

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

Lucas Barasuol Franco

UFSC: Universidade Federal de Santa Catarina

Bruna Ronchi Hermann

UFSC: Universidade Federal de Santa Catarina

Yohan Fritsche

UFSC: Universidade Federal de Santa Catarina

Valdir Marcos Stefenon (✉ valdir.stefenon@ufsc.br)

Universidade Federal de Santa Catarina <https://orcid.org/0000-0003-1091-700X>

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Abstract

The maintenance of forest formations under a scenario of climatic changes and unsustainable anthropic use of natural resources is vital for the future of mankind. In this context, there is a rising agreement 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. *Eugenia uniflora* L. (Myrtaceae) is a key species in different forest formations with ecological, economical, and medicinal importance. The present work aimed at establishing an *in vitro* culture procedure for the mass micropropagation of *E. uniflora* genotypes through the organogenesis route. Nodal segments from *in vitro* germinated seeds 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 protocol provided in this study may be helpful for the mass production of seedlings for degraded areas recovery or commercial orchard implementation, as well as in genetic improvement programs for *E. uniflora*.

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). The importance of Brazilian Myrtaceae species in this aspect is supported by the wide dispersion across the different biomes, including the species-rich Atlantic Forest and the grassland-dominated Pampa (Beise 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 sub-tropical geomorphological domains (Scarano 2002, Salgueiro et al. 2004). Their fruits are typically consumed fresh, 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 & 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 disconnected studies (Uematsu et al. 1999, Souza et al. 2008, da Silva et al. 2014, Stefenon et al. 2020b) have addressed the micropropagation of *E. uniflora*.

The objective of this study was to establish a robust *in vitro* culture procedure for the mass micropropagation of *E. uniflora* genotypes through the organogenesis route. Here we propose a four steps protocol using different strategies for the micropropagation of this species. This protocol can be employed in the mass production of seedlings for degraded areas restoration, for the establishment of commercial orchards, and in programs for the species' genetic improvement.

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 performance 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.

Plant material and *in vitro* germination

Mature seeds were obtained from four genotypes of domestic gardens in Santa Catarina State, southern Brazil. Genotypes meeting commercial demands, i.e., robustness, uniform color of the fruits, and good phytosanitary conditions, were selected for the collection of fruits.

For the experiments, ripe fruits were randomly collected in each selected tree, stored in glass flasks, and transported to the laboratory within 48 hours. The fruits were carefully pulped to obtain intact seeds.

Seeds were disinfested in a horizontal flow chamber in 70% ethanol for 10 min, 1.5% sodium hypochlorite for 20 minutes, and then rinsed three times with sterile deionized water. The seeds were inoculated individually in 180 x 20 mm test tubes containing 15 mL of germination medium (6 g L⁻¹ of agar in

distilled water). Before seed inoculation, the medium was autoclaved for 15 min at 121°C and 1.2 atm. Test tubes with inoculated seeds were kept in a growth room at $25 \pm 2^\circ\text{C}$, with a photoperiod of 16 hours and irradiance of $40\text{--}50 \text{ mol m}^{-2} \text{ s}^{-1}$ by fluorescent white lamps.

In vitro germination was monitored weekly on the number of seeds emitting roots and/or shoots, and the percentage of contaminated seeds, up to 116 days after introduction.

In vitro establishment and propagation

Hundred-twenty days after introduction, 1.0 cm long nodal segments were excised from the *in vitro* germinated seedlings. Nodal segments were immediately inoculated in test tubes containing 15 mL of verification medium composed of 6 g L^{-1} of agar and 30 g L^{-1} of sucrose (da Silva et al. 2014) and maintained in a growth room at $25 \pm 2^\circ\text{C}$, with a photoperiod of 16 hours and irradiance of $40\text{--}50 \text{ mol m}^{-2} \text{ s}^{-1}$ 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 x 20 mm), containing 15 mL of the propagation medium composed of MS basal salts (Murashige & Skoog, 1962) supplemented with 30 g L^{-1} of sucrose, 5.0 mL L^{-1} of Fe EDTA, 2.0 mL L^{-1} of Morel Vitamins (Morel and Wetmore 1951), and 6.0 g L^{-1} of agar, $0.5 \mu\text{M}$ of α -naphthalene acetic acid (NAA) and five different combinations (0.5, 1.0, 1.5, 2.0, and $2.5 \mu\text{M}$) of 6-benzylaminopurine (BAP), in pH 5.8. 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 $0.5 \mu\text{M}$ of NAA, and three different combinations (1.5, 2.0, and $2.5 \mu\text{M}$) of this cytokinin, using the MS medium or WPM medium (Lloyd & McCown 1980), both supplemented with 30 g L^{-1} of sucrose, 5.0 mL L^{-1} of Fe EDTA, 2.0 mL L^{-1} of Morel Vitamins (Morel & Wetmore 1951), and 6.0 g L^{-1} of agar. Before the explant inoculation, the medium was autoclaved for 15 min at 121°C and 1.2 atm. 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. Test tubes were maintained in a growth room at $25 \pm 2^\circ\text{C}$, with a photoperiod of 16 hours and $40\text{--}50 \text{ mol m}^{-2} \text{ s}^{-1}$ of fluorescent white lamps irradiance. 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 glass flasks 13.5 x 8.5 cm. To promote gas exchange,

the air was injected into the systems PIS/GE and TIS/TF through silicone hoses at a pressure of 0.28 bar. The injected air was sterilized through 0.2-µm filters.

All systems were filled with 100 mL of half-strength MS medium ($\frac{1}{2}$ MS), pH 5.8, without plant growth regulators, and supplemented with 5.0 mL L⁻¹ of Fe EDTA, 1.0 mL L⁻¹ of Morel vitamins, and 15 g L⁻¹ of sucrose. Flasks were sterilized through autoclaving for 20 min at 121°C and 1.2 atm. The seedlings were individually fixed in 2.5 x 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. The experiment was maintained in a growth room at 25 ± 2°C, with a photoperiod of 16 hours and irradiance of 40–50 mol m⁻² s⁻¹. After 60 days of culture, biomass accumulation, leaf area, and photosynthetic performance of the seedlings were evaluated.

Seedlings growth, rooting, and photosynthetic performance

The biomass accumulation (g) of the seedlings after culture in immersion systems was determined by measuring the fresh mass 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 cm². 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 wrapped with aluminum foil containing 1.0 mL of dimethylsulfoxide (DMSO). The microtubes were kept for 24 hours in the dark at a temperature of 25 ± 2°C and proceeded for 1 hour in a water bath at a temperature of 60°C. After the procedure, 20 µL aliquots were deposited in triplicate in a 90-cell microplate and the quantification of photosynthetic pigments was performed with the aid of a Spectramax® Paradigm® spectrophotometer. The calculations for determining the concentration of chlorophylls *a* and *b* and carotenoids were performed using spectrophotometry readings at the wavelengths 665 nm for chlorophyll *a*, 649 nm for chlorophyll *b*, and 470 nm for carotenoids. Estimation of the content of chlorophyll *a*, chlorophyll *b*, total chlorophylls, the chlorophyll *a/b* ratio, and the content of carotenoids, were performed according to Wellburn (1994):

$$\text{Chlorophyll } a = 12.47 \cdot A_{665} - 3.62 \cdot A_{649}$$

$$\text{Chlorophyll } b = 25.06 \cdot A_{649} - 6.5 \cdot A_{665}$$

$$\text{Total carotenoids: } (1000 \cdot A_{470} - 1.29 \cdot Ca - 53.78 \cdot Cb) / 220$$

Total chlorophyll = chlorophyll *a* + chlorophyll *b*,

where A_{665} is the absorbance at 665 nm, A_{649} is the absorbance at 649 nm, A_{480} is the absorbance at 470 nm, Ca is the content of chlorophyll *a*, and Cb is the content of chlorophyll *b*.

The occurrence of inter-conversion of chlorophyll *b* to chlorophyll *a* was evaluated through the estimation of the chlorophyll *a/b* ratio. This inter-conversion plays an important role in the establishment of the necessary chlorophyll *a/b* ratio during the adaptation to stresses (Martins et al. 2018).

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 1.0 mL L⁻¹ of Morel's vitamins, 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 hours 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 containers filled with commercial substrate Tropstrato HT Hortaliças® (Mogi Mirim, SP, Brazil) and sealed with plastic film. The plastic containers were maintained in a growth room at 25 ± 2°C, with a photoperiod of 16 hours and irradiance of 40–50 mol m⁻² s⁻¹. After seven days of pre-acclimatization, about 10 holes (0.5 cm Ø) were made in the plastic film, to allow gas exchange. After 14 days, the films were removed, and the plastic containers were kept in a greenhouse with automated irrigation for acclimatization. At 30 days after acclimatization, the survival rate of the plants was determined. All statistical analyses were performed using Duncan's 5% probability test and simple linear regression, with the software Genes v. 7.0 (Cruz 2009).

Results

In vitro germination provides axenic explants for in vitro introduction

The *in vitro* germination in agar:water medium provided healthy developed plantlets, with fungal contaminations occurring in around 5% of the introduced seeds. Seed germination occurred exponentially comprising the emission of root primordia and root system, with a peak occurring 30 days after inoculation. Shoot emission occurred gradually up to 100 days (Fig. 2a). On day 116 after inoculation, germination was established with approximately 81.25% of the seeds emitting roots and shoots and 11.25% of seeds emitting only roots, while 7.5% did not germinate.

The seedlings from the *in vitro* germination provided, on average, five axenic explants (nodal segments) per seed for the *in vitro* establishment and propagation. The only source of further contamination was from endogenous microorganisms (4% of the nodal segments introduced in a semi-solid medium), without indicators of exogenous contamination.

The optimal concentration of BAP ranges from 2.0 to 2.5 µM

The highest rate of *in vitro* shoot generation was obtained with 2.0 µM BAP 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. 2c-h), 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 2.60 (0.5 µM 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 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. 2b) 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.

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 ($p > 0.05$)				

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

There were no statistically significant differences between 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 cytokinin BAP (1.5, 2.0, or 2.5 μM).

Culture medium		
PGRs (μM)	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	

*Averages 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 measures related to rooting (% of rooting, number of roots, and length of the roots) did not differ statistically among the three immersion systems (Table 3), although a higher percentage of rooting (46.7%) was observed in the TIS/TF (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 acclimatized rooted seedlings survived (Fig. 3c-d). Concerning aerial development of the seedlings, the biomass accumulation and leaf area significantly differed 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 cm² of leaf area per flask. These estimations did not differ from the averages obtained in the PIS/GE but differed from measures from PIS/WE (Table 3).

Table 3

Mean values for measures of biomass accumulation (g), leaf area (cm²), rooting (%), number of roots, and root length (cm) 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

Immersion systems interfere in the carotenoids content but not in chlorophyll

Overall, there is a tendency for the highest amounts of chlorophyll *a* and *b*, carotenoids, total chlorophyll, and chlorophyll *a/b* ratio to occur in the PIS/GE. This result is possibly correlated with this immersion system's ability to accumulate biomass and generate larger leaf areas in the explants, increasing their photosynthetic capacity. As in the PIS/GE system, there is a constant exchange of the internal atmosphere of the flasks and general stimulation of the plants, which results in greater transpiration. Increased transpiration, in turn, stimulates greater absorption of nutrients from the culture medium. Consequently, as observed in the pigment analyses, a greater photosynthetic response of the explants subjected to this condition is also expected.

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).

Table 4

Mean estimations (mg g⁻¹ leaf) of chlorophyll *a*, chlorophyll *b*, total chlorophyll, chlorophyll *a/b* ratio, and carotenoids for *E. uniflora* seedlings cultivated in immersion systems

Immersion system	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Carotenoids	Total chlorophyll	Chlorophyll <i>a/b</i> 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

Discussion

Micropropagation protocols have been studied for *E. uniflora* using organogenesis (Uematsu et al. 1999, Souza et al. 2008, da Silva et al. 2014) and somatic embryogenesis (Stefenon et al. 2020b) routes. The present study promoted important advances in the organogenesis protocol for this species.

Under the concentrations of BAP used in this study, the nodal segments did not present the occurrence of necrosis and the increase of this cytokinin promoted higher shoot development up to 2.5 µM, the highest concentration tested. Reports on micropropagation of *Psidium guajava* (Ali & Ludders 2001), *Rubus idaeus* (Ering et al. 2002), *Acca sellowiana* (Oltramari et al. 2000) and *uniflora* (Souza et al. 2008) suggested 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. We observed a gradual improvement in the number of shoots formation according to the raise of the BAP concentration, from 1.0 up to 2.5 µM. A similar result was reported by Souza et al. (2008) in *E. uniflora*, reporting from 1.20 to 2.35 shoots per explant in the range of 0.0 to 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).

As far as we are aware, this is the first study using immersion systems for the *in vitro* organogenesis of *E. uniflora*. Overall, our results suggest that this strategy promotes high *in vitro* biomass accumulation and rooting. Some studies using immersion systems for the *in vitro* organogenesis in Myrtaceae [*Melaleuca alternifolia* Cheel (Scheidt 2010), *Eucalyptus grandis* x *Eucalyptus urophylla* (Oliveira et al. 2014), and *Myrtus communis* L. (Aka Kaçar et al. 2019)] have demonstrated that the gas exchange promoted by these systems is effective for the *in vitro* accumulation of biomass.

The low rooting rate has been a great challenge in the micropropagation of *E. uniflora* (Uematsu et al. 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. In turn, all rooted plants survived in the acclimatization phase. In addition to fostering a higher rooting rate, the TIS/TF system dismisses the use of agar and activated charcoal, reducing the costs of the process.

The absence of significant difference in the contents of chlorophyll *a* and *b*, and chlorophyll *a/b* ratio among the three immersion systems suggest that the culture conditions employed for the micropropagation of *E. uniflora* caused the same level of stress to the plants in all systems. The occurrence of differentiated levels of stress in the different cultivation systems would promote diverse measures for the chlorophyll *a/b* ratio since high chlorophyll *a/b* ratio may indicate less emphasis on light harvesting concerning the rates of photosystem II photochemistry under stress (Martins et al. 2018).

The protocol provided in this study may be helpful for the mass production of seedlings for degraded areas recovery or commercial orchard implementation, as well as in genetic improvement programs for *E. uniflora*. Following four main steps (Fig. 4), selected plants can be successfully propagated on a large scale. The *in vitro* germination (first step) of selected fruits can be performed from directed crosses in genetic breeding programs, or from open pollination if the large-scale mass propagation is the objective for recovery of degraded areas, for instance. The induction of buds (second step) in nodal segments using semi-solid MS medium supplemented with 0.5 µM of NAA and 2.0-2.5 µM of BAP enables the production of many clonal seedlings. The culture in twin-flasks temporary immersion system (third step) with MS medium free of plant growth regulators promotes the aerial biomass increment and rooting. The use of large TIS/TF systems with recyclable 5 liters PET (polyethylene terephthalate) flasks allows for increasing the number of seedlings produced at low cost. Finally, in the fourth step, the commercial substrate of choice can be employed for the acclimatization of the seedlings into a greenhouse.

Declarations

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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 studies with human participants or animals performed by any of the authors.

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Figures



Figure 1

Immersion systems used for the micropropagation of *E. 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.

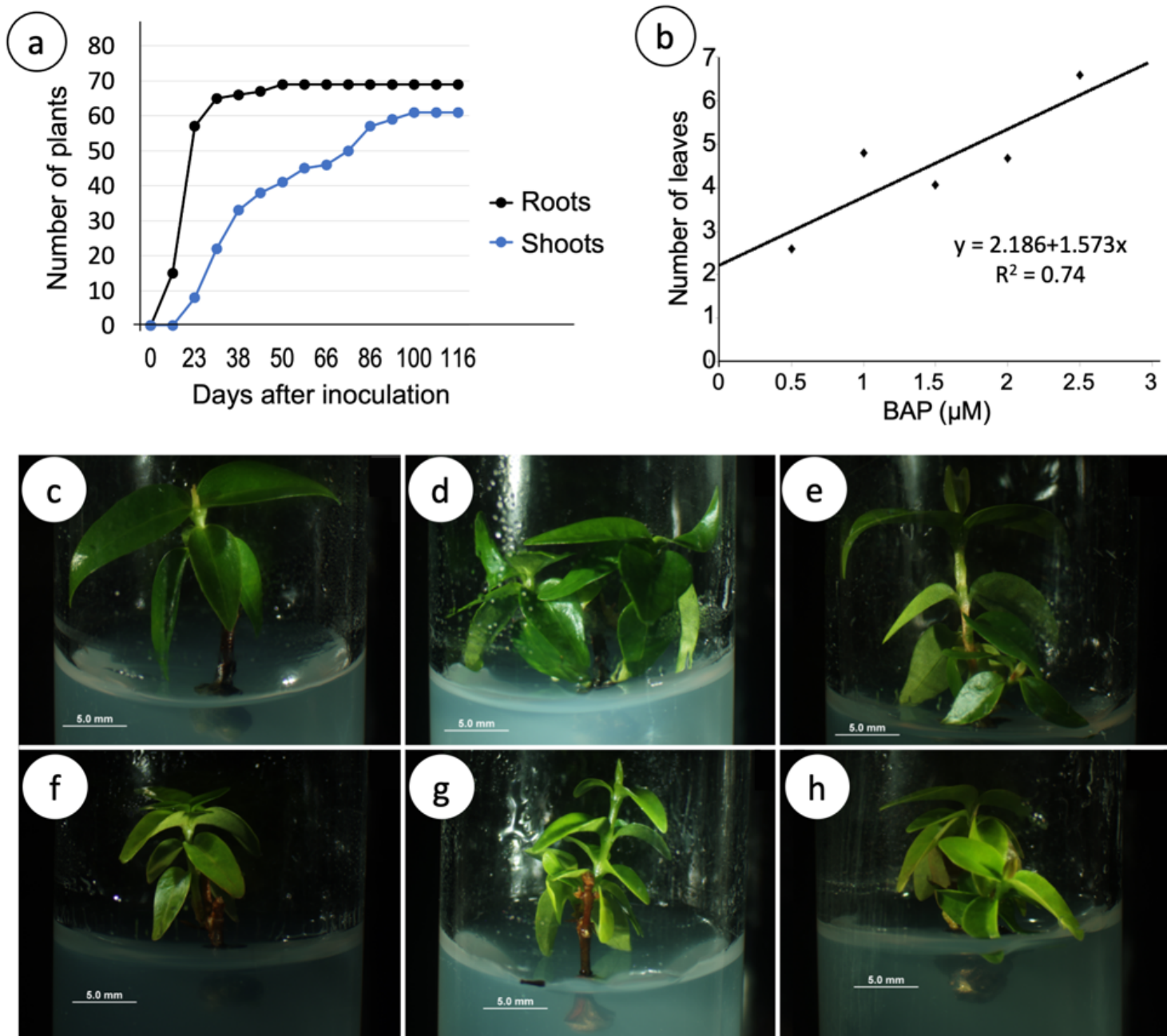


Figure 2

In vitro germination and culture of *E. uniflora* explants in semi-solid medium. (a) Emission of roots and shoots in the *in vitro* germination. (b) Regression analysis of the leaf number per explant as a function of the BAP concentration in the culture in semi-solid medium. (c) MS medium with 0.5 NAA + 1.5 BAP; (d) MS medium with 0.5 NAA + 2.0 BAP; (e) MS medium with 0.5 NAA + 2.5 BAP; (f) WPM medium with 0.5 NAA + 1.5 BAP; (g) WPM medium with 0.5 NAA + 2.0 BAP; (h) WPM medium with 0.5 NAA + 2.5 BAP

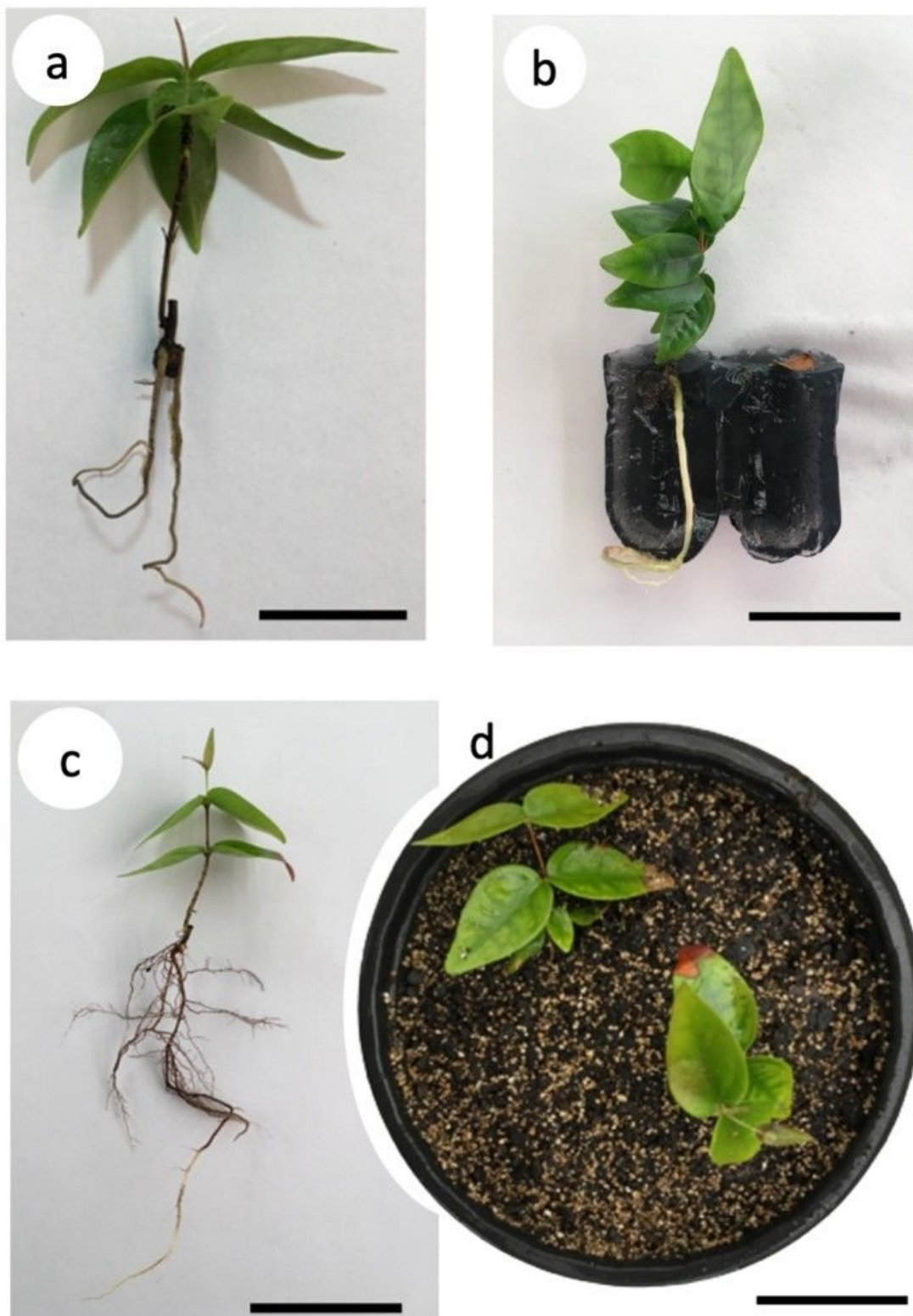


Figure 3

Rooting and acclimatization of *E. 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

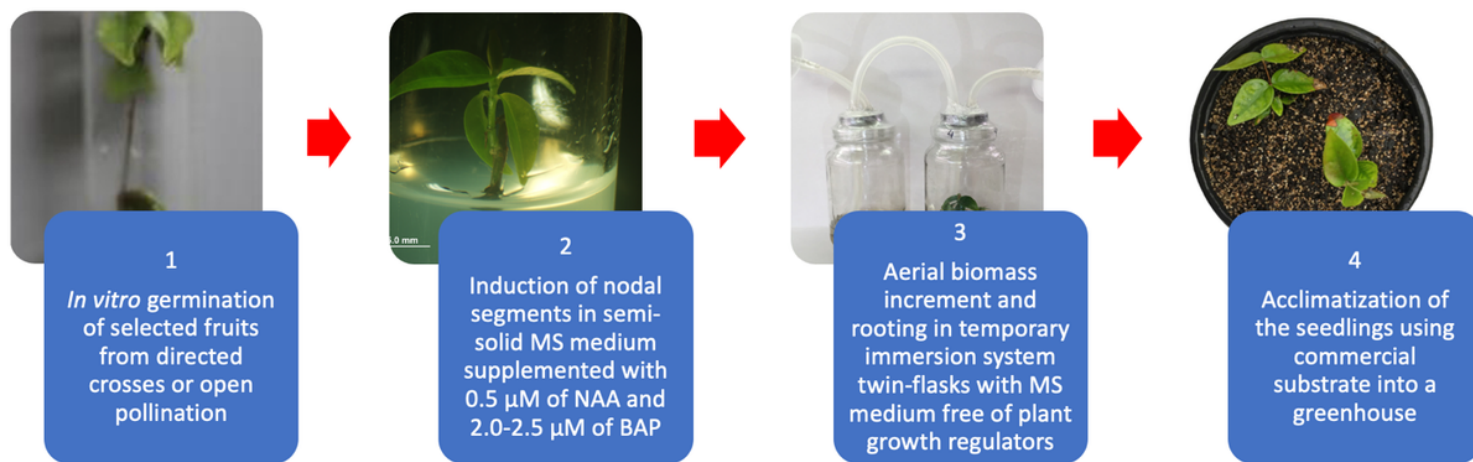


Figure 4

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