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The role of reactive oxygen species and nitric oxide in the formation of root cortical aerenchyma under cadmium contamination

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Abstract

The present study aimed to evaluate root cortical aerenchyma formation in response to Cd-driven hydrogen peroxide (H_2O_2) production and the role of nitric oxide (NO) in the alleviation of Cd oxidative stress in maize roots and its effects on aerenchyma development. Maize plants were subjected to continuous flooding for 30 days, and the following treatments were applied weekly: $Cd(NO_3)_2$ at 0, 10, and 50 μM and $Na_2[Fe(CN)_5NO]\cdot 2H_2O$ (an NO donor) at 0.5, 0.1, and 0.2 μM. The root biometrics; oxidative stress indicators H_2O_2 and malondialdehyde (MDA); and activities of catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) were analyzed. The root dry and fresh masses decreased at higher concentrations of NO and Cd. H_2O_2 also decreased at higher NO concentrations; however, MDA increased only at higher Cd levels. SOD activity decreased at higher concentrations of NO, but CAT activity increased. Aerenchyma development decreased in response to NO. Consequently, NO acts as an antagonist to Cd, decreasing the concentration of H_2O_2 by reducing SOD activity and increasing CAT activity. Although H_2O_2 is directly linked to aerenchyma formation, increased H_2O_2 concentrations are necessary for root cortical aerenchyma development.

1 | INTRODUCTION

Studies have investigated the development of root cortical aerenchyma in plants under the effects of flooding (Pereira et al., 2008; Pereira et al., 2010), soil compaction (Bergamin et al., 2010; Scapinelli et al., 2016), drought, phosphorus deficiency (Díaz et al., 2018), mineral nutrition (Coelho et al., 2006; Postma & Lynch, 2011; Steffens & Rasmussen, 2016), and Cd contamination (Li et al., 2019). The development of aerenchyma is an adaptation that allows plants to better tolerate low oxygen availability, facilitates the internal diffusion of gases (Duarte et al., 2021), and reduces metabolic costs associated with growth (Díaz et al., 2018). Moreover, the formation of aerenchyma tissue is induced by the death of cortical cells (Yamauchi et al., 2017).

The formation of lysigenous aerenchyma is stimulated by hypoxia through the production of ethylene, which induces programmed cell death (Pires et al., 2015; Yamauchi et al., 2017). However, the decrease in oxygen induces the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2; Rodríguez-González et al., 2020). At low levels, ROS act as signaling agents in stress responses, but at high concentrations, they can cause oxidative stress (Romero-Puertas et al., 2019). Environmental stress can lead to metabolic imbalance in plants (Liu et al., 2019), resulting in the production of unstable and highly reactive molecules such as H_2O_2 and nitric oxide (NO) (Barreiros et al., 2006).

Maize plants can react to excessive increases in ROS levels by activating their antioxidant system, which protects plants from cell damage (Pereira et al., 2010). Enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) are among 63 enzymatic antioxidant components that reduce oxidative stress (Moller et al., 2007). Notably, the antioxidant system of plants also

includes thioredoxins, which are small redox proteins that reduce oxidative stress caused by metals, including Cd (Kumari et al., 2021; Smiri et al., 2012). Other molecules are involved in the antioxidant system, such as glutaredoxins, which are involved in mitigating glutathione oxidative stress (Yadav et al., 2012). The presence of oxidative stress caused by abiotic factors such as Cd may also be the result of anthropogenic activities (Edelstein & Ben-Hur, 2018), affecting physiological, metabolic, and biochemical processes and causing structural changes in plants (Kasim, 2006). Cadmium can reduce the growth of non-tolerant species, but tolerant species can maintain their photosynthesis activity and anatomical structure (Pereira et al., 2016). For example, the leaf aerenchyma of the Cd-tolerant species *Typha domingensis* is not altered by Cd (Oliveira et al., 2018). However, Cd is toxic to maize plants, decreasing their growth and causing anatomical changes (Cunha et al., 2008).

Nitric oxide is a gaseous molecule capable of diffusing through cell membranes (Rodríguez-González et al., 2020) and plays a role in protection against oxidative stress, in signaling, and in response to environmental stress (Beligni & Lamattina, 1999). Nitric oxide can help decrease the effects of heavy metal toxicity (Arasimowicz-Jelonek et al., 2011; Nabaei & Amooaghaie, 2020), as it has been shown to reduce the accumulation of both lead in sesame (Amooaghaie et al., 2017) and arsenic in rice (Singh et al., 2017). Nitric oxide plays an important role in regulating ROS toxicity (Pires et al., 2016) by increasing the activity of antioxidant enzymes (Terrón-Camero et al., 2019). Moreover, ROS and NO are necessary for the activation of programmed cell death (Delledonne et al., 1998). According to Wany et al. (2017), NO and ethylene play an important role in the formation of lysigenous aerenchyma. Decreased levels of NO can, in turn, reduce aerenchyma formation in plants grown under relatively high ammonium concentrations (Wang et al., 2013).

Nitric oxide is an important multifunctional and cytoprotective molecule involved in various physiological, anatomical, and biochemical processes in plants. In this study, we hypothesized that H_2O_2 stimulates aerenchyma formation, while NO acts as an antagonist in this process by reducing ROS formation. The objective of this study was to evaluate the role of Cd in the production of H_2O_2 and its relationship with aerenchyma formation and root growth by the use of NO as an antagonist of oxidative stress in maize roots and cortical aerenchyma formation.

2 | MATERIALS AND METHODS

The plant material used was BRS 4154 Saracura maize, which is considered a flood-tolerant maize variety. Seeds were provided by Embrapa Maize and Sorghum, Sete Lagoas, Minas Gerais, Brazil. The experiment was performed in a greenhouse of the Biology Department of the Federal University of Lavras, Minas Gerais, Brazil (21°14′43″S, 45°59′59″W).

The seeds were germinated in vermiculite at room temperature under constant light and irrigated to field capacity. Seedlings that were approximately 10 cm tall were transplanted into plastic pots filled with 3 L of vermiculite and irrigated to field capacity for 15 days to allow the seedlings to acclimate. Afterward, the substrate was flooded such that a water layer was formed 1 cm above the vermiculite layer. Both $Cd(NO_3)_2$ at 0, 10, and 50 μM and $Na_2[Fe(CN)_5\ NO]\cdot 2H_2O$ (an NO donor) at 0.05, 0.1, and 0.2 μM were applied weekly. A nutrient solution (40% strength) was also applied weekly (Hoagland & Arnon, 1950). The experimental design was completely randomized in a 3 × 3 factorial arrangement with five replicates.

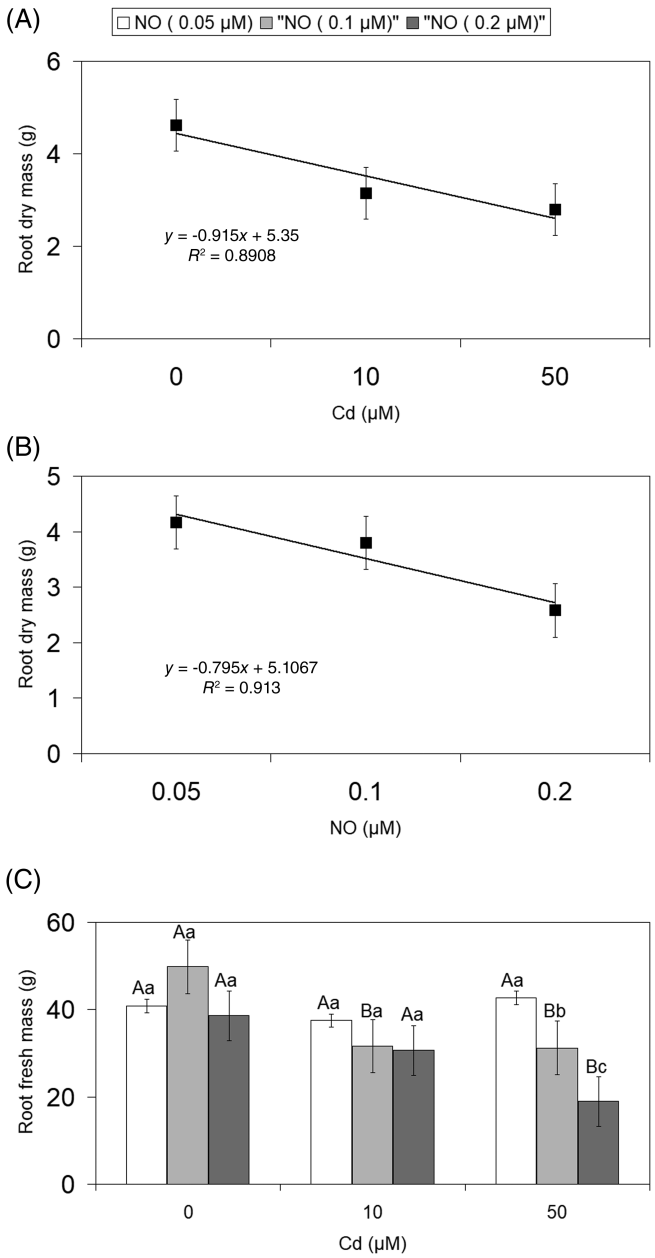

FIGURE 1 Biometrics of maize roots subjected to different concentrations of Cd (A) and NO (B) and the interactions of these factors (C). The means followed by the same letter do not differ according to the Scott–Knott test at $P \leq 0.05$ (A,B). The means followed by the same uppercase letter (Cd) and lowercase letter (NO) do not differ according to the Scott–Knott test at $P \leq 0.05$ (C). Bars: standard errors

After 30 days of the experiment, the roots were collected, and the fresh mass was measured on an analytical balance. Subsequently, the roots were dried in an oven at 60°C for 72 h, and the dry mass was measured on an analytical balance. A portion of the fresh roots was flash frozen in liquid nitrogen and then stored at −80°C until biochemical analyses, and whole roots were fixed in 70% formaldehyde: acetic acid:70% ethanol (FAA; 0.5:0.5:9.0 v/v) for 72 h (Johansen, 1940) and stored in 70% ethanol until anatomical analysis.

Afterward, 200 g of roots was macerated in a 1500 µl solution consisting of polyvinylpolypyrrolidone (PVPP) and 0.1% trichloroacetic acid (TCA). The sample was subsequently centrifuged at 12 000*g* for 15 min at 4°C, after which the supernatant was collected and stored at −20°C until analysis.

For quantification of H_2O_2, 45 µl of the supernatant was collected and added to 45 µl of 10 mM potassium phosphate buffer (pH 7) and 90 µl of 1 M potassium iodide. A standard curve of H_2O_2 was prepared via a solution of 250 µM H_2O_2. The samples were evaluated in duplicate via a spectrophotometer at 390 nm (Velikova et al., 2000).

For the quantification of malondialdehyde (MDA), 125 µl of the supernatant was added to 250 µl of a 0.5% thiobarbituric acid and 10% TCA solution and then incubated at 95°C for 30 min. The samples were placed in a plate in duplicate, the reaction was stopped by rapid cooling, and the absorbance at 535 and 600 nm was measured by a spectrophotometer (Buege & Aust, 1978).

For the extraction of CAT, APX, and SOD enzymes, 200 mg of roots was macerated in liquid nitrogen and PVPP, and the material was added to 1500 µl of extraction buffer (400 mM potassium phosphate, pH 7.8 [375 µl], 10 mM EDTA [15 µl]; 200 mM ascorbic acid [75 µl]; and distilled water [1035 µl]). The solution was then centrifuged at 13000*g* for 10 min at 4°C, after which the supernatant was stored at −20°C (Biemelt et al., 1998).

CAT activity was evaluated via a solution consisting of 200 mM potassium phosphate buffer (pH 7.0; 90 µl), distilled water (72 µl), and 250 mM H_2O_2 (9 µl) per reaction. Six microliters of the sample solution was pipetted in triplicate, and the absorbance at 240 nm was measured by a spectrophotometer every 15 s for 3 min (Havir & McHale, 1987).

APX activity was evaluated via a solution consisting of 200 mM potassium phosphate buffer (pH 7.0; 90 µl), 10 mM ascorbic acid (9 µl), distilled water (63 µl), and 2 mM H_2O_2 (9 µl). Six microliters of the sample solution was pipetted, and the absorbance at 290 nm was measured by a spectrophotometer every 15 s for 3 min (Nakano & Asada, 1981).

SOD activity was evaluated via a solution consisting of 100 mM potassium phosphate buffer (pH 7.8; 100 µl), 70 mM methionine (40 µl), 10 µM ethylenediaminetetraacetic acid (EDTA; 3 µl), distilled water (30 µl), 1 mM nitro blue tetrazolium (NBT; 15 µl), and 0.2 mM riboflavin (2 µl). Six microliters of the sample solution was added in triplicate. The samples were then illuminated for 7 min, and the absorbance at 560 nm was measured by a spectrophotometer (Giannopolitis, 1977).

For anatomical analyses, cross-sections of the piliferous region of the roots (10 cm from the apex) were obtained manually. The sections were cleared in 50% sodium hypochlorite (v/v), washed in distilled water for 10 min, and stained in safranin-Astra blue staining solution

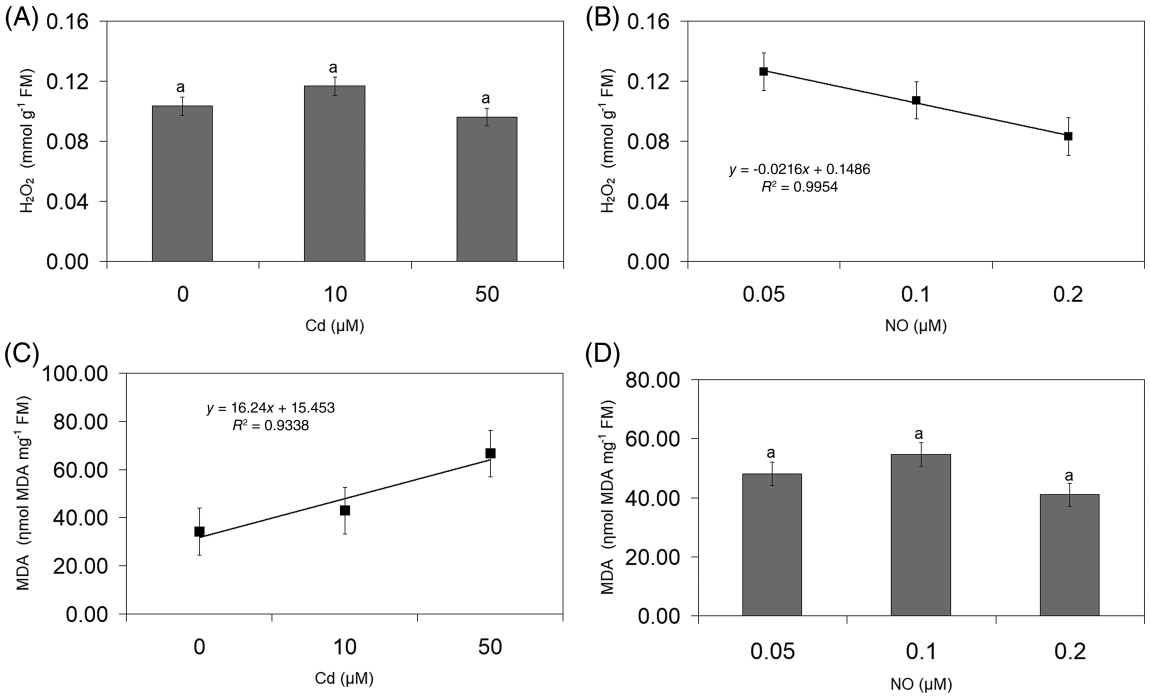

FIGURE 2 Indicators of oxidative stress in Saracura maize roots subjected to different concentrations of Cd (A,C) and NO (B,D). The means followed by the same letter do not differ according to the Scott–Knott test at $P \leq 0.05$. Bars: standard errors

(1% safranin and 0.1% Astra blue at a ratio of 3:7). Semipermanent slides were prepared with 50% glycerol (Kraus & Arduim, 1997).

One slide was prepared per replicate, and nine fields from nine different sections were analyzed. Images were obtained with a CX31 microscope (Olympus) and analyzed using the ImageTool 3.0 software (UTHSCSA). The parameters evaluated included epidermal thickness, cortical thickness, proportion of cortex, endodermal thickness, xylem vessel diameter, proportion of vascular cylinder, and proportion of root aerenchyma.

The mean was calculated for each replicate when the data were obtained in duplicate or triplicate and for the different fields for the anatomical data. The data were then subjected to analysis of variance, and the means were evaluated according to the Scott–Knott test at a significant level of 0.05 or by regression via SISVAR 5.3 software (Ferreira, 2011).

3 | RESULTS

For root dry mass, there was no interaction effect between the evaluated factors (P >0.05), and Cd and NO reduced the root dry mass of maize (Figure 1A,B). With respect to fresh mass, there was an interaction between Cd and NO. At the 0.05 μM NO concentration, there was no effect of Cd on fresh mass; however, at the highest NO concentration, higher Cd concentrations reduced the maize root fresh mass (Figure 1C). Furthermore, at Cd concentrations of 0 and 10 μM, higher concentrations of NO did not affect the fresh mass, but at a Cd concentration of 50 μM, higher concentrations of NO reduced the maize root fresh mass (Figure 1C).

There was no interaction effect of Cd and NO concentrations with H_2O_2 or MDA content ($P \geq 0.22$). Cadmium did not promote significant changes in the H_2O_2 content (Figure 2A); however, higher NO

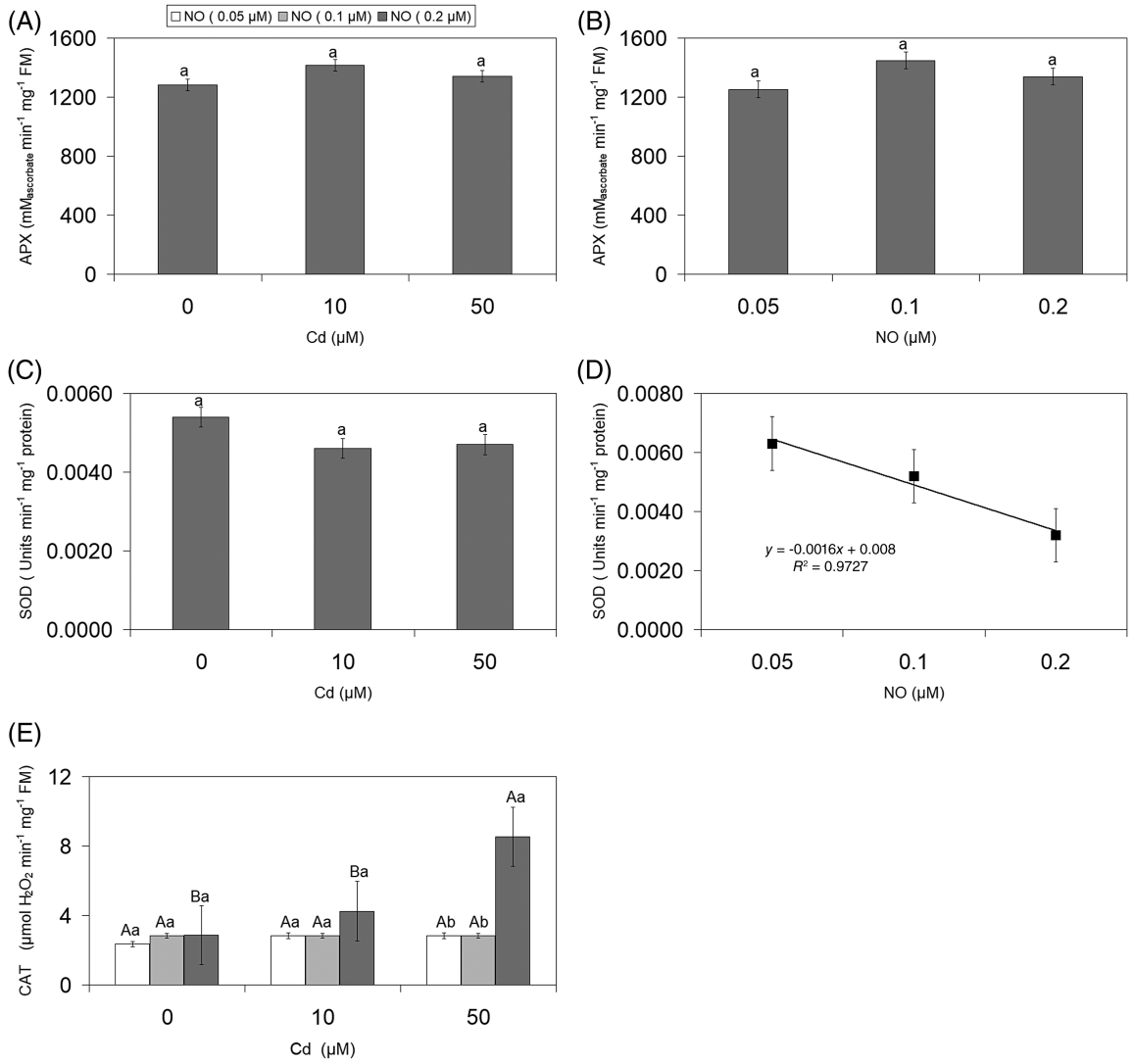

FIGURE 3 Activity of antioxidant enzymes in Saracura maize roots subjected to different concentrations of Cd (A,C) and NO (B,D) and the interactions of these factors (E). The means followed by the same letter do not differ according to the Scott–Knott test at $P \leq 0.05$ (A–D). The means followed by the same uppercase letter (Cd) and lowercase letter (NO) do not differ according to the Scott-Knott test at $P \leq 0.05$ (E). Bars: standard errors

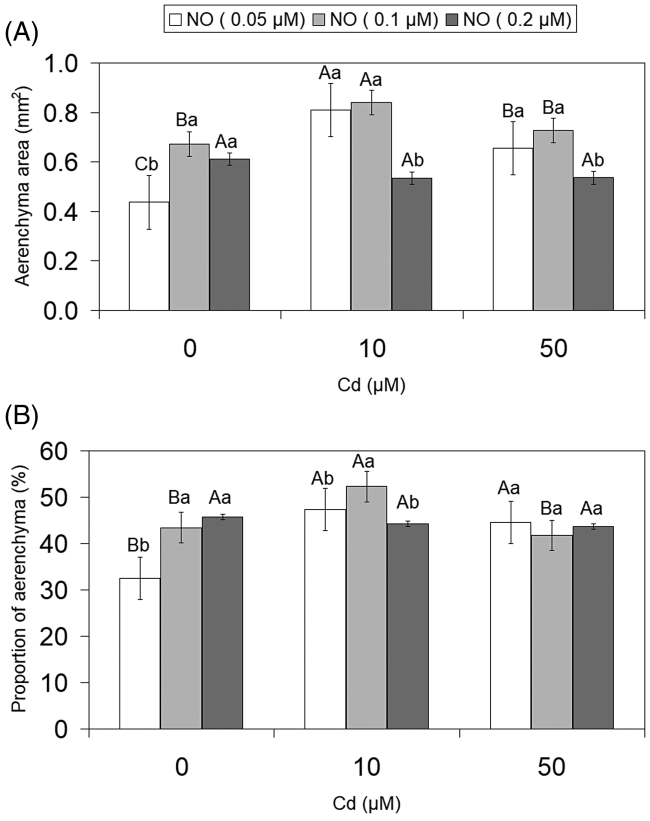

FIGURE 4 Alterations to the cortical aerenchyma of maize roots subjected to different concentrations of Cd and NO. The means followed by the same uppercase letter (Cd) and lowercase letter (NO) do not differ according to the Scott–Knott test at $P \leq 0.05$ (E). Bars: standard errors

concentrations gradually decreased H_2O_2 content (Figure 2B). The MDA content increased proportionally with increasing Cd concentration (Figure 2C), while NO had no significant effect on this parameter in maize roots (Figure 2D).

There was no interaction effect between Cd and NO concentrations on APX or SOD activity ($P \geq 0.59$), but there was an interaction effect on CAT activity ($P < 0.0001$). However, Cd and NO alone did not promote significant changes in APX activity (Figure 3A,B). Moreover, Cd did not significantly alter SOD activity (Figure 3C), but SOD activity decreased proportionally with increasing NO concentration (Figure 3D). In addition, Cd did not promote significant changes in CAT activity at NO concentrations of 0.05 and 0.1 μM but did increase enzyme activity at an NO concentration of 0.02 μM (Figure 3E). In addition, at Cd concentrations of 0 and 10 μM, increased NO concentration did not promote significant changes in CAT activity, but at 50 μM Cd, 0.02 μM NO increased CAT activity (Figure 3E).

All anatomical variables showed an interaction between Cd and NO. At an NO concentration of 0.05 μM, Cd promoted an increase in the aerenchyma area compared with that obtained in the absence of Cd (Figures 4A and 6). At a concentration of 10 μM, Cd increased the aerenchyma area at an NO concentration of 0.1 μM, but 50 μM Cd caused a reduction in this parameter to values similar to those found under 0 μM Cd (Figures 4A and 6). Interestingly, in the absence of Cd, higher NO concentrations increased the aerenchyma area, but in the presence of Cd, 0.2 μM NO reduced the aerenchyma area (Figures 4A and 6). Higher Cd concentrations also increased the proportion of aerenchyma under the lowest NO concentrations (0.05 and 0.1); however, 0.2 μM NO resulted in no increase in the proportion of aerenchyma promoted by Cd (Figures 4B and 6). Increasing concentrations of NO increased the proportion of aerenchyma in the absence of Cd; however, in the presence of 10 μM Cd, only 0.1 μM NO increased the proportion of aerenchyma, whereas, under 50 μM Cd, NO had no effect on this parameter (Figures 4B and 6).

Cadmium increased the epidermal thickness at the lowest NO concentration; however, under 0.2 μM NO, Cd reduced the epidermal thickness in maize roots (Figures 5A and 6). Higher NO concentrations increased the epidermal thickness in the absence of Cd but promoted changes in the presence of Cd, although the values were similar to those of the control (Figures 5A and 6). Higher Cd concentrations increased the thickness and proportion of the cortex at the lowest NO concentration; however, under 0.2 μM NO, Cd had no significant effect (Figures 5B,C and 6). Under 0.05 μM NO, increased concentrations of Cd did not significantly alter the cortical thickness, but Cd reduced the cortical thickness at the highest NO concentration (Figure 5B,C). Cadmium at 50 μM increased the endodermal thickness in maize roots at the lowest NO concentration; however, under 0.02 μM NO, there was no significant effect of Cd (Figures 5D and 6). In the absence of Cd, increased NO concentrations increased the endodermal thickness; however, in the presence of Cd, higher NO concentrations reduced the thickness (Figures 5D and 6). Cadmium increased the diameter of xylem vessels at the lowest NO concentrations but caused a reduction in this parameter under 0.2 μM NO (Figures 5E and 6). Nitric oxide did not alter the xylem vessel diameter in the absence of Cd; however, 0.2 μM NO caused a reduction in this parameter under 10 μM Cd, and 0.1 μM NO increased this variable under 50 μM Cd (Figures 5E and 6). Cadmium did not promote changes in the proportion of the vascular cylinder under the lowest NO concentration but did cause a reduction in this parameter at the highest concentration (Figures 5F and 6). Nitric oxide promoted an increase followed by a reduction in the proportion of the vascular cylinder at Cd concentrations of 0 and 50 μM but had no effect at a Cd concentration of 10 μM (Figures 5F and 6).

4 | DISCUSSION

Reductions in the fresh and dry mass of maize roots can be considered indicators of oxidative stress caused by the presence of high concentrations of Cd and NO. According to Boffe et al. (2017), Cd damages the cellular structure and hinders plant metabolism, negatively affecting the growth and development of the roots and shoots. However, Cd is allocated more to the root system than to other organs, reducing the amount of this pollutant that is allocated to the leaves (Rodríquez-Serrano et al., 2009). Cadmium toxicity affects root

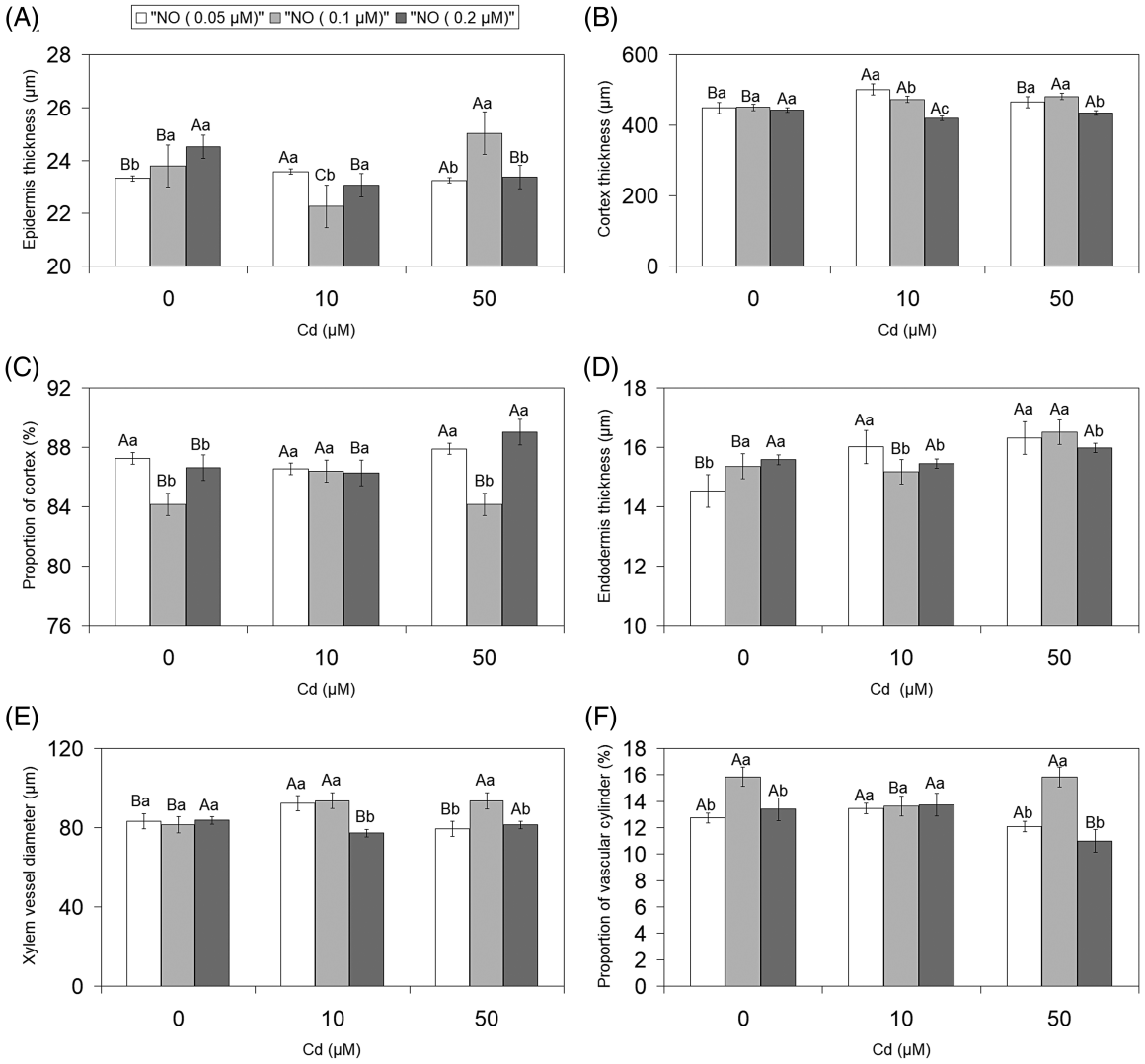

FIGURE 5 Anatomical changes in maize roots subjected to different concentrations of Cd and NO. The means followed by the same uppercase letter (Cd) and lowercase letter (NO) do not differ according to the Scott–Knott test at $P \leq 0.05$ (E). Bars: standard errors

and shoot biomass production, limiting plant growth and development (Boffe et al., 2017). Therefore, a reduction in root growth caused by the addition of Cd is an expected response in maize; however, at high concentrations, NO promoted a similar effect on dry mass.

Notably, NO at a concentration of 0.05 µM inhibited the effects of Cd toxicity on maize root fresh mass. Thus, NO at low concentrations can protect against the effects of Cd, while NO at high concentrations may act as a stressor of maize roots. The reduction in growth promoted by NO may be related to the reduced formation of root aerenchyma in maize plants, which helps to counteract hypoxic stress. According to Kotapati et al. (2017), the use of sodium nitroprusside as an NO donor inhibits nickel toxicity and increases the proportion of the fresh and dry mass of the roots and shoots of millet. Studies on rice conducted by Singh et al. (2017) and on wheat conducted by Tian and Lei (2006) showed that low NO concentrations stimulate growth and increase root biomass by reducing the production of ROS. According to Dusse et al. (2003), high NO concentrations are toxic to plants. Thus, the literature corroborates the results obtained from the present experiment involving maize, which showed that at low concentrations, NO can reduce the effects of Cd toxicity in roots but that at high concentrations, NO also causes stress; this is the first report of direct NO toxicity affecting root development. We proposed that the NO-driven growth reduction may be a secondary effect due to lower aerenchyma development because, under waterlogged conditions, this is a common response. For instance, Suralta and Yamauchi (2008) showed that rice genotypes with higher aerenchyma development exhibited higher root growth and development under waterlogged conditions. According to Mano and Omori (2013), *Zea nicaraguensis* genotypes with lower aerenchyma development showed lower root growth in flooded soils. Thus, our results indicate that NO reduced aerenchyma development, which in turn may have limited maize root growth under waterlogged conditions.

Cadmium did not significantly increase the H_2O_2 content in maize roots; this result is interesting and is related to the other evaluated

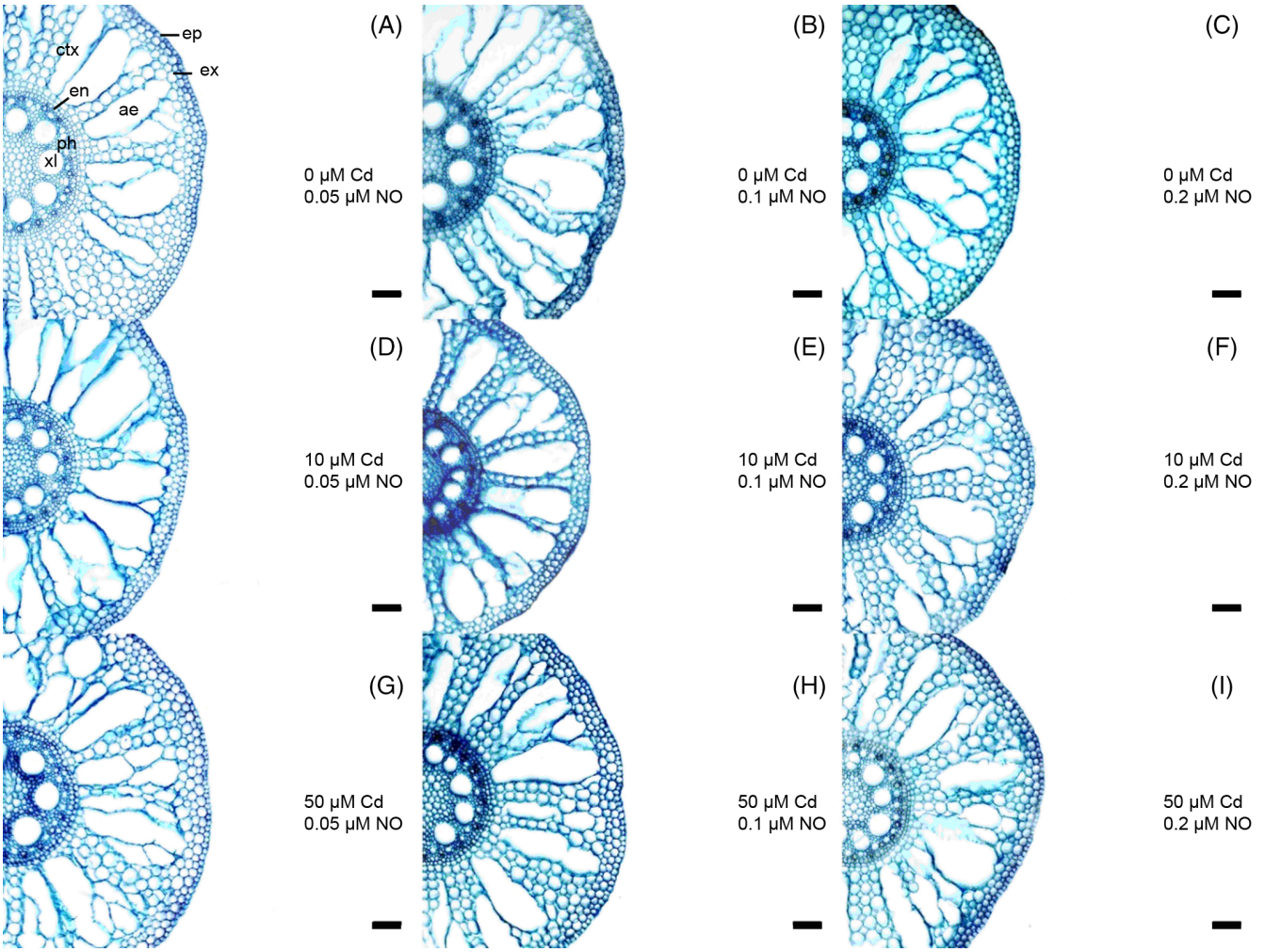

FIGURE 6 Transverse sections of roots from maize plants grown under different Cd and NO concentrations. ae, aerenchyma; ctx, cortex region; em, endodermis; ep, epidermis; ex, exodermis; ph, phloem; xl, xylem. Bars = 100 µm

parameters. Oxidative stress is caused not only by H_2O_2 but also by different ROS, such as superoxide ($O_2^{\cdot -}$), which is considered one of the main ROS produced indirectly in response to Cd (Gupta et al., 2017). Cadmium generates free radicals indirectly by binding to sulfhydryl residues, thus leading to the production of ROS (Waalkes, 2003). ROS inhibit enzyme activity and cause membrane damage, impairing the respiratory chain and subsequent growth (Gupta et al., 2017; Sandalio et al., 2012). In the present study, Cd promoted oxidative stress in maize roots because the MDA content increased significantly; MDA is related to the destruction of cell membranes by oxidative stress. Moreover, there was a significant reduction in root growth. According to Gutiérrez-Martínez et al. (2020), Cd increases the levels of MDA and H_2O_2 in bean plants as a result of oxidative stress. In the case of maize roots, the lack of an effect of Cd on H_2O_2 accumulation may be related to the presence of NO in all the treatments.

NO reduced the H_2O_2 content in maize roots but did not promote MDA accumulation. Notably, NO reduced oxidative stress by decreasing the H_2O_2 content in maize roots, indicating that the increase in MDA levels promoted by Cd is related to other ROS. According to Gadelha et al. (2017), NO also reduces the H_2O_2 content in *Jatropha curcas*. Nitric oxide can reduce the production of ROS induced through interactions with heavy metals in two ways: first, because it is a free radical, NO can neutralize ROS by reacting with them; second, NO activates signaling components that in turn stimulate the antioxidant system (Singh et al., 2017). Proteomic analyses of Cd-tolerant soybean plants revealed that Cd increases the expression of enzymes involved in the antioxidant system (Hossain et al., 2012); thus, the presence of NO may enhance Cd tolerance by favoring CAT expression under Cd contamination and then increasing CAT activity. Moreover, in the case of maize roots, NO seems to directly reduce H_2O_2 levels by stimulating the activity of CAT, which ultimately reduces the molecule.

APX activity was not affected by Cd or NO, and SOD activity was not affected by Cd; however, NO reduced SOD activity, which may also have led to a reduction in H_2O_2 content. The absence of a Cd effect on SOD was also observed in radish (Vitória et al., 2001), sunflower (Laspina et al., 2005), and coffee (Gomes-Junior et al., 2006),

among other species. SOD consumes $O_2\cdot^-$ as a substrate, yielding H_2O_2 (Moller et al., 2007), which indicates that NO reduces SOD activity, promoting a reduction in H_2O_2 content. According to Wany and Gupta (2018), the expression of the *SOD1* and *MnSOD* genes, both of which encode SOD isoforms, is upregulated 2 h after exposure of *Triticum aestivum* L. to hypoxia; however, such genes are quickly and gradually downregulated shortly afterward, at which point endogenous NO production increases. This supports our statement that reduced NO-driven SOD activity decreases the H_2O_2 content in plants since this compound is a product of SOD activity. Moreover, NO reduced the H_2O_2 content by increasing the activity of CAT, which consumes H_2O_2 as a substrate, yielding water, and oxygen (Moller et al., 2007). Therefore, NO generated two responses that clearly reduced the H_2O_2 content: a reduction in SOD activity and an increase in CAT activity. It is, therefore, possible to attribute the increase in MDA content in maize roots to the accumulation of $O_2\cdot^-$ or other ROS and not to H_2O_2.

Notably, the development of cortical aerenchyma in maize roots was directly influenced by the H_2O_2 content in this experiment. In this context, aerenchyma formation was increased by Cd and reduced by NO because of the influence of these compounds on H_2O_2 production. The formation of root aerenchyma in maize plants is essential for tolerance to hypoxia because the tissue enhances O_2 diffusion (Pereira et al., 2008; Pereira et al., 2010). Aerenchyma development is essential for plant survival under flooded conditions and occurs in tolerant crop species such as maize (Yamauchi et al., 2013). Aerenchyma development is induced by environmental stresses that stimulate ethylene synthesis, which in turn regulates the production of ROS, leading to programmed cell death (Geisler-Lee et al., 2010; Pires et al., 2015). We do not challenge this formation mechanism but instead add important information concerning the direct role of H_2O_2 in tissue formation and the fact that heavy metals or other factors that generate H_2O_2 stimulate the formation of root aerenchyma while inhibitory molecules reduce aerenchyma formation. Moreover, H_2O_2 can form in mitochondria, transferring electrons to O_2 and forming O_2^-, which subsequently receives more electrons and is converted to H_2O_2 or catalyzed by SOD, which also generates H_2O_2 as a product (Moller et al., 2007; Rajhi & Mhadhbi, 2019). H_2O_2 acts as a necessary indicator of ethylene-induced programmed cell death and can sufficiently promote this process (Ni et al., 2019; Steffens et al., 2011; Steffens & Sauter, 2009). According to Steffens et al. (2011), H_2O_2 can influence programmed cell death and, consequently, lysigenous aerenchyma formation in rice stems. According to Basu et al. (2020), ROS and reactive nitrogen species can regulate programmed cell death, facilitating the formation of lysigenous aerenchyma in rice roots. Thus, the formation of root aerenchyma in maize plants is directly related to the H_2O_2 concentration in the roots and is stimulated by Cd and inhibited by NO.

The increased epidermal and endodermal thickness in the presence of Cd and NO reflects the dynamic ability to reduce Cd uptake. The epidermis can accumulate Cd, preventing the need to allocate Cd to internal root tissues. According to Marques et al. (2011), an increase in epidermal thickness may increase the ability to biologically filter metal ions such as Cd. Therefore, increased epidermal thickness provides a barrier to reduce the amount of Cd accumulation in plants, which is beneficial for maize roots. According to Li et al. (2019), when the endodermis contains increased suberin levels, the plant retains more Cd in its roots, reducing the transport of this metal. Notably, relatively high concentrations of NO reduced the thickness of the epidermis and endodermis, demonstrating an antagonistic effect on Cd signaling that stimulates the thickening of these tissues. The thickness of the epidermis tends to increase only in the presence of relatively high concentrations of NO and in the absence of Cd, which indicates that NO has a protective function in the presence of Cd but causes stress at high concentrations in the absence of Cd.

In addition to being the largest region in roots, the cortex serves as a barrier for Cd uptake because it is a sequestration site for metals in root cells. The presence of Cd tends to increase the cortical thickness to increase the distance for the transport of ions by the roots, reducing the uptake of pollutants in addition to allocating Cd to the roots and thus avoiding Cd toxicity effects in the shoots. A similar effect was observed in plants contaminated with Cd (Qi et al., 2020) and lead (Ribeiro et al., 2015). Moreover, the thickening of apoplastic barriers is related to the accumulation of heavy metals in the roots (Sharma & Dubey, 2005). However, relatively high concentrations of NO act as inhibitors of the stress caused by Cd and consequently reduce the thickness of apoplastic barriers, and these high concentrations may favor the uptake of Cd. A reduction in the proportion of the cortex, which may increase the flow of water and nutrients, makes the cortex more functional (Corrêa et al., 2017) but facilitates Cd uptake. Studies have shown that NO can reduce Cd toxicity in addition to increasing plant tolerance to this metal (Rodríguez-González et al., 2020; Romero-Puertas et al., 2019). At relatively low Cd concentrations, NO was shown to stimulate an increased proportion and thickness of the cortex, decreasing the uptake, and transport of large amounts of Cd. Qi et al. (2020) observed high accumulations of Cd in the apoplastic barriers of the roots of rice plants. Because NO has an antagonistic effect on Cd, the root responses that would normally prevent the uptake of pollutants (increased thickness of the cortex, epidermis, and endodermis) are reversed by NO, allowing greater Cd uptake by plants under high NO concentrations. Thus, part of the reduction in root growth promoted by relatively high NO concentrations also results from this effect, which can be avoided by treatment with relatively low concentrations of NO.

The function of apoplastic barriers is to block the radial transport of water and solutes, thus reducing the translocation of dissolved ions to the xylem and, subsequently, their transport to the shoots of the plant (Kreszies et al., 2018; Qi et al., 2020). However, under relatively high Cd concentrations, the proportion of cortex tissue increases, which means that the roots have a thinner structure with a larger cortex; this increase in the cortex serves as a defense strategy to reduce the flow of Cd into maize plants. In turn, higher concentrations of NO decrease the proportion of the cortex. Nitric oxide plays an antagonistic role in the presence of Cd, in which it functions as an inhibitor of the effects of Cd and increases the susceptibility of maize roots. Studies have shown that NO plays a protective role and acts as an inhibitor

of the toxicity of Cd and other highly contaminating metals (Rodríguez-González et al., 2020). However, regulating the levels of exogenous NO is important because depending on whether NO is present in moderate or excess amounts, NO can act as a signaling molecule or a stressor molecule, respectively, in maize plants. Our results concerning the endodermis show that as Cd concentrations increased as a function of lower NO concentrations, the thickness of this tissue increased to limit the passage of Cd to the vascular cylinder and thus protect against Cd toxicity. Similar results were reported in rice plants by Qi et al. (2020), who observed an increase in the endodermis and apoplastic barriers under Cd stress. However, this increase in the thickness of the endodermis plays an important role in the sequestration of Cd in the roots, as the transport of this metal to the shoots is decreased (Kreszies et al., 2018; Qi et al., 2020). In addition, the endodermis may thicken under other stress conditions (Pereira et al., 2008); nonetheless, these changes in thickness of the apoplastic barrier are important adaptations for plant tolerance to stress (Marques et al., 2011).

At relatively high concentrations of Cd and NO, the endodermis does not show increased thickness, which facilitates increased transport of Cd to vascular tissues such as the xylem. This, in turn, increases the levels of toxicity in the plant and affects root growth and development, as shown in Saracura maize plants. The opposite was reported by Qi et al. (2020) in a study of rice plants under Cd stress, where the endodermis exhibited an increase in lignin and suberin contents; the presence of a more lignified and suberized endodermis limited the transport of Cd into the vascular cylinder, and thus, Cd was not transported to the shoots via the vascular tissue (Kreszies et al., 2018). However, the presence of NO in maize plants in the absence of Cd acts as a stressor that increases the thickness of apoplastic barriers, although it has been shown that excess NO can cause plant toxicity (Rodríguez-González et al., 2020); an increase in apoplastic barriers may be related to the accumulation of heavy metals (Ribeiro et al., 2015). Under relatively high concentrations of NO in the presence of Cd, the thickness of the endodermis tended to decrease. This reduction in apoplastic barriers means less root tissue in which Cd can be sequestered, which probably allows greater uptake and flow of Cd to the shoots, consequently affecting maize root growth.

The presence of Cd together with NO increased the diameter of xylem vessels, which allowed a greater amount of Cd to be transported to the shoots. According to a study on the accumulation of heavy metals in *Brachiaria* by Gomes et al. (2011), the vessel elements of the xylem and root cortical parenchyma have relatively thick cell walls to better bind heavy metals to the cell wall as a protective measure. Relatively concentrations of NO can be considered sufficient to inhibit Cd stimulation in Saracura maize plants. However, a reduction in apoplastic barrier thickness would allow greater Cd transport into the vascular cylinder and ultimately to the shoots of maize plants. The vascular cylinder is considered an important transporter and controller of Cd loading and transport; thus, the vascular cylinder is also considered a determining factor of variation in Cd accumulation in plants (Huang et al., 2020). A recent study by Qi et al. (2020) reported that a smaller xylem diameter and more suberized and lignified apoplastic barriers decrease xylem flow and the transfer of Cd to the shoots of rice plants. Under relatively high concentrations, NO stimulates a decrease in the diameter of xylem vessels, which indicates how NO functions as a Cd stress inhibitor in maize roots, since reduced xylem vessels diameter allows control of the flow of water to the shoots. According to Hacke and Sperry (2001), this response is one way to avoid cavitation, as suitable space for the formation of air bubbles is removed.

The proportion of the vascular cylinder was reduced under relatively high Cd concentrations together with NO, and a reduction in the vascular cylinder diameter, in turn, affected the water transport capacity of the roots. A similar reduction in the vascular cylinder diameter was observed in *Schinus molle* plants under Cd stress (Baroni et al., 2020), although only relatively high doses of NO reduced the proportion of the vascular cylinder, possibly for the purpose of reducing the transport of Cd to the shoots. However, NO acts as an antagonistic molecule in the presence of Cd; NO behaves as a signaling molecule that indicates a lack of stress in plants, thus avoiding a decrease in vascular cylinder diameter. As a result, the antagonistic involvement of NO prevents Cd uptake and the production of ROS, including H_2O_2.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.\

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