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PHYTOREMEDIATION OF POLLUTED SOILS: RECENT PROGRESS AND DEVELOPMENTS

Physiological and biochemical responses of *Dolichos lablab* L. to cadmium support its potential as a cadmium phytoremediator

Lucas A. Souza[2,3] · **Fernando A. Piotto**[1] · **Manuella N. Dourado**[1] · **Daiana Schmidt**[1] · **Mônica R. Franco**[1] · **Luis F. Boaretto**[1] · **Tiago Tezotto**[4] · **Renato R. Ferreira**[5] · **Ricardo A. Azevedo**[1]

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Abstract
Purpose This study aims to investigate the response of a high biomass producer non-hyperaccumulator legume plant species, *Dolichos lablab* L., to cadmium (Cd) stress for phytoremediation process.
Materials and methods Three individual experiments were carried out to assess physiological and biochemical parameters to support the use of this plant species as a phytoremediator. The first experiment was carried out in Cd-contaminated soil while the second and third experiments were conducted in sand in which Cd was applied to study biochemical responses. Analysis of mineral nutrition, phytoremediation parameters, antioxidant response, and protein identification by gel-based proteomics were performed.
Results and discussion Good tolerance to Cd under moderate level of contamination was observed. Mineral nutrition was little affected, and phytoremediation index was satisfactory. Additionally, biochemical responses based on antioxidant enzyme analysis were well responsive in roots, reflecting the capacity of Cd stress attenuation in this organ. A proteomic analysis revealed positive regulation of root proteins involved in carbohydrate, amino acids, nitrogen metabolism, and abiotic/biotic stress response, which together may contribute to create a scenario to overcome Cd-induced stress.
Conclusions Based on the physiological and biochemical results, we concluded that *D. lablab* L. is suitable for phytoremediation/phytostabilization purposes.

Keywords Antioxidant metabolism · Heavy metal · Oxidative stress · Phytostabilization

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✉ Ricardo A. Azevedo
raa@usp.br

[1] Departamento de Genética, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo-USP, 13400-970 Piracicaba, SP, Brazil

[2] UNICAMP/Instituto de Biologia - Depto. de Biologia Vegetal, CP. 6109, 13083-970 Campinas, SP, Brazil

[3] Instituto Federal Goiano, Rede Arco Norte/Polo de Inovação em Bioenergia e Grãos, Campus Rio Verde, CP. 66, 75901-970 Rio Verde, GO, Brazil

[4] Centro Universitário da Fundação de Ensino Octávio Bastos, Unifeob, 13874-149 São João da Boa Vista, SP, Brazil

[5] CENA, Universidade de São Paulo-USP, 13400-970 Piracicaba, SP, Brazil

1 Introduction

Cadmium (Cd) is an extremely toxic heavy metal that is naturally present in low concentrations in soil (Maksimović et al. 2007; Gratão et al. 2012). High concentrations of this metal have been found in some soils due to human activities such as mining, industrial activities, and increasing use of fertilizers and pesticides (Lux et al. 2011; Andresen and Küpper 2013).

Mechanic and chemical treatments are the most traditional methods for treating heavy metal-contaminated soils; the first removes the soil with heavy equipment, while chemical methods are based on induced chemical reactions in soils making heavy metal unavailable (Yao et al. 2012). However, these treatments may affect soil aggregation or are not efficient to remove completely the heavy metal from the soil, besides that both methods are expensive choices.

Phytoremediation is a promising technique which is based on the plant ability to uptake and accumulate toxic elements in its tissues; however, the "ideal" plant species characteristics for soil phytoremediation is still under debate, although some

basic aspects are known such as high biomass production (Zhu et al. 2012; Souza et al. 2013a).

Most of the available studies targeted hyperaccumulator plant species such as *Noccaea caerulescens* and *Arabidopsis hallery* (Huguet et al. 2012; Lovy et al. 2013). However, these plant species are slow growing and produce small amounts of biomass, making them unfeasible for field application when considering remediation purpose. On the other hand, some crop plant species whose ability to uptake toxic elements are lower than the hyperaccumulator species, have moderate to high tolerance to heavy metals, exhibit rapid growth, and produce large amounts of biomass and therefore are more suitable for phytoremediation studies (Andrade et al. 2008; de Souza et al. 2012b).

The leguminous plant species *Dolichos lablab* L. has appropriate morphological and growth characteristics for phytoremediation, but there is no information about its capacity to tolerate Cd and use in heavy metal phytoremediation. This plant species has already been shown to be tolerant to drought and saline stresses (D'Souza and Devaraj 2010; Younis 2010) and a potential phytoremediator for the herbicide trifloxysulfuron sodium (Procópio et al. 2004). Additionally, *D. lablab* L. is widely used as green manure due to its capability of nitrogen fixation.

The approach of using non-hyperaccumulator plant species that tolerate moderate concentrations of heavy metals is a very interesting strategy mainly when considering crop species that produce high amount of biomass (Bhargava et al. 2012). There are few studies carried out in this context (Andrade et al. 2008; Adhikari and Kumar 2012), especially using leguminous plants.

The potential of *D. lablab* L. for Cd phytoremediation was investigated in this work, and three distinct experiments were performed: the first to determine *D. lablab* L. ability to grow in Cd-contaminated soil, while the second evaluated its antioxidant metabolism response when exposed to Cd and the third to investigate the response in terms of protein changes in roots and leaves using a two-dimensional electrophoresis-based proteomic approach.

Proteomic analysis has been extensively used to investigate protein production patterns under several abiotic stresses (Bona et al. 2011; Arruda et al. 2011) and contributed for the understanding of how plants cope with stressful situations (Farinati et al. 2009; Ahsan et al. 2012; Arruda et al. 2013). This approach has been carried out mainly for hyperaccumulator plant species (Farinati et al. 2009; Zhao et al. 2011), whereas in the case of non-hyperaccumulator plant species, this approach may, for instance, contribute for comprehension of which proteins are being modulated by Cd presence in soil and consequent uptake and translocation in the plant system.

Therefore, the central objective of this study was to assess how *D. lablab* L. responds at biochemical and physiological levels to increasing concentrations of Cd, which may also provide information on its potential use in phytoremediation of Cd-contaminated soils.

2 Materials and methods

2.1 Experiment I

2.1.1 Plant material, soil preparation, and experimental design

Seeds of *D. lablab* L. were provided by "Piraí Sementes". The seeds were surface sterilized in 10 % hypochloride solution for 20 min, washed in running water, immersed in pure water for 60 min, and finally sown in pots filled with Cd-contaminated soil previously prepared as described below.

This experiment was carried out in soil in order to simulate a realistic contaminated environment. The soil in each pot was contaminated with $CdCl_2$ solution and allowed to rest for 15 days for stabilization. A loamy sand soil was used and its chemical characteristics were as follows: pH, 4.5; base saturation (*V*%), 24; organic matter, 2.1 %; H+Al, 47 $mmol_c\ dm^{-3}$; P, 5 mg kg^{-1}; K, 45 mg kg^{-1}; Ca, 200 mg kg^{-1}; Mg, 48 mg kg^{-1}; Cu, 0.6 mg kg^{-1}; Fe, 86 mg kg^{-1}; Mn, 5.8 mg kg^{-1}; and Zn, 2.5 mg kg^{-1}. After application of 0, 5, 10, and 15 mg kg^{-1} of Cd, the final available Cd concentrations were 0.05, 3.3, 7.0, and 14.7 mg kg^{-1}.

The experiment was carried out in a greenhouse in a completely randomized design with four $CdCl_2$ concentrations applied to the soil (0, 5, 10, and 15 mg kg^{-1}) with four biological repetitions. Seeds were planted and 45 days old plants were harvested, washed in running water, and dried with soft paper. Shoots and roots were separated and dried at 60 °C for 72 h.

2.1.2 Growth measurements and mineral analysis

After drying, shoots and roots were weighted for dry mass determination. Shoots and roots were ground and subjected to nitric-perchloric digestion for determination of P, S, Mg, Ca, Fe, Cu, Zn, Mn, and Cd by ICP-OES. K was determined in the same extract in flame photometer equipment. For total N determination, shoots and roots were digested in sulfuric acid followed by quantification by the method of semi-micro Kjeldahl. For determination of Cd concentration in soil, the metal was extracted with a DTPA solution as described by Lindsay and Norvell (1978) and determined in an atomic absorption spectrometer.

2.1.3 Phytoremediation potential evaluation

The phytoremediation potential was evaluated by analyzing tolerance index ($TI=BM_T/BM_C$), translocation index ($TI\ \%=100*Cd_{SH}/Cd_{WP}$) according to Rahman et al. (2013) and transfer factor ($TF=[Cd]_{WP}/[Cd]_S$) according to Lübben and Sauerbeck (1991), where BM_T is total biomass produced in Cd treatments, BM_C is total biomass produced in control treatment, Cd_{SH} is total microgram of Cd in shoots, Cd_{WP} is total microgram of Cd in whole plant, $[Cd]_{WP}$ is Cd concentration in whole plant (mg kg^{-1}), and $[Cd]_S$ is Cd concentration in soil (mg kg^{-1}).

2.1.4 Statistical analysis

Analysis of variance (ANOVA) and Tukey's test at 5 % of significance using the software SISVAR® were performed.

2.2 Experiment II

2.2.1 Plant material and experimental design

This experiment was carried out in sand in order to have clear samples without interference by any type of biological contaminants. For the determination of antioxidant enzyme responses of *D. lablab* L. under increasing concentrations of Cd, a sand-pot experiment in greenhouse was conducted. Seeds were germinated in bioplant®/vermiculite (1:1) substrate. Seedlings were transferred to pots filled with autoclaved sand and received complete Hoagland nutritive solution for 20 days. After this period, 0, 50, 100, and 200 μM $CdCl_2$ (corresponding to 0, 5.6, 11.2, and 22.4 mg L^{-1} Cd) were set and applied in complete Hoagland nutritive solution.

Plants were exposed to the Cd concentrations for 5 days, and complete Hoagland nutritive solution (Hoagland and Arnon 1950) without Cd was applied when necessary to supply nutrients. After 5 days of exposure, the second and third leaves as well as central root parts were collected and immediately frozen in liquid N_2. The samples were stored at −80 °C for further enzyme extraction and analyses. The remaining vegetative tissues were dried at 60 °C for 72 h, weighed, and Cd was quantified.

2.2.2 Stress indicators measurements

Oxidative stress indicators, lipid peroxidation, and hydrogen peroxide, were measured in leaves and roots. Lipid peroxidation was determined as described by Heath and Packer (1968). Hydrogen peroxide was determined as described by Alexieva et al. (2001).

2.2.3 Enzyme extraction and protein quantitation

Leaves and roots were grinded separately in a mortar with a pistil using liquid $\mathbf{N_2}$. The samples were then homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DTT and 5 % (w/v) PVPP. The homogenate was centrifuged at 12000*g* at 4 °C for 30 min. The supernatant was collected in 200 μL aliquots and stored at −80 °C for protein quantitation and enzyme activities. Total extracted protein was determined as described by Bradford (1976) using BSA as a standard.

2.2.4 Enzymes activities

Superoxide dismutase (SOD; EC 1.15.1.1) activity and isoenzymes identification were determined in non-denaturing polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970) followed by gel activity staining as described by Beauchamp and Fridovich (1971) with some modifications (Azevedo et al. 1998). Catalase (CAT; EC 1.11.1.6) activity was determined as described by Kraus et al. (1995) with some modifications (Azevedo et al. 1998). Guaiacol peroxidase (GPOX; EC 1.11.1.7) activity was determined as described by Matsuno and Uritani (1972). Ascorbate peroxidase (APX; 1.11.1.11) activity was determined as described by Nakano and Asada (1981). Glutathione reductase (GR; EC 1.6.4.2) activity was determined as described by Smith et al. (1988).

2.2.5 Statistical analysis

ANOVA and Tukey's test at 5 % of significance using the software SISVAR® were performed.

2.3 Experiment III

2.3.1 Experimental design

This experiment was carried out in sand in order to have clear samples without interference by any type of biological contaminants. Seeds were germinated in substrate containing (1:1) bioplant®/vermiculite. Seedlings were transferred to pots filled with autoclaved sand and received complete Hoagland nutritive solution (Hoagland and Arnon 1950) and at the 35th day the nutrient solution was supplemented with $CdCl_2$ at 100 μM final concentration. The plants were kept in Cd solution for 5 days. At the end of this period of exposure, leaf and root samples were collected and stored at−80 °C for proteomic analysis. Such as described for experiments I and II, the remaining vegetative tissues were dried at 60 °C for Cd determination.

2.3.2 Protein extraction, quantification and two-dimensional gel electrophoresis proteomic analysis

Foliar tissue proteins extraction was carried out with TCA/acetone solution as described by Xu et al. (2008). After precipitation, the pellet was vacuum-dried and 50 mg of dried material was added to a maximum of 1 mL of solubilization solution (7 M urea, 2 M thiourea, 2 % CHAPS and 0.4 % triton X-100). The sample was vigorously stirred using a vortex and kept in ultrasonic bath for 20 min and after 3 cycles of stirring and ultrasonic bath, the material was centrifuged at 12,100×*g* at 4 °C for 20 min and the supernatant was collected for further analysis.

Root tissue protein extraction was carried out with 100 mM Tris buffer (pH 8.5), 5 mM DTT, 1 mM EDTA, and 1 mM PMSF as described by Lee et al. (2011) followed by a precipitation step for 18 h with TCA/acetone. The pellet was washed 3 times in 0.07 % β-mercaptoethanol in pure acetone. Protein solubilization was carried out in a maximum volume of 1 mL of solubilization solution (7 M urea, 2 M thiourea, 2 % CHAPS, and 0.4 % triton X-100). Protein concentration determination was carried out as described by Bradford (1976).

2.3.3 Protein focalization—1st dimension

Isoeletric focusing was performed in 18 cm strips "Immobiline Dry Strip" non-linear pH 4–7 from GE Healthcare Life Sciences. A total amount of 800 and 500 μg of extracted protein from leaves and roots were used, respectively. After 6 h of rehydration, the proteins were subjected to step isoeletric focusing according to the following program: 30 V for 12 h, 100 V for 1 h, 200 V for 1 h, 400 V for 1 h, 700 V for 1 h, 1000 V for 1 h, 5000 V for 10 h, 8000 V for 4 h, and 100 V for 3 h for leaf proteins and 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 5000 V for 8 h, 8000 V for 3:30 h, and 200 V for 2 h for root proteins.

2.3.4 Protein separation by SDS-polyacrylamide gel electrophoresis—2nd dimension

Prior to electrophoretic protein separation, the proteins in the strips were reduced and alkylated as recommended by GE Healthcare (2004). The strips were placed on a 12.5 % polyacrylamide gel and proteins separated at 15 mA per gel for 20 min, and then at 30 mA per gel for 4:30 h. The gels were stained with colloidal Coomassie Blue G-250 as recommended by GE Healthcare (2004).

2.3.5 Gel image analysis

The gels were scanned in an ImageScanner using the software LabScan™ v 5.0 (Amershan Bioscience). The spots were detected by the software ImageMaster 2D Platinum 7.0 and the parameters contrast, smoothness, saliency, and minimal area were adjusted for automatic spot detection.

2.3.6 Protein sequencing

The selected spots were excised from the gels and processed for mass spectrometry analysis according to the procedures described by Shevchenko et al. (2007). Digested proteins were submitted to MALDI TOF-TOF MS/MS analysis for protein identification. The results were compared by BLAST against virideplantae NCBI databank through the software MASCOT®.

2.3.7 Statistical analysis

The analysis of the spots was carried out using the software ImageMaster 2D Platinum 7.0, followed by ANOVA and Student's *t* test considering $p<0.05$.

3 Results

3.1 Cadmium effects on mineral nutritional I: macronutrients

The shoot concentrations of P, S, K, and Ca were not affected by Cd presence, while the concentrations of N and Mg increased with the increase in Cd concentration in the soil (Table 1). In roots, Cd did not influence the concentrations of N, P, K, and S, but the concentration of Mg decreased as the concentration of Cd in soil increased, while Ca concentration exhibited the opposite pattern (Table 1).

3.2 Cadmium effects on mineral nutritional II: micronutrients

The concentrations of Fe and Mn were not affected by Cd in shoots, but the concentrations of Cu and Zn decreased as the concentration of Cd increased in the treatments (Table 2). In roots, the concentrations of Cu, Fe, Zn, and Mn increased with the increase in Cd concentration in the soil, with a slight reduction in the concentrations of these elements being observed only at the highest concentration of Cd used (Table 2).

3.3 Cadmium effects on growth and phytoremediation potential of *D. lablab* L

The growth and biomass accumulation of *D. lablab* L. decreased linearly according to the increase of Cd concentration in the soil (Table 3). Based on the root/shoot ratio, shoots were more sensitive to Cd than roots (Table 3). The highest Cd concentration used drastically decreased biomass yield

Table 1 Macronutrients concentration (g kg^{-1}) in shoots and roots of *Dolichos lablab* L. under increasing concentrations of cadmium

Cd (mg kg^{-1})	N	P	K	S	Mg	Ca
Shoots						
0	30.25 b	2.45 a	30.25 a	2.40 a	3.82 b	21.24 a
5	34.50 ab	2.67 a	30.88 a	2.94 a	4.03 b	19.18 a
10	37.95 a	2.84 a	30.57 a	3.38 a	4.57 ab	20.61 a
15	39.94 a	2.66 a	28.36 a	3.56 a	5.62 a	19.52 a
Roots						
0	25.36 a	2.74 a	26.08 a	7.14 a	7.46 a	7.31 b
5	26.58 a	2.65 a	24.89 a	7.82 a	4.71 b	8.02 ab
10	27.42 a	2.93 a	24.10 a	7.37 a	4.34 b	9.56 a
15	29.11 a	2.69 a	20.79 a	7.21 a	3.27 b	9.63 a

Means (n=4) with the same letter are not significantly different by Tukey's test at 5 %

(around 75 %), while the lowest Cd concentration used led to a smaller biomass reduction (around 40 %) (Table 3). Cd was mainly accumulated in the roots and the accumulation increased linearly in both, shoots (Fig. 1a) and roots (Fig. 1b), according to the increment of Cd available in the soil. The soil-plant transfer factor (TF), translocation index (TI %), and tolerance index (TI) were higher for the lowest Cd concentration used (Table 4).

3.4 Oxidative stress indicators

There was no increase in hydrogen peroxide content in both leaves and roots in all treatments (Fig. 2a). Lipid peroxidation was higher in the 200 μM Cd treatment in both leaves and roots (Fig. 2b).

Table 2 Micronutrients concentration (mg kg^{-1}) in shoots and roots of *Dolichos lablab* L. under increasing concentrations of cadmium

Cd (mg kg^{-1})	Cu	Fe	Zn	Mn
Shoots				
0	5.0 a	106.68 a	43.34 ab	10.48 a
5	4.5 ab	85.68 a	48.87 a	9.94 a
10	3.5 bc	109.80 a	45.46 ab	12.59 a
15	3.2 c	105.06 a	40.34 b	11.90 a
Roots				
0	8.63 b	2463 c	60.13 b	21.76 b
5	9.77 ab	3551 bc	66.89 b	24.07 b
10	12.53 a	5281 a	88.80 a	43.68 a
15	11.33 ab	4431 ab	75.00 ab	33.04 ab

Means (n=4) with the same letter are not significantly different by Tukey's test at 5 %

Table 3 Growth measurements of *Dolichos lablab* L. under increasing concentrations of cadmium

Cd (mg kg^{-1})	Shoot dry mass (g)	Root dry mass (g)	Root/shoot ratio	Total biomass (g)
0	3.63 a	0.62 a	0.17 b	4.25 a
5	2.08 ab	0.42 ab	0.20 b	2.49 ab
10	1.18 b	0.32 ab	0.27 a	1.51 b
15	0.86 b	0.23 b	0.28 a	1.04 b

Means (n=4) with the same letter are not significantly different by Tukey's test at 5 %

3.5 SOD isoenzymes and activity

Five SOD isoenzymes were observed in leaves (Fig. 3a) and six in roots (Fig. 3b). In leaves, total SOD activity or differential SOD isoenzyme activity patterns showed higher activity under 100 μM Cd, while in roots Cd did not affect SOD activity. In roots, the most active SOD isoenzyme was Cu/Zn-SOD.

3.6 Cadmium effects on CAT, GPOX, APX, and GR

In leaves, CAT, APX, and GPOX activities increased in response to increased Cd concentrations (Fig. 4). CAT and APX exhibited maximum activity when exposed to 100 μM Cd (Fig. 4a, c). In a similar manner to leaves, root APX activity increased under Cd stress, while GPOX activity decreased (Fig. 4c, b). APX and GPOX activities were higher in roots than in leaves, whereas CAT activity was higher in leaves than in roots. When GR activity is concerned, no differences in activity between both tissues and Cd treatments were observed (Fig. 4d).

3.7 Differentially expressed proteins under Cd stress identified by MALDI TOF-TOF MS/MS

Identified proteins, in both roots and shoot, were categorized in nine different classes: cellular signaling, carbohydrate metabolism, amino acids metabolism, secondary metabolism, energetic metabolism, abiotic and biotic stress response, protein metabolism, antioxidant metabolism, and photosynthetic metabolism, according to the function retrieved from uniprot (Table 5). Differentially expressed proteins under Cd stress (100 μM) in both, root and shoot, were distributed in almost all protein classes, except for secondary metabolism and protein metabolism classes which presented only differentially expressed protein from roots and photosynthetic metabolism class which presented only differentially expressed protein in leaves (Table 5).

The majority differentially expressed proteins from roots belonged to cellular signaling, carbohydrate metabolism,

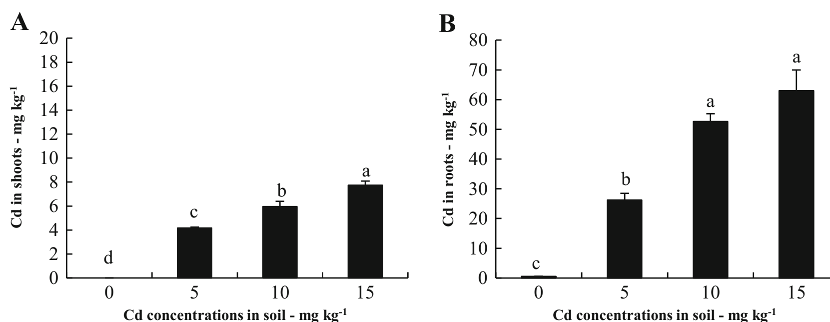

Fig. 1 Cadmium (Cd) concentration in shoots (**a**) and in roots (**b**) of *Dolichos lablab* L. under increasing concentrations of cadmium. Means (n=4) with the same letter are not significantly different by Tukey's test at 5 %

amino acids metabolism, secondary metabolism and protein metabolism classes, while those from shoot belonged to energetic metabolism, abiotic and biotic stress response, antioxidative metabolism, and photosynthetic metabolism classes (Table 5).

The same proteins were not found to be differentially expressed in both, leaves and roots, therefore each organ presented unique regulation of their proteins. In leaves, photosynthetic metabolism proteins were down regulated as well as proteins from amino acids metabolism (Table 5). However, the opposite was observed in proteins from carbohydrate metabolism which were upregulated in the presence of Cd. Moreover, proteins belonging to classes directly related to stress such as antioxidant metabolism and abiotic and biotic stress response were upregulated in the presence of Cd in leaves (Table 5). In roots, proteins belonging to abiotic and biotic stress response were shown to be upregulated in presence of Cd (Table 5).

4 Discussion

4.1 Potential of cadmium phytoremediation of *D. lablab* L

The increase in industrialization and excessive use of fertilizers contributes to increased concentration and availability of several heavy metals in the environment (Monteiro et al. 2011). Cadmium, as a highly toxic heavy metal, impairs plant growth, which may ultimately lead to plant death (Gallego et al. 2012; Azevedo et al. 2012; Gratão et al. 2015).

In the present study, the observation that macronutrients were not affected in *D. lablab* L. under increasing concentrations of Cd (Table 1) is a characteristic that is not normally observed in other plant species. Most authors have reported contrasting effects caused by Cd on the nutritional status. Tezotto et al. (2012) found that Cd decreased P content in leaves of coffee plants, although they did not observe growth reduction, while Vernay et al. (2007) reported that excess of chromium (Cr) affected Ca and Mg content in *Lolium perene* L., which was accompanied by growth reduction. It is therefore acceptable that excess of Cd may lead to impairments in macro- and micronutrient minerals and any situation disturbing their absorption reflects negatively on plant growth capability (Maathuis 2009). The results obtained in this study do not only support that the response is plant species specific, showing the importance to study each metal-plant interaction, but also suggesting that *D. lablab* L. is able to keep normal concentrations of most macronutrients under Cd-stressful situation, at least under the conditions tested in this study, in order to support its growth.

Additionally, elements that have specific roles in decontamination and energetic metabolism, such as S and Mg (Arruda et al. 2015), whose concentrations increased under Cd exposure may indicate a strategy to keep the metabolism active in a growing attempt, which may cause a dilution effect by biomass increase as observed in *Calopogonium mucunoides* (de Souza et al. 2012a). Elements whose concentration decreased under Cd exposure may be explained by inhibition or competition between Cd and other metallic nutrients for carrier proteins as observed by Bertoli et al. (2012), who observed that K levels decreased in tomato plants under increasing concentrations of Cd. As Cd slightly affected only the content of K and Mg in roots of *D. lablab* L. (Table 1), we propose that the homeostasis of these elements in this plant species was not kept only in the roots, since the translocation of these elements to the above-ground parts of the plants was not affected.

Micronutrients are also of extreme importance for cellular metabolic maintenance, mainly because they are involved in redox reactions (Hänsch and Mendel 2009). Plants have

Table 4 Phytoremediation indicator parameters for *Dolichos lablab* L. under increasing concentrations of cadmium

Cd (mg kg^{-1})	Transfer factor	Translocation index (%)	Tolerance index
0	–	–	–
5	2.44 a	44.23 a	0.60 a
10	2.28 a	29.18 b	0.40 b
15	1.37 b	30.70 b	0.27 c

Means (n=4) with the same letter are not significantly different by Tukey's test at 5 %

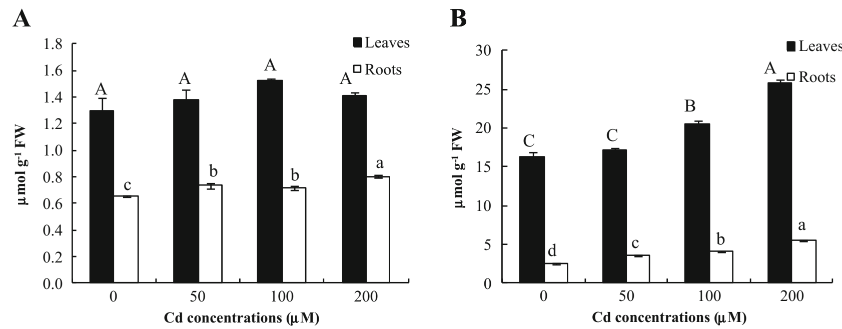

Fig. 2 Hydrogen peroxide concentration (**a**) and malondialdehyde concentration (**b**) in leaves and roots of *Dolichos lablab* L. under increasing concentrations of cadmium (Cd). Upper cases compare the means of leaves, and lower cases compare the means of roots. Means (*n*=4) with the same letter are not significantly different by Tukey's test at 5 %

different ways to respond in relation to absorption, transport and accumulation of micronutrients under heavy metal exposure. As demonstrated by Safarzadeh et al. (2013), rice plants subjected to Cd exposure resulted in diminished concentrations of Zn, Cu, Mn and Fe, and due to the essentiality of these elements, quantities below the optimum might have impact on plant development. For instance, similar results were found by Rezvani et al. (2012) in *Aeluropus littoralis*, a graminae plant species. Nevertheless, that is not the case for *D. lablab* L. whose data obtained were different revealing increment of micronutrients in the roots, whereas in the leaves no deficiency was observed (Table 2), suggesting that the nutritional status was not affected (Tables 1 and 2). Therefore, it appears that in *D. lablab* L. Cd toxicity does not have an apparent effect on macro or micronutrient acquisition or translocation, possibly pointing out to a kind of tolerance mechanism. Cadmium is generally related to growth decrease in plants (Konotop et al. 2012) especially at high concentrations (Azevedo et al. 2012), and a certain degree of growth reduction was observed in *D. lablab* L. (Table 3), nevertheless, such an effect cannot be explained or related to an impairment in mineral nutrient uptake or translocation.

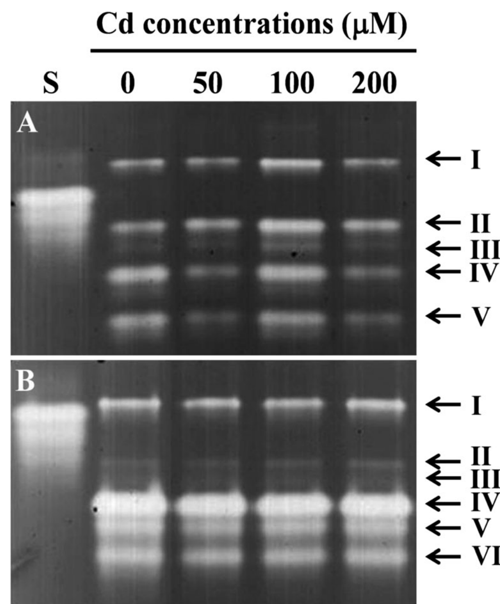

Fig. 3 Superoxide dismutase activity and biochemical characterization in leaves (**a**) and roots (**b**) under increasing cadmium (Cd) concentrations. **a** *I*, Mn-SOD; *II and III*, Fe-SOD; *IV and V*, Cu/Zn-SOD; **b** *I*, Mn-SOD; *II and III*, Fe-SOD; *IV–VI*, Cu/Zn-SOD. *S* standard bovine SOD

Some authors reported growth reduction of at least 90 % with Cd concentrations similar to those used in this study (López-Millán et al. 2009; Hediji et al. 2010). Although we have observed some growth reduction at the lowest Cd concentration (5 mg kg^{-1}) (Table 3), *D. lablab* L. was able to maintain 60 % of the growth when compared with the control (Table 4). Moreover, this concentration has been considered toxic for several plants species, for instance, for tomato plants (Dourado et al. 2014). An important aspect to be taken into account is a possible root tolerance, which can be observed by the obtained Cd root:shoot ratio (Table 3), which was similar to the control and also the amount of Cd accumulated in the roots. The higher Cd tolerance exhibited by *D. lablab* L. is important to support the use of this plant species for phytostabilization of areas with forthcoming risk of contamination, as suggested by Göhre and Paszkowski (2006), and this root tolerance may be related to a root barrier, which prevents the accumulation of excessive metals in edible aerial parts (Disla et al. 2014). As phytostabilization prevents heavy metal spreading in soil as well as leakage, it is very plausible this use for diminishing risk of contamination of uncontaminated surrounding areas.

Based on the results obtained and on the concept of Reeves and Baker (2000) in which a Cd hyperaccumulator species should accumulate in its aerial parts at least 0.01 % of its dry mass as Cd, *D. lablab* L. cannot be classified as hyperaccumulator. However, we propose the use of this plant species for phytostabilization, based on the amount of Cd accumulated in roots (Fig. 1), the high TF observed for the lowest Cd concentration (Table 4) as suggested by Kabata-Pendias and Pendias (2001), and also on the high root/shoot ratio observed at the lowest Cd concentration used (Table 3). Therefore, due to the high biomass produced by *D. lablab* L.

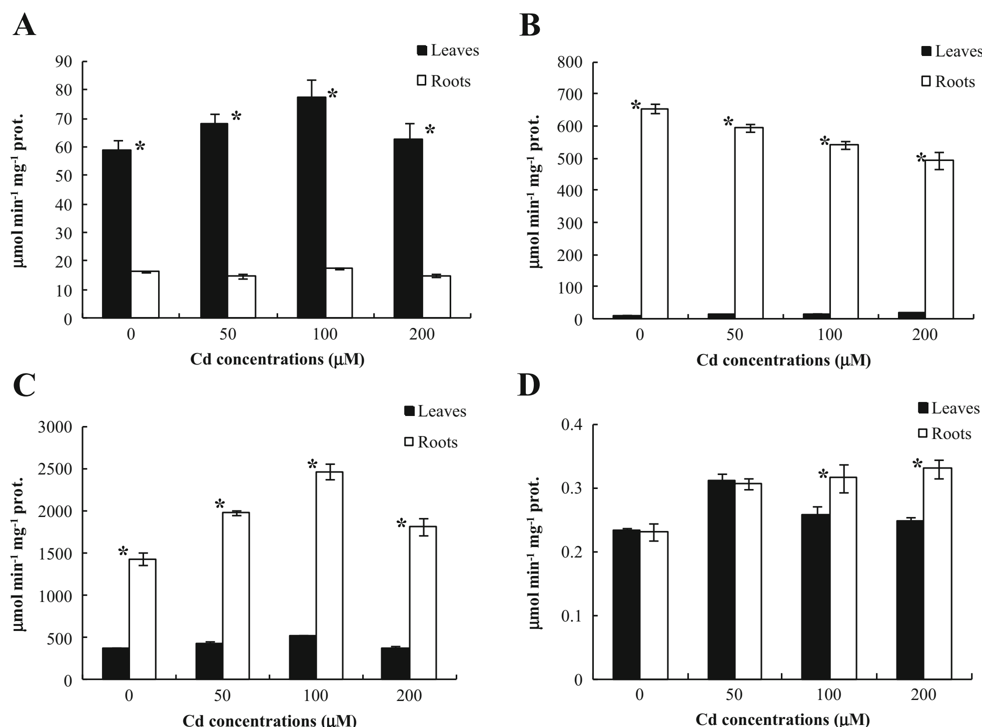

Fig. 4 Activity of the antioxidant enzymes CAT (**a**), GPOX (**b**), APX (**c**), and GR (**d**) of *Dolichos lablab* L. under increasing concentrations of cadmium (Cd). *Asterisk* indicates significant difference between means of leaf and root activity under the same Cd concentration by Tukey's test at 5 %. $n=4$

we reinforce the idea of using non-hyperccumulator plants as phytoremediators (Souza et al. 2013b) once all parameters used to support our idea allowed us to conclude that root growth was less affected by Cd, which can be an important aspect to overcome Cd stress and successfully be established as a phytoremediator.

4.2 Antioxidative response of *D. lablab* L. to cadmium

The ability of *D. lablab* L. to grow in the presence of different Cd concentrations suggested some degree of Cd tolerance, which did not influence nutrient balance (Tables 1 and 2) despite of plant growth reduction at the highest Cd concentration (Table 3). A reported mechanism involved in Cd tolerance is the maintenance of the redox balance in the cell, mediated by the enzymes of the antioxidant pathway or non-enzymatic antioxidant compounds (Gratão et al. 2005; Fidalgo et al. 2011). The SOD enzyme is involved in superoxide dismutation and the different SOD isoenzymes are located in different cell compartments (Azevedo et al. 1998), therefore responding differently depending on the plant species, genotype, organ, and cell location (Gonçalves et al. 2009).

Our results revealed the presence of up to six distinct SOD isoenzymes in the leaves and roots classified as Mn-SOD, Fe-SOD, and Cu/Zn-SOD (Fig. 3). Total SOD activity, based on the sum of SOD band intensities, was clearly higher in roots than in leaves, nonetheless, Cd exposure did not result in any major or significant change in band intensity or band distribution/presence pattern, except in leaves at 100 µM of Cd, which exhibited a slight increase (Fig. 3a, b). Yet, it is clear that the majority of the SOD activity observed in this plant species is due to Cu/Zn-SOD and also Mn-SOD activities when roots are concerned. The potential higher SOD activity already encountered in *D. lablab* L. independent of stress still have to be challenged in future studies possibly with higher Cd concentrations and also by testing other metals so that a better understanding of SOD activity thresholds and responses are determined for this plant species. Moreover, adaptive responses that can take place (Gratão et al. 2008a) cannot be ruled out and should be taken into account for *D. lablab* L.

Hydrogen peroxide is produced by the cell metabolism, including as a result of SOD activity, which can then interact with metallic ions to produce hydroxyl radicals, which is highly active and responsible for most of the cellular damage (Apel and Hirt 2004; Kim et al. 2008). Therefore, an efficient hydrogen peroxide scavenging system is required by the cells and the enzymes responsible for these processes are peroxidases (PODs) (Jouili et al. 2011). The results obtained for *D. lablab* L. suggest that the PODs tested strongly act on hydrogen peroxide degradation, besides presenting differential response between plant tissues (Fig. 4a–c). Several stressing factors such UV-radiation, ozone exposure, drought, senescence, and heavy metals lead to an increase in enzymatic activity of PODs (Gratão et al. 2008b; Cruz et al. 2013). The heavy metal-induced oxidative stress on POD activities have already been reported by some authors (Andrade et al. 2010; Dominguez et al. 2010; Pereira et al. 2011; Remans et al. 2012), which clearly indicated that the enzymes responses

Table 5 Identification and analysis of differentially expressed proteins in roots and leaves of *Dolichos lablab* under Cd stress

Spot	Protein	Organ	Organism	Access number	Score	MP[a]	CS[b]	Ratio[c]
Cellular signaling								
1	Thaumatin-like protein	Root	*Phaseolus vulgaris*	P83959.1	101	2	76	2.41
101	Auxin-induced protein	Root	*Medicago truncatula*	CBI34180.3	64	13	35	0.35
47	Phosphatidylinositol 4-phosphate 5-kinase	Leaf	*Arabidopsis lyrata*	XP_002891093.1	76	13	11	0.36
Carbohydrate metabolism								
9	Triose phosphate isomerase	Root	*Glycine max*	C6T7V6	90	13	43	1.55
70	Triose phosphate isomerase	Root	*G. max*	ABA86966 .1	109	14	50	1.65
83	Gliceraldehyde-3-phosphate dehydrogenase	Root	*G. max*	ABC75834.1	87	7	29	1.74
84	Citosolic gliceraldehyde-3-phosphate dehydrogenase	Root	*Antirrhinum majus*	G3PC_ANTMA	85	8	30	2.22
22	Enolase	Root	*G. max*	AAS18240	209	15	34	0.50
24	Phosphoglucomutase	Root	*G. max*	XP_003525238.1	78	11	13	0.35
43	UDP-glucose pirophosphorilase	Leaf	*Amorpha fruticosa*	AAL33919.1	68	11	23	3.16
Amino acids metabolism								
34	Methionine synthase	Root	*Cicer arietinum*	ACL14488.1	103	15	22	1.78
44	Cysteine synthase	Root	*Ricinus communis*	XP_002517133	76	7	28	0.45
104	Citosolic glutamine synthetase	Root	*G. max*	AAG24873.1	229	16	37	0.33
76	Plastide glutamine synthetase	Leaf	*Vigna radiata*	ADK27329.1	134	10	26	0.37
Secondary metabolism								
50	Cynnamoil CoA reductase	Root	*Corymbia citriodora*	ABQ95552.1	100	9	47	2.13
55	Isoflavone reductase	Root	*Vitis vinifera*	CAI56335.1	85	5	25	2.22
Energetic metabolism								
52	Isocitrate dehydrogenase-$NADP^+$	Root	*Passiflora edulis*	BAG84436.1	83	23	47	1.64
139	Quinone oxidoreductase	Leaf	*G. max*	ACU19769.1	82	13	54	0.34
Abiotic and biotic stress response								
120	Chitinase	Root	*M. truncatula*	CAA71402.1	88	6	18	3.09
7	Heat shock protein	Leaf	*Pisum sativum*	AAA33637.1	301	13	18	2.08
146	Lectin—galactose/*N*-acetilgalactosamine	Leaf	*Lablab purpureus*	ABM92662.2	67	4	14	1.77
Protein metabolism								
14	Acil-peptide hydrolase	Root	*M. truncatula*	XP_003600065	124	9	21	0.35
Antioxidative metabolism								
130	Monodehydroascorbate reductase	Root	*V. vinifera*	XP_002278648.1	80	14	30	0.37
225	Cytochrome P450	Leaf	*Triticum monococcum*	AAS58486.1	61	11	18	2.87
Photosynthetic metabolism								
186	Ribulose bisphosphate carboxilase—small chain	Leaf	*R. communis*	XP_002532149.1	139	15	37	0.44
190	Oxygen-evolving enhancer protein 2	Leaf	*Brassica juncea*	Q96334.1	71	5	23	0.5

[a] Matched peptides

[b] Covered sequence

[c] Treatment/control

are tissue and/or organism specific. Yu et al. (2013) reported significant increase in several antioxidant enzymes, including CAT, corroborating with our results that the excess of hydrogen peroxide is scavenged by different PODs from the antioxidant pathway. Hydrogen peroxide scavenging can also occur by the activity of APX and GPOX, as observed in this study in the root tissue (Fig. 4b, c), frequently being much more effective than CAT (Caverzan et al. 2012). APX appears to be very effective in eliminating hydrogen peroxide in roots, which can be explained by its *Km* (Mittler 2002), as well as the amount of isoenzymes and substrate available (Mittler and Poulos 2007).

Nadgorska-Socha et al. (2013) observed an increase in GPOX activity in leaves under high concentrations of Cd, a response also observed in the present study, suggesting that this pattern of response in leaves may be normal once it was also observed in other reports for other plant species (Monteiro et al. 2011; Roychoudhury et al. 2012). On the

other hand, the response of this enzyme was the opposite in roots, in which we observed a decrease in its activity (Fig. 4b), indicating that Cd can influence the response of the same enzyme differentially in each tissue. Another fact that could explain this decrease in GPOX activity in roots could be the high activity of APX in this tissue (Fig. 4c). Wang et al. (2008) working with *Thlaspi caerulescens* and *Brassica juncea* subjected to Cd stress observed a decreased CAT activity with simultaneous increase in the activity of generic PODs.

As already mentioned, the APX enzyme is very effective in hydrogen peroxide elimination, but APX is also important for the integration of the ascorbate-glutathione cycle (Suzuki et al. 2012; Baxter et al. 2014). Thus, ascorbate needs to be constantly regenerated and it mainly depends on GR activity to regenerates reduced glutathione (GSH), the electron donor for dehydroascorbate reduction (Mittler 2002; Zhao et al. 2009). GR is strongly induced under several environmental stresses (Opdenakker et al. 2012) due to its key role in keeping cellular redox homeostasis, which corroborates the suggestion that *D. lablab* L. can be considered a Cd-tolerant species in low to moderate Cd-contamination environment due to the relative good stability of GR activity (Fig. 4d).

The inter-relationship between the activities of APX and GR is clearly noticeable once an increase in GR activity was observed in roots (Fig. 4d), which probably is related to the high demand for ascorbate. In accordance with our results, Smeets et al. (2005) observed that the ascorbate-glutathione cycle efficiency is the main mechanism against Cd-induced oxidative stress in beans. Additionally, Li et al. (2013) confirmed in *Pistia estratiotes* L. that POD and SOD activities were increased under Cd stress. So, these results are in agreement with our findings and suggest that once the balance of hydrogen peroxide production in the ascorbate-glutathione cycle is altered, other PODs rapidly respond to keep the hydrogen peroxide content lower enough to avoid this molecule to induce free radicals formation that ultimately cause oxidative stress.

It is clear that enzymes from the antioxidant pathways respond differently and the decrease in activity of one type of POD may lead to an increase in the activity of another related enzyme, acting as a compensatory mechanism as reviewed by Gratão et al. (2005). Such responses may obviously differ in different organs as observed in *D. lablab* L. for CAT and APX activities between roots and leaves. Since high activities of enzymes of this pathway can be related to a better growth performance under stressful situations (Siddiqui et al. 2013), it is possible to infer that the observed behavior serves as an indicative of *D. lablab* L. versatility to cope with Cd exposure.

The pattern of response observed in roots (Fig. 4), clearly differed from that observed for leaves, which might be explained by the fact that this organ is the first to get in contact with the metal, therefore requiring a fast and effective response. In this way, we can assume that the high efficiency of the root tissue in keeping low the level of ROS such as hydrogen peroxide and superoxide is intimately related to *D. lablab* L. tolerance under Cd stress. Although we did not measure all ROS, the effect of Cd on growth, MDA, hydrogen peroxide, and enzyme activities, do allow us to argue that *D. lablab* L. root system appears to be able to face and deal with any excess of ROS eventually produced by the Cd treatment, at least under the conditions tested in this study.

4.3 Proteomic changes of *D. lablab* L. under Cd exposure

The two-dimensional proteomic-based approach was applied to elucidate which proteins were regulated by 100 μM Cd exposure in *D. lablab* L. Only recently proteomic studies have been carried out for plants under heavy metal stress and significantly contributed for the understanding of metabolic pathways that are affected, as well as identification of proteins related to tolerance (Farinati et al. 2009; Sharmin et al. 2012; Wang et al. 2012). This approach has been used mostly for hyperaccumulator plant species (Ingle et al. 2005; Tuomainen et al. 2006; Farinati et al. 2009; Walliwalagedara et al. 2010; Zhao et al. 2011), however, heavy metal-tolerant non-hyperaccumulator plant species do need to be characterized with the goal of understanding their potential as phytoremediators.

An important shift in metabolic pathways has been observed in *D. lablab* L. In roots, we could detect the upregulation and downregulation of four and two proteins, respectively, related to carbohydrate metabolism. The upregulation of enzymes involved in carbohydrate metabolism seems to be a common feature as indicated by Zhao et al. (2011) and Wang et al. (2012) in studies with Cd and Ni hyperaccumulator species. The isoenzymes of triose phosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase from roots (Table 5) exhibited more than 50 % increase in their abundance. As these two enzymes are part of the glycolytic pathway, it seems reasonable to conclude that the root is using carbon to generate energy to defend itself from Cd stress. As our work was not with a hyperaccumulator species, it may be also possible to suggest that the metabolic shift may be shared between hyperaccumulators and non-hyperaccumulators, and it is interesting because it indicates that metabolic versatility under stressful situations may integrate different metabolic pathways, such amino acid metabolism, once methionine synthase was also upregulated.

In the amino acid metabolism class, the enzyme glutamine synthetase was downregulated in both roots and leaves (Table 5) indicating that Cd directly affects N assimilation, resulting in growth disturbances in plant in contaminated soils, especially because the product of this enzyme serve as substrate for synthesis of tolerance compound, such as glutathione. However, Hradilova et al. (2010) reported an increase

in glutamine synthetase, which the authors correlated with increased tolerance of flax plants to Cd.

Tolerance of *D. lablab* L. to Cd appears to be closely related to carbohydrate and amino acids metabolism considering that molecules from primary metabolism are being converged to the energy produced by the Krebs' cycle in an attempt to overcome the stressful situation.

The upregulation of isocitrate dehydrogenase in roots under Cd stress suggests that this enzyme increased in abundance as a plant response to supply substrate for the synthesis of compounds that may confer tolerance, such as organic acids that are used as organic chelators to attenuate metal toxicity. Yet, an opposite view was reported by Sánchez-Pardo et al. (2013) who observed a 22 % decrease in abundance of this enzyme, suggesting that it may lead to drastic effects on nitrogen assimilation, as this enzyme provides α-ketoglutarate for ammonium incorporation in the GS/GOGAT pathway. For *D. lablab* L., it may not favor nitrogen assimilation, since a decrease in abundance of both isoenzymes of glutamine synthetase was observed, which may explain the effects of Cd on growth disturbance.

What is particularly interesting is the link between primary metabolism with the secondary metabolism, as the latter is not involved with energy supply, but several metabolites from primary metabolism are substrate for the synthesis of secondary metabolites, and increases in two enzymes from the secondary metabolism such cynnamoil CoA reductase and isoflavone reductase were found (Table 5).

It is known that Cd also influences some proteins from the secondary metabolism, which responded with upregulation in roots in this study and was also reported by He et al. (2011). One of the upregulated enzymes was cynnamoil CoA reductase, an enzyme involved in lignin biosynthesis, which suggests possible Cd interference in auxin metabolism, leading to higher lignification and cell shortening (Elobeid et al. 2012). Lignification is a common response under biotic and abiotic stress. It the case of biotic stresses, it appears that lignification acts as a physical barrier to biological invasions, while when abiotic stresses are concerned, Podazza et al. (2012) studying Cd response in *Citrus* concluded that the increase in lignifications is responsible for increase in Cd immobilization in root cell walls. The fact that the responses to both biotic and abiotic stresses have a point of convergence, agrees with the data obtained, which showed the increase in proteins related to both types of stresses such as chitinase in roots and heat shock proteins (HSP) and lectin in the leaves (Table 5).

Some HSPs are known as important environmental stress biomarkers (Bierkens 2000). Such as observed in this work, Basile et al. (2015) also reported increased expression of HSP in *Lemna minor* under stress by Cd and other metals, which confirms the role of this chaperone protein as a biomarker. In relation to lectin and chitinase, it is difficult to determine their roles under Cd stress since these two proteins are involved in biotic stress responses, but we may suggest that the signaling pathway may be similar in response for both biotic and abiotic stress (Smékalová et al. 2014; Prasch and Sonnewald 2015).

In leaves, despite of the upregulation of carbohydrate and amino acid metabolic proteins, photosynthesis appears to be impaired since it was observed downregulation of proteins involved in the photosynthetic process, such as the small chain of RubisCO and oxygen-evolving enhancer protein (Table 5). It is expected the decrease in photosynthesis under Cd stress as observed by Zancheta et al. (2015) studying response of sorghum and jack-bean to Cd, and even in Cd-tolerant plants, such as sunflower, the effect is the same as observed by Lopes et al. (2015) that studied Cd effects on proteins using a proteomics approach. Additionally, Cd also impairs chlorophyll content, which is directed related to photosynthesis as observed in tomato plants (Dourado et al. 2013).

Cd appears to induce a shift in the metabolism and these changes are converged to upregulation of stress response proteins and at some extent to downregulation of proteins involved in growth. This behavior is well supported under stressful situation in which organisms tend to direct their resources to overcome the stress while growth remains stunted (Prasch and Sonnewald 2015).

5 Conclusions

The approaches used in this research allowed us to support the hypothesis that non-hyperaccumulator plant species can be used as phytoremediators. Based on the minor alterations in nutritional status, the high efficiency of the enzymatic antioxidant response and positive regulation of root proteins involved in carbohydrate, amino acids, and nitrogen metabolism, a positive scenario to overcome Cd-induced stress is established, making *D. lablab* L. a good candidate for phytostabilization purposes.

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References

Adhikari T, Kumar A (2012) Phytoaccumulation and tolerance of *Riccinus communis* L. to nickel. Int J Phytorem 14:481–492

Ahsan N, Nakamura T, Komatsu S (2012) Differential responses of microsomal proteins and metabolites in two contrasting cadmium

(Cd)-accumulating soybean cultivars under Cd stress. Amino Acids 42:317–327

Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24:1337–1344

Andrade SAL, Silveira APD, Jorge RA, Abreu MF (2008) Cadmium accumulation in sunflower plants influenced by arbuscular mycorrhiza. Int J Phytorem 10:1–14

Andrade SAL, Gratão PL, Azevedo RA, Silveira APD, Schiavinato MA, Mazzafera P (2010) Biochemical and physiological changes in jack bean under mycorrhizal symbiosis growing in soil with increasing Cu concentrations. Environ Exp Bot 68:198–207

Andresen E, Küpper H (2013) Cadmium toxicity in plants. In: Sigel A, Sigel H, Sigel RKO (eds) Cadmium: from toxicity to essentiality—metal ions in life sciences 11. Springer, pp. 395–413

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399

Arruda SCC, Barbosa HS, Azevedo RA, Arruda MAZ (2011) Two-dimensional difference gel electrophoresis applied for analytical proteomics: fundamentals and applications to the study of plant proteomics. Analyst 136:4119–4126

Arruda MAZ, Azevedo RA, Barbosa HS, Mataveli LRV, Oliveira SR, Arruda SCC, Gratão PL (2013) Comparative studies involving transgenic and non-transgenic soybean: what is going on? In: Board JE (ed) A comprehensive survey of international soybean research—genetics, physiology, agronomy and nitrogen relationships. Intech, Rijeka, Croatia, pp 583–613

Arruda SCC, Silva ALD, Galazzi RM, Azevedo RA, Arruda MAZ (2015) Nanoparticles applied to plant science: a review. Talanta 131:693–705

Azevedo RA, Alas RM, Smith RJ, Lea PJ (1998) Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. Physiol Plant 104:280–292

Azevedo RA, Gratão PL, Monteiro CC, Carvalho RF (2012) What is new in the research on cadmium-induced stress in plants? Food Energ Sec 1:133–140

Basile A, Sorbo S, Cardi M, Lentini M, Castiglia D, Cianciullo P, Conte B, Loppi S, Esposito S (2015) Effects of heavy metals on ultrastructure and Hsp70 induction in *Lemna minor* L. exposed to water along the Sarno River, Italy. Ecotox Environ Safe 114:93–101

Baxter A, Mittler R, Suzuki N (2014) ROS as key players in plant stress signalling. J Exp Bot 65:1229–1240

Beauchamp CH, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287

Bertoli AC, Cannata MG, Carvalho R, Bastos ARR, Freitas MP, Augusto AS (2012) *Lycopersicon esculentum* submitted to Cd-stressful conditions in nutrition solution: nutrient contents and translocation. Ecotox Environ Safe 86:176–181

Bhargava A, Carmona FF, Bhargava M, Srivastava S (2012) Approaches for enhanced phytoextraction of heavy metals. J Environ Manag 105:103–120

Bierkens JGEA (2000) Applications and pitfalls of stress-proteins in biomonitoring. Toxicology 153:61–72

Bona E, Marsano F, Massa N, Cattaneo C, Cesaro P, Argese E, di Toppi LS, Cavaletto M, Berta G (2011) Proteomic analysis as a tool for investigating arsenic stress in *Pteris vittata* roots colonized or not by arbuscular mycorrhizal symbiosis. J Proteomics 74:1338–1350

Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 72:248–254

Caverzan A, Passaia G, Rosa SB, Ribeiro CW, Lazzarotto F, Margis-Pinheiro M (2012) Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. Genet Mol Biol 35:1011–1019

Cruz FJR, Castro GLS, Silva Junior DD, Festucci-Buselli RA, Pinheiro HA (2013) Exogenous glycine betaine modulates ascorbate peroxidase and catalase activities and prevent lipid peroxidation in mild water-stressed *Carapa guianensis* plants. Photosynthetica 51:102–108

D'Souza MR, Devaraj VR (2010) Biochemical responses of hyacinth bean (*Lablab purpureus*) to salinity stress. Acta Physiol Plant 32:341–353

de Souza LA, de Andrade SAL, de Souza SCR, Schiavinato MA (2012a) Arbuscular mycorrhiza confers Pb tolerance in *Calopogonium mucunoides*. Acta Physiol Plant 34:523–531

de Souza SCR, de Andrade SAL, de Souza LA, Schiavinato MA (2012b) Lead tolerance and phytoremediation potential of Brazilian leguminous tree species at the seedling stage. J Environl Manag 110:299–307

Disla JMS, Gómez I, Pedreño JN, Jordán M (2014) The transfer of heavy metals to barley plants from soils amended with sewage sludge with different heavy metal burdens. J Soils Sedim 14:687–696

Dominguez DM, Garcia FC, Raya AC, Santiago RT (2010) Cadmium-induced oxidative stress and the response of the antioxidative defense system in *Spartina densiflora*. Physiol Plant 139:289–302

Dourado MN, Martins PF, Quecine MC, Piotto FA, Souza LA, Franco MR, Tezotto T, Azevedo RA (2013) *Burkholderia* sp. SCMS54 reduces cadmium toxicity and promotes growth in tomato. Ann Appl Biol 163:494–507

Dourado MN, Souza LA, Martins PF, Peters LP, Piotto FA, Azevedo RA (2014) *Burkholderia* sp. SCMS54 triggers a global stress defense in tomato enhancing cadmium tolerance. Water Air Soil Pollut 225:2159

Elobeid M, Goebel C, Feussner I, Polle A (2012) Cadmium interferes with auxin physiology and lignification in poplar. J Exp Bot 63:1413–1421

Farinati S, DalCorso G, Bona E, Corbella M, Lampis S, Cecconi D, Polati R, Berta G, Vallini G, Furini A (2009) Proteomic analysis of *Arabidopsis halleri* shoots in response to the heavy metals cadmium and zinc and rhizosphere microorganisms. Proteomics 9:4837–4850

Fidalgo F, Freitas R, Ferreira R, Pessoa A, Teixeira J (2011) *Solanum nigrum* L. antioxidant defence system isoenzymes are regulated transcriptionally and posttranslationally in Cd-induced stress. Environ Exp Bot 72:312–319

Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, Zawoznik MS, Groppa MD, Benavides MP (2012) Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. Environ Exp Bot 83:33–46

GE Healthcare (2004) 2-D electrophoresis: principles and methods

Göhre V, Paszkowski U (2006) Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. Planta 223:1115–1122

Gonçalves J, Tabaldi L, Cargnelutti D, Pereira L, Maldaner J, Becker A, Rossato L, Rauber R, Bagatini M, Bisognin D, Schetinger M, Nicoloso F (2009) Cadmium-induced oxidative stress in two potato cultivars. Biometals 22:779–792

Gratão PL, Polle A, Lea PJ, Azevedo RA (2005) Making the life of heavy metal-stressed plants a little easier. Funct Plant Biol 32:481–494

Gratão PL, Monteiro CC, Antunes AM, Peres LEP, Azevedo RA (2008a) Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress. Ann Appl Biol 153:321–333

Gratão PL, Pompeu GB, Capaldi FR, Vitorello VA, Lea PJ, Azevedo RA (2008b) Antioxidant response of *Nicotiana tabacum* cv. bright yellow 2 cells to cadmium and nickel stress. Plant Cell Tiss Org Cult 94:73–83

Gratão PL, Monteiro CC, Carvalho RF, Tezotto T, Piotto FA, Peres LEP, Azevedo RA (2012) Biochemical dissection of diageotropica and never ripe tomato mutants to Cd-stressful conditions. Plant Physiol Biochem 56:79–96

Gratão PL, Monteiro CC, Tezotto T, Carvalho RF, Alves LR, Peters LP, Azevedo RA (2015) Cadmium stress antioxidant responses and root-to-shoot communication in grafted tomato plants. Biometals 28:803–816

Hänsch R, Mendel RR (2009) Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Curr Opin Plant Biol 12:259–266

He J, Qin J, Long L, Ma Y, Li H, Li K, Jiang X, Liu T, Polle A, Liang Z, Luo ZB (2011) Net cadmium flux and accumulation reveal tissue-specific oxidative stress and detoxification in *Populus x canescens*. Physiol Plant 143:50–63

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Hediji H, Djebali W, Cabasson C, Maucourt M, Baldet P, Bertrand A, Zoghlami LB, Deborde C, Moing A, Brouquisse R, Chaibi W, Gallusci P (2010) Effects of long-term cadmium exposure on growth and metabolomic profile of tomato plants. Ecotox Environ Safe 73:1965–1974

Hoagland DR, Arnon DI (1950) The water culture method for growing plants without soil. Calif Agric Exp Sta Bull 347

Hradilova J, Rehulka P, Rehulkova H, Vrbova M, Griga M, Brzobohaty B (2010) Comparative analysis of proteomic changes in contrasting flax cultivars upon cadmium exposure. Electrophoresis 31:421–431

Huguet S, Bert V, Laboudigue A, Barthes V, Marie-Pierre I, Llorens I, Schat H, Sarret G (2012) Cd speciation and localization in the hyperaccumulator *Arabidopsis halleri*. Environ Exp Bot 82:54–65

Ingle R, Smith JAC, Sweetlove L (2005) Responses to nickel in the proteome of the hyperaccumulator plant *Alyssum lesbiacum*. Biometals 18:627–641

Jouili H, Bouazizi H, El Ferjani E (2011) Plant peroxidases: biomarkers of metallic stress. Acta Physiol Plant 33:2075–2082

Kabata-Pendias A, Pendias H (2001) Trace elements in soil and plants, 3rd edn. CRC Press, Boca Raton

Kim C, Meskauskiene R, Apel K, Laloi C (2008) No single way to understand singlet oxygen signalling in plants. Embo Rep 9:435–439

Konotop Y, Meszaros P, Spiess N, Mistrikova V, Pirselova B, Libantova J, Moravcikova J, Taran N, Hauptvogel P, Matusikova I (2012) Defense responses of soybean roots during exposure to cadmium, excess of nitrogen supply and combinations of these stressors. Mol Biol Rep 39:10077–10087

Kraus TE, McKersie BD, Fletcher RA (1995) Paclobutrazol-induced tolerance of wheat leaves to paraquat may involve increased antioxidant enzyme activity. J Plant Physiol 145:570–576

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lee J, Jiang W, Qiao YL, Cho YI, Woo MO, Chin JH, Kwon SW, Hong SS, Choi IY, Koh HJ (2011) Shotgun proteomic analysis for detecting differentially expressed proteins in the reduced culm number rice. Proteomics 11:455–468

Li Y, Zhang S, Jiang W, Liu D (2013) Cadmium accumulation, activities of antioxidant enzymes, and malondialdehyde (MDA) content in *Pistia stratiotes* L. Environ Sci Pollut Res 20:1117–1123

Lindsay WL, Norvell WA (1978) Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Sci Soc Am J 42:421–428

Lopes CA, Barbosa HS, Galazzi RM, Koolen HHF, Gozzo FC, Arruda MAZ (2015) Evaluation of proteome alterations induced by cadmium stress in sunflower (*Helianthus annuus* L.) cultures. Ecotox Environ Safe 119:170–177

López-Millán AF, Sagardoy R, Solanas M, Abadía A, Abadía J (2009) Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics. Environ Exp Bot 65:376–385

Lovy L, Latt D, Sterckeman T (2013) Cadmium uptake and partitioning in the hyperaccumulator *Noccaea caerulescens* exposed to constant Cd concentrations throughout complete growth cycles. Plant Soil 362:345–354

Lübben S, Sauerbeck D (1991) The uptake and distribution of heavy metals by spring wheat. Water Air Soil Pollut 57–58:239–247

Lux A, Martinka M, Vaculík M, White PJ (2011) Root response to cadmium in the rizosphere: a review. J Exp Bot 62:21–37

Maathuis FJM (2009) Physiological functions of mineral macronutrients. Curr Opin Plant Biol 12:250–258

Maksimović I, Kastori R, Krstić L, Luković J (2007) Steady presence of cadmium and nickel affects root anatomy, accumulation and distribution of essential ions in maize seedlings. Biol Plant 51:589–592

Matsuno H, Uritani I (1972) Physiological behavior of peroxidase isoenzymes in sweet potato root tissue injured by cutting or with black root. Plant Cell Physiol 13:1091–1101

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Mittler R, Poulos TL (2007) Ascorbate peroxidase. In: Smirnoff N (ed) Antioxidants and reactive oxygen species in plants. Blackwell Publishing Ltd, pp. 87–100

Monteiro CC, Carvalho RF, Gratão PL, Carvalho G, Tezotto T, Medici LO, Peres LEP, Azevedo RA (2011) Biochemical responses of the ethylene-insensitive *Never ripe* tomato mutant subjected to cadmium and sodium stresses. Environ Exp Bot 71:306–320

Nadgorska-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. Environ Sci Pollut Res 20:1124–1134

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-especific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Opdenakker K, Remans T, Keunen E, Vangronsveld J, Cuypers A (2012) Exposure of *Arabidopsis thaliana* to Cd or Cu excess leads to oxidative stress mediated alterations in MAPKinase transcript levels. Environ Exp Bot 83:53–61

Pereira LB, Mazzanti CMA, Cargnelutti D, Rossato LV, Gonçalves JF, Calgaroto N, Dressler V, Nicoloso FT, Federizzi LC, Morsch VM, Schetinger MRC (2011) Differential responses of oat genotypes: oxidative stress provoked by aluminum. Biometals 24:73–83

Podazza G, Arias M, Prado FE (2012) Cadmium accumulation and strategies to avoid its toxicity in roots of the citrus rootstock Citrumelo. J Hazard Mater 215:83–89

Prasch CM, Sonnewald U (2015) Signaling events in plants: Stress factors in combination change the picture. Environ Exp Bot 114:4–14

Procópio SO, Santos JB, Silva AA, Pires FR, Ribeiro Júnior JI, Santos EA, Ferreira LR (2004) Seleção de plantas com potencial para fitorremediação de solos contaminados com o herbicida trifloxysulfuron sodium. Planta Daninha 22:315–322

Rahman MM, Azirun SM, Boyce AN (2013) Enhanced accumulation of copper and lead in Amaranth (*Amaranthus paniculatus*), Indian Mustard (*Brassica juncea*) and Sunflower (*Helianthus annuus*). PLoS One 8(5):e62941

Reeves RD, Baker AJM (2000) Metal-accumulating plants. In: Phytoremediation of toxic metals: using plants to clean up the environment., pp 193–230

Remans T, Opdenakker K, Guisez Y, Carleer R, Schat H, Vangronsveld J, Cuypers A (2012) Exposure of *Arabidopsis thaliana* to excess Zn reveals a Zn-specific oxidative stress signature. Environ Exp Bot 84:61–71

Rezvani M, Zaefarian F, Miransari M, Nematzadeh GA (2012) Uptake and translocation of cadmium and nutrients by *Aeluropus littoralis*. Arch Agron Soil Sci 58:1413–1425

Roychoudhury A, Basu S, Sengupta DN (2012) Antioxidants and stress-related metabolites in the seedlings of two indica rice varieties exposed to cadmium chloride toxicity. Acta Physiol Plant 34:835–847

Safarzadeh S, Ronaghi A, Karimian N (2013) Effect of cadmium toxicity on micronutrient concentration, uptake and partitioning in seven rice cultivars. Arch Agron Soil Sci 59:231–245

Sánchez-Pardo B, Carpena RO, Zornoza P (2013) Cadmium in white lupin nodules: Impact on nitrogen and carbon metabolism. J Plant Physiol 170:265–271

Sharmin SA, Alam I, Kim KH, Kim YG, Kim PJ, Bahk JD, Lee BH (2012) Chromium-induced physiological and proteomic alterations in roots of *Miscanthus sinensis*. Plant Sci 187:113–126

Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2007) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1:2856–2860

Siddiqui MH, Al-Whaibi MH, Sakran AM, Ali HM, Basalah MO, Faisal M, Alatar A, Al-Amri AA (2013) Calcium-induced amelioration of boron toxicity in radish. J Plant Growth Regul 32:61–71

Smeets K, Cuypers A, Lambrechts A, Semane B, Hoet P, Van Laere A, Vangronsveld J (2005) Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd application. Plant Physiol Biochem 43:437–444

Smékalová V, Doskočilová A, Komis G, Šamaj J (2014) Crosstalk between secondary messengers, hormones and MAPK modules during abiotic stress signalling in plants. Biotechnol Adv 32:2–11

Smith IK, Vierheller TL, Thorne CA (1988) Assay of glutathione reductase in crude tissue homogenates using 5,5′-dithilbis (2-nitrobenzoic acid). Anal Biochem 175:408–413

Souza LA, Andrade SAL, Souza SCR, Schiavinato MA (2013a) Evaluation of mycorrhizal influence on the development and phytoremediation potential of *Canavalia gladiata* in Pb-contaminated soils. Int J Phytorem 15:465–476

Souza LA, Piotto FA, Nogueirol RC, Azevedo RA (2013b) Use of non-hyperaccumulator plant species for the phytoextraction of heavy metals using chelating agents. Sci Agric 70:290–295

Suzuki N, Koussevitzky S, Mittler R, Miller G (2012) ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ 35:259–270

Tezotto T, Favarin JL, Azevedo RA, Alleoni LRF, Mazzafera P (2012) Coffee is highly tolerant to cadmium, nickel and zinc: plant and soil nutritional status, metal distribution and bean yield. Field Crop Res 125:25–34

Tuomainen MH, Nunan N, Lehesranta SJ, Tervahauta AI, Hassinen VH, Schat H, Koistinen KM, Auriola S, McNicol J, Karenlampi SO (2006) Multivariate analysis of protein profiles of metal hyperaccumulator *Thlaspi caerulescens* accessions. Proteomics 6: 3696–3706

Vernay P, Gauthier-Moussard C, Hitmi A (2007) Interaction of bioaccumulation of heavy metal chromium with water relation, mineral nutrition and photosynthesis in developed leaves of *Lolium perenne* L. Chemosphere 68:1563–1575

Walliwalagedara C, Atkinson I, van Keulen H, Cutright T, Wei R (2010) Differential expression of proteins induced by lead in the dwarf sunflower *Helianthus annuus*. Phytochemistry 71:1460–1465

Wang Z, Zhang Y, Huang Z, Huang L (2008) Antioxidative response of metal-accumulator and non-accumulator plants under cadmium stress. Plant Soil 310:137–149

Wang Y, Hu H, Zhu LY, Li XX (2012) Response to nickel in the proteome of the metal accumulator plant *Brassica juncea*. J Plant Interact 7: 230–237

Xu C, Xu Y, Huang B (2008) Protein extraction for two-dimensional gel electrophoresis of proteomic profiling in turfgrass. Crop Sci 48: 1608–1614

Yao Z, Li J, Xie H, Yu C (2012) Review on remediation technologies of soil contaminated by heavy metals. Procedia Environ Sci 16:722–729

Younis M (2010) Response of *Lablab purpureus* (L.) sweet/rhizobium symbiosis and growth to potassium supply under different water regimes. J Plant Nutr 33:1400–1409

Yu F, Liu K, Li M, Zhou Z, Deng H, Chen B (2013) Effects of cadmium on enzymatic and non-enzymatic antioxidative defences of rice (*Oriza sativa* L.). Int J Phytorem 15:513–521

Zancheta ACF, Abreu CA, Zambrosi FCB, Erismann NM, Lagôa AMMA (2015) Cadmium accumulation by jack-bean and sorghum in hydroponic culture. Int J Phytorem 17:298–303

Zhao FY, Liu W, Zhang SY (2009) Different responses of plant growth and antioxidant system to the combination of cadmium and heat stress in transgenic and non-transgenic rice. J Integr Plant Biol 51: 942–950

Zhao L, Sun YL, Cui SX, Chen M, Yang HM, Liu HM, Chai TY, Huang F (2011) Cd-induced changes in leaf proteome of the hyperaccumulator plant *Phytolacca americana*. Chemosphere 85: 56–66

Zhu Y, Bi D, Yuan L, Yin X (2012) Phytoremediation of cadmium and copper contaminated soils. In: Yin X, Yuan L (eds) Phytoremediation and Biofortification: two sides of one coin. Springer Briefs in Molecular Science. Springer, New York, NY, USA, pp 75–81