Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v41i1.35829

SOILS

Synergy between cadmium and zinc in bean plants cultivated in multi contaminated soils

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ABSTRACT. Agricultural species are subjected to a variety of biotic and abiotic stresses, which are the main limitations to crop production. In this context, contamination by trace elements is characterized as an abiotic stress that represents an environmental problem. Due to the physical and chemical similarities between cadmium and zinc, these elements may interact in the environment and may cause antagonistic or synergistic effects. In this way, physiological mechanisms to exclude, detoxify or compartmentalize trace elements that are in excess are crucial for plant survival when exposed to high concentrations of these elements. In this way, the aim of this study was to understand the physiological responses of *Phaseolus vulgaris* plants subjected to increasing doses of Cd and Zn for 21 days in different soil, Cambisol and Latosol. The activity of antioxidant enzymes, such as SOD, CAT, and APX; hydrogen peroxide content; lipid peroxidation; chlorophyll index; photosynthetic rate; stomatal conductance; and transpiration were analysed. The data obtained showed a specific behaviour of *Phaseolus vulgaris* plants in each soil analysed. Moreover, it was observed that interactions between both elements resulted in a synergistic effect, negatively affecting all of the parameters analysed.

Keywords: trace elements; antioxidante enzymes; ROS; photosynthesis; synergism.

Received on March 9, 2017.
Accepted on July 28, 2017.

Introduction

Contamination by trace elements represents a global environmental problem that puts humans, animals and plants at risk. These elements, when present at concentrations above optimum, change several metabolic functions that are fundamental to the growth and development of plants. In this way, there are alterations in cellular homeostasis by the accumulation of reactive oxygen species (ROS) in different cellular compartments (Tkalec et al., 2014). High levels of ROS can generate oxidative stress through the inactivation of enzymes and damage to proteins, lipids of membranes, photosynthetic pigments such as chlorophyll and consequently gas exchange (Sharma, Jha, Dubey, & Pessarakli, 2012). To avoid the harmful effects of ROS from cellular constituents, plants activate their enzymatic antioxidant systems, composed mainly of the enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). When the activities of these antioxidant enzymes are not enough to reduce ROS production, oxidative stress tends to occur, thus generating oxidative damage, such as increased lipid peroxidation by the accumulation of hydrogen peroxide.

The physiological effects of individual trace elements are well documented. Although combinations of trace elements are common in nature, their combined effects still need to be carefully investigated. Cadmium (Cd) in soil is often accompanied by zinc (Zn), due to the chemical and physical similarities between them. Studies about the interaction of Cd and Zn, absorption and accumulation in different plants revealed the mainly antagonistic interaction between these two elements, although synergistic effects have also been reported between them (Puga, Abreu, Melo, & Beesley, 2015). In addition, it has been found that Zn supplementation at lower concentrations may decrease Cd-induced oxidative stress, whereas elevated levels of both elements may induce increased oxidative stress (Cherif, Mediouni, Ben Ammar, & Jemal, 2011).

Phaseolus vulgaris plants are considered a sensitive species to Cd and Zn contamination according to the Organization for Economic Cooperation and Development (OECD, 2006). In this way, the aim of this study was to evaluate the physiological behaviour of *Phaseolus vulgaris* plants exposed to increases doses of Cd and Zn and cultivated in different types of soil, a Latosol and a Cambisol.

Material and methods

Growing conditions, plant material and experimental design

The experiment was conducted in a greenhouse, according to ISO 11269-2 (ISO, 2013) and OECD-208 recommendations. Two soil classes were used: a Latosol red yellow, typical dystrophic with texture medium to moderate, and a Cambisol Haplic Tb, typical dystrophic with texture medium to moderate. These soils are clean of Cd with VRQ < 0.4 mg Cd kg^{-1} dry weight (State Environmental Foundation - FEAM, 2011). Soil samples were collected, sieved and analysed in relation to chemical and physical characteristics (Table 1).

Table 1. Chemical and physical characteristics of the Cambisol and Latosol.

Soils	pH	K	P	Ca	Mg	Al	CTCef	V	MO	Clay	Texture Silt	Sand
		mg dm^{-3}		------ cmol dm^{-3} --------			---- % ----	------- % -------		----------- % ---------------		
Cambisol	5.3	34	2.6	1.6	0.4	0.5	2.5	34.0	2.8	31	22	47
Latosol	4.8	32	1.1	0.3	0.1	0.6	1.0	9.6	1.6	24	12	64

Relation 1:25; K, P: extractor Mehlich 1; Ca^{+2}, Mg^{+2} and Al^{+3}: extractor KCl 1 mol L^{-1}; CTCef: effective cation exchange capacity; V: base saturation level; MO: organic matter; oxidation Na_2CrO_7 4 N + H_2SO_4 10 N.

Fertilization was performed according to Malavolta (1981), in which 500 g of soil were weighed into a plastic receptacle and then were irrigated for one week to maintain 70% of field capacity. After this period, different levels of Cd and Zn were added, Cd using solutions $Cd(NO_3)_2.4\ (H_2O)$ and $ZnSO_4.7H_2O$ as sources of these elements (Table 2). Doses were determined using the values established by Resolution 420 of the National Environmental Council - CONAMA (2009) as parameters. The values set for this experiment is composed of multiples of 1.8 as the normative suggestion of the OECD (2006). The added doses respect a molar ratio of Cd/Zn of 64/1, given that this is a close relationship found for the concentration of these two elements in mining areas of Minas Gerais (Carvalho, Amaral, Guilherme, & Aarts, 2013).

Table 2. Cadmium and zinc concentrations used in the treatments.

Treatments	Cd	Zn
	------------------------ mg kg^{-1}	------------------------
D0	0.0	0.0
D1	0.4	14.8
D2	0.7	28.8
D3	1.2	48.0
D4	2.3	86.6
D5	4.1	152.6
D6	13.6	506.8
D7	24.4	908.6

After the addition of solutions containing Cd and Zn, 20 seeds per pot were sown. After the emergence of seedlings from 50% of the D0 treatment, the number of plants per replicate was reduced to 10. The experiment lasted twenty-one days after this emergence, according to the OECD 208-recommendations.

The experiment was conducted in a completely randomized design (CRD) in a factorial 8x2, with 8 treatments (seven increasing doses of Cd/Zn and control), two soils (Cambisol and Latosol), totalling 16 treatments with three replications. Each experimental unit consisted of a pot containing 500 g of soil and 5 plants. The data were subjected to an analysis of variance using the statistical program SISVAR 4.3 (System Analysis of Variance for Balanced Data) (Ferreira, 2011). The average between the treatments were compared by the Scott and Knott (1974) test at 0.05 probability ($p \leq 0.05$).

Cadmium and zinc content in plants

Cd and Zn doses were determined in the dry mass of shoots and roots in extracts obtained by acid digestion in a microwave oven, according to the USEPA 3051A method of the USA Environmental Protection Agency (United States Environmental Protection Agency – USEPA, 1998). The readings were taken by spectrophotometry of an atomic absorption flame or graphite furnace according to the concentrations found in the extracts. For quality control of the analysis, the BCR-482 reference material - Lichen of the Institute for Reference Materials and Measurements (Geel, Belgium) was used, with certified levels for Zn (100.6 mg kg^{-1}) and Cd (0.56 mg kg^{-1}). The recovery levels were 120 and 65% for Cd and Zn, respectively.

The limit of detection (LoD) was calculated based on the standard deviation and the average of seven readings of the blank sample, considering the value t of Student for n=7 and employing the following formula (American Public Health Association - APHA, 1998):

$$LoD = (x + t \times s) \times d$$

x: average content of the substance of interest in seven blank samples;
t: value of Student at 0.01 of probability and n-1 degrees of freedom (n = 7 e α = 0,01, t = 3,14);
s = standard deviation of the seven blank samples;
d = dilution factor of the sample employed in the digestion procedure.

After calculations, the method detection limits obtained for Cd and Zn were 1.22 mg kg^{-1} and 1.66 μg kg^{-1} for Cd and Zn, respectively.

Growth and ecophysiological analyses

A growth analysis was performed by measuring the root length and shoots with the help of a ruler and the stem diameter with the help of digital callipers. For the determination of the dry weight of shoots and roots, plant material was dried at 70°C in a forced circulation oven to constant weight. The chlorophyll index (CI) was determined by a Chlorophyll Meter (SPAD-501, Minolta Co., Japan) (Minolta Camera LTDA, 1989), with 4 readings per replicate for each treatment.

Gas exchange measurements were performed simultaneously with chlorophyll fluorescence through a portable photosynthesis system (IRGA, Model LI-6400, Li-Color, Lincoln, Nebraska, USA) with an integrated fluorescence chamber (LI-6400-40 leaf Chamber fluorometer, Li-Color). All measurements were performed in the morning between 8:00 and 11:00 am on a fully expanded leaf. The characteristics evaluated were measurements of gas exchange [photosynthetic rates (A), transpiration (E) and stomatal conductance (gs)]. All evaluations were performed between 9-10 pm with the use of artificial source of photosynthetically active radiation (PAR) in a closed chamber set at 1500 μmol photons m^{-2} s^{-1} (Blue = Red LED LI-6400-02B, LI-COR, Lincoln, USA). The CO_2 assimilation rate in the chamber was measured with a CO_2 environment concentration of 380 ± 3 μmol of CO_2 mol^{-1}.

Antioxidant enzymes activity

For enzymatic activity analyses, leaves and roots were collected, immediately frozen in liquid nitrogen and stored in a freezer at -80°C until the analyses. 0.2 g of plant material was macerated in liquid nitrogen and extracted in a buffer composed of 0.01 M EDTA, 0.4 M potassium phosphate (pH 7.8), 0.2 M ascorbic acid and water. The homogenate was centrifuged at 13,000 g for 10 minutes at 4°C, and the supernatant was collected and stored at -20°C for further analysis of the enzymes superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), according to Giannopolitis and Reis (1977), Havir and McHale (1987), and Nakano and Asada (1981), respectively.

Hydrogen peroxide and lipid peroxidation

Both for hydrogen peroxide and lipid peroxidation quantification, 0.2 g of the fresh material were first macerated in liquid nitrogen, homogenized in 1500 μL of 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 g for 15 minutes at 4°C, and the supernatant was collected and stored at -20°C. Lipid peroxidation determination was performed by quantification of thiobarbituric acid reactive species (TBA), as described by Buege and Aust (1978). To determine the hydrogen peroxide content, the methodology described by Velikova, Yordanov, and Edreva (2000) was used.

Results and discussion

Cadmium and zinc content in plants

The present research demonstrated that Cd and Zn tends to accumulate in bean plants. Higher concentration of these elements in soil resulted in an increased absorption of both trace elements. Despite the different levels of mobility of metals in plants, the contents of Cd and Zn were generally higher in roots than in shoots. In most environmental conditions, Cd accumulates in the roots as a plant strategy to reduce its translocation and damage to shoots (Clemens, Aarts, Thomine, & Verbruggen, 2013).

Zinc and cadmium contents increased in shoots and roots, according to the increase of the Cd and Zn doses (Table 3). The highest values of zinc were found in the shoots and roots of plants cultivated in the

Latosol, when compared to the Cambisol. However, for both soils, there was a greater accumulation of Zn in shoots than in roots. The presence of cadmium was more expressive in shoots of plants cultivated in the Latosol and in the roots of plants in the Cambisol. Plants cultivated in the Latosol at dose 7 did not resist these conditions. The presence of zinc in the D0 treatment plants occurred due to fertilization according to Malavolta (1981).

Table 3. Zinc and cadmium concentrations in shoots and roots of bean plants grown in a Cambisol (C) and Latosol (L) at different doses of zinc and cadmium.

Doses	Zinc (mg kg^{-1} fresh weight)				Cadmium (μg kg^{-1} fresh weight)			
	Shoot		Roots		Shoots		Roots	
	C	L	C	L	C	L	C	C
D0	132 Bh	181 Ag	100 Ag	109 Ah	<LoD	<LoD	<LoD	<LoD
D1	222 Bg	325 Af	97 Bg	225 Ag	10 Bd	15 Af	5 Ac	2 Ac
D2	472 Bf	554 Ae	223 Bf	444 Af	12 Ad	13 Af	6 Bc	11 Ab
D3	672 Be	845 Ad	303 Be	643 Ae	17 Ac	18 Ae	1 Ad	2 Ac
D4	801 Bc	834 Ad	359 Bd	808 Ad	26 Ab	21 Bd	1 Ad	3 Ac
D5	723 Bd	1061 Ac	594 Bc	3003 Ac	32 Aa	33 Ac	9 Bb	23 Aa
D6	1138 Bb	1629 Ab	4147 Ba	4434 Ab	32 Ba	54 Ab	13 Bb	21 Aa
D7	1185 Ba	-	3559 Bb	-	36 Ba	-	38 Aa	-

Upper case letters show the differences between soils, and lowercase letters show the differences between doses, according to Scott-Knott's test ($p \leq 0.05$).

Growth parameters

Since bean plants are sensitive and non-phytoremediate to trace elements (Carvalho et al., 2013), exposure of this species to increasing cadmium and zinc doses led to a reduction in growth parameters (Table 4). A growth analysis showed the plant to be sensitive to the increases of Cd and Zn doses in the soil.

Table 4. Growth parameters of bean plants grown in a Cambisol and Latosol at different doses of zinc and cadmium.

Doses	Shoot dry weight (g $plant^{-1}$)		Roots dry weight (g $plant^{-1}$)	
	Cambisol	Latosol	Cambisol	Latosol
D0	0.85 Aa	0.72 Ba	0.28 Ab	0.28 Aa
D1	0.81 Aa	0.67 Ba	0.38 Aa	0.22 Bb
D2	0.61 Ab	0.42 Bb	0.28 Ab	0.16 Bc
D3	0.52 Ac	0.43 Bb	0.25 Ab	0.16 Bc
D4	0.47 Ac	0.38 Bb	0.20 Ac	0.16 Bc
D5	0.38 Ad	0.31 Bc	0.17 Ac	0.15 Ac
D6	0.29 Ae	0.29 Ac	0.12 Ad	0.11 Ad
D7	0.22 Af	-	0.10 Ad	-

Upper case letters show the differences between soils, and lowercase letters show the differences between doses, according to Scott-Knott's test ($p \leq 0.05$).

Ecophysiological analyses

Higher values of chlorophyll were found in plants cultivated in the Cambisol than in the Latosol (Figure 1). In plants grown in both soils, increasing Cd and Zn doses resulted in a drop in chlorophyll content.

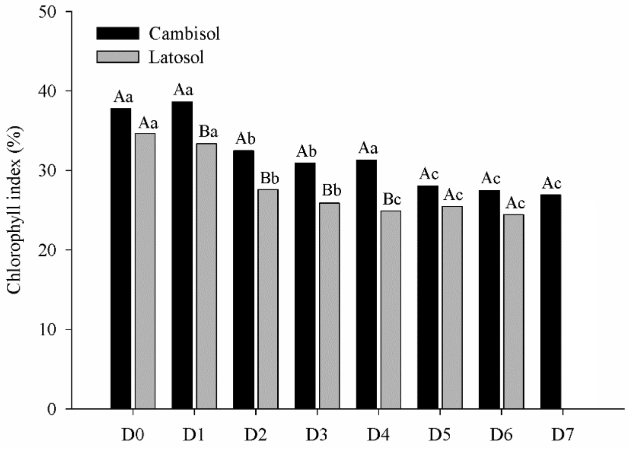

Figure 1. Chlorophyll index of bean plants grown in a Cambisol and Latosol at different doses of zinc and cadmium. Upper case letters show the differences between soils, and lowercase letters show the differences between doses, according to Scott-Knott's test ($p \leq 0.05$).

The inhibition of chlorophyll biosynthesis due to increases in doses of both trace elements had a direct effect on the photosynthetic process, since the reduction in chlorophyll levels is closely related to the reduction in light energy uptake that will be used in photosynthesis. Although Zn is considered an essential element, exposure at high concentrations causes damage to photosystem II by interacting with the thiol group of their proteins. Moreover, excess Zn can inhibit Mg absorption, reducing the photosynthetic capacity (Parmar, Freek, & Shahbaz, 2014).

As was the case with the chlorophyll index, the net photosynthetic rate, transpiration and stomatal conductance in bean plants were negatively impacted by the increase in Cd and Zn doses (Figure 2). It is important to emphasize that plants cultivated in the Latosol, at the highest dose, did not resist this condition. It was observed that plants cultivated in the Cambisol presented a higher index in gas exchanges when compared to plants cultivated in the Latosol.

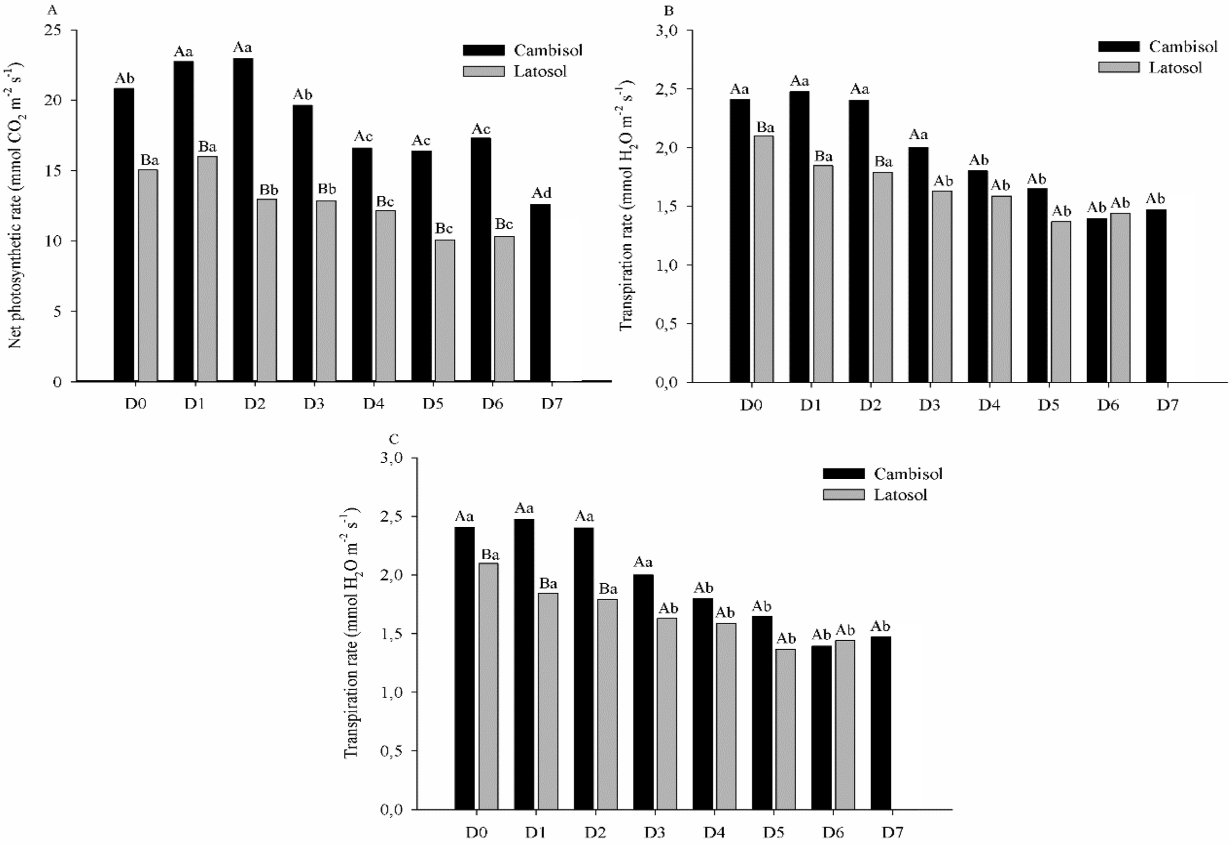

Figure 2. Photosynthetic rate (A), transpiration (B) and stomatal conductance (C) of bean plants grown in a Cambisol and Latosol at different doses of zinc and cadmium. Upper case letters show the differences between soils, and lowercase letters show the differences between doses, according to Scott-Knott's test ($p \leq 0.05$).

In response to Cd-induced oxidative stress, plants employ antioxidant defence systems to eliminate excessive production of reactive oxygen species (ROS) and prevent their destructive oxidative reactions. A reduction in SOD, CAT and APX activity was observed with increasing doses of Cd and Zn, and this reduction can be attributed to increase of H_2O_2 production, which is formed in different cell compartments (Ying et al., 2010).

When analysing the activity of antioxidant enzymes, bean plants cultivated in Cambisol showed, in general, an increase, followed by a decrease in SOD activity, not differing significantly between shoots and roots (Figure 3). Plants cultivated in Latosol, presented higher SOD activity in shoots, when compared to roots. When comparing both soils, it was observed that plants cultivated in the Cambisol showed higher activity of this enzyme independent of the dose and organ analysed.

CAT activity was influenced by different doses of Cd and Zn (Figure 4). In plants cultivated in the Cambisol, higher CAT values were observed in the roots compared to the leaves. On the other hand, when analysing the Latosol, CAT activity in leaves was significantly higher than that in roots.

When analysing the APX activity, it was possible observe a significant increase in the roots of bean plants cultivated in the Cambisol, in contrast to that observed in plants cultivated in the Latosol

(Figure 5). Regardless of the soils, it was observed that the increase of Cd and Zn doses significantly decreased the APX activity.

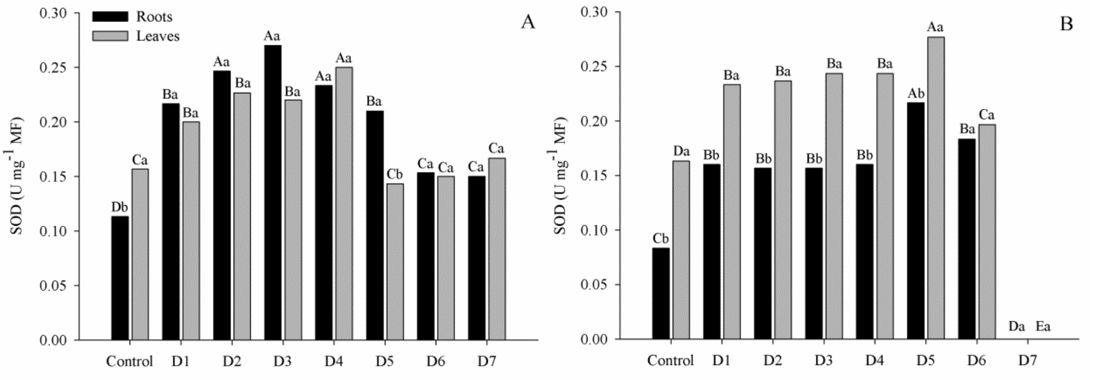

Figure 3. Superoxide dismutase activity (SOD) of bean plants grown in a Cambisol (A) and Latosol (B) at different doses of zinc and cadmium. Upper case letters show the differences between soils, and lowercase letters show the differences between doses, according to Scott-Knott's test ($p \leq 0.05$).

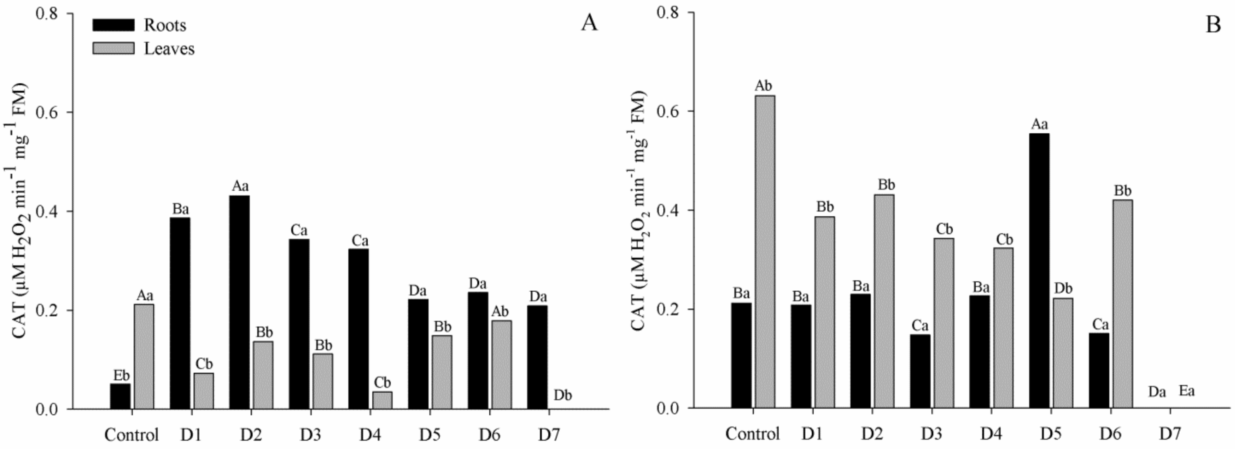

Figure 4. Catalase activity (CAT) of bean plants grown in a Cambisol (A) and Latosol (B) at different doses of zinc and cadmium. Upper case letters show the differences between soils, and lowercase letters show the differences between doses, according to Scott-Knott's test ($p \leq 0.05$).

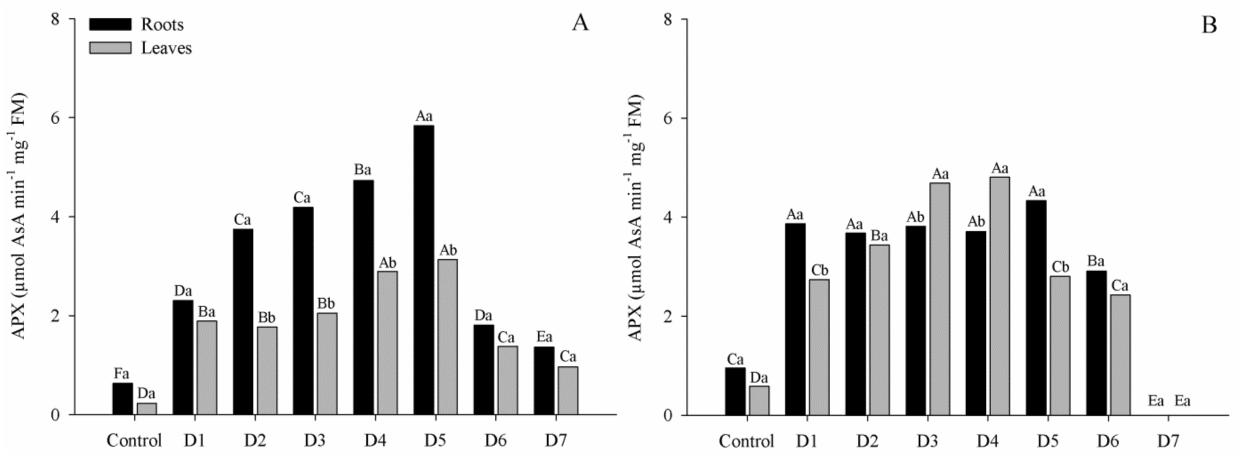

Figure 5. Ascorbate peroxidase activity (APX) of bean plants grown in a Cambisol (A) and Latosol (B) at different doses of zinc and cadmium. Upper case letters show the differences between soils, and lowercase letters show the differences between doses, according to Scott-Knott's test ($p \leq 0.05$).

With regard to the observed differences between soils, it is necessary to emphasize that the Latosol has a more acidic pH (4.8), lower values of CTC (1.08 cmol dm^{-3}), clay (24%) and organic matter (1.64%)

when compared to Cambisols, with pH of 5.3, CTC of 2.59 cmol dm^{-3}, 31% of clay and 2.87% of organic matter. These chemical and physical characteristics provide conditions that guarantee the Cambisol will have greater adsorption of Cd, providing lower contents of this element to bean plants. With lower levels of Cd available, bean plants cultivated in the Cambisol presented lower values of Cd in their tissues when compared to those cultivated in the Latosol at most doses. Lower internal Cd levels allowed to these plants to have a lower impact of this trace element on their metabolism and, therefore, presented less marked oxidative damage than that observed in plants cultivated in the Latosol.

It was observed that the amount of hydrogen peroxide was higher in the leaves of bean plants cultivated in the Latosol, and this increase was proportional to the doses of Cd and Zn (Figure 6).

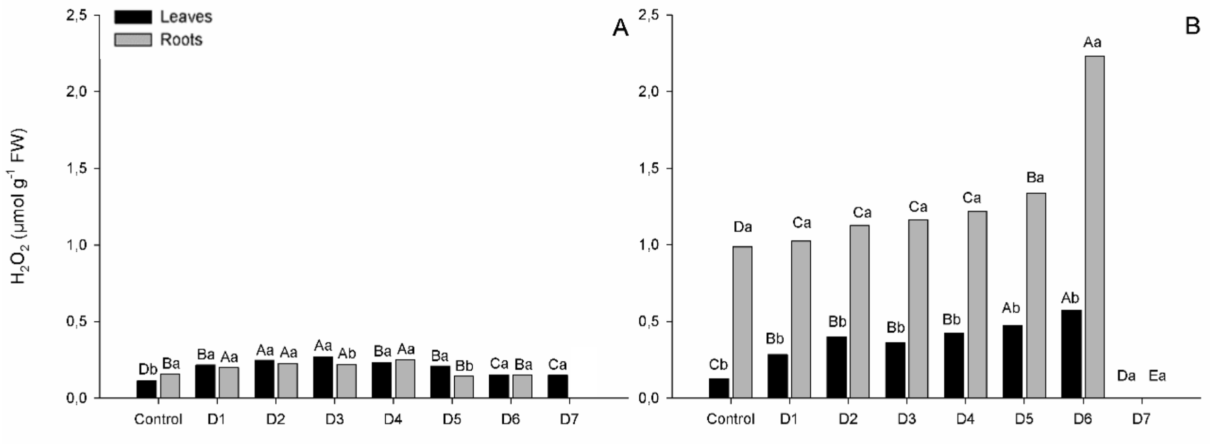

Figure 6. Hydrogen peroxide in leaves and roots of bean plants grown in a Cambisol (A) and Latosol (B) at different doses of zinc and cadmium. Upper case letters show the differences between soils, and lowercase letters show the differences between doses, according to Scott-Knott's test ($p \leqslant 0.05$).

Lipid peroxidation levels were expressed as the malonaldehyde content (MDA). The data obtained were similar, in general, between soils (Figure 7).

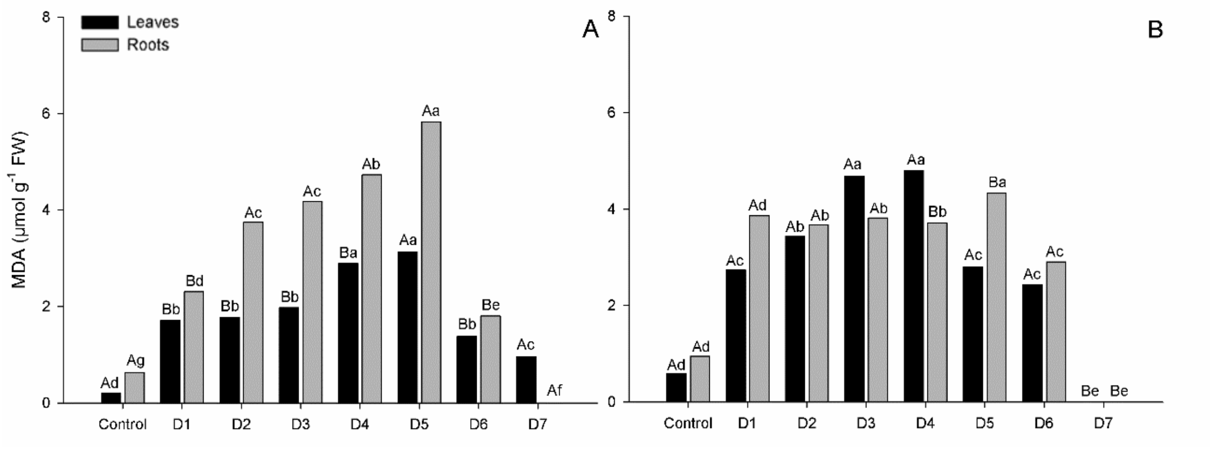

Figure 7. Malondialdehyde in leaves and roots of bean plants grown in a Cambisol (A) and Latosol (B) at different doses of zinc and cadmium. Upper case letters show the differences between soils, and lowercase letters show the differences between doses, according to Scott-Knott's test ($p \leqslant 0.05$).

Increasing Cd and Zn doses resulted in increases in the malondialdehyde content in all tissues. This is because Cd in plant tissues induces oxidative stress that is characterized by lipid peroxidation and consequent increase in malondialdehyde levels (Solti, Sárvàri, Tóth, Mészáros, & Fodor, 2016). Due to its chemical properties, Cd cannot react directly with oxygen, but can cause oxidative stress indirectly by inhibiting metabolic reactions.

In bean plants, Zn had no significant effect on the reduction of Cd contents in leaves and also had a positive effect on Cd uptake in roots of plants exposed to different doses of Cd and Zn. Higher Zn concentrations induce oxidative stress, which has been described in several plant species (Tkalec et al., 2014). ROS formation cannot be directly induced by Zn; that is, the toxic forms of oxygen, which cause lipid peroxidation, are formed as a consequence of the interaction between Zn and lipid membranes and can thus produce conformational changes capable of activating NADPH oxidase, located in the plasma membrane, which may lead to oxidative stress. High values of these parameters obtained in leaves suggest that these may be related to the photosynthetic activity, which results in ROS production (Hussain et al., 2012).

Conclusion

The interaction of Cd and Zn showed synergistic effects, regardless of doses, on bean plants, resulting in oxidative stress. The physical and chemical characteristics of both soils directly influenced the development of bean plants, thus confirming that different mechanisms are involved in the protection against oxidative stress caused by trace elements, as well as the best development of bean plants cultivated in Cambisols.

Acknowledgements

The Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for funding this project.

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