Water Air Soil Pollut (2022) 233: 263
https://doi.org/10.1007/s11270-022-05725-3

Effects of Heavy Metal-Polluted Soil (Pb, Zn, and Cd) on Seed Emergence, Seedling Growth, and Antioxidant Activity in Four *Fabaceae* Species

Chadlia Hachani · Mohammed S. Lamhamedi · Mejda Abassi · Noomene Sleimi · Zoubeir Béjaoui

Received: 1 February 2022 / Accepted: 17 June 2022 / Published online: 2 July 2022

Abstract Mine tailings can contaminate large areas of neighboring agricultural lands due to the dispersion of heavy metals, which may lead to reduction in soil fertility and toxicity in crops. The use of *Fabaceae* species as green manure to amend the soil and enhance the removal of heavy metals is a promising research approach. As part of a phytoremediation project for abandoned mining sites combining woody species and agricultural crops, this study aims to identify the most suitable species to be used. Thus, four *Fabaceae* species (*Vicia faba*, *Cicer arietinum*, *Lens culinaris*, and *Medicago arborea*) were subjected to muti-metal-contaminated soil containing high concentrations of Pb, Zn, and Cd and to control soil for 15 days. Then, the emergence rate, growth parameters, lipid peroxidation, proline and hydrogen peroxide (H_2O_2) concentrations, antioxidant enzyme activities (catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX)), and heavy metals accumulation were evaluated. Results showed that *V. faba* was the most tolerant. A relative sensitivity at the germination stage was recorded for all species with the exception of *V. faba*. Metallic stress had no significant effect on thiobarbituric acid reactive susbtances (TBARS) and electrolyte leakage rates for both *C. arietinum* and *V. faba*. The latter also showed the highest proline concentration and relatively low antioxidant enzyme activities. All species showed high Pb, Zn, and Cd root contents. *V. faba* had the lowest translocation factors of Pb and Zn and the lowest bioaccumulation factors of Zn and Cd, which underline its phytostabilizing potential and support its use as green manure for heavy metals contaminated soils amendement and rehabilitation.

Keywords Agricultural soil pollution · Heavy metal stress · Emergence · *Fabaceae* species · Oxidative damage

C. Hachani · Z. Béjaoui (✉)
Faculty of Sciences of Bizerte, University of Carthage, 7021 Jarzouna, Tunisia
e-mail: zoubeir.bejaoui@fsb.ucar.tn

C. Hachani
e-mail: chadliahachanii@gmail.com

C. Hachani · M. Abassi · Z. Béjaoui
Laboratory of Forest Ecology (LR11INRGREF03), National Institute of Research in Rural Engineering, Water and Forests (INRGREF), University of Carthage, Hédi Elkarray Street, Elmenzah IV, BP 10, 2080 Ariana, Tunisia
e-mail: mej_abassi@yahoo.fr

M. S. Lamhamedi
Center for Forest Studies, Faculty of Forestry, Geography and Geomatics, Abitibi Price Building, Laval University, Quebec, QC G1V 0A6, Canada
e-mail: mohammed-sghir.lamhamedi.1@ulaval.ca

N. Sleimi
Laboratory of Resources, Materials and Ecosystems, Faculty of Sciences of Bizerte, University of Carthage, 7021 Jarzouna, Bizerte, Tunisia
e-mail: noomene.sleimi@gmail.com

1 Introduction

Tailings in many abandoned mines are a well-known environmental problem causing pollution of soils, surface water, and groundwater (Li et al., 2014; Pinto et al., 2015) and serious health problems (Gebeyehu & Bayissa, 2020; Singh et al., 2014). Mining activity leaves high levels of heavy metals in soils (Giri et al., 2017; Nguyen et al., 2020; Wei et al., 2018), which can contaminate large areas of neighboring lands due to the dispersion of fine-grained mine wastes (Boussen et al., 2010; Cheng et al., 2018; Othmani et al., 2013). The environmental risks associated with soil contamination by heavy metals are even greater in semi-arid areas, where extended periods of severe drought, seasonal rainfall variability, and the scarcity of vegetation cover amplify soil degradation (Mendez & Maier, 2008). Most of heavy metals are non-essential elements and, over time, are toxic to plants because of their non-biodegradable nature (Giri et al., 2017; Ma et al., 2015).

Globally, the extent of heavy metal contamination is steadily increasing in agricultural soils through eolian and run-off processes (Ghorbel et al., 2010; Gutiérrez et al., 2016). This leads to their accumulation in soils at toxic levels, which reduces soil fertility and crop yields (Cheng et al., 2018; Kicińska & Wikar, 2021; Nagajyoti et al., 2010). Heavy metals can also be absorbed and accumulated in cultivated plants and eventually transferred into the food chain (Bhatti et al., 2018; Boussen et al., 2013; Gebeyehu & Bayissa, 2020). Moreover, heavy metals affect plant development processes including seed germination, metabolism, and plant growth (Khan et al., 2015). They induce oxidative stress through the accumulation of reactive oxygen species (Hasanuzzaman et al., 2020), resulting in high production of malondialdehyde and proline and increased electrolyte leakage (Filippou et al., 2014; Gill & Tuteja, 2010; Hameed et al., 2016).

Most mining tailings are deposited in inadequate facilities with the absence of proper mine land reclamation (Doumas et al., 2018; Karaca et al., 2018). Across the globe, more than 5 million sites are potentially contaminated by heavy metals (He et al., 2015). In northern Tunisia, about 50 mining districts were listed with high concentrations of heavy metals (Elouear et al., 2016). The level of heavy metals in the soils surrounding these mines indicated that the permissible limits have been significantly exceeded (Boussen et al., 2010; Chaabani et al., 2017; Ghorbel et al., 2010; Othmani et al., 2013). In Jebel Ressas, mining activity (1869–1956) generated about 2.10^6 tons of toxic wastes (Elouear et al., 2016). In addition, lead (Pb), cadmium (Cd), and zinc (Zn) are among the most widespread contaminants (Mlayah et al., 2009). The average concentrations of Pb, Cd, and Zn in the natural soil were 32 $mg.kg^{-1}$, 0.58 $mg.kg^{-1}$, and 50 $mg.kg^{-1}$, respectively (Pais & Jones, 1997). Our recent study showed high levels of heavy metals Pb (15 587 $mg.kg^{-1}$), Zn (37 766 $mg.kg^{-1}$), and Cd (181 $mg.kg^{-1}$) in soils surrounding Jebel Ressas mine, which are mostly devoted to agricultural crops (Hachani et al., 2020). Pb and Cd are considered among the most phytotoxic heavy metals (Amari et al., 2017), while Zn is regarded as one of the most mobile elements (Gutiérrez et al., 2016).

Numerous studies have suggested methods for removing heavy metals from contaminated lands (Rascio & Navari-Izzo, 2011; Khalid et al., 2017; Karaca et al., 2018). However, the availability of heavy metals is controlled by certain factors such as pH, redox potential, cation exchange capacity, soil texture, and organic matter (Khan et al., 2015). Soil organic matter is an important element that improves the physicochemical properties and fertility of soil (Feller et al., 2012; Wang et al., 2020). A positive relationship was found between the amount of organic amendment and the heavy metal detoxification efficiency (Bacchetta et al., 2015; Liu et al., 2018; Medynska-Juraszek et al. 2020). Besides, the addition of organic amendments increases the availability of metals and improves plant growth (Wang et al., 2020). However, the selection of appropriate plant species to use as amendments is essential to ensure effective removal of heavy metals. In this context, the use of *Fabaceae* species as green manure can be considered a promising path for research and a suitable tool to increase soil fertility and promote the immobilization of heavy metals. *Fabaceae* species are able to increase the organic matter content of soils (Alcántara et al., 2017), improve biological nitrogen fixation (Cooper & Scherer, 2012), immobilize toxic elements in the roots (Mahmud et al., 2020), and grow on contaminated soils (Alexander et al., 2006; Ramos et al., 2020).

This work is part of a phytoremediation project for abandoned mining sites combining woody species and agricultural crops, in agroforestry systems. Thus, two experiments are carried out simultaneously: one involving woody species (Hachani et al., 2020, 2022) and the other using *Fabaceae* species to improve phytostabilization of heavy metals. The realization of this project will contribute to improve scientific knowledge and operational applications in terms of restoration of mining sites using agroforestry in semi-arid areas in the Mediterranean area. The purpose of the present study is to identify the most suitable species to be used. This was carried out through (*i*) the evaluation of the effects of excess Pb, Zn, and Cd on emergence and seedling growth of four *Fabaceae* species during germination and early seedling stage, and (*ii*) the assessment of the effects of multi-metal (Pb, Zn, and Cd) contaminated soil on some traits of oxidative stress in order to study the relationships between metal toxicity, oxidative stress, and tolerance responses.

2 Material and Methods

2.1 Site Description, Soil Sampling, and Laboratory Analyses

The study area is the abandoned Pb–Zn mining site of "Jebel Ressas" located 30 km south of the Tunisian capital (36°36′21.4″N, 10°19′04.0″E). The region is characterized by a semi-arid climate. The mine is located near the former miners' village and surrounded by agricultural lands (Ghorbel, 2012). Near the mine and the village, there are three mine disposal dumps (tailing I, II, and III) (Fig. 1). Contaminated soil samples were collected from the slope of the tailing (II) and control soil was collected from a non-contaminated area as described by Hachani et al. (2020). Analyses of contaminated and control soils were performed using three soil composite samples (six soil samples per composite sample) as described by Hachani et al. (2020). To determine heavy metal concentrations, soil samples were subjected to acid digestion then metal concentrations were determined by ICP-OES (Perkin-Elmer Optima 4300) (Hachani et al., 2020). The analysis revealed concentrations of 15.58 ± 0.79 mg.g^{-1} for Pb, 37.76 ± 3.21 mg.g^{-1} for Zn, and 0.18 ± 0.03 mg.g^{-1} for Cd in contaminated soil. However, in control soil, concentrations were 0.009 ± 0.0004 mg.g^{-1} for Pb and 0.021 ± 0.002 mg.g^{-1} for Zn while Cd was below detection limits (Hachani et al., 2020). A physicochemical characterization of both soils (pH_{water}, pH_{CaCl2}, electrical conductivity, mineral elements, etc.) is thoroughly presented in our previous publication (Hachani et al., 2020).

2.2 Plant Material, Experimental Design, and Germination Conditions

Seeds of *Vicia faba* L (variety Badi), *Cicer arietinum* L (variety Beja 1), *Lens culinaris* Medik (variety El Kef), and *Medicago arborea* L were selected for their healthy aspects and their homogeneity. Seeds were provided by the laboratory of field crops at the National Institute of Agronomic Research of Tunisia (INRAT) with the exception of *Medicago arborea*, which was furnished by the seeds department at the National Institue of Research in Rural Engineering, Water and Forests (INRGREF, Tunisia).

The experiment was conducted at the National Institue of Research in Rural Engineering, Water and Forests (INRGREF) in Tunis. Germination tests were performed according to the recommendations of the International Seed Testing Association (ISTA) (2012). For each species (*V. faba*, *C. arietinum*, *L. culinaris*, and *M. arborea*), two treatments were applied: NCS using non-contaminated (control) soil and CS using contaminated soil. The seedlings were distributed according to an experimental design in four complete random blocks. The tests were conducted in plastic trays (length: 30 cm × width: 20 cm × depth: 7 cm) filled with 1 kg of soil (contaminated or control). Seeds were surface sterilized in a 3% sodium hypochlorite solution (v/v) for 10 min, rinsed twice with distilled water and placed on the topsoil. Then the soil was slightly turned to bury the seeds at a depth of 1 cm. Four repetitions of 100 seeds were made for each species according to the rules of ISTA (2012). Trays were irrigated to field capacity with tap water. The trays were incubated at 25 °C and 85% relative humidity for 15 days and the emergence was monitored every 24 h. Seeds were considered germinated when the cotyledon emerge on the surface of the soil.

2.3 Growth Parameters

The number of the emerged seeds was counted every 24 h for 15 days. The emergence rate was determined by the ratio of the number of emerged seeds to the total number of seeds according to the formula described by Ashraf and Abu-Shakra (1978).

Measurements of root system length, shoot height, and total dry mass of the seedlings were performed at the end of the experiment. For each parameter, 20 seedlings (5 seedlings per bloc) per treatment and per species were randomly selected. Total dry mass was determined after drying the seedlings from each treatment and each species at 60 °C for 48 h. The vigor index (VI) was calculated following the formula of Abdul-Baki and Anderson (1973):

$$VI\ (\%) = (\text{Roots length} + \text{Shoots height}) \times \text{Final germination rate}$$

Loss of vigor index (LVI) was expressed using the following formula:

$$LVI\ (\%) = \left(\frac{\text{VI of the control} - \text{VI of the treatment}}{\text{VI of the control}}\right) \times 100$$

The tolerance index (TI) was calculated using the formula described by Wilkins (1978):

$$TI\ (\%) = \left(\frac{\text{Fresh mass of stressed seedlings}}{\text{Fresh mass of control seedlings}}\right) \times 100$$

2.4 *Determination of Electrolytes Leakage, Lipid Peroxidation, and Proline and Hydrogen Peroxide (H_2O_2) Concentrations*

For each treatment and each species, the integrity of the membrane structures was evaluated on 20 randomly selected seedlings (5 shoots/bloc/treatment) according to the method described by Blum and Ebercon (1981). Thus, 20 fragments were taken from leaves located in the median part (1 cm lenght). The fragments were washed twice with 10 ml of distilled water. Then, they were soaked in sterile test tubes containing 15 ml of distilled water in the dark at 40 °C for 1 h. Free conductivity was measured using a conductivity meter (Cellox 325 model, Multiline P3 PH/LF-SET, WTW Gmbh, Weilheim, Germany) and expressed in $\mu S.cm^{-1}$. The test tubes were put in a water bath at 100 °C for 1 h to determine the maximum leakage of all electrolytes. After cooling, the total conductivity was measured. The rate of electrolytes leakage is defined as follows:

Google Earth, Landsat/ Copernicus Inst. Geogr. Nacional

Fig. 1 Localisation of Jebel Ressas abandoned mining site in North Tunisia

$$\text{Electrolyte leakage } (\%) = \left(\frac{\text{Free conductivity}}{\text{Total conductivity}}\right) \times 100$$

Lipid peroxidation was estimated by the determination of the concentration of thiobarbituric acid reactive susbtances (TBARS) according to the method described by Heath and Packer (1968) using 20 randomly selected seedlings (5 shoots/bloc) for each treatment and each species (shoots). One gram of fresh plant material was milled in the presence of 10 ml of extraction buffer consisting of 0.5% thiobarbituric acid (TBA) (w/v), 10% trichloroacetic acid (TCA) (w/v), and 0.2 mM EDTA. The homogenate was incubated in a water bath at 95 °C for 30 min then to stop the reaction, it was immediately cooled down in an ice bath. The mixture was centrifuged at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm and at 600 nm against a blank using a UV spectrophotometer (UV-1200 Model, Tomos Life Science Group Pte, Ltd, China). TBARS concentrations were calculated using the extinction coefficient ($\varepsilon = 155$ mM^{-1} cm^{-1}).

The determination of the concentration of proline was performed according to the method described by Monneveux and Nemmar (1986) using 20 randomly selected seedlings (5 shoots/bloc) for each treatment and each species (shoots). One hundred milligrams of fresh leaves was homogenized with 2 ml of 40% methanol (v/v). The mixture was incubated in a water bath at 85 °C for 1 h. After cooling, 1 ml of the mixture was added to 1 ml of acetic acid, 25 mg of ninhydrin, and 1 ml of a solution composed of 300 ml acetic acid, 30 ml orthophosphoric acid, and 120 ml distilled water. The new mixture was incubated in water bath at 100 °C for 30 min then after cooling down, 5 ml of toluene was added. The absorbance of the supernatant was measured at 528 nm using a UV spectrophotometer (UV-1200 Model, Tomos Life Science Group Pte, Ltd, China). The concentrations of proline were determined using the standard curve.

The concentration of hydrogen peroxide (H_2O_2) was determined according to the method described by Sergiev et al. (1997) using 20 randomly selected seedlings (5 seedlings/bloc) for each treatment and each species. Fresh leaves (500 mg) were cold homogenized in 5 ml of 0.1% trichloroacetic acid (TCA) solution (w/v). The homogenate was then centrifuged at 1000 rpm for 15 min. Five hundred microliters of the supernatant was added to 500 µl of 10 mM phosphate buffer ($pH = 7$) and 1 ml of a potassium iodate solution (1 M). The absorbance was measured at 390 nm using a UV spectrophotometer (UV-1200 Model, Tomos Life Science Group Pte, Ltd, China) then H_2O_2 concentrations were obtained from the standard curve.

2.5 Enzyme Assays

The determination of enzymatic activities for catalase (CAT), ascorbate peroxidase (APX), and guaïacol peroxidase (GPX) was performed at the end of the experiment using 20 randomly selected seedlings (shoots) for each treatment and each species. Four hundred milligrams of fresh shoots was homogenized at 4 °C using sterile sand and 2 ml of extraction buffer composed of 50 mM K phosphate buffer ($pH = 7$), 5 mM sodium ascorbate, and 0.2 mM EDTA. The homogenate was filtrated then centrifuged at 6000 rpm for 15 min at 4 °C. Enzyme activities were determined in the supernatant using a UV spectrophotometer (UV-1200 Model, Tomos Life Science Group Pte, Ltd, China).

CAT (EC1.11.1.6) activity was determined by monitoring the decrease in absorbance at 240 nm (molar extinction coefficient: $\varepsilon = 0.036$ mM^{-1} cm^{-1}) according to the method described by Aebi (1984). The reaction mixture consisted of 2 ml of 25 mM K phosphate buffer ($pH = 7$), 30 mM H_2O_2, and 50 µl of enzyme extract.

APX (EC1.11.1.11) activity was assayed as described by Nakano and Asada (1981) by monitoring the decrease of the absorbance at 290 nm (molar extinction coefficient: $\varepsilon = 2.8$ mM^{-1} cm^{-1}). The reaction mixture consisted of 2 ml of 50 mM K phosphate buffer ($pH = 7$), 5 mM H_2O_2, 0.5 mM sodium ascorbate, 0.1 mM EDTA, and 40 µl of enzyme extract.

GPX (EC1.11.1.7) activity was determined according to the method of Fielding and Hall (1978) by monitoring the increase of the absorbance at 470 nm (molar extinction coefficient: $\varepsilon = 26.6$ mM^{-1} cm^{-1}). The reaction mixture consisted of 2 ml of 25 mM K phosphate buffer ($pH = 7$), 10 mM H_2O_2, 9 mM guaïacol, and 10 µl of enzyme extract.

The absorbances were recorded after mixing the enzyme extracts with the substrates. Control assays were done in the absence of the enzyme extract. Enzyme activities were expressed per g fresh mass (FM). In the case of CAT and APX, 1 unit of enzyme activity was defined as the amount of enzyme necessary to decompose 1 µmol of substrate per min at 25 °C. As for GPX, 1 unit was defined as the amount of enzyme that produces 1 µmol of guaïacol per min at 25 °C.

2.6 Metal Concentrations

Twenty seedlings (5 seedlings per bloc) from each treatment and each species (shoots and roots) were dried and reduced to a fine powder. Thirty milligrams of sample was acid digested using a mixture of HNO_3: H_2SO_4: $HClO_4$ (10:1:0.5; v/v/v) during 2 h at 110 °C. After cooling, the digestion products were filtered and diluted with 0.5% nitric acid (v/v) to 50 ml. Total concentrations of Pb, Zn, and Cd were determined by atomic absorption spectrometry (Perkin Elmer PinAAcle 900 T, USA). For each species, heavy metal composition was expressed as content (concentration × dry mass) per seedling to accurately represent heavy metal accumulation (Lamhamedi et al., 2013; Timmer & Miller, 1991). The effect of metal contamination (Pb, Zn, and Cd) in the four species was assessed using bioaccumulation factor (BAF) and translocation factor (TF) (Mackay & Fraser, 2000) and calculated as follows:

$$BAF = \frac{\text{Concentration of heavy metal in plant}}{\text{Concentration of heavy metal in soil}}$$

$$FT = \frac{\text{Concentration of heavy metal in shoots}}{\text{Concentration of heavy metal in roots}}$$

2.7 Statistical Analyses

Data was analyzed with ANOVA using XLSTAT software (Addinsoft Inc., NY, USA) after testing the assumptions of homoscedasticity and normality of residuals Steel et al. (1997). The differences among treatments and species regarding all measured parameters were determined by Tukey's test at a 5% significance level.

3 Results

3.1 Growth Parameters

The presence of heavy metals in the soil significantly affected seed emergence kinetics (Fig. 2). After 15 days, the effect of heavy metals on the emergence rates of the seedlings varied depending on the species (Fig. 2). In fact, a latency period preceding seedlings emergence was longer for *V. faba* seedlings under both treatments (CS and NCS) compared to the rest of the species. A significant stimulation was observed for seedling emergence rate in stressed *V. faba* (CS) kinetics early in the experiment. However, after 15 days, no significant difference was recorded between the stressed *V. faba* seedlings (CS) and the control (NCS) (Fig. 2a). The kinetics of emergence of *C. arietinum*, *L. culinaris*, and *M. arborea* seedlings were significantly slowed compared to the controls (NCS). For *C. arietinum* and *L. culinaris*, the final emergence rates were significantly ($p < 0.05$) reduced by 35% and 22%, respectively compared to the controls (Fig. 2b and c). However, by the 10th day, the kinetics of *M. arborea* increased significantly exceeding the control (NCS). The final emergence rate significantly increased by 23% (Fig. 2d).

At the end of the experiment, seedlings subjected to contaminated soil (CS) exhibited no noticeable effect on their morphological aspect (Fig. 3). Furthermore, data revealed no significant ($p > 0.05$) difference in terms of the total dry mass regardless to the species (Fig. 4). Exposure to contaminated soil (CS) had no significant effect ($p > 0.05$) on root and shoot growth of *V. faba*, *C. arietinum*, and *L. culinaris* compared to the control soil (NCS), with the exception of *M. arborea*, which showed a significant decrease by 20% for shoots height ($p = 0.035$) and by 44% for root length ($p = 0.022$) compared to the control (NCS) (Table 1).

M. arborea exhibited the highest loss of vigor index reaching 51% while *V. faba* and *C. arietinum* showed the lowest loss of vigor index by 21% and 20%, respectively. According to the tolerance index, *M. arborea* was classified as the least tolerant species followed by *L. culinaris* then *V. faba* while *C. arietinum* is the most tolerant to contaminated soil (Table 1).

3.2 Electrolyte Leakage, Lipid Peroxidation, and Proline and Hydrogen Peroxide Concentrations

In contaminated soil (CS), electrolyte leakage increased significantly for *L. culinaris* ($p = 0.0001$) and *M. arborea* ($p = 0.041$) reaching 97% and 71%, respectively compared to the controls (CT). No

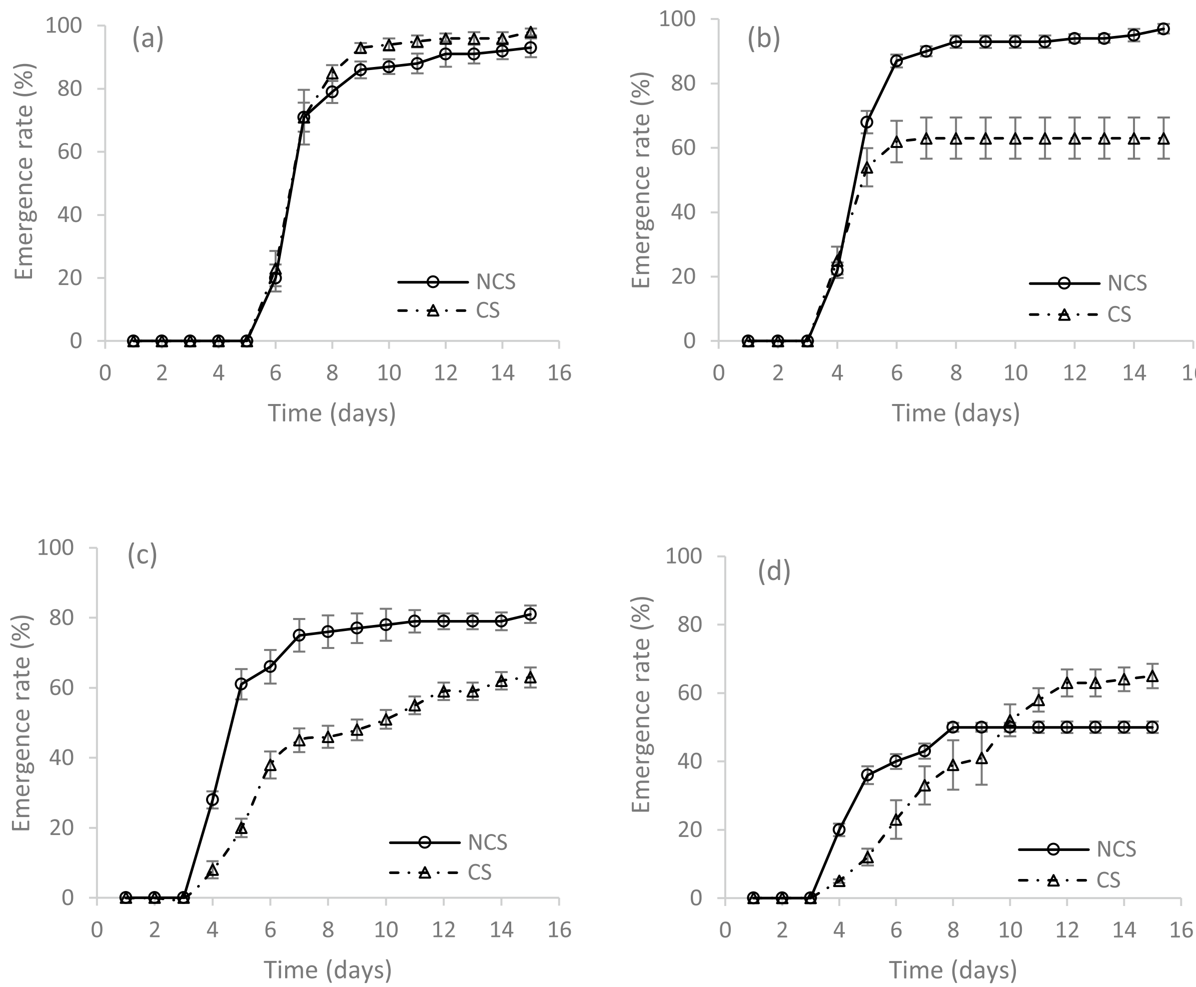

Fig. 2 Emergence rates of *Vicia faba* (**a**), *Cicer arietinum* (**b**), *Lens culinaris* (**c**), and *Medicago arborea* (**d**) seeds exposed to control soil (NCS) and Pb–Zn-Cd contaminated soil (CS) for 15 days. The values represent mean ± SD of 4 replicates of 100 seeds

statistical differences were recorded for *V. faba* ($p=0.911$) and *C. arietinum* ($p=0.997$) (Fig. 5a).

Oxidative damage to cell membranes was assessed by lipid peroxidation using an estimate of thiobarbituric acid reactive susbtances (TBARS) concentration. In respect to the controls (NCS), TBARS concentrations increased significantly for *L. culinaris* ($p=0.003$) and *M. arborea* ($p=0.0001$) reaching 86% and 84%, respectively. No significant differences were recorded for *V. faba* ($p=0.998$) and *C. arietinum* ($p=0.999$) (Fig. 5b).

Proline concentrations showed substantial increases for *M. arborea* ($p=0.036$) and *L. culinaris* ($p=0.037$) reaching 284% and 149%, respectively compared to the controls (NCS). *V. faba* and *C. arietinum* exhibited less pronounced increases reaching 101% ($p=0.0001$) and 54% ($p=0.045$), respectively in respect to the control (Fig. 5c).

In contaminated soil (CS), H_2O_2 concentrations significantly increased by 63% for *C. arietinum* ($p=0.048$) and 62% for *M. arborea* ($p=0.009$) compared to the controls (NCS). Greater increases were recorded for *L. culinaris* ($p=0.0001$) and *V. faba* ($p=0.042$) reaching 227% and 168%, respectively compared to the controls (Fig. 5d).

3.3 Enzyme Activities

In contaminated soil (CS), enzyme activities generally increased significantly but varied depending

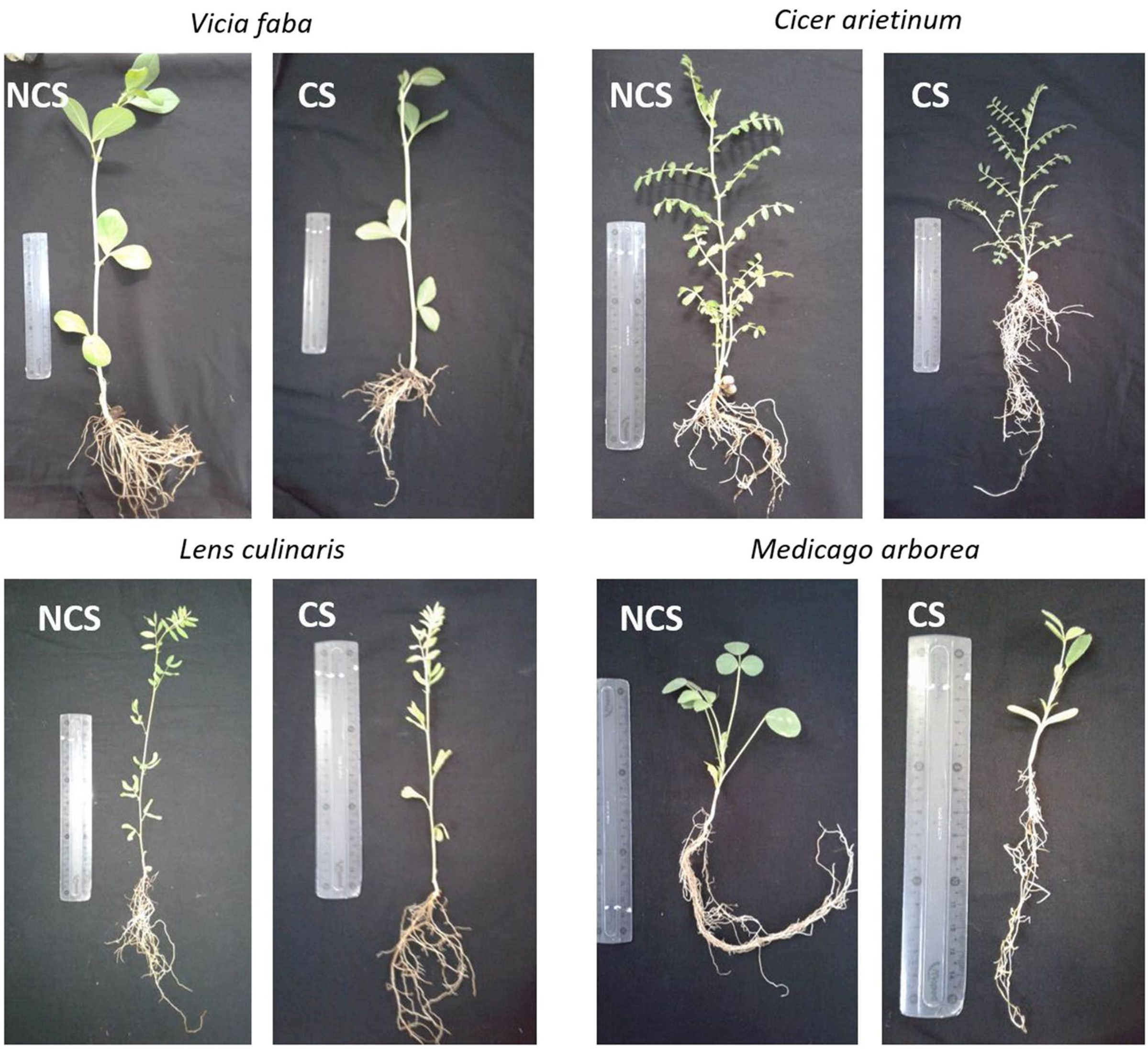

Fig. 3 Morphological aspects of *Vicia faba*, *Cicer arietinum*, *Lens culinaris*, and *Medicago arborea* seedlings after 15 days of exposure to control soil (NCS) and Pb–Zn-Cd contaminated soil (CS)

on the species (Fig. 6). *M. arborea* showed a significant rise in APX and GPX activities reaching 114% ($p = 0.008$) and 18% ($p = 0.026$) respectively. No significant difference was recorded for CAT ($p = 0.094$). Conversely, *V. faba* revealed a significant overproduction of CAT by 131% ($p = 0.033$) while no significant change was noted for either APX ($p = 0.919$) nor GPX ($p = 0.099$). *L. culinaris* and *C. arietinum* both exhibited a great overproduction of CAT up to 628% ($p = 0.0001$) and 43% ($p = 0.0001$), respectively compared to the controls (NCS), and substantial increases of GPX by 57% ($p = 0.001$) and 27% ($p = 0.015$), respectively compared to controls (NCS) (Fig. 6).

3.4 Heavy Metal Content

At the end of the experiment, significant increases ($p < 0.05$) appeared in Pb and Zn contents, especially in the roots of all species. Results showed that Zn is the most accumulated element followed by Pb, whereas only traces of Cd were detected in the roots of all species (Zn > Pb > Cd) (Table 2). Significant variations in heavy metal accumulation were noted

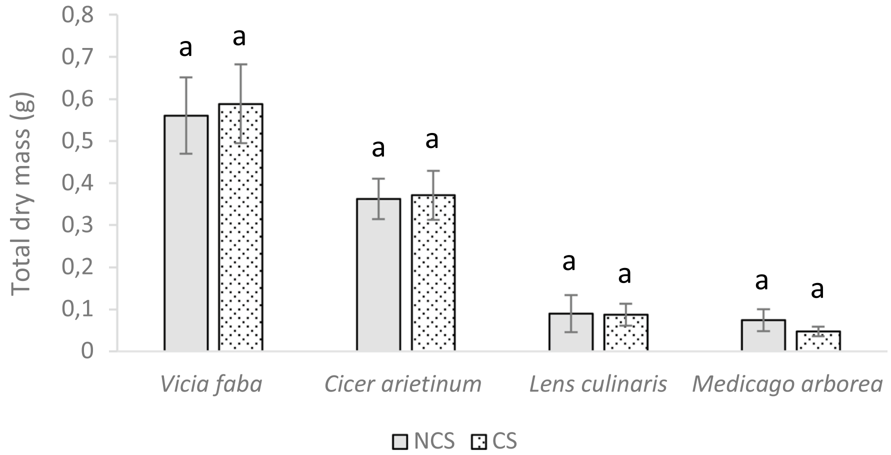

Fig. 4 Total dry masses of *Vicia faba*, *Cicer arietinum*, *Lens culinaris*, and *Medicago arborea* seedlings exposed to control soil (NCS) and Pb–Zn-Cd contaminated soil (CS). For each species, means (±SD, $n = 20$) with different letters significantly differ from each other based on Tukey's tests at $p < 0.05$

Table 1 Shoot height, root length, loss of vigor index, and tolerance index of *Vicia faba*, *Cicer arietinum*, *Lens culinaris*, and *Medicago arborea* seedlings after 15 days of growing under control soil (NCS) and Pb–Zn-Cd contaminated soil (CS) treatments

		Species			
	Treatment	*Vicia faba*	*Cicer arietinum*	*Lens culinaris*	*Medicago arborea*
Shoot height (cm)	NCS	38.75 ± 3.45a	19.21 ± 1.95a	16.87 ± 4.09a	8.60 ± 0.95a
	CS	34.80 ± 3.49a	19.03 ± 0.90a	15.52 ± 2.27a	6.85 ± 0.67b
Root length (cm)	NCS	19.32 ± 3.42a	16.47 ± 5.45a	16.96 ± 3.98a	20.32 ± 4.43a
	CS	13.50 ± 2.40a	20.67 ± 6.19a	12.84 ± 2.75a	11.26 ± 3.62b
Loss of vigor index (%)		21.06 ± 3.19c	20.51 ± 3.62c	34.79 ± 3.27b	51.80 ± 2.55a
Tolerance index (%)		91.46 ± 0.75b	99.85 ± 0.45a	89.44 ± 0.20c	63.22 ± 0.19d

For each species, means (±SD, $n = 20$) with different letters significantly differ from each other based on Tukey's tests at $p < 0.05$

between species. *C. arietinum* and *V. faba* had the highest Zn and Pb contents while *L. culinaris* and *M. arborea* exhibited the lowest contents (Table 2).

At the end of the experiment, *C. arietinum* showed significant increases in Pb shoot content by 48% ($p = 0.001$) compared to the control (NCS). No significant difference was recorded for *V. faba* ($p = 0.682$), *L. culinaris* ($p = 0.594$), and *M. arborea* ($p = 0.991$). However, Pb root contents were 24-fold higher for *V. faba* ($p = 0.0001$), 21-fold higher for *C. arietinum* ($p = 0.0001$), and 19-fold higher for *L. culinaris* ($p = 0.032$) compared to the controls (NCS). *M. arborea* exhibited lower increase in Pb root content by only eightfold ($p = 0.061$) compared to the control (Table 2).

In addition, *C. arietinum* and *V. faba* both showed significant increases in Zn shoot contents reaching 562% ($p = 0.0001$) and 470% ($p = 0.0001$), respectively compared to the controls (NCS). No statistical differences were revealed for *L. culinaris* ($p = 0.933$) and *M. arborea* ($p = 0.987$) in terms of Zn shoot contents. In contrast, Zn content in roots was substantialy increased by 89-fold for *V. faba* ($p = 0.0001$), 70-fold for *C. arietinum* ($p = 0.0001$), 43-fold for *L. culinaris* ($p = 0.043$), and 41-fold for *M. arborea* ($p = 0.028$) compared to the controls (NCS) (Table 2).

Regardless to the species, translocation factor (TF) values showed that the accumulation of Pb and Zn occurred in the roots (TF < 1). *V. faba* exhibited the lowest translocation of Pb and Zn among all species. *M. arborea* and *L. culinaris* showed the highest TF of Pb and *C. arietinum* had the highest TF of Zn. (Table 3). The bioaccumulation factor (BAF) values indicate that the accumulation of Pb, Zn, and Cd tends to take place in the soil rather than the seedlings (BAF < 1). *C. arietinum* had the highest BAF for all elements. The lowest BAF of Pb was recorded in *L. culinaris* and *M. arborea*, of Zn in *V. faba*, *L. culinaris*, and *M. arborea* and of Cd in *V. faba* and *L. culinaris* (Table 3).

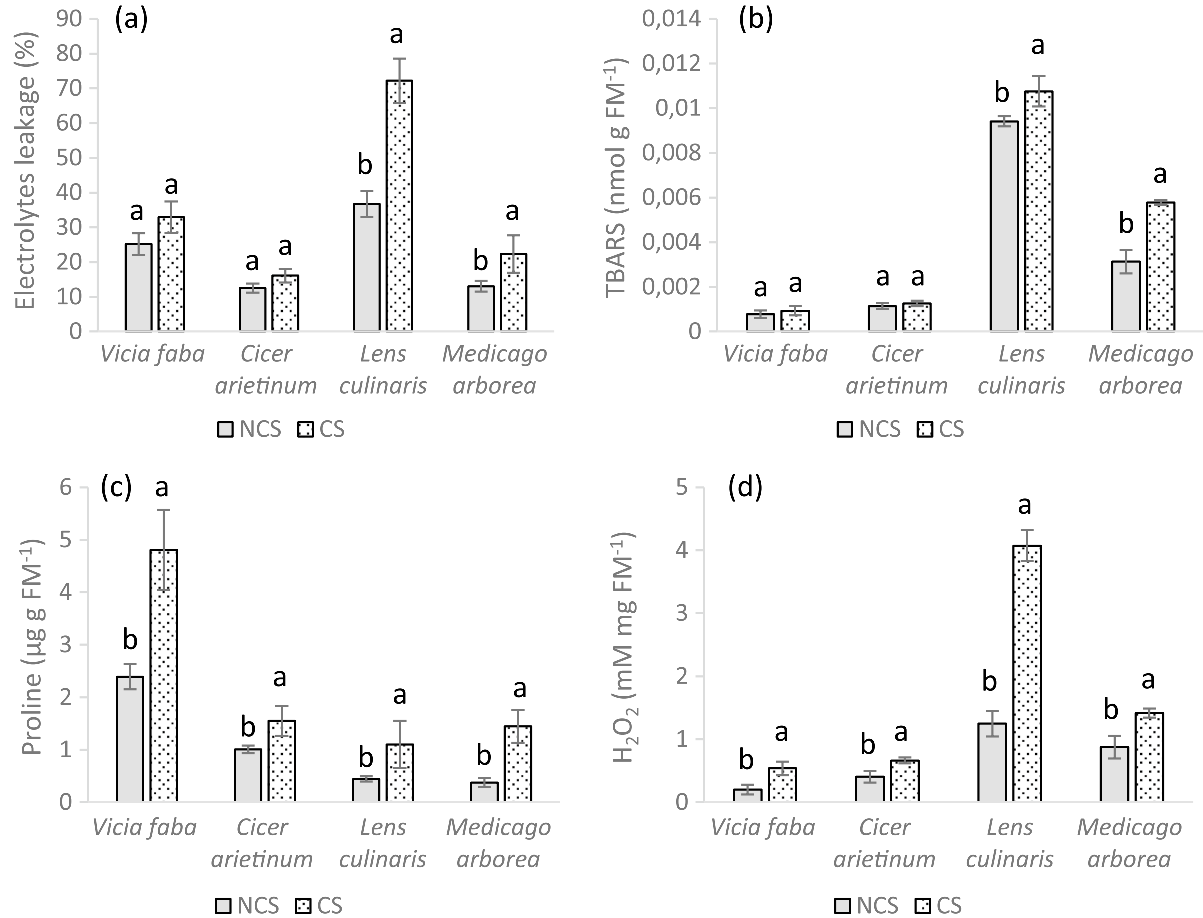

Fig. 5 Electrolytes leakage (**a**) and thiobarbituric acid reactive susbtances (TBARS) (**b**), proline (**c**) and hydrogen peroxide (H_2O_2) (**d**) concentrations in *Vicia faba*, *Cicer arietinum*, *Lens culinaris*, and *Medicago arborea* shoots exposed to control soil (NCS) and Pb–Zn-Cd contaminated soil (CS). For each species, means (±SD, $n = 3$, composite sample) with different letters significantly differ from each other based on Tukey's tests at $p < 0.05$

4 Discussion

Heavy metals are one of the major causes of environmental pollution that restrict biodiversity and plant development. However, some species have the ability to grow under restrictive conditions without a significant decline in growth and could be recommended for mining sites rehabilitation. In the present study, we evaluated the growth parameters, stress indicators, and heavy metal accumulation of four *Fabaceae* species (*V. faba*, *C. arietinum*, *L. culinaris*, and *M. arborea*) in response to heavy metal contaminated soil (Pb, Zn, and Cd).

Our findings showed that *V. faba* and *C. arietinum* were the most tolerant species; while *L. culinaris* and *M. arborea* appeared to be less tolerant (Figs. 2, 3, 4, 5, and 6; Table 1). Thus, a relative sensitivity was noted at the germination stage and was clearly manifested in *C. arietinum* and *L. culinaris* (Fig. 2b and c). Seed germination is sensitive to environmental conditions and variable among species due to disturbances in carbohydrate metabolism and reduced water and nutrient availability (Debouza et al., 2021; Liu et al., 2012). For *M. arborea*, the inhibition of seedling emergence was lifted after the 10th day exceeding the control (NCS) but without reaching total seedling

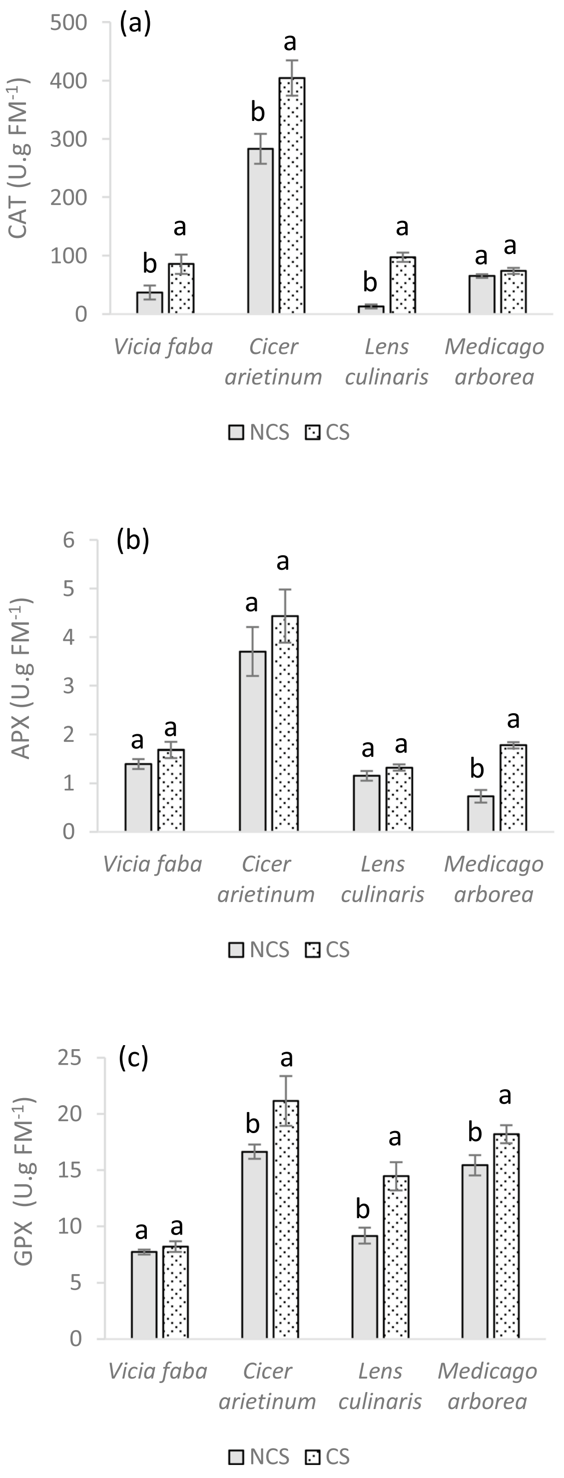

Fig. 6 Enzymatic activities of catalase (CAT) (**a**), ascorbate peroxidase (APX) (**b**), and guaïacol peroxidase (GPX) (**c**) in *Vicia faba*, *Cicer arietinum*, *Lens culinaris*, and *Medicago arborea* seedlings exposed to control soil (NCS) and Pb–Zn-Cd contaminated soil (CS). For each species, means (±SD, $n=3$, composite sample) with different letters significantly differ from each other based on Tukey's tests at $p<0.05$

emergence (Fig. 2d). Similar results were reported by Peralta et al. (2001) showing an increase of seed germination of *Medicago sativa* L in the presence of high amounts of Cd and Cr. Our results revealed that *V. faba* was notable with an emergence rate reaching 100% at the end of the experiment (Fig. 2a). This is attributed to the intrinsic capacity of the seedlings to tolerate high levels of heavy metals (Júnior et al., 2016). Furthermore, the emergence rate of *V. faba* in contaminated soil (CS) showed a slight stimulation compared to the control (Fig. 2a), which agrees with the findings of Islam et al. (2007) showing an increased germination rate of *Elsholtzia argyi* H Lév at high Pb concentration.

It was reported that early seedling growth was more sensitive to the toxicity of heavy metals in comparison to germination stage (Baruah et al., 2019; Bouslimi et al., 2021; Li et al., 2005). On the contrary, the present study showed that metallic stress had no significant effect on total dry mass of the four *Fabaceae* species (Fig. 4). Similar results showed no significant reduction in mass production of *Atriplex halimus* L under Pb-contaminated soil (Bankaji et al., 2019). Our results did not reveal any significant effect of heavy metals on the primary shoot and root growth in *V. faba*, *C. arietinum*, and *L. culinaris* (Table 1). *V. faba* stands out in terms of not only mass production but also yield (Fig. 4). It is important to note, however, that heavy metals did not affect all species with the same severity, as evidenced by the growth reduction noted in *M. arborea* (Table 1). Growth retardation could be considered a potential adaptation mechanism, which may positively influence the seedling survival by reducing their growth rate and biomass allocation (Feng et al., 2014; Grotkopp et al., 2002). In the same context, other studies showed growth inhibition of *Medicago sativa* seedlings exposed to Pb (Hattab et al., 2016) and of *Zea mays* L exposed to Zn and Cd (Xu et al., 2014).

Under heavy metals contaminated soil, *V. faba* and *C. arietinum* exhibited minimal loss of vigor index, high values of tolerance index (Table 1), and low levels of oxidative injury (Fig. 5), which shows a protection against harmful oxidative damage. On the contrary, in *L. culinaris* and *M. arborea*, electrolyte leakage and TBARS concentartions were increased compared to the control (Fig. 5). Several studies showed that exposure to high concentrations

Table 2 Heavy metal (Pb, Zn, and Cd) contents (mg.seedling.$^{-1}$) in shoots and roots of *Vicia faba*, *Cicer arietinum*, *Lens culinaris*, and *Medicago arborea* seedlings after 15 days of growing under control soil (NCS) and Pb–Zn-Cd contaminated soil (CS)

		Pb (mg.seedling^{-1})		Zn (mg.seedling^{-1})		Cd (mg.seedling^{-1})	
		NCS	CS	NCS	CS	NCS	CS
Vicia faba	Shoots	0.061 ± 0.022a	0.082 ± 0.011a	0.027 ± 0.003b	0.154 ± 0.040a	0	0
	Roots	0.055 ± 0.013b	1.351 ± 0.098a	0.015 ± 0.001b	1.343 ± 0.120a	0	0.007 ± 0.0001
Cicer arietinum	Shoots	0.062 ± 0.010b	0.092 ± 0.011a	0.027 ± 0.009b	0.179 ± 0.041a	0	0
	Roots	0.041 ± 0.011b	0.871 ± 0.102a	0.014 ± 0.005b	0.987 ± 0.103a	0	0.007 ± 0.0003
Lens culinaris	Shoots	0.012 ± 0.011a	0.015 ± 0.010a	0.004 ± 0.014a	0.020 ± 0.001a	0	0
	Roots	0.005 ± 0.009b	0.095 ± 0.020a	0.003 ± 0.001b	0.130 ± 0.030a	0	0.0007 ± 0.0002
Medicago arborea	Shoots	0.015 ± 0.012a	0.014 ± 0.012a	0.007 ± 0.004a	0.026 ± 0.019a	0	0
	Roots	0.006 ± 0.010b	0.051 ± 0.021a	0.002 ± 0.0009b	0.083 ± 0.038a	0	0.0007 ± 0.0003

For each species, each compartment (shoots and roots) and each element, means (± SD, $n=3$, composite samples) with different letters significantly differ from each other based on Tukey's tests at $p<0.05$

Table 3 Translocation (TF) and bioaccumulation (BAF) factors of *Vicia faba*, *Cicer arietinum*, *Lens culinaris*, and *Medicago arborea* exposed to Pb–Zn-Cd contaminated soil

	Species			
	Vicia faba	*Cicer arietinum*	*Lens culinaris*	*Medicago arborea*
TF				
Pb	0.048 ± 0.007c	0.098 ± 0.010b	0.134 ± 0.011a	0.132 ± 0.004a
Zn	0.091 ± 0.011c	0.168 ± 0.005a	0.131 ± 0.008b	0.147 ± 0.009b
Cd	-	-	-	-
BAF				
Pb	0.134 ± 0.014b	0.167 ± 0.012a	0.098 ± 0.010c	0.089 ± 0.005c
Zn	0.058 ± 0.003b	0.083 ± 0.009a	0.055 ± 0.005b	0.061 ± 0.007b
Cd	0.063 ± 0.009c	0.106 ± 0.011a	0.053 ± 0.008c	0.082 ± 0.006b

For each metallic element, means (± SD, $n=3$) with different letters significantly differ from each other based on Tukey's tests at $p<0.05$

of heavy metals increase the production of reactive oxygen species (ROS), leading to membrane damage and lipid peroxidation (Rucinska-Sobkowiak, 2010; Rai et al., 2016; Hasanuzzaman et al., 2020). The damaging effect of heavy metals may vary among plant species. Heavy metal-tolerant species have adaptation mechanisms such as the accumulation of compatible solutes, like proline which acts as an antioxidant and a stabilizing osmolyte of the membrane (Dar et al., 2016; Filippou et al., 2014; Gill & Tuteja, 2010). *V. faba* showed a substantial increase of proline compared to the control and the highest production of proline among all the species (Fig. 5c). Similar results have been reported by Nadgórska-Socha et al. (2013) and El-Amier et al. (2019) in *V. faba* and *Pisum sativum* L.

Hydrogen peroxide (H_2O_2) is a toxic ROS that accelerates oxidative damage in metal-stressed seedlings (Cuypers et al., 2016). This study showed a significant accumulation of H_2O_2 in the four tested species compared to the controls (Fig. 5d). The most significant increase was recorded in *L. culinaris* (Fig. 5d). Several studies have reported an increase in hydrogen peroxide contents in various plant species subjected to metallic stress (Cuypers et al., 2016; Hasanuzzaman et al., 2020). It was demonstrated that at low levels, H_2O_2 acts as a signaling regulatory component of stress-response in plants (Sofo et al., 2015; Hameed et al., 2016). Moreover, the phytotoxicity generated by heavy metals leads to oxidative stress, which is revealed by the accumulation of hydrogen peroxide (Cuypers et al., 2016). The increased production of H_2O_2 triggers the activation of antioxidant enzymes (Smirnoff & Arnaud, 2019). This occasioned an overproduction of CAT by sevenfold and 2.3-fold in *L. culinaris* and *V. faba* respectively, compared to the controls (Fig. 6). In addition, *L. culinaris* exhibited the greatest overproduction of

GPX, while *M. arborea* showed a strong increase in APX in heavy metal-stressed seedlings (Fig. 6). The patterns of enzymatic activity seem to be linked not only to the tolerance level of the seedlings but also to the enzyme itself. For example, GPX activity was increased in *Vicia faba* subjected to Pb, Zn, Cd, Ni, and Cu stress (Nadgórska-Socha et al., 2013). However, Bankaji et al. (2015) showed a decline of CAT, APX, and GPX activities in *Suaeda fruticosa* L exposed to heavy metals (Cd and Cu). Similarly, inhibition of CAT and GPX activities was observed in Pb-stressed *Jatropha curcas* L, while superoxide dimutase was the major antioxidant enzyme (Shu et al., 2012). It was shown that significant variations occur in antioxidant defense system among plants (Hasanuzzaman et al., 2020; Sachdev et al., 2021). The activation of antioxidant enzymes in response to heavy metals stress has been recognized as an early mechanism of plant adaptation (Alscher et al., 2002; Hasanuzzaman et al., 2020; Verma & Dubey, 2003). Our results indicate that an upregulation of antioxidant defense system was maintained by enzymatic antioxidants (Fig. 6) and through higher proline accumulation (Fig. 5c) to maintain cell membrane stability, particularly in *V. faba* (Fig. 5a).

The present study showed that translocation of Pb, Zn, and Cd were reduced due to their sequestration in the roots for the four species (Table 2). This effect can be simply explained by a preferential accumulation of lead, zinc, and cadmium, in the roots (Tables 2 and 3) and it was more pronounced in *V. faba* seedlings (Table 3). Our findings are in agreement with those of Souguir (2009) showing an excessive cadmium accumulation in the roots of *V. faba*. On the other hand, plants can cope with heavy metal toxicity by chelating metal ions with organic acids or amino acids and their sequestration inside the vacuoles (Pourrut et al., 2011; Rascio & Navari-Izzo, 2011). In many heavy metal-tolerant species compartmentalization is a physiological defense mechanism that restrict heavy metal movement inside roots and may also limit their transport to the shoots (Bankaji et al., 2016; Mateos-Naranjo et al., 2014; Weis & Weis, 2004).

Our results showed that the lowest TF values of lead and zinc were recorded in *V. faba* seedlings (Table 3), which underline its phytostabilization potential and support its possible use as green manure to accelerate the remediation. Indeed, the use of *Fabaceae* as green manure has been shown to increase nutrient availability, cation exchange capacity, and soil organic matter in the soil and to reduce mobility and transfer of toxic heavy metals (Aghili et al., 2014; Bai et al., 2017; Botelho and Müller 2020). These effects are associated with the ability of *Fabaceae* to develop symbiotic associations with nitrogen-fixing bacteria in the soil (Cooper & Scherer, 2012). Baghaie and Aghilizefreei (2020) revealed that the green manure amendment increased Fe uptake in wheat grown in Pb contaminated soil. In addition, the green manure amendment was shown to increase the abundance of bacteria and fungi in heavy metals contaminated soils (Ai et al., 2020; Bai et al., 2017), which can be effective in the ecological restoration of mining sites.

5 Conclusion and Research Needs

Overall, the present study showed that *V. faba* had the best performance among all the tested species, exhibiting a particular tolerance towards metallic contamination of the soil. The high phytostabilization potential, shown by the lowest TF values, together with the high production of total dry mass, makes *V. faba* a promising candidate to be used as a green manure amendment for aided phytoremediation of heavy metals contaminated soils. However, as the present study is part of a program to restore an abandoned mining site heavily contaminated by Pb, Zn, and Cd, using ectomycorrhizal *Pinus halepensis* Mill. seedlings (Hachani et al., 2020, 2022), it would be wise to combine *V. faba* with ectomycorrhizal *P. halepensis* seedlings in an agroforestry system using the intercropping model and to evaluate the efficiency of phytostabilization of heavy metals under controlled conditions. As a next step, it would be interesting to continue the long-term evaluation of this agroforestry system on a mining site to test its viability, sustainability, and economic and environmental profitability.

Acknowledgements The authors are thankful to Pr. Noura Ben Youssef, head of the Laboratory of Field Crops at the National Institute of Agronomic Research of Tunisia (INRAT), for providing the seeds used in this study. We also thank Salima Bahri, engineer in statistics and information analysis in INRGREF, for performing the statistical analyses. We are greatful to the nursery staff of INGRREF for their help during the collection of soil samples.

Funding This research was supported by the University of Carthage and the National Institue of Research in Rural Engineering, Water and Forests (INRGREF), Tunisia.

Data Availability Data are available from the corresponding author on reasonable request (zoubeir.bejaoui@fsb.ucar.tn).

This article does not contain any studies with human subjects or animals performed by any of the authors.

Declarations

Conflict of Interest The authors declare no competing interests.

References

Abdul-Baki, A. A., & Anderson, J. D. (1973). Vigor determination in soybean seed by multiple criteria. *Crop Science, 13*(6), 630–633.

Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology, 105*, 121–126.

Aghili, F., Gamper, H. A., Eikenberg, J., Khoshgoftarmanesh, A. H., Afyuni, M., Schulin, R., ... & Frossard, E. (2014). Green manure addition to soil increases grain zinc concentration in bread wheat. PloS one, 9(7), e101487 https://doi.org/10.1371/journal.pone.0101487

Ai, Y. J., Li, F. P., Gu, H. H., Chi, X. J., Yuan, X. T., & Han, D. Y. (2020). Combined effects of green manure returning and addition of sewage sludge compost on plant growth and microorganism communities in gold tailings. *Environmental Science and Pollution Research, 27*, 31686–31698. https://doi.org/10.1007/s11356-020-09118-z

Alcántara, C., Soriano, A., Saavedra, M., & Gómez, J. A. (2017). Sistemas de manejo del suelo. In D. Barranco Navero, R. Fernández Escobar, & L. Rallo Romero (Eds.), *El cultivo del olivo* (7th ed., pp 335–417). Mundi-Prensa.

Alexander, P. D., Alloway, B. J., & Dourado, A. M. (2006). Genotypic variations in the accumulation of Cd, Cu, Pb and Zn exhibited by six commonly grown vegetables. *Environmental Pollution, 144*(3), 736–745.

Alscher, R. G., Erturk, N., & Heath, L. S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany, 53*(372), 1331–1341.

Amari, T., Ghnaya, T., & Abdelly, C. (2017). Nickel, cadmium and lead phytotoxicity and potential of halophytic plants in heavy metal extraction. *South African Journal of Botany, 111*, 99–110. https://doi.org/10.1016/j.sajb.2017.03.011

Ashraf, C. M., & Abu-Shakra, S. (1978). Wheat seed germination under low temperature and moisture stress 1. *Agronomy Journal, 70*(1), 135–139.

Bacchetta, G. I. A. N. L. U. I. G. I., Cappai, G., Carucci, A., & Tamburini, E. (2015). Use of native plants for the remediation of abandoned mine sites in Mediterranean semiarid environments. *Bulletin of Environmental Contamination and Toxicology, 94*(3), 326–333. https://doi.org/10.1007/s00128-015-1467-y

Baghaie, A. H., & Aghilizefreei, A. (2020). Iron enriched green manure can increase wheat Fe concentration in Pb-polluted soil in the presence of Piriformospora indica (P. indica). *Soil and Sediment Contamination: An International Journal, 29*(7), 721–743. https://doi.org/10.1080/15320383.2020.1771274

Bai, Y. C., Zuo, W. G., Zhao, H. T., Mei, L. J., Gu, C. H., Guan, Y. X., ... & Feng, K. (2017). Distribution of heavy metals in maize and mudflat saline soil amended by sewage sludge. Journal of Soils and Sediments, 17(6), 1565-1578https://doi.org/10.1007/s11368-016-1630-z

Bankaji, I., Caçador, I., & Sleimi, N. (2015). Physiological and biochemical responses of *Suaeda fruticosa* to cadmium and copper stresses: Growth, nutrient uptake, antioxidant enzymes, phytochelatin, and glutathione levels. *Environmental Science and Pollution Research, 22*(17), 13058–13069. https://doi.org/10.1007/s11356-015-4414-x

Bankaji, I., Cacador, I., & Sleimi, N. (2016). Assessing of tolerance to metallic and saline stresses in the halophyte *Suaeda fruticosa*: The indicator role of antioxidative enzymes. *Ecological Indicators, 64*, 297–308.

Bankaji, I., Pérez-Clemente, R. M., Caçador, I., & Sleimi, N. (2019). Accumulation potential of *Atriplex halimus* to zinc and lead combined with NaCl: Effects on physiological parameters and antioxidant enzymes activities. *South African Journal of Botany, 123*, 51–61. https://doi.org/10.1016/j.sajb.2019.02.011

Baruah, N., Mondal, S. C., Farooq, M., & Gogoi, N. (2019). Influence of heavy metals on seed germination and seedling growth of wheat, pea, and tomato. *Water, Air, & Soil Pollution, 230*(12), 1–15. https://doi.org/10.1007/s11270-019-4329-0

Bhatti, S. S., Sambyal, V., & Nagpal, A. K. (2018). Analysis of genotoxicity of agricultural soils and metal (Fe, Mn, and Zn) accumulation in crops. *International Journal of Environmental Research, 12*(4), 439–449.

Blum, A., & Ebercon, A. (1981). Cell membrane stability as a measure of drought and heat tolerance in wheat 1. *Crop Science, 21*(1), 43–47.

Botelho, R. V., & Müller, M. M. L. (2020). Nutrient redistribution in fruit crops: Physiological implications. In A. K. Srivastava, & H. Chengxiao (Eds.), *Fruit crops: Diagnosis and management of nutrient constraints* (pp. 33–46). Elsevier. https://doi.org/10.1016/B978-0-12-818732-6.00003-4

Bouslimi, H., Ferreira, R., Dridi, N., Brito, P., Martins-Dias, S., Caçador, I., & Sleimi, N. (2021). Effects of barium stress in *Brassica juncea* and *Cakile maritima*: The indicator role of some antioxidant enzymes and secondary metabolites. *Phyton, 90*(1), 145. https://doi.org/10.32604/phyton.2020.011752

Boussen, S., Sebei, A., Soubrand-Colin, M., Bril, H., Chaabani, F., & Abdeljaouad, S. (2010). Mobilization of lead-zinc rich particles from mine tailings in northern Tunisia by aeolian and run-off processes. *Bulletin De La Société Géologique De France, 181*(5), 459–471.

Boussen, S., Soubrand, M., Bril, H., Ouerfelli, K., & Abdeljaouad, S. (2013). Transfer of lead, zinc and cadmium from mine tailings to wheat (*Triticum aestivum*) in carbonated

Mediterranean (Northern Tunisia) soils. *Geoderma, 192*, 227–236.
Chaabani, S., Abdelmalek-Babbou, C., Ahmed, H. B., Chaabani, A., & Sebei, A. (2017). Phytoremediation assessment of native plants growing on Pb–Zn mine site in Northern Tunisia. *Environmental Earth Sciences, 76*(16), 1–15. https://doi.org/10.1007/s12665-017-6894-0
Cheng, X., Drozdova, J., Danek, T., Huang, Q., Qi, W., Yang, S., ... & Zhao, X. (2018). Pollution assessment of trace elements in agricultural soils around copper mining area. Sustainability, 10(12), 4533https://doi.org/10.3390/su10124533
Cooper, J. E., & Scherer, H. W. (2012). Nitrogen fixation. In P. Marschner (Ed.), *Marschner's mineral nutrition of higher plants* (3rd ed., pp. 389–408). Academic Press.
Cuypers, A., Hendrix, S., Amaral dos Reis, R., De Smet, S., Deckers, J., Gielen, H., Jozefczak, M., Loix, C., Vercampt, H., Vangronsveld, J., & Keunen, E. (2016). Hydrogen peroxide, signaling in disguise during metal phytotoxicity. *Frontiers in Plant Science, 7*, 470. https://doi.org/10.3389/fpls.2016.00470
Dar, M. I., Naikoo, M. I., Rehman, F., Naushin, F., & Khan, F. A. (2016). Proline accumulation in plants: Roles in stress tolerance and plant development. In N. Iqbal, R. Nazar, & N. A. Khan (Eds.), *Osmolytes and plants acclimation to changing environment: Emerging omics technologies* (pp. 155–166). Springer. https://doi.org/10.1007/978-81-322-2616-1_9.
Debouza, N. E., Thruppoyil, S. B., Gopi, K., Zain, S., & Ksiksi, T. (2021). Plant and seed germination responses to global change, with a focus on CO_2: A review. *One Ecosystem, 6*, e74260. https://doi.org/10.3897/oneeco.6.e74260
Doumas, P., Munoz, M., Banni, M., Becerra, S., Bruneel, O., Casiot, C., Cleyet-Marel, J. C., Gardon, J., Noack, Y., & Sappin-Didier, V. (2018). Polymetallic pollution from abandoned mines in Mediterranean regions: a multidisciplinary approach to environmental risks. *Regional environmental change, 18*(3), 677–692. https://doi.org/10.1007/s10113-016-0939-x
El-Amier, Y., Elhindi, K., El-Hendawy, S., Al-Rashed, S., & Abd-ElGawad, A. (2019). Antioxidant system and biomolecules alteration in *Pisum sativum* under heavy metal stress and possible alleviation by 5-aminolevulinic acid. *Molecules, 24*(22), 4194. https://doi.org/10.3390/molecules24224194
Elouear, Z., Bouhamed, F., Boujelben, N., & Bouzid, J. (2016). Assessment of toxic metals dispersed from improperly disposed tailing, Jebel Ressas mine. *NE Tunisia. Environmental Earth Sciences, 75*(3), 254. https://doi.org/10.1007/s12665-015-5035-x
Feller, C., Blanchart, E., Bernoux, M., Lal, R., & Manlay, R. (2012). Soil fertility concepts over the past two centuries: The importance attributed to soil organic matter in developed and developing countries. *Archives of Agronomy and Soil Science, 58*(sup1), S3–S21. https://doi.org/10.1080/03650340.2012.693598
Feng, Z. T., Deng, Y. Q., Fan, H., Sun, Q. J., Sui, N., & Wang, B. S. (2014). Effects of NaCl stress on the growth and photosynthetic characteristics of Ulmus pumila L. seedlings in sand culture. *Photosynthetica, 52*(2), 313–320. https://doi.org/10.1007/s11099-014-0032-y
Fielding, J. L., & Hall, J. L. (1978). A biochemical and cytochemical study of peroxidase activity in roots of Pisum sativum: II. Distribution of enzymes in relation to root development. *Journal of Experimental Botany, 29*(4), 983–991.
Filippou, P., Bouchagier, P., Skotti, E., & Fotopoulos, V. (2014). Proline and reactive oxygen/nitrogen species metabolism is involved in the tolerant response of the invasive plant species *Ailanthus altissima* to drought and salinity. *Environmental and Experimental Botany, 97*, 1–10.
Gebeyehu, H. R., & Bayissa, L. D. (2020). Levels of heavy metals in soil and vegetables and associated health risks in Mojo area. *Ethiopia. Plos One, 15*(1), e0227883. https://doi.org/10.1371/journal.pone.0227883
Ghorbel, M. (2012). *Contamination métallique issue des dechets de l'ancien site minier de Jebel Ressas: modélisation des mécanismes de transfert et conception de cartes d'aléa post- mine dans un contexte carbonaté et sous un climat semi-aride. Evaluation du risque pour la santé humaine*. Doctoral thesis. University Paul Sabatier - Toulouse III, France. Retrieved October 19, 2017, from https://tel.archives-ouvertes.fr/tel-00760685/document
Ghorbel, M., Munoz, M., Courjault-Radé, P., Destrigneville, C., de Parseval, P., Souissi, R., ... & Abdeljaouad, S. (2010). Health risk assessment for human exposure by direct ingestion of Pb, Cd, Zn bearing dust in the former miners' village of Jebel Ressas (NE Tunisia). *European Journal of Mineralogy*, *22*(5), 639-649
Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry, 48*(12), 909–930.
Giri, S., Singh, A. K., & Mahato, M. K. (2017). Metal contamination of agricultural soils in the copper mining areas of Singhbhum shear zone in India. *Journal of Earth System Science, 126*(4), 1–13. https://doi.org/10.1007/s12040-017-0833-z
Grotkopp, E., Rejmánek, M., & Rost, T. L. (2002). Toward a causal explanation of plant invasiveness: Seedling growth and life-history strategies of 29 pine (*Pinus*) species. *The American Naturalist, 159*(4), 396–419.
Gutiérrez, M., Mickus, K., & Camacho, L. M. (2016). Abandoned PbZn mining wastes and their mobility as proxy to toxicity: A review. *Science of the Total Environment, 565*, 392–400. https://doi.org/10.1016/j.scitotenv.2016.04.143
Hachani, C., Lamhamedi, M. S., Cameselle, C., Gouveia, S., Zine El Abidine, A., Khasa, D. P., & Béjaoui, Z. (2020). Effects of ectomycorrhizal fungi and heavy metals (Pb, Zn, and Cd) on growth and mineral nutrition of *Pinus halepensis* seedlings in North Africa. *Microorganisms, 8*(12), 2033. https://doi.org/10.3390/microorganisms8122033
Hachani, C., Lamhamedi, M. S., Zine El Abidine, A., Abassi, M., Khasa, D. P., & Béjaoui, Z. (2022). Water relations, gas exchange, chlorophyll fluorescence and electrolyte leakage of ectomycorrhizal *Pinus halepensis* seedlings in response to multi-heavy metal stresses (Pb, Zn, Cd). *Microorganisms, 10*(1), 57. https://doi.org/10.3390/microorganisms10010057

Hameed, A., Rasool, S., Azooz, M. M., Hossain, M. A., Ahanger, M. A., & Ahmad, P. (2016). Heavy metal stress: Plant responses and signalling. In P. Ahmad (Ed.), *Plant metal interaction: Emerging remediation techniques* (pp. 557–583). Elsevier.

Hasanuzzaman, M., Bhuyan, M. H. M., Zulfiqar, F., Raza, A., Mohsin, S. M., Mahmud, J. A., ... & Fotopoulos, V. (2020). Reactive oxygen species and antioxidant defense in plants under abiotic stress: Revisiting the crucial role of a universal defense regulator. Antioxidants, 9(8), 681https://doi.org/10.3390/antiox9080681

Hattab, S., Hattab, S., Flores-Casseres, M. L., Boussetta, H., Doumas, P., Hernandez, L. E., & Banni, M. (2016). Characterisation of lead-induced stress molecular biomarkers in *Medicago sativa* plants. *Environmental and Experimental Botany, 123*, 1–12.

He, Z., Shentu, J., Yang, X., Baligar, V. C., Zhang, T., & Stoffella, P. J. (2015). Heavy metal contamination of soils: Sources, indicators and assessment. *Journal of Environmental Indicators, 9*, 17–18.

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of biochemistry and biophysics, 125*(1), 189–198.

International Seed Testing Association (ISTA). (2012). *International rules for seed testing*. Bassersdorf, Switzerland. Retrieved September 28, 2017, from https://www.seedtest.org/en/publications/international-rules-seed-testing-1168.html

Islam, E., Yang, X., Li, T., Liu, D., Jin, X., & Meng, F. (2007). Effect of Pb toxicity on root morphology, physiology and ultrastructure in the two ecotypes of *Elsholtzia argyi*. *Journal of Hazardous Materials, 147*(3), 806–816.

Júnior, C. A. L., Oliveira, S. R., Mazzafera, P., & Arruda, M. A. Z. (2016). Expanding the information about the influence of cadmium on the metabolism of sunflowers: Evaluation of total, bioavailable, and bioaccessible content and metallobiomolecules in sunflower seeds. *Environmental and Experimental Botany, 125*, 87–97. https://doi.org/10.1016/j.envexpbot.2016.02.003

Karaca, O., Cameselle, C., & Reddy, K. R. (2018). Mine tailing disposal sites: Contamination problems, remedial options and phytocaps for sustainable remediation. *Reviews in Environmental Science and Bio/technology, 17*(1), 205–228.

Khalid, S., Shahid, M., Niazi, N. K., Murtaza, B., Bibi, I., & Dumat, C. (2017). A comparison of technologies for remediation of heavy metal contaminated soils. *Journal of Geochemical Exploration, 182*, 247–268. https://doi.org/10.1016/j.gexplo.2016.11.021

Khan, A., Khan, S., Khan, M. A., Qamar, Z., & Waqas, M. (2015). The uptake and bioaccumulation of heavy metals by food plants, their effects on plants nutrients, and associated health risk: A review. *Environmental Science and Pollution Research, 22*(18), 13772–13799. https://doi.org/10.1007/s11356-015-4881-0

Kicińska, A., & Wikar, J. (2021). Ecological risk associated with agricultural production in soils contaminated by the activities of the metal ore mining and processing industry-example from southern Poland. *Soil and Tillage Research, 205*, 104817. https://doi.org/10.1016/j.still.2020.104817

Lamhamedi, M. S., Renaud, M., Desjardins, P., & Veilleux, L. (2013). Root growth, plug cohesion, mineral nutrition, and carbohydrate content of (1+ 0) *Picea mariana* seedlings in response to a short-day treatment. *Tree Planters' Notes, 56*(1), 35–46.

Li, W., Khan, M. A., Yamaguchi, S., & Kamiya, Y. (2005). Effects of heavy metals on seed germination and early seedling growth of Arabidopsis thaliana. *Plant Growth Regulation, 46*(1), 45–50. https://doi.org/10.1007/s10725-005-6324-2

Li, Z., Ma, Z., van der Kuijp, T. J., Yuan, Z., & Huang, L. (2014). A review of soil heavy metal pollution from mines in China: Pollution and health risk assessment. *Science of the Total Environment, 468*, 843–853.

Liu, S., Yang, C., Xie, W., Xia, C., & Fan, P. (2012). The effects of cadmium on germination and seedling growth of *Suaeda salsa*. *Procedia Environmental Sciences, 16*, 293–298. https://doi.org/10.1016/j.proenv.2012.10.041

Liu, Y., Wang, Y., Lu, H., Lonappan, L., Brar, S. K., He, L., ... & Yang, S. (2018). Biochar application as a soil amendment for decreasing cadmium availability in soil and accumulation in *Brassica chinensis*. Journal of Soils and Sediments, *18*(7), 2511-2519https://doi.org/10.1007/s11368-018-1927-1

Ma, L., Sun, J., Yang, Z., & Wang, L. (2015). Heavy metal contamination of agricultural soils affected by mining activities around the Ganxi River in Chenzhou. *Southern China. Environmental Monitoring and Assessment, 187*(12), 1–9.

Mackay, D., & Fraser, A. (2000). Bioaccumulation of persistent organic chemicals: Mechanisms and models. *Environmental Pollution, 110*(3), 375–391.

Mahmud, J. A., Bhuyan, M. B., Nahar, K., Parvin, K., & Hasanuzzaman, M. (2020). Response and tolerance of fabaceae plants to metal/metalloid toxicity. In M. Hasanuzzaman, S. Araújo, & S. Gill (Eds.), *The plant family Fabaceae* (pp. 435–482). Springer. https://doi.org/10.1007/978-981-15-4752-2_17

Mateos-Naranjo, E., Castellanos, E. M., & Perez-Martin, A. (2014). Zinc tolerance and accumulation in the halophytic species *Juncus acutus*. *Environmental and Experimental Botany, 100*, 114–121.

Medyńska-Juraszek, A., Bednik, M., & Chohura, P. (2020). Assessing the influence of compost and biochar amendments on the mobility and uptake of heavy metals by green leafy vegetables. *International Journal of Environmental Research and Public Health, 17*(21), 7861. https://doi.org/10.3390/ijerph17217861

Mendez, M. O., & Maier, R. M. (2008). Phytoremediation of mine tailings in temperate and arid environments. *Reviews in Environmental Science and Bio/technology, 7*(1), 47–59.

Mlayah, A., Da Silva, E. F., Rocha, F., Hamza, C. B., Charef, A., & Noronha, F. (2009). The Oued Mellègue: Mining activity, stream sediments and dispersion of base metals in natural environments. *North-Western Tunisia. Journal of Geochemical Exploration, 102*(1), 27–36.

Monneveux, P., & Nemmar, M. (1986). Contribution à l'étude de la résistance à la sécheresse chez le blé tendre (Triticum aestivum L.) et chez le blé dur (Triticum durum

Desf.): étude de l'accumulation de la proline au cours du cycle de développement. *Agronomie, 6*(6), 583–590.

Nadgórska-Socha, A., Kafel, A., Kandziora-Ciupa, M., Gospodarek, J., & Zawisza-Raszka, A. (2013). Accumulation of heavy metals and antioxidant responses in *Vicia faba* plants grown on monometallic contaminated soil. *Environmental Science and Pollution Research, 20*(2), 1124–1134. https://doi.org/10.1007/s11356-012-1191-7

Nagajyoti, P. C., Lee, K. D., & Sreekanth, T. V. M. (2010). Heavy metals, occurrence and toxicity for plants: A review. *Environmental Chemistry Letters, 8*(3), 199–216. https://doi.org/10.1007/s10311-010-0297-8

Nakano, Y., & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology, 22*, 867–880.

Nguyen, N. L., Vu, C. T., To, H. M., Pham, H. N., Nguyen, H. D., Nguyen, T. D., & Nguyen Thi, K. O. (2020). The interactions among the heavy metals in soils and in weeds and their antioxidant capacity under the mining activities in Thai Nguyen Province, Vietnam. Journal of Chemistry, *2020*https://doi.org/10.1155/2020/8010376

Othmani, M. A., Souissi, F., Benzaazoua, M., Bouzahzah, H., Bussiere, B., & Mansouri, A. (2013). The geochemical behaviour of mine tailings from the Touiref Pb–Zn District in Tunisia in weathering cells leaching tests. *Mine Water and the Environment, 32*(1), 28–41.

Pais, I., & Jones, J. B. (1997). *The handbook of trace elements*. CRC Press.

Peralta, J. R., Gardea-Torresdey, J. L., Tiemann, K. J., Gomez, E., Arteaga, S., Rascon, E., & Parsons, J. G. (2001). Uptake and effects of five heavy metals on seed germination and plant growth in alfalfa (*Medicago sativa* L.). *Bulletin of Environmental Contamination and Toxicology, 66*(6), 727–734.

Pinto, A. P., De Varennes, A., Fonseca, R., & Teixeira, D. M. (2015). Phytoremediation of soils contaminated with heavy metals: Techniques and strategies. In A. A. Ansari, S. S. Gill, R. Gill, G. R. Lanza, & L. Newman (Eds.), *Phytoremediation: Management of environmental contaminants* (pp. 133–155). Springer. https://doi.org/10.1007/978-3-319-10395-2_10

Pourrut, B., Shahid, M., Dumat, C., Winterton, P., & Pinelli, E. (2011). Lead uptake, toxicity, and detoxification in plants. In D. Whitacre (Ed.), *Reviews of environmental contamination and toxicology* (vol. 213, pp. 113–136). Springer. https://doi.org/10.1007/978-1-4419-9860-6_4

Rai, R., Agrawal, M., & Agrawal, S. B. (2016). Impact of heavy metals on physiological processes of plants: With special reference to photosynthetic system. In A. Singh, S. M. Prasad, & R. P. Singh (Eds.), *Plant responses to xenobiotics* (pp. 127–140). Springer. https://doi.org/10.1007/978-981-10-2860-1_6

Ramos, S. J., Gastauer, M., Mitre, S. K., Caldeira, C. F., Silva, J. R., Neto, A. E. F., ... & Siqueira, J. O. (2020). Plant growth and nutrient use efficiency of two native *Fabaceae* species for mineland revegetation in the eastern Amazon. *Journal of Forestry Research, 31*(6), 2287–2293. https://doi.org/10.1007/s11676-019-01004-w

Rascio, N., & Navari-Izzo, F. (2011). Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? *Plant Science, 180*(2), 169–181.

Rucinska-Sobkowiak, R. (2010). Stres oksydacyjny wywołany działaniem metali ciężkich na rośliny. *Postępy Biochemii, 56*(2).

Sachdev, S., Ansari, S. A., Ansari, M. I., Fujita, M., & Hasanuzzaman, M. (2021). Abiotic stress and reactive oxygen species: Generation, signaling, and defense mechanisms. *Antioxidants, 10*(2), 277. https://doi.org/10.3390/antiox10020277

Sergiev, I., Alexieva, V., & Karanov, E. (1997). Effect of spermine, atrazine and combination between them on some endogenous protective systems and stress markers in plants. *Comptes Rendus De L'academie Bulgare Des Sciences, 51*(3), 121–124.

Shu, X., Yin, L., Zhang, Q., & Wang, W. (2012). Effect of Pb toxicity on leaf growth, antioxidant enzyme activities, and photosynthesis in cuttings and seedlings of *Jatropha curcas* L. *Environmental Science and Pollution Research, 19*(3), 893–902. https://doi.org/10.1007/s11356-011-0625-y

Singh, S. N., Goyal, S. K., Rai, J. P., & Singh, S. R. (2014). Heavy metal accumulation in plants grown in peri-urban metal contaminated areas of eastern UP. *Agriways, 2*(1), 8–11.

Smirnoff, N., & Arnaud, D. (2019). Hydrogen peroxide metabolism and functions in plants. *New Phytologist, 221*(3), 1197–1214. https://doi.org/10.1111/nph.15488

Sofo, A., Scopa, A., Nuzzaci, M., & Vitti, A. (2015). Ascorbate peroxidase and catalase activities and their genetic regulation in plants subjected to drought and salinity stresses. *International Journal of Molecular Sciences, 16*(6), 13561–13578. https://doi.org/10.3390/ijms160613561

Souguir, D. (2009). *Modifications métaboliques, moléculaires et génotoxicité induites par le cadmium chez Vicia faba*. Doctoral thesis. University Blaise Pascal - Clermont-Ferrand II; University of Auvergne - Clermont-Ferrand I; University of Carthage, Tunisia. Retrieved January 07, 2019, from https://core.ac.uk/download/pdf/49293456.pdf

Steel, G. D., Torrie, J. H., & Dickey, D. A. (1997). *Principles and procedures of statistics: A biometrical approach* (3rd ed.). The McGraw-Hill Companies Inc.

Timmer, V. R., & Miller, B. D. (1991). Effects of contrasting fertilization and moisture regimes on biomass, nutrients, and water relations of container grown red pine seedlings. *New Forests, 5*(4), 335–348.

Verma, S., & Dubey, R. S. (2003). Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. *Plant Science, 164*(4), 645–655.

Wang, F., Zhang, S., Cheng, P., Zhang, S., & Sun, Y. (2020). Effects of soil amendments on heavy metal immobilization and accumulation by maize grown in a multiple-metal-contaminated soil and their potential for safe crop production. *Toxics, 8*, 102. https://doi.org/10.3390/toxics8040102

Wei, W., Ma, R., Sun, Z., Zhou, A., Bu, J., Long, X., & Liu, Y. (2018). Effects of mining activities on the release of heavy metals (HMs) in a typical mountain headwater region, the Qinghai-Tibet Plateau in China. *International Journal of*

Environmental Research and Public Health, 15(9), 1987. https://doi.org/10.3390/ijerph15091987

Weis, J. S., & Weis, P. (2004). Metal uptake, transport and release by wetland plants: Implications for phytoremediation and restoration. *Environment International, 30*(5), 685–700.

Wilkins, D. A. (1978). The measurement of tolerance to edaphic factors by means of root growth. *New Phytologist, 80*(3), 623–633.

Xu, X., Liu, C., Zhao, X., Li, R., & Deng, W. (2014). Involvement of an antioxidant defense system in the adaptive response to cadmium in maize seedlings (Zea mays L.). *Bulletin of environmental contamination and toxicology, 93*(5), 618–624.