



# Early responses to cadmium exposure in barley plants: effects on biometric and physiological parameters

Marco Lentini<sup>1</sup> · Alessia De Lillo<sup>1</sup> · Valeria Paradisone<sup>1</sup> · Daniela Liberti<sup>1</sup> · Simone Landi<sup>1</sup> · Sergio Esposito<sup>1</sup>

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## Abstract

Cadmium represents one of the most toxic pollutants in plant ecosystems: at high concentrations it can cause severe effects, such as plant growth inhibition, decrease in photosynthesis and changes in plant basal metabolism. Changes in pigments' content, RubisCO large subunit, and D1 protein indicated a severe reduction in photosynthetic efficiency. Furthermore, the decrease of nitrate reductase activity and changes in free amino acids levels show a general stress condition of nitrogen assimilation. Cadmium increased the activities of ROS scavenging enzymes; among these, ascorbate peroxidase rate was the most noticeably increased. It is worth noting that glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.64), showed changes in both activities and occurrence during cadmium stress. Interestingly, our data suggest that G6PDH would modulate redox homeostasis under metal exposure, and possibly satisfy the increased request of reductants to counteract the oxidative burst induced by cadmium. Therefore, the results suggest that APX and G6PDH may play a pivotal role to counteract the oxidative stress induced by cadmium in young barley plants.

**Keywords** Nitrate reductase · G6PDH · Amino acid levels · Heat shock proteins · APX · RWC

## Introduction

In recent years, heavy metal accumulation in soils has become a critical constraint for crop cultivation. This pollution is caused by industrial activities, irrigation using wastewater, excess of pesticides, herbicides, phosphate fertilizers, and other chemicals (Yousaf et al. 2016; Akhtar et al. 2017; Kintlova et al. 2017).

Among heavy metals, cadmium (Cd) is present worldwide in soils both from natural sources and anthropic pollution

(Choppala et al. 2014; Akhtar et al. 2017). Cd does not play any biological function and exhibits high toxicity even at low concentrations, disturbing many aspects of plant physiology, by reducing total biomass, fresh and dry weight, root length and leaf size, in both hydroponics and soil cultivation (Yousaf et al. 2016; Akhtar et al. 2017; Rizwan et al. 2017).

It has been widely shown that Cd stress affects vegetables' quality and production: potato (*Solanum tuberosum*) exposed to Cd exhibited smaller tubers; tomato (*Solanum lycopersicum*) showed a significant reduction in chlorophylls and carotenoids, affecting the plants' photosynthetic capability and resulting in no fruit production in the presence of 100 µM Cd (Rizwan et al. 2017).

Cd uptake decreases photosynthesis, conductance and water use efficiency (Rizwan et al. 2017; Zemanova et al. 2017). The reduction in chlorophyll and carotenoids' contents was reported in various crops, as well as different changes in metabolite concentrations, including significant modification in amino acids and organic acids (Rizwan et al. 2017; Zemanova et al. 2017).

Cd induces severe oxidative stress by increasing levels of reactive oxygen species (ROS), such as superoxide anion radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals ( $\cdot OH$ ) (Sandalio et al. 2001). Therefore, the

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Marco Lentini and Alessia De Lillo equally participated in this manuscript, therefore they must be considered as joint first authors.

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✉ Sergio Esposito  
sergio.esposito@unina.it

<sup>1</sup> Dipartimento di Biologia, Università di Napoli "Federico II", Via Cinthia, 80126 Naples, Italy

detoxification of ROS by scavenging enzymes plays a critical role (Gill and Tuteja 2010; Landi et al. 2017). Cadmium exposure in plants enhanced the activities of enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase and guaiacol peroxidase (GPX) (Leon et al. 2002). On the other hand, enzymes such as peroxidase (POD) and ascorbate peroxidase (APX) play diversified roles depending on species or even varieties (Gill and Tuteja 2010; Rizwan et al. 2017). Particularly, the ascorbate–glutathione cycle plays a pivotal role, maintaining an adequate photosynthetic rate even under metal stress (Gill and Tuteja 2010). Particularly, APX occurrence, activities and expression were stimulated in crops upon heavy metal stress, drought, flooding and salinity, highlighting the crucial role of APX as ROS scavenger (Gill and Tuteja 2010; Iovieno et al. 2016; Landi et al. 2017).

More specifically, a critical role in those response mechanisms triggered during oxidative burst has been defined in different crops for glucose-6-phosphate dehydrogenase (G6PDH—EC 1.1.1.49) (Yang et al. 2014; Landi et al. 2016). The reducing power produced by G6PDH activity as NADPH plays a critical role in plant physiology participating in biosynthetic pathways (e.g. ribose for nucleotides biosynthesis) (Castiglia et al. 2015; Esposito 2016); and nitrogen assimilation (Esposito et al. 2005); furthermore, these reductants are required by the ROS scavenging systems under abiotic stress (Cardi et al. 2011, 2015; Dal Santo et al. 2012; Landi et al. 2016).

Barley (*Hordeum vulgare*) represents a primary resource for food and forage demand (Landi et al. 2017), and is the fourth most important cereal crop worldwide (Shen et al. 2016). Furthermore, barley is an important cereal model, presenting genomics and bioinformatics resources including complete genome sequence, transcriptome under various environmental stresses, and several online databases (The International Barley Genome Sequencing Consortium 2011; Kintlova et al. 2017). Among cereals, barley shows a natural abiotic stress tolerance: namely, cultivated barley (*H. vulgare*) retains 40% alleles compared with the original barley progenitor (*Hordeum spontaneum*—Ellis et al. 2000). This wild ancestor showed remarkable tolerance to salt, drought and heavy metal stress, but breeding programs have produced high-yielding barley cultivars more sensitive to abiotic stress, making this aspect a critical issue in barley as well (Shen et al. 2016; Landi et al. 2017).

The aim of this work is explore the common responses of barley plants exposed to cadmium and elucidate the possible role(s) of G6PDH and other enzymes (ascorbate peroxidase—APX; and nitrate reductase—NR) under heavy metal stress.

To this purpose, barley plants were exposed to the presence of toxic levels of Cd, and enzymatic activities, HSP70s, levels of pigments, photosynthetic efficiency,

photosynthesis-related proteins, and free amino acids were determined. The possible mechanisms of Cd response in barley will be finally discussed.

## Materials and methods

### Plant material and stress treatments

Seeds of barley (*H. vulgare*, var. Nure), were supplied by “Centro di ricerca per la genomica e la postgenomica animale e vegetale” (CRA-GPG—Fiorenzuola D’Arda—PC, Italy). Seeds were germinated for 5 days in the dark on moistened paper, then seedlings were grown in hydroponic solution in darkened plastic bottles at 20 °C, at 60–80% relative humidity, under 16 h light/8 h dark regime, with approximately 180  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The composition of the medium (modified Hoagland solution), continuously bubbled with air, has been previously described (Cardi et al. 2015).

After 10 days of hydroponic culture, plants were divided into two groups: control plants were maintained in the standard growth medium; Cd-stressed plants were grown in the presence of  $10^{-5}$  M of  $\text{CdCl}_2$  added to the standard medium. Nutrient solutions were controlled for pH and daily adjusted. Leaves and roots from each group of barley plants were collected at 0 h, 6 h, 24 h, 48 h and 7 days after the stress induction.

### Growth variation and water content determination

Changes in length of barley leaves and roots exposed to Cd were measured at the end of the experimental design on five randomly chosen plants. To measure the relative water content (RWC), 15–20 plants were weighted from Cd exposed and control groups soon after hydroponic growth to determine FW. The plants were hydrated for 3–4 h by either floating in a Petri dish in distilled water and weighed to determine the turgid weight (TW); then samples were dried overnight at 70 °C for the calculation of the dried weight (DW). The plant RWC was derived from the formula (Matin et al. 1989):

$$\text{RWC \%} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100.$$

### Photosynthetic parameters’ measurements

Photosynthetic parameters: maximal photochemical efficiency (Fv/Fm); photosystem II quantum yield (QY); electron transport rate (ETR) and non-photochemical quenching (NPQ) were determined using a portable PAR-FluorPen FP 100-MAX-LM fluorimeter equipped with a light sensor (Photon System Instruments, Czech Republic) on young

barley plants exposed to 7 days to 10–5 M Cd using the default settings, as previously described (Arena et al. 2017).

### Chlorophyll and carotenoids' content

Chlorophyll and carotenoids were assayed using the method reported by Hu et al. (2013). Samples of barley leaves (500 mg) were suspended in 1.5 ml of *N,N'*-dimethylformamide (Sigma-Aldrich) and incubated for 4 h at 4 °C in the dark. Pigment levels were calculated by measuring absorbance at 664, 647 and 480 nm, using optical glass cuvettes (OG-6040 Hellma) in a Cary 60 spectrophotometer (Agilent Technologies, USA) according to Moran (1982).

### Proline content

Proline content was determined using the method of Clausen (2005). Finely ground leaf and root tissue (250 mg) was suspended in 1.5 ml of 3% sulphosalicylic acid, and filtered through a layer of glass-fiber filter (Macherey–Nagel, Ø 55 mm, Germany). 1 ml of glacial acetic acid and 1 ml ninhydrin reagent (2.5 g ninhydrin/100 ml of a 6:3:1 solution of glacial acetic acid, distilled water and 85% ortho-phosphoric acid, respectively) were added to 1 ml of the clear filtrate. After incubation at 100 °C for 1 h, optical density of samples was read at 546 nm in a Cary 60 spectrophotometer (Agilent Technologies, USA).

### Glucose-6-phosphate dehydrogenase assay

G6PDH was extracted by grinding 300 mg of barley leaves and roots. Samples were mechanical homogenized (TissueLyser, Quiagen) at 50 Hz for 2 min (5 times) using stainless steel beads (5 mM) under ice-cold conditions in 600 µl of extraction solution containing 50 mM Tris–HCl at pH 8.0, 5 mM MgCl<sub>2</sub>, 4 mM EDTA, 10% glycerol, 15 µM NADP<sup>+</sup> 10 µl of Protease Inhibitor Cocktail (Sigma P9599) were added for ml of extraction solution.

G6PDH activity was assayed as described previously (Castiglia et al. 2015), by monitoring NADP<sup>+</sup> reduction at 340 nm using a Cary 60 spectrophotometer (Agilent Technologies, USA). The assay mixture contained: 50 mM Tris–HCl pH 8.0, 50 mM MgCl<sub>2</sub>, 1.5 mM NADP<sup>+</sup>, 30 mM glucose-6P, and extract (10–100 µl). Activity was expressed as nmol reduced NADP<sup>+</sup> min<sup>-1</sup> mg<sup>-1</sup> protein.

### Western blotting

For Western blotting analysis, proteins were extracted as described previously and separated by SDS-PAGE (Cardi et al. 2011). Then, polypeptides were transferred onto a Hybond nitrocellulose membrane (GE Healthcare). The filter was incubated with primary antibodies vs G6PDH (Cyt,

P1 and P2) (Wendt et al. 2000; Cardi et al. 2015; Castiglia et al. 2015), HSP70 (Cyt, Chloroplasic and mitochondrial—Agrisera), D1 and RuBisCO large subunit (Agrisera). After incubation of the membrane with secondary antibodies, cross-reacting polypeptides were identified by enhanced chemiluminescence using the ECL Prime kit (GE Healthcare). Images were acquired by BioRad Chemidoc system (BioRad, USA).

### Ascorbate peroxidase assay

APX activity was assayed by monitoring the ascorbate oxidation at 290 nm, using a Cary 60 spectrophotometer (Agilent Technologies, USA). APX was extracted from 300 mg of leaf and root tissue. Samples were mechanical homogenized (TissueLyser, Quiagen) at 50 Hz for 2 min (5 times) using stainless steel beads (5 mM) under ice-cold conditions in 600 µl of extraction solution containing 50 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.0), 2% PVPP, 0.1 mM EDTA, 2 mM. The reaction mixture contained 30 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM Na-ascorbate and 0.06% H<sub>2</sub>O<sub>2</sub> (Nakano and Asada 1981).

### Nitrate reductase assay

Nitrate reductase (NR) was extracted from leaf and root tissue (1 g) mechanical homogenized (TissueLyser, Quiagen) at 50 Hz for 2 min (5 times) using stainless steel beads (5 mM) under ice-cold conditions in 2 ml of solution containing 25 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.2); 1 mM cystein; 25 µM FAD.

The assay mixture contained 25 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.2); 1 mM benzyl-viologen; 0.2 M KNO<sub>3</sub>; sodium dithionite (8 mg ml<sup>-1</sup> in 25 mM NaH<sub>2</sub>PO<sub>4</sub>); and 200 µl of extract. The reaction mixture was incubated for 20 min at 30 °C, then a solution containing barium acetate 0.15 M, ethanol 95% was added to stop the enzymatic reaction. The tubes were then vigorously shaken, centrifuged; nitrite formed by NR was estimated colorimetrically at 540 nm in a Cary 60 spectrophotometer (Agilent Technologies, USA) with the addition of a mixture containing 0.67 ml sulphanilamide (4% in 3 N HCl) and 0.33 ml N (1-naphthyl) ethylenediamine dihydrochloride (0.08% in water) (Vona et al. 2004). Nitrate reductase activity was expressed as nmol nitrite produced min<sup>-1</sup> mg<sup>-1</sup> protein.

### Amino acids' determination by high-performance liquid chromatography (HPLC)

Soluble amino acids were extracted in 0.85 ml of 80% ethanol for 15 min at 4 °C, and then centrifuged (Eppendorf 5415D). The supernatant was filtered using Waters Sep-Pak C18 Light Cartridges. An aliquot (50 µl) of the extract was

derivatized for 1 min with *orto*-phthaldialdehyde (*o*PA) and separated by HPLC for amino acid analysis. Chromatographic equipment was Agilent 110 HPLC series. The *o*PA derivatives were separated on a reverse-phase C18 ultraspere column (250 mm×4.6 mm). Solvent A consisted of 50 mM Na-Acetate (pH7), 1% tetrahydrofuran, and solvent B was absolute methanol (Carlo Erba). A sample (20  $\mu$ l) of the mixture was injected and eluted at a flow rate of 1 ml min<sup>-1</sup>. The eluted *o*PA derivatives were detected by a variable wavelength detector (Agilent G1314A). Quantification of single amino acids was made against a relative calibration curve and expressed as  $\mu$ mol g<sup>-1</sup> FW (Barrameda-Medina et al. 2017).

## Statistics

Values were expressed as mean  $\pm$  standard error (SE). Each experiment was made in at least three replicates. Statistical significance of biometric parameters, water content, HPLC experiments and physiological analysis between control and Cd exposed plants was evaluated through Student's *t* test ( $p \leq 0.05$ ). In addition, a one-way analysis of variance (ANOVA) was used to calculate the statistical significance of the differences occurred in enzymatic activities (G6PDH, APX and NR) and proline content between controls and Cd exposed groups in different collection points (ANOVA calculations correspond to  $\alpha = 0.05$ ). Differences between means were evaluated for significance using the Tukey–Kramer post hoc test.

## Results

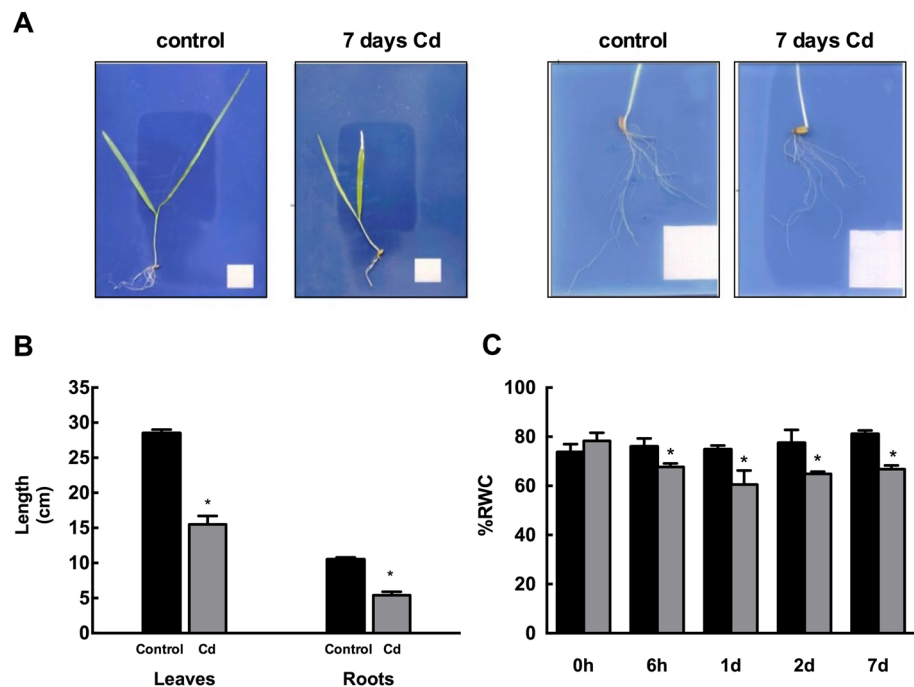
### Cd exposure induces a severe stress in barley plants

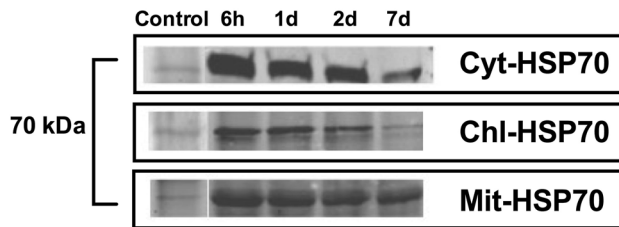
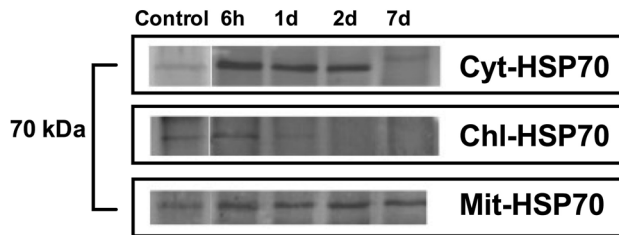
The effects of Cd stress were observed by exposing barley plants to 10<sup>-5</sup> M of CdCl<sub>2</sub> for 7 days: metal-exposed plants exhibited wilting and leaf curling (Fig. 1a); Cd stress induced a severe loss in plant biomass and growth (Supplemental Fig. 1), resulting in a 42% and 47.5% decrease in leaves and roots' length, respectively (Fig. 1b). Furthermore, Cd-stressed plants showed a significant reduction in relative water content (%RWC), from 6 h (-12.2%) to 1 day (-19.1%), 2 days (-16.3%) and 7 days (17%—Fig. 1c).

Cd exposure induced the accumulation of stress-related proteins such as HSP70s. As shown in Fig. 2, a prompt increase in cytosolic, chloroplastic and mitochondrial HSP70s levels was reported in leaves after 6 h, remaining higher than control values during the entire experimental period (Fig. 2a). On the other hand, a lower but similar increase in cytosolic and mitochondrial HSP70 was detected in barley roots exposed to Cd (Fig. 2b).

To verify possible changes in osmoregulation mechanisms, proline content was measured in the leaves. Cd-stressed plants showed significant and progressive increase in proline content from 1.8-fold after 6 h up to 2.4-fold change after 2 days, respectively: barley leaves exposed to Cd for 2 days showed a proline content of 2.64 mg g<sup>-1</sup> FW (Fig. 3a). Contrarily, barley roots exposed to Cd showed a significant increase in proline content after 6 h (+60%),

**Fig. 1** Biometric effects of Cd on barley plants. Effects of 7 days Cd exposition on barley leaves and roots (a). Changes in leaf and root length (b) in control (no Cd—black bars) and stressed (grey bars) barley plants after 7 days of cadmium exposition. Changes in relative water content (%RWC) in controls (no Cd—black bars) and stressed (grey bars) barley plants (c). Asterisks indicate significantly different values in Cd stress vs. control plants at  $p \leq 0.05$  (\*)



**A - Leaves****B - Roots**

**Fig. 2** Occurrence of HSP70 measured by Western blotting—using anti-HSP70 antibodies (Agrisera)—in leaves (a) and roots (b) of barley plants exposed to  $10^{-5}$  M Cd for 6 h, 1 day, 2 days and 7 days

then proline content decreased to the levels of control plants (Fig. 3b).

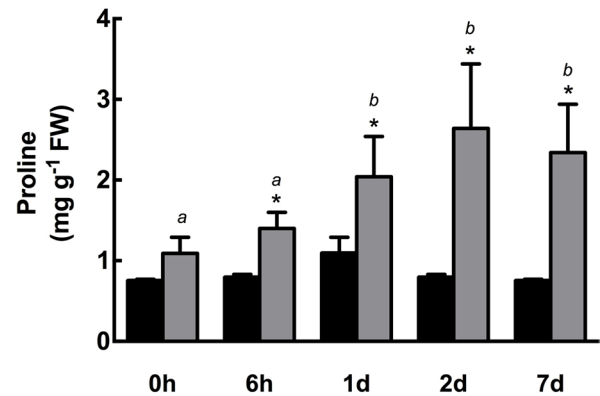
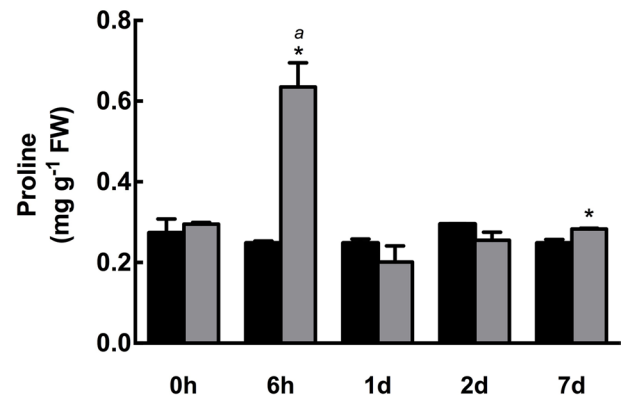
**Effects of Cd on photosynthesis**

Photosynthetic machinery was monitored by measuring changes in photosynthetic pigments, RuBisCO large subunit (LS), and D1 protein of photosystem II. As shown in Fig. 4a, critical decrease in chlorophyll A, chlorophyll B and carotenoids was reported in leaves exposed to Cd. In detail, a significant decrease about 35% and 27% was reported after 7 days for chlorophyll A and chlorophyll B upon Cd stress, respectively. Furthermore, a not significant 60% reduction was seen in the carotenoids' content as well.

Western blotting analysis suggested an increase in occurrence of both RuBisCO and D1 proteins (Fig. 4b). These results were confirmed by the data on photosynthetic efficiency parameters: HM exposure resulted in a significant slight decrease in Fv/Fm (−6.3%), QY (−5.4%) and ETR (−8.7%). Contrarily, and a 2.5-fold increase in non-photochemical quenching (NPQ) was reported (Table 1).

**G6PDH and APX exhibit a critical role during Cd stress**

The activities and the occurrence of G6PDH isoforms were investigated in barley plants exposed to  $10^{-5}$  M cadmium: this resulted in a prompt 32.5% increase in G6PDH activity with respect to the control plants after 6 h in leaves. After 1 and 2 days of cadmium exposure, G6PDH activity

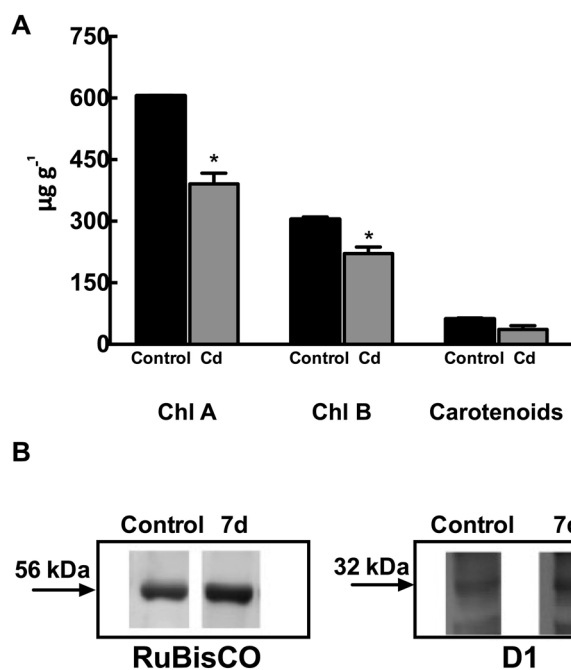
**A - Leaves****B - Roots**

**Fig. 3** Changes in the levels of proline in control (no Cd—black bars) and  $10^{-5}$  M Cd-stressed (grey bars) barley leaves (a) and roots (b). Asterisks indicate significantly different values in Cd stress vs. control plants at  $p \leq 0.05$  (\*). Differences between stressed groups were indicated by letters “a” and “b”

steadily continued to increase to about 36.1% and 27.4% in stressed plants than controls. The peak of G6PDH activity was observed after 7 days of treatment, when the activity increased up to  $51 \text{ U mg}^{-1} \text{ prot}$  (Fig. 5a). The ANOVA analysis showed no significant difference between control plants from 0 h to 7 days, while statistical changes were reported between the first 2 days and 7 days in Cd stressed plants.

Furthermore, Western blotting analyses revealed slight changes in the occurrence of the different G6PDH isoforms upon Cd stress: a light increase in the occurrence of cytosolic isoform with respect to the plastidial/chloroplastic isoforms was observed (Fig. 5b).

Interestingly, similar results were observed in roots. A 16–18% increase in G6PDH activity was observed after 1 and 2 days of Cd exposure, respectively; then, total G6PDH activity decreased in roots (Fig. 6). The ANOVA analysis indicated no significant difference between control plants from 0 h to 7 days, while statistical changes were reported between 1 and 2 days vs. 6 h and 7 days in Cd stressed plants.



**Fig. 4** Changes in the levels of chlorophyll A, chlorophyll B and carotenoids in barley plants exposed to  $10^{-5}$  M Cd for 7 days (**a**). Levels in Cd stressed plants are in grey bars; controls (no Cd—black bars). Asterisks indicate significantly different values in Cd stress vs. control plants at  $p \leq 0.05$  (\*) and  $p \leq 0.005$  (\*\*), respectively. **b** Occurrence of D1 and RuBisCO measured by Western blotting using anti-D1 and anti-RuBisCO antibodies (Agriser) in leaves of barley plants exposed to  $10^{-5}$  M Cd for 7 days

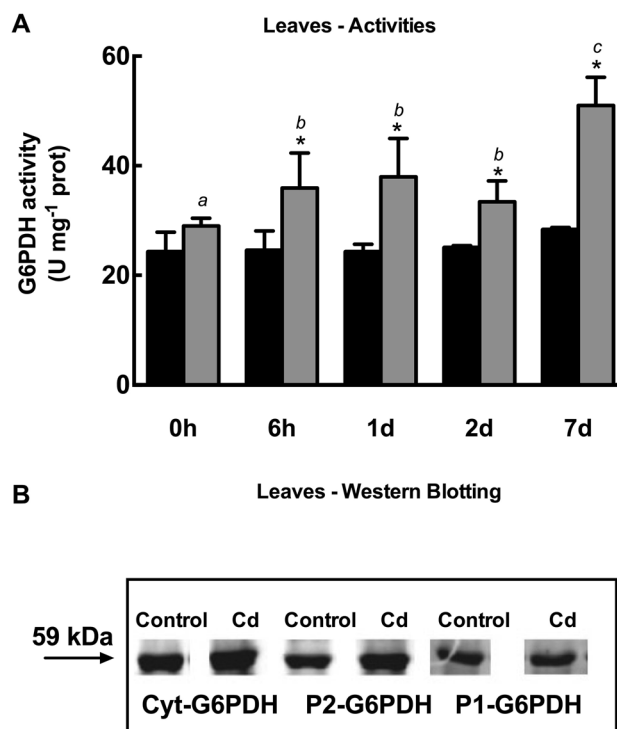
**Table 1** Effects of Cd stress on photosynthetic parameters in barley leaves

	Photosynthetic efficiency			
	Fv/Fm	QY	ETR	NPQ
Control	$0.79 \pm 0.01$	$0.75 \pm 0.01$	$36.7 \pm 1.45$	$0.07 \pm 0.01$
Cd (7 days)	$0.74 \pm 0.02^*$	$0.71 \pm 0.01^*$	$32.9 \pm 0.86^*$	$0.17 \pm 0.02^*$

Maximal photochemical efficiency (Fv/Fm); photosystem II quantum yield (QY); electron transport rate (ETR) and non-photochemical quenching (NPQ) were calculated as described in “Materials and methods” in barley plants exposed for 7 days to  $10^{-5}$  M Cd. Each value represents mean  $\pm$  standard error;  $n = 5$

Asterisks indicate significantly different values in Cd stress vs. control plants at  $p \leq 0.05$  (\*)

These results were corresponding to those obtained for ascorbate peroxidase activity in the leaves, where the enzyme rate increased about two-fold with respect to the controls within 6 h of treatment, remaining stable after 1 day (+39%) and after 2 days (32%), while the APX activity decreased only 7 days after stress imposing, at control levels ( $124 \pm 2.4$  nmol min<sup>-1</sup> mg<sup>-1</sup> prot.—Fig. 7a). ANOVA analysis showed no significant differences between control plants from 0 h to 7 days. Furthermore, significant

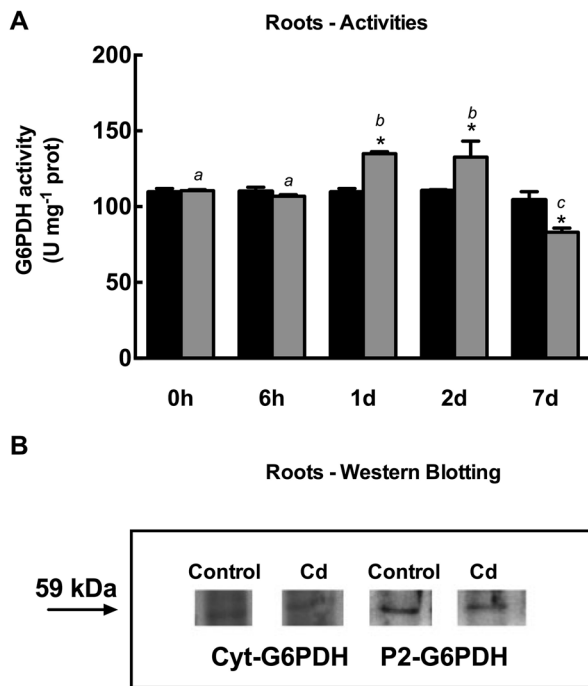


**Fig. 5** **a** Effects of Cd stress on G6PDH enzymatic activity in control (no Cd—black bars) and Cd stressed (grey bars) barley leaves. Asterisks indicate significantly different values in Cd stress vs. control samples at  $p \leq 0.05$  (\*). Differences between stressed groups were indicated by letters “a” (0 h), “b” (6 h, 1 day and 2 days) and “c” (7 days). **b** Accumulation of cytosolic, chloroplastic and plastidial G6PDH revealed by Western blotting using anti potato cyt-, P1- and P2-G6PDH antibodies

differences were reported for 6 h and 1 day in Cd-stressed plants vs. 2 days and 7 days in Cd-stressed plants. Significant differences were also reported after 2 days in stressed plants vs 7 days in stressed plants. Intriguingly, barley roots exposed to Cd showed significant and rapid increase in APX activities from 6 h (+42.6%), to 2 days (+51.6%). After 7 days of Cd exposition, APX activity remained higher—up to 121.4 nmol min<sup>-1</sup> mg<sup>-1</sup> prot.—in stressed plants with respect to controls (Fig. 7b).

### Effects of Cd on organic nitrogen metabolism

Nitrogen metabolism was monitored by measuring the activity of nitrate reductase. As shown in Table 1, barley plants exposed to Cd exhibited a significant increase in NR activities both in leaves and roots. Barley leaves exposed to Cd showed an activity of 0.05 nmol min<sup>-1</sup> mg<sup>-1</sup> prot. steadily increasing from 1.41 to 2.35-fold change after 1 day and 7 days, respectively; ANOVA analysis showed significant differences between 7 days vs. 1 and 2 days leaves in Cd exposed plants.



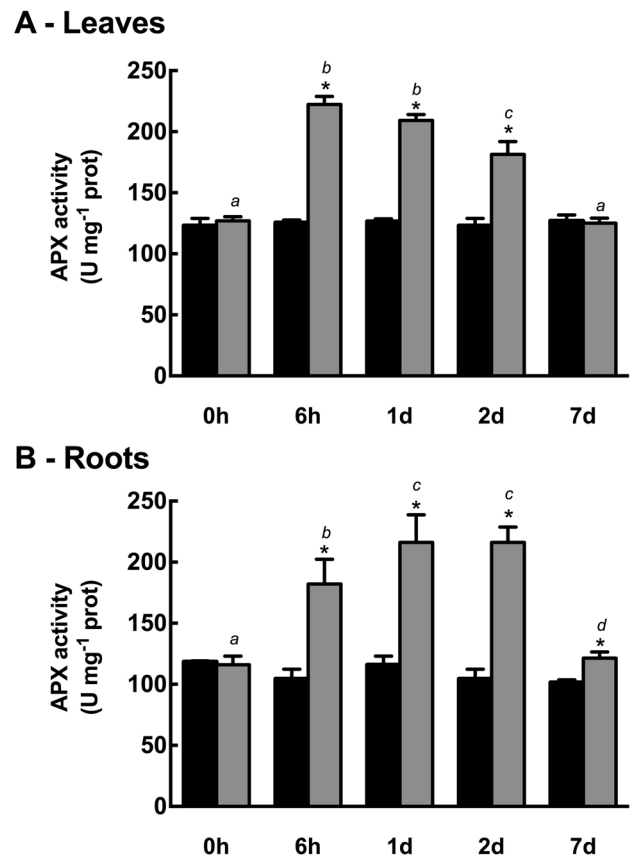
**Fig. 6** **a** Effects of Cd stress on G6PDH enzymatic activity in control (no Cd—black bars) and Cd-stressed (grey bars) barley roots. Asterisks indicate significantly different values in Cd stress vs. control samples at  $p \leq 0.05$  (\*). Differences between stressed groups were indicated by letters “a” (0 h and 6 h), “b” (1 day and 2 days) and “c” (7 days). **b** Accumulation of cytosolic, chloroplastic and plastidial G6PDH revealed by Western blotting using anti potato cyt-, and P2-G6PDH antibodies

On the other hand, roots showed an activity of  $0.17 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ prot.}$  at 0 h; no significant difference was found in control roots during the experimental period.

Cd stress induced a major increase in NR activity of three-fold change at 2 days of Cd stress. After 7 days the increase remained higher in stressed roots than control ( $0.49 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ prot.}$ —Table 2). ANOVA analysis showed significant differences in 2 days root vs. 1 and 7 days roots in Cd exposed plants; further differences were found in 7 days vs 1 day in Cd exposed roots.

Cadmium stress induced specific changes in the amino acid levels plants exposed to cadmium (Table 3). Barley roots exposed to Cd showed an increased aspartate content and a correspondent decrease in asparagine and glutamine, almost in a stoichiometric pattern. A not significant increase in glutamate content was also reported. Severe decrease in serine and valine ( $-30$ ;  $-40\%$ ) was associated with an increase in alanine levels ( $+50\%$ ). It should be underlined that levels of aromatic amino acids were increased by 20% upon Cd stress, with a particularly marked increase in tryptophan levels ( $+40\%$ ).

In leaves, a significant decrease was observed in aspartate ( $-20\%$ ), and glutamine ( $-45\%$ ), with correspondent



**Fig. 7** Effects of Cd stress on APX enzymatic activity in control (no Cd—black bars) and Cd stress (grey bars) in barley leaves (**a**) and roots (**b**). Asterisks indicate significantly different values in Cd stress vs. control plants at  $p \leq 0.05$  (\*). Differences between stressed groups were indicated by letters “a” (0 h and 7 days), “b” (6 h and 1 day) and “c” (2 days) in leaves; differences between stressed groups were indicated by letters “a” (0 h), “b” (6 h), “c” (1 day and 2 days), “d” (7 days) in roots

increase in asparagine ( $+7\%$ ) and glutamate ( $+36\%$ ). Significant reductions were observed in the levels of arginine ( $-18\%$ ), lysine ( $-15\%$ ) and valine ( $-40\%$ ). The levels of aromatic amino acids decreased by 16% in leaves exposed to Cd.

## Discussion

Heavy metals (HMs) play important and ambivalent roles in plant metabolism: some of them can represent essential micronutrients for plant growth, but, when in excess, induce severe toxicity. These conditions are generally caused by industrial and on the other anthropic activities emitting toxic heavy metals such as cadmium, chromium, nickel and others (Parrotta et al. 2015). The effects of heavy metal pollution depend on exposure time, quantity and by the specific element (Yousaf et al. 2016; Akhtar et al. 2017; Rizwan et al.

**Table 2** Effects of Cd stress on nitrate reductase enzymatic activity in barley leaves and roots

	Nitrate reductase activity (mmol min <sup>-1</sup> mg <sup>-1</sup> )							
	0 h		1 day		2 days		7 days	
	Control	Cd	Control	Cd	Control	Cd	Control	Cd
Leaves	0.0529 ± 0.01	0.0531 ± 0.02 <sup>a</sup>	0.0514 ± 0.04	0.0726 ± 0.03 <sup>*,a</sup>	0.0522 ± 0.02	0.103 ± 0.06 <sup>***,a</sup>	0.0521 ± 0.01	0.123 ± 0.01 <sup>**</sup>
Roots	0.170 ± 0.01	0.168 ± 0.003 <sup>b,c</sup>	0.228 ± 0.07	0.178 ± 0.01 <sup>b,c</sup>	0.272 ± 0.002	0.800 ± 0.06 <sup>*,c</sup>	0.214 ± 0.06	0.496 ± 0.01 <sup>**b</sup>

Values represent an average measurement ± SD

Asterisks indicate significantly different values in Cd stress vs. control plants at  $p \leq 0.005$  (\*) and  $p \leq 0.0005$  (\*\*)

<sup>a</sup>Significant differences vs 7 days in Cd exposed leaves

<sup>b</sup>Significant differences vs 2 days in exposed roots

<sup>c</sup>Significant differences vs 7 days in exposed roots

**Table 3** Amino acids concentration ( $\mu\text{mol g}^{-1}$  FW) in leaves and roots of barley plants exposed to  $10^{-5}$  M of cadmium stress for 7 days in hydroponic culture

	Leaves	Control ( $\mu\text{mol g}^{-1}$ FW)	Cd ( $\mu\text{mol g}^{-1}$ FW)	Roots	Control ( $\mu\text{mol g}^{-1}$ FW)	Cd ( $\mu\text{mol g}^{-1}$ FW)
<i>Asp</i>	1.57 ± 0.090		1.25 ± 0.032*	<i>Asp</i>	0.87 ± 0.093	0.96 ± 0.085*
<i>Glu</i>	0.36 ± 0.016		0.49 ± 0.01**	<i>Glu</i>	0.13 ± 0.084	0.18 ± 0.015
<i>Asn</i>	12.65 ± 0.851		13.62 ± 0.65*	<i>Asn</i>	4.23 ± 0.154	4.04 ± 0.384*
<i>Ser</i>	0.94 ± 0.020		0.90 ± 0.023	<i>Ser</i>	0.27 ± 0.005	0.17 ± 0.066*
<i>Gln</i>	1.23 ± 0.608		0.71 ± 0.042	<i>Gln</i>	2.35 ± 0.072	2.13 ± 0.187*
<i>His</i>	0.44 ± 0.020		0.47 ± 0.022	<i>His</i>	0.20 ± 0.009	0.19 ± 0.001
<i>Gly</i>	0.04 ± 0.003		0.05 ± 0.008	<i>Gly</i>	0.05 ± 0.005	0.04 ± 0.004**
<i>Thr</i>	0.45 ± 0.026		0.42 ± 0.018*	<i>Thr</i>	0.15 ± 0.002	0.13 ± 0.010*
<i>Arg</i>	0.41 ± 0.024		0.34 ± 0.02**	<i>Arg</i>	0.14 ± 0.014	0.14 ± 0.010
<i>Ala</i>	0.70 ± 0.095		0.70 ± 0.040	<i>Ala</i>	1.65 ± 0.104	2.43 ± 0.293*
<i>Tyr</i>	0.04 ± 0.002		0.02 ± 0.002*	<i>Tyr</i>	0.02 ± 0.001	0.02 ± 0.002
<i>Val</i>	0.27 ± 0.101		0.16 ± 0.009	<i>Val</i>	0.14 ± 0.021	0.10 ± 0.009*
<i>Trp</i>	0.36 ± 0.148		0.31 ± 0.114	<i>Trp</i>	0.41 ± 0.074	0.57 ± 0.097*
<i>Phe</i>	0.17 ± 0.025		0.15 ± 0.029*	<i>Phe</i>	0.19 ± 0.029	0.16 ± 0.010
<i>Ile</i>	0.12 ± 0.084		0.12 ± 0.050	<i>Ile</i>	0.10 ± 0.048	0.10 ± 0.052
<i>Leu</i>	0.14 ± 0.032		0.18 ± 0.061	<i>Leu</i>	0.12 ± 0.033	0.10 ± 0.031**
<i>Lys</i>	2.26 ± 0.253		1.92 ± 0.401*	<i>Lys</i>	1.53 ± 0.329	1.63 ± 0.389
<i>Cit</i>	0.03 ± 0.001		0.04 ± 0.002*	<i>Cit</i>	0.03 ± 0.001	0.03 ± 0.001*
<i>Orn</i>	0.29 ± 0.113		0.78 ± 0.102*	<i>Orn</i>	0.48 ± 0.146	0.44 ± 0.168*
Total	22.47		22.63	Total	13.099	13.59

Values represent an average measurement ± SD

Asterisks indicate significantly different values in Cd stress vs. control plants at  $p \leq 0.05$  (\*) and  $p \leq 0.005$  (\*\*), respectively

2017); among the heavy metals that contaminate the environment, cadmium causes great concerns, due to its solubility, which is able to cause serious problems to living organisms. This non-essential element is taken up from the plants through the roots and accumulates in many organs including roots, shoots, leaves and grains (Rizwan et al. 2017).

As described in this study, Cd exposition induced severe consequences in barley plants causing decrease in biomass, a reduction in leaf length, photosynthetic efficiency and a modification of the root system after Cd treatment. The reduction of these parameters is a common response

of plants to heavy metals. Recently, an interesting correlated reduction upon increasing toxic concentration of Cd was reported in biomass and chlorophyll content in *Festuca arundinacea* (Huang et al. 2017). Furthermore, the correlation between RWC, pigments, dry weight and tissue length values were previously reported in various plants (barley, bean, *Brassica* and others) after being exposed to different heavy metals as cadmium or chromium (González et al. 2015; Nahar et al. 2016; Al; Mahmud et al. 2017).

Similar results were previously shown in tomato plants exposed to cadmium and copper (Mediouni et al. 2006;



Hediji et al. 2015); these morphogenetic changes are possibly not specific symptoms of metabolic changes, generally observed under various stress such as drought (Landi et al. 2016). Furthermore, prolonged Cd stress conditions induced changes in several physiological and biochemical processes such as pigment content, osmoregulation by proline levels, and protein preservation by HSP70s. It has been reported that different isoforms of HSP70s (chloroplastic, cytosolic and mitochondrial) play a critical role in pollution stress in different types of plant bio-indicators (Basile et al. 2015). Furthermore, alterations in photosynthetic machinery have been reported: it was worth pointing out that photosynthetic efficiency parameters (e.g., Fv/Fm or QY) decreased upon heavy metal exposition (Arena et al. 2017).

Moreover, RuBisCO large subunit (LS), and D1 component of photosystem II showed an increase after 7 days of Cd exposition. Consistent with our results, both large and small RuBisCO subunits significantly increased upon Cd stress in spinach (*Spinacia oleracea*—Bagheri et al. 2017). Interestingly, heavy metal stresses showed different responses in RuBisCO large subunit (LS), and D1 in *Cynara cardunculus*: D1 proteins similarly increased upon Pb and Cd stress, while RuBisCO showed significant increase exclusively upon Cd exposure (Arena et al. 2017).

In this context, G6PDH, representing a major source of NADPH in the plant cell, would play a central role in plant stress response. Data presented in this work, together with previous results (Yang et al. 2014) suggest that a severe exposition to Cd induced an increase of G6PDH activities in leaves and roots. Thus, a central role in HM stresses response is proposed for this enzyme: in detail, in leaves a first increase of G6PDH activity was reported after 6 h of Cd exposition. The increase remained stable after 1 day and 2 days of Cd exposition, showing a crescent increase after 7 days. Similarly, our results suggest a major role for G6PDH in the first phase of Cd stress response in barley roots.

The enzymatic rate modification could also be associated to the abundance of G6PDHs isoforms observed in Western blotting. It could be argued that G6PDHs' increase is one of the main effects of heavy metals in response to the oxidative stress induced by pollution; therefore, the increase in the G6PDH activities appears involved to preserve the basal cellular metabolism (Esposito et al. 2005). It is worth to point out that a major role for cy-G6PDH has already been demonstrated upon salt stress (Dal Santo et al. 2012; Cardi et al. 2015), ABA treatments (Cardi et al. 2011) and drought (Landi et al. 2016).

G6PDH represents one of the major sources of NADPH in the plant cell, and this is the main reductant required for the ascorbate–glutathione cycle (Letierrier et al. 2007; Esposito 2016). As expected, APX activity increases under Cd stress; this increase of the APX activity in leaves plays a

crucial role to counteract the increment of  $H_2O_2$  as result of the oxidative damages induced by HM. APX and the other enzymes of the antioxidant systems (e.g., catalase, superoxide dismutase) act in first phase of the oxidative stress response (Van Oosten et al. 2016). Intriguingly, the G6PDH and APX correlation has been previously reported in various crops to reduce the negative effects induced by different stresses causing an oxidative burst (Gill and Tuteja 2010).

The activity of G6PDH was also related to nitrogen metabolism, in this study monitored by nitrate reductase activity (Esposito 2016). Plant exposed to Cd showed similar activity changes in both G6PDH and nitrate reductase. It has previously demonstrated that reducing power produced by G6PDH plays a critical role during nitrogen assimilation (Esposito et al. 2005). Furthermore, nitrate uptake and reduction play an emerging role in abiotic stress response, and nitrate reductase genes and nitrate transporters were recently identified as co-expressed with G6PDHs in *Arabidopsis thaliana* (Landi et al. 2017; Landi and Esposito 2017).

These metabolic changes induced by Cd exposition were compared with the free amino acids levels. Consistently, proline level increased upon Cd exposure in leaves, thus confirming that under stress, barley plants activate a complex range of responses to reduce the toxic effects and sustain growth under unfavourable conditions (Ashraf and Foolad 2007; Gill and Tuteja 2010; Landi et al. 2017). In response to the oxidative stress, plants activate different mechanisms, among these, proline accumulation in leaves plays a pivotal role (Ashraf and Foolad 2007).

Therefore, the nitrate assimilation increased by Cd could be related to the strong increase in proline levels possibly to counteract stress. In addition, it has been proposed that proline may represent an active  $H_2O_2$ ,  $O_2$  and  $OH^-$  scavenger (Gill and Tuteja 2010; Landi and Esposito 2017).

As expected, Cd induced changes in aminoacidic profile in both roots and leaves: plants exposed to metal toxicity accumulates specific amino acids which may play a role in the tolerance to stress and have beneficial functions such as proline and others (Xu et al. 2012; Pavlíková et al. 2014a, b).

It is worth noting that proline levels (and isoleucine and valine as well) may regulate cell osmotic potential; on the other hand, methionine, glycine, cysteine, leucine play roles in plant cell growth and cell wall organization; furthermore, levels of aromatic amino acids could modulate protein synthesis and production (Joshi et al. 2010; Pavlíková et al. 2014a; Zemanova et al. 2017).

In the roots, the increase in aspartate content corresponds to decrease in asparagine and glutamine levels, possibly limiting the transport of these amino acids to the aerial part of the plant. The accumulation of alanine in the roots indicated an overflow of pyruvate coming from increased root glycolysis induced by stress.

Barley leaves showed an opposite behaviour compared to roots in asparagine accumulation, suggesting different nitrogen re-utilization and accumulation mechanisms. Barley plants exposed to Cd showed increased levels in phenylalanine, tryptophan and tyrosine. Intriguingly, the pathways involved in aromatic amminocid synthesis (as Shikimate cycle), are strictly connected with G6PDH activity, which provides the NADPH required for these processes. Therefore, in the leaves the reported reduction of phenylalanine, tryptophan and tyrosine levels would indicate a diversion in NAPDH usage to antioxidant stress response.

## Conclusion

The data here presented suggest a primary role of G6PDH in the response to cadmium stress in barley. These results corroborated the idea that the abiotic stress response in plants involves G6PDH; this activity may play a pivotal role in assisting various physiological aspects of the stress, showing its central role played in ROS scavenging, nitrogen assimilation and basal metabolic processes.

Further studies are required to elucidate both the complete pathway(s) of signalling, from detection of stress to the increase in G6PDH activity, both the mechanisms providing a possible feedback modulation of delivery of reductants by this pivotal enzyme of cell metabolism.

**Author contribution statement** ML conceived and made the experiments, and is a joint first author along with ADL; ADL made enzymatic experiments and grew plants; VP, made WB and wrote the paper; DL grew plants and made WB; SL measured enzymatic activities, wrote and amended the paper; SE conceived the work and the experiments, wrote and amended the paper, and is the corresponding author.

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