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IN VITRO GERMINATION OF BLACK PITANGA, *Eugenia sulcata* SPRENG. EX MART. FOR THE PRODUCTION OF SEEDLINGS AIMED AT THE RECOMPOSITION OF ATLANTIC FOREST AREAS

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ABSTRACT: An important tool for the conservation of biomes is the application of technology to support biodiversity maintenance and recovery. Thus, plant tissue culture could be used as strategic tool to support the production of woody species for reforestation purposes. The use of fruit trees is particularly important because they attract seed dispersing animals that could help environment recovery. Although black pitanga (*Eugenia sulcata* Spreng ex Mart.) is difficult to find in natural habitat, it presents potential relevance for initiatives aimed at the reforestation of the Atlantic Forest because it is highly appreciated by wild birds. However, the low seed production and germination rate in natural environments impairs the maintenance of the genetic diversity. In this way, *in vitro* cultivation is an alternative to produce seedlings of arboreal species. The objective of the present work was to evaluate the potential use of *in vitro* culture for the germination of black pitanga seeds from the field to produce viable seedlings used in initiatives to recover Atlantic Forest areas. Seeds of two *E. sulcata* donor plants were cultured *in vitro* in the Wood Plant Medium (WPM) and Murashige Skoog medium (MS) with and without activated charcoal. During *in vitro* cultivation, development parameters (germination, leaf emission, root emission) and contamination were evaluated. Plants obtained were successfully acclimatized. The results demonstrated that the *in vitro* cultivation of *E. sulcata* seeds is a viable alternative to produce seedlings for reintroduction under *in vivo* conditions. However, the genetic background of donor plants could interfere in seed germination and plant development. These results are a probable reflect of the natural genetic diversity present in seeds.

KEYWORDS: biome conservation, Myrtaceae, seed germination maximization, biodiversity.

GERMINAÇÃO IN VITRO DE PITANGA PRETA, *Eugenia sulcata* SPRENG. EX MART. PARA A PRODUÇÃO DE MUDAS VISANDO A RECOMPOSIÇÃO DE ÁREAS DE MATA ATLÂNTICA

RESUMO: Uma ferramenta importante para a conservação dos biomas é a aplicação de tecnologia para apoiar a manutenção e recuperação da biodiversidade. Assim, a cultura de tecidos vegetais pode ser usada como ferramenta estratégica para apoiar a produção de plantas de algumas espécies lenhosas para fins de reflorestamento. Apesar da Pitanga negra (*Eugenia sulcata* Spreng ex Mart.) ser difícil de encontrar em habitat natural, esta espécie apresenta potencial relevância para iniciativas de reflorestamento da Mata Atlântica. No entanto, o baixo índice de produção e germinação de sementes, em ambientes naturais, dificulta a manutenção da diversidade genética. Dessa forma, a germinação de sementes in vitro é uma alternativa para a produção de mudas de espécies arbóreas. O objetivo do presente trabalho foi avaliar o potencial de uso da cultura in vitro para a germinação de sementes de pitanga preta do campo para a produção de mudas viáveis aplicadas em iniciativas de recuperação de áreas de Mata Atlântica. Sementes de duas plantas doadoras de E. sulcata encontradas foram cultivadas *in vitro* nos meios Wood Plant Medium (WPM) e Murashige Skoog (MS) com e sem carvão ativado. Durante o cultivo *in vitro*, foram avaliados os parâmetros de desenvolvimento (germinação, emissão foliar, emissão radicular) e contaminação. As plantas obtidas foram todas aclimatadas com sucesso. Os resultados demonstraram que o cultivo de sementes de *E. sulcata in vitro* é uma alternativa viável para a produção de mudas para reintrodução in vivo. No entanto, o background genético das plantas doadoras pode interferir na germinação das sementes

e no desenvolvimento das plantas. Esses resultados são provavelmente um reflexo da diversidade genética natural presente nas sementes.

PALAVRAS CHAVE: conservação de bioma, Myrtaceae, maximização da germinação de sementes, biodiversidade.

INTRODUCTION

Genetic diversity is an important factor to consider when recovering biomes. As in recent years, the growing interest in conservation initiatives has motivated the application of the Brazilian legislation (Federal Law No. 4,771), and there was an increase in the demand for seedlings and in indication of species that can be used in the recovery of biomes. However, the increase in the production of tree seedlings is seen as an important step towards the recovery of ecosystem function (Jalonen et al., 2018; Urzedo et al. 2020).

Thus, the application of techniques that allow the production of genetically varied seedlings contributes to the recovery of diversity and optimizes the efficiency of restoration and recovery projects (Neri et al., 2011).

In vitro culture techniques are pointed as important tools for the production of tree seedlings for the recovery of degraded biomes (Souza et al., 2017), currently aimed at helping the production of seedlings and maximizing the conservation of species that are potentially endangered (Wadl et al., 2011; Utami & Hariyanto, 2019). Therefore, this strategy presents potential application in the propagation of Atlantic Forest species.

Species belonging to the genus Eugenia are among those potentially efficient to assist in the restoration of forests. However, many of the species of this genus, despite their ecological importance and commercial exploitation potential, have low occurrence density (Daniel & Arruda, 2005; Prata et al., 2011; Felitto et al., 2018). This fact makes it difficult to obtain seeds in a satisfactory quantity that allows the production of seedlings in large scale to support conservation initiatives in afforestation, reforestation, or enrichment of forest fragments. These examples cited in literature reaffirm the difficulties and the need to maximize the use of obtained seeds. In addition to problems stemming from the low occurrence of seed-producing donor plants, it should be noted that most Eugenia species native to Brazil produce fruits with few seeds, often one or two (Silva et al., 2005). In this way, the use of biotechnological tools can help in the expansion of the supply of seedlings for use in the restoration of biomes.

MATERIAL AND METHODS

This research was carried out in two stages: *in vitro* germination and greenhouse acclimatization.

In vitro germination

Studies on the effects of different culture media on the germination of black pitanga were conducted at the Laboratory of Plant Tissue Culture of the Center for Strategic Technologies of Northeastern Brazil (CETENE), Recife-PE. Seeds with approximately 1.0 cm in diameter were collected from the only two donor plants found in the collection area located in Camaragibe (1) 08°01'18"S, 34°58'52"W and in Igarassu (2) 7°51'14,79"S, 34°52'58,056"W, municipalities of the state of Pernambuco.

Fruits were manually pulped and seeds were washed in running water. Seeds were disinfested in flow chamber under aseptic conditions by immersion and shaking in 70% alcohol for 1 minute and subsequently in 2.5% sodium hypochlorite solution (NaClO) for 20 minutes and rinsed in sterile distilled water for 4 times. Subsequently, in a laminar flow chamber, seeds were inoculated into test tubes (12 x 75 mm) containing 10 mL of culture media and sealed with plastic lids. Culture media were solidified with 7 $g.L^{-1}$ of agar and the pH was adjusted to 5.8 ± 0.1 before autoclaving at 121°C and 1.1 atm for 20 minutes. After inoculation, tubes were transferred to the growth room at temperature of 25 ± 2 °C, under light intensity of 2500lux (LED Lamps T08 20W Glight) and photoperiod of 16 hours.

The experiment was set up in a 2 x 2 x 2 triple factorial scheme, in which the "culture medium" factor was composed of WPM - Wood Plant Medium (Lloyd & McCown 1980) and MS Medium (Murashige and Skoog, 1962). The "antioxidant agent" factor was composed of the two treatments: addition of activated charcoal (CP) or no addition of activated charcoal (AC) and the "matrix" factor was composed of seeds collected in Matrix 1 (located in the municipality of Camaragibe) and Matrix 2 located in the municipality of Igarassu), resulting in 8 treatments (Table 1). No plant growth regulators were use in the media tested.

Table 1. Treatments resulting from the combination of factors: culture medium: WPM or MS X antioxidant agent: Presence of Active Charcoal - CP or Absence of Active Charcoal - CA X donor plants: Camaragibe matrix (1) or Igarassu matrix (2).

Treatments	Description	Initials
T1	WPM medium, absence of activated charcoal, Matrix 1	WPMCA-1
T2	WPM medium, absence of activated charcoal, Matrix 2	WPMCA-2
T3	WPM medium, presence of activated charcoal, Matrix 1	WPMCP-1
T4	WPM medium, presence of activated charcoal, Matrix 2	WPMCP-2
T5	MS medium, absence of activated charcoal, Matrix 1	MSCA-1
T6	MS medium, absence of activated charcoal, Matrix 2	MSCA-2
T7	MS medium, presence of activated charcoal, Matrix 1	MSCP-1
T8	MS medium, presence of activated charcoal, Matrix 2	MSCP-2

The design was completely randomized with 10 replicates. Variables oxidation, root emission, leaf emission, and contamination were transformed by x + 1 for statistical analysis.

For seedling height, four replicates were removed. Seedling measurements were performed 45 days after *in vitro* inoculation.

Each experimental plot was composed of a test tube with one seed and parameters were evaluated at 45 days. Data were submitted to analysis of variance and means were compared by the Tukey's test at 5% probability and ordered by the Scott Knott test. Statistical analyses were performed using the SISVAR 5.6 software (Ferreira et al., 2018). Only variable "contamination" was analyzed by the chi-square test at 5% probability.

For variables oxidation, root emission, and leaf emission, a scale of notes was adopted as described in table 2 and for plant height and data were obtained by direct measurement using a graduated ruler.

Table 2. Scale of notes used to evaluate germinative parameters.

Parameter	Scale of notes
Oxidation	1.0 = No oxidation visible in explant or culture medium 2.0 = Low oxidation in explant or culture medium 3.0 = Moderate oxidation in explant and / or culture medium 4.0 = High oxidation in culture medium and explant (explant death)
Root Emission	1.0 = No issue 2.0 = Primary root emission 3.0 = Primary and secondary root emission
Leaves Emission	1.0 = No emission of leaves 1.5 = Emission of closed leaves 2.0 = Issuing the first pair of open leaves 3.0 = Two pairs of open leaves 4.0 = More than two pairs of open leaves

Greenhouse acclimatization

After evaluations of the *in vitro* experiment, plants obtained by this process were transferred to trays containing 32 cells filled with approximately 200 mL of commercial Basaplant ® / cell substrate, being maintained in 50% shadow greenhouse and under irrigation by the micro-sprinkler system. For treatments, only culture (MS and WPM) and antioxidant agent (presence or absence of activated charcoal) factors were considered in a 2 x 2 factorial scheme, generating 4 treatments: WPM medium, absence of activated charcoal (WPMCA); WPM medium, presence of activated charcoal (WPMCP); MS medium, absence of activated charcoal (MSCA) and MS medium, presence of activated charcoal (MSCP). Plant development was evaluated in 10 replicates for each treatment at 45 days of cultivation through variables stem diameter (mm), plant height (cm), number of leaves, and canopy cover. Stem diameter was measured using a digital caliper, plant height using a graduated ruler, number of leaves by direct count, and canopy cover with the aid of a graduated ruler, measuring the distance between the ends of the apical parts of opposite larger leaves

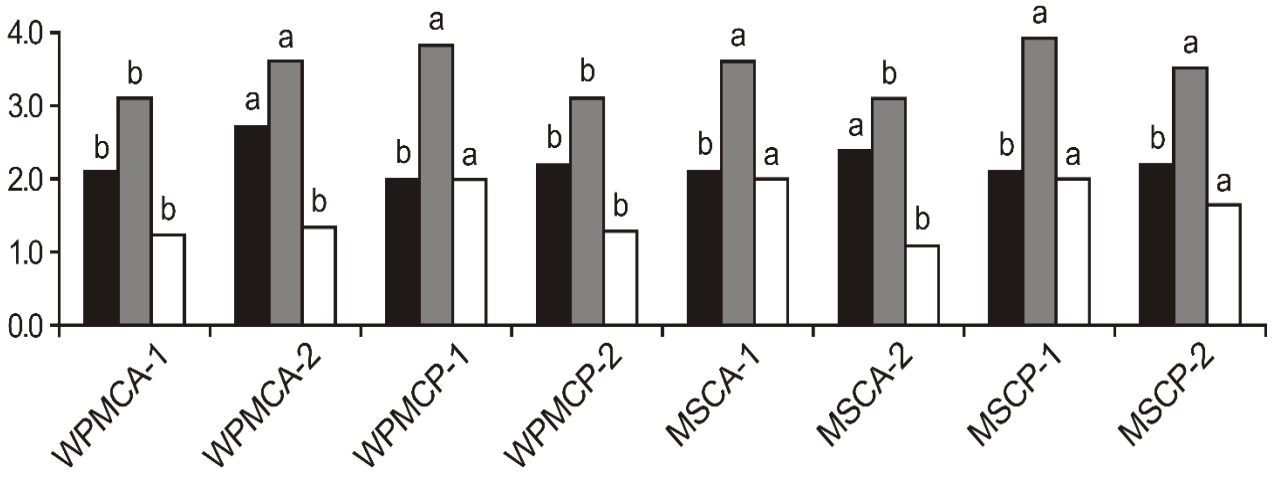
For statistical analyses, data were submitted to analysis of variance and means were compared by the Tukey test and grouped by the Scott Knott test, both at 5% probability, with the aid of SISVAR software 5.6 (Ferreira et al., 2018). Only variable contamination of the *in vitro* experiment was analyzed by the chi-square test at 5% probability.

RESULTS

Analyzing the oxidation of inoculated seeds, ANOVA detected significant difference only for factors antioxidants and matrix when isolated; however, no interaction was observed between factors.

In this case, the two highest oxidation levels occurred in the absence of the antioxidant agent and matrix 2 (Igarassu) in both media (Figure 1).

Figure 1. Oxidation level (■), emission of roots (■), and emission of leaves (■) of *Eugenia sulcata* germinated *in vitro* according to the origin matrix of seeds (1 - Camaragibe or 2 - Igarassu), composition of salts of the culture medium (WPM or MS) and the presence (CP) or absence (CA) of activated charcoal in the culture medium.



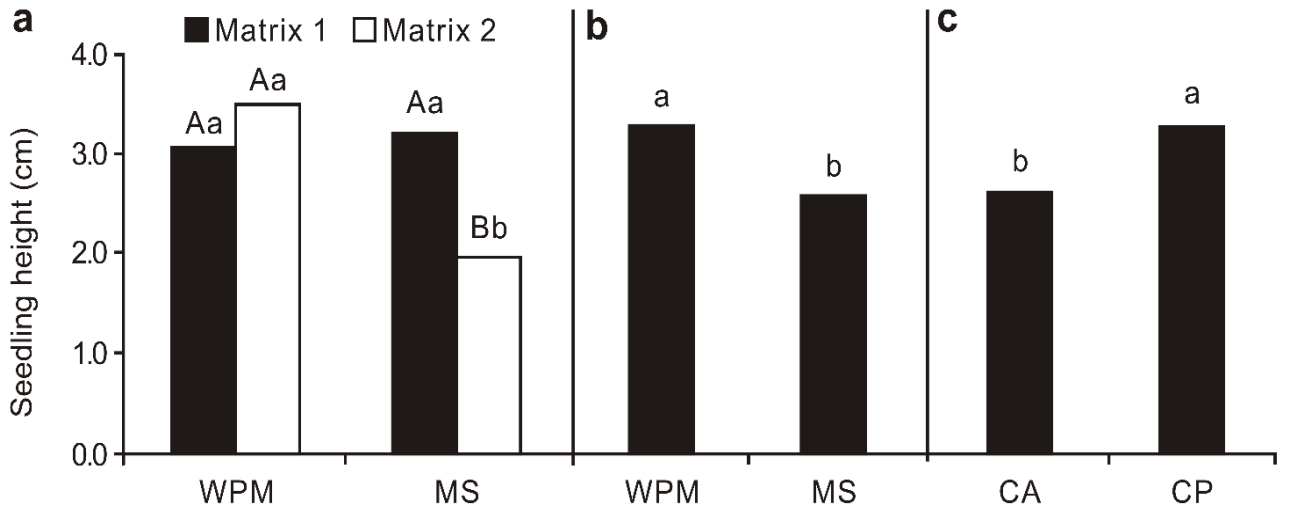
It was also observed that of the total inoculated plants, 26.3% underwent oxidation. Of these, 8.8% were grown in medium with the presence of activated charcoal and 17.5% without charcoal. Regarding the type of medium, WPM promoted 15.0% of oxidations, while the MS medium accounted for 11.1%. Of the 40 inoculated plants of matrix 1 (Camaragibe), 7.5% underwent oxidation, while in matrix 2, oxidation percentage was 45%. Of the 21 oxidized plants, 14.3% were from matrix 1, while 85% were from matrix 2.

Comparatively, it was observed that three treatments with MS media provided better development of the root system of *E. sulcata* seedlings (Figure 1). Similarly, regarding parameter oxidation, it was observed that both emission of roots and leaves were better in treatments with activated charcoal. In matrix 1, rooting was superior, since larger rooting was observed in three treatments, while in matrix 2, larger rooting was obtained only in two treatments.

Greater leaf emission was observed in three treatments where plants were cultivated in MS medium (MSCA-1, MSCP-1, and MSCP-2), whereas only one treatment with WPM medium (WPMCP-1) had higher mean values for this variable. This result demonstrates greater influence on leaf emission in seedlings cultured in MS medium compared to WPM. Similar behavior was observed regarding the matrix factor, since, of the four largest averages, three were observed in seedlings obtained from seeds of matrix 1 (Camaragibe) (Figure1).

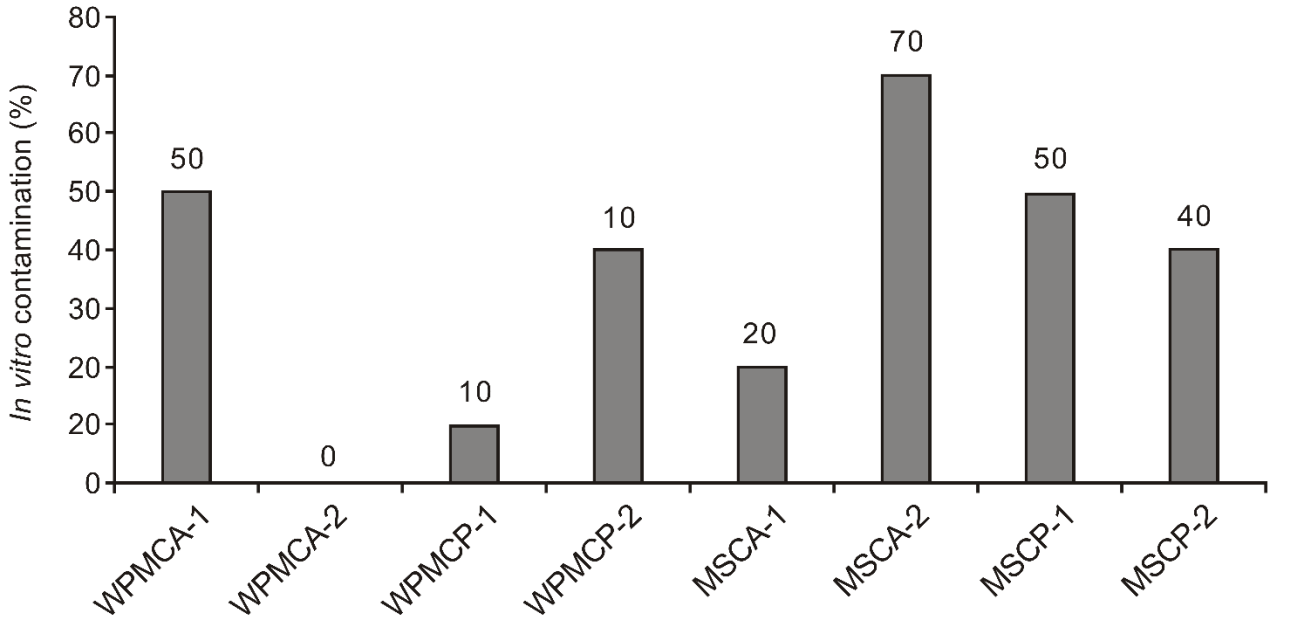
Regarding plant height, it was observed that parameters culture medium components, presence of antioxidant and matrix did not interfere in the results obtained (Figure 2). The results obtained demonstrate that the height of seedlings obtained from the seeds of matrix 2 differed statistically between culture media used (Figure 2 a). This behavior demonstrates that the influence of the culture medium composition may vary according to the seed source matrix. It was also observed that there was better development in the height of seedlings cultivated in WPM medium and with presence of activated charcoal (Figure 2b and c).

Figure 2. Height of *Eugenia sulcata* seedlings cultivated *in vitro* according to the original matrix of seeds (A), composition of salts in the culture medium (B) and absence (CA) or presence (CP) of activated charcoal in the culture medium (C).



Regarding the incidence of *in vitro* contamination, none of the evaluated parameters (culture medium composition, presence or absence of antioxidant and seed source matrix) significantly interfered with the occurrence of contaminating microorganisms. However, when comparing the *in vitro* contamination values, difference between treatments was observed. T2 treatment (WPMCA-2) was highlighted because it did not present contamination (Figure 3). T6 treatment (MSCA-2) presented the highest contamination percentage (70%). Statistically, no significant difference was observed between treatments with the lowest contamination rates (T3 with 10% and T5 with 20%) in relation to T2 treatment.

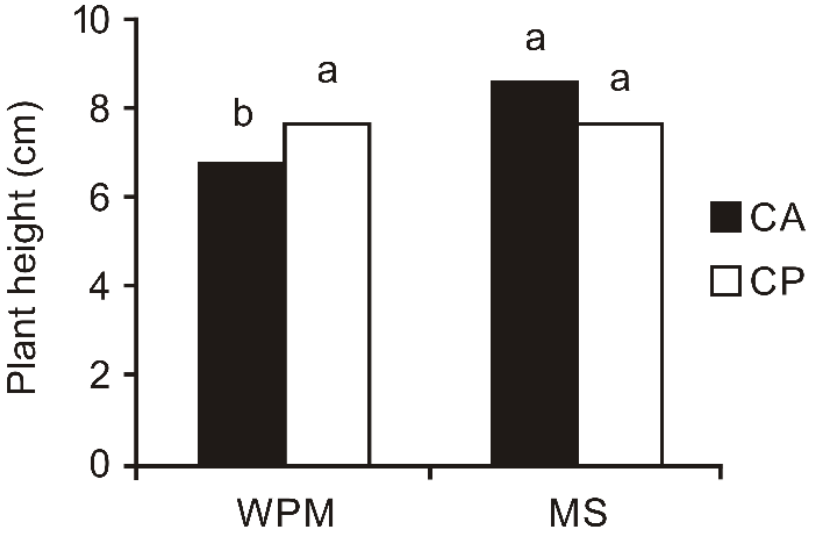
Figure 3. Contamination percentage of *Eugenia sulcata* observed *in vitro* in the different culture media.



In the acclimatization phase of plants, no significant difference was observed for variables stem diameter, plant height and number of leaves in any of factors and their associations by ANOVA at 5% probability. However, for variable canopy cover, ANOVA detected significant difference only for culture medium in isolation, in which the highest mean was reached by plants previously cultured *in vitro* in MS medium (8.7 ± 1.39 cm) compared to plants obtained in WPM culture medium (7.7 ± 1.78 cm).

When factors were implanted, significant differences were observed between the average heights of plants, where plants grown *in vitro* in MS medium in the absence of activated charcoal (Figure 4) obtained higher averages.

Figure 4. Growth in height of *Eugenia sulcata* plants after acclimatization, from *in vitro* culture in different salt compositions of WPM and MS culture media in the absence or presence of active charcoal.



DISCUSSION

The results observed for the oxidation of seeds during *in vitro* culture show that both the presence of activated charcoal antioxidant in the culture medium and the seed source are relevant factors that can influence the number of viable seeds to be *in vitro* germinated. In explants of *Sideritis* spp. (species of medicinal plant endemic to Turkey), it has also been observed that the addition of antioxidants in the culture medium plays an essential role in the control of phenolic compounds and consequently in oxidation, aiding *in vitro* development (Çördük and Aki, 2011).

In *in vitro* raspberry cultures, it was also possible to observe oxidation reduction by the addition of activated charcoal to the culture medium, reducing the absorption of phenolic compounds (Fagundes et al., 2018).

In microseps of grapia cultivated in medium containing 1.5 $g.L^{-1}$ of activated charcoal, no oxidation was observed (Lencina et al., 2018). When the rooting of Vernonia condensata buds is examined *in vitro*, and when 1% of activated charcoal is added, significant improvement is observed, avoiding the need to add auxin to the culture medium (Vicente et al., 2009).

The most used culture media are MS and WPM, where the latter is more suitable for growing woody plants. Based on results obtained, the culture media used (MS and WPM) did not influence the germination of *E. sulcata*. For the *in vitro* culture of plants, the culture medium composition (macro and micronutrients) is considered one of the main factors for the success of the *in vitro* establishment. The development of plants from the establishment of the culture medium is essential for the supply of nutrients that will support the in vitro development through the absorption of macro and micronutrients by explants (Ramage and Williams 2002).

The fact that the composition of the WPM medium is less concentrated can be seen as an economic advantage in the micropropagation of plants. Despite this characteristic, the WPM medium proved to be efficient for the cultivation of several species as *Myrciaria dubia* (Araujo et al., 2016), *Pyrus* spp (Bell et al., 2012), *Ficus carica* (Ferreira and Pasqual, 2008) and *Cordia trichotoma* (Vellozo) Arrabida ex Steudel (Mantovani et al., 2001). Although lower concentration is in some cases more recommendable for the *in vitro* cultivation of woody species, the use of different WPM concentrations would not necessarily contribute to the better development of explants. Doses above or below the original composition of WPM salts did not favor a better *in vitro* fig development ("Vermelha de Valinhos" variety) (Ferreira and Pasqual, 2008).

The occurrence of oxidation during *in vitro* culture is considered one of the most serious aspects related to the tissue culture of several plant species. The release of phenolic compounds, such as phenols, flavonoids and tannins in the culture medium and their accumulation in explants, is the main cause of oxidation, causing explant growth inhibition, reducing the germination rate and impairing the development of the root system. Apical of plants (Çördük and Aki, 2011; Preece and Compton, 1991).

Based on the results obtained in this work, regarding the oxidation and development of the root system, the WPM medium plus activated charcoal would be more suitable for the establishment and germination of *E. sulcata* seeds. The oxidation intensity varied significantly between seeds from the two donor plants used in this study. This result indicates that the propensity to produce phenolic compounds can be determined by specific genetic and physiological characteristics of different donor plants. The influence of the explant origin matrix on the *in vitro* development characteristics is similarly described for *Cedrela montana* Moritz ex Turcz (Basto et al., 2012) and *Azadirachta indica* A. Juss. (Houllou et al., 2015).

Regarding plant development, more specifically plant height after germination, one of the most important factors to consider is the choice of the matrix. According to results obtained, the growth response of plants after *in vitro* germination may be influenced or not by the culture medium composition. This difference in response may also occur according to specific genetic differences, passed on to the seed by parent plants. Thus, robustness in response to *in vitro* germination and subsequent plant development may present significant statistical differences depending on the seed matrix used. Similar result has also been reported with differences in the *in vitro* development of explants from different donor plants (Houllou et al., 2015). The authors reported statistical difference for three analyzed parameters (leaf atrophy, root system development, and leaf senescence) during the *in vitro* cultivation of *Azadirachta indica* a. juss.

Although the WPM medium presented nutrient composition with lower nitrogen concentration compared to the MS medium, it was observed that the overall plant height average was higher in the WPM medium (Stachevski et al., 2013), corroborating literature data, which indicate the WPM medium as more suitable for the development of tree species.

However, this response may vary when material rich in genetic diversity is set to develop under the same conditions. As for parameters root system oxidation and development, plant height was higher in medium added of activated charcoal. This result indicates that the reduction of phenolic compounds, responsible for oxidation, has positive gain in the germination and development of *E. sulcata*.

Although the incidence of contamination in tamarind was reduced with the presence of activated charcoal (Ferreira et al., 2018), this inhibitory effect was not observed in *Eugenia sulcata* spreng. Possibly other factors, such as seed germination stage (numbness, root system emission, root, and apical system emission) have interfered with the incidence of *in vitro* contamination. In general, the presence of contaminants in tree species is responsible for reducing seed viability, causing reduction in germination rates (Ahmadi et al., 2012). The occurrence of *in vitro* contamination in seeds is a problem also reported in other species, for example, *Zayena montana* (Sousa et al., 1999) with approximately 80% seed contamination. Thus, the adoption of more efficient decontamination procedures, such as the use of NaClO associated to Benomyl (Ahmadi et al., 2012) can be alternatives to reduce contamination and improve the establishment of plants from seeds in future experiments.

The lowest mean height observed only in plants obtained from the *in vitro* culture of *E. sulcata* without the presence of the antioxidant agent, reached by plants coming from cultivation in MS medium, may have occurred by the action of the antioxidant in the *in vitro* phase. In this sense, activated charcoal can act to promote better *in vitro* plant rooting (Simões et al., 2014), which is primordial for the development of seedlings in the acclimatization phase (Toledo and Biasi, 2018).

Successful *in vitro* germination would allow the inclusion of this pioneer species in the seedling production platform.

The platform allows the production of various species of forest essences simultaneously. This approach would allow tree seedlings to be provided regardless of phenological cycle, which restricts seed availability at specific times of the year.

The results obtained in this work indicate that the matrix plant is one of the main factors to be considered for the success of the *in vitro* establishment of *Eugenia sulcata* Spreng ex Mart. Although the genetic background of donor plants possibly influenced the parameters analyzed, the indication of the use of WPM salts associated with activated charcoal can be a basic procedure for the *in vitro* establishment of seeds of this species.

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