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RESEARCH ARTICLE

A four steps protocol for in vitro propagation of *Eugenia uniflora* L. (Myrtaceae)

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Abstract *Eugenia uniflora* L. (Myrtaceae) is a key species in different forest formations with ecological, economic, and medicinal importance. This study aimed at establishing an in vitro culture procedure for the micropropagation of *E. uniflora* genotypes through organogenesis. Nodal segments were introduced in a semi-solid medium for organogenesis establishment and posteriorly cultivated in immersion systems for shoot development and rooting. Different culture media, plant growth regulator concentrations, and immersion systems were tested. The highest rate of shoot generation was obtained with 2.0–2.5 µM BAP + 0.5 µM of NAA. The measures related to rooting did not differ among the tested immersion systems, but the biomass accumulation and leaf area were significantly higher in the twin-flasks system. There were no significant differences in chlorophyll contents among the immersion systems. A significantly higher content of carotenoids was observed in the permanent immersion system with gas exchange. The protocol provided in this study may be helpful for the mass production of seedlings for commercial orchard implementation, as well as in genetic improvement programs for *E. uniflora*.

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Introduction

Assuring the subsistence of forest formations under a scenario of climatic changes and unsustainable anthropic use of natural resources is essential for the future of mankind. Temperature rise has already shaped significant changes to natural systems, like extreme weather alterations, droughts, floods, and loss of biodiversity. In this context, tropical and subtropical forests are central ecosystems for the equilibrium of the climate and maintenance of the world's biological diversity (Stefenon et al. 2020a; Fritsche et al. 2022). However, climate change, habitat fragmentation, and pollinator failure directly impact the capacity of plant populations to reproduce effectively, generating direct consequences for their demography, evolution, and long-term persistence. In this perspective, there is a growing consensus that underutilized crops may be important for the future production of food, as they tend to be more adapted to stressful conditions than domesticated crops (Fritsche et al. 2022).

Eugenia uniflora L., also known as Surinam cherry or pitangueira, belongs to the Myrtaceae family and is one of the key species of the tropical and subtropical geomorphological domains (Scarano 2002; Salgueiro et al. 2004; Beise et al. 2020). Their fruits

are typically consumed *in natura*, or manufactured as pulp, juice, ice cream, soft drinks, jellies, liqueurs, and wines (Bezerra et al. 2004; de Almeida et al. 2012). Due to its high adaptability to grow under full sunlight or complete shade, *E. uniflora* is suitable for the reforestation of degraded areas (Stefenon et al. 2020b) and is also important for cosmetics companies due to the presence of essential oils in the leaves. This species also produces secondary metabolites that have been used in traditional medicine to treat gastrointestinal disorders (Costella et al. 2013; Teixeira et al. 2016) and has potential use in the pharmaceutical industry with antiparasitic, anti-rheumatic, anti-inflammatory, and anti-kinetoplastid activities (Auricchio and Bacchi 2003; Costa et al. 2010; da Cunha et al. 2016).

Despite the clear suitability for economic and ecological uses, *E. uniflora* has been cultivated mainly for domestic purposes, without commercial aspirations (da Silva et al. 2014). Furthermore, the seedlings' production usually occurs through free pollination, frequently leading to phytosanitary issues and problems with orchard homogeneity. Thus, developing effective techniques for *E. uniflora* micropropagation is highly relevant. Plant micropropagation is an important biotechnological tool related to the clonal mass multiplication of genotypes with desirable characteristics and superior phytosanitary quality.

Although micropropagation techniques are great supporters of germplasm banks maintenance (Pilatti et al. 2011), or even in genetic improvement programs for perennial plant species, only a few investigations have addressed the micropropagation of *E. uniflora*. Using semi-solid culture media for propagation of this species through organogenesis, Uematsu et al. (1999), reported the occurrence of shoots vitrification from 5.7 to 16.5% of the plants and abnormal rooting. A low rooting rate was also reported by da Silva et al. (2014) using the direct organogenesis route. While these two investigations reported complete protocols from the explant introduction up to the plants' acclimatization, Souza et al. (2008) only reported the effect of 6-benzylaminopurine, zeatin, and 2iP in the in vitro shoot multiplication and Stefenon et al. (2020b) published a protocol for in vitro induction of callogenesis aiming at somatic embryogenesis induction and production of secondary metabolites. Thus, in addition to being limited in number, the protocols proposed for the micropropagation of *E uniflora* still need improvements. The objective of this study was to establish an in vitro culture protocol for the micropropagation of *E. uniflora* genotypes through the organogenesis route. Here we propose a protocol that explores different strategies for the micropropagation of this species, including the use of semi-solid and liquid medium in different steps of the procedure.

Material and methods

Study layout

In this study, we first tested different concentrations of the cytokinin 6-benzylaminopurine (BAP) and two different culture media for organogenesis induction in a semi-solid medium. Following, three different immersion systems were used to study the rooting and development of the aerial part of the seedling, as well as the photosynthetic pigments content of the plants. Rooting was also tested in a semi-solid medium with activated charcoal. The acclimatization and survival of the plants were evaluated at the end of the process.

Media composition and culture conditions

The culture media used in the experiments were composed as follows:

Semi-solid germination medium: 6 g L^{-1} of agar in distilled water.

Semi-solid verification medium: 6 g L^{-1} of agar and 30 g L^{-1} of sucrose (da Silva et al. 2014).

Semi-solid propagation medium: basal salts of the MS (Murashige and Skoog 1962) or Wood Plant Medium (WPM, Lloyd and McCown 1980) supplemented with 30 g L^{-1} of sucrose and 6.0 g L^{-1} of agar.

Liquid culture medium: half-strength MS medium (½ MS), supplemented with 15 g L^{-1} of sucrose.

The culture media were autoclaved for 15 min at 121 °C and 1.2 atm and all in vitro experiments were maintained in a growth room at 25 ± 2 °C, with a photoperiod of 16 h and irradiance of 40–50 mol m^{-2} s^{-1} by fluorescent white lamps.

Plant material and in vitro germination

Mature seeds were obtained from four genotypes of domestic gardens in Santa Catarina State, southern

Brazil. Genotypes matching commercial demands, i.e., robustness, uniform color of the fruits, and good phytosanitary conditions, were selected for the collection of fruits. The selected genotypes lack genetic relatedness but have similar productivity and fruit quality.

For the experiments, ripe fruits were randomly collected in each selected tree, stored in glass flasks, and transported to the laboratory within 48 h. The fruits were carefully pulped to obtain intact seeds. Seeds were bulked and disinfested in a horizontal flow chamber in 70% ethanol for 10 min, 1.5% sodium hypochlorite for 20 min, and then rinsed three times with sterile deionized water. The seeds were inoculated individually in 180×20 mm test tubes containing 15 mL of germination medium.

In vitro establishment and propagation

Hundred-twenty days after seed introduction, 1.0 cm long nodal segments having apical or axillary buds were excised from the in vitro germinated plantlets. Nodal segments were immediately inoculated in test tubes containing 15 mL of verification medium for 14 days. This step aimed at selecting explants free of contamination for propagation.

An initial assay was performed to test different cytokinin concentrations and determine the most propitious treatments. Nodal segments from the verification step were inoculated in test tubes (180×20 mm), containing 15 mL of the MS propagation medium supplemented with 0.5 µM of α-naphthalene acetic acid (NAA) and five different combinations (0.5, 1.0, 1.5, 2.0, and 2.5 µM) of 6-benzylaminopurine (BAP), in pH 5.8. Since previous test experiments with culture medium without plant growth regulators have failed to promote the development of shoots, we excluded this control treatment in further experiments. The experiment was carried out in a completely randomized design and consisted of five treatments containing 20 replicates composed of one test tube with an inoculated nodal segment.

Based on the preliminary assay of BAP concentrations, further experiments were performed using 0.5 µM of NAA, and three different combinations (1.5, 2.0, and 2.5 µM) of this cytokinin, using the MS or the WPM propagation medium. This experiment was carried out in a completely randomized design and consisted of six treatments containing 80 replicates composed of one test tube with an inoculated nodal segment. The number of responsive explants emitting shoots, the number of shoots, and the number of leaves per explant were evaluated after 60 days.

Culture in permanent and temporary immersion systems

Sixty-day-old seedlings with shoots were introduced in three different immersion systems: Permanent Immersion System Without gas Exchange (PIS/WE; Fig. 1a), Permanent Immersion System with Gas Exchange (PIS/GE; Fig. 1b), and Temporary Immersion System Twin-Flasks (TIS/TF; Fig. 1c). All immersion systems were mounted with transparent

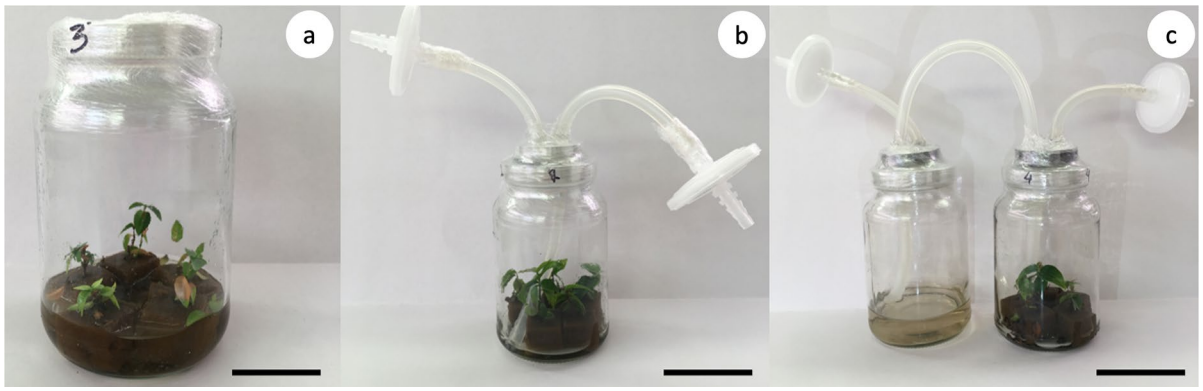

Fig. 1 Immersion systems used for the micropropagation of *EugeniaE uniflora* L. **a** permanent immersion system without gas exchange (PIS/WE), **b** permanent immersion system with gas exchange (PIS/GE), and **c** temporary immersion system twin-flasks (TIS/TF). Bar = 5 cm

glass flasks 13.5 × 8.5 cm. To promote gas exchange, the air was injected into the systems PIS/GE and TIS/TF through 0.2-µm filters at a pressure of 0.28 bar.

All systems were filled with 100 mL of liquid culture medium without plant growth regulators. Flasks were autoclaved for 20 min at 121 °C and 1.2 atm for sterilization. The seedlings were individually fixed in 2.5 × 2.5 cm phenolic foams (Green-up®). Gas exchange in the system PIS/GE and culture medium immersion in the system TIS/TF occurred for 6 min every 6 h. In the system PIS/WE, there was no gas or medium exchange.

This experiment was carried out in a completely randomized design, in triplicate, with five seedlings in each flask. After 60 days of culture, biomass accumulation, leaf area, and photosynthetic performance of the seedlings were evaluated.

Seedlings growth, rooting, and photosynthetic pigments content

The biomass accumulation of the seedlings after culture in immersion systems was determined by measuring the fresh mass (g) of each explant before its introduction in the immersion system and after 60 days of culture. Fresh mass was measured with the aid of a semi-analytical scale. Leaf area was defined from digitized images of one leaf specimen per repetition, collected from the first pair of leaves. The images were analyzed using the Software ImageJ v. 1.53t (Rasband 2018), defining the relative leaf area in cm^2. The rate of rooted seedlings and the average size and number of roots were determined for each immersion system individually.

For the quantification of chlorophyll and carotenoid contents, 10 mg of leaves were used for each repetition. The leaves were deposited in 2.0 mL microtubes containing 1.0 mL of dimethylsulfoxide (DMSO) and covered with aluminum foil. The microtubes were kept for 24 h in the dark at a temperature of 25 ± 2 °C and proceeded for 1 h in a water bath at a temperature of 60 °C (Shinano et al. 1996). The quantification of photosynthetic pigments was performed in triplicate with the aid of a Spectramax® Paradigm® spectrophotometer using 20 µL aliquots. Estimations of the content of chlorophyll *a*, chlorophyll *b*, total chlorophylls, the chlorophyll *a*/*b* ratio, and the content of carotenoids were obtained according to Wellburn (1994), using spectrophotometry readings at the wavelengths 665 nm for chlorophyll *a*, 649 nm for chlorophyll *b*, and 470 nm for carotenoids.

Rooting in semi-solid medium

Rooting was also carried out in test tubes containing 15 mL of ½ MS medium, pH 5.8, supplemented with 15 g L^{-1} of sucrose, 1.5 g L^{-1} of activated charcoal, and gelled with 6.0 g L^{-1} of agar. The plantlets were kept at 25 ± 2 °C, with a photoperiod of 16 h and an irradiance of 40–50 mol m^{-2} s^{-1}. After 120 days, the rate of rooted seedlings and the average size and number of roots were determined.

Acclimatization

Rooted plantlets were transferred from the test tubes or immersion systems into plastic vessels filled with commercial substrate Tropstrato HT Hortaliças® (Mogi Mirim, SP, Brazil) and sealed with plastic film. The plastic vessels were maintained in a growth room at 25 ± 2 °C, with a photoperiod of 16 h and irradiance of 40–50 mol m^{-2} s^{-1}. After seven days of pre-acclimatization, 10 openings (about 0.5 cm ∅) were made in the plastic film to allow gas exchange. After 14 days, the films were removed, and the plastic vessels were kept in a greenhouse with automated irrigation for acclimatization. At 30 days after acclimatization, the survival rate of the plants was determined.

Statistical analyses

The data were log(x + 2) transformed and subjected to analysis of variance (ANOVA). When ANOVA was significant, differences among treatment means at a 5% probability level were evaluated using the Tukey test (for the culture media) or Duncan's probability test (for the number of shots and leaves). A simple linear regression was used to estimate the relationship between the cytokinin concentrations and the number of developed leaves. All analyses were performed using the software Genes v. 7.0 (Cruz 2009).

Results

The optimal concentration of BAP ranges from 2.0 to 2.5 µM

The micropropagation of *E. uniflora* through organogenesis tends to be more effective according to the increase in BAP concentration up to 2.5 µM, combined with 0.5 µM of NAA. The simple linear regression equation for the number of leaves per explant (Fig. 2a) showed a positive correlation between the BAP concentration and the number of leaves ($R^2 = 0.86$; $p = 0.06$) and shoots ($R^2 = 0.72$; $p = 0.16$) per explant.

The highest rate of in vitro shoot generation was obtained with 2.0 and 2.5 µM BAP, with 75 and 70% of organogenetic response, respectively. There was no significant difference in the mean number of shoots for the different treatments (Table 1, Fig. 2b–g), ranging from 1.0 (1.5 µM BAP) to 1.47 (2.5 µM BAP). On the other hand, the mean number of leaves per explant ranged from 2.6 (0.5 µM

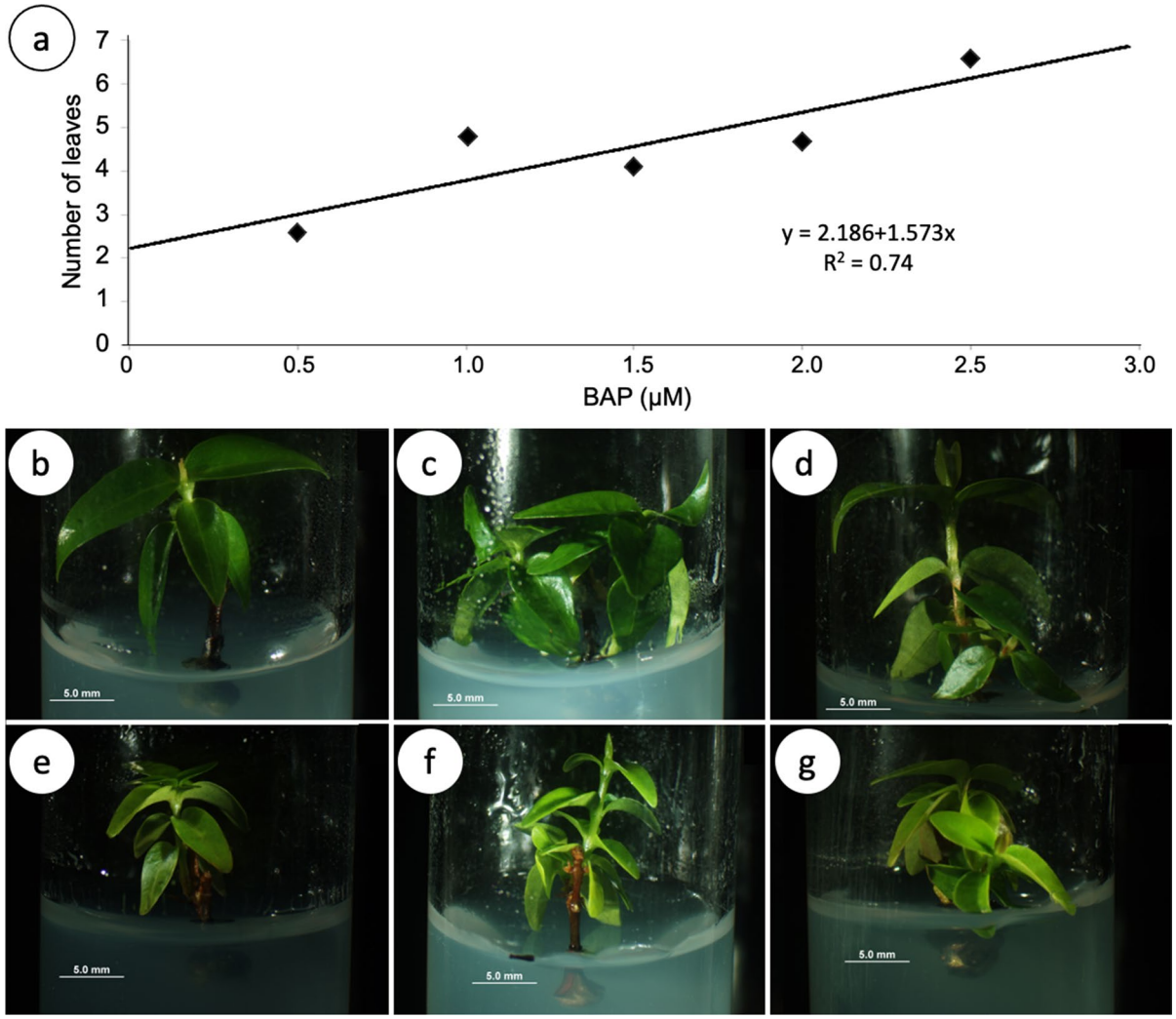

Fig. 2 In vitro culture of *Eugenia uniflora* explants in semi-solid medium. **a** Regression analysis of the leaf number per explant as a function of the BAP concentration in the culture in semi-solid medium. **b** MS medium with 0.5 µM NAA + 1.5 µM BAP; **c** MS medium with 0.5 µM NAA + 2.0 µM BAP; **d** MS medium with 0.5 µM NAA + 2.5 µM BAP; **e** WPM medium with 0.5 µM NAA + 1.5 µM BAP; **f** WPM medium with 0.5 µM NAA + µM 2.0 BAP; **g** WPM medium with 0.5 µM NAA + 2.5 µM BAP

Table 1 Mean number of shots and leaves in nodal segments of *Eugenia uniflora* after 60 days in culture

Treatment	NAA (μM)	BAP (μM)	# shoots	# leaves
T1	0.5	0.5	1.13 a	2.60 b
T2	0.5	1.0	1.20 a	4.80 ab
T3	0.5	1.5	1.00 a	4.07 b
T4	0.5	2.0	1.40 a	4.67 ab
T5	0.5	2.5	1.47 a	6.60 a
		CV (%)	48.64	67.94

Means followed by the same letter in the column statistically did not differ according to the Duncan test ($p > 0.05$)

BAP) to 6.6 (2.5 μM BAP), with a statistically significant difference ($p < 0.05$; Table 1). A high coefficient of variance (Table 1) was estimated for the number of shoots (CV = 48.64%) and the number of leaves (CV = 67.94%), likely due to the heterogeneity of the responsive repetitions concerning these variables, as well as the genotypic variability of the explants which were originated from different seeds.

The culture medium has a minor impact on the *E. uniflora* micropropagation

There were no statistically significant differences among BAP concentrations regarding the percentage of responsive explants (Table 2). On the other hand, higher doses of BAP promoted a higher response of the explants to organogenesis, although the difference is statistically not significant (Table 2).

Regarding the number of leaves, there was a statistically significant difference between the MS and WPM culture media supplemented with 2.0 μM of BAP, but no difference was observed among the other treatments (Table 2). Comparing the treatments within the same culture medium, 2.5 μM of BAP promoted the highest number of leaves, statistically differing from 1.5 μM of BAP, but not from 2.0 μM of BAP (Table 2).

The number of shoots per explant did not differ between culture media for each concentration of BAP. Similarly, there was no significant difference among the concentrations of BAP for the MS medium but the treatment with 1.5 μM of BAP was significantly lower than the other treatments for the WPM medium (Table 2).

Table 2 Organogenesis of *Eugenia uniflora* using different culture media (MS and WPM) and different concentrations of the cytokinin BAP (1.5, 2.0, or 2.5 μM)

PGRs (μM)	Culture medium	
	MS	WPM
	Responsive explants (%)	
0.5 NAA + 1.5 BAP	75.0 aA	77.5 aA
0.5 NAA + 2.0 BAP	80.0 aA	87.5 aA
0.5 NAA + 2.5 BAP	87.5 aA	92.5 aA
CV (%)	37.25	
	Leaves (n°)	
0.5 NAA + 1.5 BAP	4.4 bA	6.2 bA
0.5 NAA + 2.0 BAP	6.3 abB	9.0 abA
0.5 NAA + 2.5 BAP	7.0 aA	9.1 aA
CV (%)	32.92	
	Shoots (n°)	
0.5 NAA + 1.5 BAP	1.08 aA	1.08 bA
0.5 NAA + 2.0 BAP	1.15 aA	1.47 aA
0.5 NAA + 2.5 BAP	1.20 aA	1.45 abA
CV (%)	23.27	

Means followed by the same letter, lowercase in the column (BAP concentration) and uppercase in the rows (culture media), do not differ statistically according to Tukey's test ($P = 0.05$)

Immersion systems revealed no differences in rooting but significant differences in aerial development

The number and length of the roots and the percentage of rooting did not differ statistically among the three immersion systems according to Tukey's test (Table 3), although a higher percentage of rooting (46.7%) was observed in the TIS/TF system (Table 3, Fig. 3a). The percentage of rooting in semi-solid ½ MS with activated charcoal reached 35% with well-developed rooting systems (Fig. 3b) for acclimatization (average size of 4.5 cm and 1.4 roots/seedling). All plants that developed roots in vitro survived in the acclimatization phase (Fig. 3c–d), while only 10% of the non-rooted plants developed roots in the acclimatization substrate and survived.

Concerning aerial development of the seedlings, the biomass accumulation and leaf area were significantly different among the immersion systems, with the highest average obtained with the use of TIS/TF that accumulated, on average, 0.68 g of biomass and 2.79 cm^2 of leaf area per flask. These estimations did

Table 3 Mean values for measures of biomass accumulation, leaf area, rooting, number of roots, and root length in *E. uniflora* seedlings cultivated in different immersion systems

Immersion system	Biomass accumulation (g)	Leaf area (cm^2)	Rooting (%)	Number of roots	Root length (cm)
PIS/WG	0.09 b	0.78 b	20.0 a	1.11 a	1.06 a
PIS/GE	0.68 a	2.79 a	20.0 a	1.67 a	1.83 a
TIS/TF	0.41 ab	1.21 ab	46.7 a	1.47 a	3.37 a
CV (%)	9.37	15.13	58.83	15.89	16.87

Means followed by the same letter do not differ statistically according to Tukey's test ($P = 0.05$)

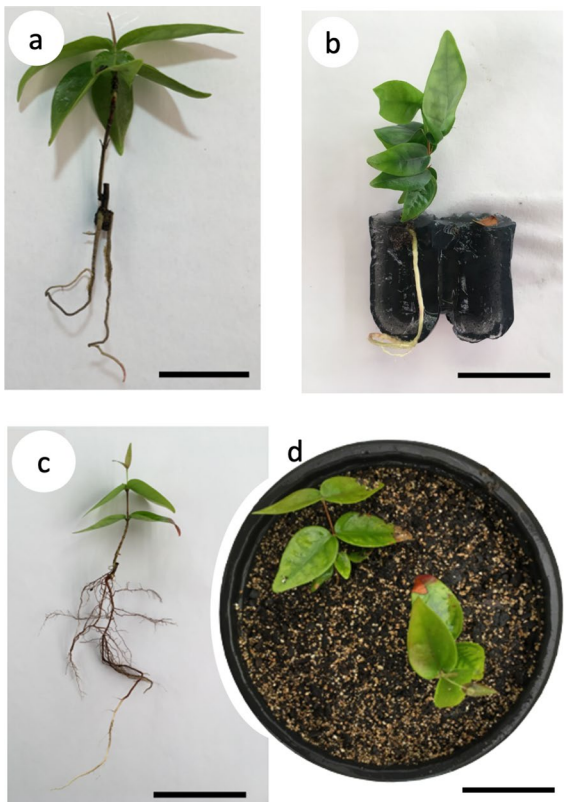

Fig. 3 Rooting and acclimatization of *Eugenia uniflora* seedlings. **a** rooted seedling from the TIS/TF immersion system. **b** rooted seedling from the semi-solid system with activated charcoal. **c** seedling after 60 days of acclimatization. **d** *Eugenia uniflora* seedlings under acclimatization in a commercial substrate. Bar = 5 cm

not differ from the averages obtained in the PIS/GE but differed from measures from PIS/WE (Table 3).

Immersion systems interfere in the carotenoids content but not in chlorophyll

There were no statistically significant differences for chlorophyll *a* and *b* contents, as well as for the content of total chlorophyll and chlorophyll *a/b* ratio among the immersion systems, while the content of carotenoids revealed statistically significant differences, with the highest mean obtained in the PIS/GE (Table 4).

Discussion

Temporary immersion systems have been found to be appropriate for shoot regeneration. The direct contact of the cultures with the medium helps an easy uptake of the nutrients, stimulating the growth rate, while the forced air supply (in TIS/TF and PIS/GE) facilitates the growth and metabolism of cultured cells and organs (Murthy et al. 2023). Accordingly, research using immersion systems for the in vitro organogenesis in Myrtaceae [*Melaleuca alternifolia* Cheel (Scheidt et al. 2011), *Eucalyptus grandis* x *urophylla* (Oliveira et al. 2014), and *Myrtus communis* L. (Aka et al. 2020)] demonstrated that the gas exchange promoted by these systems is effective for the in vitro accumulation of biomass. On the other hand, continuous immersion systems are considered unsuitable for axillary or adventitious shoot regeneration because these systems induce asphyxia and hyperhydricity of regenerated shoots (Murthy et al. 2023). Temporary immersion systems can overcome the issues of hyperhydricity in cultured plant tissues by creating conditions for optimal humidity, while the gaseous

Table 4 Mean estimations (mg g^{-1} leaf) of chlorophyll *a,* chlorophyll *b*, total chlorophyll, chlorophyll *a/b* ratio, and carotenoids for *Eugenia uniflora* seedlings cultivated in immersion systems

Immersion system	Chlorophyll *a*	Chlorophyll *b*	Carotenoids	Total chlorophyll	Chlorophyll *a/b* ratio
PIS/WG	8.41 a	3.53 a	1.33 b	11.93 a	2.35 a
PIS/GE	19.07 a	7.23 a	2.77 a	26.31 a	2.68 a
TIS/TF	12.39 a	5.21 a	1.88 ab	17.61 a	2.41 a
CV (%)	11.84	13.91	9.91	11.29	3.62

Means followed by the same letter do not differ statistically according to Tukey's test ($P = 0.05$)

environment overcomes the issue of oxygen limitation (Murthy et al. 2023).

Despite the economic and ecological importance of *E. uniflora*, only a few investigations about the micropropagation of this species have been reported, including the induction of somatic embryogenesis (Stefenon et al. 2020a, b) and in vitro organogenesis (Uematsu et al. 1999; Souza et al. 2008; da Silva et al. 2014) using semi-solid media. As far as we are aware, this is the first study using immersion systems for the in vitro organogenesis of *E. uniflora.* (Fig. 4). Overall, our results suggest that this strategy promotes high in vitro biomass accumulation and rooting.

In the in vitro establishment of the nodal segments (performed in semi-solid culture media), no necrosis was observed at the BAP concentrations utilized, and an increase in this cytokinin promoted higher shoot development up to 2.5 µM, the maximum concentration examined. Reports on micropropagation of *Psidium guajava* (Ali and Ludders 2001), *Rubus idaeus* (Erig et al. 2002), *Acca sellowiana* (Oltramari et al. 2000), and *E. uniflora* (Souza et al. 2008) suggest the use of a range of BAP concentration from 2.0 to 4.0 µM for the vegetative multiplication of the species, resulting in higher numbers of shoots and buds for higher BAP concentrations. On the other hand, much higher concentrations of BAP (50 µM) were reported as phytotoxic for the explants of *Acca sellowiana* (Oltramari et al. 2000). We observed a gradual improvement in the number of leaves according to the rise of the BAP concentration, from 1.0 up to 2.5 µM. A similar result was described by Souza et al. (2008) in *E. uniflora*, reporting from 1.20 to 2.35 shoots per explant in the range of 0.0–5.0 µM of BAP, while da Silva et al. (2014) reported 1.15 shoots per explant using 0.2 mg L^{-1} of this cytokinin in combination with 0.1 mg L^{-1} of indolebutyric acid (IBA). Since all these investigations tested somewhat limited ranges of BAP concentration, further investigations testing a wider range may improve the micropropagation of *E. uniflora* as cytokinin application has effects on the number and size of shoots and leaves.

The low rooting rate has been a great challenge in the micropropagation of *E. uniflora* (Uematsu et al.

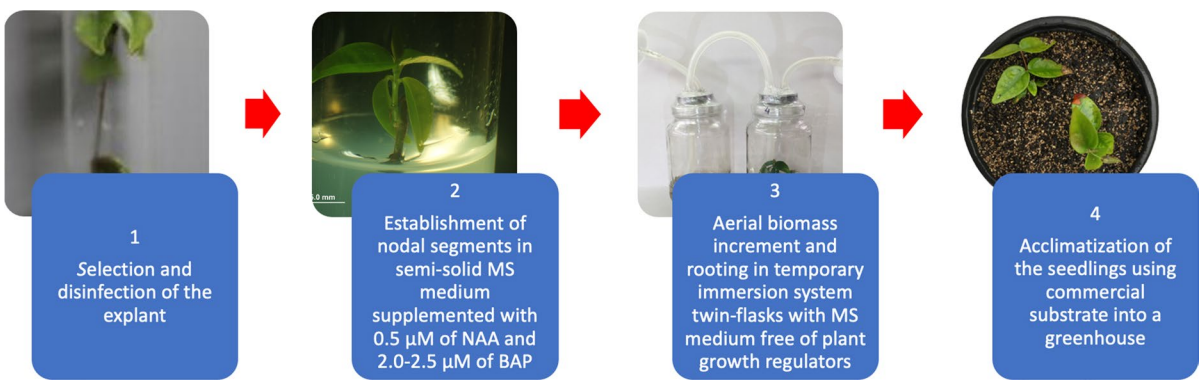

Fig. 4 Workflow of the four steps protocol for in vitro organogenesis of *Eugenia uniflora*

1999; da Silva et al. 2014). Active charcoal supplementation to the rooting medium, in the absence of plant growth regulators and with reduced concentration of salts and carbohydrate source, promoted 35% of seedlings rooting in a semi-solid medium, while the use of TIS/TF promoted 46.7% of seedlings rooting. The rooting using PIS/WG and PIS/GE reached 20%, although no statistically significant difference was estimated among the three immersion systems, as an effect of the low number of repetitions in the experiment and high coefficient of variation (C.V. = 58.83%). In turn, all rooted plants survived in the acclimatization phase. High proliferation and rooting rates were reported for woody (such as *Prunus domestica*; Gago et al. 2022) and herbaceous (such as *Centella asiatica*; Wongsa et al. 2023) species cultivated in temporary immersion systems.

Overall, there was a tendency for higher amounts of chlorophyll *a* and *b*, carotenoids, total chlorophyll, and chlorophyll *a*/*b* ratio in the PIS/GE. Such a result is possibly correlated with the capacity of this immersion system to accumulate biomass and generate larger leaf areas in the explants, increasing their photosynthetic capacity. Because the PIS/GE system promotes a constant exchange of the internal atmosphere of the flasks and general stimulation of the plants, there is also greater transpiration. Increased transpiration, in turn, stimulates greater absorption of nutrients from the culture medium. Consequently, a greater photosynthetic response of the explants is also expected, as observed in the pigment analyses. Moreover, this improved photosynthetic capability enables the decrease or even elimination of conventional carbohydrate supplementation in the culture (Vidal et al. 2019; Gago et al. 2022).

The absence of significant difference in the contents of chlorophyll *a* and *b*, and chlorophyll *a*/*b* ratio among the three immersion systems suggests that the employed culture conditions caused the same level of stress to the plants in all systems. The interconversion of chlorophyll *b* to chlorophyll *a* plays an important role in the establishment of the necessary chlorophyll *a*/*b* ratio during the adaptation to stresses (Martins et al. 2018). Thus, the occurrence of differentiated levels of stress in the different cultivation systems would promote diverse measures for the chlorophyll *a*/*b* ratio since a high ratio may indicate less emphasis on light harvesting concerning the rates of photosystem II photochemistry under stress (Martins et al. 2018).

Conclusion

The induction of shoots in nodal segments using semi-solid MS medium supplemented with 0.5 µM of NAA and 2.0–2.5 µM of BAP enabled the production of clonal seedlings and the culture of these seedlings in twin-flasks temporary immersion systems with ½ MS medium free of plant growth regulators promoted the aerial biomass increment and rooting, allowing the successful acclimatization of the seedlings into a greenhouse.

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Declarations

Conflict of interest Authors of this manuscript declare that have no conflict of interest.

Consent for publication All the authors have provided consent for publication.

Ethical approval This manuscript does not contain any research with human participants or animals performed by any of the authors.

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