

Cadmium effects on *Lupinus albus* L growth, phenolic compounds, and enzyme activities

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Abstract

Cadmium (Cd) is had received particular attention not only as potent hazards to human health but also given their constant increase in the environment and bioaccumulation throughout the food chain. The aim of this work was to evaluate the response of the white lupine plant (*Lupinus albus* L.) to different cadmium concentrations. For this purpose, seedlings were exposed in nutrient solution to 0, 10, 20, 50, 100 and 150 μM CdCl_2 for 7 days. The obtained data demonstrated that, *Lupinus albus* L. was affected especially by high Cd^{2+} doses (100 and 150 μM Cd). Cadmium exposure mainly reduced plant growth parameters and photosynthetic activity. In addition, Cd generated nutritional deficiencies, toxic accumulation of ammonium and oxidative damage in plant. However, *Lupinus albus* L. could tolerate moderate the cadmium stress by the increase of cellular metabolites like soluble sugar and proline to avoid the ionic and osmotic effects of Cd^{2+} . Furthermore, Cd^{2+} treatment increased the production of secondary metabolites in different plant organs, and also increased the activities of antioxidant enzymes. Besides its importance in food applications and health benefits, white lupin is proposed, as a candidate for cadmium phytostabilization of soil polluted by cadmium.

Introduction

The environment at contamination by heavy metals has enormously increased due to industrial activities, urban traffic and undesired some human agricultural practices (Rao et al. 2018). These pollutants are very dangerous because they do not degrade and can therefore persist for a long time in the environment. If the accumulated hazardous metals in the environment exceed the threshold level, they may not only harm plants invisibly but also offer significant health risks to animals and people who consume the poisoned plants (Mnasri et al. 2015). The term of heavy metals refers to toxic chemical elements with a density exceeding 5 g/ cm^3 , and according to legislative tests, pollution by toxic metals included seven metals and metalloids. In fact, Cadmium (Cd^{2+}) is classified as one of the most toxic heavy metals, representing a potential hazard through bioaccumulation along the food chain. It is associated with many modern industrial processes, and is taken up by plants in significant quantities from contaminated water and soil (Kan et al. 2009; Feng et al. 2010). Inhibited photosynthesis, altered mineral feeding, and water imbalance were all seen in plants exposed to Cd^{2+} . Moreover, this metal interfered with crop output (Di Toppi and Gabbrielli 1999) and stomatal function (Hossain et al. 2010, Nazar et al. 2012). In fact, Cd^{2+} ions altered the electron transfer chain in chloroplasts and mitochondria, and induced the production of active forms of oxygen, thus leading to oxidative stress followed by the peroxidation of membrane lipids. The plant's responses to cadmium are varied ranging from extreme sensitivity to a high tolerance.

One of the most challenging environmental engineering projects is the remediation of cadmium-contaminated soil. The majority of the time, conventional physiochemical approaches are highly expensive and may affect the soil. Phytoremediation, which uses plants to remove, stabilize, or degrade inorganic and organic pollutants, has been proposed as a promising, environmentally sustainable, and extremely cheap technology. Phytoremediation approach includes several types of distinct strategies such as phytoextraction, phytostabilization, phytovolatilization, phytodegradation and phytofiltration. Thereby, some plants could use in this strategies and can grow on soils contaminated by heavy metals such as *Indian mustard* (Gill et al. 2011; Wang et al. 2019), lettuce (Zorrig et al. 2013; Dawuda et al. 2019), *Sesuvium portulacastrum* (Mnasri et al. 2015). In fact, plants develop specific mechanisms to detoxify the metal or to make it unavailable (Singh et al. 2015; Chen et al. 2016).

Phytoremediation, which involves the use of plants to remove, stabilize, or degrade inorganic and organic pollutants, has been proposed as a promising, environmentally sustainable, and extremely low-cost technology. The phytoremediation approach includes several distinct strategies, including phytoextraction, phytostabilization, phytovolatilization, phytodegradation, and phytofiltration. Therefore, some plants, such as *Indian mustard* (Gill et al. 2011; Wang et al. 2019), lettuce (Zorrig et al. 2013; Dawuda et al. 2019), and *Sesuvium portulacastrum* (Mnasri et al. 2015), could use these techniques and be able to grow on soils contaminated by heavy metal.

However, *Lupinus albus* or white lupin is a very interesting species, according to Sujak et al. (2006) Lupin seeds are rich in proteins and bioactive compounds and are also rich in fibers (Bähr et al. 2014) and flavonoids (Siger et al. 2012). This species is considered for human health because of its richness in antioxidant compounds (Bähr et al. 2014). Several studies recommended

the use of lupin seed meal for people with hypertension and hyperglycemia (Bähr et al. 2014). So, it would be very interesting to understand the adaptive response of white lupin to Cd^{2+} , and to evaluate the Cd^{2+} tolerance in order to propose it as species of interest to cleaning up soils contaminated by cadmium. Indeed, with phytoextraction, of heavy metals on marginal areas represents a very relevant and promising approach to be part of the rehabilitation and enhancement of abandoned marginal areas in order to increase forage or food resources. In this context, the objective of our research is to evaluate the response of white lupine under the effect of different concentrations of cadmium through the study of the sensitivity of certain morphological, physiological and biochemical parameters.

Material and methods

Plant material and growth conditions

The plant material studied is white lupin (*Lupinus albus* L.) originating from Egypt and is an annual plant belonging to the legume family.

The seeds are disinfected by washing with 10% bleach for 15 minutes, after several rinsing with distilled water they are soaked overnight in distilled water.

The soaked seeds are germinated for 4 days on filter paper. On the 11th day of transplanting, seedlings are transferred to growth medium that contained cadmium. After germination, plants were grown hydroponically for 11 days in pots filled with 4.5 L continuously aerated Hoagland's nutrient solution (5 plants per pot) (Stephan et al. 1989)

The Hoagland's solution consisted of 5mM $\text{Ca}(\text{NO}_3)_2$, 5mM KNO_3 , 1mM KH_2PO_4 , 50 IM

H_3BO_3 , 1mM MgSO_4 , 4.5 μM MnCl_2 , 3.8 μM ZnSO_4 , 0.3 μM CuSO_4 and 0.1 mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ and 10 μM FeEDTA pH was adjusted to pH 5.8 adjusted with HCl. The total volume of the solution was kept constant by adding deionized water to compensate for water lost through plant transpiration, sampling and evaporation. The test solutions were changed every 3 days and pH was readjusted daily.

The experiment was carried out under growth chamber conditions with 25 ± 2 °C temperature, and 16 h/8 h photoperiod day/night regime. Solutions were renewed every 3 days to avoid depletion of nutrients and Cd concentrations. An initial harvest was performed just before Cd application to characterize the initial plant size and weight. Cd treatments were applied to obtain a final concentration of 0, 25, 50 and 100 μM CdCl_2 (Sigma-Aldrich) (4 pots per treatment). After 7 days, Cd-treated and control plants were harvested and used for physiological and biochemical analyses.

Growth Analysis

At the harvest, plants were divided in leaves, stems and roots. Leaves and stems were rinsed three times with cold distilled water and blotted between two layers of filter paper. Roots were immediately dipped in a 0.01 M HCl cold solution to eliminate external Cd adsorbed at the root surface according to Aldrich et al. (2003) and then washed three times with cold distilled water and blotted with filter paper. The fresh weight was immediately determined, and the dry weight was measured after 48 h of desiccation in an oven at 60 °C.

Measurement of water content

The water content (WC), expressed in ml g^{-1} DW, and was calculated according to the following equation: $\text{WC (ml H}_2\text{O g}^{-1} \text{ DW)} = (\text{FW} - \text{DW}) / \text{DW}$.

With FW: mass of fresh weight measured at final harvest, DW: mass of dry weight measured after drying at 80°C (Sconfield et al. 1988). The sensitivity index corresponds to the difference in DW production between treated and control plants. It was calculated by the ratio: $\text{SI} = ((\text{DW control} - \text{DW treated}) / \text{DW control}) * 100$.

Extraction and determination of cadmium content

Dried samples (c.a. 25 mg) were grounded to a fine powder using a porcelain mortar and a pestle and digested in 4/1 (v/v) $\text{HNO}_3/\text{HClO}_4$ (20 ml) mixture at 100°C. After cooling, the residues are solubilized by a solution of nitric acid (HNO_3) 7‰ which is followed by filtration and finished by the addition of 25 ml of nitric acid 7‰ (Chaffei et al. 2006). The cadmium concentrations were determined by atomic absorption spectrometry (Spectra AA 220 FS).

Soluble sugars, proline and malondialdehyde contents (MDA)

The extraction was carried out starting from 25 mg of dry vegetable weight in the presence of 5 ml of 80% ethanol. The samples are placed in a water bath at 70°C for 30 minutes. After cooling, the samples are centrifuged at 6000 g for 15 minutes. Subsequently, 25 µl of supernatant is removed, and added to 5 ml of anthrone solution in test tubes under a fume hood. Then these tubes are put in a water bath at 100°C for 10 minutes, and then placed in the ice. The reading of optical density is at 640 nm (Yemm and Willis 1954).

The determination of proline is a colorimetric assay based on the proline-ninhydrin complex. From a mixture of about 100 mg powdered vegetable material and 1.5 ml of sulfosalicylic acid. The mixture is centrifuged at 12000 g for 20 minutes at 4°C. The assay is carried out by mixing 500 µl with the extract with 500 µl of sulfosalicylic acid, 1 ml of ninhydrin reagent and 1 ml of concentrated acetic acid. The tubes are incubated in a water bath at 100°C for one hour and then cooled to 4°C. The acid mixture catalyzes the proline-ninhydrin reaction during which the solution turns red. 2 ml of toluene is added to separate the phases. The optical density is determined at 520 nm and the proline concentration is determined by reference to a calibration curve, which is expressed in $\mu\text{g mg}^{-1}$ FW (Bates et al. 1973).

The most commonly used method for *in vitro* estimation of lipid peroxidation in biological systems is the determination of malondialdehyde (MDA) by the reaction of thiobarbituric acid (TBA). The principle of MDA determination is based on acid and warm formation between MDA and two molecules of TBA, a red absorbing pigment at 532 nm (Hodges et al. 1999). About 250 mg of fresh leaves ground in liquid nitrogen are suspended in 5 ml TCA trichloroacetic acid (5% w/v) and centrifuged for 10 minutes at 12.000 g. Then, for 2 ml of the extract obtained is added 2 ml of thiobarbituric acid TBA (0.67%). The mixture is kept for 30 minutes in a water bath at 100°C and then cooled in ice. After that the mixture was centrifuged for one minute at 6000 g. The absorbance of the supernatant is measured at 532 and 600 nm. The amount of MDA is calculated using a molar extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$. According to Lambert Beer's law: $\text{Abs} = \epsilon \times L \times [\text{MDA}]$.

$\text{MDA (nmol g}^{-1} \text{ FW)} = ((A_{532}-A_{600}) * 100 * V) / \epsilon * \text{FW}$. With: ϵ : molar extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$

Soluble total protein

Soluble proteins are assayed according to the method of Bradford (1976). The principle of this method is the formation of complexes between Coomassie blue and the basic protein residues. $10 \mu\text{L}^{-1}$ of protein extract are added to $200 \mu\text{L}^{-1}$ of Bradford reagent. After 5 minutes incubation at room temperature and in the dark, the absorbance is measured at 595 nm and compared to that of a standard range of bovine serum albumin (BSA).

Ammonium (NH) assay and protease activity

A mass of 0.5 to 1 g of the plant material, previously frozen in liquid nitrogen, is ground at 4°C in a mortar in the presence of 2 ml of H_2SO_4 and 0.5% of polyclart AT. The ground material is centrifuged for 15 min at 30.000 g. The obtained supernatant is placed for 30 min at 37°C and the absorbance is measured at 620 nm (Weatherburn 1967).

To determine protease activity 100 mg of fresh plant material already stored in liquid nitrogen is mixed in a mortar with 1 ml of extraction buffer containing 50 mM Tris-HCl, pH = 7.5, 5‰ mercaptoethanol and 1% PVP. The centrifugation is carried out at 14.000 g for 30 minutes at 5°C. The supernatant obtained was used for the determination of proteases by the addition of reaction

medium. The whole is subjected to an incubation of 3 hours at 37°C whose reaction is stopped by the addition of 320 µl TCA 5%. The optical density is read at 330 nm following centrifugation at 15.000g for 10 min at 5°C and for enzymatic activity test.

Extraction and determination of phenolic compounds

The extraction and determination of total polyphenols was performed according to the following protocol, 1 g of dry vegetable material is mixed with 10 ml of 100% methanol. At room temperature, the mixture is stirred for 24 hours in the dark using a shaker. The extracts are then filtered with ashless filter paper and then stored at 4°C to be used for total polyphenols, flavonoids and condensed tannins. In our study, the extraction was carried out using an organic solvent methanol (MeOH) polarity from *Lupinus albus* L. plant. The determination of the total polyphenols is carried out by a colorimetric assay according to the method of Folin Cicalteu. This mixture is reduced during the oxidation of the polyphenols in a mixture of blue oxides of tungsten and molybdenum. The intensity of this blue color whose maximum absorption at 760nm provides information on the richness of the polyphenol extracts (Boizot and Charpentier 2006). The contents of the total polyphenols are determined from the linear calibration curve produced by gallic acid (0-200 µg ml⁻¹), expressed in milligrams equivalent of gallic acid per gram of dry weight (mg EAG g⁻¹DW).

For flavonoids content, a colorimetric assay based on the formation of a complex, yellow color, between flavonoids and aluminum trichloride. Soda forms another complex that makes the color pink, the intensity of which indicates the importance of the content of the extract in flavonoids. A volume of 250 µl of the methanolic extract, distilled water or hexane is mixed with 75 µl of a solution of NaNO₂ (5%). After incubation at ambient temperature, 150 µl of a 10% solution of aluminum trichloride (AlCl₃*6H₂O) freshly prepared are added. After five minutes of rest, 500 µl of sodium hydroxide (NaOH, 1M) are added and the mixture is adjusted with distilled water to 2.5 ml. Absorbance is measured at 510 nm with reference to a control devoid of the extract. The flavonoid contents are calculated using a standard range of catechin at concentrations ranging from 50 to 500 µg ml⁻¹. These flavonoid contents are expressed in mg of catechin equivalent per gram of dry weight (mg EC g⁻¹DW).

For tannins extraction, 1 ml of solution mixed with 2 ml H₂O and add 2–3 drops of 1% FeCl₃. In the presence of tannins, a blue-green (Catechism tannin) or black-blue (Gallic tannin) color develops. In the presence of 70% sulfuric acid, the hydrolyzed tannins depolymerize and by reaction with vanillin turn into red anthocyanidols measurable by spectrophotometry. An aliquot of 0.05 ml of the methanolic extract, distilled water or hexane is added to 3 ml of vanillin (4%) and 1.5 ml of 70% HCl. After 15 min of incubation at room temperature, the absorbance is measured at 500 nm. As for flavonoids, the condensed tannins content is expressed in mg of catechin equivalent per gram of dry weight (mg EC g⁻¹DW) (Ksouri et al. 2007).

Antioxidant enzymes assays

For protein extraction, 100 mg of fresh plant material already preserved in liquid nitrogen is mixed with 700 µl of extraction buffer containing (50 mM phosphate buffer, pH = 7.1 mM Na-EDTA, 20 mM MgCl₂, 50 mM KCl, 0.5 mM PMSF). Centrifugation is carried out at 14.000g for 30 minutes at 4°C. The obtained supernatant contains the soluble proteins and will be used for the determination of proteins and for enzymatic activity tests.

Superoxide dismutase (SOD) catalyze the disproportionation of superoxide into oxygen and hydrogen peroxide according to the following reaction: $2\text{O}_2^{\cdot-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$. The method of assaying SOD activity by the NBT/riboflavin assay is a method of photo reduction; in fact, the reduction of NBT by the superoxide anion $\text{O}_2^{\cdot-}$ is used as a basis for detecting the presence of SOD. The presence of SOD inhibits the oxidation of NBT. Thus, the SOD activity is determined by measuring the inhibition of NBT reduction. The absorbance measurement is carried out at 560nm by a spectrophotometer. One unit of SOD is equal to the amount needed to reduce half the reduction of NBT (Donahue et al. 1997).

Catalase (CAT) catalyzes the dissociation of hydrogen peroxide into water and molecular oxygen according to the following reaction: $\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \frac{1}{2}\text{O}_2$. The activity of this enzyme is determined by measuring the amount of H_2O_2 decomposed per unit

of time. The reaction mixture contains a volume of phosphate buffer (50 mM, pH = 7), 20 μL H_2O_2 and 20 μL of plant extract. The assay is performed by measuring the optical density at 240 nm. The activity is expressed in $\text{mmol min}^{-1} \text{mg}^{-1} \text{FW}$.

Statistical analysis

Data shown are means of 20 replicates for each treatment. Analysis of variance between treatments means was carried out with the SPSS 10.0 program. Means were compared using the Duncan's test at $P = 0.05$ (Slatni et al. 2011).

Results

Plant morphology and growth

Photo 1 shows the effect of different Cd concentrations on the Photo 1 shows the effect of different Cd concentrations on the aspects of whole plant growth and leaves of white lupin seedlings. Thus, Cd treatment has a negative effect on the morphology of the plant growth and this by the presence of visual symptoms of Cd^{2+} toxicity like chlorosis in young leaves of lupin seedlings and this effect increases with increasing cadmium concentration in the culture medium. The addition of Cd^{2+} to the culture solution affected also the growth of lupin seedlings. So, Cd^{2+} significantly decreased the dry matter in plants, especially in roots (Fig. 1) and this effect increased with increasing concentration of Cd^{2+} in the medium. The addition of cadmium to the culture solution decreased also leaf and root dry weight and this effect is more pronounced with the higher Cd^{2+} concentration (100 and 150 μM). The leaf dry weight decreases 39 and 60%, respectively under 100 and 150 μM Cd compared to control. For the root dry weight decreased by about 70% and 78% relative to the control, respectively under 100 and 150 μM Cd. Though the Cd treatment showed no significant effect on stem dry weight. Data showed a slight decrease in the length of the aerial part (Table. 1), this reduction was about 7, 14 and 31% respectively under 50, 100 and 150 μM Cd^{2+} as compared to control. This effect is more important in the roots thus the reduction of the length of the roots is reached about 41, 42 and 49% compared to the control. These results showed that the root system remained more sensitive to Cd than leaves and stems. The lowest Cd concentration had no significant effect on leaf number. However, this parameter decreased considerably under high Cd concentration (100 and 150 μM).

The estimation of the water content of various plant tissues (leaves, roots and stems) cultivated under Cd exposure was illustrated in Fig. 2. In shoot system (leaves and stems), water content decreased proportionally to Cd^{2+} dose of treatment. Similarly, root water content decreased but with lower level especially under low Cd concentration.

Cadmium accumulation

In treated plants, shoots, stem and roots Cd^{2+} concentrations increased with increasing CdCl_2 concentration in the culture medium (Fig. 3). For all concentrations of CdCl_2 the Cd^{2+} concentration in the roots tissues was higher than in the shoots (leaves and stems). The examination of Cd proportions retained in roots and those transported to leaves and stems, in relation to the total Cd quantity accumulated showed that roots accumulated the major fraction of the absorbed metal.

Photosynthetic pigments contents

CdCl_2 application induced a significant decrease in leaf chlorophyll content (Table 2). Under 150 μM Cd treatment, total chlorophyll content decreased by about 32% compared to control plant. Both the application of cadmium caused a decrease of Chl a and Chl b contents and this reduction is of the order of 30% and 37% under 150 μM of Cd Cl_2 respectively compared to control plant.

Table 1
Effects of different cadmium concentration on the shoots (aerial part of plant) and roots lengths and on the number of leaves of white lupin seedlings. Data are the means of twenty replicates. Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

CdCl_2 (μM)	Shoot length (cm)	Root length (cm)	Number of leaves
0	10.74c	37.33c	8c
10	10c	20b	8c
20	10.37c	20.26b	7bc
50	10c	20.66b	7bc
100	9.25b	21.33b	6b
150	7.40a	18.66a	4a

Table 2
Effects of different cadmium concentration on the photosynthetic pigment contents (chlorophylls and carotenoids) on leaves and stems of white lupin seedlings. Data are the means of twenty replicates. Mean values followed by the same letter are not significantly different at $p \leq 0.05$

Leaves ($\mu\text{g g}^{-1}$ FW)					Stems ($\mu\text{g g}^{-1}$ FW)			
CdCl_2 (μM)	Chltot	Chla	Chlb	Carotenoid	Chltot	Chla	Chlb	Carotenoid
0	1.09 $\pm$ 0.09b	0.78 $\pm$ 0.07c	0.31 $\pm$ 0.03c	0.21 $\pm$ 0.02a	0.07c	0.029b	0.045c	0.21 $\pm$ 0.019a
10	1.01 $\pm$ 0.09b	0.75 $\pm$ 0.07bc	0.26 $\pm$ 0.022b	1.42 $\pm$ 0.011b	0.035b	0.027b	0.01b	0.5 $\pm$ 0.043c
20	1.01 $\pm$ 0.085b	0.71 $\pm$ 0.06b	0.25 $\pm$ 0.021b	2.5 $\pm$ 0.015c	0.033b	0.025ab	0.008b	0.5 $\pm$ 0.041c
50	1.01 $\pm$ 0.08b	0.71 $\pm$ 0.056b	0.25 $\pm$ 0.02b	2.5 $\pm$ 0.012c	0.033b	0.025ab	0.006ab	0.57 $\pm$ 0.04c
100	0.96 $\pm$ 0.07b	0.70 $\pm$ 0.055b	0.24 $\pm$ 0.019b	2.57 $\pm$ 0.016c	0.029ab	0.022a	0.004a	0.51 $\pm$ 0.034c
150	0.75 $\pm$ 0.06a	0.56 $\pm$ 0.045a	0.18 $\pm$ 0.011a	2.28 $\pm$ 0.014c	0.027a	0.02a	0.006ab	0.56 $\pm$ 0.035c

Moreover, the stem chlorophyll content also decreased significantly with the higher concentration of Cd^{2+} . Thus under 150 μM of Cd Cl_2 treatment, total chlorophyll decreased by about 62% referring to control. Chl a and Chl b contents decreased too, respectively by 22% and 85% under 150 μM CdCl_2 treatment. Results showed that Cd treatment, even in moderate concentration, decreased the chlorophyll contents.

Results in Table 2 showed a significant increase in carotenoid levels under 10 μM CdCl_2 treatment. In leaves, under 100 μM Cd treatment, carotenoid level increase was about 10 times compared to control plants. While, in stems it was about 2 times compared to control.

Total soluble sugar, proline and malondialdehyde contents

CdCl_2 exposure increased carbohydrate content in roots, stems and leaves of white lupin plant (Fig. 4a). Results showed an increase in sugar content level by 456%, 151% and 259% compared to control plants, respectively in leaves, stems and roots under 150 μM CdCl_2 .

A significant accumulation of proline was observed (Fig. 4b). This accumulation depends on the concentration of CdCl_2 in the culture medium and plant organ. Thereby, the proline accumulation was higher in aerial parts (especially leaves) than in roots and this effect increases with increasing the concentration of CdCl_2 in the medium. Thus, the increase in the level of proline reached

263% in the leaves compared to the control. While this increase reached 174% and 145% respectively in the stems and roots compared to the control.

Addition of Cd^{2+} to the nutrient solution significantly increased the production of malondialdehyde (MDA) which is an indicator of the peroxidation of membrane lipids. Results showed that MDA level increase was proportional to CdCl_2 concentration in the medium. The more significant MDA content was obtained mainly in leaves, then stems and finally in roots. The more important increase as compared to control plants was obtained mainly in roots. In fact, under 150 μM Cd treatment, the increase of MDA content was about 1456% in roots, 351% in leaves, and 253% in stems of those measured in control plants. (Fig. 4c).

Secondary metabolites production

Figure 5a illustrated the effect of different cadmium concentrations in the total polyphenol contents in white lupin seedling. The analysis of the effect of different cadmium concentrations in the total polyphenol contents in white lupin seedling confirmed that the methanolic extract of roots was richer in total phenols compared to leaves and stems. thus, Cd treatment induced a strong increase in total polyphenol content in all plant organs. compared to the control plants, the polyphenol content increase was about 192%, 429%, and 182% respectively in leaves, stems, and roots under 150 μM Cd treatment. In control plants, total flavonoid levels were quite close in different plant organs. However, a significant increase of total flavonoid content in different organs of the white lupin plant with increasing Cd^{2+} concentration in the culture solution (Fig. 5b). Thus, in the Cd-treated plants, total flavonoid content was more significant in roots than in stems and leaves. In fact, under 100 μM Cd treatment, total flavonoid content increased by 190% in leaves and stems. While, in roots, the total flavonoid content increase reached 346% of those measured in control plants. cadmium induced a significant increase of the tannin content hydrolyzed in the various organs of the plants (Fig. 5c). In fact, results showed a significant increase in leaves, stems and roots by about 174%, 171% and 167%, respectively, under 150 μM Cd treatment of those measured in control plants.

Antioxidant Enzymes Activities

In control plants, the catalase activity was mainly obtained in aerial parts (leaves and stems) (Fig. 6a). In leaves, catalase activity increased under Cd treatment. But catalase activity increase remained stable independently of different CdCl_2 concentration in the medium. In fact, the increase in catalase activity is 26% from 10 μM to 150 μM CdCl_2 . While in stems and roots, catalase activity enhancement was more significant under the high cadmium concentration. Thus, under 150 μM CdCl_2 , the catalase activity increase of 77% and 61.9% respectively in stems and roots than in untreated ones. Superoxide dismutase activity (SOD) in leaves and roots in control plants was less significant than that in stems (Fig. 6b). Under 150 μM CdCl_2 , SOD activity increased considerably mainly in stems. whereas, in roots, a less significant SOD activity enhancement was reported.

Soluble Protein, Ammonium Contents and Protease Activity in Plant

In control seedlings, the total soluble protein (SP) content was significant in the aerial parts (leaves and stems) as well as in roots. However, cadmium induced a significant decrease of the total soluble protein and this effect was more pronounced with increased the CdCl_2 concentration in the medium. Thereby SP content decreased by about 18.4%, 46.2% and 83.8% respectively in leaves, stems and roots under 150 of μM CdCl_2 as compared to control (Table 3).

Table 3

Effects of different cadmium concentration on soluble protein and ammonium contents and protease activity in leaves stems and roots of white lupin seedlings. Data are the means of twenty replicates. Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

	Protein content			Ammonium content			Protease activity		
	(mg g ⁻¹ FW)			(μmol g ⁻¹ FW)			(Umg ⁻¹ protein)		
CdCl ₂ (μM)	leaves	stems	roots	leaves	stems	roots	leaves	stems	roots
0	4.9d	5.2d	5.73de	0.312a	0.875b	0.562a	374.14cd	304.48c	130.89a
10	4.8d	3.86c	4.66cd	0.375a	0.906b	0.937b	468.75d	410.18d	196.7b
20	5.06d	3.33bc	5d	0.406a	1.001b	1.125b	559.94e	525.52e	202.83b
50	4.66cd	3.34bc	4.93d	0.562a	1.026b	2.187c	609.79f	548.9e	219.74b
100	4.13c	3.2b	4.26c	0.875b	1.125b	2.437c	706.21g	575.51ef	332.54c
150	4c	2.8b	0.93a	1.812b	1.137b	2.187c	833.33h	565.47ef	170.25ab

SP content decrease could be related to proteolytic pathway stimulation. In control seedlings, the ammonium content (NH₄⁺) was important in the aerial parts (leaves and stems) and in the roots. In stressed seedlings, NH₄⁺ content increased by about 481%, 30% and 289% in leaves, stems and roots, respectively, under 150 μM of CdCl₂ of those measured in control plants.

In fact, the increase of NH₄⁺ content could be explained by the activation of protease pathways and the disruption of NH₄⁺ incorporation. In control plants, the protease activity in aerial parts appeared to be greater than that in roots. Under 150 μM CdCl₂ the protease activity increased by about 122.7%, 30.1% and 85.8% respectively in leaves, roots and stems as compared with control plants (Table 3).

Discussion

Based in a previous study, in white lupin the roots were the preferential organs for cadmium accumulation. Cd uptake was correlated to endodermal apoplastic barriers which may restrict or derestrict the Cd loading into xylem (Feng et al. 2018). In fact, Cd²⁺ concentration in the roots tissues was higher than in the leaves and stems shoots. Similar results have been observed in other species such as *Brassica napus* (Ben Youssef et al. 2005) and *Ageratum conyzoides* L (Zhu et al. 2018). Several studies revealed that roots appear as trap organs, ensuring the accumulation of high part of the metal, thus limiting its export to shoot (Pereira et al. 2002). Root Cd accumulation could be explained by the parietal retention of Cd and the strong precipitation of the metal in apoplastic spaces or in xylem. Other studies showed that endocellular sequestration of the pollutant could be carried out by sulfur peptides (Ben Ammar et al. 2008, Yang et al. 2019). The present results showed that the high concentration of Cd develop significant physiological and metabolic disorders in white lupin seedling. In fact, many visual symptoms of toxicity were detected in white lupin plants. These symptoms were manifested by necrosis and chlorosis spots in leaves and by browning in roots. These effects were also associated with leaf drop especially for the doses 100 and 150 μM CdCl₂. In addition to ionic Cd toxicity, these symptoms have been attributed to a mineral deficiency including manganese, magnesium and iron (Ben Youssef et al. 2005; Ben Ammar et al. 2008). The plant growth was significantly reduced in the presence of high Cd concentration; this effect was linked to ionic Cd toxicity in shoots and roots (Zorrig et al. 2010). Many studies explained the reduction of biomass by the loss of cell turgescence and by a nutritional deficiency caused by the heavy metal (Gouia et al. 2003).

These negative effects could also be in related to balance disturbances of some growth hormones notably auxin and the deterioration on the cell walls composition (Chaoui and El Ferjani 2005). This study showed that the growth reduction of white lupin was accompanied by dehydration especially in shoots. The water content decrease in tissue reveals the disturbance of water absorption and transport under Cd exposure (El Khateeb and Al-Qwasemeh 2014). In fact, the reduction of the root

absorption surface following the fall of root growth caused an imbalance between the shoot water demand and the root absorption capacity (Chamseddine et al. 2009). This effect may be due also to the inhibition of the xylem cell division and elongation by cadmium. The Cd treatment induced too, a lowering of the total chlorophyll content, chl a and chl b. This is consistent with many studies that report a decrease in chlorophyll content under the effect of Cd (Farooq et al. 2016). The reduction of chlorophyll is one of the primary events in plants exposed to metal stress and results from the inhibition of the enzymes responsible for chlorophyll biosynthesis. Consequently, the decrease in photosynthetic activity led to decrease in the chlorophyll content. In *Brassica napus* studies, chlorosis is due to decrease in the density of chloroplasts and an increase in the size of mesophylls cells. This effect is linked to the genesis disruption of chloroplasts by Cd^{2+} ions (Barylá et al. 2001). Photosynthesis decrease could be related to a block in photosystem II (PSII) acceptor and donor side electron transport under Cd treatment and/or a decrease in quantum yield of PSII (Chu et al. 2018). On the other hand, the malonyldialdehyde (MDA) content in white lupin seedlings increased proportionally to CdCl_2 concentration in the nutrient solution. This effect is an indicator of lipids membrane peroxidation. In fact, MDA levels increased simultaneously with membrane instability and decrease in membrane integrity followed by a stimulation of the lipoxygenase activity. Indeed, several studies showed that lipid peroxidation was accompanied by hydroxyl and peroxide accumulation, hence the destruction of the membrane and cellular components (Ben Ammar et al. 2005; Manquían-Cerda et al. 2016). Several studies also showed also that MDA production is followed by a change in the activity of antioxidant enzymes such as catalase (CAT) and/or superoxide dismutase (SOD) (Fidalgo et al. 2011). To tolerate and reduce oxidative damage, plants set up enzymatic (CAT, SOD) and non-enzymatic antioxidant defense systems (carotenoids and polyphenols). These systems play an important role in regulating ROS concentrations in plant. Catalase and superoxide dismutase enzymes allowed the destruction of free radical species by catalyzing the transformation of the $\text{O}_2^{\cdot -}$ radical into H_2O_2 . Our results revealed a stimulation of SOD and CAT activities in leaves, stems and roots of white lupin under Cd treatment. These results were confirmed by several studied which showed a significant increase in SOD activity in Cd-treated plants (Zouari et al. 2016; Bashir et al. 2018). Furthermore, several studies showed that ascorbate peroxidase (APX) act as $\text{H}_2\text{O}_2^{\cdot -}$ scavenging enzyme. Also, it is indispensable for the protection of chloroplasts and other cell constituents. Heavy metals stimulated the expression of APX in plants. In fact, Cd stress enhanced leaf APX activity in *Brassica juncea* (Mobin and Khan 2007; Ahmad et al. 2011) and *aestivum* (Khan et al. 2007). Also, Cd treatment, increased APX activity in pea seedlings (Dixit et al. 2001) and maize seedlings (Rellán-Álvarez et al. 2006).

On the other hand, high Cd^{2+} concentrations induced the increase of the levels of non-enzymatic antioxidant molecules such as carotenoids, polyphenols, flavonoids and tannins compounds in the leaves, stems and roots. This effect in carotenoid levels provided protection against oxidative stress due to its ability to trap singlet oxygen and neutralize peroxy radicals. In addition to preventing Reactive oxygen species (ROS) production, either by inactivating of chlorophylls or by dissipating excess energy through the xanthophyll cycle (Farooq et al. 2016). In Cd-treated seedlings, the polyphenol rise was explained by the increase scavenging of enzymes involved in phenolic metabolism. Also, flavonoids and tannins could act as ROS scanning compounds. Indeed, phenolic compounds could chelate transition metal ions, eliminate ROS and thus inhibit lipid peroxidation. Similar results, including an increase in total polyphenol contents were reported in olive tree (*Olea Europaea*) and in *Cakile maritimum* subjected to water deficit and salt stress (Ksouri et al. 2007). In addition, the present study showed an increase in proline concentration in different organs of plant. In fact, proline played a major role in plant osmoregulation and osmotolerance to various abiotic constraints. Recent studies showed too, that Proline improve growth and yield in Cd-treated common bean plants and improve enzymatic and non-enzymatic antioxidant activities (Rady et al. 2019). Some studies showed that proline could protect enzymes from inactivation and also induced mechanism of Cd chelation and detoxification in tomato plants, by forming a non-toxic Cd-proline complex (Hayat et al. 2011). Proline accumulation could lead to direct impact manifested by the improvement of photosynthetic activity and mineral nutrition, while the indirect impact of this metabolite resulted from improved disease tolerance (Shahid et al. 2014). Cd stress induced a significant increase in soluble sugar levels in shoots and roots of treated lupin seedlings. Various metals caused an increase in soluble sugar content of *Lemna polyrrhiza* plants (John et al. 2008). The soluble sugars accumulation is positively correlated with metal tolerance in *Synara scolymus* plants (Karimi et al. 2012). The accumulation of soluble sugars lead to the improved permeability of the membrane, the homeostasis of the maintained water and the reinforced antioxidant defense mechanisms. A pea (*P. sativum*) study showed that soluble sugars act as an osmoprotectants, which maintained turgor and induced tolerance in stressed plants (Shahid et al. 2014).

We could clearly observe that white lupin maintained higher growth in the aerial part of plant and photosynthetic activity under moderate Cd doses. It's also clear that roots accumulated more Cd than leaves and stems for that white lupin tolerate moderate concentration of Cd and could be used in Cd-phytostabilization.

The present study confirmed Vázquez et al. (2006) who have shown strong retention of Cd in roots in white lupin and Cd-tolerance of this species was linked to Cd phytochelatin production and demonstrate that the cell-wall binding of Cd. The same study recommended the use of white lupin in the phytostabilization of heavy metal like Cd and As. Vázquez et al. (2006) showed many others benefits of this species especially the revegetation of polluted soils. Our results agree with Vázquez study, strongly encourages the use of white lupin to improve the rehabilitation of marginal abandoned areas in order to increase forage and food resources.

The capacity of white lupin to tolerate moderate Cd doses and to survive in the presence of high Cd doses was linked to many adaptive mechanisms and protective compounds. In fact, the synthesis of protective metabolites like soluble sugars and proline and the activation of the antioxidant system like CAT and SOD conferred this species the possibility to be a promoter for establishment of plant cover in polluted soil by heavy metal. Consequently, this species with multiple interests, in fact, it could be used firstly as forage and food interest, secondly used in many health benefits, then this species could be also interesting for phytostabilization since its roots accumulate high levels of cadmium.

Results showed that plant subjected to Cd accumulated high levels of ammonium in their organs. This result confirmed that Cd affect nitrogen metabolism in white lupin seedling. The accumulation of NH_4^+ correlated with a decrease of protein content and an increase of proteolytic activity in different organs of plant. Others works showed a correlation between protein degradation and proteolytic activity with respect to oxidative stress has also been proposed, suggesting an increase in protein degradation as an index of oxidative stress. Cadmium treatment induced ammonium accumulation in several plant species such as *Nicotiana tabaccum* (Maaroufi-Dguimi et al. 2019) and *Medicago sativa* (Yang et al. 2019).

In addition, many studies showed that toxic accumulation of ammonium in plant is linked to the disruption of the incorporation of NH_4^+ in the carbon skeletons by the decrease of enzymatic activities of glutamine synthetase (GS) and glutamate synthase (GOGAT) in white lupin plant subjected to cadmium (Chaffei et al. 2006). Also, the same work showed that cadmium induces symptoms of senescence in lupin plants. Previous studies showed that white lupin nodules are sensitive to cadmium. In fact, the Cd affected nodules cortex and infected zones and decreased P, K, leghemoglobin, malate and soluble protein in nodules (Carpena et al. 2003; Sánchez-Pardo et al. 2013).

Conclusion

Based on obtained results, we can conclude that *Lupinus albus* L. was affected by high doses of Cd (100 and 150 μM CdCl_2). Cd exposure mainly reduced plant growth parameters and photosynthetic activity. In addition, cadmium generated nutritional deficiencies, toxic accumulation of ammonium, and oxidative damage in plants. Nevertheless, white lupin could survive at high Cd doses and could tolerate moderate Cd doses by the increase of cellular metabolites like soluble sugar and proline to overcome the ionic and osmotic effect of Cd. In addition, to overcome oxidative stress, Cd induced the production of secondary metabolites such as polyphenols, tannin compounds, and flavonoids. Also, the cadmium increased the activities of antioxidant enzymes like CAT and SOD.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary material.

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Authors' contributions: Sabeh M'rah and Ghazoua Boudali., conducted the experimental Work (Culture, analysis), Sabeh M'rah and Hanen Zaier, writing the MS and Chiraz Chaffei-Haouari., is the supervisors of the work and responsible for the conception and the writing-review and editing of the MS.

Conflicts of Interest: The authors declare no conflict of interest.

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Photo

Photo 1 is available in the Supplementary Files section

Figures

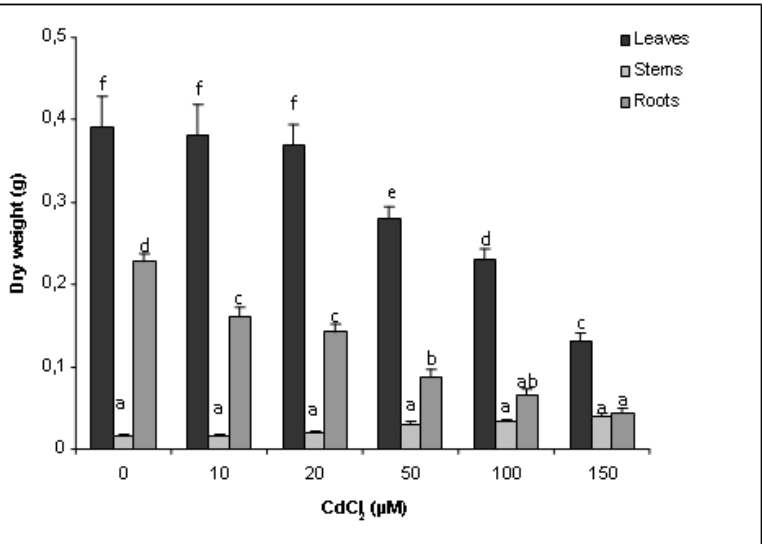


Figure 1

Accumulation of cadmium at the leaves, stems and roots of white lupin according to the cadmium concentration in the medium.

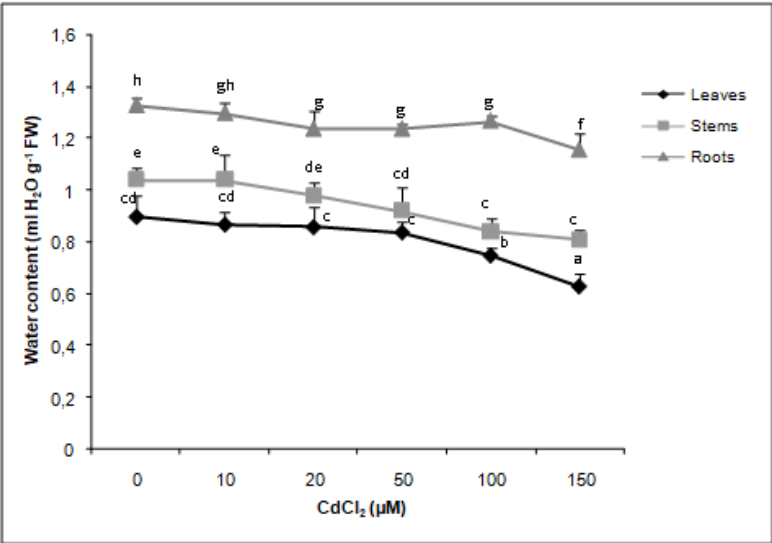


Figure 2

Effect of different cadmium concentrations on roots, stems and leaves dry weight in white lupin seedling. Data are the means of twenty replicates. Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

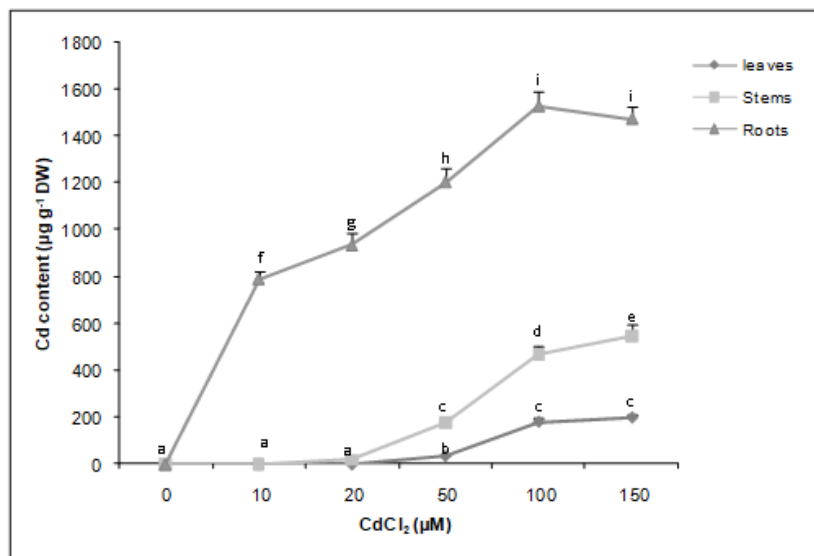


Figure 3

Effect of different cadmium concentrations on roots, stems and leaves water content in lupin seedling. Data are the means of twenty replicates. Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

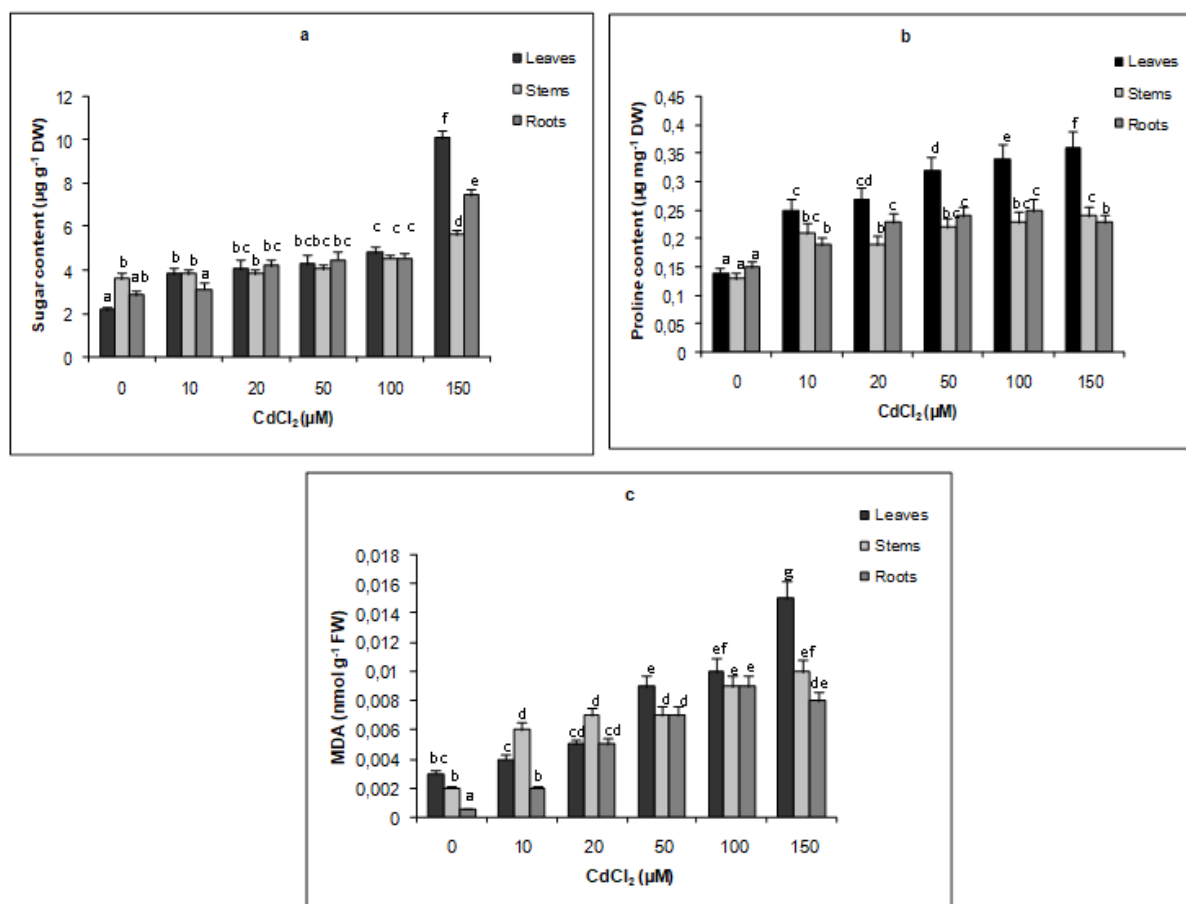


Figure 4

Effect of different cadmium concentrations on metabolites production in white lupin seedling. Total sugar content (a), proline content (b) and MDA content (c). Data are the means of twenty replicates. Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

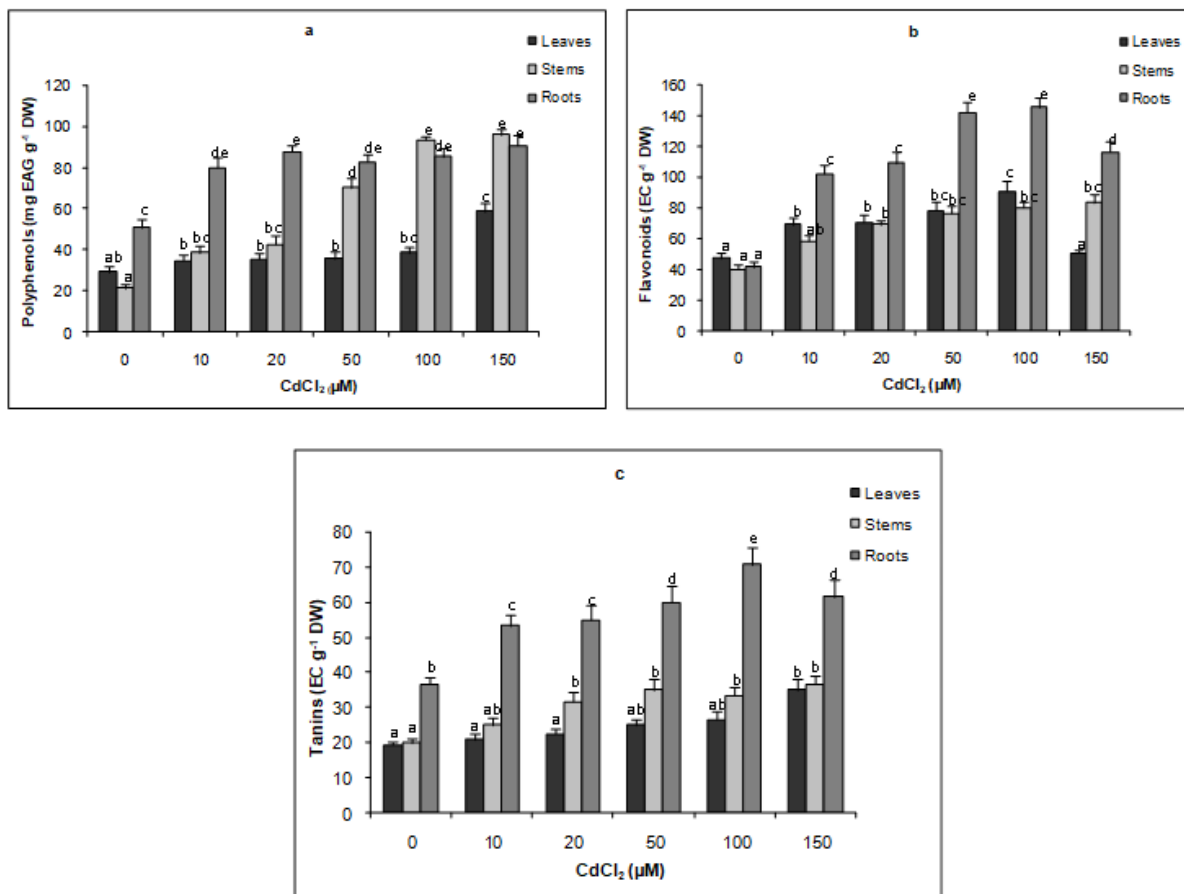


Figure 5

Effect of different cadmium concentrations on antioxidant enzymes activities in white lupin seedling. Catalase activity (a) and superoxide dismutase activity (b). Data are the means of twenty replicates. Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

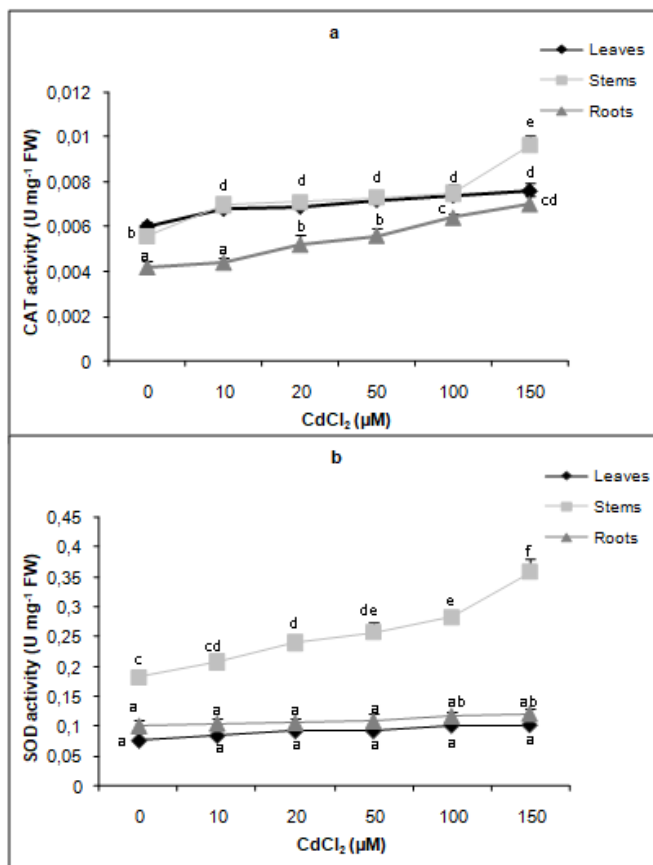


Figure 6

Effect of different cadmium concentrations on antioxidant enzymes activities in 770 white lupin seedlings. Catalase activity (a) and superoxide dismutase activity (b). Data are the means of twenty replicates. Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

Supplementary Files

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- [P1.png](#)