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RESPONSES OF A LEGUME (*CICER ARIETINUM*) TO CADMIC STRESS IN THE PRESENCE OF TWO SOIL FUNGI

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Abstract: Metal trace elements, by their non-biodegradable nature, are ecotoxic and could be involved in many pathologies. It is therefore important to fully understand their effects on living organisms, but also to implement sustainable solutions to limit their risks. In our work, the impact of cadmium chloride on the chickpea (*Cicer arietinum)* is to be assessed via the determination of certain biomarkers of stress at the root level (glutathione (GSH), malondialdehyde (MDA), lipid, ascorbate peroxidase (APX) and glutathione peroxidase (GPX) activities) in the presence or absence of fungal strains. The results obtained show a fluctuation of the various parameters studied according to the increase in xenobiotic doses, suggesting the installation of oxidative stress and the induction of the antioxidant system. However, all values recorded in treated plants appear to be lower in the presence of *Fusarium oxysporum* and *Aspergillus niger*, suggesting that these fungi block the passage of metals through the plant by accumulating them, thereby reducing the effect of cadmium on plant metabolism.

Keywords: Metals, Oxidative stress, Bioremediation, *Fusarium oxysporum, Aspergillus niger.*

INTRODUCTION

The main sources of soil pollution by metal trace elements (MTE) are related to human activities (Benhamadi, 2014) and more specifically metallurgical industries, which generate large quantities of waste with high metal content, as well as the misuse of pesticides and phosphate fertilizers (Thi My Dung Huynh, 2010). These contaminated soils represent a major environmental and health risk due to the persistence, accumulation and possible transfer of metals to the food chain or groundwater, thus disrupting the various ecosystems.

Plants, continuously in contact with the soil, can be exposed to high levels of metals, which induces an oxidative burst causing a cascade of reactions that damage biological macromolecules (Ali *et al.*, 2011). In general, the toxicity of ETMs on plants is due to the formation of reactive oxygen species (ROS), thus affecting a wide variety of cellular, physiological and biochemical functions, such as plasma membrane rupture, lipid peroxidation, protein denaturation, and DNA and RNA destruction (Bose *et al.*,2013; Li *et al*.,2018). This has been observed in countless studies such as Lotmani and Menoua (2011), which have demonstrated the induction of the antioxidant system (peroxidases and catalase) in *Atriplex halimus* L. under metallic stress. Similarly, in Park *et al*., (2017), where the overexpression of AtCYP21-4 from potatoes (*Solanum tuberosum* L.), a protein involved in tolerance to oxidative stress, resulted in heavier tubers.

To remedy these stressful conditions and reduce the harmful effects of metals, various soil decontamination techniques have been carried out, physical, chemical and biological (Yadav and Hassanizadeh, 2011; Lipińska *et al*., 2014). Several authors have conducted studies on biological remediation techniques such as Kavamura & Esposito (2010) and Wu *et al*. (2010), which have worked on the different bioremediation strategies (phyto- and microremediation) applicable *in situ* for the decontamination of soils with ETM.

Fungi are known for their biodegradation capacity attributed to the enzymes they produce, which degrade a large number of pollutants such as polycyclic aromatic hydrocarbons and heavy metals (Bumpus *et al*., 1985). In this context, the impact of Cadmium on *Cicer arietinum* defense system must be assessed in order to demonstrate the species tolerance to applied metallic stress. On the other hand, determine the health of the plant by using biological pollution control processes that limit the absorption of metals by the plant in the presence of fungi and therefore reduce the intensity of oxidative stress.

MATERIALS AND METHODS

Plant material

The plant material used in our work is represented by a variety of chickpeas (*Cicer arietinium*). The seeds were provided by the Algerian Interprofessional Cereals Office (OAIC) El Hadjar Annaba, Algeria.

Chemical equipment

This work focused on cadmium chloride, three concentrations were chosen.

Fungal species

These are two fungal strains, *Fusarium oxysporum* and *Aspergillus niger*, provided by the National Plant Protection Institute, the Laboratory of Mycology, Annaba-Algeria.

Growing conditions

Chickpea seeds (*Cicer arietinum*) are disinfected with a 3% sodium hypochlorite solution for 5 minutes and then rinsed with distilled water. Chickpeas are grown in plastic pots filled with 250 g of disinfected soil. The seeds are sown in holes about 2 cm deep, made with a pencil, each pot contains four seeds. The

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inoculation of the two fungal strains (*Fusarium oxysporum* and *Aspergillus niger)* was carried out separately using the following technique: In Petri dishes, containing the solid PDA medium, a 6mm disc of each fungal strain is placed in the center of each dish and incubated until the mycelial growth reaches the edges of the dishes. From these boxes, 10 ml of 0.9% sterile physiological water is poured to obtain a homogeneous spore suspension which is read at 625 nm. This measured density is assumed to be equivalent to "10^6 spores/ml" (Braga *et al*., 2007). Then, 1 ml of the sporal suspension is injected on peat that will coat the seed. Metallic stress is applied at the seedling stage using different concentrations as shown in Table 1. All dosages will be done after 1 month and 2 months of exposure, part of it remains irrigated periodically and without treatment: this is the control.

Table 1.

Cadmium concentrations applied

Cadmium chloride (g/l) Cd (1 month)	**C 0**	**C1 4.10^{-4}**	**C2 5.10^{-4}**	**C3 8.10^{-4}**
Cadmium/*Aspergillus niger* Cd/A (1month)	0	C1A	C2A	C3A
Cadmium/*Fusarium oxysporum* Cd/F (1month)	0	C1F	C2F	C3F
Cadmium chloride (g/l) Cd' (2 months)	0	C1 4.10^{-4}	C2 5.10^{-4}	C3 8.10^{-4}
Cadmium/*Aspergillus niger* Cd'/A' (2months)	0	C1A'	C2A'	C3A'
Cadmium/*Fusarium oxysporum* Cd'/F' (2months)	0	C1F'	C2F'	C3F'

A: *Aspergillus niger*, **F:** *Fusarium oxysporum*, **C:** control

Analytical techniques

Lipid determination

Total lipids are determined by the method of Goldsworthy *et al*., (1972). Each sample consists of 0.5g of fresh root material, is cut and macerated in 10ml of trichloroacetic acid TCA (20%). After grinding and filtration in test tubes, 1ml of the extract is taken and centrifuged at 5000 rpm for 10 minutes. The supernatant is poured in and the pellet containing the lipids is kept in the same tube. To the latter is added 1ml of the Ether/Chloroform mixture (1/1), then a second centrifugation (5000 rpm for 10 min) is carried out, which gives two phases: a pellet (containing the proteins) and a supernatant (containing the lipids). 100µl is removed from the supernatant to which 1ml of sulphuric acid is added and the tubes are placed, after stirring, in a water bath at 100°C for 10 minutes. After cooling, 200µl is removed again from the extract, to which 2.5ml of the 85% sulfophospho-vanillinic mixture is added (0.38g Vanillin + 195ml orthophosphoric acid + 55ml H2 O). This reaction develops a pink colour. Thus, the spectrophotometric reading is performed at a wavelength of 530nm.

Dosage of malondialdehyde (MDA)

Homogenization of the plant tissue in trichloroacetic acid (TCA 5%) at a rate of 10 ml per 1 g of plant material, followed by centrifugation for 15 min at 12000 g. To the supernatant is added an equal volume of thiobarbituric acid (TBA) at 0.5% in the 20% TCA, and the mixture is then incubated for 30 min at 100 ° C. The absorbance of the supernatant obtained after centrifugation at 10000 g for 5 min is read at 532 nm (Draper *et al.*,1990).

Glutathione Assay (GSH)

The enzyme extract is homogenized in a Tris / EDTA solution and deproteinized with 0.25% sulfo-salicylic acid. After centrifugation at 2000 g for 10 minutes, the supernatant is used for the assay with DTNB reagent at 0.01M to 412 nm. The concentrations of GSH are assayed by the method of (Weckbecker *et al.*,1988).

Ascorbate-Peroxidase Activity Assay (APX)

The determination of the ascorbate peroxidase activity is carried out according to the method of Manivannan *et al*., (2007). The final reaction volume of 3 ml contains: 100µl of enzymatic extract, 50µl H_2O_2 at 0.3% and 2850µl of NaK buffer Ascorbate (pH = 7.2). The reading is carried out at a wavelength of 290 nm for 1 min and is expressed in nmol/min/mg of Proteins.

Guaiacol Peroxidase Activity Assay (GPX)

For a final volume of 3 ml, the reaction mixture contains: 100 µl of enzymatic extract, 50 µl of 0.3% H_2O_2 and 2,850 ml of Na K-guaiacol buffer (pH 7.2). The GPX activity is determined at 470 nm and expressed in nmol/min/mg of proteins (Hiner *et al*., 2002).

Statistical analysis

The results obtained are expressed by the mean standard deviation. Using the statistical test, analysis of variance with two controlled factors (ANOVA) as a function of time and concentrations applied, using data analysis software: Minitab (version 16.0).

RESULTS

Effect of Cd on lipid levels in *Cicer arietinum* roots after one and two months of treatment, with or without fungi

The lipid assay revealed a very highly significant decrease (P≤0.000) in the levels of lipids in cadmium-treated chickpea roots after one and two months of treatment in the absence of fungal strains. The lowest value is observed at the highest concentration which reaches 1.6 mg/g FM after 1 month and 0.6 mg/g FM at the end of treatment, a decrease of half.

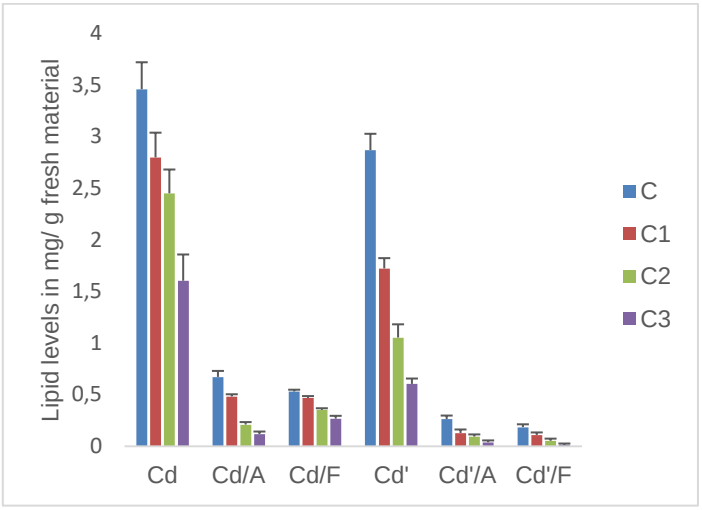

Fig. 1. Effect of Cd on lipid levels after one month and two months of exposure with or without fungi.

At the same time, the effect of cadmium on lipid levels in the presence of *Aspergillus niger* and *Fusarium oxysporum* shows a very highly significant decrease (P≤0.000) in the latter after one month of treatment with Cd. However, it should be noted that this decrease is more very highly significant in roots treated with *Aspergillus* than in roots treated with *Fusarium*. At the end of the treatment, we recorded a significant decrease in the same rate in the controls as well as in the roots treated with two fungi. It should be noted that this decrease is more significant in the 2nd months compared to the one after the first month.

Cadmium effect on the level of MDA in the roots of *Cicer arietinum* after one and two months of treatment and in the presence or absence of fungi

MDA levels increased significantly (P≤0.001) with dose in treated roots with increasing concentrations of cadmium after one month of treatment compared to the control roots (Fig.2) and reached their maximum (0.14 µmol / mg protein) for C3 is twice the control value. Although this rate decreases during month 2.

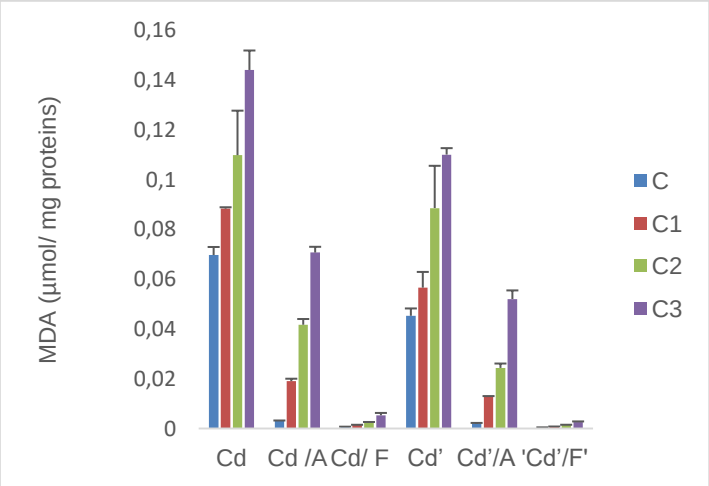

Fig. 2. Effect of Cd on MDA levels after one month and two months of exposure with or without fungi.

At the same time, when the two fungi are added separately to the soil, the MDA level decreases significantly according to the increasing concentrations of cadmium after one month compared to the control

roots (without fungi). The highest value of MDA is recorded for C3 (0,0052µmol / mg Protein) which is eight times the control value in the presence of *Aspergillus niger*, whereas this same rate is very low in the roots exposed to Cd during a month and in the presence of *Fusarium oxysporum*. It should be noted that all levels of MDA recorded after 2 months of treatment and in the presence of both fungal strains are lower compared to those obtained after 1 month.

Cadmium effect on the level of GSH in *Cicer arietinum* roots after one and two months of treatment, with or without fungi

From Figure 3, there is a significant decrease (P≤0.001) of GSH levels in roots treated with Cd after 1 and 2 months in the absence of the fungus. The set of recorded values are lower than those of the control (0,06nmole / min/mg Proteins).

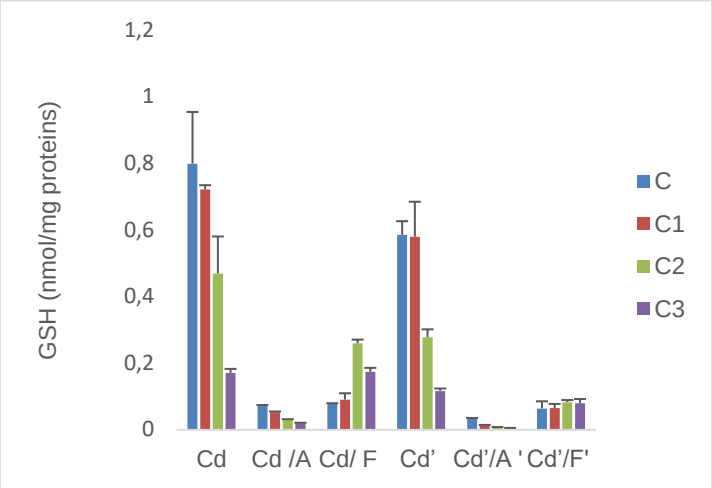

Fig. 3. Effect of Cd on GSH levels after one month and two months of exposure with or without fungi.

At the same time, it is noted that after 1 and 2 months of treatment and in the presence of *Aspergillus* and *Fusarium*, the GSH level decreases significantly according to the Cd concentrations compared to the treatment without fungi. Note that the decrease in the second month is always lower compared to the first month.

Cadmium effect on APX activity of *Cicer arietinum* roots after one or two months of treatment, with or without fungi

The APX activity monitoring results recorded during the whole treatment (Fig. 4) show that the latter increases significantly (P≤0.001) as a function of increasing concentrations of Cd. A peak is observed at C2 in the first month which equals four times the control value in the absence of fungi.

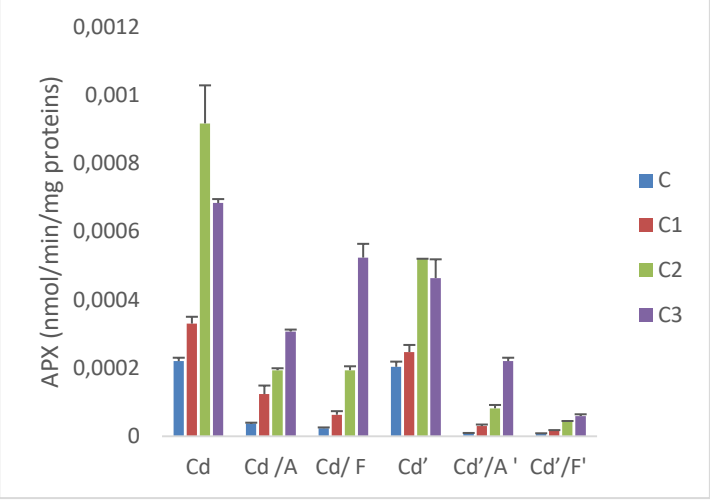

Fig. 4. Effect of Cd on APX activity after one and two months of exposure in the presence or absence of fungi.

APX activity decreased significantly in cadmium-treated chickpea roots after 1 and 2 months in the presence of both fungal strains compared to roots treated in the absence of fungi. However, it is observed that in the presence of Fusarium and after one month of treatment, APX activity increases to its maximum at the highest concentration, which is twice that observed in treated roots in the presence of *Aspergillus*. However, after two months of treatment and in the presence of *Fusarium*, all APX values recorded are

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lower than those observed in the first month but also lower than those obtained in the presence of *Aspergillus*.

Cadmium effect on GPX activity in *Cicer arietinum* roots after one and two months of treatment, with or without fungi

From fig. 5, the GPX activity increases significantly in Cd-treated chickpea roots and reaches a maximum of 80% for C3 compared to the control. Same at the end of treatment where there is a dose-dependent increase in this activity with a peak recorded at the highest dose. However, we report that all values obtained after two months of exposure are lower compared to those obtained after one month of treatment.

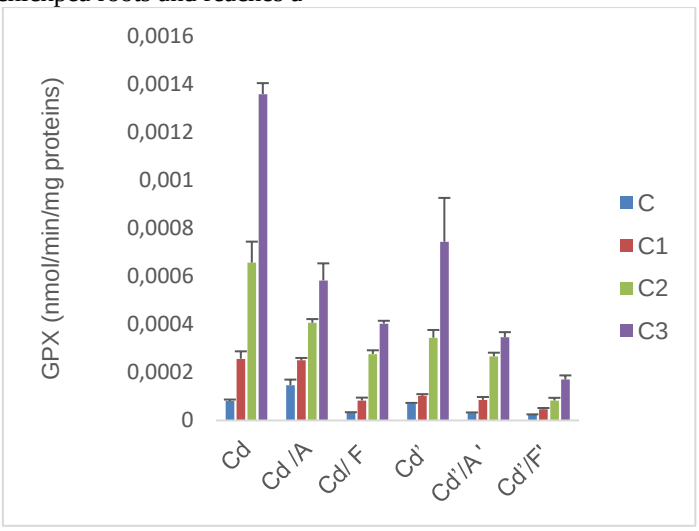

Fig. 5. Effect of Cd on GPX activity after one and two months of exposure in the presence or absence of fungi.

In general, all the GPX activity values obtained in Cd-treated chickpea roots after 1 and 2 months and in the presence of *Fusarium* and *Aspergillus* are dose-dependent with a peak recorded at the highest C3 concentration. It should be noted that this activity is lower in the second month than in the first month compared to controls, but it is also lower in the presence of *Fusarium oxysporum* than in the presence of *Aspergillus niger*

DISCUSSION

The results recorded in our study revealed an increase in MDA levels as well as a decrease in lipid levels in roots treated with different concentrations of cadmium, which is due to the interaction of polyunsaturated fatty acids (PUFAs) in membrane lipids with ROS or toxic oxygenated intermediates accumulated during metal stress to form lipid radicals and reactive aldehydes (Lyubenova *et al.*, 2009). Ben Youssef *et al.*, (2002, 2005) reported a decrease in lipid and PUFA levels in rapeseed leaves treated with 50µM Cd and a reduction in phospholipid and neutral lipid levels in the roots. Similarly, Bouchama (2012) reported a decrease in lipid levels in *Phragmites australis* treated with Cd. At the same time, the results obtained for MDA levels corroborate those observed by Shutzendubel and Polle (2002) and Gill and Tuteja (2010) who report an increase in MDA levels in the presence of cadmium and other heavy metals. Similarly, work by Rao *et al.* (2014) and Salah *et al.* (2015) showed an increase in MDA in roots and leaves of *Brassica juncea* and *Oryza sativa L.* exposed to ZnO nanoparticles (NPS) and in roots of *Zea mays L.* treated with Fe_2O_3 NP_S compared to control roots (Li *et al.*, 2016).

It should also be noted that in roots treated in the presence of fungal strains, the level of MDA decreased as a result of metal accumulation by the inoculated fungi compared to those treated in the absence of fungi. Our results support those reported by Hashem *et al.* (2015) who observed a decrease in this level in mycorrhized tomatoes grown in cadmium-polluted soil and by Drai *et al.* (2016) who showed that mycorrhization of tomato plants and wheat causes fluctuations in the production of MDA in both compartments (leaves and roots). Similarly, these results have also been reported by Redon *et al.* (2008); Redondo-Gómez *et al.* (2014), Chen *et al.* (2015); Firmin *et al.* (2015) and Zhu *et al.* (2015), who explain that the presence of fungi makes the plant more resistant to stress because fungi have the capacity to provide many benefits to the plant, including improved growth and stress tolerance.

At the same time, the level of GSH recorded during our experiment decreases in chickpea roots treated with cadmium which is probably due to its use in the scavenging of free radicals produced during its oxidation to oxidized glutathione (Kadry *et al.*, 2012) but also to an increased use of the latter for the regeneration of ascorbic acid from dehydroascorbic acid or for the synthesis of phytochelatins (Hossain *et al.*, 2012). Several authors have reported the same observations in different species such as Barrameda-Medina *et al.* (2014) in *Lactuca sativa* and *Brassica*

following exposure to high concentrations of zinc and Yaiche *et al.* (2017) in copper-treated *Triticum durum* leaves who attribute this decrease to the binding of GSH to the metal.

However, in the presence of the two fungi, all measured GSH values are lower than those observed in the absence of these fungi. According to (Thorsen *et al.*,2012), GSH-SH groups have the ability to bind to metallic trace elements such as arsenic, thus forming As / GSH complexes in the extracellular space and acting as a chemical barrier to the entry of the pollutant. Similar results were obtained with lead in peas (Malecka *et al.*, 2009), cadmium in *Ceratophylhum* (Aravind *et al.*, 2005) and mercury in *Pfaffia Glomerate* (Calgaroto *et al.*, 2010).

Concerning the induction of recorded APX activity, our results are in agreement with the work of Kisa, (2017) and Sbartai *et al.*, (2012) who demonstrated the induction of APX activity in the presence of Cd in tomato roots. Lannone *et al* (2016) also showed a high induction of this activity in the presence of NP_S in *Triticum aestivum*. Indeed, in the presence of Cd, the dismutation of the superoxide anion leads to an accumulation of hydrogen peroxide (Noctor *et al.*, 1998; .Gill and Tuteja, 2010) hence the induction of APX in chickpea roots treated with Cd indicating its high affinity for H_2O_2.

At the same time, the induction of GPX activity, whose role is to eliminate excess H_2O_2, observed in our study under the effect of metals has been reported by several authors (Ederli *et al.*, 2003) in *Phragmites australis* and (Yaiche *et al.*,2017) in durum wheat. Also, the results of Labrada *et al.* (2019) showed an increase of this activity in leaf tissue of tomato plants subjected to saline stress and foliar application of copper NP_S.

On the other hand, several studies have reported inhibition of APX and GPX activities in the presence of mycorrhizal fungi in plants exposed to heavy metals (Garg & Singla, 2012; Chen *et al*., 2015; Firmin *et al*., 2015), which corroborates our results, thus expressing the plant's resistance to applied stress. The latter is linked to extracellular mechanisms that occur directly outside fungal cells through the excretion of chelating molecules that bind metals in the soil near the hyphae because these fungi have the ability to bind them to their fungal walls by non-specific biosorption allowing a decrease in the import of ETM into the cell (Bellion *et al*., 2006; Zhao *et al*., 2015).

CONCLUSION

Our results reveal that the applied xenobiotic acts on certain stress biomarkers, resulting in a decrease in lipid levels, an increase in MDA levels, a decrease in GSH levels and the induction of certain enzymatic activities (APX and GPX) involved in the antioxidant defense system, suggesting the installation of oxidative stress. When fungal strains are inoculated, all the parameters measured are lower than those obtained in their absence, once again proving their role in chelating metals and consequently protecting the plant by minimizing the intensity of oxidative stress. However, *Fusarium oxysporum* has a better ability to limit the effects of cadmium compared to *Aspergillus niger*, which allows us to suggest it as a solution for the bioremediation of cadmium contaminated soils.

AUTHORS CONTRIBUTIONS

Conceptualization: B. Laib, I. Sbartai, H. Sbartai; Methodology: B. Laib; Data collection: B. Laib; Data validation: I. Sbartai, H. Sbartai; Data processing: B. Laib; Writing—original draft preparation: B. Laib, I. Sbartai, H.Sbartai; Writing—review and editing: B. Laib, I. Sbartai, H.Sbartai.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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