Comparative responses to cadmium toxicity of *Arabidopsis thaliana* mutant seedling (*cad2*) and wild type (*col0*)

CHAFFEI HAOUARI CHIRAZ[1], SLATNI TARAK[2], DJEMAL FATMA[1], GOUIA HOUDA[1]

(Company / Faculté des Sciences de Tunis)

*1*Université Tunis El Manr, Faculté des Sciences de Tunis, Département de Biologie, 1060, Tunis, Tunisia

2 (Company / Centre de Biotechnologie Borj-Cédria)

*1*Université Tunis El Manr, Faculté des Sciences de Tunis, Département de Biologie, 1060,

*1*Université Tunis El Manr, Faculté des Sciences de Tunis, Département de Biologie, 1060,

Abstract.

The present study revealed the effects of cadmium on plant growth, anti-oxidant system characteristics, co-factors such as nicotinamide adenine dinucleotide reduced (NADH) and oxided (NAD^+), cadmium uptake and translocation and physiological responses of two *Arabidopsis thaliana*: the *cad2* mutant and the *col0* wild type. The inhibitory effect of cadmium on growth characteristics (dry and fresh weight and length of aerial and root parts) was more evident for the cad2 mutant, whereas least for the *col0* wild type. The resistance of *A. thaliana* wild type (*col0*) to cadmium was mainly due to a lower cadmium absorption and translocation, thus keeping more effective activities of anti-oxidative enzymes and better co-factor balance state. Furthermore, hyperactivity of anti-oxidant enzymes also played an important role in protecting wild *A*rabidopsis *thaliana* from cadmium toxicity.

Keywords*: Arabidopsis thaliana, cadmium oxidative stress, nicotinamide adenine dinucleotide.*

1. Introduction

The direct impact of the contamination of grounds by heavy metals such as cadmium (Cd). Cd is one of the naturally occurring toxic heavy metal and negatively affects the plant growth and development. Plants (Yu Song et al., 2017) readily absorb Cd. These last years, the interest is turned to the study of the answer of plants towards the soil pollution to heavy metals to understand mechanisms and processes involved in the fight against the toxic effects of these metals by plants.

The excess of cadmium in plants can perturbs nutrient homeostasis, change physiological processes (Sharma and Kumar, 2002) with the appearance of visual symptoms of toxicity as the reduction of the growth and the appearance of chlorosis and of necrosis at the foliar and root part (Song et al., 2017; Wang et al., 2018) and induce massively toxic reactive oxygen species (ROS) (Daoud Khan et al., 2013; Li et al., 2015), all of which are associated with the damage of proteins, nucleic acids and lipids of plasma membrane (Daud Khan et al., 2013). Furthermore, plants have an antioxidant defence system, which tries to limit the damages engendered by the ROS at the level of the cellular constituents (Wojcik and Tukiendorf, 2004). Many plant species have developed diverse mechanisms to deal with the issues by cadmium (Cd) efflux, chelation, sequestration or detoxification (Singh et al., 2011; Hang et al., 2012). For example, most of brassicacea species or Arabidopsis generate phytochelatins, when challenged to environmental cadmium (Wang et al., 2018).

Arabidopsis wild plants (*col0*) enhanced Cd tolerance, while mutation by glutathione deficiency led to more sensitivity of plants to Cd (Wojcik and Tukiendorf, 2004). Glutathione was found to activate genes involved in PCs production, suggesting that Cd-induced glutathione was able to positively regulate Cd tolerance through the PCs synthesis-mediated pathway (Abozeid et al., 2017).

The objective of our work is to understand the influence of the cadmium on the functioning of the antioxidant system and the interaction between its various components to *Arabidopsis thaliana* savage *Col0* plants and mutant *cad2* characterised by glutathione deficiency. To date, the majority of studies are focusing on identification of essential function of phytochelatins

(PCs) by stabilization of the PC-Cd complex and sequestration to the cell vacuole (Choppala et al., 2014; Song et al., 2017).

2. Materials and methods

2.1. Plant material and growth conditions

The plant material concerned by this study is *Arabidopsis thaliana* wild type (*col0*) and mutant type (*cad2*), glutathione deficiency. The seeds are disinfected by washing with 10% bleach for 15 minutes, then rinsed thoroughly with distilled water; as a result, they are germinated in petri dishes on filter paper soaked in water at room temperature and in the dark.

Five days after germination, seedlings are transplanted at bucket of nutrient solution containing: KNO_3 3 mM, $Ca(NO_3)_2$ 1 mM, KH_2PO_4 2 mM, $MgSO_4$ 0.5 mM, Fe-Ethylenediaminetetraacetic acid (EDTA) 32.9 µM, and micronutrients: H_3BO_4 30 µM, $MnSO_4$ 5 µM, $CuSO_4$ 1 µM, $ZnSO_4$ 1 µM, and $(NH_4)6Mo_7O$ 1 µM. The cultures are conducted in air-conditioned room, the photoperiod is 8 hours of light / 16 hours of darkness.

After 20 days of culture on basic nutrient medium, *A. thaliana* seedlings are placed in nutritive solutions with different concentration of cadmium: 0, 25 and 50 µM.

Harvests are made after 6 days of treatment. The plants are subdivided into leaves and roots. Part of the samples are used for the determination of their fresh material (FM) and placed in the oven at 60 ° C for 3 days and reweighed to determine the mass of dry matter (DM) and another part is kept at -80 ° C for enzymatic assays or certain metabolites.

2.2. Cadmium content

Cadmium content in various plant tissues were analyzed by digestion of dried samples with an acid mixture ($HNO_3/HClO_4$, 4/1 v/v). Metal concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer, AAnalyst 300).

2.3 Malondialdehyde content (MDA)

Lipid peroxidation was measured according to malondialdehyde (MDA) content was measured photometrically. Data was collected from three independent repetitions (Daud Khan et al., 2013)

2.4. Nicotinamide adenine dinucleotide

Nicotinamide adenine dinucleotide reduced (NADH) and oxyded (NAD^+) content were estimated spectrophotometrocally on crude leaf extracts (Mika-Russak et al., 2012).

2.5. Antioxidative enzymes assay

Leaves part of *A. thaliana* (control and treated plants) were homogenized in liquid nitrogen using a mortar. For superoxide dismutase (SOD), the resulting powder was added to 100 mg of a 50 mM extraction medium of potassium phosphate buffer (pH 7) containing 1 mM EDTA and 5% (V/V) polyvinylpolypyrrolidone (PVP). For ascorbate peroxidase (APX), 5 mM ascorbate was added to the extraction medium. The crude extracts were centrifuged at 14,000 g for 30 min at 4°C and the supernatants were used for assays. SOD activity was determined according to Abozeid et al, (2017), absorbance was measured at 340 nm. GR activity was assayed according to Rao et al (1996). The activity of glutathione reductase was measured from the oxidation of NADPH at 340 nm.

2.6. Statistical analysis

The results are the means±S.E. of at least three independent replicates. The analyses of variance were computed on statistically significant differences determined based on the appropriate F-tests. The mean differences were compared utilizing Duncan's multiple range tests.

3. Results and discussion

3.1. Growth and cadmium accumulation

The morphological observation (Fig. 1A) of plants shows toxicity due to the cadmium, which shows by the reduction of the size of the plant, the appearance of chlorosis on, leaves part and the reduction of the length of the root, as well at the savage *col0* as the mutant *cad2*. We notice that the dose 50 µM of $CdCl_2$ induces more chlorosis on mutant plant (*cad2*) than on wild type (col0). We also note that the phytotoxic effects of cadmium are dependent on the used dose. The effects of cadmium on the biomass production of the plants of *Arabidopsis thaliana* in wild (*col0*) and mutant (*cad2*) type, shows that the production of the biomass of leaves (Fig. 1B) and roots (Fig. 1C) was affected especially with the higher dose of Cd (50µM). The sensibility was more important with the mutant (*cad2*) than the wild type (*col0*).

However, in the two rang of plant such as wild (*col0*) or mutant type (*cad2*), the leaves part appear more sensitive than the root part.

The accumulation of Cd in leaves and roots of wild (*col0*) and mutant type (cad2) of *A. Thaliana* differed with increasing Cd concentrations (Fig. 2). At lower dose of Cd (25µM), the endogenous accumulation was similarly such as in wild (*col0*) or in mutant (*cad2*) seedlings. This similitude was common in leaves and roots of the two types of *A. thaliana* seedlings. Compared to lower dose, the leaves of wild type (col0) increased by 2.5 fold the endogenous accumulation at 50 µM Cd (Fig. 2A). However, when the plants were treated with Cd at higher dose (50 µM), the roots of mutant (*cad2*) type shows the high intensity accumulation of cadmium (Fig. 2B). This accumulation was 4 fold than that is at 25µM of Cd. This may be correlated with the high decrease of dry weight at the dose of 50µM Cd. Glutathione (GSH) also plays a critical role in detoxification of heavy metal because of its capacity to chelate heavy metals and act as substrate of phytochelatine (PC) and then PC-metal complexes are transported across the tonoplast into vacuoles (Li et al., 2012; Anjum et al., 2015; Cahoon et al., 2015).

Cadmium as an environmental stress may increase or decrease metabolite production such as lipid peroxidation (MDA) (Daud Khan et al., 2013), nicotinamide adenine dinucleotide oxydaded (NAD^+) or reduced (NADH), parallely to the enzymatic response of key enzymes of oxidative stress likely superoxyde dismutase (SOD) (Zawznik et al., 2007), ascorbate peroxydase (APX) (Zawznik et al., 2007) and glutathione reductase (GR).

3.2. Antioxidative response of mutant (*cad2*) and wild (*col0*) type to cadmium

As figure 3 shows the extent of oxidative damage of membrane lipids, in both *Arabidopsis* genotypes Cd altered not only the malondialdehyde content in the leaves but also all antioxidant enzymes activities. For the leaves of mutant genotype (*cad2*), a significant increase in malondialdehyde content (Fig. 3A) compared to untreated plants was observed after Cd treatment. However, no significant differences in MDA contents were found treated and non- cadmium treated in wild type (*col0*). An induction of lipid peroxidation upon cadmium treatment was reported especially for Arabidopsis mutant (*cad2*) seedlings (Radeva-Ivanova et al., 2010). Data show the significant increase in SOD activity in leaves of mutant type (cad2), and no increase in APX and GR activities, key enzymes of glutathione-ascorbate cycle. In figure 3C, APX activity was significantly inhibited. To investigate the high increase

of SOD (Fig. 3B) activity without complementary induction of H_2O_2 scavenging, amplify and accelerate the cellular damage (Dubreuil-Maurizi et al., 2011). Plants have developed effective defence system against H_2O_2 accumulation, which include the induction of GSH-AsA cycle, necessarily to send a systemic signal for APX induction activity (Sandalio et al., 2001). This data can explain the high toxicity effect of cadmium in leaves of mutant type (*cad2*). Hydrogen peroxide (H_2O_2) accumulation removal the indication the cadmium toxicity on APX activity in mutant type glutathione (GSH) deficiency. In glutathion-ascorbate cycle, the GSH pool ply a primordial role in the ascorbate generation, which the substrate of APX. This generation was blocked in mutant type (*cad2*), induced the significant decrease of APX activity.

In addition, in absence of glutathione (GSH) pool in mutant (*cad2*) type, the phytochelatine biosynthesis was blocked, wish responsible to a high cytoplasmic accumulation of cadmium ion (Cd^{2+}) induced ROS generation accompanied of APX inhibition activity and hydrogen peroxide accumulation (H_2O_2). The no stimulation of glutathione reductase (GR) (Fig. 3D) activity in mutant type was explained the deleterious effect of glutathione deficiency in defence metabolites. To validate that more high cellular oxidative stress observed in mutant type (cad2), as an indicator that mutant type was more sensitive to cadmium than wild type (col0).

The significant stimulation of APX activity in wild type (Col0), key enzyme of glutathion-ascorbate cycle, induces the generation of dehydroascorbate (DHA). The generated DHA will reduced on ascorbate AsA catalysed by monodehydroascorbate reductase, which that is NADPH or NADH dependent (Singh et al., 2017).

3.3. Cadmium effects on NAD(H) content

The effects of cadmium on the NAD^+ and NADH contents derived from both the wild (*col0*) and mutant (*cad2*) leaves of *Arabidopsis thaliana* were also measured (Fig. 4A and B). Under control condition, the NAD^+ content was similar in wild (*col0*) and mutant (*cad2*) seedlings. In contrast, the NADH content was 2 fold greater in wild type than in mutant type. In treated plant (with 25µM of cadmium), NAD^+ content decreased in both wild (*col0*) and mutant type (*cad2*) (Fig. 4A). The result of NADH pool, unchanged in wild type (*col0*) under cadmium stress conditions (Fig. 4B). In mutant type (*cad2*), the NADH pool significantly increased in

presence of 25µM Cd. The kinetic activity of monodehydroascorbate reductase (MDHAR) needs the presencec of NADH or NADPH co-factor. Although, the glutathione-ascorbate cycle was inhibited in mutant type (*cad2*) in presence of cadmium, the MDHAR activity wich catalyses the ascorbate synthesis by NADH oxidation was completely inhibited. These data can explain the high accumulation of NADH in mutant type (*cad2*).

To investigate the increase pool of NADH will affected another reaction, whereas resulted a strong production of NADH induced a rise of equilibrium on cellular redox state, which could to increased ROS and intensity of oxidative stress, observed in our result with the mutant type (*cad2*) (Li et al., 2012).

In summary, it can be concluded that the glutathione deficiency in mutant type (*cad2*) is related to distubed growth, which directly reduced biomass production and the accumulation of endogenous cadmium more than in wild type (*col0*), associated with the redox state modification and more important in this type. Our results highlight that glutathione modulate and enhanced the sensivity of Arabidopsis thaliana seedlings to cadmium stress.

Literature cited

Abouzeid A., Ying Z., Lin Y., Zhang Z., Tang Z. (2017). Ethylene improves root system developmentunder cadmium stress by modeling superoxide anion concentration in *Arabidopsis thaliana*. Frontiers in Plant Sciencers. 8: 1-15.

Anjump NA., Hasanuzzaman M., Hossain MA., Thangavel P., Roychoudhury A., Gill SS., Rodrigo M., Adam V., Fujita M., Duarte AC., Preira E., Ahmad I. (2015). Jacks of metal/metalloid chelation trade in plants an overview. Front Plant Sciences. 6: 192-199.

Cahoon RE., Lutke WK., Cameron JC., Chen S., Lee SG., Rivard RS., Rea PA., Jez JM. (2015). The J. Biol. Chem. 29: 17321-17329.

Choppala G.n, Bolan SN., Bibi S., Iqbal M., Rengel Z. (2014). Cellular mechanisms in higher plants governing tolerance to cadmium toxivity. Critical Reviews in Plant Sciences. 33(5): 374-391.

Daud Khan M., Mei L., Ali B., Chen Y., Cheng X., Zhu SJ. (2013). Cadmium induced upregulation of lipid peroxidation and reactive oxygen species caused physiological,

biochemical and ultrastructural changes in Upland Cotton seedlings. Biomedical Research. Inter. 2013: 1-10.

Dubreuil-Mauriz C., Vitecek J., Marty L., Branciard L., Frettinger P., Wendhenne D., Meyer AJ., Mauch F., Poinssot B. (2011). Glutathione deficiency of the Arabidopsis mutant pad2-1 affects oxidative stress related event, defense gene expression and the hypersensitive response. Plant Physiol. 157: 2000-2012.

Huang J., Zhang Y., Peng JS., Zhong C., Hy Y., Dw O., Gong JM. (2012). Fusion yeast HMT1 lowers seed cadmium through phytochelatin-dependent vacuolar sequestration in Arabidopsis. Plant Physiol. 158(4): 1779-1788.

Li Y., Gong Z., Mu Y., Zhang Y., Qiao Z., Zhang L., Jin Z., Li H., Pei Y. (2012). An Arabidopsis mutant atcrs-2 exhibits high cadmium stress sensivity involved in the restriction of H_2S emission. J. Biomedecine and Biotechnology. 13(12): 1006-1014.

NIKA-RUSSAK M., KABABA K., BURZYNSKI M. (2012). DIFFERENT EFFECT OF CADMIUM AND COPPER ON H^+-ATPASE ACTIVITY INN PLASMA MEMBRANE VESICLES FROM *CUCUMIS SATIVUM* ROOTS. J. EXP. BOT. 63(11): 4133-4142.

Radeva V., Petrov V., Minkov I., Toneva V., Gechev T. (2010). Effect of cadmium on Arabidopsis thaliana mutants tolerant to oxidative stress. Biotechnology and Biotech. Equipments. 24: 113-116.

Rao, M.V., Paliyath, G., Ormrod, D.P. (1996). Ultraviolet-B- and Ozone- Induced Biochemical Changes in Antioxidant Enzymes of *Arabidopsis thaliana*. *Plant Physiology*, 110(1), 125-136.

Sandalio L.M., Dalurzo H.C., Gomez M.3, Romero-Puertas M.C., Del Rio L.A, (2001). Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J. Exp. Bot*. 52, 2115-2126.

Sharma SS., Kumar V. (2002). Responses of wild type and abscisic mutants of Arabidopsis thaliana to cadmium.J. Plant Physiology; 159: 1323-1327.

Singh GS., Tuteja N. (2011). Cadmium stress tolerance in crop plants: probing the role of sulphur. Plant Signaling and Behaviour. 6(2): 215-222.

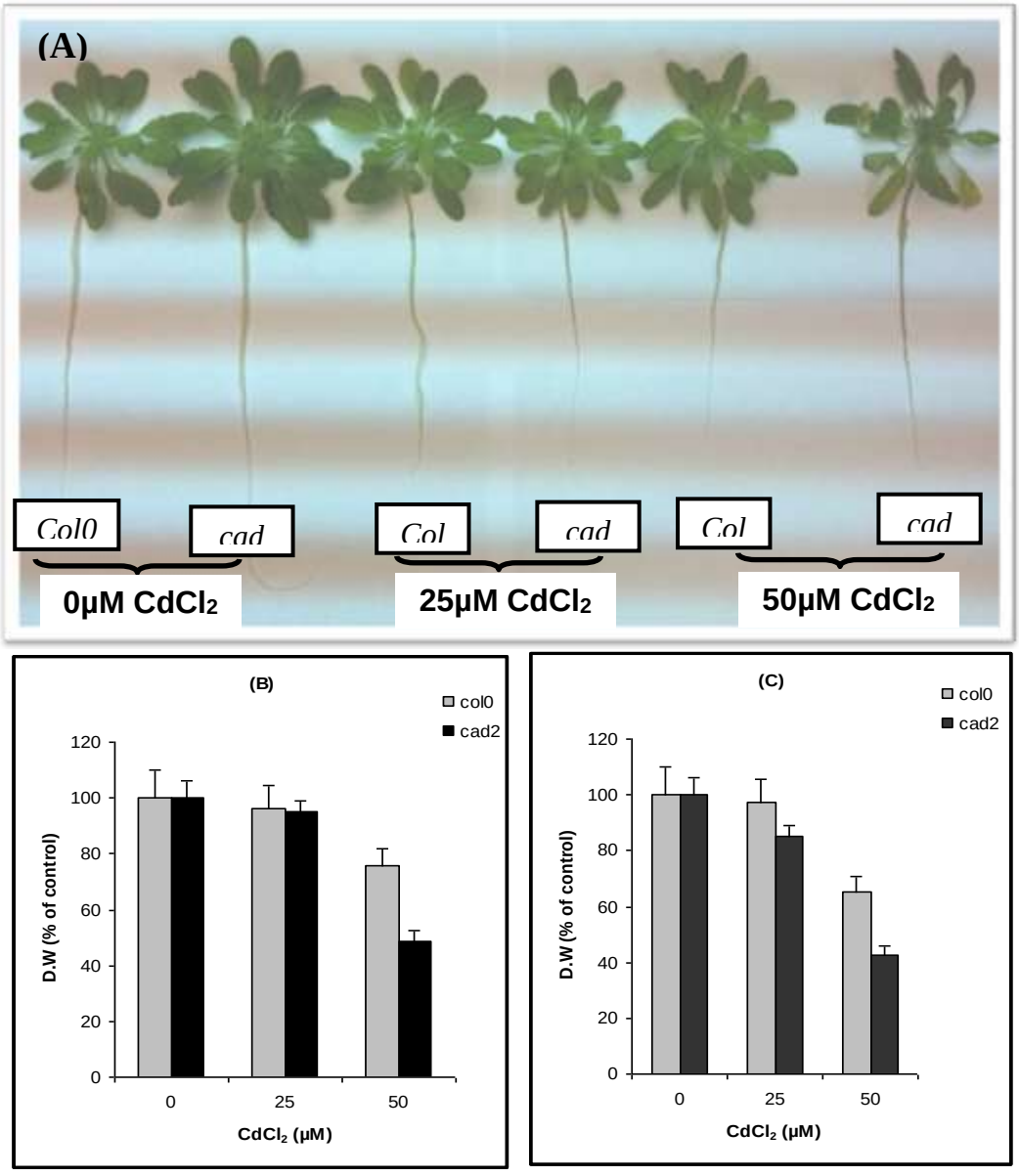
Singh S., Singh A., Srivastava PK., Prasad SM. (2017). Cadmium toxicity and its amelioration by kinetin in tomato seedlings via Ascorbate-glutathione cycle. J. Photochemistry and Photobiology. 178: 1-12.

Song Y., Jin L., Wang X. (2017). Cadmium absorption and transportation pathways in plants. Inter. J. Phytoremediation. 119(2): 133-141.

Wang T., Hua Y., Chen M., Zhang J., Guan C.N., Zhang Z. (2018). Mechanisms enhancing Arabidopsis resistance to cadmium: the role of NRT1.5 and proton pump. Frontiers in Plant Sciences. 9: 1-12.

Wojcik M., Tukiendorf A. (2004). Phychelatin synthesis and cadmium localization in wild type of Arabidopsis thaliana. Plant Growth Regulation. 44: 71-80.

Figures list



CHAFFEI HAOUARI CHIRAZ[1], SLATNI TARAK[2], DJEMAL FATMA[1], GOUIA HOUDA[1]

Figure 1: Effects of cadmium dose on morphological aspect of wild type (*col0*) and mutant (*cad2*) of *Arabidopsis thaliana* seedlings.

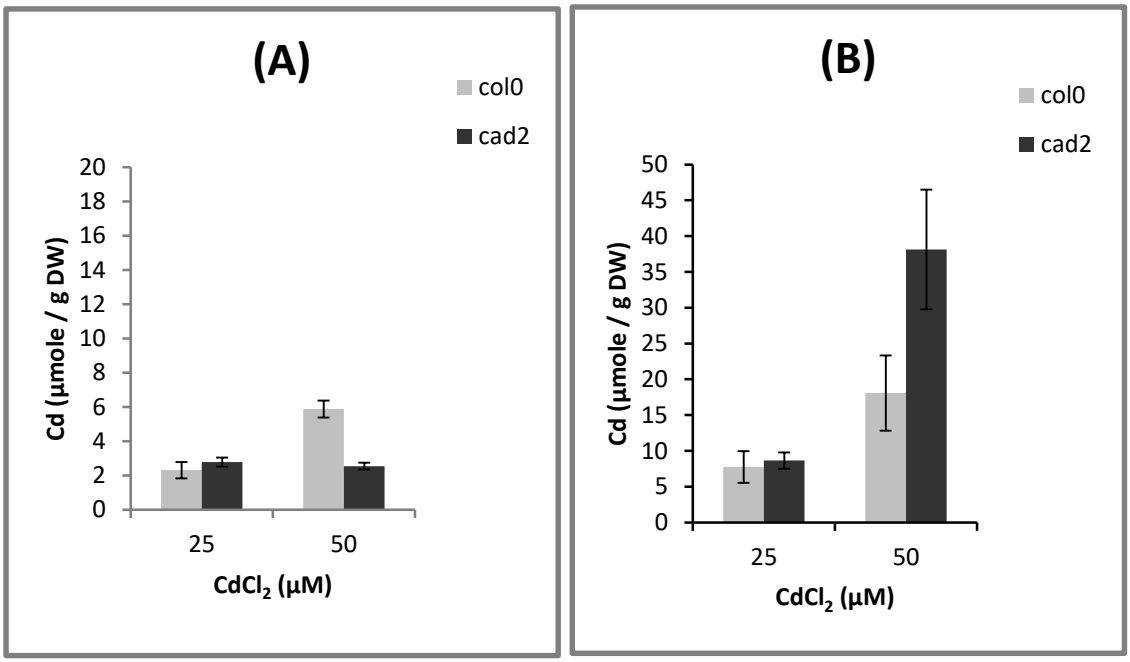


Figure 2. Cadmium content in leaves (A) and roots (B) of the *cad2* mutant and the *col0* wild type of *Arabidopsis thaliana* seedlings.

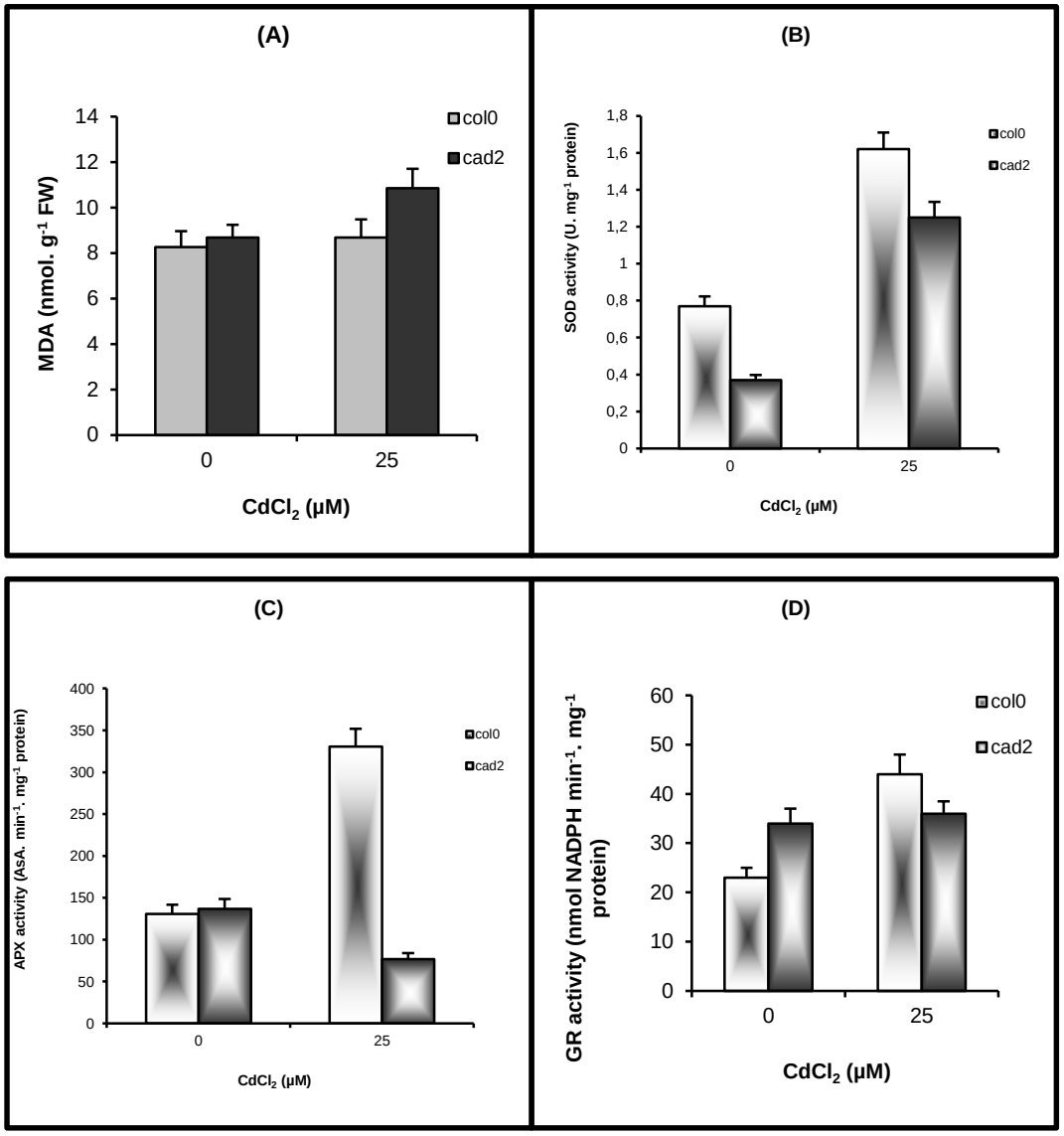


Figure 3: Malonyldialdehyde (MDA) production (A), the specific activities of superoxyde dismutase (SOD) (B), of ascorbate peroxydase (APX) (C) and the the glutathion reductase (GR) (D) on leaves of the *cad2* mutant and the *clo0* wild type of *Arabidopsis thaliana* seedlings under cadmium treatment after four days.

CHAFFEI HAOUARI CHIRAZ[1], SLATNI TARAK[2], DJEMAL FATMA[1], GOUIA HOUDA[1]

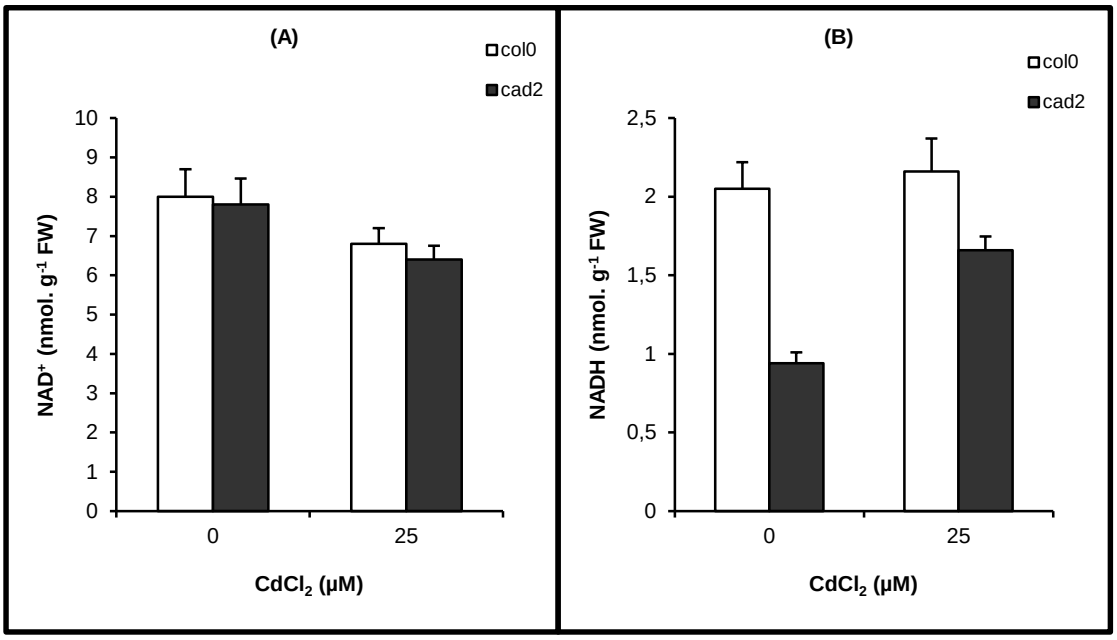


Figure 4: Nicotinamide adenine dinucleotide oxide form (A) and reduced form (B) on leaves of the *cad2* mutant and the *clo0* wild type of *Arabidopsis thaliana* seedlings under cadmium treatment after four days.