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Cellular localization of cadmium and structural changes in maize plants grown on a cadmium contaminated soil with and without liming

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The effects of different concentrations of soil cadmium (0, 1, 3, 5, 10, and 20 mg kg^{-1}) on growth, structural changes and cadmium cellular localization in leaves of maize plants (*Zea mays* L.) were investigated in a pot experiment. The results showed that the structural changes observed in maize leaves were not only a response to the Cd-induced stress but also a cellular mechanism to reduce the free Cd^{+2} in the cytoplasm. However, this mechanism seems to be efficient only up to a Cd concentration in leaves between 27 and 35 mg kg^{-1} for soils without and with liming, respectively. The cellular response varied with both the Cd concentration in soil and liming. For limed soil, Cd was preferentially accumulated in the apoplast while for unlimed soils Cd was more evenly distributed into the cells. The ability of Cd accumulation depended on the leaf tissue considered. The apoplast collenchyma presented the highest Cd concentration followed by the endodermis, perycicle, xylem, and epidermis. On the other hand, symplast Cd accumulated mainly in the endodermis, bundle sheath cells, parenchyma, and phloem. Based on the structural changes and growth reduction, the critical toxic concentration of soil Cd to maize plants is between 5 and 10 mg kg^{-1}.
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1. Introduction

Cadmium is one of the most widespread and toxic metals in soils. It is mainly produced by industrial activities, mining, and zinc refining. This metal has been reported to be the one with the highest rate of global emission to soil [1]. The entry of Cd into agricultural soils occurs through sewage sludge application and extensive use of herbicides and fertilizers [2]. This is a growing concern because Cd can be absorbed by plants and threat animal and human health due to trophic chain contamination. Thus, soils contaminated with Cd are an environmental problem that requires an effective and affordable solution. In this context, cost-effective methods such as phytoremediation have been proposed to decontaminate Cd polluted soils [3,4], but the high Cd phytotoxicity is a serious drawback for efficient phytoextraction. The phytoextraction effectiveness relies on the accumulation of metals in shoots of high biomass plants [5] and is therefore dependent on efficient tolerance mechanisms to heavy metals [6].

Cadmium toxicity to plants is often associated with alterations on uptake and transport of water and nutrients [7], photosynthesis inhibition via chlorophyll degradation [8], and disruption of enzymes involved on CO_2 fixation [9]. Although tolerance of plants to heavy metals is likely associated with vacuole compartmentalization and phytochelatin synthesis [6,10,11], cell wall immobilization also plays a role in alleviating metal stress, notably for non-hyperaccumulating species [12–14]. The vacuole compartmentalization relies on an increased synthesis of organic acids and S-rich peptides such as phytochelatins [10]. Immobilization of metals in the cell wall, on the other hand, is linked to the accumulation of soluble (tannins) and insoluble (lignin) polyphenol compounds [14–16]. Such compounds promote an increased cation exchangeable capacity due to production of anionic polygalacturonic acids [17]. It is important to bear in mind that various defense mechanisms against metals might operate simultaneously. Thus, a holistic approach is needed in the study of the response of plants to cadmium [10].

Cadmium localization and structural changes in cells are essential to understand the Cd tolerance in plants. Several anatomical and structural changes in plant tissues are related to Cd toxicity such as cell wall impregnation by phenolic compounds, degeneration of phloem sieve tubes, thickening of cell wall support tissues, decreased cambial activity, and chloroplast alterations [14,18]. Rapid senescence, as indicated by increase in reactive oxygen species, is also regarded as an indirect effect of Cd stress [8,19].

Most previous works on Cd phytotoxicity and structural changes in plants were carried out in hydroponics [8,11,12]. Studies on soil are necessary to determine whether the same results can be

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reproduced in pot experiments. In addition, such studies can be used to propose a toxic critical level of soil Cd based not only on the Cd concentration in soil but also on the plant response to metal stress. Thus, the work aimed at integrating the Cd availability in soil to the cellular localization, structural changes and growth reduction in maize plants grown on a contaminated soil with and without liming.

2. Material and methods

2.1. Pot experiment

The soil used in this study, classified as Typic Kandiudult according to the U.S. Soil Taxonomy [20], was collected at the 0–20-cm depth. Selected chemical and physical properties of the soil studied are pH 4.9, Al^{+3} 0.92 cmolc dm^{-3}, Ca^{+2} 0.97 cmolc dm^{-3}, Mg^{+2} 0.08 cmolc dm^{-3}, K^{+} 0.08 cmolc dm^{-3}, P 9 mg dm^{-3}, organic carbon 3.45 g kg^{-1}, sand 628 g kg^{-1}, silt 32 g kg^{-1}, and clay 340 g kg^{-1}.

The soil was air dried, passed through a 2 mm sieve and half of the samples were limed to pH 6.0 with carbonates of Ca and Mg (3:1 molar ratio) before further use, whereas the second half was left at the original pH. Samples of 2 kg of soil were amended with $CdCl_2$ to achieve the concentrations of 0, 1, 3, 5, 10, and 20 mg kg^{-1} of cadmium. The Cd-amended samples were kept incubated for a 30-day period at 80% of the water holding capacity. Availability of Cd in soil was assessed by DTPA extractant [21]. The soil samples were fertilized as follows: 250, 240, 150, and 100 mg kg^{-1} of N, P, K, and S, respectively, added as NH_2SO_4, $NH_4H_2PO_4$, and KNO_3. The Micronutrients Fe ($FeSO_4 \cdot 7H_2O$), Mn ($MnCl_2 \cdot 4H_2O$), Zn ($ZnSO_4 \cdot 7H_2O$), Cu ($CuSO_4$), B (H_3BO_3), and Mo ($Na_2MoO_4 \cdot 2H_2O$) were applied at the concentrations of 2, 4, 4, 1.5, 1, and 0.2 mg kg^{-1}, respectively. Then, the soil samples were transferred into plastic pots to conduct the greenhouse experiment.

Five maize seeds were sown directly onto the soil and thinned to 2 plants per pot 5 days after germination. Plants were watered to 80% of the soil water holding capacity on a daily basis by weighing the pots and adding water to compensate for any weight loss. After a 30-day period of cultivation, the shoots were dried in an oven at 65 °C and further ground and digested in a mixture of HNO_3:$HClO_4$ (3:1 v/v). The concentration of Cd in the extracts was determined by atomic absorption spectrometry.

2.2. Microscopical analysis

Samples of mature leaves were collected and immediately fixed in a FAA 50 solution (50 mL of formaldehyde 40% + 50 mL of acetic acid +900 mL of alcohol 50%) for at least 24–48 h until preparation of semi permanent histological layers [22]. Leaf sections of the nervure and mesophyll were clarified in a sodium hypochlorite 30% solution neutralized with acetic water 1:500. The sections were then washed with distilled water, stained with astra blue and safranin [23] and mounted on glycerin 50% [24].

Sections of leaves were also used to histochemical analysis for lignin with acid fluoroglucine [25]. For histochemical detection of Cd in leaves was used the technique developed by Seregin and Ivanov [12], based on the ability of dithizone (30 mg diphenilthiocarbazone dissolved in 60 mL of acetone and 20 mL of distilled water) in producing a reddish color compound after reacting with cadmium. Transversal sections of leaves were maintained in layers with dithizone for 90 min. After that time, the sections were washed in distilled water and immediately analyzed in an optical microscopy. The images were then digitalized and analyzed for lumen diameter of the xylem cells, mesophyll and epidermis thickness, transversal area of the foliar nervure, phloem, and collenchyma using the software Image Tool [26].

2.3. Statistical analysis

The experiment was a two-factorial one replicated in three randomized complete blocks. Data were subjected to ANOVA with soil (with or without liming) and six rates of cadmium. Regression analysis was employed to study the dependence of Cd concentrations in shoots and biomass production from the metal rates in soil samples. The coefficients were tested at the 5 and 1% probability levels. The structural changes in plants were compared by Tukey test ($p < 0.05$). The statistical analysis was accomplished with software SAS [27].

3. Results and discussion

3.1. Cadmium localization in the plant tissues

Cadmium was detected in the cells of maize leaves thanks to the brownish-red color developed under dithizone influence (Figs. 1 and 2). The color was attributed to Cd by comparing the rates 0 and 20 mg kg^{-1} of the metal (Fig. 1C and D).

Cadmium was located in both inside and outside cells of maize leaves (Figs. 1 and 2). For the mesophyll, the endodermis cells were important sites for Cd binding. They displayed intense dithizone coloration even for the lowest Cd concentration in plants (Fig. 2A and B). The immobilization of Cd in the endodermis is important to avoid its transfer to the bundle sheath cells where the intense photosynthesis activity could be affected (Fig. 2B vs. D). However, this apparent protection imparted by endodermis cells seems to be dependent on the Cd concentration in leaves. In Cd concentrations higher than 10 mg kg^{-1}, there was a transport of Cd to the bundle sheath cells, notably to chloroplast cells, and neighbor parenchyma cells (Fig. 2C and D).

It is worth mentioning that the inflexation point in the biomass response-curve occurred close to the 10 mg kg^{-1} concentration of Cd in soil. At this concentration, there was a 30% reduction for dry matter yield (Fig. 3C) and the plants accumulated 35 and 27 mg kg^{-1} of Cd in leaves for soils with and without liming, respectively. Lin et al. [28] reported that Cd can induce oxidative stress in wheat seedlings at Cd concentration in soil between 3.3 and 10 mg kg^{-1}. Based on our results, the toxic critical value of DTPA-available soil Cd for maize plants is similarly between 5 and 10 mg kg^{-1}.

Taking in account the Cd localization in the mesophyll cells, there was an intracellular distribution gradient as a function of Cd concentration in soil. The metal was initially immobilized in the endodermis and then accumulated in the bundle sheath cells and other parenchyma cells. This preferential Cd localization in the bundle sheath cells rather than in other parenchyma cells can be explained by its nearness to the vessels that transport Cd via xylem [19] as well as by the high chloroplast density found in bundle sheath cells. Both the bundle sheath and vessel cells displayed an intense brownish color. This indicates a complete disorganization of the cytoplasm as a consequence of Cd toxicity (Fig. 2C and D).

A distribution gradient was also observed to extra cellular Cd, especially for the cell wall of the abaxial and adaxial collenchyma in nervures (Figs. 1 and 4Figs. 1B and 4B). This result corroborates the hypothesis that Cd is first distributed from tissues around the vessels, which are the less sensitive to Cd toxicity [19].

Liming was an important factor on determining Cd availability. The Cd concentration in soil was decreased 13% by pH increasing (Fig. 3a). Plants grown on limed soil presented higher Cd accumulation (Fig. 3b and d) as well as higher dry matter yield than plants cultivated in unlimed soil (Fig. 3c). The highest Cd absorption in limed soil seems to be related to the higher Cd allocation in the collenchyma cell wall on such soil as compared to unlimed soil (Fig. 1C vs. H). This indicates that plants on limed soil managed to

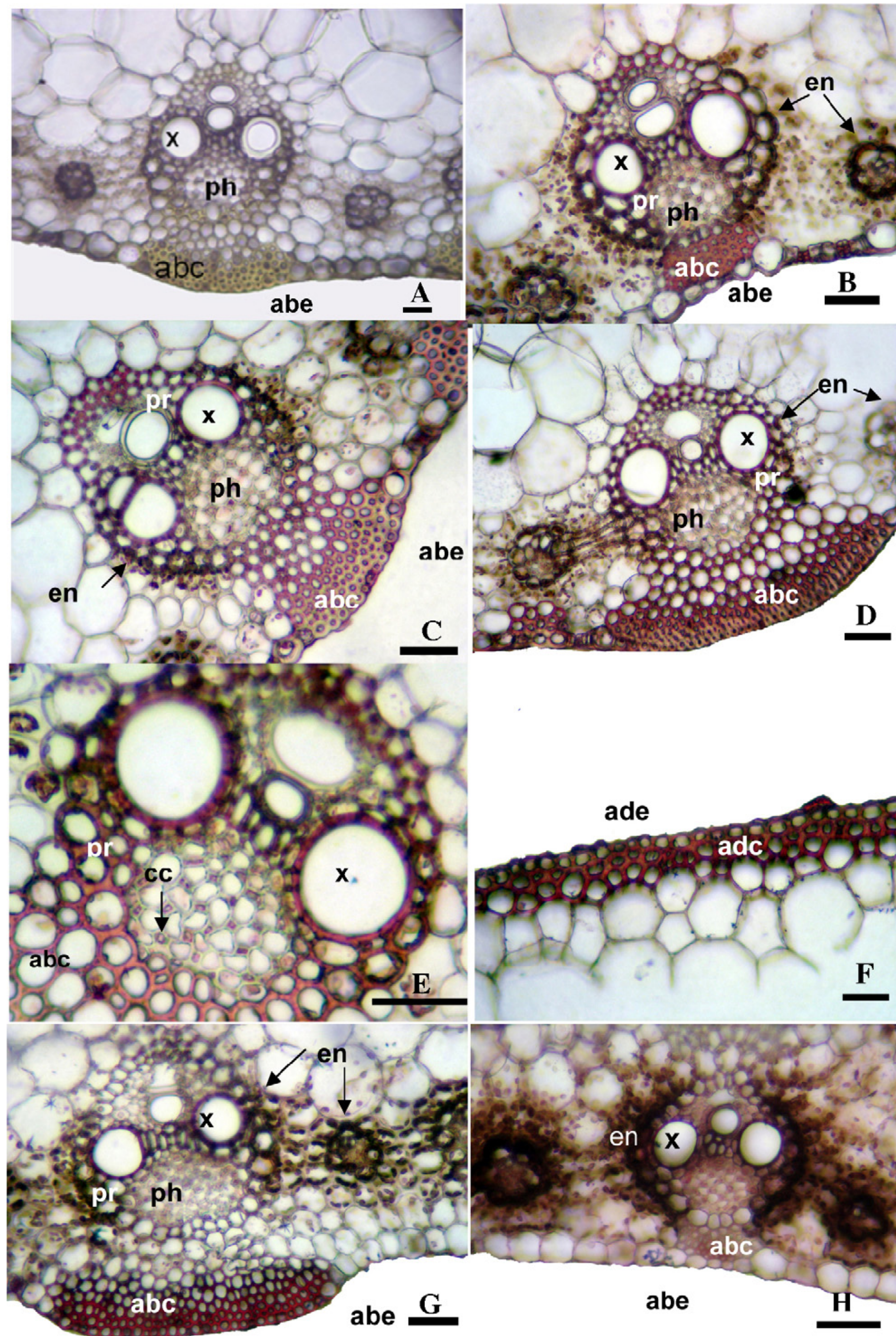

Fig. 1. Localization of Cd in the leaf veins of maize plants exposed to 0, 1, 5, and 20 mg kg^{-1} of Cd in limed soil samples (A–D, respectively). Cd in companion cells (E) and adaxial collenchyma (F). (G) and (H): leaves of plants exposed to 3 and 20 mg kg^{-1} of Cd in soil without liming. x, xylem; abc, abaxial collenchyma; adc, adaxial collenchyma; abe, abaxial epiderm; ade, adaxial epiderm; en, endoderm; pr, perycicle; ph, phloem. Bar: 50 μm.

restrain the Cd movement to cytoplasm and thus ameliorated the Cd toxic effects that affected the plants grown on unlimed soil samples. The differences between treatments with and without liming are mainly due to the acidity effects on plants. It is likely that plants grown on limed soil are more nutritionally balanced than plants cultivated in acidic soil. It seems that these healthier plants were more efficient to both detoxify Cd and promote structural modifications. For instance, leaves exposed to 20 mg kg^{-1} of Cd in unlimed soil did not present collenchyma's lignification (Fig. 4D). Besides, there was a dilution effect on the Cd concentration owing to the higher biomass of plants grown on limed soil (Fig. 3). This can also diminish the metal toxic effects.

Plants cultivated in limed soil accumulated more Cd in collenchyma cells than plants on soil without liming (Fig. 1C vs. H). Such difference demonstrates that not only endodermis retention but also cell wall immobilization is dependent on the Cd stress level. Both mechanisms seem to be efficient for Cd detoxification at low level of metal stress (in the present work, up to 10 mg kg^{-1}

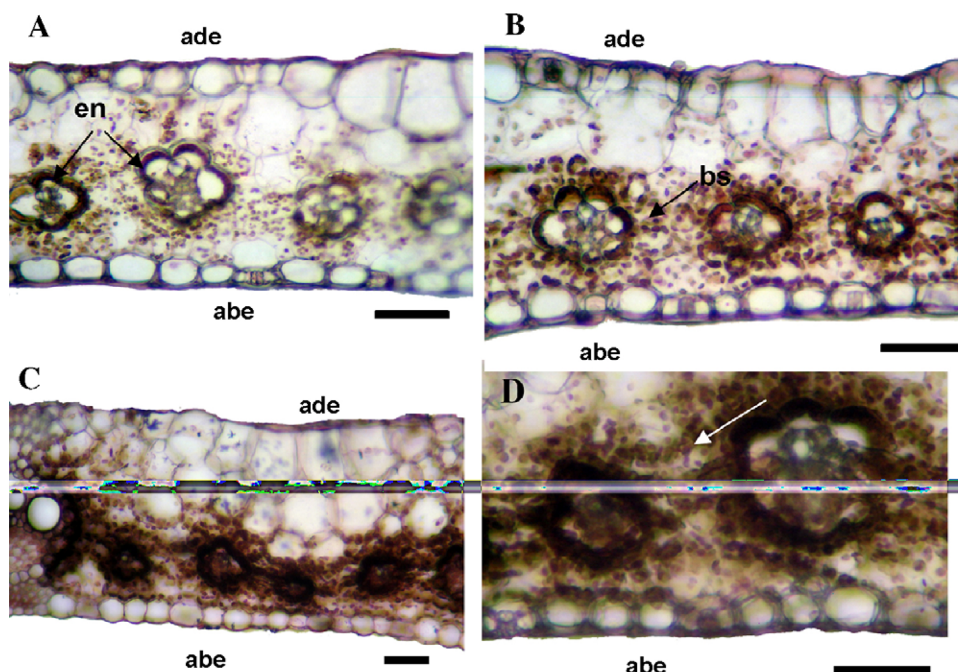

Fig. 2. Localization of Cd in mesophyll cells of maize plants exposed to 1 and 20 mg kg^{-1} of Cd in limed soil (A and B). Without liming treatments showing deleterious effect in mesophyll cells, especially in bundle sheath cells (C and D). abe, abaxial epiderm; ade, adaxial epiderm; en, endoderm; bs, bundle sheath cells. Bar: 50 μm.

of Cd in soil). This result corroborates the role of cell wall immobilization in increasing tolerance of plants to heavy metals [19]. As pointed out by Toppi and Gabrielli [10], the importance of cell wall immobilization as a tolerance mechanism may vary accordingly to metal concentration in tissues, plant species and exposure time. Vazquez et al. [29] found that cell wall immobilization plays a key role in the Cd-detoxification strategy of white lupin grown in perlite contaminated with up to 150 μmol L^{-1} of cadmium.

For plants grown on unlimed soil, the low Cd detection in the collenchyma was associated with low biomass production (Figs. 1H vs. 3c). This growth reduction might be related to Cd effects on the chloroplasts (Fig. 2D). Chlorophyll loss is one of the most common symptoms of Cd phytotoxicity. It occurs due to the Cd interference in root Fe uptake [30]. Kupper et al. [31] reported that Cd can replace Fe in chlorophyll making it either unstable or functionally degraded. Pietrini et al. [32] suggested that chlorophyll loss induced by Cd can diminish the photochemical capacity of *Phragmites australis* (Cav.).

3.2. Structural changes in plants induced by cadmium

Structural changes on cells were observed as a function of exposure to Cd. There was an increase of the transversal area occupied by collenchyma in the foliar nervure as well as of the cell wall lignification (Table 1, Fig. 4A vs. C). The lignification of cell walls in metal stressed plants has been shown to have a close relation with cellular oxidative stress and peroxides content [13,15,16,33,34]. It suggests that the oxygen peroxide (H_2O_2) could be the signalizing molecule

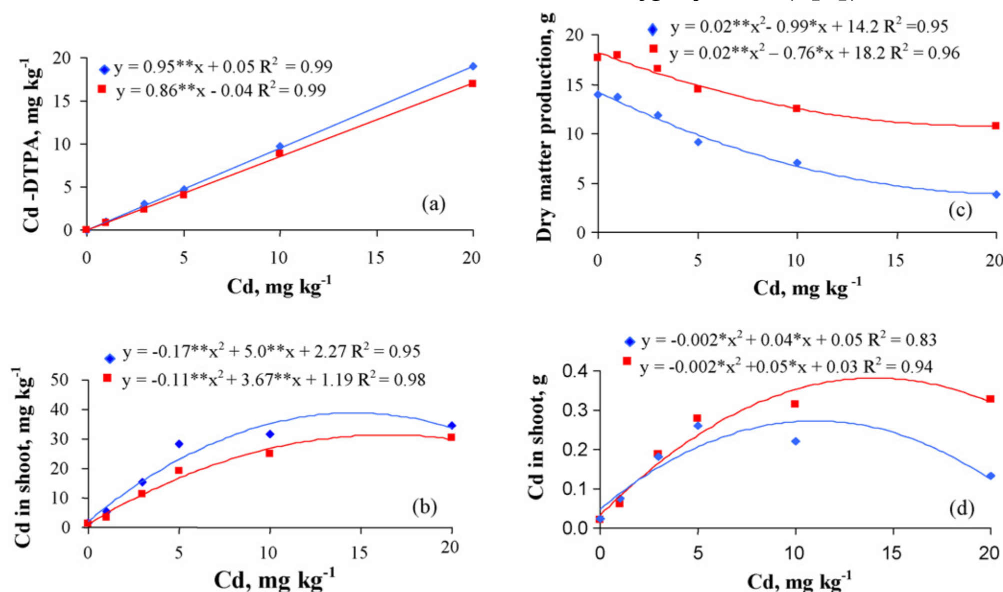

Fig. 3. Concentration of Cd in soil (a), in shoot (b), shoot dry matter production (c) and accumulation of Cd (d) in maize plants exposed to increasing doses of metal in soil with (■) and without liming (♦).

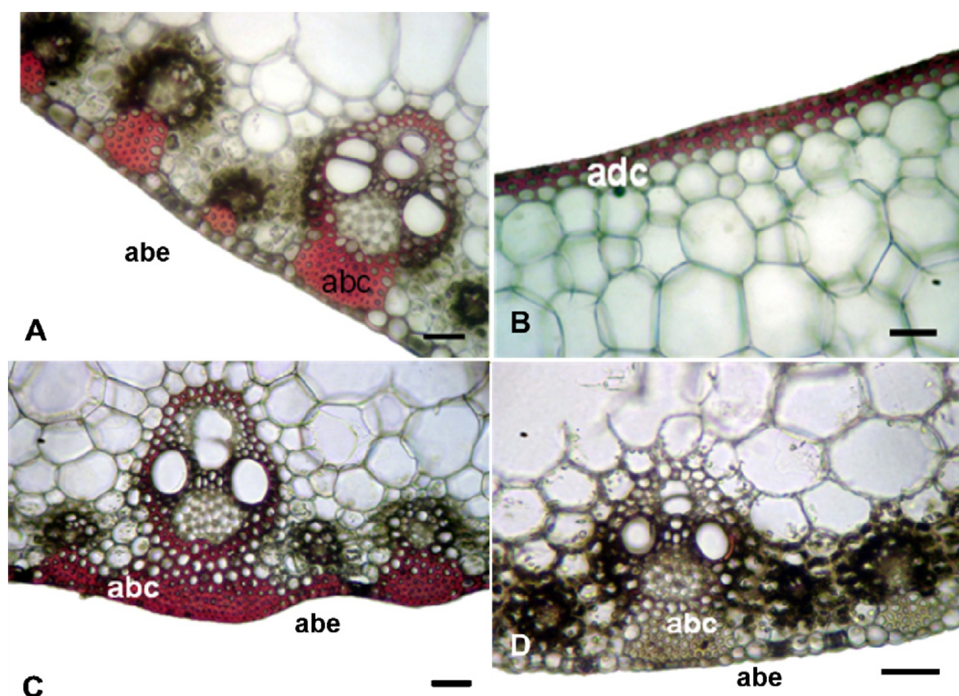

Fig. 4. Structural changes in leaves of maize grown on a Cd contaminated soil with and without liming. Sections of leaf exposed to 0 mg kg^{-1} of Cd (A) and 5 mg kg^{-1} of Cd (B and C) in limed soil. Leaf exposed to 20 mg kg^{-1} of Cd in unlimed soil (D) did not display collenchyma's lignification. abc: abaxial collenchyma; adc: adaxial collenchyma; abe: abaxial epiderm; ade: adaxial epiderm. Bar: 50 μm.

for the lignification process. It is well known that peroxides are needed to oxidation and polymerization of lignin precursors. They also favor lignin consolidation on the proteins and carbohydrates of cell wall at the same time that decreases the cell H_2O_2 levels. Schutzendubel et al. [13] sustain that plants under stress activate the lignin metabolism in order to ameliorate the unbalanced cellular oxidative system. This provides new bond sites for metal at the cell wall. Thus, lignin deposition in the collenchyma cell wall allows for a high Cd accumulation in this tissue (Fig. 1C and G).

It is important to point out that the changes on the cell oxidative balance is regarded as an indirect effect of Cd toxicity. Cadmium is not a redox metal, such as Cu and Fe, and therefore it cannot catalyze Fenton reactions and produce reactive oxygen species. However, the alterations provoked by Cd in chloroplasts [18,31] can disturb the electron transportation rates of the photossystems I and II that overproduces oxygen free radicals in cells [18]. Therefore, the activation of lignin metabolism resulting in lignification of the collenchyma cell wall can be explained by the accumulation of oxygen reactive species triggered by Cd in the chloroplasts (Fig. 2A–D).

For the plants grown on limed soil, the increasing of Cd concentration in the collenchyma paralleled the 5-fold increasing in the area occupied by such tissue and the high lignification of its cell wall (Figs. 1C and 4C). A positive correlation ($r = 0.88^{**}$) between soil Cd and collenchyma area (Table 2) supports such an assumption. On the other hand, for the unlimed soils, the correlation was inverse ($r = -0.45^{*}$). This reinforces the idea that cell wall immobilization as a tolerance mechanism depends on plant metal stress. In soil without liming, Cd availability increased while the collenchyma

Table 1
Anatomical characteristics of leaves of maize plants grown on a contaminated soil with increasing doses of Cd[a]

Dose (mg kg^{-1})	Diameter xylem (μm)	Thickness (μm)		Area (μm^2)		
		Epiderm	Mesophyll	Vein	Phloem	Collenchyma
Without liming						
0	30 b	62 a	203 a	22159 b	4123 ab	5288 c
1	38 ab	64 a	250 a	27952 a	4916 a	9091 b
3	46 a	62 a	259 a	29687 a	4497 b	16274 a
5	32 b	64 a	264 a	20411 b	3495 b	6131 bc
10	34 b	60 a	245 a	20747 b	3211 b	3955 c
20	35 ab	52 a	182 a	19560 b	3678 ab	4018 c
Mean	36	61	234	23419	3987	7459
With liming						
0	45 a	71 a	226 a	32,246 ab	6173 ab	4214 c
1	41 a	66 a	197 ab	25,296 bc	4619 bc	4556 c
3	38 a	67 a	218 a	18,153 c	3242 c	4424 c
5	42 a	65 a	167 b	24,657 bc	5322 ab	14,533 b
10	46a	66 a	226 a	25,905 bc	5172 ab	15,862 b
20	47 a	65 a	226 a	34,284 a	6462 a	20,138 a
Mean	43	67	210	26,757	5165	10621

[a] Means followed by the same letters are not significantly different by Tukey Test at $p < 0.05$.

Table 2
Correlation coefficient between anatomical characteristics of maize leaves, shoot dry matter production, content and concentration of Cd in shoot and concentration of Cd in soil[a]

	Dry matter	Content	Concentration	
			Shoot	Soil
Without liming				
Diameter of xylem	0.23 ns	−0.23 ns	−0.12 ns	−0.10 ns
Thickness of epiderm	0.27 ns	−0.13 ns	−0.34 ns	−0.53*
Thickness of mesophyll	0.16 ns	0.20 ns	−0.03 ns	−0.44 ns
Area of veins	0.60**	−0.50*	−0.57*	−0.54*
Area of phloem	0.63**	−0.64**	−0.60**	−0.47*
Area of collenchyma	0.48*	−0.10 ns	−0.36 ns	−0.45*
With liming				
Diameter of xylem	0.12 ns	0.03 ns	0.29 ns	0.42 ns
Thickness of epiderm	0.36ns	−0.55**	−0.24 ns	−0.25ns
Thickness of mesophyll	−0.14 ns	−0.10 ns	0.09 ns	0.26 ns
Area of vei ns	−0.05 ns	0.06 ns	0.20 ns	0.43 ns
Area of phloem	−0.07 ns	0.12 ns	0.27 ns	−0.25 ns
Area of collenchyma	−0.81**	0.79**	0.95**	0.88**

[a] (*), (**) and ns: significant at <5%, <1%, and not significant, respectively. $n = 36$.

area was decreased. This resulted in decreased binding sites for Cd immobilization (Table 1 and Fig. 4C).

The xylem lumen diameter and the transversal area occupied by the central nervure, phloem and collenchyma were 16, 12, 23, and 23% lower, respectively, in the unlimed soil samples as compared to limed ones. This is corroborated by the negative correlation between Cd content in plants and these structural changes (Table 2). There was no correlation between characteristics for plants cultivated on limed soil samples. This result demonstrates that the slight structural changes observed in these treatments did not cause reduction on the growth and development of the maize plants (Table 2) and corroborates the role of liming on diminishing Cd uptake.

Degeneration of phloem cells was observed as an effect of increasing Cd concentration in soils without liming (Fig. 1E). It can result from the Cd allocation to companion cells. The sieve tubes and companion cells are sensitive to the oxidative stress and are severely injured by Cd [14]. Therefore, photosynthate exportation is diminished [35]. This is probably the cause for the 72% growth reduction of plants grown on unlimed soil. There was a highly significant correlation between the phloem area and the maize dry matter production ($r = 0.63^{**}$) for acidic soil.

The negative and highly significant correlation between collenchyma area and shoot biomass ($r = -0.81^{**}$) suggests that the secondary metabolism responsible for Cd cellular detoxification reduced the plants growth and development. This outcome is attributed to the high demand of cellular energy needed to the long reaction chain that culminates in the lignin synthesis [36]. Furthermore, the lignification decreases the cell plasticity and therefore reduces the cell elongation and plant growth [33].

Both the content and concentration of Cd in plants were correlated with collenchyma area ($r = 0.95^{**}$ and $r = 0.79^{**}$, respectively). This indicates that the process of collenchyma cell wall lignification provided an increased concentration of Cd in shoots. Such finding corroborates once more the important role of cell wall immobilization on detoxifying Cd in plants [14,29].

4. Conclusions

The structural changes observed in maize leaves were not only a response to the Cd-induced stress but also a cellular mechanism to reduce the free Cd^{+2} in the cytoplasm. However, this mechanism seems to be efficient only up to a certain Cd concentration in leaves. The cellular response varied with the Cd concentration in soil. For limed soil, Cd was preferentially accumulated in the apoplast while for unlimed soils Cd was more evenly distributed into the cells. The ability of Cd accumulation depended on the considered tissue. The apoplast collenchyma presented the highest Cd concentration followed by the endodermis, perycicle, xylem, and epidermis. On the other hand, symplast Cd accumulated mainly in the endodermis, bundle sheath cells, parenchyma, and phloem. Based on the structural changes and growth reduction, the critical toxic concentration of soil Cd to maize plants is between 5 and 10 mg kg^{-1}.

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