Reliable callus-induced plantlet regeneration from leaf explants of Lagerstroemia speciosa and genetic fidelity assessment through ISSR markers

Bin Wu
bin.wu@tamu.edu
Texas A&M University https://orcid.org/0000-0003-3212-5812
Nicholas S Zhang
Texas A&M University
Benjamin Dixon
Texas A&M University
Ivan Sierra
Texas A&M University
Sofya Kan
Texas A&M University
Alanna Layton
Texas A&M University
Mengmeng Gu
Colorado State University
Margaret R Pooler
USDA-ARS USNA: USDA-ARS United States National Arboretum
Hui Duan
USDA-ARS USNA: USDA-ARS United States National Arboretum
Hongmin Qin
Texas A&M University https://orcid.org/0000-0002-0045-5780

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Abstract

Crapemyrtle (*Lagerstroemia* sp.) is the top-selling flowering tree in the U.S. However, threats from arthropod pests, including the recently emerged crapemyrtle bark scale (CMBS; *Acanthococcus lagerstroemiae*), severely jeopardize the aesthetic and production attributes of crapemyrtle. A tropical species, *L. speciosa* (L.) Pers. ("Queen's Crapemyrtle") exhibits partial resistance to CMBS and other pests, but conventional breeding to incorporate the characteristics of *L. speciosa* into existing hybrids remains challenging. Recognizing the potential of tissue culture in facilitating molecular breeding, but also the possibility of undesirable somaclonal variations from *in-vitro* organogenesis, we utilized leaf explants of *L. speciosa* to develop a callus-induced regeneration protocol and assessed genetic fidelity of regenerated plantlets using inter-simple sequence repeat (ISSR) markers. Using woody plant medium (WPM) supplemented with 0.2 mg/L 2,4-D and 1.0 mg/L 6-BA achieved 97.9% callus induction. Shifting the growth regulators to 10.0 mg/L 6-BA and 0.5 mg/L NAA resulted in 32.4% of callus explants differentiating into adventitious buds. Finally, nodal segment proliferation (94.6%) and new shoot growth was maximized by using WPM supplemented with 1.0 mg/L 6-BA and 0.02 mg/L NAA. Explants rooted 100% using half-strength WPM supplemented with 0.2 mg/L IBA, and acclimatization survival was 98.3%. The ISSR primer analysis revealed 98.7% monomorphic markers, confirming the genetic integrity of the regenerated plantlets. We describe a reliable callus-induced regeneration system for *L. speciosa*, which will facilitate future molecular breeding and biotechnology to enhance cold hardiness, pest resistance, and other desired traits in this important genus.

INTRODUCTION

Crapemyrtle (*Lagerstroemia* L.) is renowned for its mid-summer bloom, diversity of plant sizes and flower color, and ease of cultivation and maintenance as a landscape tree or shrub. It is the top-selling deciduous flowering tree in the U.S. with over 3 million plants sold annually at a market value of $69.6M (Stats 2020). Crapemyrtles originated in China and Southeast Asia and were introduced to the southeastern U.S. beginning with *Lagerstroemia indica* over a century and a half ago (Chappell et al. 2012). Since then, public and private breeding programs incorporated additional *Lagerstroemia* species, including *L. fauriei* Koehne, *L. speciosa* Pers., *L. subcostata* Koehne, and *L. limii* Merr. leading to diversity in plant habit, cold hardiness, flower color, panicle structure, disease resistance, and bark features (Chappell et al. 2012; Dirr 2002; Egolf 1981; Egolf 1986; Egolf 1990; Egolf and Andrick 1978; Pooler 2006; Pooler and Dix 1999; Reichard 1997).

Despite its prominence in the landscape and its economic impact on the nursery industry, the crapemyrtle faces considerable threats from arthropod pests, which can severely impact nursery production. In addition to damage caused by prevalent insect pests such as the crapemyrtle aphid (*Tinocallis kahawaluokalani*), flea beetle (*Altica* sp.), and Japanese beetle (*Popillia japonica*) (Cabrera et al. 2008; Herbert et al. 2009; Mizell III and Knox 1993; Pettis et al. 2004), a new invasive species, the crapemyrtle bark scale (CMBS; *Acanthococcus lagerstroemiae*), is causing rapid and unprecedented damage to trees across the U.S. This sap-sucking hemipteran pest has been found in 17 U.S. states and Washington D.C. since it was first reported in Texas in 2004 (EDDMapS 2021; Gu et al. 2014; Heather 2021; Pooler 2006; Skvarla and Schneider 2022; Wang et al. 2016). Severe CMBS infestations affect crapemyrtle growth, flowering, and market value by weakening the plant through insect feeding and by accumulation of "sooty mold" on the bark, resulting in up to 50% loss of production, with the estimated annual cost to control the insect exceeding $34M in the U.S. (Chen and Diaz 2022; Marwah et al. 2021; Marwah et al. 2022; Merchant et al. 2018). Developing new varieties of crapemyrtle that are resistant to CMBS is therefore a priority based on the impacts on the industry, the landscape, and the environment (Boutigny et al. 2020; Datta 2021; Smith 2021).

In searching for sources of resistance to CMBS, comprehensive greenhouse assays and insect feeding behavior analyses revealed that *L. speciosa* (Queen's Crapemyrtle), a deciduous tropical tree native to Southeast Asia, did not serve as a host for crapemyrtle aphids and exhibited partial resistance to CMBS compared to other tested *Lagerstroemia* species (Gilman and Watson 2014; Herbert et al. 2009; Klein et al. 2007; Rojas-Sandoval 2017; Wu et al. 2022; Wu et al. 2021). The *L. speciosa* is characterized by broader leaves and more prominent flowers with less crinkled petals than *L. indica* and its hybrids (Pounders et al. 2007). However, *L. speciosa* is cold hardy only to USDA Zone 10b-11, whereas most cultivars in production in the U.S. exhibit root hardiness up to Zone 6 (Gilman and Watson 2014; Pooler 2006). Combining the pest resistance and ornamental characteristics of *L. speciosa* with the cold-hardiness and landscape attributes of the more familiar and widely planted crapemyrtle species holds considerable promise to enhance genetic diversity, increase pest resistance, and introduce novel ornamental traits into commercial cultivars. Despite decades of rigorous conventional breeding research, limited success has been achieved in obtaining fertile interspecific hybrids between *L. indica* and *L. speciosa* (Pounders et al. 2007) which restricts introgression of complex traits and significantly limits the adaptability and production range of crapemyrtle (Pounders et al. 2007). Recent advances in genome editing technologies provide promising avenues for synergizing conventional breeding techniques with molecular biology insights, positioning plant tissue culture as an essential component for expediting molecular breeding in crapemyrtles (Bruegmann et al. 2019; Cardi et al. 2023; Osakabe et al. 2016; Pooler 2006).

Plant regeneration in tissue culture is predominantly driven by somatic embryogenesis or *de novo* organogenesis (Duclercq et al. 2011; Phillips and Garda 2019). Plant tissues *in vitro* can generate various primordia through a *de novo* cellular dedifferentiation process, followed by a series of organogenic events that culminate in the formation of embryos, flowers, leaves, shoots, and roots (Schwarz and Beaty 2018). Auxin, cytokinin, or other plant growth regulators (PGRs) synergistically and dynamically modulate plant developmental processes through

coordinated cell division and differentiation, exhibiting both antagonistic and supportive roles (Alayón-Luaces et al. 2008; Sasamoto et al. 2002; Schaller et al. 2015). However, many woody plants, including crapemyrtle, exhibit recalcitrance in the *in-vitro* organogenic process, necessitating resource-intensive experiments to find the optimal balance of PGRs to promote organogenesis (Benson 2000; Bonga 2017; Long et al. 2022; Tanimoto and Harada 1984; Thorpe and Harry 1990). Previous studies on micropropagation of *L. speciosa*, focused on the effects of various PGRs including thidiazuron (TDZ), 6-benzyladenine (6-BA), α-naphthalene acetic acid (NAA), and N6-(3-hydroxybenzylamino purine) (*meta*-Topolin) (Ahmad et al. 2022a; Ahmad et al. 2022b; Lim-Ho and Lee 1985; Vijayan et al. 2015), but few studies have examined callus-induced regeneration from leaf explants (Rahman et al. 2010). A comprehensive investigation to optimize PGR combinations for callus induction, adventitious bud differentiation, shoot proliferation, and the subsequent rooting of regenerated plantlets is a crucial first step in developing methodologies to apply modern breeding tools to *L. speciosa* and other taxa.

Although tissue culture regeneration is critical in many plant biotechnology applications, the process can lead to genetic aberrations, commonly termed 'somaclonal variation', encompassing genetic and phenotypic changes (Bairu et al. 2011). While morphological changes are occasionally observable, molecular-level variations are more pervasive and subtle, requiring comprehensive evaluation tools for detection to discern the locations and magnitude of variations from the reference clone plant (Cloutier and Landry 1994; Evans et al. 1984; Israeli et al. 1991; Sultana et al. 2022; Thakur et al. 2021). Somaclonal variation or genetic instability can result in adverse phenotypes in horticultural crops, including floral sterility, poor fruit set, or undesirable morphological changes, impeding the successful commercialization of regeneration protocols for mass production in the horticulture industry (Bhojwani et al. 2003). Molecular marker technologies can facilitate the identification of variation dispersed throughout the plant genome (Gostimsky et al. 2005), and arbitrary markers such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) offer an efficient approach to assess genetic fidelity in various in-vitro regenerated plants, including micropropagated *L. speciosa* (Ahmad et al. 2022a; Ahmad et al. 2022b; Kader et al. 2022; Saha et al. 2016; Sultana et al. 2022; Thakur et al. 2021). The ISSR primers have been shown to be exceptionally reproducible and informative for this purpose (Bairu et al. 2011; Gostimsky et al. 2005; Kaeppler et al. 2000; Reddy et al. 2002), although these markers have not been tested in callus-induced regenerated *L. speciosa.*

The objective of this research was to advance the opportunities to apply new biotechnological breeding applications to crapemyrtle by 1) establishing a highly efficient and stable callus-mediated regeneration system for *L. speciosa*, and 2) assessing the genetic integrity of regenerated plantlets using ISSR markers.

Materials and methods

Plant material and tissue culture conditions

Plants of *L. speciosa* were provided by Dr. Gary Knox at the North Florida Research and Education Center (Quincy, FL). The plants were grown in 11.4-liter pots containing potting mix (Jolly Gardener® Pro-Line C/GP growing mixture, Oldcastle Lawn & Garden Inc., Poland Spring, ME) and maintained in the greenhouse (25 ℃ with a photoperiod of 10.5:13.5 (light: dark) h).

Healthy juvenile stems were collected as explants from the greenhouse-grown plants in early June 2021. The explants were disinfected using 70% (v/v) ethanol for 10 s and 20% commercial bleach solution (Clorox®, 6% sodium hypochlorite, Oakland, CA) for 15 min, followed by 4–6 rinses with sterile double-distilled water. The disinfected explants were cut into 1.5-2.0 cm nodal segments and cultured (2–5 per jar) onto half-strength woody plant medium (WPM; (Lloyd and McCown 1980) supplemented with 3.0% (w/v) sucrose, 0.65% (w/v) agar, 0.05% (w/v) ascorbic acid, 0.05% (w/v) polyvinylpyrrolidone (PVP-40). Aseptic plantlets that developed from each node were maintained in this medium and used as a source of explants for further studies.

Unless specified otherwise, all tissue culture experiments took place in CONVIRON® growth chambers (Controlled Environments Ltd., Winnipeg, Canada) maintained at 25 $\backslash\mathrm{varvec}^{\circ}\mathrm{C}$, 60% relative humidity, and 16:8h light: dark photoperiod with a light intensity of 1500–2000 lux.

All culture media, PGRs, agar, and other reagents were sourced from PhytoTech Labs, Inc. (Lenexa, KS, USA). The pH of all media formulations was adjusted to 5.8 prior to autoclaving, which was carried out at 121 $\backslash\mathrm{varvec}^{\circ}\mathrm{C}$ and 15 psi for 20 min using an AMSCO® autoclave (STERIS Corporation, Mentor, OH, USA). The media were dispensed in quantities of 30 mL or 50 mL into a CultureJar™ G9 culture jar or a PTL-100™ culture vessel (PhytoTech Labs, Inc.).

Microscopic assessment of in vitro differentiation

For tissue culture experiments, each phase of plant tissue morphogenesis, including callus induction, adventitious bud formation and proliferation, and regenerated plantlet rooting, was examined using an Olympus SZX7 stereomicroscope equipped with a high-definition LC30 color camera (Olympus Corporation, Waltham, MA, USA). For enhanced detail and clarity, images were manually focused along the z-axis, with subsequent focus stacking conducted using Helicon Focus software (Helicon Soft Ltd., Kharkiv, Ukraine). This approach facilitated a robust and detailed analysis and allowed us to accurately determine the stage of differentiation and growth for each experiment.

Callus induction from leaf explants

Leaf explants were collected from the aseptic plantlets described above, cut into smaller (1.5-2.0 cm long × 0.5-1.0 cm wide) pieces, and cultured on WPM supplemented with different PGR treatments (Table 1). The callus induction experiment was repeated three times with ten replicates (culture jars) for each PGR treatment. Each culture jar contained 4–9 explants. The number of leaf pieces that formed callus was recorded 14 days after culturing on the induction medium and converted to a percentage by dividing by the total number of leaf pieces.

Table 1
Effects of different PGR combinations on induction and growth of callus in leaf explants of *L. speciosa* after 14 days on induction medium

PGR combination (mg/L)			Percentage of leaf pieces with callus two weeks after induction
NAA	2,4-D	6-BA	
0.5	-	-	7.2 ± 4.1[z] e[y]
2.0	-	-	31.0 ± 2.0 d
0.1	-	5.0	24.1 ± 3.6 de
-	0.2	-	75.4 ± 12.5 abc
-	0.2	1.0	97.9 ± 1.0 a
-	0.5	-	70.4 ± 14.8 bc
-	0.5	2.0	87.0 ± 4.4 ab
-	-	2.0	55.0 ± 6.1 d
Statistical analysis			$F_{7, 23} = 17.9305$; $p < 0.0001$

[z] Values represent means ± standard error

[y] Means followed by different letters within the same column are significantly different as determined by Tukey's Honestly Significant Difference test ($\alpha = 0.05$)

Callus differentiation into adventitious buds

Leaf-derived callus was transferred to WPM supplemented with different PGR combinations to optimize formation of adventitious buds (Table 2). The callus differentiation experiment was repeated three times with four replicates (culture jars) for each PGR treatment. Each culture jar contained 3–6 callus pieces. 50 days after the emergence of initial shoot buds, the number of adventitious buds per differentiated callus within each PGR combination was recorded.

Table 2
Effects of different PGR combinations on adventitious bud differentiation from leaf callus of *L. speciosa*

PGR combination (mg/L)				Number of replications	Percent differentiated callus [z]	Number of newly developed buds	Differentiation ratio
6-BA	ZT	TDZ	NAA				
-	1.0	-	0.1	5	2.8 ± 1.7[y] c[x]	2.4 ± 1.9 c	1.1 ± 0.7 bc
-	1.0	-	0.2	5	2.5 ± 1.6 c	1.4 ± 0.9 c	1.4 ± 0.9 bc
-	1.0	-	0.5	3	2.2 ± 2.2 c	0.7 ± 3.7 c	0.7 ± 0.7 bc
-	1.0	-	1.0	3	2.1 ± 2.1 c	0.3 ± 0.3 c	0.3 ± 0.3 c
8.0	-	-	0.5	6	16.9 ± 2.7 b	7.2 ± 1.4 b	2.2 ± 0.3 b
10.0	-	-	0.1	4	5.8 ± 3.4 c	1.5 ± 1.0 c	0.5 ± 0.5 c
10.0	-	-	0.5	5	32.4 ± 3.2 a	23.4 ± 3.4 a	3.9 ± 0.1 a
-	-	1.0	0.1	4	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
Statistical analysis					$F_{7, 34}$ = 21.609; p < 0.0001	$F_{7, 34}$ = 20.2731; p < 0.0001	$F_{7, 34}$ = 5.9199; p = 0.0003

[z] The percent differentiated callus was calculated as (number of callus clumps that differentiated adventitious buds) ÷ (total number of callus clumps in that treatment). The differentiation ratio was calculated as (number of newly differentiated adventitious buds) ÷ (total number of differentiated callus clumps)

[y] Values represent means ± standard error

[x] Means followed by different letters within the same column are significantly different as determined by Tukey's Honestly Significant Difference test (α = 0.05)

Micropropagation/ in vitro shoot proliferation

Once the differentiated buds developed into 2–3 cm long shoot clumps, the clumps were split and cut into 1-1.5 cm long nodal segments and transferred to WPM supplemented with four different PGR combinations to optimize shoot proliferation (Table 3). The micropropagation experiment was repeated five times with 5–33 culture jars for each PGR treatment. Each culture jar contained 3–6 nodal segments. The number and length of newly developed shoots per nodal segment within each PGR combination were recorded 28 days after transferring to shoot proliferation media.

Table 3
Effects of different PGR combinations on shoot proliferation of *L. speciosa* 28 days after culture

PGR combination (mg/L)			Percent with shoots[z]	Average number of newly developed shoots	Average shoot length (cm)	Proliferation ratio
6-BA	ZT	NAA				
0.2	-	0.02	80.8 ± 8.1[y] a[x]	23.4 ± 5.3 b	5.6 ± 1.0 a	1.9 ± 0.1 b
1.0	-	0.02	94.6 ± 4.0 a	80.4 ± 15.2 a	4.1 ± 0.9 ab	4.5 ± 0.3 a
5.0	-	0.02	87.8 ± 6.7 a	84.6 ± 8.3 a	1.6 ± 0.3 b	5.4 ± 0.6 a
1.0	0.2	-	81.0 ± 16.1 a	60.0 ± 15.8 a	3.4 ± 0.8 ab	4.5 ± 0.9 a
Statistical analysis			$F_{3, 19}$ = 0.450; p = 0.7209	$F_{3, 19}$ = 5.431; p = 0.0091	$F_{3, 19}$ = 4.506; p = 0.0179	$F_{3, 19}$ = 6.874; p = 0.0035

[z] The percentage of explants with shoots was calculated as (number of nodal segments initiated) ÷ (total number nodal segments cultured). The proliferation ratio was calculated as (total number of newly developed shoots) ÷ (total number of cultured nodal segments)

[y] Values represent means ± standard error

[x] Means followed by different letters within the same column are significantly different as determined by Tukey's Honestly Significant Difference test (α = 0.05)

Rooting and acclimatization

After shoots elongated on WPM supplemented with 0.5 mg/L 6-BA for four weeks, 3–5 cm long shoots were excised and cultured on half-strength WPM supplemented with 3.0% (w/v) sucrose, 0.65% (w/v) agar, 0.05% (w/v) ascorbic acid, 0.05% (w/v) PVP-40, and varying concentrations of IBA. After roots had formed (approximate 30 days), culture media was gently washed off, and plantlets were transplanted into 6-cell plug trays covered with a dome to maintain humidity. The plug trays contained 70% potting mix (Jolly Gardener® Pro-Line C/GP growing mixture) and 30% perlite (Dicaperl®, Dicalite Management Group, Inc., West Conshohocken, PA, USA). Following 90 days of acclimatization, regenerated plantlets were transplanted into 3.78-liter containers with the potting mix described above and maintained in the greenhouse. Upon the emergence of floral buds on the regenerated plants, the developmental progression was closely monitored until full bloom. Concurrently, key morphological characteristics, such as the color and diameter of the fully-bloomed flowers, the shape and arrangement of the leaves, and their respective lengths and widths, were documented. For comparative purposes, six specimens of *L. speciosa*, provided by Dr. Knox, were employed as a reference control to ascertain the baseline morphological characteristics of the leaves.

The rooting experiment was repeated five times with 2–14 regenerated shoots for each PGR treatment. The number and length of newly developed roots (longer than 1 cm) per regenerated plantlet and the total number of rooted plantlets that survived 28 days after transplanting in covered chambers were recorded. To ascertain the floral and foliar characteristics, seven transplanted plants were morphologically analyzed at the peak of their bloom.

Genetic fidelity assessment

DNA extraction and quantification

Young leaf tissue was collected from one donor plant and five randomly chosen regenerated plantlets. For each sample, 0.2 g of leaf tissue was finely ground in liquid nitrogen using a pre-chilled mortar and pestle. Genomic DNA was isolated from the resultant tissue powder using a modified cetyltrimethyl ammonium bromide protocol (Doyle and Doyle 1987). The DNA pellet was resuspended in sterile double-distilled water and preserved at -20 $\varvec{}$°C until further analysis. The concentration of the isolated DNA was determined using a Nanodrop® ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

PCR amplification

The extracted DNA samples were diluted with sterile double-distilled water to achieve a working concentration between 11.4 ng/$\varvec{}\mu$L and 32.4 ng/$\varvec{}\mu$L. Amplification was carried out using ten ISSR primers (Integrated DNA Technologies, Inc., Morrisville, NC, USA; Table 5), chosen based on their unambiguous, reproducible, and clear banding patterns as reported in earlier studies (Ahmad et al. 2022b; Godwin et al. 1997; Reddy et al. 2002; Thakur et al. 2021). The PCR reactions were conducted in a 2720 thermal cycler (Applied Biosystems, Waltham, MA,

USA) with a 20 \varvecμL reaction mixture containing 40.0 ng of the diluted genomic DNA as template, 1.25 \varvecμL of 10 \varvecμM ISSR primer, 10 \varvecμL of 2 × GoTaq® Green Master Mix (Promega, Madison, USA), in a total volume of 20 \varvecμL. The amplification program was initiated with a denaturation step at 95 \varvec°C for 4 min, followed by 45 cycles of 95 \varvec°C for 45 s, annealing (temperature based on the Tm of the respective primer) for 1 min, and a 72 \varvec°C extension for 1 min, with a final elongation at 72 \varvec°C for 10 min. To ensure accuracy and reproducibility, PCR amplification for each primer was repeated three times.

Table 4 Effects of different PGR combinations on rooting of regenerated plantlets of *L. speciosa* 28 days after transplantation

IBA (mg/L)	Percent rooted [z]	Average number of newly developed roots (>1cm)	Average root length (cm)	Survival rate (%)
0.0	90.0 10.0[y] a[x]	6.3 1.6 b	2.5 0.3 ab	51.9 3.9 c
0.2	100.0 0.0 a	10.4 1.1 a	3.6 0.7 a	98.3 1.7 a
1.0	100.0 0.0 a	7.1 0.9 ab	2.5 0.4 ab	79.7 12.6 ab
5.0	90.7 5.7 a	4.9 1.0 b	1.4 0.2 b	71.4 9.6 bc
Statistical analysis	$F_{3, 19}$ = 0.9372; p = 0.4457	$F_{3, 19}$ = 3.8277; p = 0.0305	$F_{3, 19}$ = 4.2890; p = 0.0212	$F_{3, 19}$ = 5.496; p = 0.0087

[z] The percent rooted was calculated as (number of shoots with roots) (total number of inoculated shoots). The survival rate was calculated as (number of surviving plantlets) (total number of transplanted plantlets)

[y] Values represent means standard error

[x] Means followed by different letters within the same column are significantly different as determined by Tukey's Honestly Significant Difference test (α = 0.05)

Table 5
Genetic fidelity assessment of *in vitro* regenerated *L. speciosa* using ten ISSR markers

ISSR primer code	Tm (°C)	Total bands amplified	Average bands/ individual	Fragment size range (bp)	Number of bands amplified		Number of regenerated plants displaying polymorphic bands	Percentage (%)	
					Monomorphic	Polymorphic		Monomorphism	Polymorphism
811	46.8	36	6	500–1500	35	1	1	97.2	2.8
834	49.2	24	4	400–1000	24	0	0	100.0	0.0
836	48.9	48	8	200–1500	48	0	0	100.0	0.0
847	53.1	12	2	900–1500	12	0	0	100.0	0.0
855	53.1	42	7	400–1500	41	1	1	97.6	2.4
868	43.2	24	4	300–1100	24	0	0	100.0	0.0
880	47.9	48	8	200–900	46	2	2	95.8	4.2
889	50.1	78	13	250–1500	77	1	1	98.7	1.3
891	50.3	42	7	400–1500	41	1	1	97.6	2.4
900	56.2	6	1	300–400	6	0	0	100.0	0.0
Total		360	60		354	6	2	98.7	1.3

Gel Electrophoresis

The resulting PCR products were subjected to electrophoresis on a 0.8% (w/v) agarose gel prepared in 1 × TAE buffer, followed by staining with ethidium bromide. The molecular weight of the separated PCR fragments was estimated by comparison to 100 bp and 1kb GeneRuler™ DNA Ladders (Thermo Fisher Scientific). The electrophoretic separation was performed at 82 volts for 60–100 min. Bands were visualized and documented using the Gel Doc™ EZ Imager system (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Tissue culture experiments were set up as completely randomized designs with 3–6 replications. The data from the callus induction, callus differentiation into adventitious buds, shoot proliferation, and rooting experiments were analyzed by one-way ANOVA with Tukey's Honestly Significant Difference (HSD) test (α = 0.05) using JMP® (SAS Institute Inc., Cary, NC, USA) to estimate the effects of PGRs on the *in vitro* organogenesis from leaf-derived callus.

For the ISSR analysis, the genetic fidelity assessment was conducted as described by (Sherif et al. 2018). Only reproducible and well-resolved bands ranging from 100 to 3000 bp were recorded. Data on amplified PCR fragment size (bp), total number of bands amplified, average number of bands per sample (one donor plant and five regenerated plantlets), monomorphic and polymorphic bands, and percentage of monomorphism and polymorphism were manually calculated from each ISSR primer and tabulated for genetic fidelity assessment (Table 5). Bands were scored as present (1) or absent (0) for the ISSR primer in each DNA sample.

Results

Callus induction from leaf explants

We observed significant variation in callus induction in *L. speciosa* in response to the eight selected PGR combinations ($F = 17.9305$; $df = 7, 23$; $p < 0.0001$; Table 1), highlighting the importance of individual PGR combinations in facilitating callus induction. The highest callus induction efficiency (97.9%) was achieved on medium supplemented with 0.2 mg/L 2,4-D and 1.0 mg/L 6-BA. Similar results were seen using 0.5 mg/L 2,4-D and 2.0 mg/L 6-BA (87.0% efficiency) and 0.2 mg/L 2,4-D alone (75.4% efficiency).

Visual observations taken 14 days after inoculation indicated that 0.5 mg/L NAA induced minimal callus formation, yet stimulated root formation in *L. speciosa* leaf explants (Fig. 1-a). A higher concentration of NAA (2.0 mg/L) promoted formation of whitish friable callus (Fig. 1-b). A combination of NAA (0.1 mg/L) and 6-BA (5.0 mg/L) initiated the formation of yellowish slightly compact callus (Fig. 1-c). Medium supplemented with 0.2 mg/L 2,4-D resulted in the formation of yellowish friable callus and flocky roots (Fig. 1-d), whereas increased concentration of 2,4-D (0.5 mg/L) led to the formation of yellowish friable callus (Fig. 1-e). A combination of 2,4-D (0.2 mg/L) and 6-BA (1.0 mg/L) facilitated the production of whitish granular callus (Fig. 1-f), while elevated concentration of 2,4-D (0.5 mg/L) and 6-BA (2.0 mg/L) resulted in whitish slightly compact callus (Fig. 1-g). Medium supplemented with only 2.0 mg/L 6-BA induced green compact callus (Fig. 1-h). Based on these morphological observations and the results presented in Table 1, we conclude that the combination of 0.2 mg/L 2,4-D with 1.0 mg/L 6-BA is the optimal PGR combination for proficient callus induction from leaf explants of *L. speciosa*.

Callus differentiation into adventitious buds

There were also significant differences in callus differentiation of *L. speciosa* in response to different PGR combinations ($F = 21.609$; $df = 7, 34$; $p < 0.0001$; Table 2). The highest differentiation percentage occurred on WPM supplemented with 10.0 mg/L 6-BA and 0.5 mg/L NAA, which induced 32.4% callus differentiation. All other PGR combinations resulted in significantly lower responses, including the use of TDZ, which did not promote any callus differentiation (Table 2). This combination of PGRs also resulted in the highest number of newly formed buds and the highest differentiation ratio.

Visual observations showed a clear sequence of stages in the differentiation process. A noticeable transition was observed after 45–90 days on induction medium, where the yellowish friable callus developed green meristematic callus (Fig. 2-a), followed by the emergence of discernible protuberances, denoting the initiation of adventitious shoot buds 35–50 days later (Fig. 2-b). This signaled the onset of structural differentiation, which led to the development of shoot buds 25–45 days later (Fig. 2-c). 40–60 days after this significant morphological transition, fully matured adventitious buds proliferated with elongating leaves (Fig. 2-d). Concurrently, protuberances from the green meristematic callus continued to differentiate, resulting in vigorous proliferation of adventitious shoots (Fig. 2-e,f,g,h). This dynamic progression illustrates the potential of sustained culture in the callus differentiation medium, leading to optimal shoot regeneration. Based on these observations and the results summarized in Table 2, we conclude that the combination of 10.0 mg/L 6-BA and 0.5 mg/L NAA is the optimal combination to induce callus differentiation into adventitious buds.

Micropropagation/ in vitro shoot proliferation

Our experiments to determine the most effective PGRs to proliferate adventitious shoots indicated that the response did not differ significantly among the varied PGR combinations ($F = 0.450$; $df = 3, 19$; $p = 0.7209$; Table 3). The combination of 6-BA (0.2-5.0 mg/L) with NAA (0.02 mg/L) or ZT (0.2 mg/L) induced micropropagation equally well. However, we did see differences in both the number ($F = 5.431$; $df = 3, 19$; $p = 0.0091$; Table 3) and the length ($F = 4.506$; $df = 3, 19$; $p = 0.0179$; Table 3) of the emergent shoots across the diverse PGR combinations tested. Furthermore, a significant difference was seen in the proliferation ratios ($F = 6.874$; $df = 3, 19$; $p = 0.0035$; Table 3).

Following the development of well-formed adventitious shoots, the clusters were split and cut into nodal segments prior to culture on fresh medium supplemented with different PGR combinations for micropropagation. 28 days after culture, nodal segments cultured on WPM containing 0.2 mg/L 6-BA and 0.02 mg/L NAA generated 2–3 new shoots per segment, with individual shoots reaching a length of 5–7 cm (Fig. 3-a; Table 3). Increasing the 6-BA concentration to 1.0 mg/L, while retaining NAA levels at 0.02 mg/L, increased shoot development to 4–5 new shoots per segment (Fig. 3-b). These shoots demonstrated variable growth, with lengths ranging from 3–5 cm (Table 3). Using WPM supplemented with 6-BA at 5.0 mg/L and 0.02 mg/L NAA led to the emergence of 5–6 new shoots per segment, with each shoot 1–2 cm in length (Fig. 3-c; Table 3). Medium containing 1.0 mg/L 6-BA and 0.2 mg/L ZT yielded 3–6 new shoots per segment, with lengths of 3–4 cm (Fig. 3-d; Table 3). Based on these results, we conclude that the combination of 1.0 mg/L 6-BA and 0.02 mg/L NAA is the most effective for micropropagation by shoot proliferation in *L. speciosa*.

Regenerated plantlet rooting and acclimatization

As with shoot proliferation, we did not see significant differences among treatments in the percentage of rooting success ($F = 0.4457$; $df = 3, 19$; $p = 0.9372$; Table 4). However, we did see significant differences in the number of roots initiated ($F = 3.8277$; $df = 3, 19$; $p = 0.0305$; Table 4), the length of roots ($F = 4.2890$; $df = 3, 19$; $p = 0.0212$; Table 4), and the survival rate ($F = 5.496$; $df = 3, 19$; $p = 0.0087$; Table 4) after acclimatation. Figure 4 representatively illustrated the morphological characteristics of regenerated plantlets rooted under various concentrations of IBA. In the absence of IBA, plantlets yielded an average of 6.3 roots, each with a mean length of 2.5 cm (Table 4; Fig. 4-a). Supplementation with 0.2 mg/L IBA enhanced root production to an average of 10.4 roots, with a mean length of 3.6 cm (Table 4; Fig. 4-b). At an IBA concentration of 1.0 mg/L, the average number of roots increased slightly to 7.1, whereas the mean root length decreased to 2.5 cm (Table 4; Fig. 4-c). However, at a concentration of 5.0 mg/L IBA, there was a reduction to an average of 4.9 roots, with an average length of 1.4 cm (Table 4; Fig. 4-d). Considering the survival rate (Table 4) and morphological observations (Fig. 4-a,b,c,d), we conclude that 0.2 mg/L IBA leads to the best combination of root number, length, and survival of plantlets compared to other treatments.

One year after transplanted into 3.5-liter pots, the regenerated plants started to bloom. Bright pink or lavender flowers with a 61.9 $\pm$ 1.8 mm diameter clustered on terminal panicles, and elliptic leaves, measuring 139.6 $\pm$ 19.2 mm by 49.6 $\pm$ 7.5 mm, were oppositely or sub-oppositely arranged (Fig. 5). These leaf dimensions bear a close resemblance to those of the control plant leaves, measuring 128.17 $\pm$ 2.8 mm by 51.08 $\pm$ 4.2 mm.

ISSR analysis

In assessing the genetic fidelity of *in vitro* regenerated plantlets, we observed a total of 360 reproducible bands from ten ISSR markers, with a size range of 200-1,500 bp (Table 5). This included 355 monomorphic and 5 polymorphic bands, averaging 60 bands per six samples analyzed. The number of bands amplified by each ISSR marker ranged from 1 to 13. Overall, 98.7% of the bands were identical between parent plants and regenerated plantlets, while 1.3% showed genetic polymorphism (Table 5). The five polymorphic bands were predominantly observed in two of the plantlets, yielding a cumulative count of 2, as detailed in Table 5 and Fig. 6.

Discussion

The prevailing theory in plant tissue culture suggests that exogenous auxin promotes callus formation, whereas cytokinin supports callus differentiation (Gaba 2005). Empirical evidence indicates a more nuanced and sometimes less predictable interaction between auxin and cytokinin. For example, Bhaskaran and Smith (1990) found that 2,4-D promoted the formation of regenerable callus particularly when combined with cytokinin. However, in leeks (*Allium ampeloprasum*), the amount of 2,4-D determined whether crystalline or gelatinous callus would form of leek (Silvertand et al. 1996). In our research, the interplay between auxin and cytokinin notably influenced callus initiation and proliferation in *L. speciosa* leaf explants, as shown in Table 1 and Fig. 1. Utilizing WPM supplemented with 0.1 mg/L NAA and 5.0 mg/L 6-BA facilitated the development of yellowish, moderately compact callus in approximately 24.1% of the explants. In contrast, WPM supplemented with 0.2 mg/L 2,4-D and 1.0 mg/L 6-BA supported the formation of whitish granular callus in 97.9% of the explants. In studies involving other crapemyrtle species, an MS basal medium containing 0.5 mg/L 2,4-D and 1 mg/L 6-BA stimulated somatic embryogenesis from *L. indica* leaf explants (Niranjan and Sudarshana 2005). Furthermore, a 70.33% induction rate of whitish-green callus from 'Dynamite' (*L. indica*) leaf explants occurred 20 days after inoculation on WPM supplemented with 0.5 mg/L NAA and 0.8 mg/L 6-BA (Jiang et al. 2023). Additionally, 46.5% of *L. fauriei* cotyledon explants exhibited callus formation when cultured on half-strength MS medium with 0.5 mg/L 2,4-D and 1.0 mg/L 6-BA (Chen et al.

2018). The different results from these studies can be attributed to differences in endogenous phytohormone levels of explants, different plant genotypes, variability in explant age, differences in basal culture medium, and distinct culture environments (Hu et al. 2017; Long et al. 2022).

The intricate process of adventitious bud differentiation from callus into a full plantlet is significantly influenced by the interaction between cytokinin and auxin levels (Schaller et al. 2015; Su et al. 2011). Emerging literature underscores that the relative cytokinin concentration or cytokinin-to-auxin ratio, rather than their absolute levels, profoundly influences *de novo* organogenesis, including meristem initiation and the development of shoots and roots (Cammarata et al. 2022; Ćosić and Raspor 2022; Dermastia et al. 1994). Studies have shown that elevated concentrations of cytokinin, coupled with low auxin levels, facilitate shoot organogenesis and elongation (Raspor et al. 2021; Skoog and Miller 1957; Šmeringai et al. 2023; Tu et al. 2021). For instance, de Oliveira et al. (2022) observed no adventitious bud differentiation in cotyledonary leaf callus of *Eucalyptus cloeziana* on MS medium supplemented with 0.5 mg/L 6-BA and 0.5 mg/L NAA, in contrast to a notable 10.0% differentiation rate achieved with 1.0 mg/L 6-BA and the same NAA concentration. Our study supports these findings in that we observed increased callus differentiation when the concentration of 6-BA was increased to10.0 mg/L, while maintaining NAA at 0.5 mg/L (Table 2). Furthermore, research with other crapemyrtle species revealed an 18.7% differentiation yield in cotyledonary callus of *L. fauriei* on WPM containing 1.0 mg/L 6-BA and 0.5 mg/L IBA (Chen et al. 2018). Intriguingly, Jiang et al. (2023) recorded decreased callus regeneration in 'Dynamite' (*L. indica*) with an increase in 6-BA concentration. These findings underscore the importance of determining an optimized cytokinin-to-auxin ratio for each genotype in a plant tissue culture regeneration and differentiation process.

Given the critical role the cytokinin-to-auxin ratio plays in plant developmental processes, conducting detailed morphological analyses becomes essential in determining when and how PGRs affect the developmental stages (Galán-Ávila et al. 2020; Morinaka et al. 2023; Peng et al. 2022; Saitou et al. 1992; Tanimoto and Harada 1984). Our study is the first to delineate the morphological transitions occurring from callus initiation to adventitious shoot development in *L. speciosa*. The progression of developmental milestones in *L. speciosa*, as described above in the Results section, documents differentiation in response to various PGRs that can be used to optimize cytokinin-to-auxin ratios. It also serves as a foundational reference for future research aimed at enhancing the efficiency of *L. speciosa* regeneration and facilitating the establishment of robust plant regeneration systems for other crapemyrtle species or cultivars.

The integration of artificial intelligence tools into plant tissue culture has witnessed a significant upsurge in recent years, with promising applications in the optimization of PGRs through predictive modeling (Ayuso et al. 2017; García-Pérez et al. 2020; Hameg et al. 2020; Hesami et al. 2019). For example, applying machine learning models for PGR optimization in *Phaseolus vulgaris*, a recalcitrant crop, lead to significant increases in shoot regeneration frequency, shoot number, and shoot length (Aasim et al. 2022). In *L. speciosa*, the morphological progression documented in our study can serve as vital parameters in deploying machine learning strategies, potentially utilizing acritical neural network algorithms to precisely calibrate PGR combinations, thereby increasing the *in vitro* regeneration efficiency.

Tissue-culture induced somaclonal variation is a challenge in regeneration-based clonal propagation, and has historically hampered the commercialization of plant tissue culture techniques, especially in the case of recalcitrant woody plants (Bhojwani and Dantu 2013; Bhojwani et al. 2003). For instance, oil palm (*Elaeis guineensis*) and date palm (*Phoenix dactylifera*), when propagated through somatic embryogenesis utilizing leaf or inflorescence tissues, have encountered critical setbacks, including floral sterility and aberrant fruit setting (Cohen et al. 2004; Jaligot et al. 2011). Ascertaining the genetic and morphological stability of regenerated plantlets is critical in facilitating the integration of plant tissue culture techniques into the ornamental plant industry. The results presented in our study demonstrate, for the first time, a successful protocol for regenerating *L. speciosa* plantlets through callus-induced shoot organogenesis using leaf explants, with genetic stability evaluated using ISSR primers. While our plants did exhibit some level of variation (1.3% polymorphism) between donor plants and regenerated plantlets (Table 5 and Fig. 6), the plantlets exhibited normal floral development when transplanted and maintained in the greenhouse (Fig. 5). These results are similar to previous studies in *L. speciosa* micropropagation (Ahmad et al. 2022b; Gilman and Watson 2014; Unnikrishnan 2023). Research in other crops has indicated that a genetic similarity exceeding 90% between the micropropagated plantlets and donor plant is generally regarded as acceptable, assuming no phenotypic differences between the propagules and the parent plants (Bhattacharyya et al. 2017; Sherif et al. 2020; Tikendra et al. 2019). Therefore, this protocol offers a reliable platform for mass propagation by growers and for conducting genetic transformation in *L. speciosa*. It could facilitate the introgression of valuable traits such as cold hardiness and disease and pest resistance from other species, using callus cultures, protoplast fusion, gene editing, or other technologies that circumvent traditional breeding barriers between species (Herbert et al. 2009; Pooler 2006; Pounders et al. 2007). Consequently, our research marks a vital step towards the precise and reliable targeted improvement of crapemyrtle cultivars, substantially enhancing their commercial potential.

Conclusion

This study is the first to successfully regenerate genetically stable plantlets from callus using leaf explants of *L. speciosa*, thereby facilitating molecular breeding in this species to improve cold hardiness, pest resistance, and other desired traits. This feat was realized through meticulous investigations to determine the optimal PGRs conducive to callus induction (0.2 mg/L 2,4-D and 1.0 mg/L 6-BA), callus differentiation (10.0 mg/L 6-BA and 0.5 mg/L NAA), shoot proliferation (1.0 mg/L 6-BA and 0.02 mg/L NAA), and root induction (0.2 mg/L IBA), supplemented by genetic fidelity assessment using ISSR primers (98.7% identity). Moreover, this is the first report that offers detailed

morphological observations of the developmental transitions occurring within *L. speciosa* callus during differentiation into adventitious shoots. This work provides critical insights that could contribute to the optimization of PGRs for enhancing shoot organogenesis in other crapemyrtles, laying a foundation for future research in this important genus.

Declarations

Author contributions **H.Q., M.G.,** and **H.D.** acquired funding; **H.Q.** and **B.W.** conceived and designed the study; **H.Q.** supervised its execution; **B.W.**, **N.Z.**, **B.D.**, **I.S.**, **S.K.**, and **A.L.** carried out the experiments and collected data; **B.W., H.Q.,** and **M.P.** analyzed the obtained data; **B.W.** drafted the manuscript; **H.Q, M.P.,** and **H.D.** performed review and editing. All the authors have read and agreed to the published version of the manuscript.

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Data availability All data generated and/or analyzed during this study are included in this published article.

Conflict of interest All authors declare no conflict of interest.

Ethical approval Not applicable.

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Consent for publication Not applicable.

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Figures

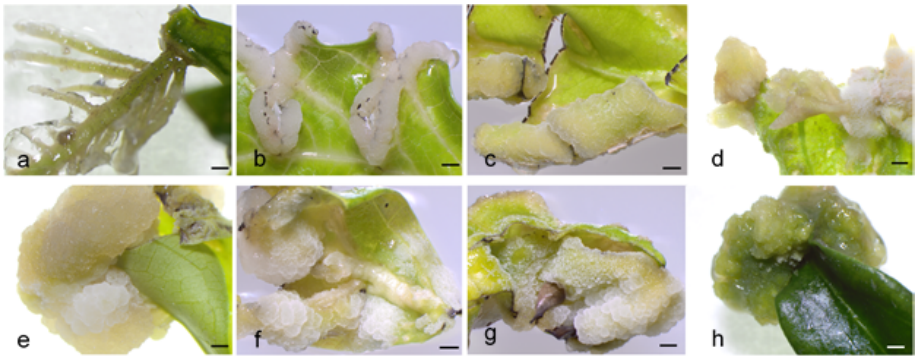

Figure 1

Effects of different PGR combinations on callus induction and morphogenesis in leaf explants of *L. speciosa*. Observations recorded 14 days after inoculation on WPM demonstrated: **(a)** 0.5 mg/L NAA facilitated limited callus induction but promoted root formation in the leaf explants. **(b)**NAA at 2.0 mg/L promoted the development of whitish friable calli. **(c)** A combination of 0.1 mg/L NAA and 5.0 mg/L 6-BA induced yellowish, slightly compact calli. **(d)** 0.2 mg/L 2,4-D resulted in yellowish, friable callus formation with the development of flocky roots. **(e)** 2,4-D at 0.5 mg/L induced yellowish friable calli. **(f)** 1.0 mg/L 6-BA and 0.2 mg/L 2,4-D facilitated the formation of whitish granular calli. **(g)** 2.0 mg/L6-BA and 0.5 mg/L 2,4-D led to the development of whitish slightly compact calli. **(h)**2.0 mg/L 6-BA induced green compact calli. Scale bars represent 1 mm

Figure 2

Adventitious bud formation and proliferation from leaf callus of *L. speciosa* on WPM supplemented with 6-BA (8.0-10.0 mg/L) and NAA (0.1-0.5 mg/L). **(a)** A transition from yellowish friable callus to green meristematic callus (inside dashed-line box) was observed 45-90 days after inoculation. **(b)** Following the establishment of green meristematic callus, shoot bud initials or protuberances (inside dashed-line box) became evident after 35-50 days, marking the onset of structural differentiation. **(c)** 25-45 days after the emergence of the protuberances, shoot buds enclosed by outer primordial leaves developed. **(d)**40-60 days following shoot bud emergence, numerous fully-developed adventitious buds accompanied by elongating leaves had formed. **(e-h)** A persistent differentiation of shoot initials (inside dashed-line box) from the meristematic callus resulted in proliferation of adventitious shoots upon sustained culture in the callus differentiation medium. Scale bars represent 1 mm

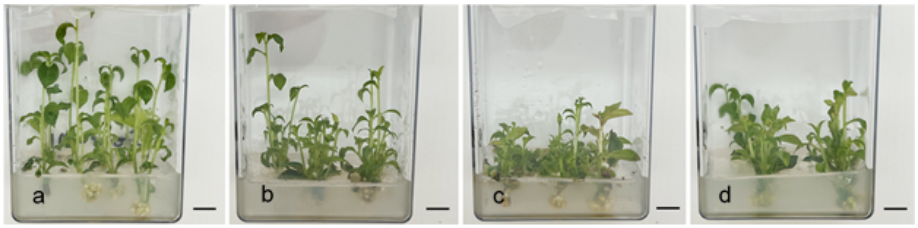

Figure 3

Effects of different PGR combinations on shoot proliferation after 28 days on induction media. (a) Each nodal segment cultured on WPM supplemented with 0.2 mg/L 6-BA and 0.02 mg/L NAA exhibited proliferation into 2-3 new shoots, each measuring 5-7 cm in length. (b) 6-BA at 1.0 mg/L and 0.02 mg/L NAA fostered the development of 4-5 new shoots per segment, with lengths of 3-5cm. (c) WPM supplemented with 5.0 mg/L 6-BA and 0.02 mg/L NAA resulted in 5-6 new shoots per segment, each 1-2 cm in length. (d) WPM supplemented with 1.0 mg/L 6-BA and 0.2 mg/L ZT resulted in 3-6 new shoots per segment, each 3-4 cm in length. Scale bars represent 10 mm

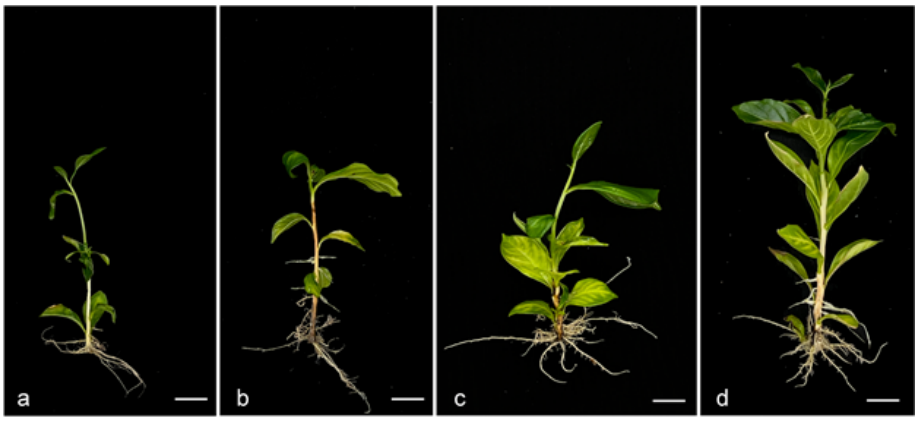

Figure 4

Rooting for regenerated plantlets of *L. speciosa*. Observations recorded at 28 days after inoculation on half-strength WPM revealed: (a) Plants grown on medium with no IBA generated an average of 6.3 roots with an average length of 2.5 cm. (b) Plants grown on medium supplemented with 0.2 mg/L IBA generated an average of 10.4 roots with an average length of 3.6 cm. (c) Plants grown on medium supplemented with 1.0 mg/L IBA generated an average of 7.1 roots with an average length of 2.5 cm. (d) Medium supplemented with 5.0 mg/L IBA generated an average of 4.9 roots, with an average length of 1.4 cm. Scale bars represent 10 mm

Figure 5

Acclimatization and morphological observation for regenerated plantlets of *L. speciosa*. **(a)**Plantlet with healthy root system after removal of agar were transplanted in plug trays for acclimatization. **(b)** Fully acclimatized regenerated plantlets in 3.78-liter pots in the greenhouse. **(c)** Floral buds emerged from regenerated plantlet of *L. speciosa* 12 months after transfer to the greenhouse. **(d)** Regenerated plantlet of *L. speciosa* fully bloomed within 7 days after the floral buds' emergence. Scale bars represent 10 mm

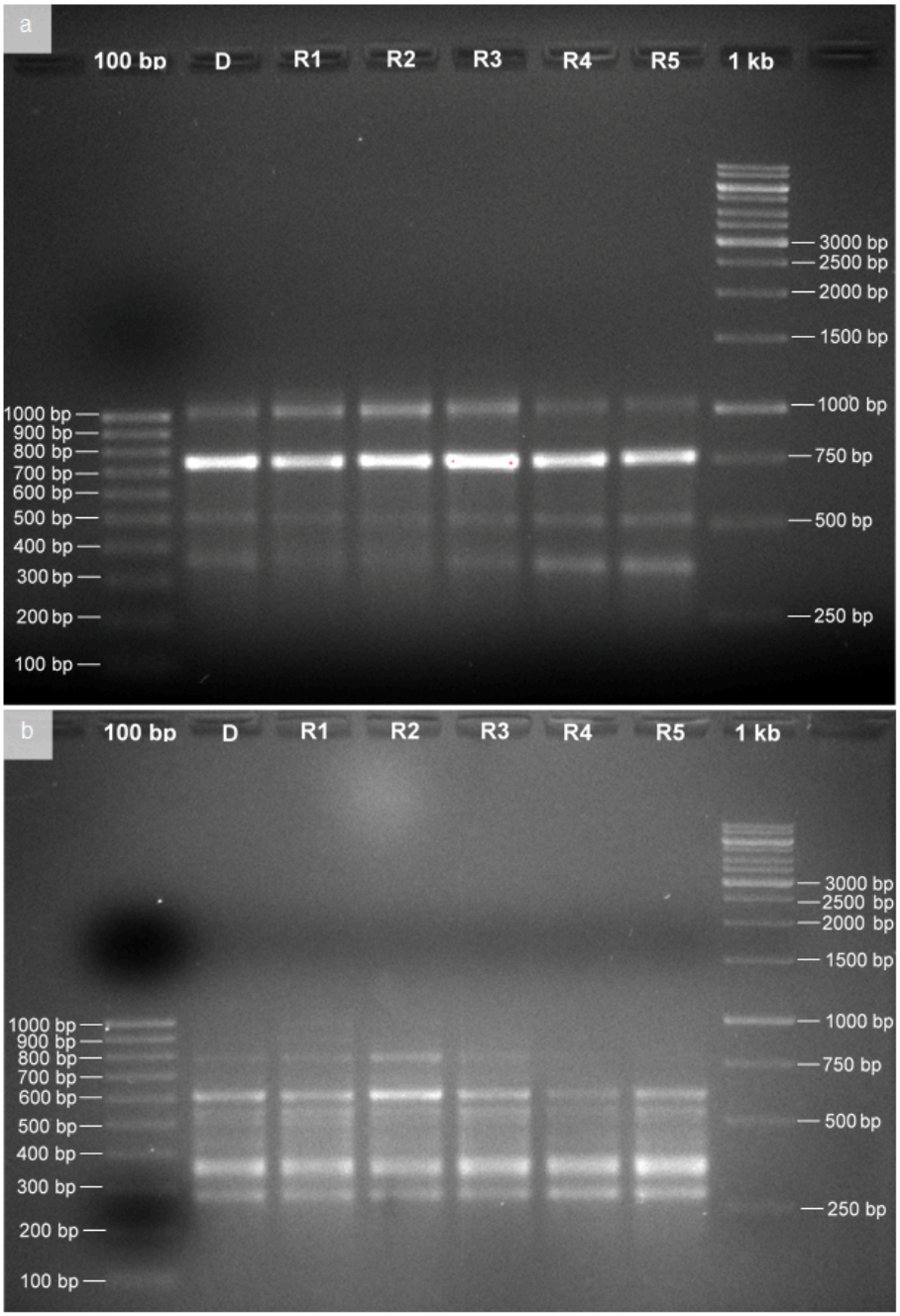

Figure 6

Assessment of genetic fidelity in *in vitro* regenerated *L. speciosa* using ISSR markers. **(a)** Amplified profile from ISSR primer UBC-868 revealed 24 monomorphic bands and 0 polymorphic bands between donor plant (D) and randomly selected regenerated plantlets. **(b)** Amplified profile from UBC-880 revealed 46 monomorphic bands and 2 polymorphic bands between the donor plant and the regenerated plantlets. Lanes leaved empty were devoid of DNA and primers