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Cadmium toxicity and sensitivity responses in *Enterolobium contortisiliquum*

Toxicidade de cádmio e respostas de sensibilidade em *Enterolobium contortisiliquum*

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ABSTRACT - Soil contamination with heavy metals brings with it several environmental problems. Among these metals, cadmium (Cd) stands out as an extremely harmful element to plant development and may even cause possible loss or suppression of vegetation in various soils worldwide. Thus, it is necessary to identify tolerant species to reestablish the ecological conditions of the environment. The present study aimed to evaluate Cd tolerance in *Enterolobium contortisiliquum* seedlings by assessing the effects of Cd on morphophysiological and biochemical variables and determining its potential as a phytoremediator species. The experimental design was completely randomized with four replications. Five Cd concentrations (0, 25, 50, 75, and 100 μmol L^{-1}) were assessed. At the end of the exposure period to the treatments, we measured photosynthetic, morphological (shoot and root dry weight and root morphology), and biochemical (concentration of photosynthetic pigments, hydrogen peroxide content, membrane lipid peroxidation, and guaiacol peroxidase and superoxide dismutase activity) variables in plants. Even with the activation of antioxidant enzymes, cadmium concentrations negatively affected the photosynthetic pigments and photosynthetic rate of *Enterolobium contortisiliquum, which* reduced biomass production and photosystem functions, evidencing its sensitivity to excess Cd. Based on these characteristics, *E. contortisiliquum* seedlings can be used as a bioindicator for cadmium -contaminated areas.

Keywords: Antioxidant enzymes. Cadmium excess. Gas exchange. Oxidative stress. Timbaúva.

RESUMO - A contaminação do solo por metais pesados traz consigo diversos problemas ambientais. Dentre esses metais, destaca-se o cádmio (Cd) como um elemento extremamente prejudicial ao desenvolvimento das plantas, podendo inclusive causar possível perda ou supressão da vegetação em diversos solos ao redor do mundo. Assim, é necessário identificar espécies tolerantes para restabelecer as condições ecológicas do ambiente. Neste estudo, o objetivo foi avaliar a tolerância ao Cd em mudas de *Enterolobium contortisiliquum*, avaliando os efeitos do Cd sobre variáveis morfofisiológicas e bioquímicas para determinar seu potencial como espécie fitorremediadora. O delineamento experimental utilizado foi o inteiramente casualizado com quatro repetições, composto por cinco concentrações de Cd: 0, 25, 50, 75 e 100 μmol L^{-1}. Ao final do período de exposição aos tratamentos, foram medidas as variáveis fotossintéticas, morfológicas (massa seca da parte aérea e da raiz e morfologia da raiz) e bioquímicas (concentração de pigmentos fotossintéticos, teor de peróxido de hidrogênio, peroxidação lipídica da membrana e atividade da guaiacol peroxidase e superóxido dismutase). Mesmo com a ativação de enzimas antioxidantes, as concentrações de cádmio afetaram negativamente os pigmentos fotossintéticos e a taxa fotossintética de *Enterolobium contortisiliquum*. Isso resultou em uma redução na produção de biomassa e nas funções do fotossistema, fato que indica sua sensibilidade ao excesso de Cd. Devido a essas características, mudas de *E. contortisiliquum* podem ser utilizadas como bioindicadoras de áreas contaminadas por cádmio.

Palavras-chave: Enzimas antioxidantes. Excesso de cádmio. Trocas gasosas. Estresse oxidativo. Timbaúva.

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INTRODUCTION

The significant increase in industrialization and urbanization processes has generated a high accumulation of pollutants in the air, water, and soil that directly impacts the quality of life of living beings (OUABO; SANGODOYIN; OGUNDIRAN, 2020). Among these pollutants, cadmium (Cd) stands out as a non -essential element and is considered one of the main soil pollutants in several parts of the world (ONO et al., 2019).

In addition to reducing crop yields, the presence of Cd in agricultural soils is a great danger to human and animal health due to the risk of contamination via the food chain (KUINCHTNER et al., 2021). In plants, excess Cd promotes a reduction in photosynthetic pigments, contributing to a decrease in the photosynthetic rate, which leads to reduced shoot and root growth, and a subsequent decrease in dry biomass (SHAARI et al., 2024). In addition, excess Cd causes oxidative stress, leading to increased production of reactive oxygen species (ROS) (BAMAGOOS; ALHARBY; ABBAS, 2022). Increased ROS levels also cause protein and lipid oxidation, increasing lipid peroxidation levels and damage to nucleic acids, eventually leading to cell death (LIU et al., 2021).

However, Cd-tolerant plants can develop mechanisms to mitigate the toxic effects of this metal, such as the activation of the antioxidant enzymes superoxide dismutase (SOD) and guaiacol peroxidase (POD) to try to reestablish the balance inside of these plants (ZHAO et al., 2021). Thus, understanding the morpho-physiological and biochemical responses of plants exposed to heavy metals is paramount to assisting in the recovery of contaminated areas.

Developing and applying technologies involving physical and chemical processes to remediate Cd-contaminated soils tend to be costly and result in harmful changes to soil functions and properties. Therefore, a viable alternative for soil decontamination would be phytoremediation, a practical, economical, and environmentally ecological tool (CHAMBA-ERAS et al., 2022).

Thus, the selection of plant species to be used in phytoremediation is fundamental, and tree species represent a viable alternative in this context because, in addition to the risk of contamination via the food chain is almost null, these organisms are generally more tolerant to metal toxicity (JANG et al., 2020). Woody crops can immobilize metals absorbed in plant tissues for a longer period, delaying their return to the soil (CHAMBA-ERAS et al., 2022).

Among the potential species for use in polluted areas is *Enterolobium contortisiliquum* (Vell.) Morong. It belongs to the Leguminosae family and is popularly known as “timbaúva”, “tamburil”, and “orelha de macaco” (SILVA et al., 2018). In addition to presenting fast initial growth, it is highly effective in the regeneration of polluted areas (BRASIL, 2011). In studies involving tree species and metals such as copper, aluminum, and zinc, *E. contortisiliquum* showed excellent results (SILVA et al., 2018; PRESOTTO et al., 2018). However, no studies to date explain the biochemical and physiological behavior of this species when exposed to Cd.

Thus, we hypothesized that *E. contortisiliquum* seedlings adopt cadmium tolerance mechanisms to mitigate its toxic effects, preventing or alleviating the negative effects on plant growth and simultaneously inhibiting oxidative damage. Therefore, we aimed to evaluate Cd tolerance in *Enterolobium contortisiliquum* seedlings, evaluating the effects of Cd on morphophysiological and biochemical variables to determine its potential as a phytoremediation species.

MATERIAL AND METHODS

Study area and experiment

The experiment was conducted in the Laboratory of Plant Physiology and Nutrition and the greenhouse of the Biology Department of the Federal University of Santa Maria, located in Santa Maria, Rio Grande do Sul (RS). Santa Maria is located between 29°43'15” S and 53°43'18” W, in the physiographic region of the Central Depression of the state of Rio Grande do Sul. The climate is classified as Cfa-type according to Köppen and has an altitude of approximately 90 meters (ALVARES et al., 2013).

Seeds of *Enterolobium contortisiliquum* were obtained from the Forest Research Center of the Department of Agricultural Diagnosis and Research (DDPA) in Santa Maria, RS.

Firstly, seeds underwent acid scarification in sulfuric acid (H_2SO_4) for 40 minutes (SILVA et al., 2014) to overcome dormancy. Subsequently, the seeds were taken to the germination room at 25°C ± 1 and photoperiod of constant light. Afterward, the seeds were placed in Petri dishes containing germitest® paper moistened (2.5 times its weight) with distilled water until radicle emission.

Subsequently, the seeds were sent to the greenhouse, organized in groups of 30 units, and sown in plastic trays (38 cm x 56 cm) as cultivation containers. Carolina Soil® substrate composed of *Sphagnum* sp. and vermiculite plus 30% of carbonized rice husk was used to produce the seedlings of the study.

The seedlings were irrigated daily, and every five days, Hoagland’s nutrient solution (50 ml per seedling) was added with pH 4.5 ± 0.1, composed of (µmol L^{-1}): 6090.5 of N; 974.3 of Mg; 4986.76 of Cl; 2679.2 of K; 2436.2 of Ca; 359.9 of S; 243.592 of P; 0.47 of Cu; 2.00 of Mn; 1.99 of Zn; 0.17 of Ni; 24.97 of B; 0.52 of Mo; 47.99 of Fe ($FeSO_4$/Na-EDTA). Nutrients sources added were NH_4Cl, $MgSO_4.7H_2O$, $MgCl_2.6H_2O$, KH_2PO_4, KCl, $Ca(NO_3)_2.4H_2O$, $CuSO_4.5H_2O$, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $NiSO_4.6H_2O$, H_3BO_3, $H_2MoO_4.H_2O$ and FeSO4.7H_2O.

When the seedlings measured approximately 10 cm in height (60 days after sowing), they were selected considering the shoot and root growth homogeneity, then transferred to the hydroponic system.

The hydroponic system consisted of 20 plastic trays (16L) with 16 seedlings each, containing Hoagland’s nutrient solution (HOAGLAND; ARNON, 1950), and kept under constant aeration. Seedlings were left to acclimate in Hoagland's nutrient solution for seven days at 100% of its original concentration and electrical conductivity of 1.9 dS m^{-1} (DELDEN; NAZARIDELJOU; MARCELIS, 2020). After this period, five Cd concentrations were added (as $CdCl_2.H_2O$): 0, 25, 50, 75, and 100 µM, equivalent to 2.81, 5.62, 8.43, and 11.24 mg L^{-1} Cd, respectively. The concentrations were defined based on the scientific literature (DORNELES et al., 2019; PEREIRA et al., 2018) and preliminary tests. The experiment was conducted in a completely randomized design with four replications. The solutions were changed every seven days, and the pH was adjusted to 4.5 ± 0.1, a value also based on the scientific literature (DORNELES et al., 2019; PEREIRA et al., 2018). Plants were exposed to the treatments for ten days, a period needed for the plants to show visual symptoms of toxicity, especially at the highest concentration of Cd (100 µM L^{-1}).

Morphophysiological variables

After the acclimatization period and before treatment application, taproot length and shoot height in four plants per experimental unit was measured with a millimeter ruler, totaling 16 plants per treatment. After ten days of exposure to Cd, the shoot height and taproot length were measured in the same plants to determine the increase in both traits.

Subsequently, these plants were collected and sectioned, and the roots went through a digitalization process with the aid of the Epson 11000XL scanner, where the images were analyzed in WinRHIZO Pro software to assess root surface area (cm^2 $plant^{-1}$) and total root length (cm $plant^{-1}$). After this process, shoots and roots were placed in an air-forced circulation oven at 65°C until a constant weight was reached. Lastly, samples were weighed on a precision analytical balance to estimate root and shoot dry weight.

Photosynthetic variables

After seven days of treatment exposure, the physiological variables related to the photosynthetic apparatus were analyzed in one plant from each tray. This was done in the morning, between 8 am and 11 am, with a portable infrared CO_2 meter (LI-COR, model LI-6400XT). The following measures were taken: net CO_2 assimilation rate/ photosynthetic rate (A), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), transpiration rate (E), water use efficiency (WUE), and Rubisco carboxylation efficiency (A/Ci) at an ambient CO_2 concentration of 400 µmol mol^{-1} at 20-25°C, 50 ± 5% relative humidity and a photon flux density of 1500 µmol m^{-2} s^{-1}.

Biochemical variables

After ten days of treatment exposure, in addition to the seedlings used for growth analysis, the others were also collected and separated into shoots and roots. This fresh material was frozen in an ultrafreezer (-80°C) for subsequent maceration in nitrogen liquid. This material was then used to determine pigment concentration, membrane lipid peroxidation, guaiacol peroxidase and superoxide dismutase activity, and hydrogen peroxide content.

The concentration of pigments (chlorophyll *a*, *b,* and carotenoids) was measured using fresh leaf samples, following the Hiscox and Israelstam (1979) method, and the results were estimated by the Lichtenthaler equation (1987). Total chlorophyll is the sum of chlorophyll *a* + chlorophyll *b*. Membrane lipid peroxidation was determined according to the method described by El-Moshaty et al. (1993), where the degree of lipid peroxidation was given as nmol malondialdehyde (MDA) mg^{-1} of protein.

According to Zeraik, Souza, and Fatibello-Filho (2008), guaiacol peroxidase (POD) activity was determined using guaiacol as a substrate. A molar extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ was used for the calculation, and the results were expressed in enzyme units per mg of protein (U mg^{-1} protein). The spectrophotometric method described by Giannopolitis and Ries (1977) served as the basis for measuring superoxide dismutase (SOD) activity, where one unit of SOD is defined as the amount of enzyme that inhibits the photoreduction of NBT by 50% (BEAUCHAMP; FRIDOVICH, 1971).

The analysis of hydrogen peroxide content was performed according to the method described by Loreto and Velikova (2001), where the evaluation of H_2O_2 concentration in each sample was performed by comparing the absorbance curve to a standard calibration curve, and the concentration was expressed by µmol g^{-1} fresh weight.

Statistical analysis

The data were checked for the normality of errors by the Shapiro-Wilk test and the homogeneity of variances by the Bartlett test. Given these assumptions, the effect of Cd concentrations was analyzed using regression analysis, with only the equations whose coefficients of the highest degree were significant ($p < 0.05$) being presented using the Sisvar software (FERREIRA, 2019).

RESULTS AND DISCUSSION

The taproot length values decreased linearly with increasing Cd concentrations ($p \leq 0.05$) (Figure 1a). The severe effect of Cd on shoot and root dry weight was also observed (Figures 1b and 1c). This decrease in growth may be associated with the direct harmful effect of Cd on cells or the reduction in the uptake of mineral nutrients such as Zn, Cu, and Ca (RIZWAN et al., 2017). This is due to increased competition between Cd and mineral elements at the root surface during plant uptake, reducing root growth and biomass production (BELGHITH et al., 2016).

It was observed that the surface area of roots responded quadratically to the Cd concentrations in the nutrient solution, with a slight increase in lower Cd concentrations and subsequent reduction of this variable in higher concentrations. The total length of roots reduced linearly with exposure to Cd (Figures 1d and 1e). This response may have occurred due to decrease in the number of secondary roots emitted by *E. contortisiliquum* seedlings, indicating a root system with less adaptability to Cd stress. This suggests that *E. contortisiliquum* seedlings are sensitive to this metal. Thus, root morphological parameters are essential in selecting potential species for the phytoremediation of contaminated soils. This is because Cd-tolerant plants tend to present tolerance mechanisms, preventing the harmful effect of Cd on root length and surface area. This allows plants to continue exploring a greater soil volume looking for less contaminated sites. With this, the plants will be able to adapt to soils with higher Cd levels, which is desired. In this work, analyzing the morphological variables, it was possible to observe that the species *E. contortisiliquum* does not present these mechanisms of tolerance to Cd.

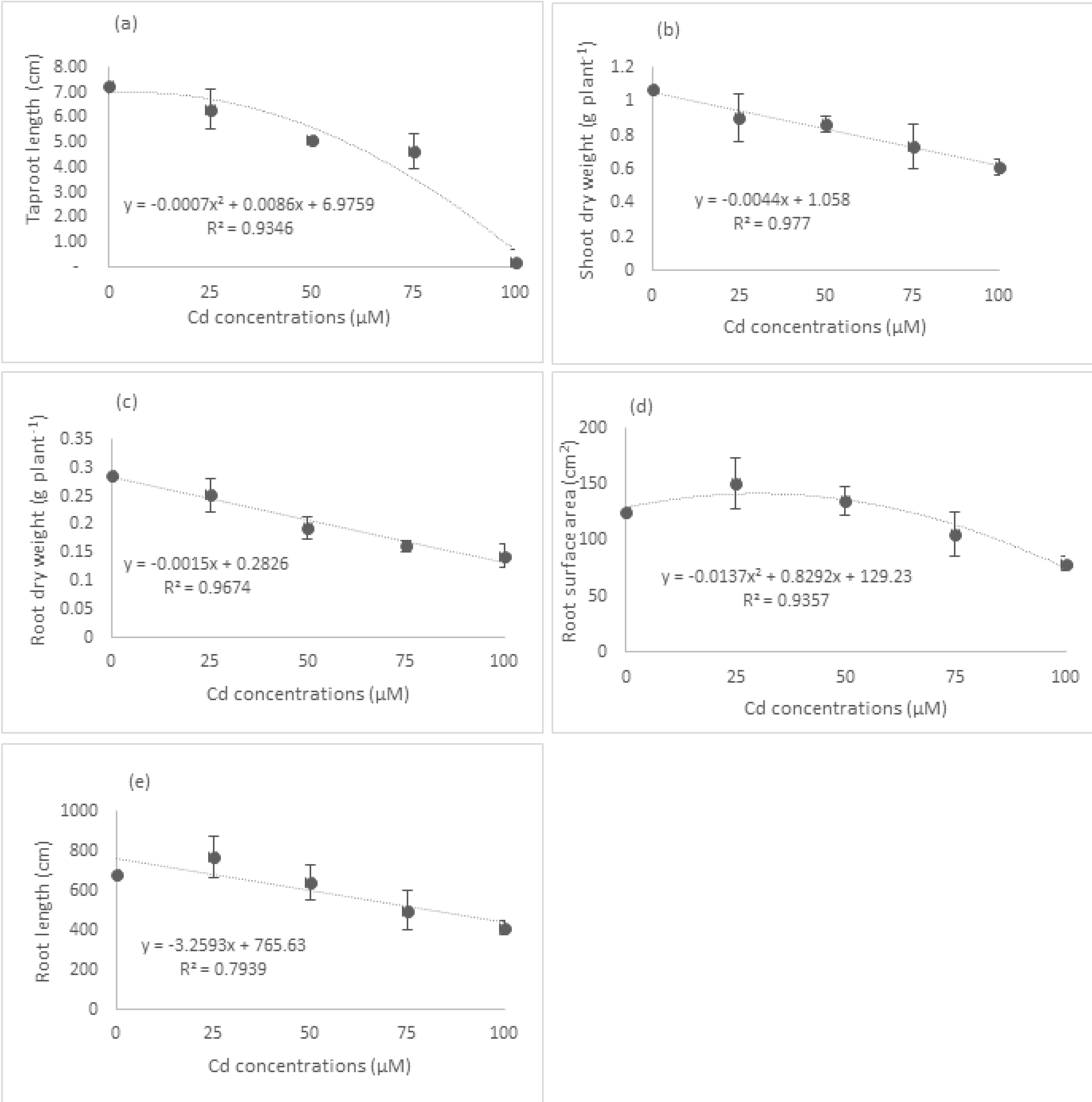

Figure 1. Mean values recorded for the increase in taproot length (a), shoot dry weight (b), root dry weight (c), root surface area (d), and root length (e) of *Enterolobium contortisiliquum* seedlings grown in different Cd concentrations.

Plants adjust their allocation and relative biomass distribution in organs when subjected to stress conditions, referred to as allocation plasticity (ZHANG et al., 2020). As the stress caused by heavy metals affects growth, there is also a consequent change in basic processes such as photosynthesis and respiration (ZHAO et al., 2021). Thus, changes in plant growth under heavy metal contamination can be attributed to reduced net photosynthetic rate.

Rubisco photosynthetic rate and carboxylation efficiency results responded quadratically to Cd concentrations, where the lowest values were observed at 50 and 100 µmol L^{-1} Cd (Figures 2a and 2b). This was because Cd^{2+} ions may have bound to the functional groups of some enzymes (e.g., sulfhydryl–SH groups) and replaced essential metal elements in these proteins. This type of replacement can change the conformation of biological macromolecules and inhibit activity, decreasing photosynthetic capacity (HUIHUI et al., 2020). In addition, Cd ions decrease ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) activity and directly damage its structure by replacing Mg ions, which are essential cofactors in carboxylation reactions, and changing Rubisco activity for oxygenation reactions (CONCEIÇÃO et al., 2020).

Total chlorophyll values decreased linearly with the addition of Cd to the nutrient solution ($p \leq 0.05$) (Figure 2c). Furthermore, a reduction in carotenoid values was observed at higher Cd concentrations (Figure 2d). Possible reasons for the decrease in major photosynthetic pigments under Cd stress include oxidative stress, the direct influence of metal ions on pigment biosynthesis pathways, and the replacement of Mg^{2+} by metal ions within the chlorophyll molecule (BAMAGOOS; ALHARBY; ABBAS, 2022). The reduction in pigment levels can be used to indicate the harmful effects induced by Cd, as the decrease in pigments is one of the main factors for the reduction of photosynthesis and growth (LI et al., 2016).

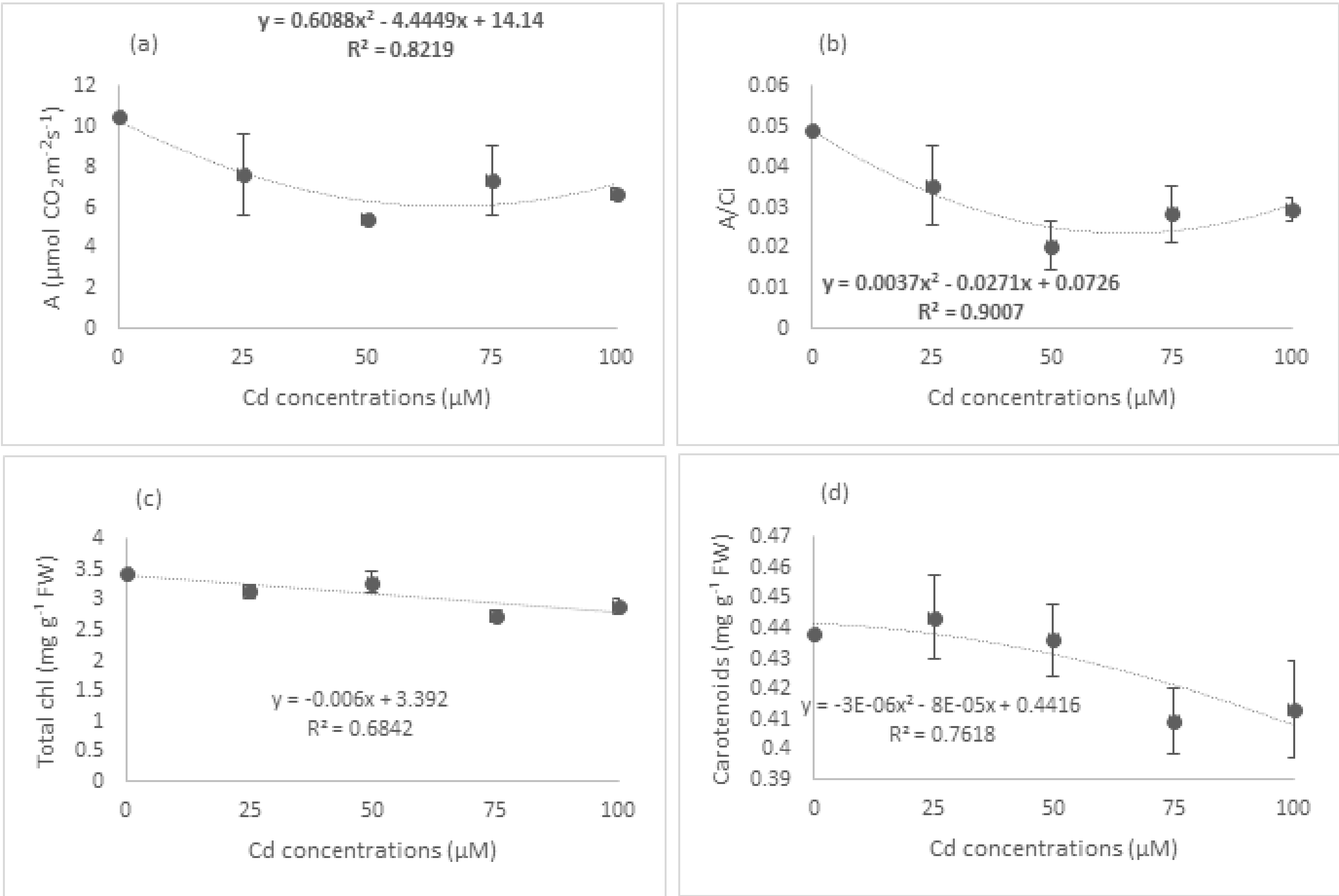

Figure 2. Mean values recorded for net CO_2 assimilation rate (A) (a), instantaneous carboxylation efficiency (by Rubisco) (A/Ci) (b), total chlorophyll (c), and carotenoids (d) of *Enterolobium contortisiliquum* seedlings grown in different Cd concentrations.

Excess heavy metals stimulate plants to produce more reactive oxygen species (ROS), which can react with lipids, proteins, nucleic acids, and other substances, causing lipid peroxidation and membrane damage, thus affecting cell performance and viability (ZHAO et al., 2021). To deal with the stress caused by ROS, one of the possible strategies used by plants is the activation of the antioxidant enzyme system, such as superoxide dismutase (SOD) and guaiacol peroxidase (POD) (LIU et al., 2021).

SOD and POD are considered the main antioxidant enzymes that play critical roles in eliminating ROS and maintaining homeostasis in plant cells (SHAARI et al., 2024). SOD catalyzes the dismutation of the superoxide radical into O_2 and H_2O_2. POD helps in the conversion of H_2O_2 into water and oxygen by dissociating H_2O_2, playing a key role in providing plant tolerance to unfavorable conditions (KUINCHTNER et al., 2021).

In general, SOD and POD activity in leaves increased quadratically ($p \leq 0.05$) with increasing Cd availability in the nutrient solution. This occurs because some plants subjected to stress by heavy metals tend to gradually increase the activity of antioxidant enzymes with the increase in the concentration of these toxic metals (GUTIÉRREZ-MARTÍNEZ et al., 2020), aiming to combat the oxidative stress caused by excess metal. But, when the concentration of heavy metals becomes too high, the protective enzyme system can be damaged or inhibited, and the enzyme activity decreases (DORNELES et al., 2019). However, only a reduction in shoot H_2O_2 concentrations was observed at low Cd concentrations (Figure 3d), while at higher concentrations, H_2O_2 levels were similar to the control treatment. This result may be linked to POD activity in the shoot, which increased its activity with increasing Cd concentrations in the nutrient solution (Figure 3c). Thus, POD was activated to alleviate oxidative stress promoted by Cd and dismutate and/or transform ROS molecules into less reactive forms (LI et al., 2016; RIZWAN et al., 2017).

The POD enzyme activity in the roots did not show a significant difference regardless of the applied Cd concentrations (data not shown). On the other hand, the SOD enzyme in the roots showed less activity at intermediate Cd concentrations (Figure 3b). This suggests that Cd stress caused damage to the internal balance of the roots of these plants, which may have triggered the inhibition of the SOD enzyme. On the other hand, the H_2O_2 content in the roots showed a linear reduction upon exposure to Cd ($p \leq 0.05$) (Figure 3e), making it possible to infer that the low activity of the SOD enzyme may also be related to the low content of H_2O_2 in the roots. In addition, other peroxidase enzymes may be acting in the plants, being responsible for this reduction in H_2O_2 concentrations since the POD enzyme was not activated in this organ.

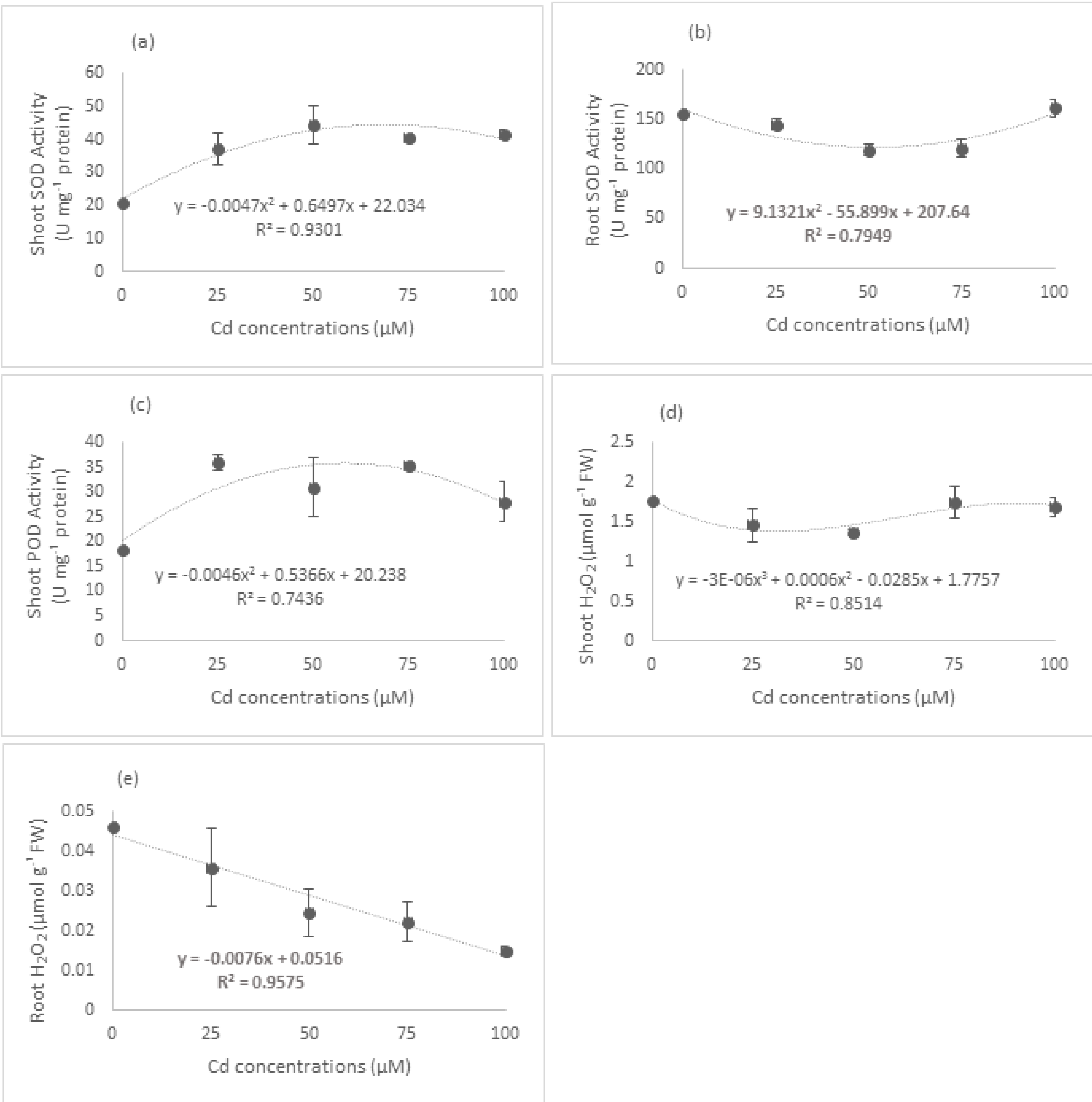

Figure 3. Mean values recorded for superoxide dismutase (SOD) activity in shoots (a) and roots (b), guaiacol peroxidase (POD) activity in shoots (c), and hydrogen peroxide (H_2O_2) concentration in shoots (d) and roots (e) of *Enterolobium contortisiliquum* seedlings grown in different Cd concentrations.

Among ROS, H_2O_2 is one of the most stable and versatile molecules, which plays a dual role concerning oxidative stress. In low concentrations, H_2O_2 plays a signaling role (KIJOWSKA-OBERC; STASZAK; RATAJCZAK, 2021), while in high concentrations, H_2O_2 can cause toxicity and participate in the metabolism in an oxidative way, which triggers lipid peroxidation related to increased levels of malondialdehyde (MDA) (KUINCHTNER et al., 2021).

MDA is an oxidized product of membrane lipids and accumulates when plants are exposed to oxidative stress (ZHAO et al., 2021). MDA can serve as a biomarker of membrane integrity and lipid peroxidation, and it has been used to assess plant oxidative damage caused by metal pollutant stress (LI et al., 2016).

However, no significant difference was observed for MDA levels in roots and shoots of *E. contortisiliquum* compared to the control (data not shown). This was possible because the rate of ROS elimination in plants exposed to Cd was faster than accumulation, preventing the increase in MDA levels and subsequent damage to the cell membrane. Thus, antioxidant enzyme activity was consistent with changing MDA levels, suggesting that SOD and POD played an important regulatory role. However, it was observed that this maintenance of homeostasis had a cost for *E. contortisiliquum*. This cost can be verified in the reduction of dry mass production in these plants, showing that the plant possibly needed more energy to maintain the antioxidant system.

Due to the high degree of toxicity and the different sources and means of contamination by Cd, numerous countries worldwide have established maximum values of this metal in the most diverse situations through resolutions. In Brazil, the National Environment Council established such values through Resolution nº 420/2009 (BRASIL, 2009). The soil is at risk from 1.3 mg kg^{-1} of Cd, and 3 mg kg^{-1} is classified as contaminated. The average level of Cd in Brazilian soils considered uncontaminated is 0.18 mg Kg^{-1}, and contaminated soils worldwide range from 5.9 to 531 mg Kg^{-1}, depending on the cause of the pollution (KUBIER; WILKIN; PICHLER, 2019). In this study, the lowest concentration of Cd used was 25 µmol L^{-1}, corresponding to 2.81 mg L^{-1} Cd.

Therefore, it is possible to observe that, even with the activation of the antioxidant enzymes, the *E. contortisiliquum* plants were not able to defend themselves against the toxic effects caused by the excess of Cd, revoking our initial hypothesis. Therefore, *E. contortisiliquum* seedlings can be used as bioindicators of Cd-contaminated soils. Using plants that are bioindicators of toxic metals makes it possible to verify the impact of pollution on the soil, thus enabling the evaluation of additive and synergistic effects of metals. In addition, it is possible to detect cadmium stress at low pollution levels and can act for prolonged periods.

CONCLUSION

Even with the activation of antioxidant enzymes, cadmium concentrations negatively affected the photosynthetic pigments and photosynthetic rate of *Enterolobium contortisiliquum*. This resulted in a reduction in biomass production and photosystem functions, indicating its sensitivity to excess Cd. Because of these characteristics, *E. contortisiliquum* seedlings can be used as a bioindicator for cadmium-contaminated areas.

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