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Phloroglucinol improves morphometry, biochemical attributes and *ex vitro* growth of micropropagated plantlets of *Coccoloba uvifera* L.

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ABSTRACT

***In vitro* regeneration enables efficient clonal propagation of woody plants which are difficult to propagate via conventional methods. *Coccoloba uvifera* (Polygonaceae) is a stress-tolerant species and prevents erosion of coastal areas. The present investigation was executed to recognize the effect of phloroglucinol (PG) on morpho-biochemical traits during *in vitro* propagation of *C. uvifera*. The cultures were grown on Murashige and Skoog's (MS) medium supplemented with 1.0 mg L^{-1} 6-benzylaminopurine (BAP), 0.5 mg L^{-1} of α-naphthalene acetic acid (NAA), and different concentrations of phloroglucinol (0.5-2.0 mM). Morphometric and biochemical analysis of the *in vitro* proliferated shootlets revealed that PG at 1.0 mM in medium improved morphology, shoot length, foliar biomass, photosynthetic pigments, carbohydrates, and protein contents. The total amount of biochemicals was decreased except for free amino acids when the PG was used beyond the optimized concentrations. Phloroglucinol-derived shoots exhibited better rooting and a higher acclimatization rate. The present findings show that supplementation of PG in the medium helps to develop plantlets with improved morphometric and biochemical traits under *in vitro* conditions, to withstand adverse conditions under *ex vitro* and *in vivo* environments.**

INTRODUCTION

The genus *Coccoloba* P. Browne (buckwheat family/Polygonaceae) is distributed in the coastal regions of the world with about 150 species and stands out for its wide range of stress-tolerant potential, owing to its varied ecophysiological abilities (Melo, 2004). Among these, *Coccoloba uvifera* L. gained a lot of attention and was planted along with the coastal areas due to its saline adaptation and coastal land reclamation property (Bâ et al., 2014). It is native to tropical America and the Caribbean and is popularly known as bay-grape or sea-grape (Melo, 2004). Sea-grape has various ethno-pharmacological applications in treating intestinal disorders, and venereal and skin diseases

(Usvat, 2015). It is a rich source of phytochemicals like tannins, emodin, chrysophanol, physcion, royleanone, rhein, α-amyrin, and β-sitosterol, and exhibits antiviral, antihypertensive, antioxidant, anti-tyrosinase, photoprotective, and anti-hyperglycemic activities (Silveira et al., 2008; Rodriguexz-Garcia et al., 2019).

Coccoloba uvifera faces a poor seed set and seed germination issues (Duran et al., 2000), and vegetative propagation eventually results in its failure to meet the demand (Séne et al., 2018). Being a woody species, the *in vitro* propagation of sea-grape is challenging (Manokari et al., 2021a). The past decades have witnessed enormous progress in the development of effective micropropagation systems for woody plant species using novel tools and chemicals (Kher et al., 2021). Despite the success, the physiochemical parameters optimized during *in vitro* propagation may affect the developmental and physiological state of *in vitro* grown shoots and plantlets (Ruffoni and Savona, 2013; Manokari et al., 2021b), and the success of *in vitro* propagation protocol depends on the maximum survival of plantlets at low cost (Bairu and Kane, 2011).

Phloroglucinol (1,3,5-trihydroxybenzene) (PG) is a phenolic compound and is widely used in tissue culture as a supplement to growth regulators due to its stimulatory effect of cell division, differentiation, and regeneration properties (Teixeira da Silva et al., 2013; Aremu et al., 2015). It also alleviates *in vitro* induced physiological disorders such as controlling hyperhydricity and tissue browning by improving lignifications processes (Ross and Grasso, 2010; Naidoo et al. 2017). It is a degradation product of phloridzin, and a precursor of lignin biosynthesis (Londe et al., 2016; Teixeira da Silva et al., 2019). Recent literature revealed that phloroglucinol acts as auxin synergists and may be used as growth regulators. Nutrient medium supplemented with PG improves organogenesis and proliferation of shoots (Londe et al., 2016), shoot elongation (Bairwa et al., 2012), reduces hyperhydricity (Ross and Grasso, 2010), prevents callus interventions, promotes rooting (Teixeira da Silva et al., 2013; Tchouga et al., 2020), *in vitro* acclimatization (Pérez et al., 2016), recovery of cryopreserved protocorms (Vendrame and Faria, 2011), and stimulates somatic embryogenesis (Petti, 2020). The addition of PG to growth mediums can significantly improve the quality and physiological state of micropropagated plants (Aremu et al., 2015). As PG promotes the lignin biosynthesis pathway, it effectively controls hyperhydricity and improves the rate of shoot multiplication in *Decalepis hamiltonii* (Gururaj et al., 2004) These studies suggested that PG opened up new avenues in the improvement of plant tissue culture protocols (Teixeira da Silva et al., 2019).

The present investigation aims to evaluate the effect of phloroglucinol (PG) supplementation in the optimized nutrient medium on the *in vitro* qualitative morphometry, metabolic state (photosynthetic pigments, carbohydrates, and proteins), rooting, and acclimatization potential of plantlets of *C. uvifera*.

MATERIALS AND METHODS

*In vitro establishment of cultures***.** Nodal segments of *C. uvifera* were collected at vegetative growth phase from a 10 years old tree (Figures 1A and 1B) growing in the coastal areas of Puducherry, India (11° 56′ 01″ N, 79° 49′ 47″ E) to establish the cultures. The explants were sterilized in three sequential steps using 1.0% (v/v) of sodium hypochlorite for 5 min, 1.0% (w/v) Bavistin for 7 min, 0.1% (w/v) mercuric chloride for 5 min and rinsed thoroughly with autoclaved water. The protocol for the establishment of cultures was done following a previous report on *in vitro* propagation of *C. uvifera* (Manokari et al., 2021a). The sterilized nodal explants were inoculated on Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium containing 3.0 mg L^{-1} 6-benzylaminopurine (BAP) for bud breaking. The freshly emerged shoots were subcultured on MS medium supplemented with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} α-naphthalene acetic acid (NAA) for 4 weeks. The physical parameters of the growth chamber were set at 50 μmol m^{-2} s^{-1} spectral flux photon density (SFPD), 16-h photoperiod, and 25 ± 2 °C temperatures.

Effect of phloroglucinol on assessment of growth parameters. The 4-week-old *in vitro* proliferated

shootlets were subcultured on MS medium containing optimized growth regulators (1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA) and various concentrations (0.5, 1.0, 1.5, and 2.0 mM) of phloroglucinol. The cultures were selected with the same weight of shootlets (1.5 g) and culture vessels (350 ml capacity culture bottles) containing 50 ml culture medium. After 4 weeks, the effect of PG on morphometric traits (rate of shoot multiplication and shoot length) was analyzed. The cultures devoid of PG in the culture medium were used as control.

Analysis of fresh and dry biomass of shoots. The proliferated shootlets were removed from the culture vessels, washed with sterile distilled water to remove the adhered nutrient medium and the excess water was dried using filter paper (Whatman No.1) to estimate the fresh weight (FW). The dry weight (DW) of the shootlets was determined after drying them in a hot-air oven at 70 °C for 24 h. To determine the fresh and dry weight of the control and PG treated shootlets (g/shootlet/culture bottle), the following formula was adopted (Manokari et al., 2020):

$$\text{Growth Ratio (GR)} = \frac{\text{Harvested fresh weight (g)} - \text{Inoculated fresh weight (g)}}{\text{Inoculated fresh weight (g)}}$$

Determination of photosynthetic pigments. The middle fresh leaf samples (200 mg) of *in vitro* proliferated shoots from PG and control treatments were collected and soaked in 25 ml of acetone and absolute ethanol [in 1:1 (v/v) ratio] and incubated in dark for 24 h at 25 °C temperatures. The chlorophylls (Chl '*a*' and Chl '*b*') and carotenoid (C_{x+c}) contents in the supernatants were determined spectrophotometerically at 663 nm, 645 nm, and 470 nm (Lichtenthaler and Wellburn, 1983; Hendry and Price, 1993). The photopigment estimation was carried out using UV-Vis double beam spectrophotometer (Model 2202, Systronics India Ltd, India).

Influence of phloroglucinol on foliar carbohydrate and soluble protein contents. The application of PG on total soluble sugars (TSS), total reducing sugars (TRS), and soluble proteins from middle leaves were determined and compared with the leaves developed from the control experiment. About 10 gm of leaves from each experiment were homogenized using 100 ml of 80% (v/v) ethanol. The mixture was heated for 20 min at 60°C and then centrifuged at 10000 rpm for 10 min. the supernatant of each sample was used for quantification of primary metabolites. Total soluble sugars were extracted following Homme et al. (1992) and the absorbance of the color complex was recorded at 420 nm (Yemm and Willis, 1954). Total reducing sugars were estimated using the DNS method and the absorbance of the color complex was recorded at 540 nm (Miller et al., 1959). Glucose was used as a standard to quantify carbohydrates. The concentration of soluble proteins was determined using a standard curve of bovine serum albumin (Bradford, 1976).

Effect of PG on ex vitro rooting and acclimatization percentage. Rooting was carried out under *ex vitro* conditions using optimized auxin treatments described earlier by Manokari et al., (2021a). The cut ends of *in vitro* proliferated shootlets were pulse treated with 400 mg L^{-1} NAA for five min and planted into soilrite® (perlite + peat moss + exfoliated vermiculite, Keltech Energies Ltd., Bangalore, India) moistened with one-fourth strength of MS macro-salts solution and covered by disposable transparent plastic cups to ensure high humidity (70-80%) at 28 ± 2°C and maintained in a greenhouse for 5 weeks. The plantlets were further hardened for 5 weeks in nursery polybags containing an equal ratio (1:1:1) (w/w) of soilrite®, cocopeat, and garden soil.

Experimental design and statistical analysis. The experiments (shoot, induction, proliferation, rooting, analysis of photosynthetic pigments, carbohydrates, protein, and biomass) were arranged in a completely randomized design with 20 replicates and each treatment was repeated thrice. The statistical analyses were performed using the statistical program SPSS version 16.0 (SPSS Inc., Chicago, USA). Data were subjected to analysis of variance (ANOVA) and mean values were compared using Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

RESULTS

Effect of phloroglucinol on in vitro morphometric traits of C. uvifera.

*a. Shoot number and length***.** Phloroglucinol concentrations were not found much effective in the *in vitro* proliferation of shoots of *C. uvifera* as compared with control, but it improved shoot elongation and foliar biomass (Table 1). Shoots cultured on the optimized nutrient medium (MS medium with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA) and 1.0 mM PG recorded enhanced shoot length (8.2 cm) within 4 weeks (Table 1; Fig. 1A). Nevertheless, the lower (0.5 mM) and higher (2.0 mM) concentrations of PG resulted in reduced morphometric traits in comparison with the control plants (Table 1).

*b. Fresh and dry biomass***.** Phloroglucinol treatment indicated significant (*P<0.05*) improvement over the control in fresh and dry biomass production. The incorporation of 1.0 mM PG in the optimized MS medium showed a gain in fresh weight (3.80 ± 0.9 g) and dry weight (1.0 ± 0.5 g) of *in vitro* proliferated shootlets. The shootlets proliferated in the control experiment showed lower fresh (2.3 g) and dry weights (0.75 g) as compared to PG-treated shoots at the same culture conditions (Table 1).

Table 1. Influence of phloroglucinol (PG) with optimized growth regulators on *in vitro* growth of *C. uvifera*.

PG (mM)	No. of shoots	Shoot length (cm)	Fresh weight (g)	Dry weight (g)
0 (Control)	11.4 ± 0.51^{b}	7.0 ± 0.43^{c}	2.30 ± 0.67^{e}	0.75 ± 0.29^{d}
0.5	10.0 ± 0.70^{d}	6.0 ± 0.29^{d}	3.00 ± 0.40^{c}	0.80 ± 0.35^{c}
1.0	12.0 ± 0.65^{a}	8.2 ± 0.10^{a}	3.80 ± 0.90^{a}	1.00 ± 0.22^{a}
1.5	11.0 ± 0.40^{c}	7.5 ± 0.33^{b}	3.40 ± 0.58^{b}	0.90 ± 0.15^{b}
2.0	9.0 ± 0.34^{e}	5.8 ± 0.50^{d}	2.90 ± 0.30^{d}	0.80 ± 0.28^{c}

Data in each column represent the mean ± standard error of 20 replicates in each treatment were repeated three times. Means values followed by the different letters are significantly different at $P = 0.05$ using Duncan's multiple range test. Medium: MS medium with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA

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Influence of PG on foliar biochemical attributes.

a. Determination of photosynthetic pigments. The impact of different PG treatments on the photosynthetic pigments of the *in vitro* proliferated shoots is presented in Table 2. It could be seen that the three photosynthetic pigments, Chl '*a*', '*b*' and carotenoids were maximum in the leaves developed on 1.0 mM PG containing medium (Chl *a* : 300.0 µg g^{-1} FW, Chl *b*: 277.0 µg g^{-1} FW, Carotenoids: 81.0 µg g^{-1} FW), which was about 30% more than the control leaves (Table 2). The level of chlorophyll '*a*' and '*b*' decreased with the increased concentrations of PG, whereas, carotenoids increased in response to the higher concentrations of PG.

*b. Analysis of foliar carbohydrate and protein contents***.** Significant differences were observed in the carbohydrate contents measured as total reducing sugar (TRS), total soluble sugar (TSS), and soluble protein contents in leaves of *C. uvifera* derived from various PG treatments (Table 2). The highest carbohydrates (TRS: 230.0 mg g^{-1} DW; TSS: 218.4 mg g^{-1} DW) and soluble proteins (19.0 mg g^{-1} FW) were observed with the 1.0 mM PG treatment, which was followed by 1.5 mM PG (TRS: 198.0 mg g^{-1} DW; TSS: 211.0 mg g^{-1} DW; SP: 15.8 mg g^{-1} FW), while the lower amount of carbohydrates (TRS: 120.0 mg g^{-1} DW; TSS: 145.0 mg g^{-1} DW) and soluble protein (12.0 mg g^{-1} FW) were detected in the higher concentrations of PG

(2.0 mM) and control experiments (TRS: 75.0 mg g^{-1}; TSS: 82.0 mg g^{-1}; SP:11.0 mg g^{-1} FW), though the tissues receiving 2.0 mM PG treatment accumulated significantly higher amount than control.

Effect of PG on rooting and acclimatization of plantlets. Phloroglucinol facilitated improvement in *ex vitro* rooting and acclimatization percentage (Table 3). Rooting efficiency and shoot morphology differed greatly among the plants grown in PG treatments in comparison to the control shoots. The shoots derived from 1.0 mM PG treatment pulsed with 400 mg L^{-1} NAA grew more vigorously and significantly increased the rooting percentage (100%), the number of roots (18.0), and root length (3.0 cm) (Table 3; Fig. 1B). The control treatment-derived shoots grew slow as compared to PG-treated shoots (Table 3; Fig. 1B). After 4 weeks, the PG-derived shoots showed appreciable *ex vitro* root growth, produced longer shoots and more leaves. The survival rate of plantlets generated through PG treated shoots during acclimatization was 100%, significantly higher than that of the control-derived plantlets (86.4%) (Table 3). After acclimatization, PG-mediated plantlets possessed increased plant height, number of leaves, and internodes than the controlled plantlets (Fig. 1C).

Table 2. Biochemical traits of *C. uvifera* during *in vitro* growth on various concentrations of PG.

PG (mM)	Chl 'a' (µg g^{-1} FW)	Chl 'b' (µg g^{-1} FW)	Carotenoids (µg g^{-1} FW)	Total reducing sugars (mg g^{-1} DW)	Total soluble sugar (mg g^{-1} DW)	Soluble protein (mg g^{-1} FW)
0	90.0±0.22^{e}	84.0±0.50^{e}	30.0±0.27^{e}	75.0±0.32^{e}	82.0±0.19^{e}	11.0±0.37^{e}
0.5	178.0±0.40^{c}	190.0±0.27^{d}	50.0±0.32^{d}	150.0±0.18^{c}	190.0±0.23^{c}	14.0±0.20^{c}
1.0	300.0±0.35^{a}	277.0±0.19^{a}	81.0±0.30^{c}	230.0±0.44^{a}	218.4±0.40^{a}	19.0±0.35^{a}
1.5	263.0±0.26^{b}	240.0±0.30^{b}	89.0±0.53^{b}	198.0±0.30^{b}	211.0±0.12^{b}	15.8±0.41^{b}
2.0	190.0±0.11^{d}	206.0±0.48^{c}	98.0±0.20^{a}	120.0±0.17^{d}	145.0±0.25^{d}	12.0±0.29^{d}

Data in each column represent the mean ± standard error of 20 replicates in each treatment were repeated three times. Means values followed by the different letters are significantly different at $P = 0.05$ using Duncan's multiple range test. Medium: MS medium with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA.

Table 3. Influence of PG supplementation on *ex vitro* rooting of shoots and acclimatization percentage in *C. uvifera*.

PG (mM)	*Ex vitro* rooting on 400 mg L^{-1} NAA (%)	No. of roots	Root length (cm)	Survival rate (%)
0	90.0 ± 0.13^{c}	13.0 ±0.19^{d}	1.8 ± 0.10^{d}	86.4 ± 0.29^{d}
0.5	93.0 ± 0.25^{b}	15.4 ±0.40^{b}	2.0 ± 0.31^{c}	90.0 ± 0.10^{c}
1.0	100 ± 0.21^{a}	18.0 ±0.27^{a}	3.0 ± 0.20^{a}	98.0 ± 0.25^{a}
1.5	89.5 ± 0.18^{c}	14.0 ±0.11^{c}	2.4 ± 0.35^{b}	93.0 ± 0.12^{b}
2.0	85.0 ± 0.10^{d}	10.0 ±0.25^{e}	1.5 ± 0.20^{e}	80.0 ± 0.31^{e}

Data in each column represent the mean ± standard error of 20 replicates in each treatment were repeated three times. Means values followed by the different letters are significantly different at $P = 0.05$ using Duncan's multiple range test.

Figure 1. Morphology of plant and effect of phloroglucinol supplementation on *in vitro* propagation of *Coccoloba uvifera.* A. Morphology of *Coccoloba uvifera* growing near the coastal area of Puducherry, India. B. Morphology of leaf (platter leaf) along with fruits (sea grape). C. Efficacy of 1.0 mM PG with optimized growth regulators (1.0 mg L-1 BAP and 0.5 mg L-1 NAA) on shoot growth. D. Comparative *ex vitro* rooting of shoots derived from control and PG incorporated medium (shoots pulsed with 400 mg L-1 NAA for five minutes). E. The morphology of *in vitro* propagated plantlets after acclimatization.

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DISCUSSION

The elongation of *in vitro* shoots of *C. uvifera* in the presence of phloroglucinol could be due to its growth-promoting property. Application of PG in nutrient medium revives the meristematic activity and promotes shoot induction and multiplication *in vitro* (Teixeira da Silva et al., 2013). The optimal concentrations of PG play a pivotal role in modulating axillary shoot proliferation in Potato (Sarkar and Naik, 2000) and *Musa acuminata* cv. Grand Naine (Londe et al., 2016). Similarly, the fortification of medium with PG aided in shoots bud elongation of *Wrightia tomentosa* (Jain et al., 2009) and *Capsicum annum* (Bairwa et al., 2012). Additionally, Naidoo et al. (2017) reported

improved bulblets development in *Scadoxus puniceus* upon the exposure of cultures to a combination of PG and NAA.

Another noticeable finding of the current study was the improvement of foliar biomass and the biochemical profile of *C. uvifera* shoots. Interaction of PG and sucrose in the medium has been observed to improve shoot proliferation and elongation, which ultimately improved the fresh weight in *Solanum tuberosum* (Sarkar and Naik, 2000). Phloroglucinol, being the precursor of the lignin biosynthesis pathway controls hyperhydricity by improving the lignification process and enhancing the rate of proliferation of woody plant species (Ross, 2006; Teixeira da Silva et al., 2013). Exogenous application of PG improves lignin deposition in cell walls with xylem development, which was evidenced by structural analysis in *Vaccinium corymbosum* (Ross and Castillo, 2009) and *Achyrocline flaccida* (Ross and Castillo, 2010). Aremu et al. (2015) reported that PG improved morphology, bioactive compounds, and enhanced bulblets formation in *Ecklonia maxima*, which indicates the positive role of PG in the development of storage organs in plants.

The nutrient medium enriched with a ready-made source of carbon often results in reduced concentrations of photosynthetic pigments in the *in vitro* plants (Hazarika, 2006). The increased or optimal development of photo-pigments in micropropagated plants improves carbon metabolism, which assists in the acclimatization of plants (Bairu and Kane, 2011). It is considered that sucrose and starch partitioning would be optimum when there are sufficient photosynthetic pigments developed *in vitro*, and the level of foliar photosynthetic pigments reveals the physiological status of the plantlet (Yavari et al., 2021). The accumulation of organic solutes (osmolytes) such as reducing and soluble sugars and soluble proteins is essential to avoid the adverse effect during water stress (Darko et al., 2019; Živanović et al., 2020). Thus, the presence of reducing sugars is an important physiological adaptation against the new circumstances of water loss, via contributing the osmotic adjustment for the plant cell and maintaining their growth and metabolism (Pantin et al., 2012). Moreover, plantlets may restore these accumulated sugars in their persistent leaves as a source of energy, particularly in the first few days after transplanting to *ex vitro* conditions (Zein El Din et al., 2020).

Similar to the present findings, incorporation of 79 µM PG in the medium improved the rate of photosynthesis in *Carica papaya* (Pérez et al., 2016). The foliar chlorophyll contents are the key indicator of the photosynthetic activity, and the carotenoids are involved in the defense mechanism against oxidative stress (Shah et al., 2017). It can be assumed that abiotic stresses caused by the higher concentrations of phloroglucinol in the nutrient medium negatively affected the chlorophyll pigments and the carotenoids were increased to protect the photosynthetic apparatus against the stress impact of increased PG.

Phloroglucinol is an auxin promoter, stimulates the induction of new roots, root development, and elongation. The positive effects of PG on *in vitro* rooting have been documented in economically important species like almond (Ainsley et al., 2001), papaya (Pérez et al., 2016), banana (Londe et al., 2016), apple (Kim et al., 2020), and *Diospyros crassiflora* (Tchouga et al., 2020). Physiologically, PG shows auxin synergism and protects auxins during rhizogenesis (Dobránszki and Teixeira da Silva, 2010; Daud et al., 2013). The phenolic nature of PG mocks decarboxylation in woody apple shoots and protects indole-3-acetic acid (IAA) (De Klerk et al., 2011). Auxins in combination with PG improved root growth during acclimatization of *Pyrus calleryana* (Berardi et al., 1993), and the PG-derived plantlets of *Ornithogalum dubium* developed tolerance to transplant shock (Petti, 2020).

From these results, it can be suggested that the addition of PG makes the *in vitro* plant production cost-effective and efficient process to develop healthy plantlets with improved survival.

CONCLUSIONS

The present results indicate that the optimal phloroglucinol enhanced *in vitro* growth performance of plantlets of *Coccoloba uvifera*, by increasing shoot length, biomass, photo-pigments, carbohydrates, and protein contents. Further, a significant improvement in *ex vitro* rooting and acclimatization was also observed in PG-treated shoots and plantlets, respectively.

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