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IMPACT OF CADMIUM TOXICITY ON LEAF AREA AND STOMATAL CHARACTERISTICS IN FABA BEAN

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ABSTRACT

Although changes in stomatal response to elevated doses of cadmium has already been observed in several plants, few studies have focused on this phenomenon in more detail. The effects of different doses of cadmium Cd^{2+} (50 and 100 $mg.kg^{-1}$ soil) on leaf area and stomatal characteristics (number of stomata, number of epidermal cells, stomatal index and size of stomata) of faba bean (*Vicia faba* L. cultivar Aštar) were studied after 15 days of heavy metal treatment. No visual changes on adaxial or abaxial side of leaves of the tested variety of faba bean were found. The leaf area was higher by 14.15 % at lower and by 12.23 % at the higher doses of Cd. The tested doses of Cd did not lead to a change in the number of stomata. The number of epidermal cells was increased on adaxial side by 4.98 % at dose Cd50. Stomatal index was decreased due to the effect of higher dose of cadmium by 11.38 % on adaxial side of leaves. In stomatal widths, decrease by 2.04 % (adaxial side) and 2.26 % (abaxial side) was observed at higher doses of Cd. In stomatal length decrease by 1.85 % was observed at higher dose of Cd on adaxial and decrease by 3.89 % (Cd50) and 4.63 % (Cd100) on abaxial side of leaves respectively. The variations of response could be signals of tolerance or adaptive mechanisms of the leaves of tested bean cultivar under the used concentrations of cadmium.

Keywords: plant defense, faba bean, cadmium, stomata, leaf area

INTRODUCTION

Cadmium (Cd) does not have an essential function in any living organism; thus even a low concentration of this metal is toxic to organisms. Due to high accumulation potential of cadmium, the single links of the food chain get contaminated relatively fast (**Demková *et al.*, 2017**). Agricultural crops show varied levels of sensitivity to the given metal; with majority of them accumulating Cd in roots (**Vamerali *et al.*, 2012, Wu 1990**). In plants, exposure to Cd causes oxidative stress, inhibition of growth, nutritional imbalances, changes in the activity of many enzymes, reduction of a transpiration rate and water content (**Barceló and Poschenrieder, 1988; Benavides *et al.*, 2005; Kuklová *et al.*, 2017**). In addition, it causes stomatal closure due to entry of Cd into the guard cells in competition to Ca^{+2} (**Perfus-Barbeoch *et al.*, 2002**). Decrease in stomatal density is also characteristic symptom of Cd stress resulting in lesser conductance to CO_2 (**Pietrini *et al.*, 2010**) which consequently leads to inhibition of photosynthesis. However, it is accepted that the factors limiting photosynthesis have stomatal and non-stomatal nature (**Vassilev and Yordanov, 1997**). Reduced photosynthetic activity is often due to iron deficiency in cadmium treated leaves (**Zhang *et al.*, 2011**). The changes in the stomatal density, size of stomata and epidermal cells as a response to environmental stress are important means of regulating the rate of absorption of risk elements by plants, while the stomatal index often remains unchanged (**Gostin, 2009**). Several authors suggest that leaves of resistant species show xeromorph characteristics, which most probably help plants adapt to the increased concentrations of heavy metals and gases (**Kutschera-Mitter *et al.*, 1982; Nikolaevkij, 1989**). Many studies have attempted to clarify the mechanism of heavy metal toxicity in plants (**Benavides *et al.*, 2005; Procházková *et al.*, 2014; Kohanová *et al.*, 2018**). However, the high variability in the response of different genotypes to ions of Cd often results in contradictory relations between toxicity and physiological processes occurring in plants (**Vassilev and Yordanov, 1997**). In addition, Cd can interfere in several ways on the parameters that affect this physiological processes in leaves (**Poschenrieder and Barceló, 2004**).
Although fabaceae are relatively sensitive to higher doses of metals (**Inohue *et al.*, 1994**), results of several studies suggest that plants such as *Lupinus albus*, *Vicia faba* and *Trifolium repens* show tolerance to cadmium and may be used in re-vegetation and phytostabilization of cadmium contaminated soils (**Verma *et al.*, 2006, Pichtel and Bradway, 2008; Bidar *et al.*, 2009**).
In the present article, the influence of different concentrations of cadmium ions (50 and 100 $mg.kg^{-1}$ soil) on leaf area, stomata size and frequency in faba bean cv. Aštar are presented.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of beans (*Vicia faba* cv. Aštar) were surface-sterilized with 5 % sodium hypochloride for 5 min and planted in pots containing mix of soil (BORA, pH 6; 1,0 % N; 0,3 % P_2O_5; 0,4 % K_2O) and perlite (4:1). The plants were cultivated in a growth chamber at 20 °C, 12 hours light/12 hours dark period (illumination of 400 lux), and relative humidity 60 – 70 %. Pots were watered daily to 60 % water-holding capacity of the soil. When the first assimilating leaves were developed, plants were supplied with distilled water (control) or two Cd^{2+} ion solutions with concentrations of 50 and 100 $mg.kg^{-1}$ of soil (Cd50 and Cd100). Cadmium was added as $Cd(NO_3)_2.4H_2O$ (**Piršelová *et al.*, 2016**). On day 15 after application of metal solutions, the following characteristics were determined: leaf area, stomatal density, stomatal index, length and width of stomata in both adaxial (upper) and abaxial (lower) surface of leaves. Three replicates were used per treatment and 8 plants per pot were analyzed (altogether 24 plants).

Number of stomata, size of stomata and stomatal index determination

The number and size of stomata were assessed after 15 days of growth in contaminated soil on upper (adaxial) and lower (abaxial) sides of leaves using clear nail polish, tape, and a glass slide. Leaves of similar size and maturity were used. The stomatal samples were collected at conditions with a temperature of 25 °C during 9:30-11:00 am. In total 24 microscopic fields of each epidermis and variant of experiments were randomly selected and examined using the Zeiss Axioplan II optical microscope and then they were counted. The number of stomata was expressed per mm^2 of leaf area. To determine stomatal length and width, 50 randomly chosen stomata on each leaf (variant of experiments) were measured at 400× magnification. Images were obtained using Sony DXCS500 digital camera and analysed with AxioVision AC software (Zeiss, Germany). Stomatal density was defined as the number of stomata per square millimeter of leaf surface. Stomatal index (SI) was calculated using the equation of **Salisbury (1927)**. It is defined as:

$$SI\ (\%)\ \frac{\text{stomatal density}}{\text{stomatal density} - \text{epidermal cell density}}\ \text{x}\ 100$$

Determination of leaf area

Leaf area was determined gravimetrically.

Measurements of cadmium content in leaves

Dried plant material (0.5 g roots and shoots) was digested in the mixture of 5 mL water, 5 mL of concentrated HNO_3 p.a. (Merck, Darmstadt, Germany), and 1.5 mL of H_2O_2 p.a. (Slavus, Bratislava) by using the microwave oven Mars Xpress (CEM Corporation, Matthews, USA). Decomposition temperature was 140 °C, ramp time 15 min, and hold time 13 min. After digestion, the solution was diluted to 25 mL with deionised water and filtered through an acid-resistant cellulose filter (Whatman No. 42). Blank samples were prepared in a similar way. The cadmium was determined by electrothermal atomic absorption spectroscopy (AAS Perkin Elmer 1100B, Nor-walk, Connecticut, USA) (**Dobroviczká *et al.*, 2013, Piršelová *et al.*, 2016**).

Statistical analysis

Data were analyzed by one-way ANOVA or Kruskal-Wallis tests using XLSTAT software. Data are expressed as the means the replicates ± standard deviation.

RESULTS AND DISCUSSION

Plant growth

Plants grown for 15 days in soil contaminated with cadmium did not show any visual symptoms of metal toxicity, such as chlorosis, necrotic lesions or wilting. Similar conclusions were presented in a study by **Dobroviczká *et al.* (2013)** with soybeans exposed to dose of Cd of 50 $mg.kg^{-1}$ soil. Changes in the evaluated parameters of shoots (fresh and dry weight of shoots, shoot length) were statistically insignificant compared to the control; a decrease in the content of root biomass was noted (**Piršelová *et al*., 2016**).

Cadmium accumulation

The rate of cadmium accumulation in the roots and shoots was proportional to the dose of cadmium applied (Table 1). The accumulation of Cd in the roots was more pronounced, the reduced transport of Cd to the shoots may contribute to the plant tolerance to higher doses of Cd. (**Zornoza *et al*., 2002**).

Table 1 Cadmium content in roots and shoots ($\mu g.g^{-1}$ dry weight).

Variant of experiment	Root	Shoot
Control	0.50 ± 0.01 Aa	0.10 ± 0.03 Ab
Cd50	62.26 ± 9.60 Ba	13.73 ± 3.27 Bb
Cd100	86.40 ± 0.99 Ba	16.53 ± 4.37 Bb

Legend: Different upper case letters within a column and different lower case letters within a row indicate results *significant* at $p < 0.05$ (Kruskal–Wallis test).

Since the level of translocation of Cd from the roots to the shoots proved to be low, we assume its translocation to the seeds to be also low. However, single varieties of faba bean can have different levels of accumulation of Cd in their tissues. The selection of plant genotypes with low cadmium accumulation using genetic markers can contribute to rapid identification of plants suitable for growing in soils with a certain metallic load (**Socha *et al*., 2015**; **Vollmann *et al.*, 2015**).

Number of stomata, size of stomata and stomatal index determination

Changes in the size and number of stomata are a manifestation of the plants' response to changes in the environment and are an important tool in regulating the absorption of pollutants by plants (**Gostin, 2009**). Compared with guard cell length, stomatal density is relatively plastic and potentially adaptive to environmental changes (**Sekiya and Yano, 2008**). Leaves treated with Cd50 and Cd100 showed decreased number of stomata by 5.72 % and 2.9 % respectively on the adaxial side and by 5.13 and 1.0 % respectively on the abaxial side; these changes were insignificant (Figure 1).
The number of epidermal cells was increased significantly on adaxial surfaces (by 4.98 %) and decreased, but not significantly (by 13.37 %) on abaxial surfaces at doses Cd50 and Cd100 respectively (Figure 1). Stomatal index decreased significantly on adaxial side of leaves (Figure 2).

Results of the studies aimed at observing the number of stomata and epidermal cells affected by heavy metals are contradictory. Several authors (**Kastori *et al*., 1992; Chwil, 2005; Shi and Caia, 2009**) mentioned that number of stomata of epidermis increased with increased concentration of heavy metals. In contrast, however, cadmium decreased number of stomata on abaxial side of leaves of sorghum (*Sorghum bicolor*) **Kasim (2006)**. Decrease in the number of stomata caused by the effect of cadmium was observed also on the leaves of oilseed rape by **Baryla *et al*. (2001)**. Decrease in the number of epidermal cells due to metal contamination was also recorded by **Makovníková (2001)** and **Gostin (2009)**. The given contradictions can be caused also by the differing reactions of the single parts of leaves to the different types of stress (**Saidulu *et al*., 2014**). Positive and negative correlations were also noticed among the metal concentrations and the number of stomata on the both sides of the leaves (**Chwil, 2005**).
Stomatal index was decreased due to the effect of both doses of cadmium by 9.83 % and 11.38 % (adaxial side) and by 3.07 % and 1.00 % (abaxial side), however only decrease on adaxial side of leaf at higher dose of cadmium was statistically significant (Figure 3). These changes may be the result of increased oxidative stress in the leaves of the test variety of beans at a dose of cadmium 100 $mg.kg^{-1}$ soil (**Piršelová *et al*., 2016**).

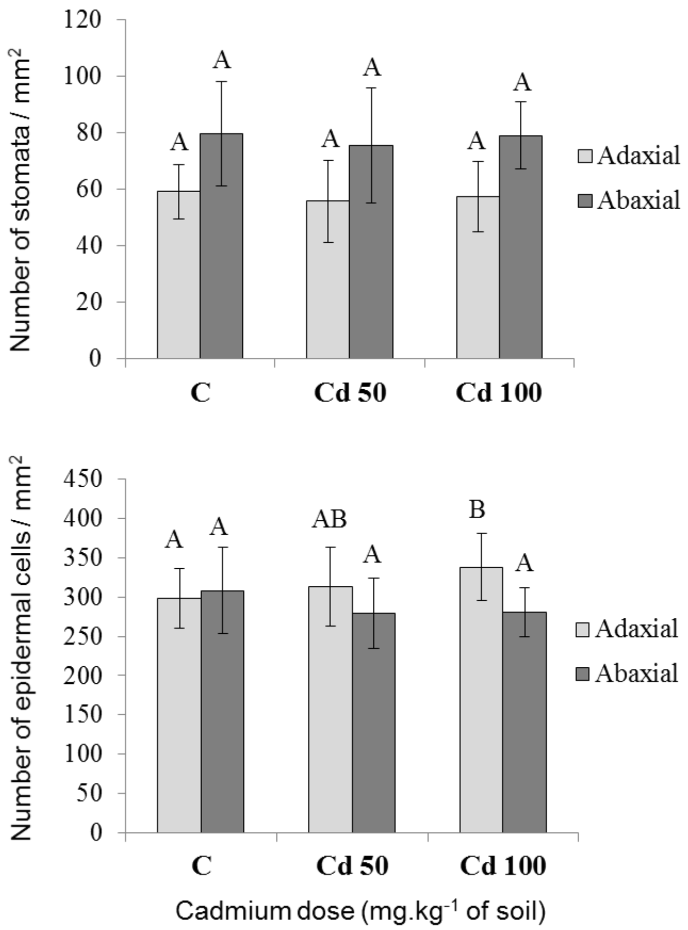

Figure 1 Number of stomata and epidermal cells determined for adaxial and abaxial sides of leaves exposed to ions of Cd in concentrations of 0 (control – C), 50 or 100 $mg.kg^{-1}$ of soil (Cd50 and Cd100). Bars indicate ± standard deviation of mean values (n = 25). Columns with the same letter are *not significantly different* ($p < 0.05$, Kruskal–Wallis test).

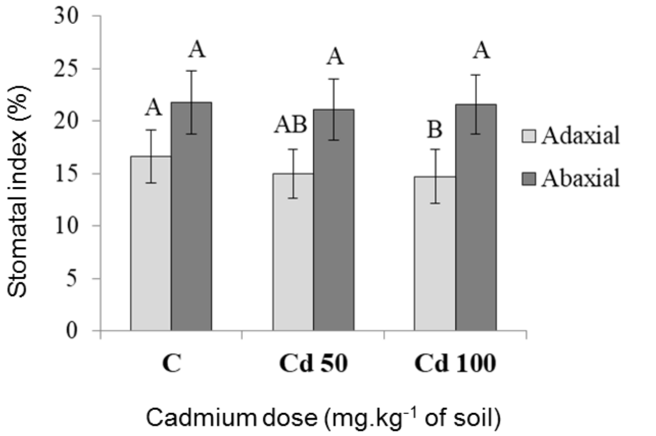

Figure 2 Stomatal indexes determined for adaxial and abaxial sides of leaves exposed to ions of Cd in concentrations of 0 (control – C), 50 or 100 mg.kg^{-1} of soil (Cd50 and Cd100). Bars indicate ± standard deviation of mean values (n = 25). Columns with the same letter are not significantly different ($p < 0.05$, Kruskal–Wallis test).

Similar findings were also reached by **Verma *et al*.** (**2006**) in their experiments with cadmium who observed decrease in stomatal index in upper epidermis in *Ipomea pes-tigridis*. In contrast, however, an increase in stomatal index was noted on the abaxial side of *Trifolium montanum* and *T. repens* and no significant changes were noted on the abaxial side of *T. pratense* and *Lotus corniculatus* (**Gostin, 2009**).
In stomatal widths, statistically significant decrease by 2.04 % (adaxial side) and 2.26 % (abaxial side) was observed at higher doses of Cd (Figure 3).

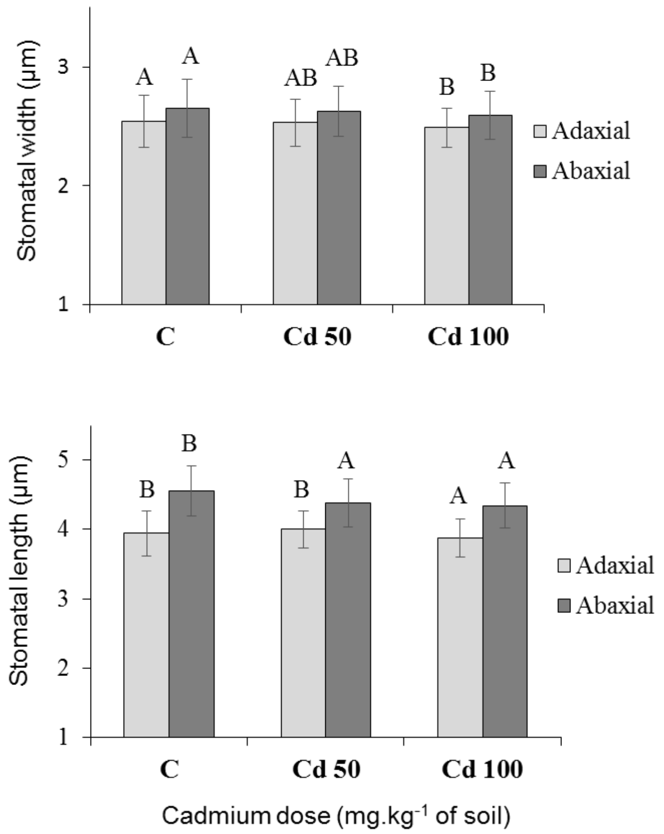

Figure 3 Changes in length and width of stomata on adaxial and abaxial surface of leaves exposed to ions of Cd in concentrations of 0 (control - C), 50 or 100 mg.kg^{-1} of soil. Bars indicate ± standard deviation of mean values (n = 190). Columns with the same letter are *not significantly different* ($p < 0.05$, Kruskal–Wallis test).
In stomatal length decrease by 1.85 % was observed at higher dose of Cd on adaxial surface and decrease by 3.89 % (Cd50) and 4.63 % (Cd100) on abaxial surfaces respectively (Figure 3).

Application of the lower doses of Cd resulted in less pronounced changes, while these mostly reflect the negative impact of Cd on the size of stomata, especially the length on abaxial leaf side (Figure 3). Shortening the stomata on the adaxial side of leaves of *Arachis hypogaea* L. after application of cadmium is reported also by **Shi and Caia** (**2009**) in their study.
Several other authors reported decreasing size of stomata with increasing concentrations of Cd ions applied in the form of $CdCl_2$ solution (**Gostin, 2009; Pereira *et al*., 2016**). Increase in stomatal density, the number of stomata and reduction in the size of guard cells per unit area represent a self-defense system, which is developed in plants under stress conditions and helps them survive in the contaminated environment (**Azmat *et al*., 2009**).

Effect of cadmium on leaf area

The change in leaf area due to cadmium is a common phenomenon associated with a reduction in the *transpiration rate* (**Lai *et al*., 2015**). In our experiments the leaf area was higher by 14.15 % at lower and by 12.23 % at the higher doses of cadmium (Figure 4).
The stimulatory effect of low doses of chemicals on plant growth is referred to as hormesis (**Calabrese, 2009**). Although the knowledge about mechanisms of hormesis is growing, the phenomenon is not sufficiently explained so far. Stimulation tends to increase plant defense and is mainly due to induction of synthesis of defense molecules (stress proteins), secondary metabolites, alteration of antioxidant enzyme activity and reduction of oxidative stress by inhibiting lipid peroxidation of membranes (**Allender *et al*., 1997**). In the tested cultivar enhanced accumulation of PR proteins (chitinases) was recorded in roots treated of cadmium, lead and arsenic (**Békésiová *et al*., 2008**).

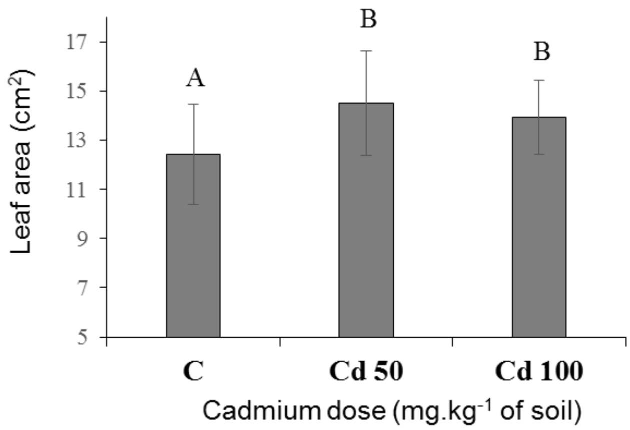

Figure 4 Effect of cadmium (50 or 100 mg.kg^{-1} of soil) on leaf area. Columns with the same letter are not significantly different ($p < 0.05$, Kruskal–Wallis test). C – control.

CONCLUSION

We observed no visual symptoms of toxicity on the leaves of the tested variety of faba bean caused by the test doses of cadmium. The leaf area was higher by 14.15 % at lower and by 12,23 % at the higher doses of cadmium. We recorded no statistically significant changes in the monitored parameters of stomata caused by the lower dose of cadmium (Cd50) except of the length of stomata on the bottom side of leaves. Higher dose (Cd100) caused decrease in the size of stomata on both sides of leaves and increase of the number of epidermal cells on adaxial side at the unchanged number of stomata, which lead to a decrease of the stomatal index. The variations of response could be signals of tolerance or adaptive mechanisms of the leaves under the determined concentrations. Deeper anatomic and physiological studies can contribute to the explanation of the role of cell epidermis of faba bean in its adaptation to ions of cadmium.

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