Evaluation of different antioxidants during in vitro establishment of allspice (*Pimenta dioica* L. Merrill): a recalcitrant species

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Abstract

**Background:** *Pimenta dioica* L. Merrill is a tree whose fruits are used as a spice due to their culinary and therapeutic uses. Conventional propagation techniques using seeds and cuttings do not guarantee the phytosanitary quality of this crop. Therefore, the use of Plant Tissue Culture techniques are an option for in vitro establishment. The aim of this study was to evaluate the effect of different antioxidant agents (Methylene blue, L-cysteine and silver nanoparticles) added to MS (Murashige and Skoog) culture medium at different concentrations (0, 50, 100 and 200 mg L\(^{-1}\)) during axenic establishment of buds used as explants of *P. dioica*.

**Results:** The percentage of survival, oxidation and contamination was determined, as well as the content of soluble phenols, cell wall-linked phenols, antioxidant capacity and lipid peroxidation. Results showed significant differences among the different antioxidants for the evaluated variables; the highest survival occurred in the treatments with the addition of L-cysteine with percentages greater than 40 %, while the lowest survival occurred in the control treatment, 100 and 200 mg L\(^{-1}\) methylene blue, with 0, 3.3 and 0% survival, respectively. The highest percentage of oxidation was observed in the control treatments, 100 and 200 mg L\(^{-1}\) methylene blue with 96.67% oxidation, while the lowest percentages were observed in explants treated with L-cysteine, with 30% oxidation. Treatments with 100 and 200 mg L\(^{-1}\) AgNPs had the lowest contamination values, with 20%.

Biochemical determinations showed that L-cysteine and 50 and 100 mgL\(^{-1}\) AgNPs resulted in an increase in the content of soluble phenols. The highest contents of cell wall-linked phenols were obtained in treatments with 200 mg L\(^{-1}\) methylene blue, L-cysteine, and 200 mg L\(^{-1}\) AgNPs. Analysis of antioxidant capacity revealed that all treatments had a reaction of scavenging / reduction mechanisms free radicals. Regarding lipid peroxidation, the highest content of malondialdehydes was observed in the control treatment and 200 mgL\(^{-1}\) methylene blue.

**Conclusion:** the addition of L-cysteine to the culture medium showed a higher survival rate, decreased oxidation, greater production of phenolic compounds, increased antioxidant capacity and decreased lipid peroxidation, this amino acid being an alternative to reduce oxidation during *in vitro*.
introduction of allspice and other species that exhibit recalcitrance in vitro during establishment.

Background
Allspice (Pimenta dioica L. Merrill), also called Jamaica pepper, is native to Mexico and Central America and has been domesticated in several tropical countries of the world [1]. The cultivation of this species plays an important role in the agro-food and pharmaceutical industry because its fruits are used as a spice and in therapies due to their high level of eugenol [2, 3].

Allspice is traditionally propagated through seeds and cuttings; however, being a dioecious species, male trees do not produce seeds, while the propagation method by cuttings does not guarantee phytosanitary quality for planting. The use of biotechnological techniques through Plant Tissue Culture is an alternative for in vitro production of pest and disease free clonal seedlings [4]. However, the in vitro establishment stage is the main constraint on initiating commercial micropropagation of plants, especially when the explants come from woody plants [5]. Wounds made to the explants excrete a large amount of phenolic compounds, which on contact with the atmosphere tend to be oxidized, causing a darkening of the tissue [6]. Oxidation of explants in woody plants is one of the causes of recalcitrance during in vitro culture. This oxidation leads to the generation of Reactive Oxygen Species (ROS) that cause damage to the cellular structure [7, 8] resulting in plant tissue necrosis [9, 10]. Oxidative stress can be triggered by the production of ROS that promote changes in phenolic content, antioxidant capacity and lipid peroxidation, among other things [11, 12].

The use of compounds with antioxidant activity such as methylene blue, L-cysteine and silver nanoparticles is an alternative means of reducing oxidation and necrosis of explants during in vitro establishment. By decreasing oxidation, it is possible to increase the survival rates and response capacity of plant tissues in species recalcitrant to in vitro morphogenesis. The aim of this study was to evaluate different antioxidant agents to reduce oxidation, as well as the phenolic content, antioxidant capacity and lipid peroxidation of P. dioica explants during in vitro establishment.

Results
Treatments with antioxidant agents
The evaluation of different antioxidant agents showed significant differences for the variables survival, oxidation and contamination in vitro in P. dioica nodal explants (Table 1). The highest
survival was observed with the addition of L-cysteine, with percentages greater than 40%, while the lowest survival occurred in the control treatment, 100 and 200 mg L\(^{-1}\) methylene blue, with 0, 3.3 and 0% survival, respectively. The highest percentage of oxidation was observed in the control treatment, 100 and 200 mg L\(^{-1}\) methylene blue with 96.67% oxidation, while the lowest percentages of oxidation were observed in explants treated with L-cysteine, with 30% oxidation. Regarding the percentage of contamination, treatments with 100 and 200 mg L\(^{-1}\) AgNPs had the lowest contamination values, with only 20.00%, while the rest of the treatments had contamination values between 30.00 and 46.67%.

Figure 1 shows the appearance of the explants under the different treatments with antioxidant agents.

Content of soluble phenols and cell wall-linked phenols

\(P. \textit{dioica}\) buds cultured in medium with methylene blue showed no differences in soluble phenol content; however, treatments with L-cysteine and 50 and 100 mg L\(^{-1}\) AgNPs had a significant increase in soluble phenol content, with values above 3.843 mg GAE / g DW, while the rest of the treatments showed the lowest values (Figure 2a). Regarding cell wall-linked phenolic compounds, the highest contents were obtained in the treatments with 200 mg L\(^{-1}\) methylene blue, L-cysteine, and 200 mg L\(^{-1}\) AgNPs, obtaining concentrations higher than 7.185 mg GAE / g DW, while the control and the rest of the treatments did not show significant statistical differences (Figure 2b).

Antioxidant capacity

The evaluation of antioxidant capacity showed that all antioxidant treatments had a reaction of DPPH free radical capture, with values greater than 990 TE / g DW, differing significantly from the control treatment (Figure 2c).

Lipid peroxidation

Lipid peroxidation showed significant differences in malondialdehyde (MDA) content among treatments with antioxidant agents. The highest MDA content was observed in the control treatment and 200 mg L\(^{-1}\) methylene blue, with values higher than 1.72x10\(^{-4}\) nmol MDA / g fresh weight, while the lowest content was obtained in the treatments with the addition of L-cysteine and AgNPs, with values lower than 9.07x10\(^{-5}\) nmol MDA / g fresh weight (Figure 2d).
Discussion
Treatments with antioxidant agents
Antioxidants are compounds that prevent the oxidation of molecules. Oxidation is any chemical reaction that involves transferring electrons from a substance to an oxidizing agent. In this study, the use of antioxidant agents had an effect on the survival, oxidation, and contamination in vitro of *P. dioica* nodal explants. Adding methylene blue to the culture medium had a negative effect on survival in the explants; the decrease in survival was probably caused by the high percentages of oxidation observed. No effect on the percentage of contamination was observed because methylene blue has no microbicidal action. Waranusantigui et al. [13] state that methylene blue reduces light penetration and thus prevents oxidation, while Bruchey and Gonzalez-Lima [14] point out that, at low doses, it decreases superoxide radicals produced in oxidative phosphorylation.

Regarding L-cysteine, it increased survival and decreased the percentage of oxidation, without having an effect on contamination. The reduction of oxidation caused an increase in the survival rate. L-cysteine is an amino acid that has been used to reduce the oxidation of phenolic compounds. In this regard, Richard-Forget et al. [15] report that the thiol group of L-cysteine traps free radicals and quinones by inhibiting the enzyme polyphenol oxidase. The use of this amino acid to reduce *in vitro* oxidation has been reported by Hussain et al. [16] in black pepper (*Piper Nigrum*), Oberschelp and Gonçalves [17] in eucalyptus (*Eucalyptus dunnii* Maiden) and Akhtar and Shahzad [18] in sandalwood (*Santalum album* L.).

AgNPs did not show a drastic effect on survival rate or oxidation; however, they caused a reduction in contamination rates. The use of AgNPs to reduce *in vitro* contamination has been reported by Cancino-Escalante et al. [19] in blackberry (*Rubus glaucus* Benth), Spinoso-Castillo et al. [20] in vanilla (*Vanilla planifolia* Jacks. ex Andrews) and by por Vitali et al. [21] in black poplar (*Populus nigra* L.). AgNPs have been reported to bind to bacteria producing cell cycle arrest [22], caused by toxicity due to ROS production [23]. In addition, Kim et al. [24] demonstrated that AgNPs have an antimicrobial effect bacterial cell membrane surface and modifying the cell potential. Picazo-Vela and Hernández [25] report that the adsorption of AgNPs in the extracellular wall of the bacteria is the
main mechanism of toxicity, having an effect against organisms such as *Escherichia coli*, *Bacillus subtilis* and *Salmonella tifus* [26]. In fungi, AgNPs break the cell membrane of hyphae altering the mechanisms of infection [27]. Lee et al. [22] report that AgNPs that penetrate the cell increase Ag+ cations, which could affect the electrical potential of the membrane, denaturing proteins, leading to cell cycle arrest. On the other hand, Rónavári et al. [28] report growth inhibition of fungi such as *Candida*, *Criptococcus*, *Microsporum* and *Trichophyton*.

Because *P. dioca* is a woody species, it has a high susceptibility to oxidation during *in vitro* establishment; this effect is caused by mechanical damage to the explants, resulting in the presence of phenolic compounds that oxidize rapidly on contact with the *in vitro* environment [29, 30]. Oxidation problems have been reported in other woody species such as cedar of Lebanon (*Cedrus libani*) [31], black pepper (*Piper nigrum* L.) [32], guava (*Psidium guajava* L.) [33], and pistachio (*Pistacia vera*) [34].

**Soluble phenols and cell wall-linked phenols**

The production of phenolic compounds is an indicator of defense against the mechanical damage involved in the healing process. The addition of methylene blue showed no effect on the content of soluble phenols; however, changes were observed in the content of cell wall-linked phenols at the highest concentration evaluated, probably because some dyes have a toxic effect on plant tissues [35], due to the formation of chelates that produce toxicity [36]. On the other hand, when L-cysteine was added, an effect on the content of phenolic compounds was observed. Pinedo and Reyna [37] propose that the increase in phenolic compound content caused by L-cysteine is due to its ability to remove quinones and participation in membrane stability by neutralizing oxidizing agents [38]. The use of L-cysteine as an *in vitro* antioxidant agent has been reported by Ricco et al. [39] in mistletoe and the release of exudates. Regarding the use of AgNPs, they did not decrease *Ligaria cuneifolia*, obtaining greater production of phenolic compounds, preventing their oxidation the production of soluble phenols probably due to the reaction of Ag+ with the thiol, carboxylate, phosphate, hydroxyl, amine, imidazole and indol groups of some enzymes, producing their inactivation and cell death [40]. However, the addition of AgNPs increased the content of cell wall-linked phenols at the highest
evaluated concentration, due to possible toxic damage caused by an excess in the Ag+ ion in the culture medium. Toxicity at high concentrations of AgNPs has been reported by Bello-Bello et al. [41, 42] in sugar cane (Saccharum spp.) and vanilla (Vanilla planifolia Jacks. Ex Andrews), respectively, and by Hussain et al. [43] in tangerine (Citrus reticulata).

**Antioxidant capacity**

Antioxidant capacity is a defense mechanism against oxidative stress. All treatments with antioxidant agents showed antioxidant capacity. Callaway et al. [44] report that methylene blue at low concentrations can function as a free radical scavenger. However, at high concentrations it can lead to oxidative stress at the cellular level. Bilodeau et al. [45] state that L-cysteine acts as an intracellular precursor of glutathione biosynthesis, whose function is to protect cells from oxidation, combating damage from free radicals. Haase et al. [46] point out that exposure to AgNPs induces the synthesis of antioxidant enzymes, such as catalase and superoxide dismutase. Pace et al. [47] report that the addition of L-cysteine in lettuce (Lactuca sativa) plants increases antioxidant capacity. Regarding the effect of AgNPs, Chung et al. [48] report their antioxidant capacity in cucumber (Cucumis anguria) roots.

**Lipid peroxidation**

An increase in the oxidative degradation of lipids is the result of the capture of electrons that make up the fatty acids present in the cell membrane. The increase in MDA content in the control treatment was due to oxidative stress caused by the lack of antioxidants as reducing agents to prevent and protect oxidative damage. In this regard, Mostofa et al. [49] point out that malondialdehydes produced because of some type of stress can act as molecules that inhibit development. The addition of methylene blue at low concentrations resulted in a decrease in MDA production because it acts as an electron cycler [14], having a redox reaction avoiding the oxidative stress of tissues; however, at the highest concentration evaluated it produced greater stress in the P. dioica explants, resulting in higher MDA production. The effect on lipid peroxidation using methylene blue has not yet been reported; however, this fact was probably due to toxic damage caused by the high concentration of methylene blue.
The addition of L-cysteine resulted in a decrease in MDA content, probably because this amino acid is an intracellular precursor of glutathione biosynthesis [45], which reduces lipid hydroperoxides to fatty acids by binding to coenzyme A, the acyl group transporter [50]. Ali et al. [51] report decreased MDA production in lychee (*Litchi chinensis*) fruits with the addition of L-cysteine. Regarding the addition of AgNPs, like L-cysteine, they reduced lipid peroxidation. In our study, the decrease in MDA content was probably due to the low effect of silver ions [52, 53] on the electrostatic interaction inside and outside the cell [54]. Spinoso-Castillo et al. [20] report the use of 50 mgL$^{-1}$ of AgNPs in *V. planifolia* for MDA depletion.

**Conclusion**

The present study found that the addition of L-cysteine in the *in vitro* culture medium of *P. dioica* increases survival and reduces oxidation of explants without showing an effect on contamination, while AgNPs had an effect on *in vitro* contamination. Methylene blue was not efficient during *in vitro* establishment. However, it was shown that different antioxidants have an effect on the accumulation of phenolic compounds, antioxidant capacity and lipid peroxidation, with L-cysteine being an alternative to reduce oxidation of *P. dioica* and other species with *in vitro* recalcitrance caused by oxidation during establishment.

**Methods**

**Plant material and *in vitro* establishment**

Cuttings of female plants of *P. dioica* ecotype Totonacapan from the Totonacapan region, Veracruz, Mexico, was provided by La Joya del Totonacapan, S. de P. R. de R. L., were collected and grown for two months in controlled greenhouse conditions under irradiation with natural light of 130 μmol m$^{-2}$ s$^{-1}$, 30 ± 2 °C and 60 ± 5% RH. The leaves were removed from the new shoots and 2 cm high nodal segments were taken as explants. The explants were washed with running water and Tween® 20 soap (Sigma-Aldrich®, St. Louis, MO) in continuous circulation for 30 minutes. Subsequently, they were rinsed with drinking water. The explants were transferred to a laminar flow hood where they were immersed in 70% (v/v) ethanol for 1 minute and rinsed three times with sterile distilled water; they were then immersed in sodium hypochlorite (6% active ingredient Clorox®, NL, MX) at 0.6 and 0.9%
(v/v) for 15 and 10 minutes, respectively, adding 2 drops of Tween® 20 (Sigma-Aldrich®, St. Louis, MO) per 100 mL of solution. The explants were rinsed three times with sterile distilled water. Finally, the internodes were cultured in different treatments with different antioxidant agents.

**Treatments with antioxidant agents**

The antioxidant response of methylene blue (Merck, Darmstadt, Germany), L-cysteine (Sigma-Aldrich®, St. Louis, MO) and Silver nanoparticles (Argovit™) at concentrations of 0, 50, 100 and 200 mg L⁻¹ was evaluated. The antioxidant agents were added to MS [55] culture medium, supplemented with 3% sucrose (w / v) (Fermont® Chemical Company, NL, MX) and 2.5 g L⁻¹ phytage (Sigma-Aldrich®, St. Louis, MO) as gelling agent. The pH was adjusted to 5.8 ± 0.1, with 0.1 N HCl (Fermont®, Chemical Company, NL, MX) and 0.1 N NaOH (Fermont®, Chemical Company, NL, MX), sterilized in an autoclave at 120 °C and 115 kPa for 20 min. Finally, 10 explants per treatment were seeded individually in test tubes (15 x 2 cm) containing 10 mL of culture medium. After 30 days of culture, the percentage of survival, oxidation and contamination was evaluated. In addition, phenol content, antioxidant capacity and lipid peroxidation were evaluated. Explants were incubated at 25 ± 1 °C with an irradiance of 45 μmolm⁻² s⁻¹ provided by 60 W fluorescent lamps (Osram®, Munich, Germany) with a photoperiod of 16 h light.

**Determination of soluble phenols and cell wall-linked phenols**

**Soluble phenols.** Phenolic content was determined according to Payet et al. [56]. First, 18 mg dry weight of plant tissue was used in the different treatments with antioxidant agents. Extraction was performed with methanol: water (50:50) and 180 μL of the supernatant were taken by adding 100 μL of 10% Folin-Ciocalteu reagent (E. Merck, Darmstadt, Germany); it was homogenized in a vortex (Corning® LSE™, U.S. A.), 30 μL of 20% Calcium Carbonate (Sigma-Aldrich®) were added and then it was incubated for 2 hours at 26 °C. Finally, the absorbance was measured at 765 nm using distilled water as a blank. Phenolic content was calculated from a gallic acid calibration curve (0–10000 μg / mL) and expressed as milligrams of gallic acid equivalents (GAE) per g of dry weight (g DW) of *P. dioica* nodal explants.
**Cell wall-linked phenols:** The determination was carried out according to Payet et al. [56]. First, 250 µL of 1M NaOH (Fermont®, Chemical Company, NL, MX) were added to the pellet obtained from the TPC determination, homogenized in a vortex (Corning® LSE™, U.S. A.) and then incubated in a water bath at 70 °C for 16 hours. Subsequently, 250 µL of 2 M HCl (Fermont®, Chemical Company, NL, MX) were added and 100 µL of extract, 900 µL of distilled water and 100 µL of 10% Folin-Ciocalteu reagent (E. Merck, Darmstadt, Germany) were taken; after 5 min, 600 µL of 1 M NaOH (Fermont®, Chemical Company, NL, MX) were added, saturated with Calcium carbonate (Sigma-Aldrich®) and incubated at 26 °C for 1 hour. Absorbance and calibration conditions were the same as those used in the determination of soluble phenol content.

**Determination of antioxidant capacity**
The determination of DPPH (2, 2-Diphenyl-1-picrylhydrazyl) was performed by the methodology proposed by Huang et al. [57]. An aliquot of 3900 µL of DPPH and 100 µL of methanolic extract obtained in the TPC determination was taken. A calibration curve with Trolox (Sigma-Aldrich®, St. Louis MO) was used at different concentrations. The mixture was incubated at 30 °C for 30 minutes and the absorbance was measured at 515 nm. Data were expressed as Trolox equivalents (TE) per g of dry weight (g DW) of *P. dioica* nodal explants.

**Determination of lipid peroxidation**
The analysis of lipid peroxidation was performed according to the methodology proposed by Heath and Packer [58]. First, 50 mg of fresh plant material were used by adding 1 mL of 0.1% trichloroacetic acid (TCA) (Sigma-Aldrich®, St. Louis MO) and homogenizing it in a vortex (Corning® LSE™, USA), after which it was centrifuged at 10,000 xg for 15 minutes. Next, 500 µL of the supernatant was taken and 1 mL of 20% TCA plus 1 mL of 0.5% thiobarbituric acid (TBA) (Sigma-Aldrich®, St. Louis MO) were added, leading to homogenization in a vortex mixer (Corning® LSE™, USA). The supernatant was incubated in a water bath at 95 °C for 30 minutes followed by an ice bath. MDA content was calculated by the absorbance difference at 532 and 600 nm using an extinction coefficient of 155 mM⁻¹ cm⁻¹.
To evaluate lipid peroxidation, 200 mg of freezedried vanilla shoots were homogenized in 4 ml of 0.1% Trichloroacetic Acid (TCA). Then the extract was centrifuged at 10,000×g for 15 min and the supernatant (1 ml) was collected and mixed with 2 ml of 20% TCA and 2 ml of 0.5% Thiobarbituric acid (TBA). The mixture was heated at 95 °C for 30 min in a fume hood and later cooled on ice. The absorbance of supernatant was read at 532 and 600 nm.

**Experimental design and statistical analysis**

A completely randomized experimental design was used. All trials were performed in triplicate. Data were processed with the Statistical Package for the Social Sciences (SPSS) version 22 software for Windows and statistical analysis was carried out using an analysis of variance (ANOVA) and a comparison of means using Tukey’s test (p ≤ 0.05). Values expressed in percentages were transformed by the Arcsine function before performing the analysis.

**Abbreviations**

AgNPs: Silver nanoparticles; DPPH: 2, 2-Diphenyl–1-picrylhydrazyl, MDA: Malondialdehyde; MS: Murashige and Skoog; *P. dioica*: *Pimenta dioica*.

**Declaration**

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**Authors’ contributions**

SVH and JJBB designed the experiments, analysis the data, data interpretation and drafting the manuscript, SVH conducted all the experimental work, CACC, MSS and JJBB contributed to the conceptualization of the experiment, and revising the manuscript.

All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Table 1 Effect of different antioxidant agents on the survival, oxidation and contamination in vitro of P. dioica (L.) Merrill nodal explants

| Treatment    | Concentration (mg L⁻¹) | Survival (%) | Oxidation (%) |
|--------------|------------------------|--------------|---------------|
| Control      | 0                      | 0.00 ± 0.00⁰⁷  | 96.67 ± 3.33⁸  |
| Methylene blue| 50                     | 13.33 ± 3.33⁰⁷ | 66.67 ± 3.33⁰⁷ |
|              | 100                    | 3.33 ± 3.33⁰⁷  | 96.67 ± 3.33⁰⁷ |
|              | 200                    | 0.00 ± 0.00⁰⁷  | 96.67 ± 3.33⁰⁷ |
| L-Cysteine   | 50                     | 43.33 ± 3.33⁰⁷ | 30.00 ± 0.00⁰⁷ |
|              | 100                    | 43.33 ± 3.33⁰⁷ | 30.00 ± 5.77⁰⁷ |
|              | 200                    | 40.00 ± 5.77⁰⁷ | 30.00 ± 5.77⁰⁷ |
| AgNPs        | 50                     | 13.33 ± 3.33⁰⁷ | 66.67 ± 3.33⁰⁷ |
|              | 100                    | 33.33 ± 3.33⁰⁷ | 83.33 ± 3.33⁰⁷ |
|              | 200                    | 33.33 ± 3.33⁰⁷ | 83.33 ± 3.33⁰⁷ |

Means ± standard error within a column followed by the same letter are not significantly different according to Tukey’s L.S.D. mean separation test at 30 days of in vitro culture. AgNPs: Silver nanoparticles.

Figures
Effect of different antioxidant agents on the in vitro response of allspice (Pimenta dioica L. Merrill) explants. a) No antioxidants; b, c and d) 50, 100 and 200 mg L-1 methylene blue; e, f, g) 50, 100 and 200 mg L-1 L-cysteine; h, i, j) 50, 100 and 200 mg L-1 AgNPs. Bar= 1 cm.
Figure 2

Effect of different antioxidant agents on a) soluble phenols, b) cell wall-linked phenols, c) antioxidant capacity and d) lipid peroxidation in P. dioica after 30 days of in vitro culture. Phenolic content, expressed in milligrams of gallic acid equivalents per gram of dry weight (mg GAE / g DW). Antioxidant capacity, quantified by oxygen radical absorbance capacity expressed as trolox equivalents per gram of dry weight (TE / g DW). Lipid peroxidation quantified by malondialdehyde (MDA) assay, expressed as nanomol per gram of dry weight (nmol / g FW). Different letters denote statistically significant differences according to Tukey’s test (p ≤ 0.05).