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Ativação de enzimas antioxidantes como mecanismo contra estresse por cádmio em *Mimosa scabrella*

Activation of antioxidant enzymes as a mechanism against cadmium stress in Mimosa scabrella

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RESUMO: Dentre os elementos que acarretam sérias consequências ao meio ambiente e aos seres vivos, o cádmio (Cd) é considerado um dos mais danosos. Ao atingir altos níveis de contaminação de Cd em uma área, pode ocorrer a supressão da vegetação. Portanto, identificar espécies resistentes a esta contaminação auxiliará no processo de revegetação e descontaminação destes locais.Assim, objetivou-se analisar os efeitos do Cd sobre variáveis morfológicas e bioquímicas de *Mimosa scabrella*, a fim de avaliar o seu potencial de utilização em ambientes poluídos por Cd. As mudas de *M. scabrella* foram cultivadas em cinco concentrações de Cd (0, 25, 50, 75 e 100 μM). Avaliou-se as variáveis morfológicas de folhas e de raízes, pigmentos fotossintéticos, atividade das enzimas antioxidantes, bem como o conteúdo de peróxido de hidrogênio (H_2O_2) e peroxidação lipídica (MDA). Não se observou efeitos negativos para o comprimento total e área superficial de raízes nas concentrações menores que 75 μM Cd. O estresse por Cd ativou antioxidantes enzimáticos, principalmente em raízes de mudas de *M. scabrella*, ocasionando, com isso, a manutenção na produção de biomassa de raízes e parte aérea das mudas. Assim, a espécie tolerou concentrações elevadas de Cd, podendo ser indicada para fitorremediação de solos contaminados com cádmio.

Palavras-chave: Áreas contaminadas; Bracatinga; Sistema de defesa antioxidante.

ABSTRACT: Among the elements that cause serious consequences to the environment and to living beings, cadmium (Cd) is considered one of the most harmful. When reaching high levels of Cd contamination in an area, vegetation suppression can occur. Thus, the objective was to analyze the effects of Cd on morphological and biochemical variables of *Mimosa scabrella*, in order to evaluate its potential for use in environments polluted by Cd. *M. scabrella* seedlings were grown in five concentrations of Cd (0, 25, 50, 75 and 100 μM). The morphological variables of leaves and roots, photosynthetic pigments, antioxidant enzyme activity, as well as the content of hydrogen peroxide and lipid peroxidation (MDA) were evaluated. No negative effects were observed on the total length and surface area of roots at concentrations lower than 75 μM Cd. Cd stress activated enzymatic antioxidants, mainly in roots of *M. scabrella seedlings*, thus causing maintenance in the production of biomass of roots and shoot of seedlings. Thus, the species tolerated high concentrations of Cd and could be recommended for phytoremediation of soils contaminated with cadmium.

Keywords: Contaminated areas; Bracatinga; Antioxidant defense system.

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INTRODUCTION

High content and availability of heavy metals in the soil stand out among the main aggravating factors for environmental pollution in different soil types, worldwide (Alsheri *et al*., 2022). Mining, industrial effluent discharge, rapid urbanization and agricultural practices are the human activities mostly contributing to this scenario (Bamagoos; Alharby; Abbas, 2022).

Among the toxic metals, cadmium (Cd) stands out as an element that cannot be degraded and is stable in the environment, being easily absorbed by plants and distributed to the shoot, where it can accumulate at high levels. Thus, Cd can easily enter the food chain, being bioaccumulative, and becoming very harmful to humans as well as animal health (Subaši *et al*., 2022). Cd toxicity can affect several organs in the human body, but it mainly accumulates in the kidneys and leads to severe damages, such as pulmonary emphysema, renal tubular damage, and kidney stones (Haider *et al*., 2021). Given the several potential risks caused by heavy metals to human health through the food chain, it is necessary to develop research aboutboth the excess of heavy metals and their effects on plant growth, more specifically, on plants' tolerance to Cd stress (Zhang *et al*., 2020).

Excess Cd in plants leads to several toxicity symptoms. It happens because Cd competes with membrane transporters of other cations, causes leaf chlorosis, as well as reduces the amount of photosynthetic pigments and consequently,it inhibits these plants' growth and biomass production (Woraharn *et al*., 2021). Furthermore, plants grown under excess Cd conditions often show oxidative stress resulting from imbalance between antioxidant responses and increased production of reactive oxygen species (ROS) (liu *et al*., 2021).

However, Cd-tolerant plants can develop mechanisms to help mitigating the toxic effects of this metal, such as activating antioxidant enzymes like superoxide dismutase (SOD) and guaiacol peroxidase (POD) to reestablish balance within their cells (Zhao *et al*., 2021). In addition, plants capable of toleranting toxic metals tend to show high biomass accumulation and dense root system, which implies greater immobilization of metals in plant tissues delaying their return to the soil, or greater metal removal due to high biomass production (Covre *et al*., 2020).

Therefore, studies focused on investigating the growth potential of plant species subjected to excess metal conditions, as well as the physiological effects of such condictions on plants, such as oxidative damage and antioxidant enzyme activity in the presence of toxic metals, are extremely useful to helpin identifying species suitable to be used for phytoremediation purposes. It is so, because results recorded for the aforementioned variables enabled inferring the nature of plants' adaptation toexcess metal conditions.

Among the promising species used for the recovery of degraded areas, we can mention *Mimosa scabrella*Benth. (Fockink *et al*., 2022), which belongsto Fabaceae family and is popularlyknown as "bracatinga" (Ferreira *et al*., 2019). This species has fast growth and is undemanding regarding the physical and chemical conditions of the soil, which is the reason why it is widely used in revegetation programs implemented in degraded environments (Silva *et al*., 2019). The tolerance of *M. scabrella*plants grownin places contaminated with other metals and organic pollutants waspreviously investigated in other studies (Gonçalves *et al*., 2012; Silva *et al*., 2019). However, the literature still lacks studies focused on investigating the biochemical/physiological behavior of *M. scabrella*. plants exposed to Cd. Thus, the aim of the current study was to analyze Cd effects on morphological and biochemical variables of species *M. scabrella* in order to assess its potential to be usedfor phytoremediation purposes in Cd-polluted environments. The herein advocated hypothesis is that *M. scabrella* seedlings are capable of tolerating high Cd levels by activating antioxidant mechanisms, as well as by simultaneously maintaining the growth of their shoot and root system, due to their strong adaptability to contaminated environments.

2. MATERIALS AND METHODS

2.1 STUDY SITE

The study was conducted in greenhouse of Federal University of Santa Maria (UFSM) - Santa Maria Campus – RS (29°42'56.35"S e 53°43'12.64" W), under controlled temperature of approximately 25ºC, and mean humidity of 60%. Analyses were carried out at the Plant Physiology and Nutrition Laboratory of the Biology Department.

2.2 CONDUCTING THE EXPERIMENT

M. scabrella seeds acquired at Santa Maria Forest Research Center (DDPA) were subjected to dormancy breaking process, in compliance with*"Instruções Para Análise de Sementes de EspéciesFlorestais"* [Instructions for the Analysis of Forest Species' Seeds] (BRASIL, 2013), before they were sown in substrate. Seeds were immersed in distilled water at 80ºC; subsequently, the water was removed from the heat source and seeds were left to soak in it for 24 h. After dormancy was overcome, seeds were directly sown in commercial substrate (Bioplant®) comprising pine bark, ash, coconut fiber, rice husk and vermiculite. Plastic trays (38 cm x 56 cm) were used as cultivation containers for *M. scabrella* seedling germination and initial growth. During this period, seedlings were irrigated on a daily basis until the experiment was set up.

Seedlings with approximately 15 cm in height were removed from the substrate, washed in running water and transferred to hydroponic system subjected to constant aeration at 50 days after sowing (DAS). *M. scabrella* plants were left to acclimate to this system for three weeks. The seedling acclimation process took place in Hoagland and Arnon's (1950) nutrient solution comprising (in mg L^{-1}): NO_3^- = 196; NH4 = 14; P = 31; K = 234; Ca = 160; Mg = 48.6; S = 70; Fe-EDTA = 5; Cu = 0.02; Zn = 0.15; Mn = 0.5; B = 0.5; and Mo = 0.01.

After the acclimation period, the seedlings were exposed to five concentrations of Cd (0, 25, 50, 75 and 100 μM), which were added to the nutrient solution in the form of cadmium chloride ($CdCl_2$. H_2O). These are equivalent to 0 (control treatment), 2.81, 5.62, 8.43 and 11.24 mg L^{-1} Cd, respectively. The aforementioned Cd concentrations were defined based on preliminary tests carried out by the study group with other woody and perennial species, as well as studies in the scientific literature (Paiva *et al*, 2004; Pereira *et al*., 2018; Hassan *et al*., 2020; Kuinchtner *et al*., 2021; Senhor *et al*., 2023; Wertonge *et al*., 2024).

The experiment has followed a completely randomized design, with four repetitions. Each sample unit comprised a tray (16 L capacity) with 16 plants, and it totaled 64 plants per treatment. The solution was changed every 7 days and its pH was adjusted to 4.5 ± 0.1. Plants remained exposed to the herein defined treatments for 14 days, which was thetime necessary for them to show visible toxicity symptoms, mainly at the highest Cd concentration (100 μM).

2.3 DETERMINING GROWTH VARIABLES

Seedlings' shoot height and main root length were measured in six plants per tray, with millimeter ruler. Measurements were taken before (0 days) and after plants' total exposure to Cd (14 days) - plant growth was defined as increment observed during this period.

Three (3) plants were collected from each tray after 14 experimental days in order to assessshoot and root biomass (g $plant^{-1}$) and root morphology. Plants were divided into shoot and roots, dried in forced air circulation oven at 65ºC, and weighed on precision scale (0.0001g) until they reached constant weight.

Roots' morphological featuring was based on digitized images. This procedure was carried out inWinRhizo Pro 2013 software coupled to EPSON Expression 11000 scanner equipped with additional light (TPU), at 600-DPI resolution. Root surface area (cm^2 $plant^{-1}$), root volume (cm^3 $plant^{-1}$) and total root length (cm) were measured.

2.4 BIOCHEMICAL VARIABLE DETERMINATION

Part of the seedlings were collected, sectioned and had their leaves and roots washed in distilled water, added to aluminum foil envelopes and frozen in liquid nitrogen (N) right away in order to avoid sample degradation. These samples were kept in ultrafreezer at -80° C, until analysis time, when they were macerated in liquid N, homogenized in specific buffer and analyzed, later on.

Chlorophylls *a* and *b* and carotenoids were extracted according to the method of Hiscox and Israelstan (1979) and estimated using the Lichtenthaler equation (1987). The absorbance of the solution was measured in a spectrophotometer at 663, 645, and 470 nm for chlorophyll *a*, chlorophyll *b*, and carotenoids, respectively. Total chlorophyll is the sum of chlorophyll *a* + chlorophyll *b*.

Hydrogen peroxide content was determined according to Loreto and Velikova (2001). The H_2O_2 concentration of the supernatant was evaluated by comparing its readings with a standard calibration curve at 390 nm. The concentration of H_2O_2 was expressed as μmol g^{-1} fresh weight.

Lipid peroxidation was determined by the concentration of malondialdehyde (MDA) following the method of El-Moshaty *et al*. (1993). The absorbance of the supernatant was read at 532 and 600 nm (to correct non-specific turbidity). Lipid peroxidation was expressed as nmol of MDA mg^{-1} protein.

The roots and leaves samples (0.5 g) were homogenized in 3.0 mL of 0.05 M sodium phosphate buffer (pH 7.8) according to the method of Zhu *et al*. (2004). Afterwards, the homogenate was centrifuged and the supernatant used to determine the activity of the guaiacol peroxidase (POD), according to Zeraik, Souza and Fatibello-Filho (2008), and superoxide dismutase (SOD) enzymes, according to Giannopolitis and Ries (1977).

2.5 STATISTICAL ANALYSIS OF THE DATA

The results obtained were submitted to error normality analysis by the Shapiro-Wilk test and Homogeneity of Variances using Bartlett's test (Storck *et al*., 2016). After meeting the assumptions, the data were submitted to analysis of variance and differentiated means by Tukey's test in 5% probability of error using the statistical software Sisvar (Ferreira, D., 2019).

3 RESULTS AND DISCUSSION

3.1 MORPHOLOGICAL VARIABLES

There was significant effect ($p \leq 0.05$) of different cadmium (Cd) concentrations on variables "total root length" and "root surface area". Thus, the lowest values recorded for root length and surface area were observed at 100 μM Cd (Figure 1a and Figure 1b). It may have happened because the root system is the first organ to have contact with this contaminant and, overall, it is the organ mostly damaged by it, likely due to higher Cd accumulation in its tissues (Subaši *et al*., 2022). Cadmium, when in contact with meristems, can

lead to inhibition of cell multiplication and division and consequently, it can reduce root growth (Haider et al., 2021). This behavior was even observed in Cd-tolerant species, such as *Acmella oleracea* (L.) R.K. Jansen, *Jatropha curca* L. and *Hevea brasiliensis* (Willd. ex A. Juss.) Müll.Arg., which showed morphological damage in the root system after they were exposed to Cd stress (Hungria *et al*., 2019; Yamada *et al*., 2018; Cupertino, 2006).

However, the highest values recorded for root surface area were observed at Cd concentrations of 0 (control treatment), 25 and 50 μM (Figure 1b). This behavior was also observed for total root length, although there was not significant difference between the control treatment and Cd concentration of 75 μM (Figure 1a). This result is interesting, since 75 μM is considered a high Cd concentration (Ullah *et al*., 2020) and corresponds to 8.43 mg Cd L^{-1} in the solution. Soils containing 1.3 mg Kg^{-1} Cd indicate risk of contamination, whereas soils presenting 3 mg Kg^{-1} Cd are classified as contaminated (Akbar *et al.,* 2006; Brasil, 2009). Mean Cd concentrations in non-contaminated soils in Brazil correspond to 0.18 mg Kg^{-1}, whereas contaminated soils worldwide tend to present concentrations ranging from 5.9 to 531 mg Kg^{-1} Cd, depending on the pollution source (Kubier; Wilkin; Pichler, 2019). The minimum Cd concentration used in the current study was 25 μM, which corresponds to 2.81 mg L^{-1} Cd. In other words, this concentration is considered high, mainly if one takes into consideration plants' exposure to it in nutrient solution, where Cd bioavailability is higher than that of the soil.

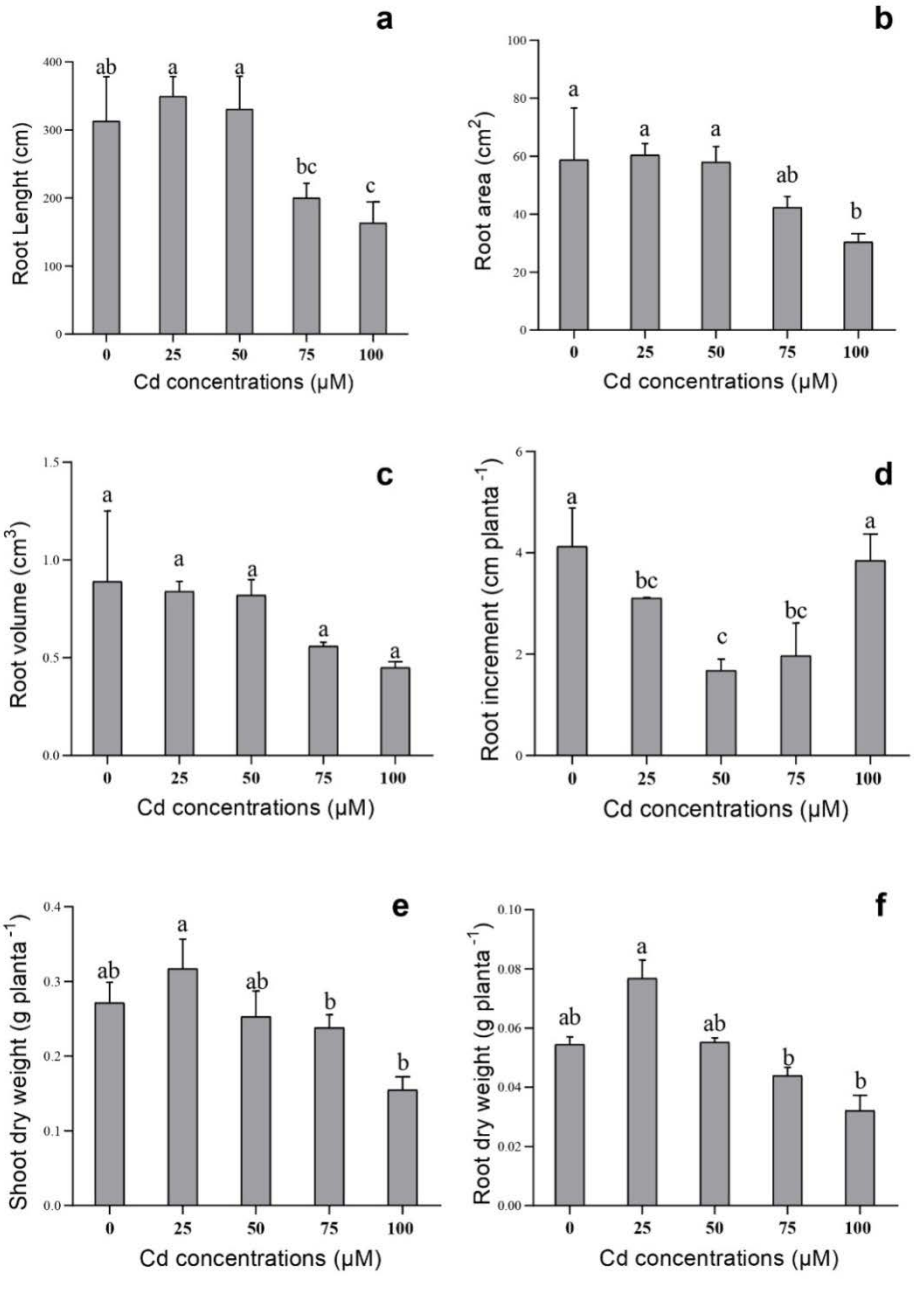

Figure 1. Mean values recorded forroot length (a) root surface area (b), root volume (c), main root (RI) (d) shoot dry weight (e), root dry weight (f) in *M. scabrella*seedlings grown under different Cd concentrations. Different letters between treatments represent statistically significant difference in the Tukey test. Bars represent the mean ± standard deviation.

Reduction in root increment (RI) was observed at 25, 50 and 75 μM Cd. However, RI values recorded at the highest Cd concentration were statistically equal to that recorded for the control (Figure 1d). However, these treatments did not show significant difference in root volume, orin shoot and root dry mass,in*M. scabrella* seedlings (Figure 1c, Figure 1e, and Figure 1f).

Therefore, plants' response through total root length and surface area, at concentrations lower than 75 μM Cd (Figure. 1a and Figure 1b), may be associated with increased number of secondary roots emitted by the *M. scabrella*, a fact that indicated root system's higher adaptability to Cd stress. This outcome suggested species' tolerance to Cd, since main root increase was the only root variable affected by high Cd concentrations. In addition, plants may also have mitigated toxic Cd effects by accumulating it in vacuoles

(Woraharn *et al*., 2021), or by compartmentalizing it in metabolically less-active plant tissues in order to reduce its bioavailability and toxic effect of this metal (Subaši *et al*., 2022).

Furthermore, no significant difference was observed for root volume, regardless of the applied Cd concentrations (Figure 1c). It may have happened due to lack of inhibition in total root length (Figure 1a), and to increased fine root and root hair emissions, which contributed to increase root volume. Plants' ability to maintain root length and volume in the presence of toxic metals can be an important strategy used by them to adapt to metals, since plants maintain the area of soil/solution explored, with low carbon investment (Hoekstra *et al*., 2014). This factor helps explaining the fact that the biomass of *M. scabrella* seedlings was not affected by different Cd concentrations (Figure 1e and Figure 1f). Thus, it is possible inferring that water and nutrient absorptionby roots was not impaired by Cd stress and, consequently, it did not impair plant growth and development (Shaari *et al*., 2022).

3.2 BIOCHEMICAL VARIABLES

The herein investigated different Cd concentrations have shown significant effect ($p \leq 0.05$) on some biochemical variables analyzed in the present study. Cadmium concentrations did not significantly affect total chlorophyll and carotenoid contents in comparison to the control (Figure 2a and Figure 2b). Biomass production maintenance at toxic Cd levels can also be explained by lack of damage to photosynthetic pigments (Figure 2a and Figure 2b). It happened because increased pigment amounts enabled plants to absorb more light radiation and to convert it into carbohydrate, which results in increased biomass production (Roca *et al*., 2018).

Thus, chlorophyll and carotenoid levels were not negatively affected by Cd addition to the solution; this outcome has evidenced species' tolerance to Cd application (Figure 2a and Figure 2b). Carotenoids not only work as antenna pigments for light absorption purposes, but also as photoprotective pigments in the photosynthetic system. They protect chlorophylls by preventing singlet oxygen (reactive oxygen species - ROS) formation and they are non-enzymatic antioxidants due to their ability to suppress ROS and free radicals (Niedzwiedzki *et al.,* 2020). Increasing carotenoid contents is likely a strategic plant mechanism adopted to mitigate the toxic effects of Cd-generated oxidative stress.

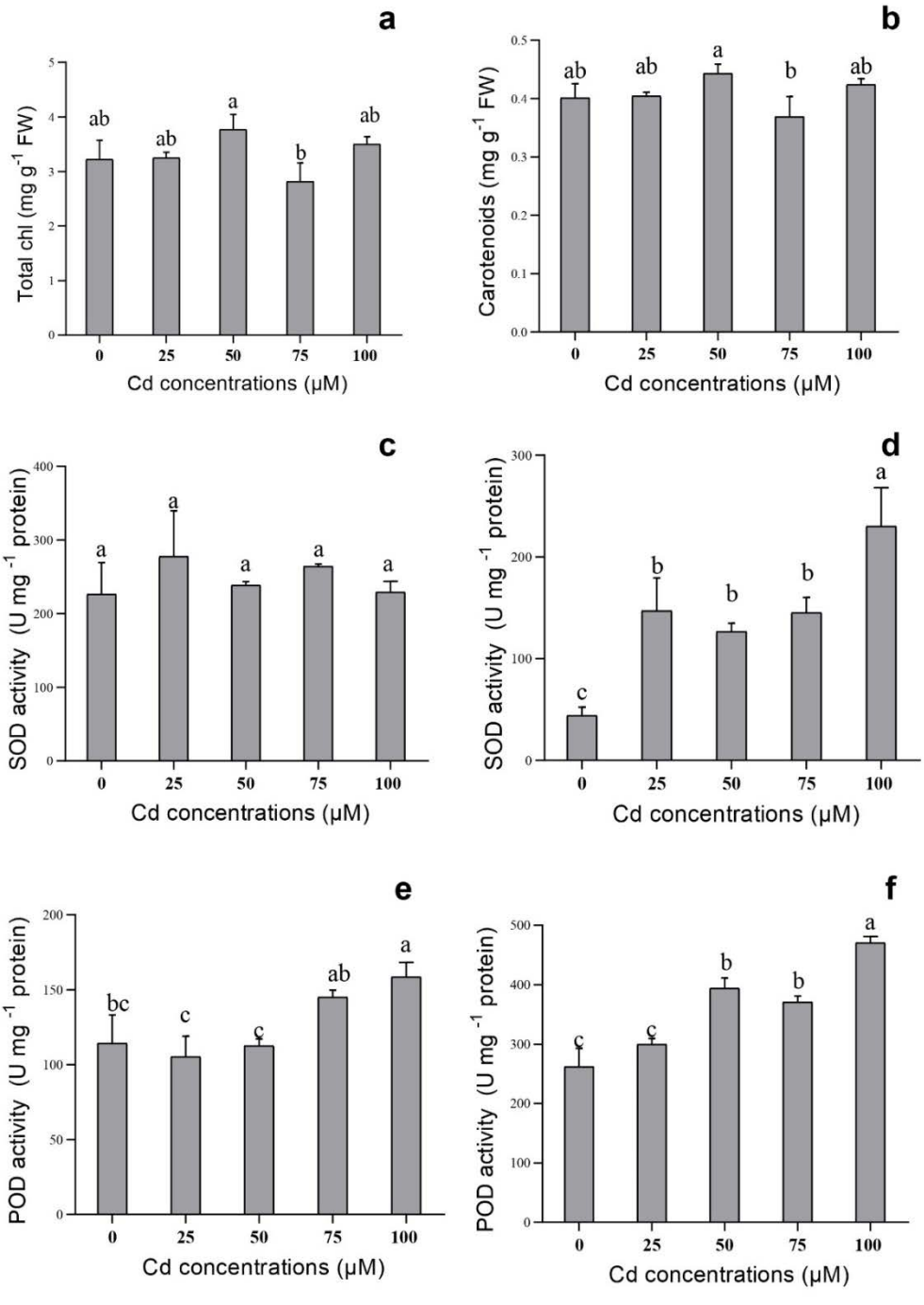

Figure 2. Mean total chlorophyll (a), carotenoids (b), superoxide dismutase (SOD) enzyme activity in shoot (c) and roots (d) and guaiacol peroxidase enzyme (POD) in shoot (e) and roots (f) in *M. scabrella*seedlings grown under different Cd concentrations. Different letters between treatments represent statistically significant difference in the Tukey test. Bars represent the mean ± standard deviation.

However, excess of heavy metals stimulate plants to produce more ROS, which can react to lipids, proteins and nucleic acids, among other substances, and cause lipid peroxidation and membrane damage, thus affecting cell performance and viability (Zhao *et al*. 2021). Plants often activate antioxidant defenses and alter cellular metabolism to maintain cellular redox homeostasis and to mitigate oxidative damage caused by excess of metals (Zhu *et al*. 2021). Thus, one of the possible strategies used by plants to restore the internal balance lies on activating antioxidant enzymes, such as guaiacol peroxidase (POD) and superoxide dismutase (SOD) (Alsherif; Al-shaikh; Abdelgawad, 2022).

POD and SOD stand out among the main antioxidant enzymes playing critical role in ROS elimination and in protecting plants against likely cellular damage and tissue dysfunction (Hassan et al., 2020). There was no significant difference in superoxide dismutase (SOD) enzyme activity in the shoot, regardless of the applied Cd concentrations (Figure 2c). However, increased SOD activity was observed in the roots of plants exposed to all tested Cd concentrations, except for the control (Figure 2d).

Increased SOD activity has indicated its potential to be used to mitigate oxidative damage, since SOD converts superoxide anion ($O_2^{\bullet-}$) into H_2O_2, which is often correlated to increased plant tolerance to excess metal (Schmitt *et al*., 2020).H_2O_2 is the most abundant and stable ROS in plant cells; thus, low H_2O_2 concentrations play fundamental regulatory role in plants' organs and protect them from damage caused by abiotic stress (Zhao *et al*., 2021).On the other hand, high H_2O_2 concentrations in plant tissues can have harmful effects such as lipid peroxidation and decreased cell membrane stability (Bamagoos; Alharby; Abbas, 2022).

As for the SOD enzyme, the activity of the enzyme guaiacol peroxidase (POD) also increased in shoots by 100 μM and in roots by 50, 75 and 100 μM (Figure 2d, 2e and Figure 2f). Thus, the highest means recorded for POD activity in plants' shoot and roots were observed at 100 μM Cd (Figure 2e and Figure 2f). POD acts in H_2O_2 conversion into water and oxygen due to H_2O_2 dissociation; thus it plays essential role in providing plants with tolerance to unfavorable conditions (bamagoos; Alharby; Abbas, 2022).Increased POD activity in the shoot may have happend due to increased H_2O_2 content in the shoot at the same Cd concentration (100 μM).

H_2O_2 content in the shoot and malondialdehyde (MDA) content in the roots have significantly increased at 100 μM Cd, in comparison to the control (Figure 3a and Figure. 3d). On the other hand, there was not significant difference in H_2O_2 production in the roots in relation to the control (Figure 3b). However, lipid peroxidation in the shoot was already high at 25 μM, differing from the control at all Cd concentrations (Figure 3c). Lipid peroxidation in *M. scabrella* roots has only significantly increased at Cd concentration of 100 μM, in comparison to the control (Figure 3d). Thus, increased POD activity in the shoot was not enough to prevent higher H_2O_2 production in *M. scabrella* seedlings at 100 μM Cd (Figure 2e and Figure 3a). In addition to H_2O_2, other ROS were likely formed in the shoot, since MDA (lipid peroxidation) content increased at all Cd concentrations (Figure 3c and Figure 3d). This behavior may also be explained by lack of SOD activation in the shoot. However, although Cd caused lipid peroxidation in seedlings' shoot, it did not have negative effect on biomass production in this organ, indicating that other defense or repair mechanisms were activated.

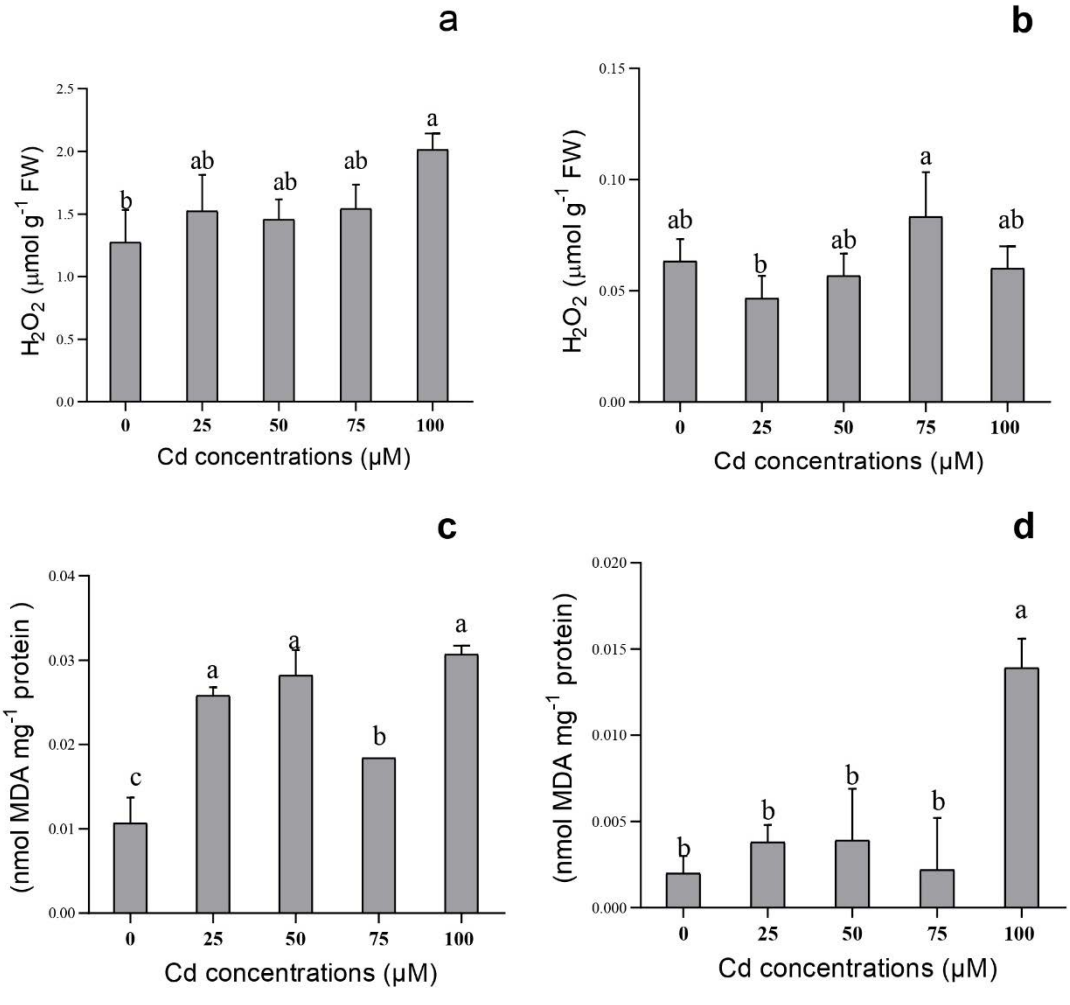

Figure 3. Mean values recorded forhydrogen peroxide (H_2O_2) content in shoot (a) and roots (b), and membrane lipid peroxidation in shoot (c) and roots (d) in *M. scabrella*seedlings grown under different Cd concentrations. Different letters between treatments represent statistically significant difference in the Tukey test. Bars represent the mean ± standard deviation

Increase in SOD and POD activity in the roots was observed depending on the Cd concentrations applied to the nutrient solution (Figure 2d and Figure 2f). Thus, there was association between increased SOD and POD enzyme activity and reduced H_2O_2 contents in *M. scabrella* roots, a fact that avoided excessive H_2O_2 accumulation in them (Figure 2d, Figure 2f and Figure 3b). Although there was not H_2O_2 content increase in the roots, there was increase in MDA levels in them at 100 μM Cd (Figure 3d). Thus, the action of other ROS may have impaired lipid peroxidation in cell membranes, since it did not have significant effect on H_2O_2 content in the roots, although MDA content increased at 100 μM Cd.

Therefore, increased activity of antioxidant enzymes, mainly in the roots of plants subjected to Cd addition, suggested the role played by these enzymes in minimizing H_2O_2 contents and in helping plants to maintain normal root and shoot growth, without affecting biomass production, proving our initial hypothesisof the current study. In addition, the investigated plants may have also triggered some Cd-tolerance mechanisms, such as compartmentalization and subcellular chelation, in order to mitigate toxic Cd levels in their tissues (Haider *et al*., 2021).

4 CONCLUSIONS

Cadmium stress has activated the antioxidant enzyme system in the roots of *M. scabrella* seedlings to help maintaining biomass production both in their roots and shoot. Thus, the investigated species was capable of tolerating high Cd concentrations and may be indicated for phytoremediation in cadmium-contaminated soils.

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