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# A regenerative route for *Eugenia uniflora* L. (Myrtaceae) through in vitro germination and micropropagation

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**Abstract.** *Eugenia uniflora* is a tree species native from Central and South America, largely employed in the popular medicine, in the cosmetics and pharmaceutical industries and also consumed in natura. Aiming to provide plant material with high sanity and genetic uniformity for the establishment of commercial plantations, we developed a protocol for seeds disinfestation, in vitro germination and in vitro propagation of this species through organogenesis. Fruits of *E. uniflora* were obtained from wild trees growing in the Pampa biome, Southern Brazil. Seeds were disinfested using ethanol 70% (10 min) and NaOCl 1.25% (10 or 25 min). Shoot apexes and nodal segments of non-contaminated plantlets were cultivated in verification medium $AS_{30}$ during 20 days, posteriorly in ½MS medium supplemented with sucrose, IBA and BAP during 45 days and acclimatized in greenhouse. Disinfesting seeds with ethanol 70% (10 min) and NaOCl 1.25% (25 min) allowed germination with significantly lower contamination (2.0%) and production of healthy explants for the micropropagation. No difference concerning size and contamination was observed for the propagation using shoot apexes or nodal segments as explant. Acclimatized plants revealed normal phenotype and healthy appearance. This regenerative route can be applied for mass clonal propagation from seeds of cross-pollinated or self-pollinated selected trees aiming the establishment of commercial plantations of *E. uniflora* and other Myrtaceae species.
**Keywords** Brazilian cherry, organogenesis, tree breeding, conservation of genetic resources.

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## Introduction

*Eugenia uniflora* L. (Myrtaceae, Myrtoideae) is a tree species native to Central and South America. In Brazil, it is found in different biomes along rivers in riparian forests, in coastal forest formations and in semidecidual forests (Margis et al. 2002, Salgueiro et al. 2004). This species is an important source of food for a variety of birds and mammals in the forests and has major significance in the natural regeneration (Almeida et al. 2012).

Due to the small to medium size and the multi-branched stem, *E. uniflora* lacks value as a timber species, but is largely employed in the popular medicine, in the cosmetics and pharmaceutical industries and also consumed in natura, as juice and ice cream (Almeida et al. 2012, Costella et al. 2012). In addition, this light-demanding tree species can be employed in the restoration of degraded areas and deforested zones. The potential of *E. uniflora* for forestry includes a growing concern in collecting and conserving the species germplasm across the world. Over 300 selected genotypes are conserved in germplasm banks in Brazil, USA, Australia, Africa, Central America and Asia (Lattuada 2008).

Despite the broad utility and commercial perspectives for this species, the establishment of *E. uniflora* orchards in South Brazil has been practiced mainly for household consumption, without commercial aspirations. Moreover, the nursery stocks of E. *uniflora* are typically propagated through randomly collected seedlings, so that such plantations lack morphological, physiological and genetic uniformity, making the commercialization of the fruits difficult. The in vitro micropropagation is a viable method for mass propagation of tree species, providing plants with high sanity and clonal homogeneity. Thus, the development of in vitro regenerative protocols in *E. uniflora* has direct applications for clonal mass propagation of selected plants and may be integrated into breeding programs, restoration of degraded and deforested areas, as well as in urban forestry and in non-wood products forestry.

Although in vitro techniques have been recommended for the reproduction and germplasm conservation of *E. uniflora* (Pilatti et al. 2011), only a few studies (Uematsu et al. 1999, Souza et al. 2008, Lattuada 2010) have focused on the micropropagation of shoots from plants growing in greenhouse. In addition, obtaining explants free of bacterial and fungal contamination is a major concern for the micropropagation of selected plants of *E. uniflora* (Lattuada 2008).

Since, in developing countries, rural population derive a significant part of their food requirements and economic profits from forest species (FAO 1986), the micropropagation may advance the forestry use of *E. uniflora* in South America, through the establishment of clonal plantations of selected trees. In such a case, the development of genetic improvement programs is important and the use of controlled cross-pollination or self-pollination of selected trees followed by in vitro germination of the seeds and micropropagation of the plantlets is an attractive alternative.

Following this rationality, towards the advance of genetic improvement programs for *E. uniflora* and the establishment of commercial plantations of this species, this study aimed to develop a protocol for disinfestation of selected seeds, in vitro germination and in vitro multiplication of *E. uniflora* through organogenesis.

## Material and methods

### Studied species and sample collection

*Eugenia uniflora* is a rather versatile species and grows in several different habitats including riparian forests, arid and semiarid environments. It can be found as a medium tree 4-5 m tall, reaching up to 12 m. The plants are hermaphrodites, with small white flowers having

four petals and several stamens. Insects pollinate the species and flowering happens twice a year, in January and September. Fruit ripening occurs in February and October, approximately five to six weeks after flowering. Seeds are recalcitrant (Pilatti et al. 2011) and their dispersion occurs through animals, mainly birds (Almeida et al. 2012). Natural self-pollination seems to occur, but the fruit development is not effective, reaching less than 7.0% of the self-pollinated flowers (Franzon 2008).

The seeds employed in this study were obtained from four wild genotypes growing in the Brazilian Pampa, Rio Grande do Sul State, Southern Brazil. These genotypes were selected based on the large size and appealing color of the fruits, which reach commercial requirements. For this experiment, 100 healthy ripe fruits were randomly collected during the summer season of the year 2012, stored in paper bags and transported to the laboratory within 24 hours.

### Seeds disinfestation and in vitro germination

After removed from the fruits, seeds were divided in two samples of 50 seeds each. Seeds were surface disinfected in a flow chamber with ethanol 70% (1 min), 1.25% sodium hypochlorite solution (NaOCl) during 10 min (50 seeds) or 25 min (50 seeds) and rinsed three times in sterile distillated water. Each seed was individually inoculated in test tubes containing 10 mL of germination medium (Figure 1A), composed by 6.0 g $L^{-1}$ agar (Sigma-Aldrich, St Louis, MO) in ultrapure water. Before explant inoculation, the germination medium was distributed in 180 x 20 mm test tubes (10 mL/tube), which were sealed with plastic film and autoclaved during 17 min at 121ºC and 1.2 atm. Test tubes were kept at 25 ± 2°C, 16 h photoperiod with an irradiance of 40 – 50 mol $m^{-2}$ $s^{-1}$. The experimental design followed a completely randomized blocks scheme, with two treatments and 50 seeds/treatment.

### In vitro multiplication

After 45 days, the non-contaminated plantlets from each treatment (10 min and 25 min immersion in NaOCl) that presented more than 3.0 cm length were incised in 1.5 cm segments as proposed by Souza et al. (2007). The explants (shoot apex or nodal segment) were inoculated in 180 x 20 mm test tubes containing 10 mL of verification medium $AS_{30}$, composed by agar 7.5 g $L^{-1}$ and sucrose 30 g $L^{-1}$ (Figure 1B). Before explant inoculation, test tubes were sealed with plastic film and autoclaved during 17 min at 121ºC and 1.2 atm. This medium was employed to verify the existence of endogenous bacteria and fungi, which could emerge after germination. The explants were evaluated after one, nine and 20 days of inoculation in this medium. The size of the explants and the number of buds were registered in the $20^{th}$ day of culture.

After 20 days in the verification medium, non-contaminated shoot apices and nodal segments were transferred to test tubes containing 10 mL of ½MS medium (Murashig & Skoog 1962) pH 5.8 (Figure 1C). Based on our prior tests of the culture medium composition for *E. uniflora*, the ½MS medium was supplemented with 30 g $L^{-1}$ of sucrose, 0.1 mg $L^{-1}$ indolebutyric acid (IBA), 0.2 mg $L^{-1}$ 6-benzylaminopurine (BAP) and solidified with 6.0 g $L^{-1}$ of agar. All reagents were purchased from Sigma-Aldrich. The ½MS medium was distributed in 180x20 mm test tubes (10 mL/tube), tubes were sealed with plastic film and autoclaved as described above.

Explants were cultured during 45 days in a completely randomized blocks design, with two treatments (shoot apices and nodal segments) and 40 replicates. During the culture in verification medium and in ½MS medium, the test tubes were kept at 25±2°C, 16 h photoperiod with an irradiance of 40-50 mol $m^{-2}$ $s^{-1}$.

Unrooted plantlets cultivated during 45 days in the ½ MS medium were transferred to pots with autoclaved sand and maintained in

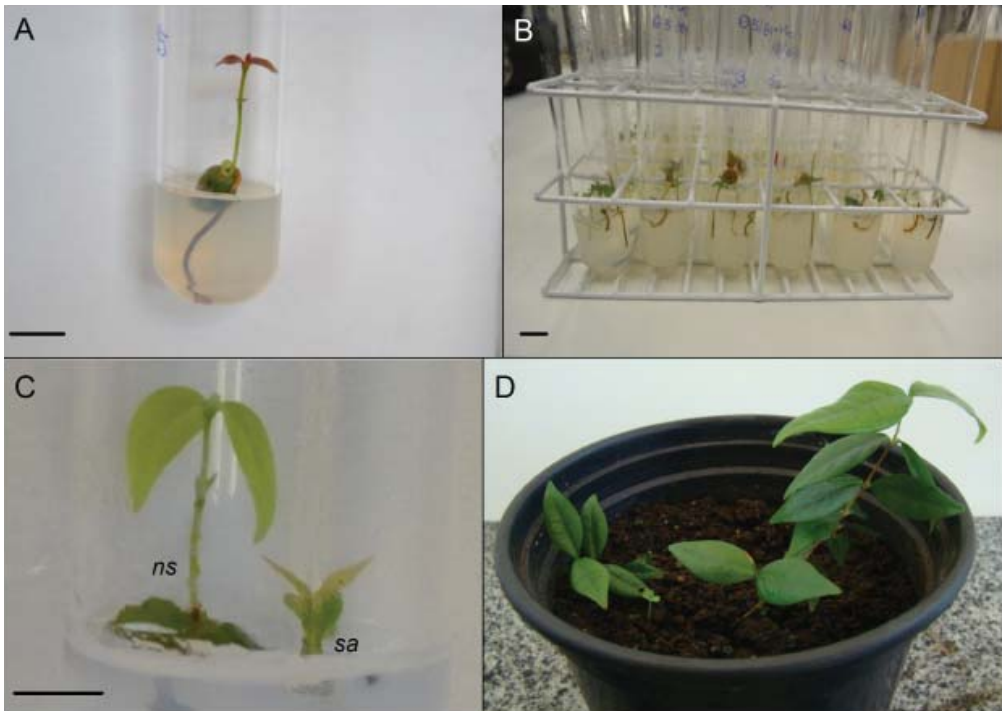


**Figure 1** In vitro germination and propagation of *Eugenia uniflora*. (A) Germinated seed in germination medium after 45 days of inoculation. Scale bar: 10 mm. (B) Explants after nine days in verification medium $AS_{30}$. Scale bar: 10 mm. (C) Nodal segment (*ns*) and shoot apex (*sa*) inoculated in ½MS medium supplemented with IBA (0.1 mg $L^{-1}$) and BAP (0.2 mg $L^{-1}$). Scale bar: 5 mm. (D) *Ex vitro* acclimatized seedling of *E. uniflora* after six months in greenhouse environment

nursery environment in greenhouse with daily watering during 60 days. After this period, the plants were transferred to pots with sand: organic soil (1:1 v/v) and cultivated in greenhouse with daily watering.

All statistical analyses were performed through the Fisher's exact test using the software GraphPad®.

## Results

For the in vitro germination, the use of 10 min of immersion in NaOCl revealed significantly higher contamination ($p < 0.001$) in comparison to 25 min of immersion. After 45 days in the germination medium, 74.0% of the seeds from the shorter treatment revealed bacterial contamination, while just one seed (2.0%) originated from the longer treatment revealed contamination (Table 1). All non-contaminated seeds germinated after 45 days, presenting 26% of germination in seeds treated with 10 min immersion in NaOCl and 98% of germination for seeds treated during 25 min.

In the verification medium $AS_{30}$, bacterial contamination was observed in 2.0% of the inoculated explants after nine days of culture. The mean size of the explants was 2.05 cm (±0.69 cm) and the mean number of developed buds was 1.15 (±0.55) buds/explant.

After 45 days of inoculation in the ½MS medium, 98.73% of survival was observed, with 2.17% of the explants revealing bacterial contamination. There was no difference concerning survival rate when comparing the use of shoot apexes or nodal segments as explants ($p = 0.12$, Table 1).

The unrooted plantlets cultivated in pots with autoclaved sand presented normal develop-

**Table 1** Percentage of contamination[1] in *E. uniflora* explants. The effect of the NaOCl treatment was evaluated after 45 days in germination medium and the effect of the explant was evaluated after 20 days in ½MS medium supplemented with IBA (0.1 mg $L^{-1}$) and BAP (0.2 mg $L^{-1}$)

| | Contamination (%) |
|---|---|
| NaOCl treatment | |
| 10 min (n = 50) | 74.0[a] |
| 25 min (n = 50) | 2.0[b] |
| Explant | |
| Shoot apex (n = 40) | 0.0[a] |
| Nodal segment (n = 40) | 3.9[a] |

Note. [1]Only bacterial contamination, since no fungal contamination was observed. Values followed by different letters revealed significant difference according to the Fisher's exact test ($p < 0.05$).

ment of roots and buds after 60 days of culture in greenhouse, generating completely healthy seedlings. After six months in pots with sand: organic soil as substrate, these plants presented normal development and mean height of 10.0 cm (Figure 1D).

## Discussion

Our study presents a procedure for in vitro germination and clonal propagation of *E. uniflora*, which allowed obtaining low fungal/bacterial contamination and high rates of reproduction through an organogenetic pathway. The main advance proposed in this study is the use of selected seeds instead of selected trees as start material for the mass propagation of *E. uniflora*. It enables the conduction of controlled crosses and the propagation of the improved genotypes.

Martinoto et al. (2007) observed 88.25% of germination after 71 days for scarified seeds of *E. dysenteryca* (Myrtoideae) in MS medium. For *Myrcianthes pungens* (Myrtoideae), Souza et al. (2011) obtained from 68 to 98% of germination and from 2.0% to 14.0% of contamination after 31 days of culture in wood plant medium (WPM). Considering the observed germination rate for *E. uniflora* (98% after 45 days), the use of agar: water medium as substrate revealed to be efficient, in addition to allowing reduction of costs in comparison to the MS and WPM media. Given that the conditions of temperature and light incidence were quite similar in the referred studies, our high rate of germination seems to be related mainly to genetic factors and seed ripeness. In a greenhouse experiment, physiological stage of the seeds and genetic traits significantly influenced the germination rate, the number of leaves and the size of seedlings of *E. uniflora* germinated and cultivated in different substrata (Antunes et al. 2012).

Before the inoculation of the explants in the propagation medium we employed a verification medium ($AS_{30}$) to evaluate the persistence of contaminants. Although such verification step usually is not employed, the use of the verification medium $AS_{30}$ is an efficient, low-cost and simple alternative to identify the presence of contamination in explants from in vitro germinated seeds. It prevents the contamination of the micropropagation medium, which presents higher costs in the mass propagation process.

Lower concentrations of the MS medium were found to be more effective for the micropropagation of *Acca sellowiana* (Dal Vesco & Guerra 1999). Concerning the growth regulators, higher concentrations of BAP promoted negative effects in the organogenic response of *E. uniflora* (Lattuada et al. 2008, Souza et al. 2008) and *E. pyriformis* (Nascimento et al. 2008), while lower concentrations of IBA were more effective for the root development in *Rubus* spp (Leitzke et al. 2009). Corroborating these results, the ½MS medium supplemented

with IBA 0.1 mg $L^{-1}$ and BAP 0.2 mg $L^{-1}$ revealed to be effective for the survival and in vitro development of shot apexes and nodal segments of *E. uniflora*, although no root development was observed in these conditions. The root development in absence of growth regulators may occur in micropropagated plants as effect of the endogenous auxin produced and its translocation to the explant basis. Ex vitro or in vitro rooting in absence of plant growth regulators are desired due to the economical aspects and the possibility of automation of the process (Cuzzuol et al. 1996). Although ex vitro rooting leads to a decrease in fresh aerial mass in comparison to in vitro rooted plants, this strategy reduces costs and promotes de development of a more complete and functional root system (Cuzzuol et al. 1996).

## Conclusions

The in vitro germination of selected seeds and the in vitro propagation of shoot apexes or nodal segments in ½MS medium supplemented with low concentration of growth regulators is a viable alternative to develop the mass propagation of high sanity plants of *E. uniflora*, as well as other *Eugenia* species. Studies conducted in our laboratory have shown high efficiency of this protocol for the mass clonal propagation of *E. involucrata*. This regenerative route can be applied for the mass clonal propagation from seeds of cross-pollinated or self-pollinated selected trees aiming the establishment of uniform high sanity orchards. The procedure may also be effective for the micropropagation of further tree species with importance for traditional and urban forestry, as well as for forestry based on non-wood forest products.

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