 indicate that the embryogenesis rate was notably low across all BAP treatments when sucrose was absent. The introduction of sucrose significantly enhanced this rate, peaking at 53.33% with 30 g L^{-1} sucrose and 2.0 mg L^{-1} BAP at six weeks, and further increasing to 60.00% with 50 g L^{-1} sucrose and 1.5 mg L^{-1} BAP. Morphogenesis mirrored these trends; in the absence of sucrose, all samples experienced growth arrest by 10 weeks. In contrast, sucrose treatments of 30 g L^{-1} and 50 g L^{-1} initially supported the development of globular callus and embryos by six weeks, but these progressed to abnormal formations by 10 weeks. Statistical analysis revealed a significant enhancement in embryogenesis rates with increasing sucrose concentrations ($p < 0.05$), highlighting the complex interactions between sucrose and BAP in promoting embryogenesis.

Table 4. Effect of sucrose and BAP on somatic embryogenesis from leaf callus cultured on WPM

Sucrose (g L^{-1})	BAP (mg L^{-1})	Embryogenesis rate (%) at 6 weeks	Morphogenesis	
			6 weeks	10 weeks
0	0.0	0.00 ± 0.00 d	Growth arrest	-
0	0.5	6.67 ± 3.33 d	Callogenesis	Callogenesis
0	1.0	16.67 ± 3.33 cd	Callogenesis, abnormal structure	Callogenesis, abnormal structure
0	1.5	13.33 ± 3.33 cd	Callogenesis, abnormal structure	Callogenesis, abnormal structure
0	2.0	20.00 ± 5.77 bcd	Callogenesis	Callogenesis
30	0.0	36.67 ± 3.33 abc	Callogenesis	Callogenesis
30	0.5	43.33 ± 6.67 ab	Callogenesis	Callogenesis
30	1.0	46.67 ± 3.33 a	Globular callus and embryos	Abnormal embryos
30	1.5	50.00 ± 5.77 a	Globular callus and embryos	Abnormal embryos
30	2.0	53.33 ± 3.33 a	Callogenesis	Callogenesis
50	0.0	46.67 ± 3.33 a	Callogenesis	Callogenesis
50	0.5	53.33 ± 6.67 a	Globular callus and embryos	Abnormal embryos
50	1.0	56.67 ± 3.33 a	Callogenesis	Callogenesis
50	1.5	60.00 ± 5.77 a	Callogenesis	Callogenesis
50	2.0	50.00 ± 5.77 a	Callogenesis	Callogenesis

Values (mean ± SE) with the same letter are not significantly different ($p < 0.05$), Duncan's test

Effect of carbon sources, malt extract, and BAP on somatic embryogenesis

Callus cultures grown on WPM supplemented with 30 g L^{-1} sucrose and 500 mg L^{-1} ME showed varying developmental responses based on BAP concentration. At 1 mg L^{-1} BAP, the cultures primarily underwent callogenesis with persistent abnormal embryos observed for up to six weeks. Increasing BAP to 2 and 3 mg L^{-1} led to the early formation of green, globular calluses by the second week, but these continued to exhibit callogenesis and abnormal embryos by the fourth week. A higher BAP concentration of 4 mg L^{-1} effectively transitioned from globular callus formation in the first week to embryoid development by the fourth week, eventually forming shoots and buds by the sixth week (Figure 3). Conversely, the highest concentration of 5 mg L^{-1} BAP resulted in early globular calluses and abnormal embryos, progressing to extensive callusing and significant necrosis by the eighteenth week (Figure 4) (Table 5). Epicotyl of shoots is cultured on MS medium for multiplication. After three months, these shoots are individually separated and transferred to an MS medium supplemented with Indole-3-butyric acid (IBA) to induce root development. Following a month of root formation, the plantlets are relocated to a nursery for acclimatization (Figure 5).

In contrast, cultures grown on WPM with 30 g L^{-1} maltose and supplemented with 500 mg L^{-1} ME showed limited development, confined to callogenesis by the second week. Doubling the ME to 1000 mg L^{-1} initiated globular callus formation, which transitioned to green callogenesis by the second week, with no further development noted.

Table 5. Somatic embryogenesis from leaf callus on WPM medium added carbohydrate sources, malt extract, and different concentrations of BAP

Medium composition	BAP (mg L^{-1})	Morphogenesis						
		1 w	2 w	4 w	6 w	10 w	14 w	18 w
WPM + 30 g L^{-1} sucrose + 500 mg L^{-1} ME	1	Callogenesis and abnormal embryos	Globular, and abnormal embryos					
	2	Callogenesis and abnormal embryos	Green, globular callus			Callogenesis and abnormal embryos		
	3	Callogenesis, and abnormal embryos	Green, globular callus			Callogenesis and abnormal embryos		
	4	Globular callus		Globular embryos		Embryoids formation		Shoots and buds clumps
	5	Globular callus, abnormal embryos		Callusing, abnormal embryos, necrosis at 18 weeks				
WPM + 30 g L^{-1} maltose + 500 mg L^{-1} ME	-	Callogenesis		Callogenesis, white		-	-	-
WPM + 30 g L^{-1} maltose + 1000 mg L^{-1} ME	-	Callogenesis, globular callus		Callogenesis, green		-	-	-

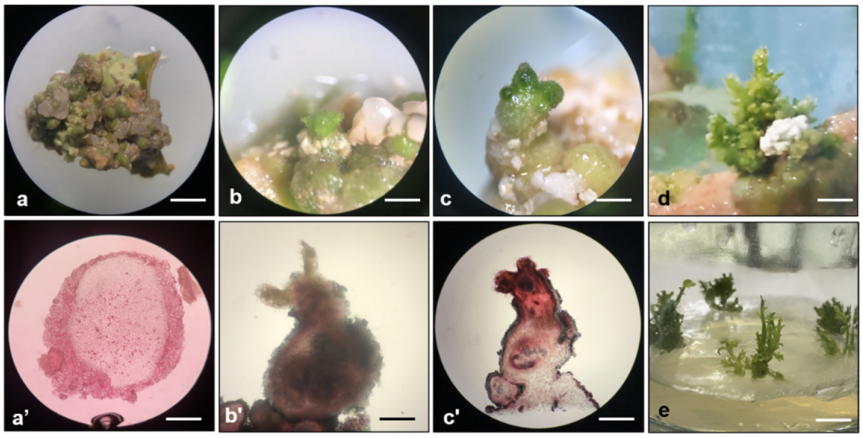

Figure 3. Somatic embryogenesis from leaf-callus on WPM + 30 g L^{-1} sucrose + 500 mg L^{-1} ME + 4 mg L^{-1} BAP and histological sections. (a) globular embryos induced for 6 weeks; (b, c) embryoid with leaf primordia development stage for 10, and 14 weeks, respectively; (d) clump of buds formed for 18 weeks; (e) elongated shoots on WPM medium for 4 weeks; (a') globular embryo section; (b', c') embryoids section. (bar= 5 mm (a), 1 cm (d, e), 1 mm (b, c, a', b', c')

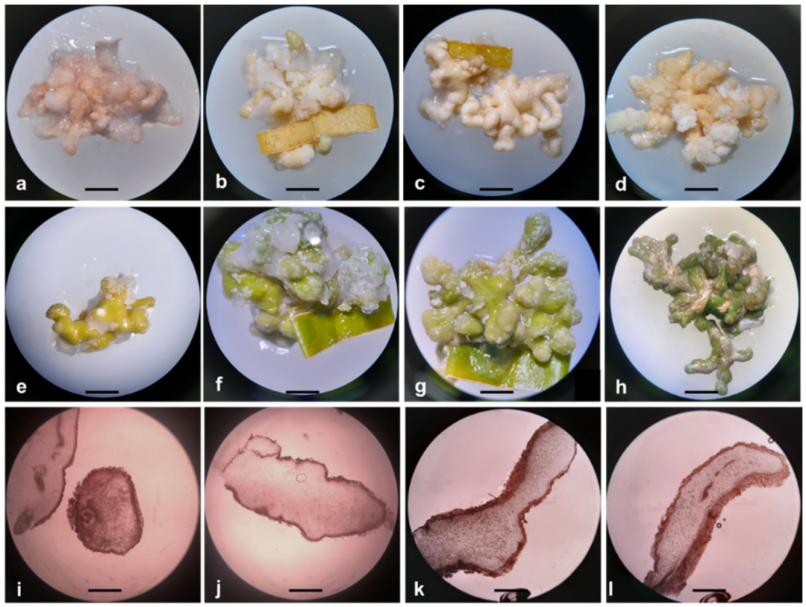

Figure 4. Various abnormal embryos formed (a-h) and histological sections (i-l). (i) globular stage embryo with proliferation of cells in protoderm; (j-l) various malformation including elongated embryo axis, proliferation of cell in protoderm. bar= 5 mm (a-h), 0.5 mm (i-l)

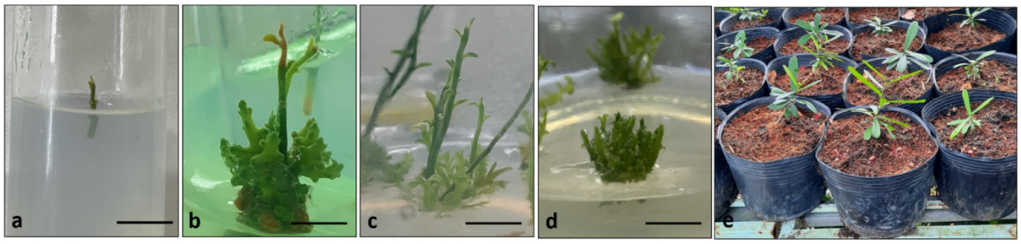

Figure 5. Shoots multiplication from epicotyl of the somatic embryo on MS + 2 mg L^{-1} NAA. (a) Epicotyl of bud-derived embryoid; (b-d) bud and shoots multiplication at 1, 2, 3 months; (e) plantlet in nursery after 4 months.

Effect of TDZ on shoot organogenesis of leaf callus

Table 6 demonstrates the effects of different concentrations of TDZ on shoot organogenesis from callus cultures grown on WPM supplemented with sucrose. No embryogenesis was observed at the lowest concentrations (0.00 and 0.01 mg L^{-1} TDZ), where growth arrest, defined as a cessation in developmental progress, was noted at 4 weeks. A slight increase in embryogenesis rates was observed with 0.02 mg L^{-1} TDZ, but this was accompanied by necrosis, characterized by tissue degradation, by 14 weeks. At concentrations of 0.03 and 0.04 mg L^{-1} TDZ, embryogenesis rates improved, yet necrosis still ensued.

A concentration of 0.07 mg L^{-1} TDZ shown the highest rates of embryogenesis (77.78% at 4 weeks and 88.89% at 8 weeks) and subsequent bud development at later stages (Figure 6). However, higher TDZ levels (0.10, 0.30, 0.70, 1.00 mg L^{-1}) led to increased embryogenesis initially but resulted in necrosis by 14 weeks. These data indicate that while TDZ can effectively stimulate embryogenesis, optimal concentrations are crucial to avoid adverse effects such as growth arrest and necrosis, with 0.07 mg L^{-1} being identified as the most effective concentration for sustained organogenesis.

Table 6. Shoot organogenesis from callus on WPM medium added sucrose (30 g L^{-1}) and different concentrations of TDZ

TDZ (mg L^{-1})	Embryo formation (%)		Morphogenesis				
	4 weeks	8 weeks	4 weeks	8 weeks	14 weeks	18 weeks	22 weeks
0.00	0.00 ± 0.00 g	0.0 ± 0.00 1.0 h	Growth arrest	-			
0.01	0.00 ± 0.00 g	0.00 ± 0.00 h	Growth arrest	-			
0.02	11.11 ± 4.59 f	16.67 ± 3.49 g	Callus and globular structure			Growth arrest	Necrotic
0.03	27.78 ± 4.04 e	38.89 ± 4.49 f	Callus and globular structure			Growth arrest	Necrotic
0.04	38.89 ± 3.18 cd	50.00 ± 3.19 de	Callus and abnormal structure			Growth arrest	Necrotic
0.07	77.78 ± 1.73 a	88.89 ± 4.25 a	Callus and globular structure			Bud primordia	Bud development
0.10	16.67 ± 0.93 f	61.11 ± 2.63 c	Callus and globular structure			Growth arrest	Necrotic
0.30	55.56 ± 2.93 b	55.56 ± 4.04 cd	Callus and abnormal structure			Growth arrest	Necrotic
0.70	44.44 ± 2.03 c	72.22 ± 0.18 b	Callus and abnormal structure			Growth arrest	Necrotic
1.00	33.33 ± 1.14 de	44.44 ± 3.29 ef	Callus and abnormal structure			Growth arrest	Necrotic

Values (mean ± SE) with the same letter are not significantly different ($p < 0.05$), Duncan's test.

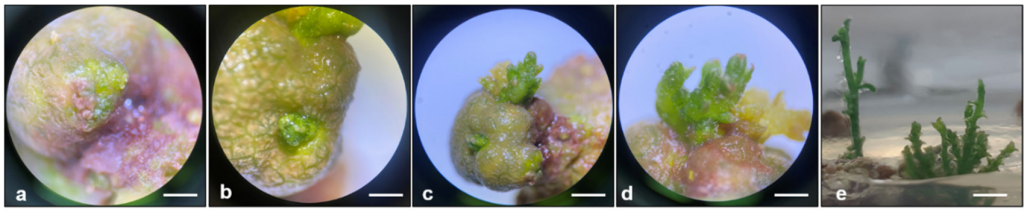

Figure 6. Shoot regeneration from leaf-callus after 18 weeks of culture. (a) bud primordia; (b-d) bud development at 19, 20, 22 weeks of culture; (e) adventitious shoots formed at 26 weeks of culture. bar = 1 mm (a-d), 5 mm (e)

Genetic stability of embryo-derived plantlets

Genetic homogeneity analysis was performed on 9 randomly selected *in vitro* somatic embryos and the mother plant, using SCoT markers. A total of 52 bands were produced by the 12 SCoT primers, including 10 polymorphic bands, with an average of 4.16 bands per primer. SCoT2 produced a maximum of 7 bands, whereas SCoT4, 9, 10, and 13 produced the lowest number of 3 bands (Table 7). The amplified bands were clear, unambiguous, and reproducible. The electrophoresis profiles indicated a monomorphic banding pattern, validating the genetic purity of the regenerated somatic embryos raised *in vitro* with their mother plant (Figure 7).

Table 7. List of SCoT primers and DNA bands produced

Primer code	Sequences (5′–3′)	GC (%)	Ta (°C)	Range of DNA bands (bp)	Scorable bands	Polymorphic bands	Polymorphic ratio (%)
SCoT-01	CAACAATGGCTACCACCA	50.0	48	500-3000	5	0	0
SCoT-02	CAACAATGGCTACCACCC	55.6	48	500-3000	7	6	86
SCoT-03	CAACAATGGCTACCACCG	55.6	50	1000-2500	4	0	0
SCoT-04	CAACAATGGCTACCACCT	50.0	48	750-1500	3	0	0
SCoT-05	CAACAATGGCTACCACGA	50.0	50	250-1500	5	0	0
SCoT-07	CAACAATGGCTACCACGG	55.6	50	750-2500	4	2	50
SCoT-08	CAACAATGGCTACCACGT	50.0	50	1000-3000	5	1	20
SCoT-09	CAACAATGGCTACCAGCA	50.0	50	750-1500	3	0	0
SCoT-10	CAACAATGGCTACCAGCC	55.6	50	1000-3500	3	0	0
SCoT-11	AAGCAATGGCTACCACCA	50.0	50	500-2000	5	1	20
SCoT-12	ACGACATGGCGACCAACG	61.1	50	250-1500	5	0	0
SCoT-13	ACGACATGGCGACCATCG	61.1	50	500-2000	3	0	0
	Total				52	10	

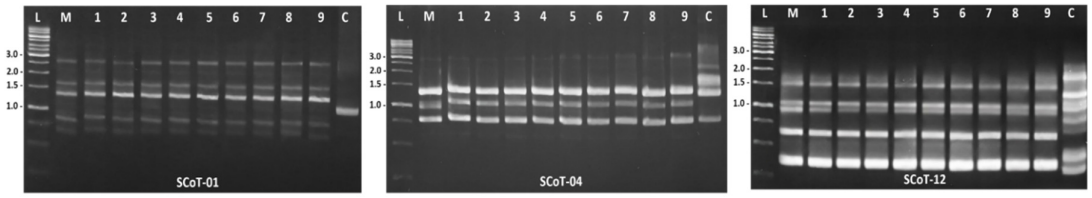

Figure 7. SCoT products with a monomorphic pattern of SCoT-01, SCoT-04, and SCoT-12 primers. Lane (L): DNA ladder (1 kb); Lane (M): mother plant; Lane (1-9): embryo-regenerated plantlets; Lane (C): *Citrus maxima*

Discussion

This study provides a comprehensive examination of factors influencing plant tissue culture, encompassing species identification, callogenesis, embryogenesis, and the genetic stability of regenerated plantlets. The integration of morphological assessment and DNA barcoding has effectively confirmed the identity of the *Paramignya trimera*, with high sequence similarity across several genetic markers (*rbc*L, *mat*K, ITS1, *trn*H-*psb*A) establishing a robust methodological framework for species verification in botanic studies.

Effect of explant types, basal salts, plant growth regulators to callogenesis

In *Citrus* somatic embryogenesis, explants from ovular origins such as ovules and nucellus are more effective than non-ovular types (Savita *et al.,* 2015). Ovular explants typically lead to direct embryogenesis while non-ovular types often require an intermediate callus phase for indirect embryogenesis (Carimi *et al.,* 1998). Research on leaf explants for callus formation and embryogenesis in *Citrus* is limited. The age of leaf

explants significantly affects callogenesis, with older leaves demonstrating higher callus production potential, highlighting the importance of leaf age in successful callogenesis in *Citrus* (Carli *et al.*, 2018).

In the genus *Paramignya* (formerly *Atalantia*), only one instance of successful embryogenesis from immature seeds of *Atalantia ceylanica* has been reported (Jing-Tian and Iwamasa, 1997). This study used leaf samples from one-year-old and four-year-old plants. Our findings underscore the significant influence of explant age, basal salts, and PGRs concentrations on callogenesis. The choice of basal salt and PGRs also critically affects embryogenesis rates. WPM medium supplemented with 4 mg L^{-1} BAP was especially effective, indicating that its nutrient composition and hormonal balance enhance embryonic development more than MT or MS mediums. These insights are crucial for refining medium formulations to enhance somatic embryogenesis outcomes in this species.

Effect of carbon sources, malt extract, and bap on somatic embryogenesis

The differential impacts of sucrose and maltose on somatic embryogenesis underline the need to choose a carbon source that matches the specific metabolic requirements of the cultured plant species. Sucrose is particularly effective, not only as an energy source but also for its role in osmotic regulation which is crucial for cell viability and differentiation, as supported by studies like Karami *et al.* (2006) and Mauri and Manzanera (2004). While higher sucrose concentrations generally boost embryogenesis rates (Silveira *et al.*, 2013), they can also lead to the production of abnormal embryos, indicating that other factors also play roles in embryonic development.

The cytokinin BAP significantly enhances embryogenesis, affirming its effectiveness in the process (Asghar *et al.,* 2023). However, the relationship between BAP concentration and embryo quality is complex; high levels of BAP may cause stress that disrupts normal embryogenic pathways (Zavattieri *et al.*, 2010). Notably, the highest embryogenesis rates were not correlated with the healthiest embryonic development, suggesting that conditions fostering cell proliferation might not adequately support proper differentiation (Nieves *et al.*, 2003).

Somatic embryos need specific cultural conditions and nutrients to germinate and develop into complete plantlets with shoots and roots. Sometimes, the medium used for somatic embryogenesis also supports embryoid germination. For example, Gill *et al.* (1994) successfully germinated mandarin somatic embryos on MS medium supplemented with BAP, NAA, and malt extract, the same medium used for their initial creation. Tomaz *et al.* (2001) saw growth and germination in somatic embryos from nucellar callus across several *Citrus* types using a carbohydrate-rich medium. Malt extract proved crucial, with a 500 mg L^{-1} dose sufficing to induce germination in 11 *Citrus* genotypes (Rodriguez *et al.,* 1999). Although somatic embryos generally develop into small plants on PGRs-free mediums, adding growth regulators can boost germination in *Citrus*. Plantlets have been produced from somatic embryos in mediums with GA3 (Kunitake *et al.,* 1991; Kayim and Koc, 2006). Additionally, Deng *et al.,* (1991) transformed embryoids of *C. reticulata* and *C. sinensis* into complete plantlets using MT medium supplemented with 0.5% glycerol and 10 mg L^{-1} ABA. Auxins also play a critical role, as demonstrated by Pasquale *et al.* (1994), who used MS medium containing 0.27 µM NAA and malt extract to successfully develop plantlets from three lemon cultivars.

This study reveals that combination of sucrose and ME greatly influences somatic embryogenesis than maltose, particularly at higher BAP concentrations. Optimal BAP levels, around 4 mg L^{-1}, promote embryoid formation and the progression to shoots and buds, indicating a favorable hormonal balance for differentiation and maturation. However, increasing BAP to 5 mg L^{-1} leads to detrimental effects, including increased necrosis by week 18, underscoring the need for precise optimization of BAP levels to prevent cytotoxic effects and promote healthy development. The limited embryogenic progression in maltose-based media, even with increased ME, suggests that maltose may not support embryogenesis as effectively as sucrose, possibly due to differences in sugar metabolism pathways and their roles in osmotic regulation, energy provision, and signalling during callus culture.

This study highlights how various components of the culture medium interact to affect the efficiency and quality of somatic embryogenesis. These insights are critical for refining tissue culture techniques to enhance plant regeneration capabilities, essential for plant biotechnology applications and crop improvement programs. Further research should delve into the biochemical and genetic mechanisms underlying these responses to optimize culture conditions further.

Effect of thidiazuron in shoot regeneration from callus

In several *Citrus* species, cytokinins either alone or combined with other growth regulators have proven beneficial for the differentiation and development of callus cultures into shoots. Kumar *et al.* (2011) reported that shoot regeneration and the number of shoots per callus were highest when the callus of *C. jambhiri* was cultured on MS medium supplemented with 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} kinetin, and 3.0 mg L^{-1} BAP. Hasan *et al.* (2016) observed that small pieces of green and healthy friable callus from *C. japonica* margarita cultured on MS medium with various concentrations of BA and kinetin alone or in combination showed significant shoot development after 35 days at 13.0 μM BA. According to Kaur (2018), the most effective regeneration occurred in calli on MS medium supplemented with 0.5 mg L^{-1} NAA, 3.0 mg L^{-1} BAP, and 1.0 mg L^{-1} kinetin, while the maximum number of shoots was recorded in MS medium with 0.5 mg L^{-1} NAA, 2.5 mg L^{-1} BAP, and 0.5 mg L^{-1} kinetin. Hussain *et al.* (2018) reported that callus from nucellus tissue, shoot apical meristems, and nodal segments showed promising shoot regeneration on N7 medium containing 1.5 mg L^{-1} kinetin and 500 mg L^{-1} malt extract.

In this study, no adventitious shoots were formed from leaf callus even after 18 weeks of culture on basal salts supplemented with BA and malt extract (refer to Table 2 and Table 5). This lack of shoot formation may be attributed to the genetic characteristics of *P. trimera*, the leaf callus origin, and the absence of other PGRs such as NAA and kinetin in the medium. Further research is necessary to enhance shoot induction from callus, streamline the process, and improve the multiplication rate.

Thidiazuron (TDZ) has been extensively studied for its role in inducing shoot organogenesis in woody plants. TDZ has shown superiority over natural cytokinins in promoting shoot regeneration in various tree species by stimulating axillary shoot proliferation, adventitious shoot organogenesis, and somatic embryogenesis (Novikova and Zaytseva, 2018; Vinoth and Ravindhran, 2018). Different concentrations of TDZ have been found to evoke varying regeneration routes from the same explant, with factors like explant type, maturity, and TDZ concentration influencing the outcomes (Shyam *et al.,* 2018). Additionally, TDZ has been observed to induce shoot bud formation and adventitious shoot induction in *Pterocarpus marsupium*, highlighting its potential for conservation efforts through rapid multiplication of regenerants (Tippani and Thammidala, 2021).

Our results reveal that TDZ is effective in stimulating shoot regeneration, with specific concentrations (notably 0.07 mg L^{-1}) yielding the best outcomes in terms of high embryogenesis rates without consequent necrosis. This finding contributes to a deeper understanding of the role of TDZ in manipulating plant developmental pathways, advocating for a balanced approach to its application to avoid cytotoxic effects. Further research might explore the underlying mechanisms of TDZ's action at cellular and molecular levels to better understand its role in plant tissue culture.

Genetic stability of embryo-derived plantlets

SCoT markers play a crucial role in assessing the genetic fidelity of plantlets regenerated *in vitro* (Biswas *et al.,* 2022). These markers aid in confirming the genetic uniformity and stability of *in vitro* raised plants, ensuring that they retain the desired characteristics without unwanted genetic changes (Bansal *et al.,* 2022). By utilizing SCoT markers, researchers can effectively evaluate the clonal fidelity of regenerants, detecting any somaclonal variations that may arise during the tissue culture process (Gawronski *et al.,* 2022). Studies on various plant species, including Dutch Iris, *Digitalis purpurea*, and *Scutellaria baicalensis*, have demonstrated

the utility of SCoT markers in revealing genetic diversity among regenerants and confirming the genetic stability of *in vitro* propagated plants. In this study, the genetic stability of embryo-derived plantlets, as confirmed by SCoT marker analysis, validates the integrity of the somatic embryos produced. This aspect of the study is particularly important for applications in conservation biology and agriculture, where maintaining genetic fidelity in cloned plants is crucial. Overall, SCoT markers serve as valuable tools in confirming the genetic integrity of *in vitro* regenerated plantlets.

Conclusions

The study successfully established a reliable protocol for the *in vitro* propagation of *Paramignya trimera* through somatic embryogenesis and organogenesis, demonstrating the significant influence of explant type, age, and specific culture conditions on the outcomes of callogenesis and embryogenesis. Key results highlighted the improved callogenesis response in younger explants when treated with plant growth regulators and the superiority of WPM medium over MT medium in enhancing embryogenesis rates, particularly with higher concentrations of BAP. Furthermore, the use of SCoT markers confirmed the genetic stability of the regenerated plantlets, ensuring that the *in vitro* propagation did not introduce genetic variations. These findings emphasize the potential of advanced tissue culture techniques in conserving and sustainably utilizing *Paramignya trimera*, providing a foundation for future research to explore molecular mechanisms underlying these responses and adapt these protocols to other valuable plant species.

Authors' Contributions

Conceived and designed the experiments: NVP. Performed the experiments: DHTV, CTCH, TNHT, TTCV, LHMQ, THL, and NVP. Analysed the data: NVP, DHTV, CTCH, TNHT, THL. Wrote or proofread the paper: DHTV, TNHT, NVP. All authors have read and approved the final manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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