Explants sterilization through metal nanoparticles for *in vitro* mass propagation of *Eugenia involucrata*

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ABSTRACT

An efficient method for the sterilization of explants is essential for the establishment of plant micropropagation. In this study we aimed to evaluate the efficacy of treatments using silver and gold nanoparticles as disinfectant agents of explants of *Eugenia involucrata*, in comparison to results from three other sterilization procedures. The sterilization procedure based on serial immersions in ethanol, sodium hypochlorite, and silver nanoparticles was quite effective, providing a very low proportion of explants contamination in nodal segments and apical buds of *E. involucrata*. The same protocol using gold instead of silver nanoparticles did not control fungi contamination of the explants. The immersion of explants in an *in-house* prepared solution of silver nanoparticles revealed to be an easy, cheap and efficient method for sterilization of nodal segments and shoot apices of *E. involucrata*, while the gold nanoparticles treatment was ineffective for controlling fungal contamination in this study.

Keywords: Cerejeira-do-Rio-Grande; *in vitro* propagation; silver nanoparticles; gold nanoparticles; Myrtaceae.

INTRODUCTION

Eugenia involucrata D.C. (Figure 1A) is a fruit tree species from the family Myrtaceae, native to tropical and subtropical regions of Central and Southern Brazil, Argentina and Paraguay. The fruits of *E. involucrata* are consumed *in natura*, as juice and as jellies, and are also an important source of feed for local fauna. This species can also be employed for the regeneration of degraded environments and has potential use in the cosmetic and pharmaceutical industries (Sardi et al., 2017; Nicácio et al., 2017). Despite the recognized ecological and economic potential of *E. involucrata*, it is still underexplored, and few investigated.

Physiological studies have shown that seeds of *E. involucrata* do not endure reductions in water content to values under 50% (Maluf et al., 2003), losing viability shortly after harvest (Golle et al., 2012; Cripa et al., 2014). As a consequence of the seeds' recalcitrance, the seminiferous propagation of this species at large scale has low viability. Initiatives towards the propagation of *E. involucrata* through grafting or cutting also revealed to be unsuccessful (Alegretti et al., 2015). Therefore, *in vitro* establishment of *E. involucrata* may be a convenient alternative for its large-scale propagation (Golle et al., 2012). However, fungal and bacterial contamination rates higher than 39% have been reported in experiments aiming the *in vitro* propagation of this species (Golle et al., 2013).

The explant sterilization is the first step to be considered for the achievement of an optimal *in vitro* establishment in woody plant species (Pierik, 1997). Contamination levels tend to be higher when the parent plants used as the source of explants come from the field. However, even plants submitted to strict phytosanitary control and kept in a protected nursery or greenhouse are potential sources of microorganisms (mainly bacteria and fungi), which may become a limiting factor to *in vitro* culture procedures (Ray; Ali, 2017), being considered a serious threat to the success of plant micropropagation (Thomas; Prakash, 2004). Contaminated cultures may survive for several years but they grow poorly and are difficult to proliferate (Drew, 2013). In order to reduce contamination in the culture medium, treatments of the explant with ethanol, sodium chloride, hydrogen peroxide, or mercuric compounds have traditionally been used (Kumar; Reddy, 2011). Nevertheless, such treatments may be not so efficient for some species and the use of alternative methods based on metal nanoparticles has been tested. Silver nanoparticles were used for disinfestation of tomato (Mahna et al., 2013), carnation (Babaei et al., 2015), rose (Shokri et al.,

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2015), olive (Rostami; Shahsavar, 2009), Norfolk-Island pine (Sarmast et al., 2011) and an almond *x* peach hybrid (Arab et al., 2014). Titanium nanoparticles were employed for explants sterilization in micropropagation of potato and tomato (Safavi, 2014). The outcome of these studies revealed a high potential for using metal nanoparticles as disinfectant agent in plant micropropagation. In addition, recent studies have suggested the absence of phytotoxicity of silver nanoparticles in plant micropropagation when added at low concentrations in the *in vitro* culture medium (Timoteo et al., 2018).

Aiming to overcome the difficulties concerning explants disinfection for *in vitro* establishment of *E. involucrata*, we evaluated this study, the efficacy of treatments using silver and gold nanoparticles as disinfectant agents of explants of this species. We compared the results of the metal nanoparticle treatments with results from three other sterilization procedures, evaluating the proportion of contaminated explants and the shoots' development after 30 days of *in vitro* culture.

MATERIALS AND METHODS

Plant material

Ripe fruits of *Eugenia involucrata* (Figure 1B) were collected during the spring season from four adult trees previously selected within the urban perimeter of the city of São Gabriel, Rio Grande do Sul State, Brazil (30° 20'S, 54° 19'W). A total of 600 fruits were washed in running water and their seeds were removed from the pulp. The seeds were germinated indoor, in plastic trays containing an autoclaved combination of 50% vermiculite and 50% sand as substrate. Watering was achieved daily with distilled water and biweekly with a solution of 1/4 MS medium (Murashige; Skoog, 1962). Plantlets with about 10 cm in height were used as the source of the explants employed in the study.

In vitro culture and acclimatization

The experiment consisted of five disinfestation treatments (Table 1), each treatment with twelve test tubes and four replicates, totaling forty-eight test tubes per treatment with two explants/tube (i.e. 96 explants/treatment). In aseptic conditions, shoot apices and nodal segments with about 1.5 cm in length were

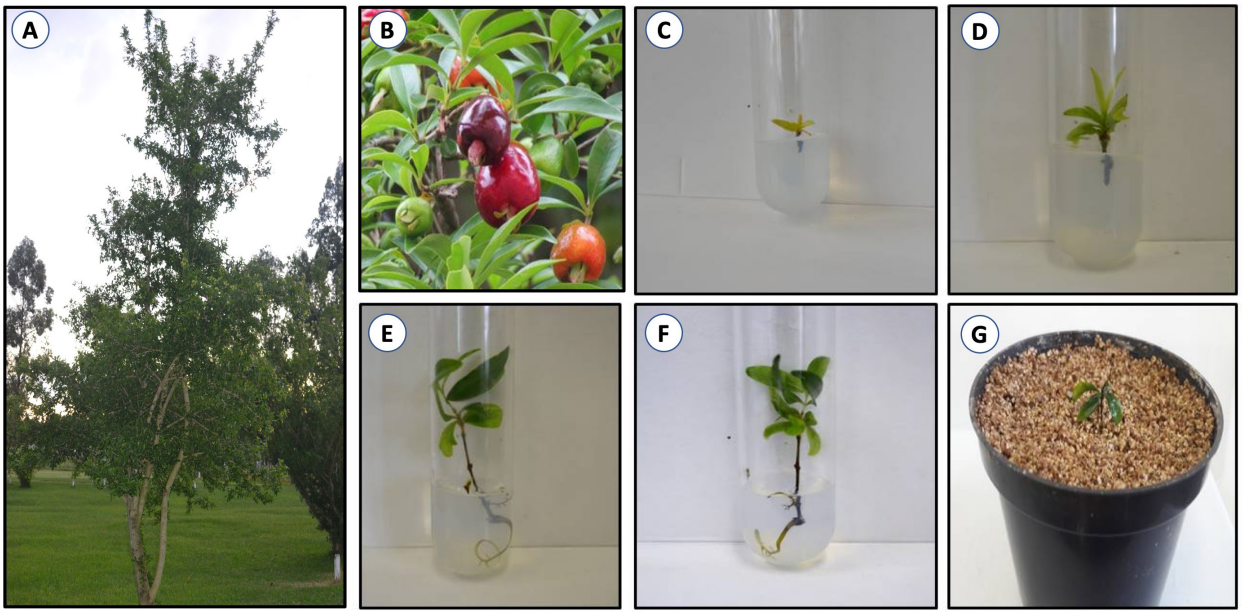

Figure 1 – Characteristics of the plant, fruits and different stages of the *in vitro* propagation of *Eugenia involucrata* explants treated with silver nanoparticles. (A) Adult tree of *E. involucrata*. (B) Fruits of *E. involucrata* at different stages of ripeness. (C) Shoot apex after 30 days of culture. (D) Seedling after 45 days of culture. (E) Seedling after 60 days of culture with developed leaves. (F) Seedling after 90 days of culture, presenting leaves and root system. (G) Seedling under acclimatization in an outdoor shaded environment.

incised from the plantlets and disinfected using one of the five treatments before inoculation. All procedures were performed in a sterile environment, within a flow-chamber.

Treatment 1 (ethanol:hypochlorite) is the sterilization method more frequently used for the *in vitro* micropropagation of plants. Treatment 2 (dichloro) uses sodium dichloroisocyanurate, a source of chlorine suggested as a potential disinfectant for plant micropropagation (Parkinson et al., 1996), but with few reports of use. Treatment 3 (antibiotic) is based on the use of chloramphenicol, a broad-spectrum antibiotic aging over both gram-positive and gram-negative bacteria. Treatments 4 (gold nanoparticles) and 5 (silver nanoparticles) are based on the use of house-made solutions of metal nanoparticles.

Gold nanoparticles (AuNps) and silver nanoparticles (AgNps) were synthesized through the reduction of the chlorauric acid (for the synthesis of AuNps) or of the silver nitrate (for the synthesis of AgNps) by sodium citrate (Turkevich et al., 1951). Shortly, 95 mL of a solution containing 5 mg of chlorauric acid or of silver nitrate was heated into a water bath at 90° C, and 5 mL of a solution of sodium citrate 1% was added to the boiling solution under constant shake until reaching a purple coloration for gold nanoparticles (Figure 2A) or a yellowish coloration for silver nanoparticles (Figure 2C). All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). AuNps were further characterized by their UV-Vis absorption in the 500-600 nm range (Figure 2B), while AgNps were characterized by their UV-Vis absorption in the 420-440 nm range (Figure 2D) using a Cary 60 UV-VIS spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA).

After the sterilization treatment, the explants were inoculated in 180x20 mm test tubes containing 10 mL of solid MS culture medium (Murashige; Skoog, 1962) with half of the salt composition and vitamins (½ MS medium), supplemented with 0.1 mg L^{-1} of indole-butyric acid (IBA), and 0.2 mg L^{-1} of 6-benzylaminopurine (BAP) and gelled with 7.0 g L^{-1} of agar (Figure 1). The culture medium was adjusted to pH 5.8 prior to adding the gelling agent and was autoclaved for 20 min at 1.0 kgf cm^{-2}. All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

The test tubes were maintained in growth incubators under temperature conditions of 25 ± 2 °C and a photoperiod of 16 light hours, with a luminous intensity of 100 W m^{-2} provided by white LED lamps. After 30 days of *in vitro* culture, bacterial and/or fungal contamination, size of the seedlings and number and size of the developed shoots were evaluated.

After 120 days of *in vitro* culture, seedlings were transferred to plastic containers containing a moistened substrate composed of 50% vermiculite and 50% sand. These containers were packed with transparent plastic bags, closed and placed for indoor acclimatization for one week. After this period, containers were unpacked and placed in an outdoor shaded environment for further acclimatization (Figure 1G).

Statistical analysis

Data collected after 30 days of culture were transformed by (percentage of contamination) or by (size

Table 1 – Treatments used for the disinfestation of the *E. involucrata* explants.

Compound	Time of immersion				
	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5
Ethanol 70%	1 min	1 min	1 min	1 min	1 min
Sodium hypochlorite 1.25%	15 min	15 min	15 min	15 min	15 min
Autoclaved distilled water	rinsed three times	rinsed three times	rinsed three times	rinsed three times	rinsed three times
Sodium dichloroisocyanurate 2 g L^{-1}	--	10 min	--	--	--
Chloramphenicol 10 µg mL^{-1}	--	--	10 min	--	--
Gold nanoparticles	--	--	--	10 min	--
Silver nanoparticles	--	--	--	--	10 min

of the seedlings and number and size of the developed shoots) and submitted to analysis of variance (ANOVA) followed by a post-hoc Tukey test, using the Statistic 7.0 software.

RESULTS AND DISCUSSION

A statistically significant difference ($p < 0.05$) was observed among treatments concerning the rate of contamination after 30 days of *in vitro* culture. Among the five sterilization treatments tested, two were inefficient for the micropropagation of *E. involucrata*. All explants disinfected using the dichloro treatment and the gold nanoparticles treatment presented high fungal contamination and mortality after one week of cultivation. The lowest level of contamination was obtained using the silver nanoparticles treatment, with only four explants contaminated (4.16%). The treatments with ethanol:hypochlorite and antibiotic presented 25 (26.04%) and 38 (39.58%) contaminated explants, respectively (Table 2).

Since no explant survived in the dichloro and the gold nanoparticles treatments, further evaluations were performed just for the ethanol:hypochlorite, the antibiotic and the silver nanoparticles treatments. The mean size of the seedlings did not differ among these

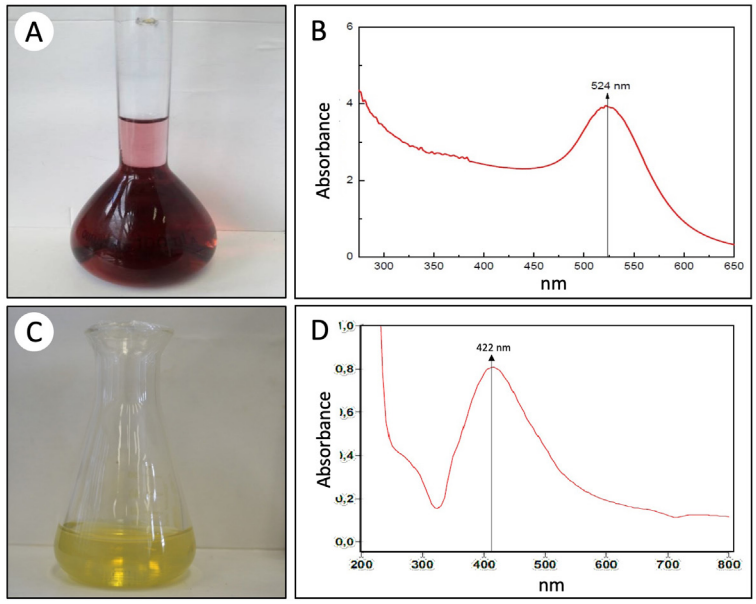

Figure 2 – Characterization of the metal nanoparticles. (A) Solution of gold nanoparticles with the characteristic purple coloration. (B) UV-Vis spectrum of the gold nanoparticles solution. (C) Solution of silver nanoparticles with the characteristic yellowish coloration. (D) UV-Vis spectrum of the silver nanoparticles solution.

Table 2 – Contamination rate and growth of *E. involucrata* after explant disinfection using different treatments.

Treatment	Contamination rate (%)§*	Seedlings size (cm)*	Number of shoots*	Size of the shoots (mm)*
Ethanol:hypochlorite	26.04 c	5.35 (2.6) a	25 b	1.67 (0.48) b
Dichloro	100.00 a	--	--	--
Antibiotic	39.58 b	4.8 (2.0) a	16 c	2.43 (0.8) a
Gold nanoparticles	100.00 a	--	--	--
Silver nanoparticles	4.16 d	5.3 (2.25) a	60 a	2.20 (1.0) a

§ Contamination rate includes both bacterial and/or fungal growth in the culture medium.
*Means followed by the same letter in the column, do not differ statistically by Tukey test at 5% probability. The numbers in parentheses correspond to the standard error.

three treatments (p = 0.916), with 5.35 ± 2.6 cm for the ethanol:hypochlorite treatment, 4.8 ± 2.0 cm for the antibiotic treatment and 5.3 ± 2.25 cm for the silver nanoparticles treatment. On the other hand, the size of the shoots in the ethanol:hypochlorite treatment (1.67 ± 0.48 mm) was significantly smaller (p < 0.05) in comparison to treatments using antibiotic (2.43 ± 0.8 mm) and silver nanoparticles (2.20 ± 1.0 mm). The number of shoots developed in the explants also differed across the three treatments. The total number of shoots observed in the treatment silver nanoparticles (60 shoots) was significantly higher (p < 0.05) than in the ethanol:hypochlorite (25 shoots) antibiotic (16 shoots) treatments (Table 2).

While gold nanoparticles failed in controlling fungi contamination, resulting in fungal proliferation (mainly *Fusarium* sp) and mortality of 100% of the explants, the treatment with silver nanoparticles promoted the best results among the five procedures tested, with only 4.16% of explants contaminated. The biosynthesis of gold nanoparticles in the range from 8-40 nm by the fungus *Fusarium oxysporum* has been reported (Mukherjee et al., 2002). The fungal resistance to the sterilization process using gold nanoparticles may be related to the size range of the nanoparticle that we employed (20-70 nm), that overlaps the range produced by the fungus. *Fusarium oxysporum* is also capable of producing silver nanoparticles in the range from 5 to 15 nm (Ahmad et al., 2003). However, these nanoparticles are smaller than the nanoparticles found in our solution (20-70 nm), which was efficient in controlling fungi contamination. Thus, it seems that the success of using metal nanoparticles as a disinfectant agent is directly related to the size of the synthesized nanoparticles.

The ethanol:hypochlorite treatment, widely used in plant micropropagation, presented a contamination rate of explants within the values reported in several studies, with 26% of contaminated explants after 30 days of *in vitro* culture. The treatment with dichloro was effective for controlling bacterial infection but failed in controlling fungi contamination. Similarly, the antibiotic treatment successfully controlled bacterial infection and failed concerning fungi contamination. In addition, the seedlings regenerated from the antibiotic treatment presented the lowest size and number of shoots, since these compounds are frequently phytotoxic delaying or even inhibiting explants growth (Safavi, 2014).

Explants sterilization is a crucial step in the *in vitro* micropropagation of plant species. The use of metal nanoparticles as the disinfectant agent in plant micropropagation was tested for the first time in *Valeriana officinalis* with quite satisfactory results (Abdi et al., 2008), and subsequently employed in some cultivated species. In this study, silver nanoparticles proved to be an efficient method for sterilization of shoot apices and nodal segments of *E. involucrata*. Previous studies on micropropagation of this species have reported difficulties in the sterilization step, with levels of contamination ranging from 39% to near 90% for nodal segments (Golle et al., 2013). Although few studies report the bacterial and fungal contamination in micropropagation of *Eugenia* species (Montalvo et al., 2010; Silva et al. 2014; Assis et al., 2018), the experience of our laboratory and reports from research partners suggest that this problem is recurrent also for other species from this genus. The treatment using silver nanoparticles presented here allowed the micropropagation of nodal segments of *E. involucrata* with only 4.16% of contamination. Ongoing studies in our laboratory have demonstrated such effective results of silver nanoparticles also for *Eugenia uniflora*.

Another important methodological aspect to consider is the way the metal nanoparticles are introduced in the sterilization process. The use of metal nanoparticles may be performed through immersion of the explants in a solution of nanoparticles or by adding the nanoparticles to the culture medium. The immersion of explants into a solution of silver nanoparticles caused injuries and browning in nodal segments of olive (Rostami; Shahsavar, 2009). However, the immersion into metal nanoparticles has been reported as efficient in other plant species, suggesting that the response to the treatment may be adverse depending on the species or the tissue in turn. For *E. involucrata*, no damage was observed after the immersion of the explants in the solution of silver nanoparticles, neither the *in vitro* development was prejudiced.

CONCLUSIONS

The immersion of explants in an *in-house* prepared solution of silver nanoparticles is an easy, cheap and efficient method for sterilization of nodal segments and shoot apices of *Eugenia involucrata*. On the other hand, the gold nanoparticles treatment was ineffective for controlling fungal contamination in this study.

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