Original Article

# ANTIOXIDANTS IN THE CONTROL OF MICROORGANISM CONTAMINATION AND PHENOL OXIDATION IN *Eugenia pyriformis*

## *ANTIOXIDANTES NO CONTROLE DA CONTAMINAÇÃO MICROBIANA E DA OXIDAÇÃO FENÓLICA EM* Eugenia pyriformis

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**ABSTRACT:** Uvaia (*Eugenia pyriformis)* is a fruit tree of the Myrtaceae family. It has recalcitrant seeds of limited longevity, making seed propagation difficult. Micropropagation is an alternative method to obtain a large quantity of progeny plants in a short period of time, by using any part of the plant as explant. The high concentration of phenols associated with the chemical composition of the Myrtaceae, and the presence of microorganisms in the plant material or culture media, can make *in vitro* propagation difficult and/or impossible. The objective was to evaluate various concentrations of antioxidants affecting the control of microbial contamination and phenol oxidation *in vitro* in uvaia. A completely randomized design was used, with a 3 (antioxidants PVP, L-cysteine, and ascorbic acid) × 3 (antioxidant concentrations 100, 200, and 300 mg $L^{-1}$) × 2 (activated charcoal at 0 and 2 g $L^{-1}$) factorial arrangement + 2 additional variables (absence of antioxidants and activated charcoal; absence of antioxidants with 2 g $L^{-1}$ activated charcoal), with three repetitions comprising four plants each. The percentage of bacterial and fungal contaminations, along with the number of oxidized explants, was evaluated after 7, 14 and 21 days of *in vitro* cultivation. It was concluded that, where bacterial and fungal contaminations were concerned, *in vitro* cultivation of uvaia can be performed without the use of antioxidants. PVP or ascorbic acid must, however be used in the process, at a concentration of 300 mg $L^{-1}$, along with 2 g $L^{-1}$ of activated charcoal. This helps to minimize phenol oxidation.

**KEYWORDS:** Ascorbic acid. Activated charcoal. L-cysteine. Polyvinylpyrrolidone, Uvaia.

## INTRODUCTION

*Eugenia pyriformis* (Cambess.), known commonly as "uvaia", is a perennial fruit tree of the Myrtaceae family. It is prized for its fleshy and velvety fruits, the pulp of which is rich in antioxidants and vitamin C, thus contributing to its inclusion in diets (SILVA et al., 2014). Seed propagation is, however, difficult owing to the short natural longevity of the seeds, together with the recalcitrant behavioral characteristics of the species (SCALON et al., 2012), which instigated the research on other propagation methods.

Micropropagation is a viable alternative, since it allows for a large quantity of healthy progeny plants to be obtained in a short period of time and because any part of the plant can be used as explant (NASCIMENTO et al., 2008; GARCÍA-GONZALES et al., 2010).

Micropropagation protocols are already defined for a great diversity of plant species, from fruit species (RODRIGUES et al., 2013) to medicinal plants (MORAIS et al., 2012), which ensures the sustainable exploitation of species and allows increasing the supply of compounds biologically active, decreasing the price of phytochemical active ingredients derived from medicinal plants (PLETSCH, 1998). Through this technique it is also possible to produce secondary metabolites *in vitro*, thus ensuring alternative forms of exploitation, especially in endangered ecosystems.

The *in vitro* establishment phase is considered the most difficult, since the success of subsequent procedures, such as multiplication and acclimatization, depends on the morpho-physiological and phytosanitary state of the explants maintained in the growth media (NASCIMENTO et al., 2008; PINHAL et al., 2011).

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A serious problem associated with micropropagation is contamination by microorganisms (bacteria and fungal). These agents establish themselves in the growth media and/or plant material and may lead to the death of the explants by lowering the pH of the media, by the production of toxic metabolites or via competition for nutrients (SOUSA et al., 2007; LEONE et al., 2016).

During the *in vitro* establishment phase there is also a need to control oxidation caused by phenolic exudation at the sites where the explants were cut, which are responsible for darkening the explants via the activity of peroxidase enzymes (LAUKKANEN et al., 2000) and polyphenol oxidase (WILLADINO et al., 2013).

Uvaia, like other Myrtaceae, has a high concentration of phenolic acids in its chemical composition (HAMINIUK et al., 2014). Phenols and other exudates in *in vitro* cultivation systems can compromise the micropropagation process, since they inhibit the initial growth of the explants, which may lead to its death (BASSAN et al., 2006). The concentration of such compounds is related to the type of explant used, with nodal segments usually presenting lower concentrations than do apical segments (GOLLE et al., 2012), which justifies the addition of antioxidant substances to the culture media.

Research is currently being developed regarding the effects of antioxidants PVP, L-cysteine, ascorbic acid and activated charcoal on guava (*Psidium guajava* Linnaeus) (AGUILAR et al., 2016), lupin (*Lupinus mutabilis* Sweet) (MAMANI et al., 2014), 'alecrim-de-tabuleiro' (*Lippia gracilis* Schauer) (MARINHO et al., 2011), 'jacarandá da Bahia' *Dalbergia nigra* (Vell.) Allemao ex Benth. (SARTOR et al., 2013) respectively, in addition to other species, with the aim of controlling oxidative processes during micropropagation. These substances are effective in the antioxidant action and their cost is relatively smaller when compared to other substances, of equal action, available in the biotechnology market. In this way, the aim of the present research was to explore, besides the antioxidant potential, the antimicrobial action, because there is no reports in the literature on the action of these substances as antimicrobial/antioxidant agents in *E. pyriformis*, which highlights the relevance of the present study for the micropropagation of this fruit tree.

The aim of this study was, therefore, to evaluate the types and concentrations of antioxidants that control microbial contamination and phenol oxidation *in vitro* in *E. pyriformis.*

## MATERIAL AND METHODS

Nodal segments of *E. pyriformis* removed from adult plants were used as explants. The plants were previously sprayed with the antibiotic penicillin (1 g $L^{-1}$), three times per week, for a period of four weeks. In the second week, the systemic fungicide Cerconil WP® (thiophanate-methyl and chlorothalonil) (Iharabras S.A. Chemical Industries, Sorocaba, São Paulo, Brazil) was added to the pulverizations at a concentration of 2 g of commercial product per liter of water (adapted from RAMOS et al., 2013).

After the pulverizations, the young nodal segments were collected and taken to the Plant Tissue Culture Laboratory, Department of Agriculture, Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil. Where they were washed with a neutral detergent and rinsed for 5 minutes in running water. The explants were then disinfected using a 70% alcohol solution with Tween 20 (one drop per 100 mL) for 90 seconds and immersed in a solution of sodium hypochlorite (1% active chlorine) for a period of 20 minutes. The explants were subsequently subjected to a triple wash in autoclaved distilled water inside a laminar flow cabinet.

Test tubes (20 × 150 mm) containing 15 mL of Murashige and Skoog (MS) culture medium, comprised of salts and vitamins (MURASHIGE and SKOOG, 1962), and with the addition of 30 g $L^{-1}$ of sucrose and 5.5 g $L^{-1}$ of agar, were used in this study. The pH was adjusted to 5.8 and the medium autoclaved at 121°C and 1.2 atm for 20 minutes.

Methodology described by Golle et al. (2013) was used for inoculation *in vitro*, with the removal of existing leaf remains in each explant, each composed of two buds. In addition, the basal cut was performed at a slant, to increase contact surface area with the nutrient medium. All test tubes were kept in the dark in a plant growing room at a temperature of 25 ± 2 °C for the duration of the study so as to minimize the effects of oxidation.

Evaluations were performed on days 7, 14 and 21 following *in vitro* cultivation, measuring the percentage of bacterial (presence of bacterial colonies near the explants) and fungal contamination (contamination formed of fungal mycelia near the explants), and phenol oxidation (explant darkening).

A completely randomized design was used, in a 3 × 3 × 2 + 2 factorial trial, in which the factors were: antioxidants [(Polyvinylpyrrolidone (PVP), L-cysteine and ascorbic acid)], concentrations (100, 200 and 300 mg $L^{-1}$), activated charcoal (0 and 2 g

$L^{-1}$) and additional factors (absence of antioxidants and activated charcoal; absence of antioxidants with 2 g $L^{-1}$ activated charcoal), in a total of 20 treatments, with three repetitions of four explants, with one in each test tube.

The treatments were: T1: absence of antioxidant and activated charcoal; T2: absence of antioxidant and 2 g $L^{-1}$ activated charcoal; T3: 100 mg $L^{-1}$ PVP and 0 g $L^{-1}$ activated charcoal; T4: 100 mg $L^{-1}$ PVP and 2 g $L^{-1}$ activated charcoal; T5: 200 mg $L^{-1}$ PVP and 0 g $L^{-1}$ activated charcoal; T6: 200 mg $L^{-1}$ PVP and 2 g $L^{-1}$ activated charcoal; T7: 300 mg $L^{-1}$ PVP and 0 g $L^{-1}$ activated charcoal; T8: 300 mg $L^{-1}$ PVP and 2 g $L^{-1}$ activated charcoal; T9: 100 mg $L^{-1}$ L-cysteine and 0 g $L^{-1}$ activated charcoal; T10: 100 mg $L^{-1}$ L-cysteine and 2 g $L^{-1}$ activated charcoal; T11: 200 mg $L^{-1}$ L-cysteine and 0 g $L^{-1}$ activated charcoal; T12: 200 mg $L^{-1}$ L-cysteine and 2 g $L^{-1}$ activated charcoal; T13: 300 mg $L^{-1}$ L-cysteine and 0 g $L^{-1}$ activated charcoal; T14: 300 mg $L^{-1}$ L-cysteine and 2 g $L^{-1}$ activated charcoal; T15: 100 mg $L^{-1}$ ascorbic acid and 0 g $L^{-1}$ activated charcoal; T16: 100 mg $L^{-1}$ ascorbic acid and 2 g $L^{-1}$ activated charcoal; T17: 200 mg $L^{-1}$ ascorbic acid and 0 g $L^{-1}$ activated charcoal; T18: 200 mg $L^{-1}$ ascorbic acid and 2 g $L^{-1}$ activated charcoal; T19: 300 mg $L^{-1}$ ascorbic acid and 0 g $L^{-1}$ activated charcoal; T20: 300 mg $L^{-1}$ ascorbic acid and 2 g $L^{-1}$ activated charcoal.

Data normality was analyzed using the Shapiro-Wilk test at the 5% significance level. An analysis of variance was performed on the data using R® (R DEVELOPMENT CORE TEAM, 2014) statistics software, while the means were compared using Tukey's test with 5% probability.

## RESULTS AND DISCUSSION

All treatments were effective in controlling microbial activity (bacterial and fungal), with no contaminations seven days after inoculation. However, the following antioxidant treatments resulted in 50% or more of the uvaia explants being oxidized: PVP at all concentrations in the absence of activated charcoal (T3, T5 and T7); L-cysteine at the lowest and highest concentrations tested, in the absence (T9) and presence (T14) of activated charcoal, respectively; and ascorbic acid at the lowest concentration tested in the absence of activated charcoal (T15). No oxidation was observed in the T2, T4, T6 and T10 trials (Figure 1).

Fourteen days after inoculation, the explants exhibited similar behaviors to those observed at day 7 of bacterial and fungal contamination, except in T1 and T9 trials, where the absence of antioxidants (T1) or the use of 300 mg $L^{-1}$ ascorbic acid (T19), both in the absence of activated charcoal, induced the appearance of bacterial colonies, even though they occurred in only 8.3% of the explants (Figure 2).

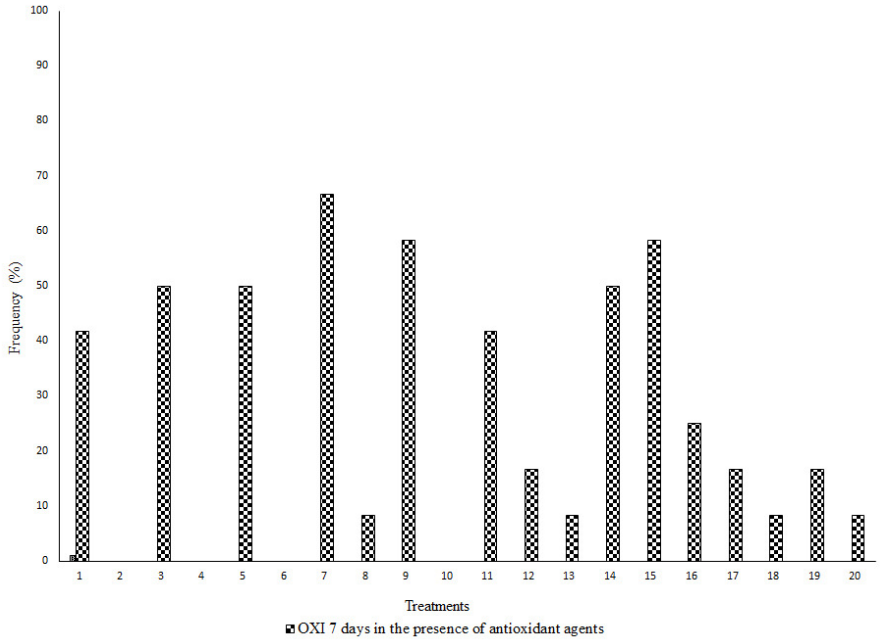


**Figure 1.** Frequency distribution (%) of oxidation (OXI) seven days after *in vitro* inoculation of *Eugenia pyriformis* with and without the use of antioxidant agents.

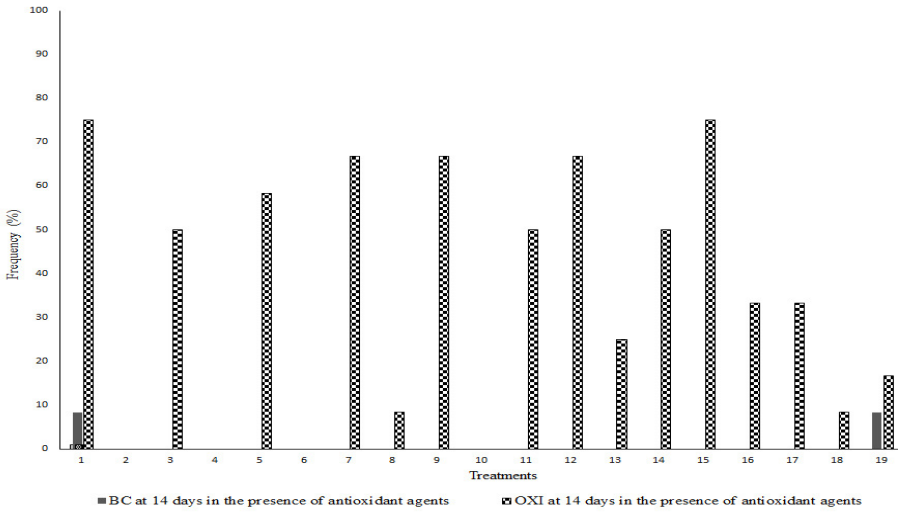


**Figure 2.** Frequency distribution (%) of bacterial contamination (BC) and oxidation (OXI) 14 days after *in vitro* inoculation of *Eugenia pyriformis* with and without antioxidant agents.

Where oxidation was concerned, treatments T1, T3, T5, T7, T9, T11, T12, T14 and T15 did not confer satisfactory protection of the plant material 14 days after *in vitro* inoculation. This was based on the occurrence of phenol oxidation and darkening in 50% or more of the explants. This occurred mainly when the antioxidants were not in the presence of activated charcoal (Figure 2).

After 21 days of inoculation (Figure 3), bacterial colonies developed in treatments T5 and T19 (Figure 4). Conversely, the appearance of fungal mycelia (Figure 5) was observed in T13. This was despite no more than 8.3% of explants being contaminated.

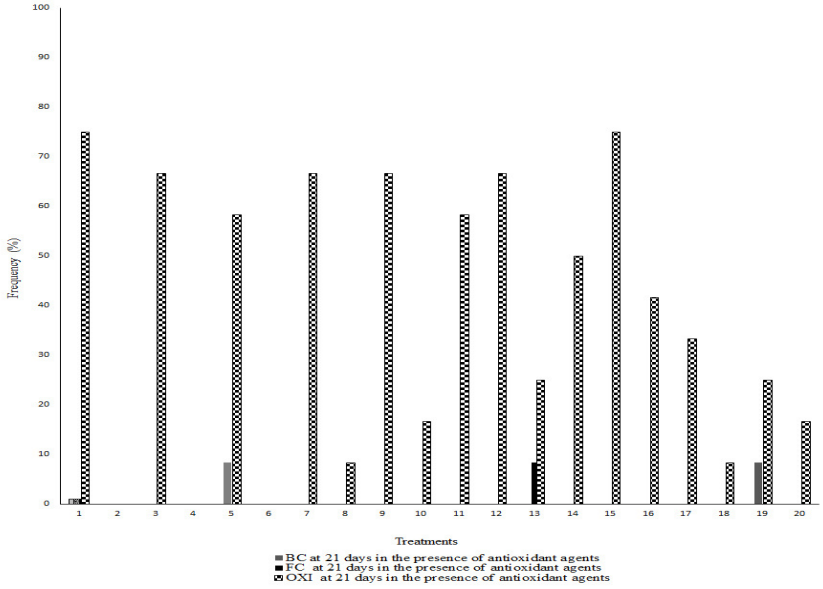


**Figure 3.** Frequency distribution (%) of bacterial (BC) and fungal (FC) contaminations, and oxidation (OXI) after 21 days of *in vitro* inoculation of *Eugenia pyriformis* with and without antioxidant agents.

**Figure 4.** Bacterial contaminations of *in vitro* cultivations of *Eugenia pyriformis.*

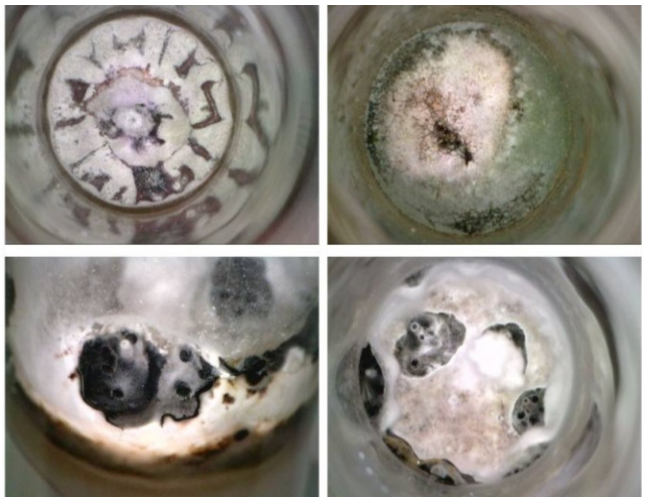

**Figure 5.** Fungal contaminations of *in vitro* cultivations of *Eugenia pyriformis.*

More than 50% of the explants in the treatments T1, T3, T5, T7, T9, T11, T12, T14 and T15 exhibited oxidation 21 days after *in vitro* inoculation (Figure 3).

The formation of bacterial colonies and/or fungal mycelia near the explants was negligible 7, 14 and 21 days after inoculation. This may have been due to the antimicrobial activity of the antioxidant agents tested (PVP, L-cysteine and activated charcoal) during micropropagation. Another explanation for the low frequency of bacterial contamination may be the result of the aseptic treatment of plants in the field. Both bactericides and fungicides were used prior to the inoculation, which may also have contributed to the protection of the plants following *in vitro* inoculation. This may have been due to the residual effects of those substances on the plants.

Even though the occurrence of microbial contamination was low in this study, it is important to control these microorganisms, as these contaminants can compete with the explants for the nutrients in the culture medium, releasing toxic metabolites that can lead to the death of the plant (PEREIRA et al., 2003). This creates obstacles for the tissue culture of plant species, especially ligneous plants (GOLLE et al., 2013).

In a similar study, Sousa et al. (2007) reported that supplementing culture medium with 0.1% activated charcoal (1 g $L^{-1}$) was effective in reducing bacterial and fungal contaminations by 60% (without activated charcoal) to 20% (with activated charcoal) during the micropropagation of the orchid, *Cattleya walkeriana*. In addition, the use of ascorbic acid (150 mg $L^{-1}$), when used in conjunction with citric acid (200 mg $L^{-1}$), helped to reduce bacterial contamination in guava buds, *Psidium guajava* L. (Myrtaceae) 72 hours after *in vitro* inoculation (CONCEPCIÓN et al., 2005).

Since oxidation was observed in all the time periods analyzed (Figure 6), the interaction between different types of antioxidants (PVP, L-cysteine and ascorbic acid), as well as their concentrations and activated charcoal, had a significant effect ($p$<0.05) on the reduction of phenolic exudation and darkening of the explants 7 days after inoculation.

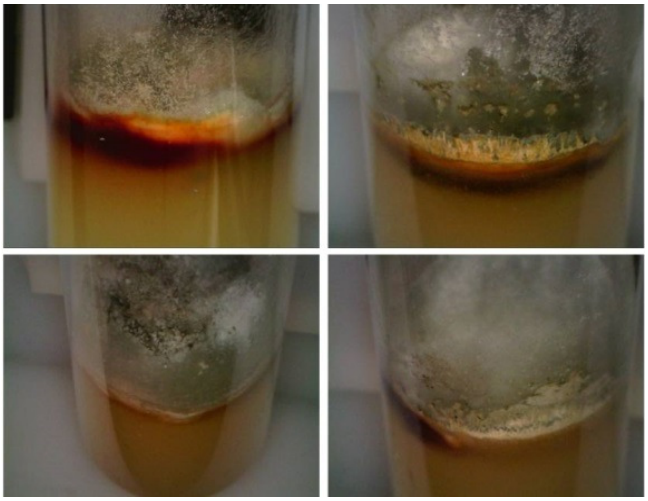

**Figure 6.** Oxidation in *Eugenia pyriformis in vitro* cultivation.

The lowest percentage of *E. pyriformis* oxidation resulted from the antioxidants L-cysteine or ascorbic acid used at a concentration of 300 mg $L^{-1}$ without the addition of activated charcoal, when compared to that of PVP. The use of PVP or ascorbic acid, when used at the same concentration, as well as 2 g $L^{-1}$ activated charcoal, also decreased the oxidative process in uvaia more than the use of L-cysteine did (Table 1).

**Table 1.** Percentage of oxidation of *Eugenia pyriformis* explants treated with different types and concentrations of antioxidants, with and without activated charcoal, 7 days after *in vitro* inoculation.

| Antioxidant | Concentration 100 mg $L^{-1}$ | | Concentration 200 mg $L^{-1}$ | | Concentration 300 mg $L^{-1}$ | |
|---|---|---|---|---|---|---|
| | Activated charcoal 0 g $L^{-1}$ | Activated charcoal 2 g $L^{-1}$ | Activated charcoal 0 g $L^{-1}$ | Activated charcoal 2 g $L^{-1}$ | Activated charcoal 0 g $L^{-1}$ | Activated charcoal 2 g $L^{-1}$ |
| PVP | 50.0 a | 0.0 a | 50.0 a | 0.0 a | 66.7 a | 8.3 b |
| L-cysteine | 58.3 a | 0.0 a | 41.7 a | 16.7 a | 8.3 b | 50.0 a |
| Ascorbic acid | 58.3 a | 25.0 a | 16.7 a | 8.3 a | 16.7 b | 8.3 b |

Means followed by the same letter in the column do not significantly differ between each other, according to Tukey's test ($p \leq 0.05$).

Phenol oxidation, which is associated with the dark coloring of the explant, is another impediment to be overcome during the establishment of ligneous plants *in vitro*. According to Golle et al. (2012), the presence of oxidation in the explants, though undesirable, do not appear to hinder the establishment and development of the cultures *in vitro*. This was not confirmed in the present study because the oxidation compromised *E. pyriformis* micropropagation in a way that did not allow us to evaluate the development of the plants *in vitro*. The use of antioxidant agents is therefore justified, especially for the cultivation of Myrtaceae. This is due to the elevated release of phenolic compound exudates in the culture medium (AGUILAR et al., 2016).

Similarly, the following the development of an *in vitro* protocol for the guava tree, Aguilar et al. (2016) reported that the best treatment with an antioxidant and disinfectant was obtained using 0.5% PVP, 5% chlorine and 3 drops of tween 20. This ensured the survival of 90% of the explants. Likewise, the addition of 15 mL $L^{-1}$ ascorbic acid to the MS culture medium was efficient in preventing oxidation in banana tree explants (*Musa* spp.). This helped to maintain the green color of the pseudostem, indicating that the quality of the plant material used as explant was preserved (ANICEZIO, 2012).

The interaction between antioxidants and their concentrations had a significant effect ($p<0.05$) on explant oxidation 14 days after *in vitro* inoculation. The use of 200 mg $L^{-1}$ ascorbic acid led to more than 50% reduction in the percentage of phenol oxidation over that observed when using L-cysteine (Table 2).

**Table 2.** Percentage of oxidation of *Eugenia pyriformis* explants subjected to different types and concentrations of antioxidants 14 days following *in vitro* inoculation.

| | Concentrations | | |
|---|---|---|---|
| Antioxidant | 100 mg $L^{-1}$ | 200 mg $L^{-1}$ | 300 mg $L^{-1}$ |
| PVP | 8.3 a | 29.2 ab | 37.5 a |
| L-cysteine | 16.7 a | 58.3 a | 37.5 a |
| Ascorbic acid | 37.5 a | 20.8 b | 16.7 a |

Means followed by the same letter in the column do not significantly differ between each other, according to Tukey's test ($p \leq 0.05$).

With regard to the same period of time, there was a significant interaction ($p<0.05$) between antioxidants and activated charcoal. The use of PVP, regardless of the concentration, when used in conjunction with 2 g $L^{-1}$ activated charcoal, led to a reduction in phenolic exudation that was 13.8 times less that that caused by the use of L-cysteine (Table 3).

**Table 3.** Percentage of oxidation of *Eugenia pyriformis* explants subjected to different levels of activated charcoal and types of antioxidant, 14 days following *in vitro* inoculation.

| | Activated charcoal | |
|---|---|---|
| Antioxidant | 0 g $L^{-1}$ | 2 g $L^{-1}$ |
| PVP | 47.2 a | 2.8 b |
| L-cysteine | 36.1 a | 38.9 a |
| Ascorbic acid | 30.5 a | 19.4 ab |

Means followed by the same letter in the column do not significantly differ between each other, according to Tukey's test ($p \leq 0.05$).

Similarly, Costa et al. (2007) also reported that PVP and activated charcoal were agents capable of reducing phenol oxidation. The addition of 0.5 g $L^{-1}$ PVP or 3.0 g $L^{-1}$ activated charcoal to the MS culture medium contributed to the lower percentage of oxidation of pepper-rosmarin (*Lippia sidoides* Cham.) *in vitro,* increasing survival rates of the explants, when compared to the controls (absence of antioxidants).

Twenty-one days after *in vitro* inoculation, there was a significant ($p<0.05$) interaction between antioxidants and activated charcoal. It was concluded that the use of PVP, regardless of the concentrations used, and when used in with the presence of 2 g $L^{-1}$ of activated charcoal, appeared to reduce the extent of oxidation *in vitro* more than L-cysteine did. As a result, there was 41.6% more oxidation in the explants when L-cysteine was used than in those in which PVP was used (Table 4).

**Table 4.** Percentage of oxidation of *Eugenia pyriformis* explants treated with different levels of activated charcoal and types of antioxidant 21 days after *in vitro* inoculation.

| | Activated charcoal | |
|---|---|---|
| Antioxidant | 0 g $L^{-1}$ | 2 g $L^{-1}$ |
| PVP | 52.8 a | 2.8 b |
| L-cysteine | 50.0 a | 44.4 a |
| Ascorbic acid | 33.3 a | 22.2 ab |

Means followed by the same letter in the column do not significantly differ between each other, according to Tukey's test ($p \leq 0.05$).

The use of PVP (10 g $L^{-1}$ in semi-solid medium or 5 g $L^{-1}$ in liquid medium) and activated charcoal (250 or 500 mg $L^{-1}$ in semi-solid culture medium) resulted in less explant oxidation of the forest species *Terminalia amazonia* (Gmel.) Excell. This was observed only in the upper and lower parts, with no color alteration of the culture medium, in addition to increased survival of the plant material established *in vitro* (MÉNDEZ-ÁLVAREZ and ABDELNOUR-ESQUIVEL, 2014).

The results of PVP use are related to the properties of this polymer, which has the ability to absorb organic compounds using hydrogen molecules, such as phenols, thereby reducing or avoiding oxidation (CONCEPCIÓN et al., 2005).

Even though the use of antioxidant agents is not necessary for the control of microbial contaminants, its use is useful for minimizing oxidative processes, making the cultivation of *E. pyriformis in vitro* viable.

## CONCLUSION

With regard to bacterial and fungal contaminations, the *in vitro* cultivation of *E. pyriformis* can be performed without the use of antioxidant agents. However, PVP or ascorbic acid, at a concentration of 300 mg $L^{-1}$, when used in conjunction with 2 g $L^{-1}$ activated charcoal, is recommended for minimizing the effects of phenol oxidation.

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**RESUMO:** A uvaia *Eugenia pyriformis* é uma frutífera da família das mirtáceas cujas sementes apresentam longevidade curta e aspecto recalcitrante, fato que dificulta a propagação seminífera. A micropropagação surge como alternativa para obtenção de grande quantidade de mudas em curto período de tempo, por meio da utilização de qualquer parte da planta como explante. A elevada concentração de fenóis associados à composição química das mirtáceas e a presença de microrganismos no material vegetal ou no meio de cultura podem dificultar e/ou impossibilitar a propagação *in vitro*. Objetivou-se avaliar tipos e concentrações de antioxidantes no controle da contaminação microbiana e da oxidação fenólica *in vitro* de *E. pyriformis.* Utilizou-se o delineamento inteiramente casualizado em esquema fatorial 3 (antioxidantes – PVP, L-cisteína e ácido ascórbico) x 3 (concentrações - 100, 200 e 300 mg $L^{-1}$) x 2 (carvão ativado – 0 e 2 g $L^{-1}$) + 2 adicionais (ausência de antioxidantes e de carvão ativado; ausência de antioxidantes com 2 g $L^{-1}$ de carvão ativado), com três repetições constituídas por quatro plantas. Após sete, 14 e 21 dias do cultivo *in vitro* foram avaliadas a porcentagem de contaminação bacteriana, fúngica e de explantes oxidados. Conclui-se que o cultivo *in vitro* de *E. pyriformis*, em relação as contaminações bacterianas e fúngicas, pode ser efetuado sem a utilização de agentes antioxidantes. Entretanto, para reduzir a oxidação fenólica deve ser utilizado o PVP ou ácido ascórbico, ambos na concentração de 300 mg $L^{-1}$, associados a 2 g $L^{-1}$ de carvão ativado.

**PALAVRAS-CHAVE:** Ácido ascórbico. Carvão ativado. L-cisteína. Polivinilpirrolidona. Uvaia.

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