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Root system and antioxidant mechanisms of *Eugenia uniflora* plants promote greater tolerance to aluminum stress

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ABSTRACT: Aluminum (Al) toxicity is an issue in many cultivated areas in the world, affecting the productivity of the most diverse crops. Therefore, it is necessary to assess whether excess Al has negative effects on the growth of tree species such as *Eugenia uniflora*, which has great ecological, economic, food and medicinal potential. Thus, this study aimed to evaluate Al tolerance of *E. uniflora* seedlings, based on the effects of Al on physiological, biochemical and morphological variables. We used a completely randomized experimental design, consisting of five Al concentrations (0, 15, 30, 45 and 60 µM) and four replications. At the end of the period of exposure to the treatments, morphological (shoot and root dry weight and root morphology), physiological (chlorophyll a fluorescence) and biochemical (photosynthetic pigment concentration, hydrogen peroxide content (H_2O_2), guaiacol peroxidase (POD) and superoxide dismutase (SOD) activity)) variables were evaluated. The absence of significant differences in shoot and, root dry weight of *E. uniflora* seedlings, regardless of Al concentrations, suggest that *E. uniflora* is aluminum tolerant. The maintenance of the content of photosynthetic pigments and the values of Fv/Fm, as well as the low H_2O_2 content in roots also confirm the tolerance of *E. uniflora* to aluminum.

Key words: excess aluminum; oxidative stress; photosynthetic pigments; pitangueira; toxic metals

Sistema radicular e mecanismos antioxidantes de plantas de *Eugenia uniflora* promovem maior tolerância ao estresse por alumínio

RESUMO: A toxicidade do alumínio (Al) é um problema que ocorre em muitas áreas cultivadas no mundo, afetando a produtividade das mais diversas culturas. Assim, torna-se necessário verificar se o excesso de Al causa efeitos negativos no crescimento de espécies arbóreas, como a *Eugenia uniflora*, que apresenta grande potencial ecológico, econômico, alimentício e medicinal. Desta forma, objetivou-se verificar a tolerância ao Al em mudas de *E. uniflora*, verificando os efeitos do Al nas variáveis fisiológicas, bioquímicas e morfológicas. Adotou-se o delineamento experimental inteiramente casualizado, composto por cinco concentrações de Al: 0, 15, 30, 45 e 60 µM, e quatro repetições. Ao final do período de exposição aos tratamentos avaliou-se as variáveis morfológicas (massa seca da parte aérea e de raízes e morfologia de raízes), fisiológicas (fluorescência da clorofila *a*) e bioquímicas (concentração de pigmentos fotossintéticos, conteúdo de peróxido de hidrogênio (H_2O_2), e atividade das enzimas guaiacol peroxidase (POD) e superóxido dismutase (SOD)) nas plantas. A ausência de diferenças significativas no peso seco da parte aérea e da raiz de mudas de *E. uniflora*, independentemente das concentrações de Al, sugere que *E. uniflora* é tolerante ao alumínio. A manutenção do teor de pigmentos fotossintéticos e dos valores de Fv/Fm, bem como o baixo teor de H_2O_2 nas raízes também confirmam a tolerância de *E. uniflora* ao alumínio.

Palavras-chave: estresse oxidative; excesso de alumínio; metais tóxicos; pigmentos fotossintéticos; pitangueira

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Introduction

Acidic soils (pH ≤ 5.5) are widely distributed in numerous food and forest product producing regions, including tropical and subtropical areas of the world, making up most of the world's arable land (approximately 50%) (Shetty et al., 2021). These soils typically have phytotoxic levels of aluminum (Al) and low levels of exchangeable calcium and magnesium, resulting in unfavorable conditions for the development of several vegetable crops (Basílio et al., 2022). Thus, Al toxicity is one of the greatest limitations to crop production. As the soil pH decreases, Al activity in the soil solution increases and there is consequently a rise in its harmful and toxic effects on the crops (Dorneles et al., 2019).

Aluminum in the solution can impair cell division and elongation, increasing root diameter, decreasing growth and consequently root volume and surface area (Aguilar et al., 2023a). As a result, roots will likely reduce water and nutrient uptake, which may decrease plant growth and biomass production (Mayer & Ueno, 2021). However, Al tolerant plants can develop mechanisms to mitigate the toxic effects of this metal, such as the activation of superoxide dismutase (SOD) and guaiacol peroxidase (POD) to try to reestablish the balance within these plants (Dorneles et al., 2019). In addition, plants tolerant to Al usually show increased concentrations of photosynthetic pigments in the leaves, which reduce the loss of energy by chlorophyll fluorescence and result in higher values of dry matter, as adaptive mechanisms to excess Al.

Still, there is little information in the literature about the effects of excess Al on tropical plant species such as *E. uniflora* L. *Eugenia uniflora*, popularly known as *pitangueira*, is a small tree species (Fidelis et al., 2022) found in Guyana, Brazil and Uruguay (Jucoski et al., 2016). In Brazil, this species has a wide geographic distribution, including regions with high levels of soil acidity.

Given that Al in acidic soils is a phytotoxic element that damages plants and contaminates environments, there is a need for research to evaluate excess Al in tree species such as *E. uniflora*, which has high ecological, economic, food and medicinal potential. Thus, the aim of the study was to assess Al tolerance of *E. uniflora* seedlings, based on the effects of Al on physiological, biochemical and morphological variables. The study of Al toxicity may contribute to understanding the survival of this species in environments with high levels of this metal.

Materials and Methods

Plant material and growing conditions

The study was carried out in a greenhouse (29°42'56.35"S e 53°43'12.64" W) located in the city of Santa Maria, RS, southern Brazil. Inside the greenhouse, relative humidity was 60% and average temperature was 25 °C. *Eugenia uniflora* seeds were collected from 10 mother trees located in forest fragments in the cities of Santa Maria and Nova Palma. Seeds were planted in plastic trays (38 x 56 cm) containing Carolina Soil® substrate, composed of peat moss *Sphagnum* spp. and vermiculite, with the addition of 30% carbonized rice husk. Base fertilization was carried out with Osmocote® controlled release fertilizer (CRF) with a nutrient release time of six months.

After the emergence and initial growth of the seedlings (about 6 months), homogeneous plants, approximately 10 cm tall, were transferred to the hydroponic system. Plants were fixed using sponges and polystyrene (Styrofoam) plates with holes, placed in plastic buckets containing five liters of full-strength nutrient solution, so that plant roots were in contact with the solution. The plants were acclimatized for 15 days in nutrient solution. The nutrient solution had the following composition (in µM): 6,090.5 N; 974.3 Mg; 4986.76 Cl; 2679.2 of K; 2436.2 Ca; 359.9; 50 of P; 0.47 Cu; 2.00 of Mn; 1.99 Zn; 0.17 Ni; 24.97 B; 0.52 Mo; 47.99 Fe (FeSO4/ Na-EDTA).

The experimental design was completely randomized with four replications. Seedlings of *E. uniflora* were grown in nutrient solution with five Al concentrations of 0, 15, 30, 45 and 60 µM. The concentrations and the pH of the solution were defined based on preliminary tests and reviewing scientific literature. Each sampling unit consisted of a pot with five plants, totaling 20 experimental units and 100 plants. Seedlings were kept in the Al concentrations for 31 days, totaling 46 days in the hydroponic system. The nutrient solution in each pot was replaced twice a week and the pH was adjusted daily to 4.5±0.2 with 1.0 mol L^{-1} HCl or 1.0 mol L^{-1} NaOH.

Morphological variables

After 31 days of exposure to Al, eight (8) plants were collected from each treatment to assess growth variables. Plants were separated into shoots and roots, subsequently leaf area and root morphology were determined from digitized images, using WinRhizo Pro 2013 software coupled to an EPSON Expression 11000 scanner equipped with additional light (TPU), with resolutions of 200 and 600 dpi for leaves and roots, respectively. Root surface area (cm^2), root volume (cm^3), average root diameter (mm) and total root length (cm) were measured. After morphological characterization, the leaves and roots were dried in an oven with forced air circulation (65 ± 1 °C) until constant mass was reached. Finally, samples were weighed on a precision digital scale to determine shoot and root dry weight.

Physiological variables

Chlorophyll *a* fluorescence was determined after 45 days of cultivation in a hydroponic system using a portable fluorometer with modulated light (Junior-Pam Chlorophyll Fluorometer Walz Mess-und-Regeltechnik, Germany). Measurements were taken on fully expanded leaves of each plant, on a sunny day, between 8:00 am and 10:30 am. Prior to the measurements, the leaves were preadapted to the dark for 30 min. to determine initial fluorescence (Fo). Then, the leaves were subjected to a saturating light pulse (10,000

µmol m^{-2} s^{-1}) for 0.6 s to detemine maximum fluorescence (Fm). Through the fluorescence of the induction curve (1,500 mmol m^{-2} s^{-1}), we determined electron transport rate (ETRm). The maximum potential quantum yield of PSII (Fv/Fm) was obtained by the ratio between variable fluorescence (Fv = Fm-Fo) and maximum fluorescence.

Biochemical variables

For biochemical analysis, 12 plants were collected from each treatment, totaling 60 plants. First, the plants were separated into leaves and roots, which were then washed in distilled water, stored in aluminum foil envelopes and immediately frozen in liquid N to avoid sample degradation. The samples were kept in an ultra freezer at -80 °C until preparation for analysis. Then, the samples were prepared by manual maceration with liquid N until a fine powder was obtained. Subsequently, the specific quantity for analysis was weighed on a precision digital scale: 0.05 g of fresh sample to determine foliar pigments, 0.5 g for antioxidant enzymes and 0.3 g for hydrogen peroxide.

Pigment content

The Hiscox & Israelstan (1979) method was used for the extraction of total chlorophylls and carotenoids, while the Lichtenthaler (1987) equation was used for estimatation. The samples, previously weighed in 15 mL falcon tubes, were arranged in grids and 5 mL of dimethyl sulfoxide (DMSO) was added. The tubes were incubated at 65 °C for approximately 1.5 h until the pigments were completely detached, resulting in a dark green solution. Then, this solution was separated into two repetitions of 2 mL each. The solution absorbances were measured in a UV-visible spectrophotometer (1105, Bel Photonics) at 663, 645, and 470 nm for chlorophyll *a*, chlorophyll *b* and carotenoids, respectively.

Antioxidant enzyme activity

Antioxidant enzymes were determined by adding 0.5 g of sample in 3 mL of 0.05 M homogenate extraction buffer (pH 7.8) containing 1 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 13,000 x g for 20 min at 4 °C in a centrifuge (High-Speed Refrigerated Centrifuge - CR22 N) and the supernatant was used to determine enzyme activity and protein concentration (Zhu et al., 2004).

Guaiacol peroxidase (POD) activity was determined according to Zeraik et al. (2008), using guaiacol as substrate. The reaction mixture contained 1.0 mL of potassium phosphate buffer (100 mM, pH 6.5), 1.0 mL of guaiacol (15 mM) and 1.0 mL of H_2O_2 (3 mM) in the quartz cuvette. After homogenization, 50 µL of plant extract was added to this solution. Enzyme activity was measured by the oxidation of guaiacol to tetraguaiacol with the increase in absorbance at 470 nm, at 15-second reading intervals. The results were expressed as enzyme units per mg protein (U mg^{-1} protein). A molar extinction coefficient of 26.6 mM^{-1} cm^{-1} was used for calculation.

Superoxide dismutase (SOD) activity was determined according to the spectrophotometric method described by Giannopolitis & Ries (1977). The reaction mixture (MIX) that was kept in the dark contained 50 mM of potassium phosphate buffer (pH 7.8), 13 mM of methionine, 0.1 Mm of EDTA, 75 µM of nitrobluetetrazolium (NBT) and 2 µM of riboflavin. The photochemical production of blue formazan from NBT was monitored by the increase in absorbance at 560 nm.

The reaction was carried out at 25 °C in test tubes (13 x 100 mm), each containing 2.8 mL of reaction mixture (MIX) and 200 µL of enzyme extract of the respective samples. After pipetting, the tubes were placed in a reaction chamber with a 15 W fluorescent lamp. The reaction was started by turning on the light and after two minutes it was stopped by turning off the light. Finally, the samples were read in the UV-visible spectrophotometer (1105, Bel Photonics).

One unit of SOD was defined as the amount of enzyme that inhibits the photoreduction of NBT by 50% (Beauchamp & Fridovich, 1971). In the assay, methionine reduces photochemically excited riboflavin to semiquinone, which donates an electron to oxygen, forming the superoxide radical, which then converts NBT to blue formazan. Superoxide dismutase catalyzes this reaction.

Hydrogen peroxide content

Hydrogen peroxide content was determined according to Loreto & Velikova (2001). Root and leaf samples (0.3 g) were homogenized in 3.0 mL of 0.1% trichloroacetic acid (TCA). Samples were centrifuged after homogenization. We added 0.5 mL of the supernatant to 0.5 mL of potassium phosphate buffer (10 mM) and 1 mL of KI (1M) and then measured absorbance in a spectrophotometer at 390 nm. The H_2O_2 concentration of the supernatant was evaluated by comparing its readings to a standard calibration curve. H_2O_2 concentration was expressed as µmol g^{-1} fresh weight.

Membrane lipid peroxidation

Lipid peroxidation was determined by malondialdehyde (MDA) concentration, according to the method of El-Moshaty et al. (1993). Leaf and root samples (0.5 g) were homogenized in 4.0 mL of sodium citrate buffer (pH 6.5) and centrifuged. One mL of the supernatant was added to 1 mL of 20% (w/v) trichloroacetic acid (TCA) containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was heated to 95 °C for 40 min, then cooled in an ice bath for 15 min and finally centrifuged at 10,000 x g for 15 min. The absorbance of the supernatant was read at 532 and 600 nm (to correct for non-specific turbidity). Lipid peroxidation was expressed as MDA mg^{-1} protein nmol.

Statistical analysis

The experimental data were checked for Normality of errors by the Shapiro-Wilk test and for Homogeneity of Variances by the Bartlett test. Contemplated these assumptions, analysis of variance (ANOVA) and the significant

difference of the means by the Tukey test at 5% probability of error were analyzed using SISVAR software (Ferreira 2019).

Results and Discussion

There was no significant difference for total root length, root surface area, average root diameter and root volume of *E. uniflora* seedlings, even with the high concentrations of Al used in our study (Table 1). This possibly happened because Al did not bind strongly to negatively charged carboxyl groups in the cell wall of cortical and epidermal root cells, and thus did not change ion binding and distribution in the apoplast (Zhu et al., 2014), which directly influenced the growth of the organ. As a result, there was also a possible maintained of secondary roots emitted by *E. uniflora* seedlings in the presence of Al, indicating a root system with greater adaptability to Al stress. Plants without toxic effects of Al on the root system tend to absorb more water and nutrients and consequently contribute to maintained production of root and leaf biomass (Aguilar et al., 2023a), as observed in this experiment (Table 1). This is evidence of the tolerance potential of *E. uniflora* seedlings to Al stress.

For leaf dry weight and leaf area, no significant differences were observed, regardless of the Al concentration (Table 1). This most likely happened because *E. uniflora* has an internal defense system or mechanisms to prevent Al from being absorbed by the roots and then transported to the shoots (Kuinchtner et al., 2021). This result may be interesting, as plants that have greater leaf area per unit of leaf dry mass can increase the ability to capture and assimilate CO_2 per plant and as a result its ability to produce new leaves (Aguilar et al., 2023b). Leaf area is an indicator of productivity, as the photosynthetic process depends on the interception of light energy and its conversion into chemical energy, which is influenced by the size of the photoassimilation system (Santos & Ferreira, 2020).

Lower values of initial fluorescence (Fo) and maximum fluorescence (Fm) were found in the control and in concentrations of 15 and 30 µM Al (Figures 1A and 1B). Despite the higher values of Fo and Fm observed at 45 and 60 µM Al, there was no significant difference for the electron transport rate (ETRm) and maximum quantum yield of PSII (Fv/Fm) with the exposure to Al (Figure 1). This possibly occurred because there were no negative effects of Al on photosynthetic pigment content, even with increasing Al concentrations in the nutrient solution (Figures 1E and 1F).

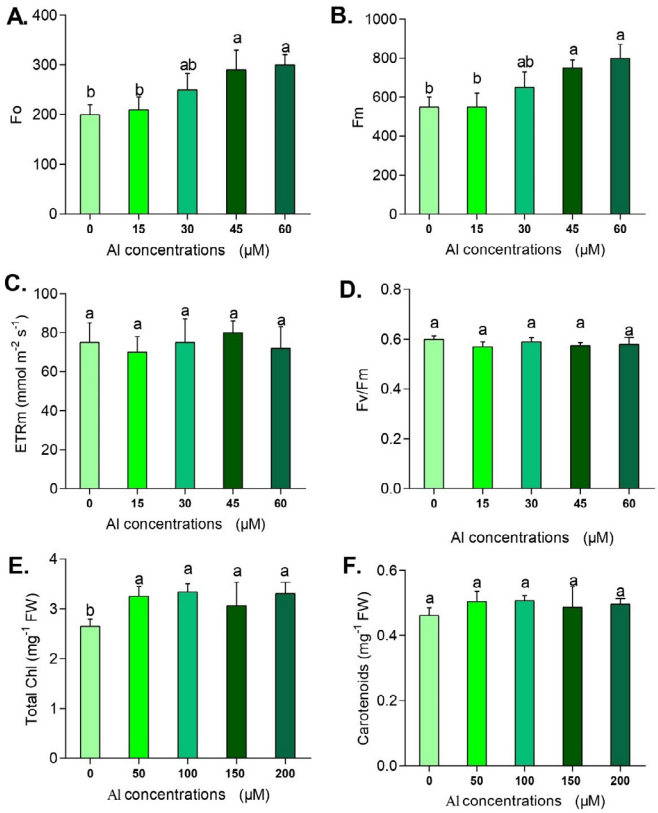

Figure 1. Mean values of initial fluorescence (Fo) (a), maximum fluorescence (Fm) (b), electron transport rate (ETRm) (c), maximum quantum yield of PSII (Fv/Fm) (d) total chlorophyll (Total chl) (e) and carotenoids (f) in *Eugenia uniflora* seedlings grown in nutrient solution with five Al concentrations. Different letters between treatments represent statistical difference by the Tukey test. Bars represent mean ± standard deviation.

Thus, the higher levels of chlorophylls in *E. uniflora* seedlings may have shown greater efficiency in absorbing and transferring energy to the photosynthetic processes. This is because the increase in total chlorophylls and carotenoids in the leaves promoted greater capture and absorption of light in different regions of the spectrum in the early stages of photosynthesis (Roca et al., 2018). Therefore, there was a greater transfer of energy by resonance in the antenna complexes to the reaction centers where energy can be used for the photochemical reactions responsible for biomass production (Berghetti et al., 2021).

Table 1. Morphological variables of *Eugenia uniflora* seedlings grown in nutrient solution with increasing Al concentrations.

Variables	Al concentrations (µM)				
	0	15	30	45	60
Root length	285±29^{ns}	258±56	240±34	203±42	207±24
Root surface	57±10^{ns}	61±9.1	56±8	43±5.8	47±2.9
Root volume	0.934±0.3^{ns}	1.20±0.11	1.07±0.17	0.752±0.06	0.874±0.034
Root diameter	0.636±0.04^{ns}	0.779±0.8	0.758±0.83	0.678±0.37	0.737±0.66
Shoot dry weight	0.529±0.1^{ns}	0.420±0.14	0.612±0.13	0.458±0.034	0.465±0.1
Root dry weight	0.173±0.0^{ns}	0.169±0.024	0.202±0.02	0.147±0.01	0.145±0.012
Leaf area	105±0.0^{ns}	80±0.024	115±0.02	90±0.01	70±0.012

^{ns}Not significant at 5% error probability.

The Fv/Fm values found for *E. uniflora* plants were around 0.580 (Figure 1D). Similar results were observed in research carried out in southern Brazil with seedlings of native tree species which indicated that Fv/Fm values between 0.55 and 0.70 can be considered good indicators of initial growth (Turchetto et al., 2016; Berghetti et al., 2021). Thus, the Fv/Fm values found in the study show that most of the light energy is being directed to the photochemical stage of photosynthesis instead of being lost by chlorophyll *a* fluorescence (Santos & Ferreira, 2020). As a result, it was possible to observe constant biomass production even when *E. uniflora* seedlings were exposed to high concentrations of Al.

However, excess Al in plants can trigger an increase in the production of reactive oxygen species (ROS) (Shetty et al., 2021) which can react with lipids, proteins, nucleic acids and other cellular structures, causing lipid peroxidation and membrane damage, thus affecting cell performance and viability (Dorneles et al., 2019). To mitigate this oxidative damage caused by excess metals, plants usually activate antioxidant defenses and change cellular metabolism to maintain cellular redox homeostasis (Kuinchtner et al., 2021). Thus, one of the possible strategies used by plants is the activation of guaiacol peroxidase (POD) and superoxide dismutase (SOD) (Alsherif et al., 2022).

Superoxide dismutase (SOD) plays the most important role in the antioxidant defense mechanism, as it is the most effective enzyme in stress resistance, involved in the dismutation of $O_2^{\bullet-}$ into H_2O_2 and molecular oxygen in plants under stress conditions (Rajput et al., 2021). On the other hand, guaiacol peroxidase (POD) acts in the conversion of H_2O_2 into water and oxygen by H_2O_2 dissociation, playing an essential role in providing tolerance to unfavorable conditions in plants. H_2O_2 is considered the most abundant and stable ROS in plant cells and when found in low concentrations, it plays a key regulatory role in their organs and protects plants from damage caused by abiotic stress (Kuinchtner et al., 2021). However, high concentrations of H_2O_2 in plant tissues can have harmful effects and cause lipid peroxidation, decreasing the stability of cell membranes (Aguilar et al., 2023b).

Higher values of SOD activity in leaves were found at 15, 45 and 60 µM Al (Figure 2A). This increase in SOD activity in the shoots indicates that the free radicals produced by stress are being neutralized, resulting in a high accumulation of H_2O_2, as SOD is involved in the dismutation of the superoxide free radical into H_2O_2, effectively preventing cell damage. Furthermore, we observed that the increase in POD activity in leaves was not enough to prevent a greater production of H_2O_2 in shoots, since the H_2O_2 content increased significantly in this organ with increasing Al concentrations (Figures 2C and 2E). With the reduction of POD activity in leaves at concentrations of 45 and 60 µM Al, a possible delay in the removal of ROS can be inferred. As a result, this contributed to the increase in H_2O_2 content in the shoots, as SOD was activated in the presence of Al, releasing H_2O_2 in the reaction.

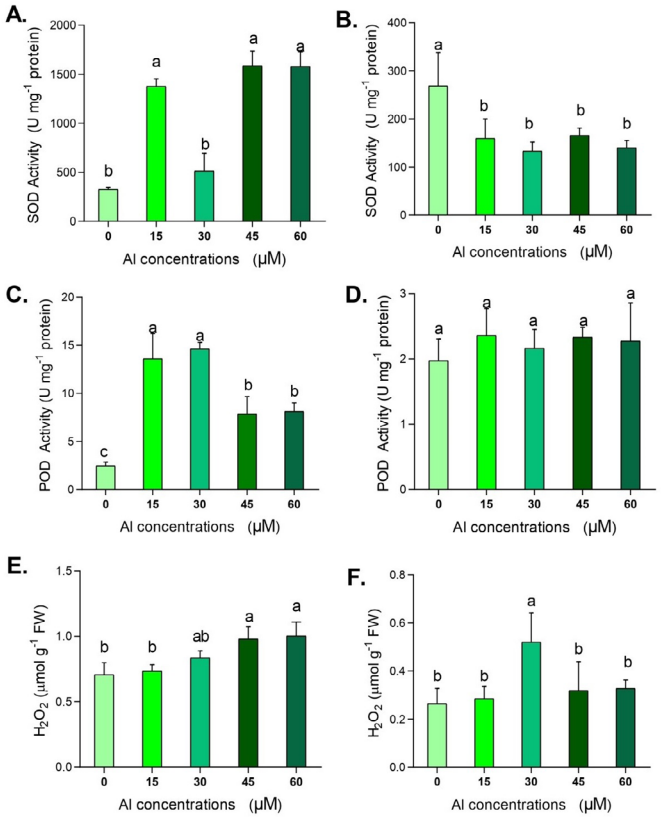

Figure 2. Mean values of superoxide dismutase (SOD) activity in shoots (a) and roots (b), guaiacol peroxidase (POD) activity in shoots (c) and roots (d), hydrogen peroxide content (H_2O_2) in shoots (e) and roots (f) of *Eugenia uniflora* seedlings grown in nutrient solution with five Al concentrations. Different letters between treatments represent statistical difference by the Tukey test. The bars represent the mean ± standard deviation.

However, even with the detection of higher levels of H_2O_2 in shoots with the presence of Al, no significant effect was observed on biomass production, possibly indicating that higher levels of H_2O_2 in shoots were not significant or enough to cause cellular damage.

Still, we observed decreased SOD activity in roots upon exposure to Al. Consequently, there was no increase in H_2O_2 levels and POD did not need to be activated (Figures 2B, 2D and 2F). Thus, the roots were generally less affected by Al levels, as the roots did not show an increase in oxidative stress induced by ROS, and even though Al inhibited SOD activity at all concentrations, H_2O_2 content only increased by 30µM. Therefore, the reduction of H_2O_2 levels in roots had a positive effect on the growth of this organ and as a result increased biomass production of *E. uniflora*.

Plants, when subjected to stressors, do not change morphology at first. However, metabolism damage can be seen with the evaluation of physiological characteristics, which can be observed later morphologically. At this time, interference through silvicultural practices is possible, minimizing loss in productivity. Thus, evaluating pigment content, chlorophyll *a* fluorescence, oxidative (H_2O_2) stress

and antioxidant enzyme (SOD and POD) activity allow us to understand the effect of different types of stress on photosynthesis and plant defense system caused by nutritional deficiency or metal toxicity.

Al stress interfered with the mechanisms of regulation and/or functioning of the SOD and POD enzymes and the H_2O_2 levels of *E. uniflora* seedlings, sometimes increasing or reducing the activities of the enzymes according to the homeostasis needs in plant cells. Consequently, plants also developed other response mechanisms to Al stress, such as an increase in total chlorophylls, avoiding the loss of energy due to chlorophyll a fluorescence, which culminated in the maintenance of *E. uniflora* biomass.

Conclusion

The absence of significant differences in shoot and root dry weight of *Eugenia uniflora* seedlings, regardless of Al concentrations, suggest that *E. uniflora* is aluminum tolerant.

The maintenance of the content of photosynthetic pigments and the values of Fv/Fm, as well as the low H_2O_2 content in roots also confirm the tolerance of *E. uniflora* to aluminum.

Compliance with Ethical Standards

Author contributions: Conceptualization: GRS, LAT; Data curation: GRS, MVMA; Formal analysis: GRS, MVMA, LAT; Funding acquisition: GRS, MVMA, LAT; Investigation: TWP, DVV, TDA; Methodology: GRS, MVMA; Project administration: TWP, DVV, TDA; Resources: GRS, MVMA, TWP, DVV, TDA, LAT; Software: GRS, MVMA, TWP, DVV, TDA. LAT; Supervision: TWP, DVV, TDA; Validation: GRS, MVMA, TWP, DVV, TDA, LAT; Visualization: TWP, DVV, TDA; Writing - original draft: GRS, LAT; Writing - review and editing: GRS, MVMA, LAT.

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