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High-efficiency plant regeneration via callus-induced organogenesis from leaf explants of Queen's crapemyrtle (*Lagerstroemia speciosa*)

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Summary

Crapemyrtle (*Lagerstroemia* sp.) is the best-selling flowering tree and provides excellent pollen sources for pollinators in the U.S. However, the market's most commercially available crapemyrtle cultivars are easily infested by a recently invasive insect, crapemyrtle bark scale (CMBS; *Acanthococcus lagerstroemiae*), which jeopardizes the production and esthetic value of crapemyrtles anticipated by the Green Industry. Therefore, breeding CMBS-resistant cultivars is in great need on the market. Our previous study revealed that Queen's crapemyrtle (*L. speciosa*) was resistant to CMBS among all available crapemyrtle species. Establishing a highly efficient regeneration system for Queen's crapemyrtle is essential to molecular plant breeding for resistance improvement. Here, our study found that 97.9 ± 1.0% of leaf

explants were induced callus when cultured on Lloyd & McCown woody plant medium (WPM) supplemented with 0.20 mg/L 2,4-D and 1.00 mg/L 6-BA. After transferring to the WMP medium supplemented with 10.00 mg/L 6-BA with 0.50 mg/L NAA, 32.4 ± 3.2% of callus successfully differentiated as the largest number of adventitious buds (23.4 ± 3.4) at the highest differentiation ratio (3.9 ± 0.1). The WPM medium supplemented with 1.00 mg/L 6-BA and 0.02 mg/L NAA induced 94.6 ± 4.0% of nodal segments of the regenerated shoots to produce 80.40 ± 15.16 new shoots (4.1 ± 0.9 cm in length) at the proliferation ratio of 4.5 ± 0.3. Half-strength WPM supplemented with 0.20 mg/L IBA induced 100.0 ± 0.0% of the regenerated shoots to produce 10.4 ± 1.1 roots (3.6 ± 0.7 cm in length) per shoot, and 98.3 ± 1.7% of the rooted plantlets survived after transplanting into the pots containing Jolly Gardener ® Pro-Line C/GP soil and 30% perlite for acclimatization. The successful establishment of the highly efficient callus-induced regeneration system lays a critical foundation for the genetic engineering of crapemyrtle to improve plant resistance or other desired traits, which meet priority needs of the nursery production or Green Industry.

INTRODUCTION

Crapemyrtle bark scale (*Acanthococcus lagerstroemiae*) is a recently invasive sap-sucking hemipteran initially reported on crapemyrtle (*Lagerstroemia* sp.) in the U.S. Heavy infestations of *A. lagerstroemiae* significantly reduce crapemyrtle growth and flowering, negatively impacting the production and landscape aesthetic value of crapemyrtles (Marwah et al., 2021; Merchant et al., 2018). A new crapemyrtle species or cultivar resistant to *A. lagerstroemiae* is in great need for plant resistance improvement and commercial on the U.S. market (Boutigny et al., 2020; Datta, 2021; Smith, 2021).

Greenhouse assays for host confirmation and plant susceptibility/resistance evaluation found that *Lagerstroemia speciosa* was relatively resistant to *A. lagerstroemiae* among the tested crapemyrtle species (Wu et al., 2021; Wu et al., 2022). *Lagerstroemia speciosa*, commonly known as 'Queen's crapemyrtle', is a tropical deciduous tree native to southeast Asia (Gilman and Watson, 2014; Klein et al., 2007; Rojas-Sandoval, 2017). Currently, most of the research on *in vitro* propagation of Queen's crapemyrtle focused on improving the micropropagation system by investigating different plant growth regulator (PGR) combinations for shoot proliferation and rooting, such as 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). Although one publication mentioned shoot organogenesis-based regeneration from leaf callus, it is beneficial to have a comprehensive investigation to optimize the PGR combination for callus initiation, differentiation, micropropagation, and rooting, which allows to establish a highly efficient and stable callus-induced regeneration system of Queen's crapemyrtle (Rahman et al., 2010).

In this study, aseptic leaf explants of Queen's crapemyrtle were utilized to induce callus, and different types and concentrations of auxins, including 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA), and NAA, and cytokinin,

including 6-BA, TDZ, zeatin (ZT) were inspected to determine the most efficient PGR combination for each step of callus-induced plant regeneration.

MATERIALS AND METHODS

Collection and disinfection of plant materials. Queen's crapemyrtle plants were provided by Dr. Gary Knox at North Florida Research and Education Center (Quincy, FL 32351). The plants were transplanted in 11.4 L pots containing potting soil (Jolly Gardener® Pro-Line C/GP growing mixture, Oldcastle Lawn & Garden Inc., Poland Spring, ME) and maintained in the Greenhouse (at 25 ± 5 °C, 50% ± 10% relative humidity (RH), and a photoperiod of 10.5:13.5 (light: dark) h) at the Department of Horticultural Sciences of Texas A&M University (lat. 30°36'31.9"N, long. 96°21'1.9"W).

Healthy and juvenile stems were collected as explants from the potted Queen's crapemyrtle in early June 2021 (**Fig. 4A**). 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 being thoroughly rinsed with sterile distilled water for 4-6 times. The disinfected explants were cut into nodal segments and cultured onto Lloyd and McCown woody plant medium (WPM; (Lloyd and McCown, 1980)) supplemented with 3.0% (w/v) sucrose, 0.65% (w/v) agar, and 0.5 mg/L 6-BA to acquire and maintain aseptic plantlets from axillary shoot formation and micropropagation/proliferation. Each culture jar contained 2 to 5 nodal segments.

Callus initiation. Aseptic leaf explants collected from the aseptic plantlets were cut into smaller pieces and cultured on WPM medium supplemented with different PGR treatments (**Table 1**). The callus initiation experiment was repeated thrice and 10 replicates (culture jars/vessels) for each PGR treatment per time. And each culture jar/vessel contained 4-9 explants. The percentage of leaf pieces successfully formed callus (total number of leaf pieces that formed callus divided by the total leaf pieces) 2 weeks after the 1st initiation were recorded to optimize the PGR combination for callus initiation/formation from leaf pieces of Queen's crapemyrtle.

Callus differentiation. Leaf-derived callus was subcultured on WPM medium supplemented with different PGR combinations (**Table 2**). The callus differentiation experiment was repeated at least thrice and 4 replicates (culture jars/vessels) for each PGR treatment per time. Each culture jar/vessel contained 3-6 callus. The total number of newly developed adventitious buds per differentiated callus within each PGR combination was recorded to optimize the PGR combination for callus differentiation from the leaf-derived callus.

Micropropagation/shoot proliferation. When the differentiated buds developed as 2-3 cm long shoot clumps, the clumps were split, cut into 1-1.5 cm long nodal segments, and proliferated on WPM medium supplemented with four different PGR combinations (**Table 3**). The micropropagation experiment was repeated 5 times and 5-33 replicates (culture jars/vessels) for each PGR treatment per time.

Each culture jar/vessel contained 3-6 nodal segments. The number and length of newly developed shoots per nodal segment within each PGR combination were recorded 4 weeks after transfer to optimize the PGR combination for micropropagation/shoot proliferation.

Rooting and acclimatization. After elongating and hardening the shoots on WPM medium added with 0.5 mg/L 6-BA for 4 weeks, 3-5 cm long shoots were excised and cultured on half-strength WPM medium 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 different concentrations of IBA. Each well-rooted plantlet was gently washed off the medium residuals and separately transplanted into 6-cell plug trays capped with a humidify dome for hardening and acclimatization. The plug trays were potted with soil (Jolly Gardener® Pro-Line C/GP growing mixture, Oldcastle Lawn & Garden Inc., Poland Spring, ME) and 30% perlite (Dicaperl®, Dicalite Management Group, Inc., West Conshohocken, PA).

The rooting experiment was repeated 5 times and 2-14 replicates (regenerated shoots) for each PGR treatment per time. The number and length of newly developed roots (longer than 1 cm) per regenerated plantlet, and the total number of rooted plantlets survive 4 weeks after transplanting in covered chambers were recorded to optimize the IBA concentration for rooting regenerated plantlets of Queen's crapemyrtle *in vitro*.

Culture condition and statistical analysis. Unless further clarification, after inoculation, all plant materials were incubated in a walk-in growth chamber (CONVIRON®, Controlled Environments Ltd., Winnipeg, Manitoba, Canada) at 25 ± 1 ℃ under 60 ± 5 % RH and a 16:8 h (L:D) light intensity of 1500 – 2000 lux. All culture media, PGRs, agar, and other reagents used for crapemyrtle regeneration experiment were purchased from PhytoTech Labs, Inc., Lenexa, KS, and all the culture media were WPM media supplemented with 3.0% (w/v) sucrose, 0.65% (w/v) agar, 0.05% (w/v) ascorbic acid, and 0.05% (w/v) polyvinylpyrrolidone-40 (PVP-40) with pH value adjusted to 5.8 before being autoclaved at 121 ℃ and 15 psi pressure for 20 min (AMSCO®, STERIS Corporation, Mentor, OH). Every 30 mL or 50 mL media were, respectively, distributed per culture jar (CultureJar™ G9, volume 220 mL; inside diameter 43 mm, height 95 mm; PhytoTech Labs, Inc., Lenexa, KS) or culture vessel (PTL-100™, volume 372 mL; length 75 mm, width 75 mm, height 98 mm; PhytoTech Labs, Inc., Lenexa, KS).

All the experiments were completely randomized design with different numbers of replications as mentioned above. The data related to the callus initiation, callus differentiation, shoot proliferation, and rooting experiments were, respectively, analyzed by one-way ANOVA with Tukey's Honestly Significant Difference (HSD) test (α = 0.05) to estimate the effects of PGRs on the organogenesis from leaf-derived callus of Queen's crapemyrtle.

RESULTS AND DISCUSSION

Callus initiation. The percentage of response in callus initiation from leaf pieces of Queen's crapemyrtle differed among the eight PGR combinations (F = 17.9305; df = 7, 23; p < 0.0001; **Table 1**). 0.20 mg/L 2,4-D with 1.00 mg/L 6-BA induced the highest

percentage of callus initiation (97.94 ± 1.04%), followed by 0.50 mg/L 2,4-D with 2.00 6-BA combination (87.1 ± 4.4%), 0.2 mg/L 2,4-D alone (75.4 ± 12.5%), which was higher than using 0.50 mg/L and 2.00 mg/L NAA alone (7.2 ± 4.1% and 31.00 ± 2.0%, respectively) or 0.10 mg/L NAA with 5.00 mg/L 6-BA (24.1 ± 3.6%).

Together with the growth status of induced callus in each PGR combination (**Fig. 1**), the optimum one was 0.20 mg/L 2,4-D with 1.00 mg/L 6-BA.

Table 1. Effects of different PGR combinations on initiation and growth of callus derived from leaf pieces of Queen's crapemyrtle (*Lagerstroemia speciosa*).

PGR combination (mg/L)			Percentage of successfully initiated callus two weeks after 1st initiation (%)
NAA	2,4-D	6-BA	
0.50	-	-	7.2 ± 4.1[z] e[y]
2.00	-	-	31.0 ± 2.0 d
0.10	-	5.00	24.1 ± 3.6 de
-	0.20	-	75.4 ± 12.5 abc
-	0.20	1.0	97.9 ± 1.0 a
-	0.50	-	70.4 ± 14.8 bc
-	0.50	2.00	87.0 ± 4.4 ab
-	-	2.00	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 ($\alpha = 0.05$).

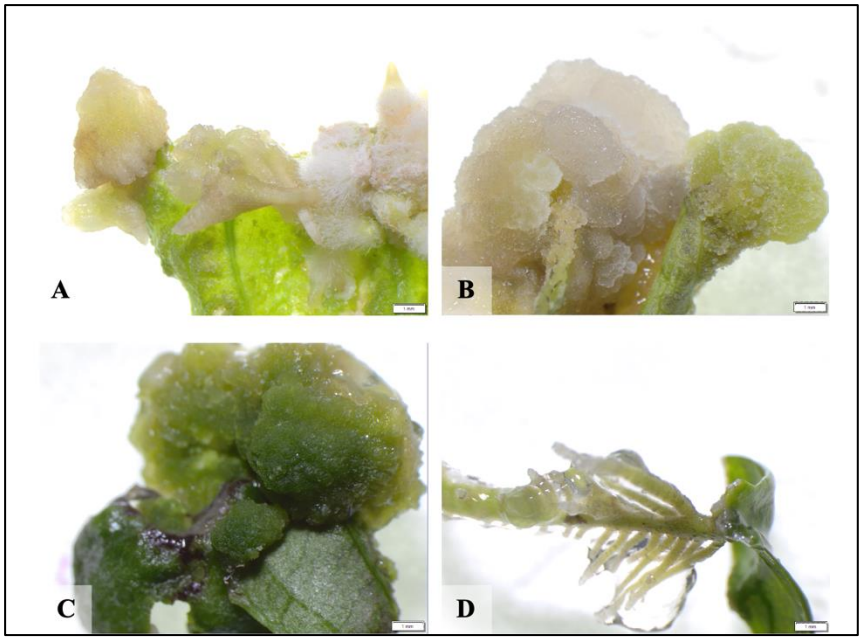

Figure 1. Effects of different PGR combinations on initiation and growth of callus derived from leaf pieces of Queen's crapemyrtle (*Lagerstroemia speciosa*). A: Yellowish, granular, and friable callus along with short flocky roots formed on WPM medium supplemented with 0.20 mg/L 2,4-D. B: Yellowish green, granular, and friable callus formed on WPM medium supplemented with 0.50 mg/L 2,4-D. C: Dark green, poor-granular, and compact callus formed on WPM medium supplemented with 2.00 mg/L 6-BA. D: Leaf pieces were directly rooted on WPM medium supplemented with 0.50 mg/L NAA.

Callus differentiation. The percentage of response in callus differentiation of Queen's crapemyrtle differed among different PGR combinations (F = 21.609; df = 7, 34; $p < 0.0001$; **Table 2**). 10.00 mg/L 6-BA with 0.50 mg/L NAA initiated the highest percentage of callus differentiation (32.4 ± 3.2%), followed by 8.00 mg/L 6-BA with 0.50 mg/L NAA (16.9 ± 2.7%), which was higher than 10.00 mg/L 6-BA with 0.10 mg/L NAA (5.8 ± 3.4%) or the combinations using ZT and NAA (less than 2.8 ± 1.7). 1.00 mg/L TDZ with 0.1 mg/L NAA did not initiate the callus differentiation.

The number of newly developed adventitious buds (F = 20.2731; df = 7, 34; $p < 0.0001$; **Table 2**) and the differentiation ratio (F = 5.9199; df = 7, 34; p = 0.0003; Table 2) differed among different PGR combinations. Together with the number of newly differentiated buds and the differentiation ratio, the optimum PGR combination for callus differentiation of Queen's crapemyrtle was 10.00 mg/L 6-BA with 0.50 mg/L NAA, which produced the largest number of newly developed adventitious buds (23.4 ± 3.4) and the highest differentiation ratio (3.9 ± 0.1).

Table 2. Effects of different PGR combinations on callus differentiation of Queen's crapemyrtle (*Lagerstroemia speciosa*).

PGR combination (mg/L)				Repeated number	Percentage of response (%)[z]	Number of newly developed buds	Differentiation ratio
6-BA	ZT	TDZ	NAA				
-	1.00	-	0.10	5	2.8 ± 1.7[y] c[x]	2.4 ± 1.9 c	1.1 ± 0.7 bc
-	1.00	-	0.20	5	2.5 ± 1.6 c	1.4 ± 0.9 c	1.4 ± 0.9 bc
-	1.00	-	0.50	3	2.2 ± 2.2 c	0.7 ± 3.7 c	0.7 ± 0.7 bc
-	1.00	-	1.00	3	2.1 ± 2.1 c	0.3 ± 0.3 c	0.3 ± 0.3 c
8.00	-	-	0.50	6	16.9 ± 2.7 b	7.2 ± 1.4 b	2.2 ± 0.3 b
10.00	-	-	0.10	4	5.8 ± 3.4 c	1.5 ± 1.0 c	0.5 ± 0.5 c
10.00	-	-	0.50	5	32.4 ± 3.2 a	23.4 ± 3.4 a	3.9 ± 0.1 a
-	-	1.00	0.10	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 percentage of response for callus differentiation was calculated as (total amount of differentiated callus) ÷ (total amount of inoculated callus) × 100%. The differentiation ratio was calculated as (total amount of newly differentiated adventitious buds) ÷ (total amount of differentiated callus). [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/shoot proliferation. The percentage of response in micropropagation of Queen's crapemyrtle did not differ among different PGR combinations (F = 0.450; df = 3, 19; p = 0.7209; **Table 3**), which indicated that 6-BA (0.20-5.00 mg/L) combined with NAA (0.02 mg/L) or ZT (0.2 mg/L) initiated the micropropagation with equal effectiveness. However, 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 newly developed shoots and the proliferation ratio (F = 6.874; df = 3, 19; p = 0.0035l; **Table 3**) differed among the PGR combinations. Together with the growth status of newly shoots in each PGR combination (**Fig. 2**), the optimum PGR combination for the micropropagation of Queen's crapemyrtle was 1.00 mg/L 6-BA with 0.02 mg/L NAA, which induced 94.6 ± 4.0% of nodal segments to produce 80.4 ± 15.2 new shoots (4.1 ± 0.9 cm in length) at the proliferation ratio of 4.5 ± 0.3.

Table 3. Effects of different PGR combinations on micropropagation of Queen's crapemyrtle (*Lagerstroemia speciosa*) at four weeks after inoculation.

PGR combination (mg/L)			Percentage of response (%)[z]	Number of newly developed shoots	Shoot length (cm)	Proliferation ratio
6-BA	ZT	NAA				
0.20	-	0.02	80.8 ± 8.1[y] a[x]	23.4 ± 5.3 b	5.6 ± 1.0 a	1.9 ± 0.1 b
1.00	-	0.02	94.6 ± 4.0 a	80.4 ± 15.2 a	4.1 ± 0.9 ab	4.5 ± 0.3 a
5.00	-	0.02	87.8 ± 6.7 a	84.6 ± 8.3 a	1.6 ± 0.3 b	5.4 ± 0.6 a
1.00	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 response for micropropagation was calculated as (total amount of nodal segments initiated micropropagation) ÷ (total amount of nodal segments inoculated) × 100%. The proliferation ratio was calculated as (total amount of newly developed shoots) ÷ (total amount of inoculated 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).

Regenerated plantlet rooting and acclimatation. The percentage of response in rooting of Queen's crapemyrtle did not differ among different PGR combinations (F = 0.4457; df = 3, 19; p = 0.9372; **Table 4**), which indicated that IBA (0.00, 0.20, 1.00, and 5.00 mg/L) all initiated the rooting with equal effectiveness. However, the number (F = 3.8277; df = 3, 19; p = 0.0305; **Table 4**) and the length (F = 4.2890; df = 3, 19; p = 0.0212; **Table 4**) of newly developed roots and the survival rate (F = 5.496; df = 3, 19; p = 0.0087; **Table 4**) differed among different IBA concentrations. Together with the growth status of regenerated plantlets in each treatment **(Fig. 3**), the optimum IBA concentration for rooting Queen's crapemyrtle was 0.20 mg/L IBA, which induced 100.0 ± 0.0% shoots to produce 10.4 ± 1.1 roots (3.6 ± 0.7 cm in length) per shoot. After the transplanting, the survival rate of the 0.2 mg/L IBA treated plantlets was 98.3 ± 1.7%, which was significantly higher than the plantlets treated with 0.00 and 5.00 mg/L IBA.

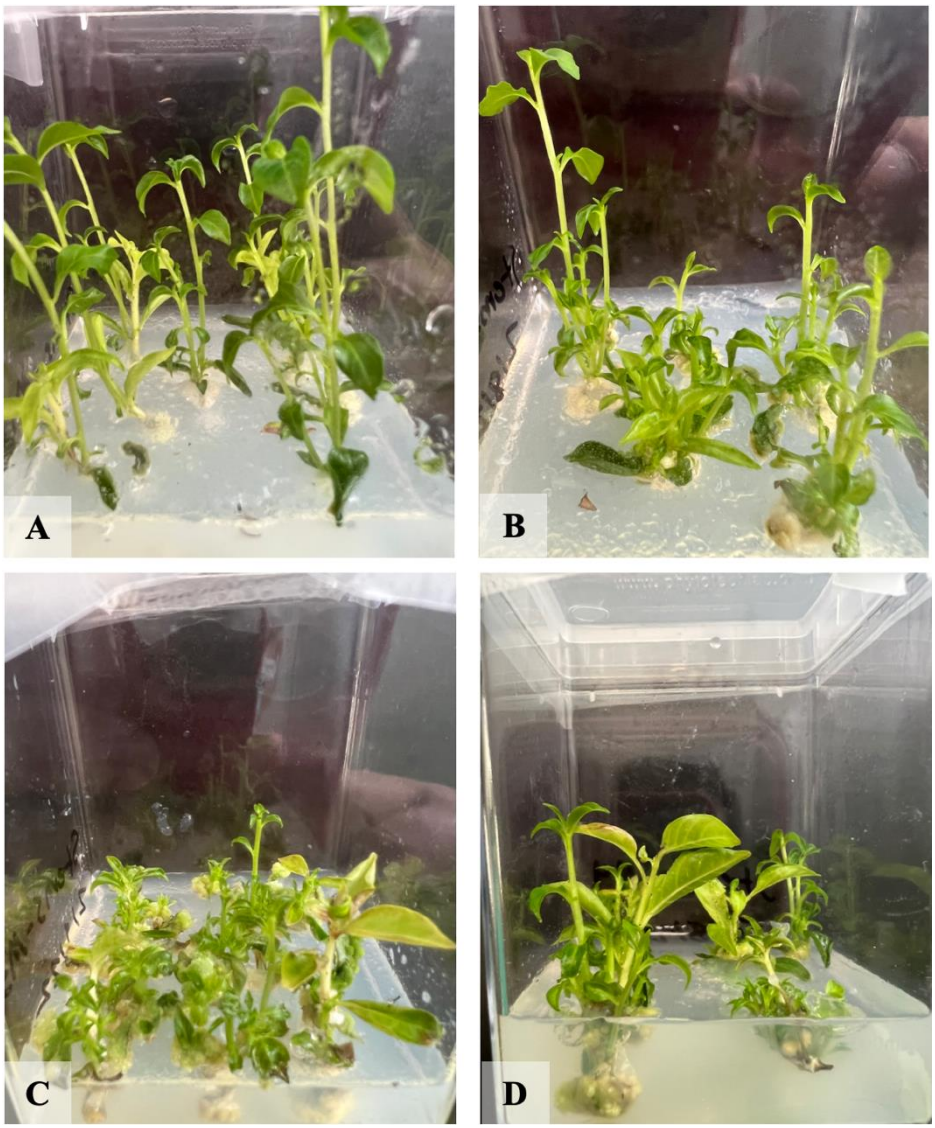

Figure 2. Effects of different PGR combinations micropropagation of Queen's crapemyrtle (*Lagerstroemia speciosa*) at four weeks after inoculation. A: Each nodal segment of Queen's crapemyrtle proliferated into 2-3 new shoots in 7-9 cm length on WPM medium supplemented with 0.20 mg/L 6-BA and 0.02 mg/L NAA. B: Each nodal segment proliferated into 4-5 new shoots in 3-7cm length with small amounts of callus formed on WPM medium supplemented with 1.00 mg/L 6-BA and 0.02 mg/L NAA. C: Each nodal segment proliferated into 3-5 new shoots in 2-4 cm length with relatively large amounts of callus formed on WPM medium supplemented with 5.00 mg/L 6-BA and 0.02 mg/L NAA. D: Each nodal segment proliferated into 3-6 new shoots in 3-5cm length with relatively some callus formed on WPM medium supplemented with 1.00 mg/L 6-BA and 0.20 mg/L ZT.

Table 4. Effects of different PGR combinations on rooting of regenerated plantlets of Queen's crapemyrtle (*Lagerstroemia speciosa*).

IBA (mg/L)	Percentage of response (%)[z]	Number of newly developed roots (>1cm)	Root length (cm)	Survival rate (%)
0	90.0 ± 10.0[y] a[x]	6.3 ± 1.6 b	2.5 ± 0.3 ab	51.9 ± 3.9 c
0.20	100.0 ± 0.0 a	10.4 ± 1.1 a	3.6 ± 0.7 a	98.3 ± 1.7 a
1.00	100.0 ± 0.0 a	7.1 ± 0.9 ab	2.5 ± 0.4 ab	79.7 ± 12.6 ab
5.00	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 percentage of response for rooting was calculated as (total amount of rooted shoots) ÷ (total amount of inoculated shoots) × 100%. The survival rate was calculated as (total amount of survived plantlets) ÷ (total amount of transplanted plantlets) × 100%. [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).

Figure 3. Effects of different PGR combinations rooting of regenerated plantlets of Queen's crapemyrtle (*Lagerstroemia speciosa*). Four weeks after inoculation, A: 4.3 roots in 1.0 cm long developed on half-strength WPM medium supplemented with 0.00 mg/L IBA. B: 7.3 roots in 2.6 cm long developed on half-strength WPM medium supplemented with 0.20 mg/L IBA. C: 7.6 roots in 2.2 cm long developed on half-strength WPM medium supplemented with 1.00 mg/L IBA. D: 3.8 roots in 1.1 cm long developed on half-strength WPM medium supplemented with 5.00 mg/L IBA.

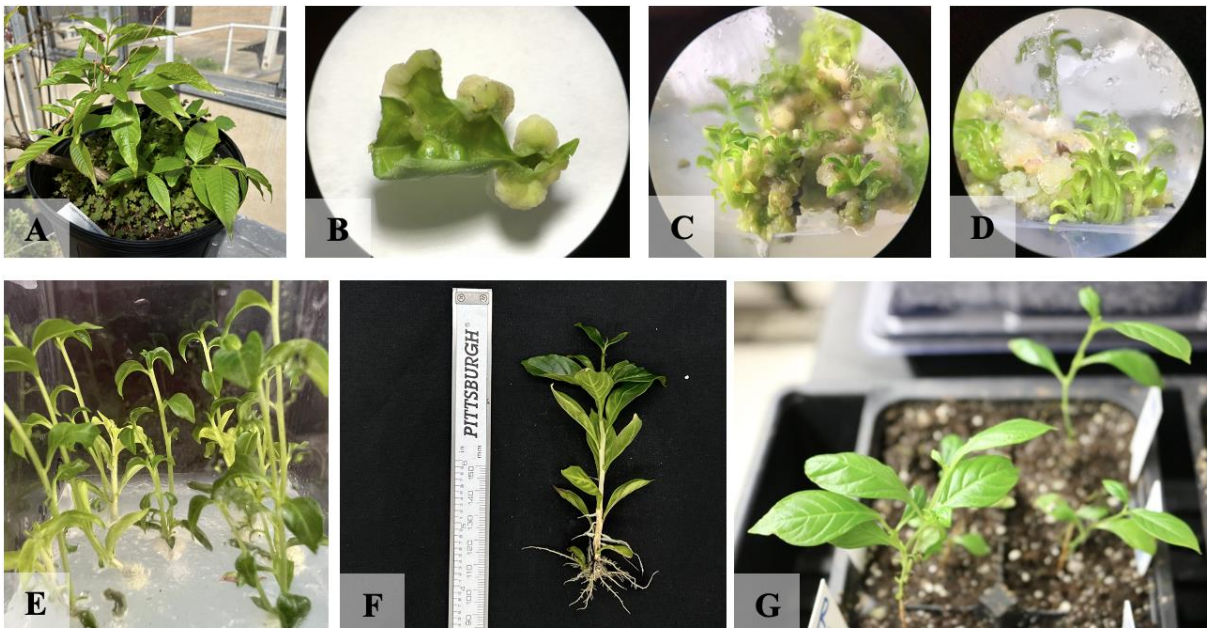

Figure 4. Establishment of callus-induced regeneration system from leaf pieces of Queen's crapemyrtle (*Lagerstroemia speciosa*). A: Initial leaf explants used for callus initiation were collected from Queen's crapemyrtle (L. speciosa) provided by Dr. Gary Knox from UF/IFAS North Florida Research and Education Center (Quincy, FL 32351). B: Leaf-derived callus was initiated on Lloyd & McCown woody plant medium (WPM) supplemented with 0.20 mg/L 2,4-D with 1.00 mg/L 6-BA at 5 days after inoculation. C: Adventitious buds differentiated from the leaf-derived callus on WPM medium supplemented 10.00 mg/L 6-BA with 0.50 mg/L NAA at 4 weeks after inoculation. D: More shoots developed and elongated on WPM medium supplemented with 10.00 mg/L 6-BA with 0.50 mg/L NAA in 14-28 days after inoculation. E: Regenerated shoots were in vitro proliferated on WPM medium supplemented with 1.00 mg/L 6-BA with 0.02 mg/L NAA in 5 weeks after inoculation. F: Strong root system was developed on half-strength WPM supplemented with 0.20 mg/L IBA in 4 weeks after inoculation. G: Rooted plants were transplanted into growth chamber for acclimatization. All the WPM medium (full-strength and half-strength) were also supplemented with 3.0% sucrose, 0.65% agar, 0.05% ascorbic acid, 0.05% PVP-40.

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