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RESEARCH ARTICLE

Cadmium and Copper Stress affect seedling growth and Enzymatic activities in Germinating Barley Seeds

[1,*]Tawba Kalai, [2]Abdellileh Chaoui, [3]Khalil Khamassi, Jaime A., [4]Teixeira da Silva, [1]M'barek Ben Naceur, Houda Gouia, and [1]Leila Bettaieb Ben-Kaab**

[1]Unité de recherche: «Nutrition et métabolisme azotés et protéines de stress» (99 UR /09-20) Département de Biologie et Physiologie végétale, Faculté des sciences de Tunis, Université Tunis EL Manar, Campus universitaire, Tunis 1060, Tunisia

[2]Unité de recherche: «Bio-Physiologie cellulaires», Département des sciences de la vie, Faculté des sciences de Bizerte, 7021 Zarzouna, Tunisia

[3]Institut National de Recherche Agronomique de Tunisie, Ariana, Tunisia, 43, avenue Charles Nicolle 1082 Tunis-Mahrajène Tunisia

[4]Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan

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ABSTRACT

To assess Cd and Cu phytotoxicity, experiments focusing on germination of barley (*Hordeum vulgare* L. var. 'Manel') seeds were germinated for two days in a solution containing $CdCl_2$ (25, 50 and 100 µM) or $CuSO_4$ (100, 300 and 500 µM) . The growth of radicles and shoots decreased while the water content in stressed seeds remained near control values. A decline in α-amylase, acid phosphatase and alkaline phosphatase activities was also observed in endosperms while β-amylase activity was only slightly modified by heavy-metal treatments. However, Cd and Cu increased lipid peroxydation, enhanced soluble protein and sugar content even at the lowest dose and induced a significant accumulation of proline essentially in radicles. These results suggest that the inhibition of seed germination after exposure to Cd or Cu is not the consequence of reduced water uptake by seed tissues but may be due to a failure in reserve mobilization from the endosperm.

INTRODUCTION

Barley (*Hordeum vulgare* L.) is an important cereal crop in Tunisia where it is used mainly as a grain for human and animal nutrition. It has been the subject of many research investigations in Tunisia (Bettaieb-Ben Kaab and Attias, 1992; Bettaieb-Ben Kaab *et al*., 2005; Abdellaoui *et al*., 2007; 2010; Bchini *et al*., 2010). Heavy metals are among the major pollutants contaminating our environment and can severely restrict plant growth (Yadav, 2010; Kranner and Colville, 2011), especially of barley plants. Metal sensitivity and toxicity to a plant are influenced not only by the concentration and toxicant type, but are also influenced by the developmental stage (Aina *et al*., 2007; Liu *et al*., 2007; He *et al*., 2008). Seed germination is one of the most highly sensitive physiological process in plants ,because of a lack of some defense mechanisms (Ben Naceur *et al*., 2007; He *et al*., 2008), which start with the crucial stage of water absorption by seed (Bradford, 1995) and end with the elongation of the embryonic axis and the emergence of the radical through structures surrounding the embryo (Bewley, 1997). At the cellular level, seed germination is essentially characterized by (a) resumption of respiratory activity through the reactivation of glycolysis, Kreb's cycle and the respiratory chain (Müntz *et al*., 2001), (b) reserve mobilization by the secretion of hydrolytic enzymes (Sfaxi-Bousbih, 2010), depolymerization of reserves and transport of metabolites released into the growing embryonic cells (Smiri *et al*., 2009) and (c) the decrease in strength imposed by tissues surrounding the embryo, mainly by increasing the activity of several cell wall hydrolases (Bewley, 1997; Welbaum *et al*., 1998). Inhibition of this process appears to depend, however, on the metal and its concentration, duration of exposure of seeds, plant species, and even the grain integuments (Carlson *et al*., 1991; Munzuroglu and Geckil, 2002). Thus, extensive research has already been conducted on the effects of heavy metals on inhibition of metabolic activities in germinating seeds of cereal plants, particularly rice (Shah and Dubey, 1998; Ahsan *et al*., 2007; Aina *et al*., 2007; He *et al*., 2008; Mishra and Dubey, 2008) and wheat (Lahmadi *et al*., 2011). However, the effects of heavy metals on metabolic activities of germinating barley seeds are not well documented, especially at an early stage of germination.

Thus, the purpose of this study is to contribute to an understanding of the biochemical changes in germinating barley seeds exposed to heavy metals, specifically cadmium (Cd) and copper (Cu). In particular, this work aims to evaluate the degree to which exogenous Cd and Cu are able to affect germination during the two first days of *H. vulgare* seed germination and to better understand the mobilization of organic reserves degraded by two hydrolytic

Corresponding author: k_tawba@live.com

enzymes, amylase and phosphatase, during the germination of barley seeds 48 h after Cd and Cu treatments. Thus, an attempt was made to provide a mechanism by which Cd and Cu might impair seed germination.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MI, USA) and were of the highest purity available.

Plant material and germination conditions

The barley material used was var. 'Manel'. This variety, which was officially registered in 1996, has contributed significantly to barley national (i.e., Tunisian) production because it has a high degree of disease (brown rust, helmintosporiose, ouidium) resistance (Deghais, 1999). Prior to starting the experiment, seed viability was tested, revealing 92% viability, i.e. 92% germination. The seeds (200 in total) were placed in liquid nitrogen for 2 to 3 min, disinfected with 2% sodium hypochlorite for 10 min, then rinsed thoroughly with distilled water. Seeds were germinated in a vacuum oven (Memmert, Schwabach, Germany). Fifty seeds were placed on two layers of Whatman filter paper (0.5 mm) (ISTA 1999) in 15-cm diameter glass Petri dishes soaked with H_2O or solutions of $CdCl_2$ at 25, 50 and 100 µM, or $CuSO_4$ at 100, 300 and 500 µM. The control in all cases was the absence of Cd and Cu. Seeds were germinated in the dark at 25°C. After 48 h, young sprouts were harvested and rinsed quickly in three successive baths of distilled water. Germination was studied until 48 h and not beyond because from the third day onwards there is insufficient endosperm to physically analyze the enzymes related to reserve mobilization. After removing the integument and plumule of the embryonic axis, the samples were divided into radicles, shoots and endosperms. The length of the radicles and shoots was measured then each was separately weighted to determine the fresh weight (FW). The same samples were then placed in liquid nitrogen or dried at 70°C for at least 3 days to determine dry weight (DW). Water content (WC) of the germinated seeds during treatments was determined using the following formula: WC (%) = [(FW-DW)/FW]×100.

Determination of protein, sugar and proline contents

Total soluble protein (TSP) content was quantified using Coomassie Brilliant blue (Bradford, 1976) with bovine serum albumin as the protein standard. Endosperm were powdered and homogenized in 80% ethanol, boiled for 30 min at 70°C and then centrifuged at 6000 × *g* for 15 min at 4°C. The supernatants were tested for total soluble sugar (TSS) (McCready *et al*., 1950). Proline content was determined using a colorimetric method modified from Li (2000) with minor modifications. The fine powder of fresh seedling tissues (0.1 g) was treated with 5ml of 3% Sulphosalicylic acid and maintained at 100 NC for 10minutes. The supernatant (2ml) was added to a solution of 2 ml of glacial Acetic plus 2 ml of 2.5% (w/v) acidic Ninhydrin, and kept at 100 NC for 25minutes. After the liquid was cooled down, it was added to 4 ml of Toluene. The photometric absorbance of the Toluene extract was read at 520nm.

Determination of amylase and phosphatase activities

Protein extraction and measurement of hydrolytic activities were carried out using the following established methods: α- and β-amylases (Dure, 1960), acid phosphatase (Ikawa *et al*., 1964) and alkaline phosphatase (Torriani, 1967).

Determination of Cd and Cu content

Cd and Cu were extracted from dry matter (HE *et al*., 1998) and assayed by atomic absorption spectrophotometry (Analyst 300, Perkin Elmer, Norwalk, USA).

Determination of lipid peroxydation

Barley roots and shoots (100mg) were separately homogenized in 1ml ice cold extraction buffer containing 0,1M Na- phosphate buffer (pH7), 1mM EDTA, 1mM PMSF and 0,5 % (w/v) of PVP. The homogenate was centrifuged at 9000g for 20 minutes. The supernatants were used at the crude extract for determination of lipid peroxidation. Accumulation of lipid peroxides in tissues was determined in terms of thiobarbituric acid reactive substances (TBARS), by estimation of malondialdehyde (MDA) content based on the method of Health and Packer (1968). Supernatant (100 µl) was mixed with 1ml of 0,1 % (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 10000g for 5minutes and 200µl of the supernatant was mixed with 0,8 ml of 0,5% (w/v) thiobarbituric acid (TBA) in 20% TCA. The mixture was heated at 95°C for 30 minutes, chilled in ice, and centrifuged at 10000g for 5 minutes. The absorbance of the supernatant was measured at 532 nm. The value for non specific absorbance at 600 nm was substracted. The amount of TBARS was calculated by using the extinction coefficient of 155/mM/cm.

Statistical analysis

One-way analysis of variance (ANOVA or Kruskal-Wallis test) was used to test for overall differences between controls and treatments.

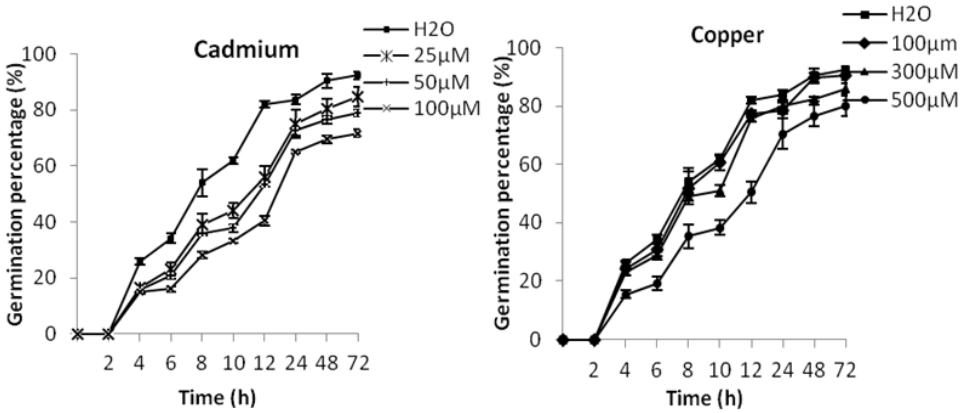

Fig. 1. Evolution over time of germination percent of barley grain treated with inreasing doses of Cd or Cu in the germination medium. Data represent mean ± SE, *n* = 3

The Tukey HSD multiple-comparison test was used in pair-wise comparisons of treatments and controls. In all statistical tests for significance, a $P < 0.05$ value was assumed.

RESULTS

Effects of Cd and Cu on barley seed germination

Germination is defined as the emergence of the radicle from the testa as observed by eye. It is expressed as a percentage of total germination relative to the control. Seed germination of barley var.

'Manel' decreased significantly as Cu concentration increased, except at 500 µM (the reduction was 15% relative to controls). However, for Cd, the highest dose, i.e., 100 µM, affected the germination percentage significantly (23% reduction relative to the control). The average time for seed germination in controls was 9 h and this period increased in the presence of metal stress, reaching 18 h for 100 µM Cd and 12 h for 500 µM Cu (Fig. 1). Thus, overall, Cd and Cu negatively impacted barley seed germination.

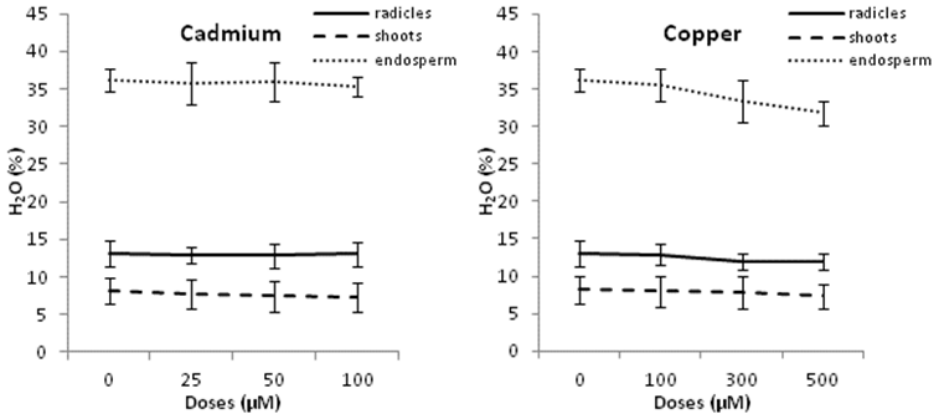

Fig. 2. Effects of Cd and Cu applied during 48 h of germination on water content of radicles, shoots and endosperm of germinating barley. Data represent mean ± SE, *n* = 3.

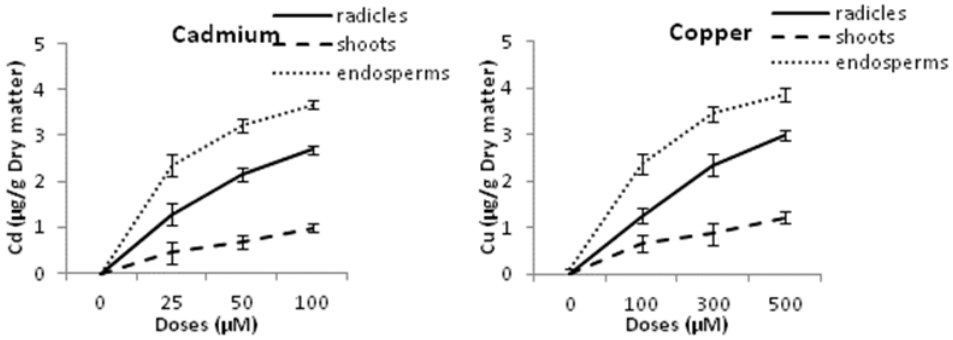

Fig. 3. Effects of Cd and Cu applied during 48 h of germination on Cd and Cu content of radicles, shoots and endosperm of germinating barley. Data represent mean ± SE, *n* = 3.

Bioaccumulation of Cd and Cu, and water status in germinating barley seeds

Water absorption is the major factor determining whether a seed germinates, or not. However, many factors play a major role in inhibiting water uptake by seeds. In order to determine if Cd and Cu supplied in the germination medium produced an osmotic effect on germinating seeds, water content WC was measured. The WC of Cd- and Cu-treated radicles, shoots and endosperms was not significantly affected in the presence of metal stress (Fig. 2). Cd and Cu accumulated exponentially in the endosperms but linearly in the radicles and shoots of germinating barley seeds when the concentration of Cd or Cu in the medium increased (Fig. 3).

Effects of Cd and Cu on seedling growth

Plant growth in this study is expressed as length and dry weight (DW) of radicles and shoots (Fig. 4 and 5). The main radicles and shoots were significantly shorter in Cd- and Cu-treated seedlings than in the control, especially at higher doses (Fig.4). The highest dose of Cd (100 µM) affected radicle elongation the most, reducing it by 63% while, for the highest dose of Cu (500 µM), there was a 47% reduction. Therefore, relative to the control, shoots were stunted by 58 and 20%, respectively even at lower doses of Cd (25 µM) and Cu (100 µM) (Fig. 4). As expected, high doses of Cd (100 µM) and Cu (500 µM) resulted in even more accentuated stunting (75 and 54% decrease in shoot length, respectively). DW (Fig. 5) followed a similar trend as shoot length (Fig. 4). DW in Cd- and Cu-treated radicles and shoots decreased compared to the controls. High doses of Cd (50 and 100 µM) and Cu (500 µM) decreased radicle DW of radicles by 52, 65 and 38%, respectively.

Total soluble protein content

The total soluble protein (TSP) of radicles, shoots and endosperms exposed to Cd and Cu are shown in Fig. 6. Relative to the control, a significant reduction in protein content in two organs was observed after germinating seeds were exposed to metal stress. The decrease was greater in radicles than in shoots but only at high doses of Cd and Cu: 31 and 12% reduction relative to the control for 100 µM Cd and 500 µM Cu, respectively (19 and 11% in shoots). There was a significant accumulation of TSP in Cd- and Cu-treated endosperm, especially at high doses (100 µM Cd and 500 µM Cu). In summary, in the presence of Cd or Cu, TSP decreases significantly compared with controls in radicles and shoots but increases in endosperms (Fig. 6).

Effects of Cd and Cu on soluble sugar content of endosperm

Metal stress induced the accumulation of soluble sugar in seed (Fig. 7) especially at higher doses of Cd (100 µM) and Cu (500 µM). Furthermore, compared to the control, the greatest increase in total soluble sugar (TSS) was in the endosperm after exposure to 100 µM Cd and 500 µM Cu.

Effects of Cd and Cu on proline content

Proline content of the radicles was greatly enhanced with Cd and Cu especially with high concentration; by contrast, the shoots appeared less sensitive to Cd and Cu exposure (Fig.8). The increase of proline concentration in the radicles is dose-dependent, and it increase by 160 and 119 %in the radicles treated with 100µM Cd and 500µM Cu, respectively, compared to the controls.

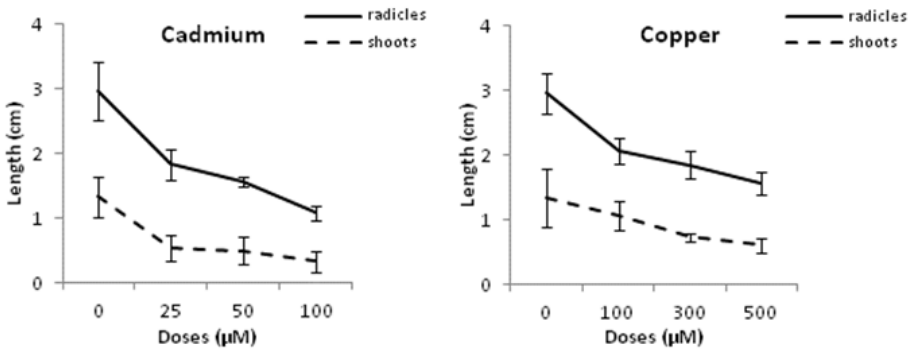

Fig. 4. Effects of Cd and Cu applied during 48 h of germination on radicle and shoot length of germinating barley. Data represent mean ± SE, *n* = 3.

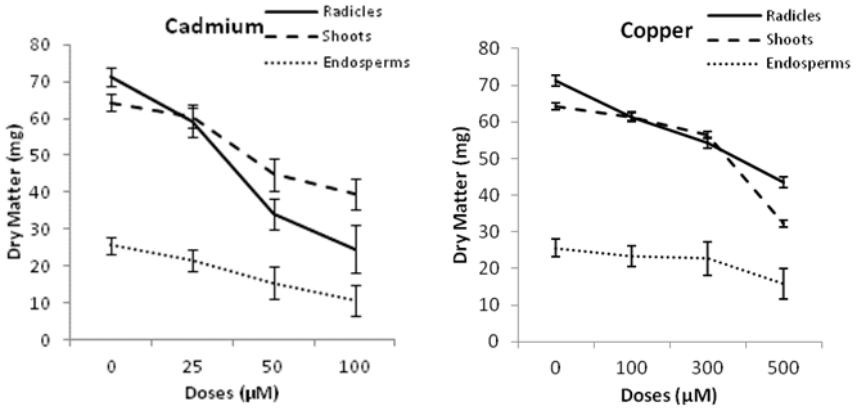

Fig. 5. Effects of Cd and Cu applied during 48 h of germination on radicle dry weight, shoot dry weight and endosperm dry weight production of germinating barley. Data represent mean ± SE, *n* = 3.

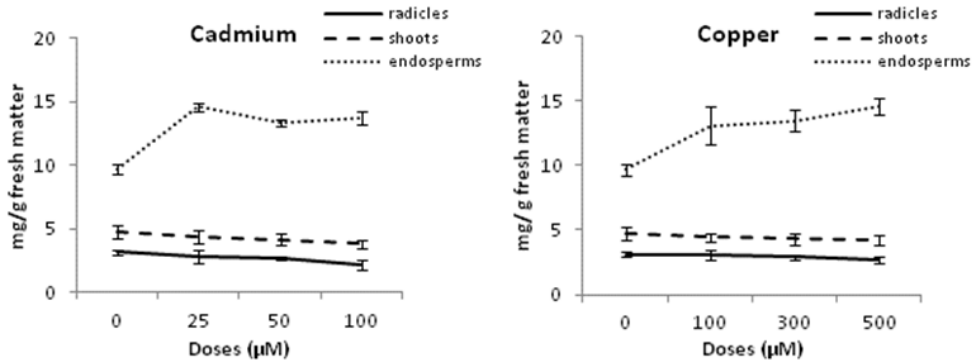

Fig. 6. Changes in soluble protein content in radicles, shoots and endosperm of barley grains exposed to Cd and Cu during 48 h of germination. Data represent mean ± SE, *n* = 3.

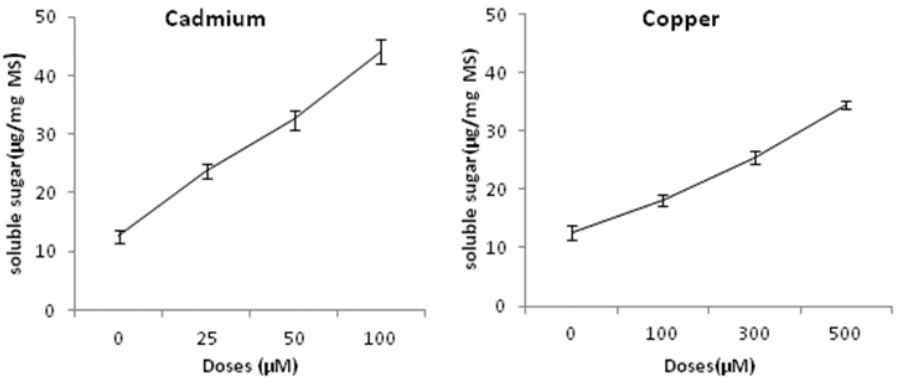

Fig. 7. Effects of Cd and Cu applied during 48 h of germination on sugar content of germinating barley endosperm. Data represent mean ± SE, *n* = 3.

Effects of Cd and Cu on the activities of amylases and phosphatases in endosperm

α-Amylase activity decreased as Cd and Cu concentration increased (Fig. 9). At 100 µM Cd and 500 µM Cu, α-amylase activity decreased by 68 and 50%, respectively. In contrast, β-amylase activity in barley seeds remained at the same level as the control, even as Cd or Cu concentration increased (Fig. 9). Alkaline phosphatase activity in barley endosperm was lower than acid phosphatase activity in control seed (Fig. 10). The application of high doses of Cd and Cu (100 µM Cd, 500 µM Cu) during germination reduced alkaline phosphatase activity by about 77 and 60 % relative to controls but acid phosphatase was more sensitive to Cd and Cu treatment (the reduction was approximately 85 and 73% with 100 µM Cd and 500 µM Cu, respectively).

Effects of Cd and Cu on MDA content

Oxidative stress due to the existence of the toxic metals can be demonstrated by MDA content. Significant increases in MDA content were observed in both radicles as well as shoots after exposure to stress environments (Fig.11). The highest increases in MDA content was seen in radicles under 100µM Cd and 500µM Cu (616 and 535% respectively). Under both control and stress treatments, radicles maintained higher MDA content than shoots.

DISCUSSION

Contamination of agricultural soil by heavy metals has become a critical environmental concern due to their potential adverse ecological effects. Such toxic elements are considered as soil

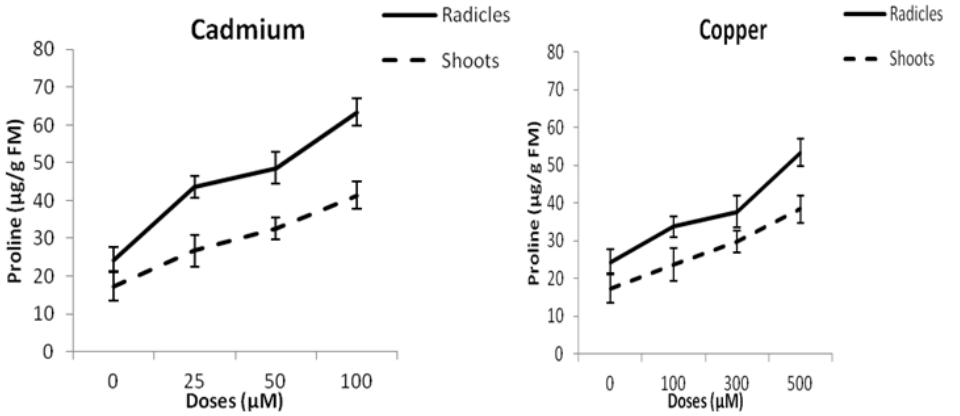

Fig. 8. Changes in proline content in radicles and shoots of barley grains exposed to Cd and Cu during 48 h of germination. Data represent mean ± SE, *n* = 3.

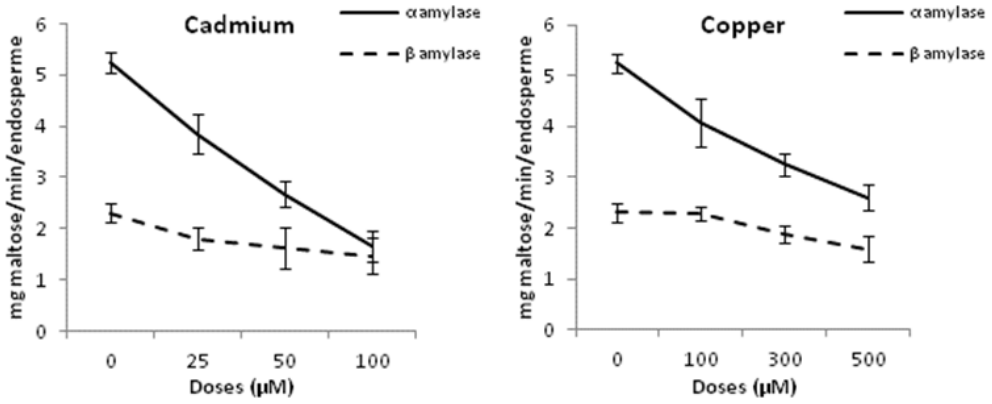

Fig. 9. Effects of Cd and Cu applied during 48 h of germination on amylases (alpha and beta) activity of germinating barley endosperm. Data represent mean ± SE, *n* = 3

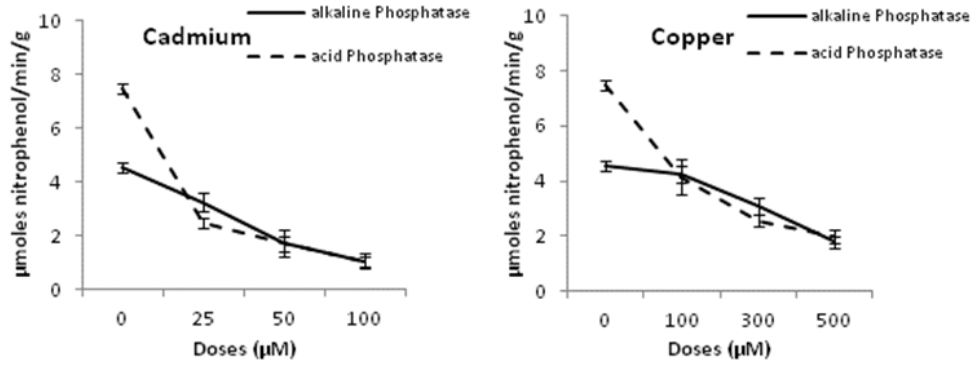

Fig. 10. Effects of Cd and Cu applied during 48 h of germination on phosphatase activity of germinating barley endosperm. Data represent mean ± SE, *n* = 3.

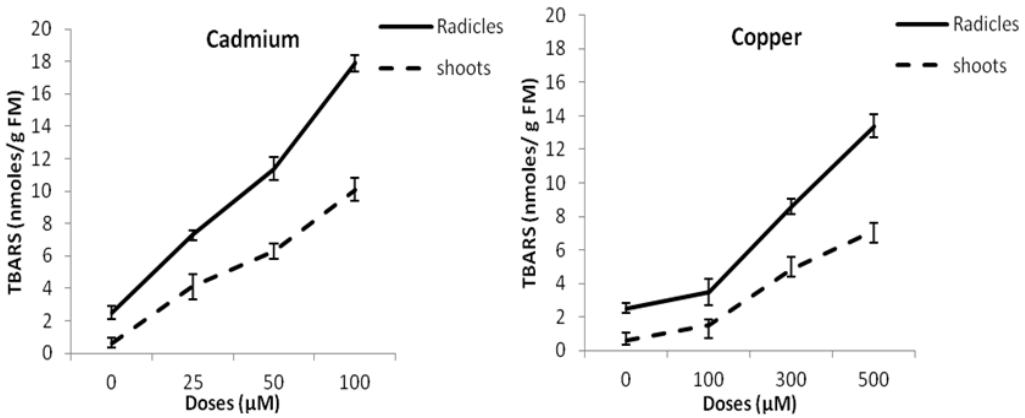

Fig. 11. Changes in MDA content in radicles and shoots of barley grains exposed to Cd and Cu during 48 h of germination. Data represent mean ± SE, *n* = 3.

pollutants due to their widespread occurrence, and their acute and chronic toxic effect on plants grown of such soils (Yadav, 2010). The regulatory limit of Cd in agricultural soil is 100 mg/kg soil (Salt *et al*., 1995) although this threshold is continuously being exceeded because of several human activities (Yadav, 2010). When plants are exposed to a high level of Cd, this causes a reduction in photosynthesis, water uptake, and nutrient uptake. Plants grown in soil containing high levels of Cd show visible symptoms of injury reflected in terms of chlorosis, growth inhibition, browning of root tips, and finally death (Wojcik and Tukiendorf, 2004; Mohampuria *et al*., 2007). Soil is also contaminated with Cu, which is considered as a micronutrient for plants (Thomas *et al*., 1998) and plays important role in CO_2 assimilation and ATP synthesis. Cu is also an essential component of various proteins like plastocyanin of the photosynthetic system and cytochrome oxidase of the respiratory electron transport chain (Demirevska-Kepova *et al*., 2004). However, enhanced industrial and mining activities have contributed to the increased occurrence of Cu in ecosystems (Yadav, 2010). Cu is also added to soils from different anthropogenic activities, including mining and smelting of Cu-containing ores. Mining activities generate a large amount of waste rocks and tailings, which get deposited at the surface. Excess Cu in soil plays a cytotoxic role, induces stress and causes injury to plants which ultimately leads to plant growth retardation and leaf chlorosis (Lewis *et al*., 2001). Exposure of plants to excess Cu generates oxidative stress and ROS. Oxidative stress disturbs metabolic pathways and damages macromolecules (Hegedus *et al*., 2001).

In the present work we have focused on the effects of Cd and Cu on some physiological processes involved in early (two days) germinative metabolism in barley seeds and seedling growth. The germination percentage suffered significant differences, compared to the control, under both Cd and Cu treatment, mainly at the highest concentrations (Fig. 1). A similar trend was observed by Mihoub *et al*. (2005) on pea (*Pisum sativum* L.) and Liu *et al*. (2007) on six wheat varieties (*Triticum aestivum* L.). The inhibition of seed germination is among the well known effects of toxic impacts of heavy metals found in many plants (Ernest, 1998). Figure 1 confirms that the inhibition caused by Cd and Cu started early, as soon as the four first hours of germination. However, some studies on pea (Siddiqui *et al*., 2009) and sorghum (*Sorghum bicolor*) (Kuriakose and Prasad, 2008) have related this inhibition of germinating seeds to a decrease in osmotic potential of the germination medium, particularly in the presence of a high concentration of Cd and Cu (Ahsan *et al*., 2007) making the absorption of water by grain rather difficult.

Ours results, in contrast, showed that during germination, water content of germination seeds (i.e., in the endosperm) was not affected by either Cd or Cu (Fig. 2). Previous studies conducted on pea seeds (Mihoub *et al*., 2005) exposed to high doses of Cd and Cu (5 mM) showed a similar trend after 4 days of germination. Thus, it is plausible, in the present investigation, that water cotyledonary reserves were sufficient to imbibe seed during the early stages of germination (i.e., 48 h) as also found for pea (Mihoub *et al*., 2005). Consequently, the germinating seeds did not need to absorb water from the germination medium. Moreover, the water content of radicles and shoots varied little under Cd and Cu treatment, following a similar pattern as the water content of seeds (Fig. 2). Cd and Cu accumulated in both radicles and barley grain at 48 h when exposed to the highest dose of Cd and Cu (Fig. 3). Seed coats can be impermeable to heavy metals following imbibitions in different plant species (25 species) in the presence of lead (Wierzbicka and Obidzinska, 1998). This might avoid the over-accumulation of a pollutant in the germinating seed. However, this assumption contrasts with the mechanism presented by Sfaxi-Bousbih *et al*. (2010) in bean seeds (*Phaseolus vulgaris* L.) showing that the extent to which a metal is avoided by the testa appears to decrease as treatment time increased, evidenced by the disappearance of the testa. Thus, it is possible that the testa becomes more permeable to a heavy metal allowing it to penetrate into endosperm tissue and delay or inhibit germination. The accumulation of that heavy metal might continue, resulting in a significant decrease in the growth of radicles and shoot length (Fig. 4) and dry weight production (Fig. 5).

Related results have been described by several authors working on cereals. He *et al*. (2008) suggested that heavy metal inhibition can be due to many anomalies of mitoses which directly affect the cell division and growth of rice seedlings exposed to Cd during germination. Liu *et al*. (2007) associated growth inhibition of wheat seedlings exposed to Cd and As to damage in membrane lipids due to the production of toxic oxygen free radicals which caused the per oxidation of membrane lipids. In addition, because plant roots are the first point of contact for heavy metal toxic factors, the reduction in root length was more prominent in wheat plants when exposed to different doses of Pb (0; 1; 2 and 4 mM) relative to the growth of shoots (Yang *et al*., 2010).

From a separate point of view, ours results indicate that in the presence of Cd or Cu, TSP suffered a small decrease compared with the control in the radicles and shoots but a significant increase in the endosperm (Fig. 6). Maheshwari and Dubey (2008), working on rice seeds, suggest that such a response could be the result of a decrease in protease activity in the grain in which RNases are secreted by the aleurone layer of cereal grains and are required for the hydrolysis and mobilization of RNA in seed tissues. Likewise, proteases are expressed during seed development and germination to facilitate hydrolysis and mobilization of storage proteins to supply the growing embryonic axis (Maheshwari and Dubey, 2008). Protease activity is sensitive to metals such as Cu, Cd, As and Ni, which may inhibit enzyme activity by binding to the functional groups of proteins, thus the decline in protease activity could be responsible for the observed concomitant accumulation of soluble protein, through disruption of

hydrolysis of endospermic storage reserves (Kranner and Colville, 2011), as also clearly evidenced in barley (Fig. 6).

When Cd and Cu accumulated substantially in both radicles and endosperms, sugar mobilization was obstructed, i.e., soluble sugar content accumulated considerably in the endosperm of germinating seeds exposed to Cd and Cu, especially at the highest levels of heavy metals (Fig. 7). In fact, these two metals reduced the transport of sugar from storage tissues of the endosperm to the growing embryonic cells of common bean (*Phaseolus vulgaris* L.) (Rahoui *et al*., 2008), pea (Mihoub *et al*., 2005; Smiri *et al*., 2009) and in the presence of Cd, Cu and Al in rice (Mishra and Dubey, 2008). Sugar content increased considerably in the embryonic axes of germinating bean seeds following H_2O imbibition, while almost no carbohydrates were available in the embryo tissues after Cd treatment (Sfaxi-Bousbih *et al*., 2010). This should be directly associated with an alteration in the freeing mechanism from cotyledons, notably TSS and fructose, which increased in Cd-treated cotyledons. The availability of sugars is regulated by several parameters such as the activity of carbohydrase, respiratory catabolism, biosynthesis and transport to growing embryonic axes (Monerri *et al*., 1986). For example, the freeing of fructose is closely controlled by the activity of invertase, which is the main supplier of this hexose during seed germination and Cd impairs the capacity of all invertase isoforms, which in turn, cannot correctly provide fructose (Sfaxi-Bousbih *et al*., 2010).

As shown in Fig. , increasing the concentrations of Cd and Cu in the growth medium resulted in a pronounced increase in Proline. In many plants, unfavorable environmental effects bring about the accumulation of Proline, which is, by itself, one of the most universal poly-functional stress-protective substances (Ashraf *et al*., 2007). Proline is known to accumulate under heavy metal exposure and is considered to be involved in the particular stress resistance (Chen *et al*.,2004; Gouia *et al*., 2003). The Proline accumulation in Cd or Cu-treated seedlings can be regarded as one of the most sensitive responses to water deficiency and osmotic stress (Ashraf et Harris, 2004). The capability of plants for a heavy-metal induced Proline accumulation could be brought about not only by a direct effect of Cd or Cu ions, but also by water deficiency (Shevyakova *et al*., 2003). This deficiency develops in the plant tissues under the conditions of metal stress due to damage to the water-absorbing capacity of roots. Schat *et al*. (1997) considered that Proline accumulation is mainly induced by the water stress component of Cd toxicity while Kastori *et al*. (1992) argued that Proline accumulation occurred as a result of Cd toxicity independent of any water-stress component. From an experimental point of view, however, causes and consequences are quite difficult to distinguish. Besides its putative impact on osmotic adjustment processes, Proline was shown to protect enzymes and cellular structures against heavy metal damages as a consequence of the formation of Cd–Proline complexes (Sharma, 2006) or against maintenance of the glutathione redox state, thus indirectly acting as an antioxidant (Siripornadulsil, 2002). So, greater Proline content in Omid may be a major factor involved in the comparatively higher degree of resistance of this variety.

Seed germination relies almost exclusively on seed reserves for the supply of metabolites for respiration, as well as other anabolic reactions (Liu *et al*. 2005). Starch is quantitatively the most abundant storage material in all seeds, and available evidence indicates that in germinating seeds starch is degraded predominantly via the amylolytic pathway (Dua and Shawney, 1991). The inhibition of these enzymes within this pathway might explain the inhibition of germination or growth (Lahmadi *et al*. 2011). In this study, amylase activity, particularly α-amylase activity, was significantly repressed by Cd and Cu, while β-activity did not vary significantly under the impact of metallic stress. Liu *et al*. (2005) found that α-amylase activity decreased as the dose of As increased over the whole concentration range but that β-amylase decreased only for the higher doses of As (> 2 mg/l). It is possible that this inhibition resulted from a direct effect of Cd ions on the enzymes, by displacing the Ca^{2+} ions that are essential for amylasic activities, as suggested by He *et al*. (2008) in germinating rice seeds under Cd stress and by Lahmadi *et al*. (2011) in germinating wheat seeds under Pb stress. Phosphatases are responsible for the release of phosphate and are thus important for the production of energy (ATP). Mihoub *et al*. (2005) found, in agreement with ours results, that the activity of phosphatases, especially the activity of acid phosphatase, was negatively affected by increasing the concentration of Cd and Cu in medium. In the same way, Kuriakose and Prasad (2008) reported that the activities of hydrolyzing enzymes such as acid phosphatase and α-amylase in sorghum seeds exhibited a significant decrease as Cd concentration increased in the medium.

It is well documented that excess level of heavy metals (Ahsan *et al*., 2006; Guo *et al*., 2007; Sfaxi-Bousbih *et al*., 2010; Lamhamdi *et al*., 2011) are harmful due to the production of reactive oxygen species (ROS) by autoxidation and Feton reactions (Jonak *et al*., 2004). ROS are regulated as the initiators of peroxidative cell damage. TBARS formation in plants exposed to adverse environmental conditions is a reliable indicator of cellular free radical generation. Therefore, we measured the cellular TBARS concentrations as an indicator of Cd and Cu-induced ROS formation. Greater accumulation of TBARS was observed in the presence of highest concentration of Cd and Cu (100µMCd and 500µM Cu) that were significant compared to control. TBARS measurement has been routinely used in heavy metal-treated plant samples as an indicator of lipid peroxidation caused by oxidative stress (Cho and Seo, 2005; Kim *et al*., 2005). Our observation is in good agreement with previous research indicating that ROS formation was caused by excess cadmium and copper treatment (Jonak *et al*., 2004).

Conclusions

Taken together, our finding suggest that the inhibition of seed germination, after exposure to Cd and Cu, is not the consequence of starvation in water uptake by tissues, but may be due to a failure in the organic and mineral reserves mobilization process from barley endosperm in which we have noted a high decline of α-amylase, acid phosphatase and alkaline phosphatase activities associated to a high accumulation of soluble protein, soluble sugar, proline and mineral element contents. This fact, will testified that early stage of germination (two days) seems, in the present work, a very sensitive stage of plant development. Hence, it is conceivable that the investigation of the respiratory activity might provide deeper insights about this sensitive stage of seed germination of barley under metallic stress.

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