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

Sugey Vásquez-Hernández
Universidad Veracruzana Facultad de Ciencias Quimicas
Carlos Alberto Cruz-Cruz
Universidad Veracruzana Facultad de Ciencias Quimicas
Maricela Santiago-Santiago
La Joya del Totonacapan
Jericó Jabín Bello-Bello (jericobello@gmail.com)
Colegio de Postgraduados Campus Cordoba https://orcid.org/0000-0002-2617-3079

Research article

Keywords: methylene blue, L-cysteine, silver nanoparticles, polyphenols, antioxidant capacity, lipid perioxidation

Posted Date: April 7th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-21190/v1

Version of Record: A version of this preprint was published at Agro Productividad on November 26th, 2021. See the published version at https://doi.org/10.32854/agrop.v14i11.2167.

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. dioica buds cultured in medium with methylene blue showed no differences in soluble phenol content; however, treatments with L-cysteine and 50 and 100 mgL^{-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* Maideny) 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 (*Robus 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 call 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} phytagel (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-Ciocalteual 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, theabsorbance 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 ($p \leq 0.05$). 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.*

Declarations

Acknowledgements

The authors wish to thank the Universidad Veracruzana and the Colegio de Postgraduados Campus Córdoba, as well as CONACyT Mexico.

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.

Funding

This work was funded by La Joya del Totonacapan, S. de P.R. de R.L., with the support of the Stimulus Program for Research, Technological Development and Innovation [PEI-2018, Project No. 251642], PROINNOVA (Innovation-oriented network projects) modality through the project OPTIMIZATION OF THE LARGE-SCALE MICROPROPAGATION PROCESS FOR *Pimienta dioica* (L.) MERRIL PLANTS IN BIORREACTOR SYSTEMS. The funders had no role in the analysis and interpretation of data, or in the writing of the report.

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.

Author details

[1] Facultad de Ciencias Químicas, Universidad Veracruzana, Prolongación de Avenida Oriente 6, 1009, Rafael Alvarado, 94340, Orizaba, Veracruz, Mexico, [2] La Joya del Totonacapan, calle sin nombre s/n, localidad El Progreso, 93680, Atzalan, Veracruz, Mexico, [3] CONACYT-Colegio de Postgraduados Campus Córdoba, Km 348 de la carretera Federal Córdoba- Veracruz, Congregación Manuel León, Amatlán de los Reyes, Veracruz, Mexico.

*For correspondence. Email jericobello@gmail.com

References

1. da Rocha Voris DG, dos Santos Dias L, Lima JA, Lima KDSC, Lima JBP, dos Santos Lima AL. Evaluation of larvicidal, adulticidal, and anticholinesterase activities of essential oils of Illicium verum Hook. f., Pimenta dioica (L.) Merr. and Myristica fragrans Houtt. against Zika virus vectors. Environ Sci Pollut Res. 2018; 25: 22541–22551.
2. Marques CS, Carvalho SG, Bertoli LD, Villanova JCO, Pinheiro PF, dos Santos DCM and Bernardes, P. C. β-Cyclodextrin inclusion complexes with essential oils: Obtention, characterization, antimicrobial activity and potential application for food preservative sachets. Food Res Int. 2019; 119: 499–509.
3. Lorenzo-Leal AC, Palou E, López-Malo A, Bach H. Antimicrobial, Cytotoxic, and Anti-Inflammatory Activities of Pimenta dioica and Rosmarinus officinalis Essential Oils. Biomed Res Int. 2019; 8.
4. Cardoso JC, Gerald LTS, da Silva JAT. Micropropagation in the Twenty-First Century. Plant Cell Culture Protocols. Methods in Molecular Biology. Loyola-Vargas V., Ochoa-Alejo N. (ed) Humana Press, New York, NY. 2018; 17–46.
5. Ramírez M, León de Sierralta S, Urdaneta A. Evaluación de desinfectantes superficiales en el establecimiento in vitro de Psidium guajava L. y Psidium friedrichsthalianum Nierdz. Rev Fac Agron Luz. 1999; 16: 243–255.
6. Sarropoulou V, Dimassi-Theriou K, Therios I. Effect of the ethylene inhibitors silver nitrate, silver sulfate, and cobalt chloride on micropropagation and biochemical parameters in the cherry rootstocks CAB–6P and Gisela 6. Turk J Biol. 2016; 40: 670–683.
7. Mittler R. ROS are good. Trends Plant Sci. 2017; 22: 11–19.

8. Singla P, Bhardwaj RD, Kaur S, Kaur J. Antioxidant potential of barley genotypes inoculated with five different pathotypes of Puccinia striiformis f. sp. hordei. Physiol Mol Biol Plants. 2019; 25: 145–157.
9. Azofeifa, Á. Problemas de oxidación y oscurecimiento de explantes cultivados in vitro. Agron Mesoam. 2009; 20: 1.
10. Sharry SE, Adema M, Abedini W. Problemas en el cultivo de tejidos vegetales. Plantas de probeta. Universidad Nacional de La Plata (eds). 2015; 7: 102–112.
11. Xu H, Sun X, Wang X, Shi Q, Yang X, Yang F. Involvement of a cucumber MAPK gene (CsNMAPK) in positive regulation of ROS scavengence and osmotic adjustment under salt stress. Sci Hortic. 2011; 127: 488–493.
12. Wei Z, Shi X, Wei F, Fan Z, Mei L, Tian B, Shi G. The cotton endocycle-involved protein SPO11-3 functions in salt stress via integrating leaf stomatal response, ROS scavenging and root growth. Physiol Plan. 2018; 167: 127–141.
13. Waranusantigui P, Pokethitiyook P, Kruatrachue M, Upatham ES. Kinetic of basic dye (methylene blue) biosorption by giant duckweed (Spirodela polyrrhiza). Environ Pollut. 2003; 125: 385–392.
14. Bruchey AK, Gonzalez-Lima F Behavioral, physiological and biochemical hormetic responses to the autoxidizable dye methylene blue. Am J Pharmacol Toxicol. 2008; 3: 72–79.
15. Richard-Forget FC, Goupy P, Nicolas J. Cysteine as an inhibitor of enzymatic browning. J Agric Food Chem. 1992; 40: 2108–2113.
16. Hussain A, Naz S, Nazir H, Shinwari ZK. Tissue culture of black pepper (Piper nigrum L.) in Pakistan. Pak J Bot. 2011; 43: 1069–1078.
17. Oberschelp GPJ, Gonçalves AN. Assessing the effects of basal media on the in vitro propagation and nutritional status of Eucalyptus dunnii Maiden. In Vitro Cell Dev Biol-Plant. 2016; 52: 28–37.
18. Akhtar R, Shahzad A. Morphology and ontogeny of directly differentiating shoot buds and somatic embryos in Santalum album L. J For Res. 2019; 30: 1179–1189.
19. Cancino-Escalante GO, Quevedo García E, Villamizar CE, Díaz-Carvajal C. Propagation in vitro of selected materials of Robus glaucus Benth (mora de castilla) in the province of Pamplona, northeastern Colombia. Rev colomb Biotecnol. 2015; 17: 7–15.
20. Spinoso-Castillo JL, Chavez-Santoscoy RA, Bogdanchikova N, Pérez-Sato JA, Morales-Ramos V, Bello-Bello JJ. Antimicrobial and hormetic effects of silver nanoparticles on in vitro regeneration of vanilla (Vanilla planifolia Jacks. ex Andrews) using a temporary immersion system. Plant Cell Tiss Organ Cult. 2017; 129: 195–207.
21. Vitali F, Raio A, Sebastiani F, Cherubini P, Cavalieri D, Cocozza C. Environmental pollution effects on plant microbiota: the case study of poplar bacterial-fungal response to silver nanoparticles. Appl Microbiol Biotechnol. 2019; 103: 8215–8227.
22. Lee B, Lee MJ, Yun SJ, Kim K, Choi IH, Park S Silver nanoparticles induce reactive oxygen species-mediated cell cycle delay and synergistic cytotoxicity with 3-bromopyruvate in Candida albicans, but not in Saccharomyces cerevisiae. Int J Nanomedicina. 2019; 14: 4801–4816.

23. Park HJ, Kim JY, Lee JH, Hahn JS, Gu MB Yoon J. Silver-ion-mediated reactive oxygen species generation affecting bactericidal activity. Water Res. 2009; 43: 1027- 1032.
24. Kim KJ, Sung WS, Suh BK, Moon SK, Choi JS, Kim JG, Lee DG. Antifungal activity and mode of action of silver nano-particles on Candida albicans. BioMetals. 2009; 22: 235- 242.
25. Picazo-Vela S, Hernández LR. A New Era without Antibiotics. Technology, Science and Culture-A Global Vision. Intech Open. 2019; 1–22.
26. Sarsar V, Selwal KK, Selwal MK. Nanosilver: potent antimicrobial agent and its biosynthesis. Afr J Biotechnol. 2014; 13: 546–554.
27. Bocate KP, Reisa GF, de Souza PC, Oliveira Junior AG, Durán N, Nakazatoa G, Furlanetoa MC, de Almeida RC, Panagio LA. Antifungal activity of silver nanoparticles and simvastatin against toxigenic species of Aspergillus. Int J Food Microbiol. 2019; 291: 79–86.
28. Rónavári A, Igaz N, Gopisetty MK, Szerencsés B, Kovács D, Papp C, Pfeiffer I Biosynthesized silver and gold nanoparticles are potent antimycotics against opportunistic pathogenic yeasts and dermatophytes. Int J nanomedicine 2018; 13: 695- 703.
29. Abdelwahd R, Hakam N, Labhilili M, Udupa SM. Use of an adsorbent and antioxidants to reduce the effects of leached phenolics in in vitro plantlet regeneration of faba bean. Afr J Biotechnol. 2008; 7 (8): 997–1002.
30. Nieto Vargas VE, Valdiviezo Menéndez ML. Establecimiento de un protocolo de regeneración in vitro y aclimatación para Fuchsia pilaloensis y Fuchsia hybrida para su conservación. Bachelor's. 2013.
31. Khuri S, Shmoury MR, Baalbaki R, Maunder M, Talhouk SN Conservation of the Cedrus libani populations in Lebanon: history, current status and experimental application of somatic embryogenesis. Biodivers Conserv. 2000; 9: 1261–1273.
32. Ahmad N, Abbasi BH, Fazal H Khan MA, Afridi MS. Effect of reverse photoperiod on in vitro regeneration and piperine production in Piper nigrum L. C R Biologies. 2014; 337 (1): 19–28.
33. Perales Aguilar L, Silos Espino H, Montero V, Lorenzo L, Perales Segovia C, Flores Benítez S. Propagación in vitro de guayaba (Psidium guajava L.) a partir de segmento nodales. Rev Mexicana cienc Agricola. 2016; 7: 375–386.
34. Nezami-Alanagh E, Garoosi GA, Landín M, Gallego PP. Computer-based tools provide new insight into the key factors that cause physiological disorders of pistachio rootstocks cultured in vitro. Sci Rep. 2019; 9: 9740.
35. Wang S, Boyjoo Y, Choueib A. A comparative study of dye removal using fly ash treated by different methods. Chemosph. 2005; 60: 1401–1407.
36. Castellar G, Angulo E, Zambrano A, Charris D. Equilibrio de adsorción del colorante azul de metileno sobre carbón activado. Rev UDCA Actualidad & Divulgación Científica. 2018; 16: 263–271.
37. Pinedo S, Reyna M. Efecto de la suplementación de L-cisteína en el dilutor Tes-Tris-Yema, sobre la calidad espermática post-descongelamiento en alpaca (Vicugna pacos). 2014.

38. Bedascarrasbure E, Maldonado L, Álvarez A, Rodríguez E. Contenido de fenoles y flavonoides del propoleos argentino. Acta Farm Bonaerense. 2004; 23, 369–372.
39. Ricco MV, Bari ML, Ricco RA, Wagner ML, Álvarez MA. Análisis de polifenoles e iniciación de cultivos in vitro de Ligaria cuneifolia (R. et P.) Tiegh (Loranthaceae) de ejemplares provenientes de la localidad La Población, provincia de Córdoba, Argentina. Dominguezia. 2018; 34: 53–64.
40. Ashraf S, Abbasi AZ, Pfeiffer C, Hussain SZ, Khalid ZM, Gil PR, Parak, WJ, Hussain I. Protein-Mediated Synthesis, pH-Induced Reversible Agglomeration, Toxicity and Cellular Interaction of Silver Nanoparticles. Colloids Surf B. 2013; 102: 511–51.
41. Bello-Bello JJ, Chavez-Santoscoy RA, Lecona-Guzman CA, Bogdanchikova N, Salinas-Ruíz J, Gómez-Merino FC, Pestryakov A. Hormetic response by silver nanoparticles on in vitro multiplication of sugarcane (Saccharum spp. Cv. Mex 69–290) using a temporary immersion system. Dose-Response. 2017; 15: 1559325817744945.
42. Bello-Bello JJ, Spinoso-Castillo J, Arano-Avalos S, Martínez-Estrada E, Arellano-García M, Pestryakov A, Bogdanchikova N. Cytotoxic, genotoxic, and polymorphism effects on Vanilla planifolia jacks ex andrews after long-term exposure to argovit® silver nanoparticles. Nanomaterials. 2018; (8): 754.
43. Hussain M, Raja NI, Naz F, Iqbal M, Aslam S. Green synthesis and characterisation of silver nanoparticles and their effects on antimicrobial efficacy and biochemical profiling in Citrus reticulata. IET Nanobiotechnology. 2018; 12: 514–519.
44. Callaway NL, Riha PD, Bruchey AK, Munshi Z, Gonzalez-Lima F. Methylene blue improves brain oxidative metabolism and memory retention in rats. Pharmacol Biochem Behav. 2004; 77: 175–181.
45. Bilodeau JF, Blanchette S, Gagnon C, Sirard M A. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology. 2001; 56: 275–286.
46. Haase A, Rott S, Mantion A, Graf P, Plendl J, Thünemann AF, Reiser G. Effects of silver nanoparticles on primary mixed neural cell cultures: uptake, oxidative stress and acute calcium responses. Toxicol Sci. 2012; 126: 457–468.
47. Pace B, Capotorto I, Ventura M, Cefola M. Evaluation of L-cysteine as anti-browning agent in fresh-cut lettuce processing. J Food Process Preserv. 2015; 39: 985–993.
48. Chung IM, Rajakumar G, Thiruvengadam M. Effect of silver nanoparticles on phenolic compounds production and biological activities in hairy root cultures of Cucumis anguria. Acta Biol Hung. 2018; 69: 97–109.
49. Mostofa MG, Hossain MA, Fujita M. Trehalose pretreatment induces salt tolerance in rice (Oryza sativa L.) seedlings: oxidative damage and co-induction of antioxidant defense and glyoxalase systems. Protoplasma. 2015; 252: 461–475.
50. Ursini F, Bindoli A. The role of selenium peroxidases in the protection against oxidative damage of membranes. Chemistry and physics of lipids. 1987; 44: 255–276.
51. Ali S, Khan AS, Malik AU. Postharvest L-cysteine application delayed pericarp browning, suppressed lipid peroxidation and maintained antioxidative activities of litchi fruit. Postharvest Biol Technol. 2016; 121: 135–142.

52. Gómez GL. Nanopartículas de plata: tecnología para su obtención, caracterización y actividad biológica. Investigación en Discapacidad. 2013; 2: 18–22.
53. Nuñez-Anita RE, Acosta-Torres LS, Vilar-Pineda J, Martínez-Espinosa JC, de la Fuente-Hernández J, Castaño VM. Toxicology of antimicrobial nanoparticles for prosthetic devices. Int J nanomedicine. 2014; 9: 3999.
54. Singh RP, Shukla VK, Yadav RS, Sharma PK, Singh PK, Pandey AC. Biological approach of zinc oxide nanoparticles formation and its characterization. Advanced materials Letters. 2011; 2:4 313–317.
55. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plan. 1962; 15: 473–497.
56. Payet B, Shum Cheong Sing A, Smadja J. Comparison of the concentrations of phenolic constituents in cane sugar manufacturing products with their antioxidant activities. J Agric Food Chem. 2006; 54: 7270–7276.
57. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J Agric Food Chem. 2002; 50: 4437- 4444.
58. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 1968; 125: 189–198.

Table

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 (%)	Contamination (%)
Control	0	0.00 ± 0.00^{c}	96.67 ± 3.33^{a}	46.67 ± 3.33^{a}
Methylene blue	50	13.33 ± 3.33^{b}	66.67 ± 3.33^{b}	43.33 ± 3.33^{a}
	100	3.33 ± 3.33^{c}	96.67 ± 3.33^{a}	46.67 ± 3.33^{a}
	200	0.00 ± 0.00^{c}	96.67 ± 3.33^{a}	43.33 ± 3.33^{a}
L-Cysteine	50	43.33 ± 3.33^{a}	30.00 ± 0.00^{c}	43.33 ± 3.33^{a}
	100	43.33 ± 3.33^{a}	30.00 ± 5.77^{c}	46.67 ± 3.33^{a}
	200	40.00 ± 5.77^{a}	30.00 ± 5.77^{c}	43.33 ± 3.33^{a}
AgNPs	50	13.33 ± 3.33^{b}	66.67 ± 3.33^{b}	30.00 ± 0.00^{ab}
	100	33.33 ± 3.33^{ab}	83.33 ± 3.33^{ab}	20.00 ± 5.77^{b}
	200	33.33 ± 3.33^{ab}	83.33 ± 3.33^{ab}	20.00 ± 5.77^{b}

Means ± standard error within a column followed by the same letter are not significantly different according to Tukey's test at ($p \leq 0.05$), at 30 days of *in vitro* culture. AgNPs: Silver nanoparticles.

Figures

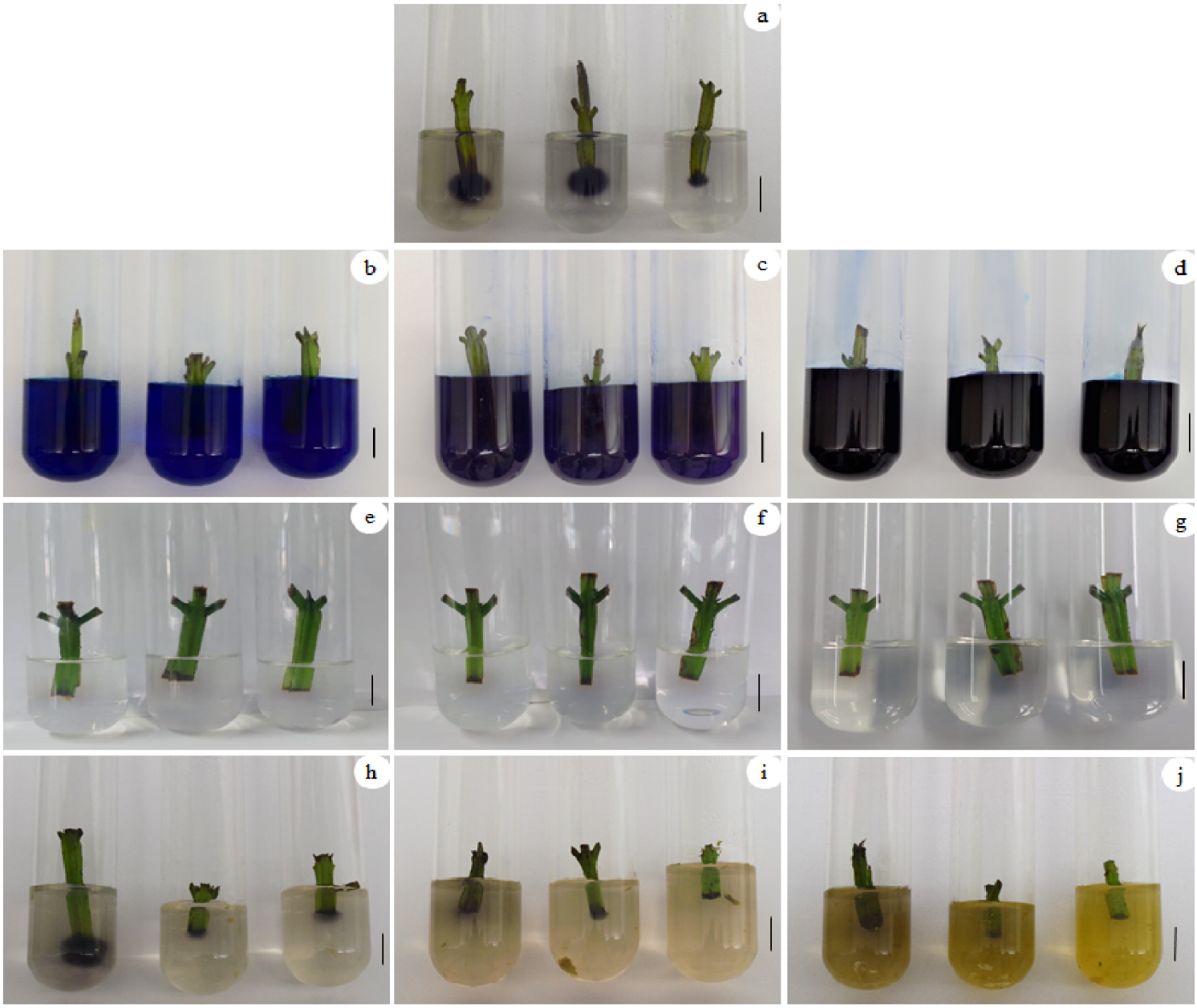

Figure 1

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.

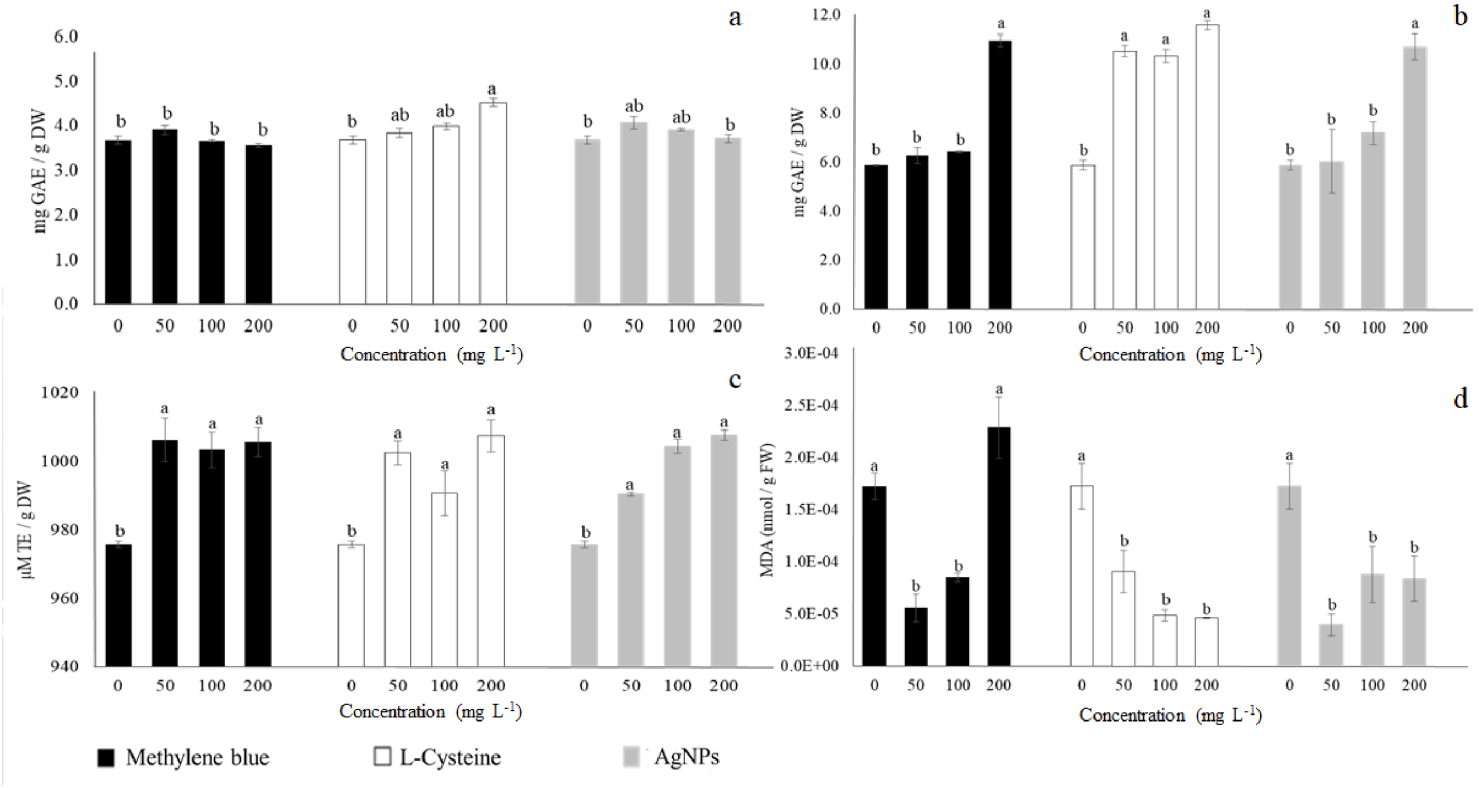

Figure 2

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 ($p \leq 0.05$).