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Full Length Research Paper

Alteration of cotyledonary globulins and albumins mobilization in pea exposed to cadmium

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Seed germination and post germination events are complex physiological processes which can be seriously affected by heavy metals. Cadmium (Cd) is an environmental pollutant extremely toxic to plants and other living organisms including humans. To assess Cd phytotoxicity, pea seeds (*Pisum sativum* L. var. douce province) were germinated in distilled water or 200 µM $CdCl_2$. Cadmium treatment induces a deleterious inhibition in embryonic axis growth by limiting the biomass transfer from cotyledons, particularly the hydrolysis of the major storage proteins and, thereby, the amino acids freeing. The electrophoretic analysis of albumins and globulins showed that heavy metal alters the breakdown of 33 and 19 kDa polypeptides (alkaline legumin and small vicilin, respectively), as well as other peptides (17-19 kDa) which may be produced by a previous hydrolysis of albumins.

Key words: Albumin, amino acid, cadmium, germination, globulin, *Pisum sativum* L.

INTRODUCTION

Heavy metals contamination has disastrous effects on plant productivity and both animal and human health, particularly, cadmium (Cd) is recognized as one of the most phytotoxic pollutant found in the air, water and soil (Wagner, 1993; Mendiola et al., 2011). It can interrupt a wide range of physiological processes, including photosynthesis (Baryla et al., 2001), plant water status (Barcelo and Poschenrieder, 1990), mineral nutrition (Chaoui et al., 1997a) and antioxydative state (Chaoui et al., 1997b, 2004; Chaoui and El Ferjani, 2005; Maksymiec, 2007).

Seeds are crucial organs for plant life dispersal and survival, and the germination time control is a strong advantage under adverse environmental conditions (Bewley, 1997). Seed germination is triggered by tissue imbibition (uptake of water) and, after an apparent lag phase, is followed by the elongation of the radicle and thereafter of the whole embryonic axis (Bewley and Black, 1994; Bewley, 1997).

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Recognized explanations for the impact of Cd on the seed germination are that it produces respiratory disturbances (Smiri et al., 2009, 2010a, b) and limitation in nutriment (minerals and carbohydrates) availability (Rahoui et al., 2010; Sfaxi-Bousbih et al., 2010) but not an osmotic effect delaying the seed tissues hydration capacity (Mihoub et al., 2005; Raouhi et al., 2008). Nevertheless, little is known about the behaviour of breakdown of storage proteins under Cd stress, and the effects of heavy metals on proteolysis during seed germination and post-germination have not been studied thus far.

Pea seeds (*Pisum sativum* L.) represent great nutritional importance due to their high-quality source of protein and starch (Hedley, 2001). The main storage proteins of pea are water-soluble albumins and salt-soluble globulins consisting, respectively, in 25 to 35 and 50% of total proteins recovered in the seeds of several lines (Schroeder, 1982). Two major groups of globulins have been identified on the basis of their sedimentation coefficients; the first one is 7S fraction: convicilin (70 kDa trimer) and vicilin (71 kDa trimer) and the second is 11S fraction: legumin (380-410 kDa) with 6 subunits (α acid 40 kDa and β basic 20 kDa linked by a disulfide bridge)

(Gatehouse et al., 1984; Gueguen, 1991). Regarding albumins, two dimers PA1 (PA1a; 6 kDa and PA1b; 4 kDa), PA2 (26 kDa) and tetramer lectin (50 kDa) have been characterized (Higgins et al., 1986; Masson et al., 1986).

Therefore, the aim of the present work was to investigate the influence of 200 µM $CdCl_2$ on the status of major storage proteins (albumins and globulins) and amino acids in reserve tissues of pea. The working hypothesis is to verify that Cd stress can inhibit the mobilization of cotyledonary biomass, particularly; nitrogen starvation in embryonic axis should be associated with Cd-imposed delay in post-germination events.

MATERIALS AND METHODS

Seeds of *Pisum sativum* L. (cv. douce province) were surface-sterilized with 2% of sodium hypochlorite for 10 min and then rinsed several times and soaked in distilled water at 4°C for 30 min to obtain an initial stage (Murray, 1979). Seeds were germinated at 25°C in the dark over two sheets of filter paper moistened continuously with the same volume of distilled water or 200 µM $CdCl_2$.

Seedlings were transferred to plastic beakers filled with aerated solutions of calcium chloride (100 µM) (Guardiola and Sutcliffe, 1971; Monerri et al., 1986) in the presence or absence of 200 µM CdCl2 in a growth chamber (16 h light–8 h dark) under a light intensity of 150 µmol s^{-1} m^{-2}, day/night temperature of 25/20°C and 65 (±5%) relative humidity. At appropriate intervals, cotyledons were sampled for the assays.

Cotyledons were powdered with quartz sand in a pre-cooled mortar and pestle at 4°C and homogenized with H_2O. Homogenate was stirred for 30 min and centrifuged at 20,000 g for 30 min at 4°C. The supernatant was referred to as “Albumin”. The pellet was suspended in 50 mM Tris-HCl buffer (pH 8.6) containing 1 M NaCl and centrifugated at 20,000 g for 30 min at 4°C. The supernatant was used for “Globulin” analysis. Proteins were quantified according to Bradford (1976) using bovine serum albumin as standard.

Albumin and globulin fractions (50 µg) were subjected to 15% SDS-PAGE (Laemmli, 1970). The gels were stained overnight with 0.125% Coomassie blue R-250 in methanol:acetic acid:water (5:1:4) and proteins were fixed with 7% acetic acid. The mixture of molecular weight markers consisted of BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.2 kDa).

Cotyledons were powdered and homogenized in 80% ethanol, boiled for 30 min at 70°C, and then the homogenate was centrifuged at 8,000 g for 20 min at 4°C. Amino acids were determined in the supernatant by ninhydrin staining according to Moore and Stein (1954) using glycine as standard.

The data reported are the mean values ± SE of several replicates independently. Statistical analysis was subjected to one-way ANOVA test. A significant level of 0.05 was used for all statistical tests.

RESULTS

Effect on embryonic axis growth

The ultimate event following the germination process; that is protrusion of radical via seed coat is the embryo growth. Figure 1a shows higher sensitivity to Cd exhibiting decrease in dry weight of embryonic axis (53% from control after 9 days of germination). The cotyledonary biomass mobilization reached 62 and 54%, respectively, in control and treated seeds after 9 days as compared to the dry weight of ungerminated seeds (Figure 1b). However, after 15 days of mobilization, cotyledons retained only 7% (9 mg) of initial dry weight (128 mg) under control conditions, while heavy metal treatment inhibited the freeing of 37% of storage compounds (Figure 1b and 1c). So, prolonging the experience duration was required and explained by the “absence” of Cd effect on cotyledon biomass allocation during the *sensu stricto* period of germination (9 days).

Effect on globulins and albumins

The breakdown of globulins and albumins in Cd-treated cotyledons is compared to the kinetic of control patterns (Figures 2 and 3). Since it is difficult to get a resolving model of the different subunits of “Albumin” and “Globulin” rich fractions after SDS-PAGE analysis, only changes in the major polypeptide bands are emphasized.

Globulins were resolved into five major polypeptides referred to as A, B, C, D and E with apparent molecular weight of 69, 50, 40, 33 and 19 kDa, respectively (Figure 2). The largest band A has rapidly disappeared after 6 days of H_2O germination, and Cd treatment did not influence significantly its hydrolysis. Instead, the polypeptides in B and D seem to be tough to hydrolysis and disappeared after 15 days under control conditions, but not in the presence of Cd. The later did not affect the mobilization of C, while persistence in band intensity of low molecular weight peptides (E) was seen.

The electrophoresis of the albumins fraction showed that it was composed of three major polypeptides (A, B and C) (Figure 3). Those of molecular weights > 45 kDa (A) were rapidly mobilized after 6 days and disappeared even after 9 days in control conditions, but the degradation was reduced as transitional way as response to Cd toxicity. The same observation is true for B. However, C began to breakdown after 6 days of germination in H_2O until an absolute disappearance occurring after 15 days, whereas it persists until the end of the test duration in the presence of Cd (*Asterix*; Figure 3). It seems that some bands disappeared during germination but there was also an accumulation of new peptides as indicated by the high colored band (C; 17 kDa).

An adjusting model of correlation between the distribution of storage proteins (albumin + globulin) content and whole biomass of cotyledons in the absence and presence of Cd is showed in Figure 4. At 0.05 error risk, the correlation coefficients r, obtained from the determination coefficients r^2, suggest highly significant correlation between biomass mobilization and proteolysis.

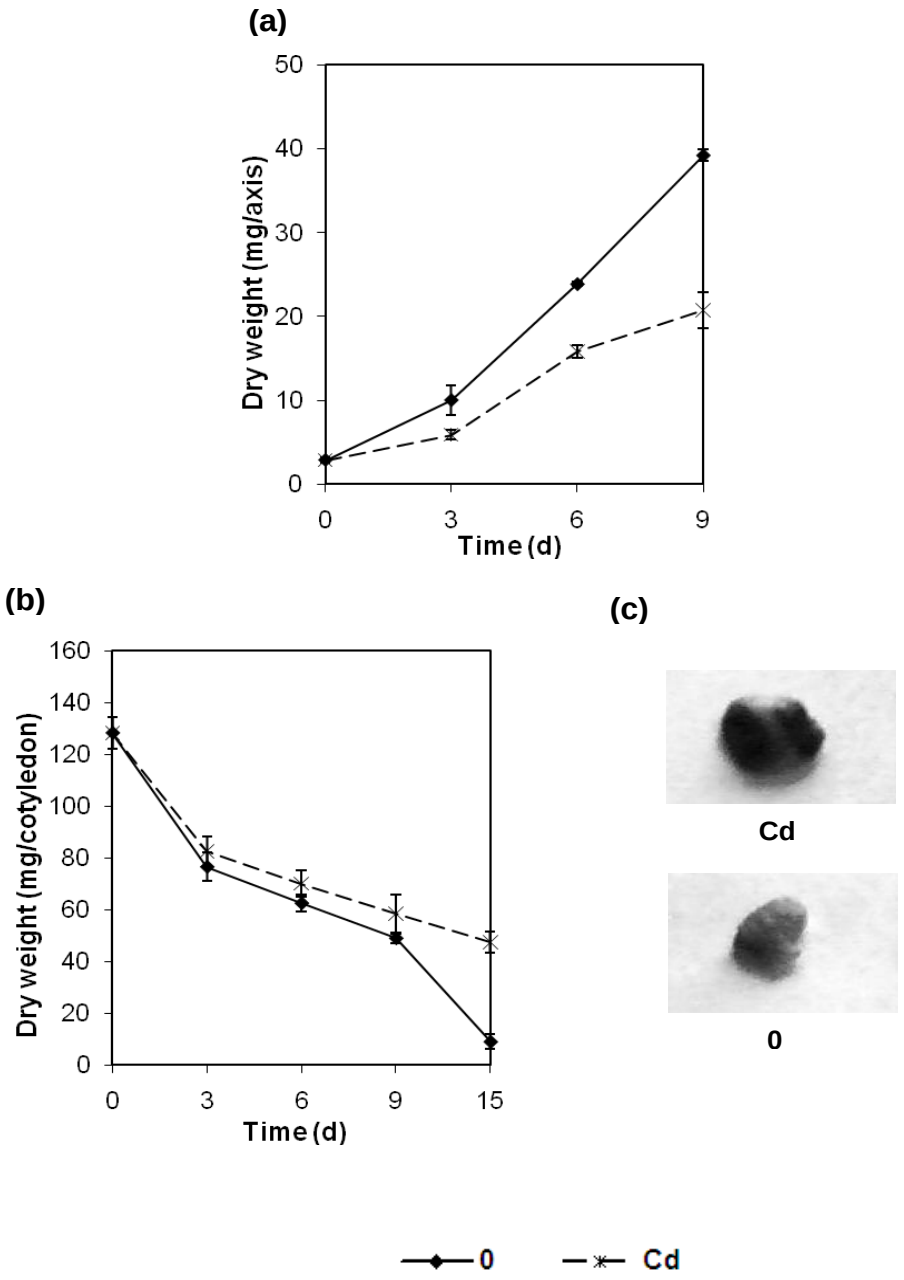

Figure 1. Embryo (a) and cotyledon (b) dry weight of pea treated with H_2O (0) or 200 µM Cd. Values are the means of five measurements. Each measurement was performed with 80 germinating seeds. Error bars (SE) indicated when large enough to be shown. (c) Cotyledons of 15-days-old seedlings.

Thus, Cd should alter similarly the mobilization of storage proteins, as well as other reserves categories, notably sugars.

Effect on free amino acids

The quantitative changes of free amino acids in reserve tissues are showed in Figure 5. In control, an increase to 5-fold was noticed after 3 days. Then no significant variation was recorded, followed by a drastic decrease since 9th day. However, in Cd-treated cotyledons, the content of amino acids was always higher.

DISCUSSION

The present work has pointed out that Cd exposure

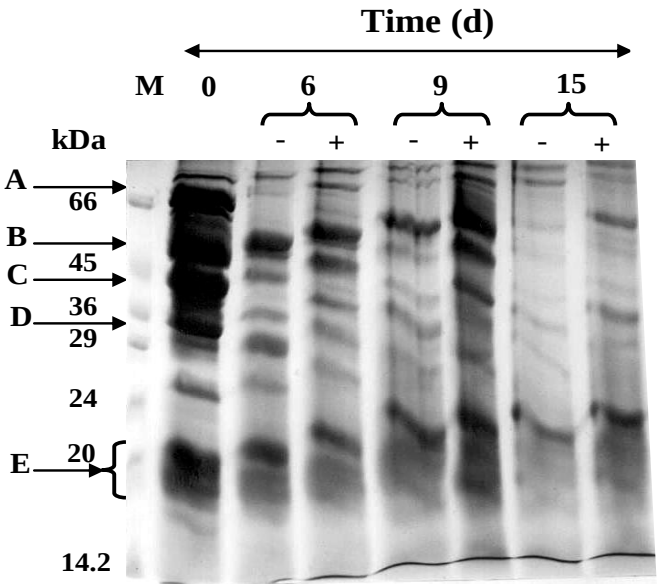

Figure 2. Electrophoregram of cotyledonary globulin (50 µg) of ungerminated seed (0) of pea treated with H_2O (-) or 200 µM Cd (+). M: Molecular weight marker (kDa). A, B, C, D, E: Major polypeptide bands. Gel was stained with Coomassie Blue. Experiment was performed in duplicate.

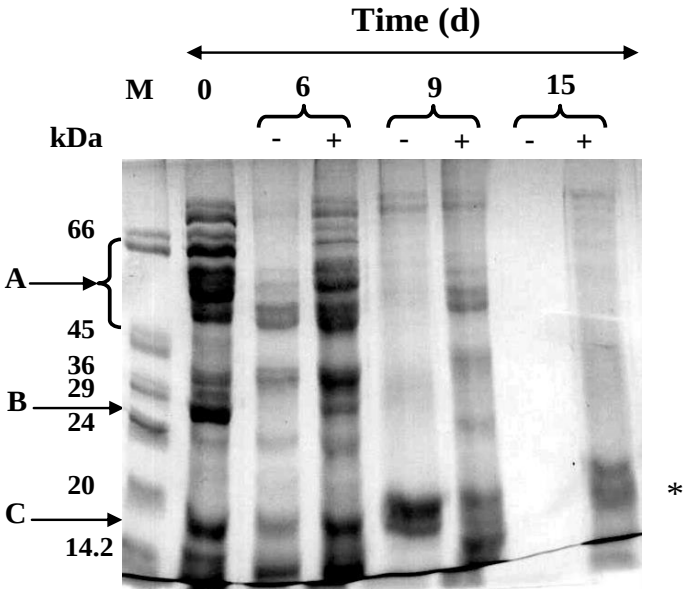

Figure 3. Electrophoregram of cotyledonary albumin (50 µg) of ungerminated seed (0) of pea treated with H_2O (-) or 200 µM Cd (+). M: Molecular weight marker (kDa). A, B, C: Major polypeptide bands. *Asterix* denote bands which appear to represent the degradation products. Gel was stained with Coomassie Blue. Experiment was performed in duplicate.

disturbs the mobilization of biomass from reserve tissues and, consequently, delays the growth of embryonic axis (Figure 1). Heavy metals alter seed germination metabolism through respiratory disorders, either by

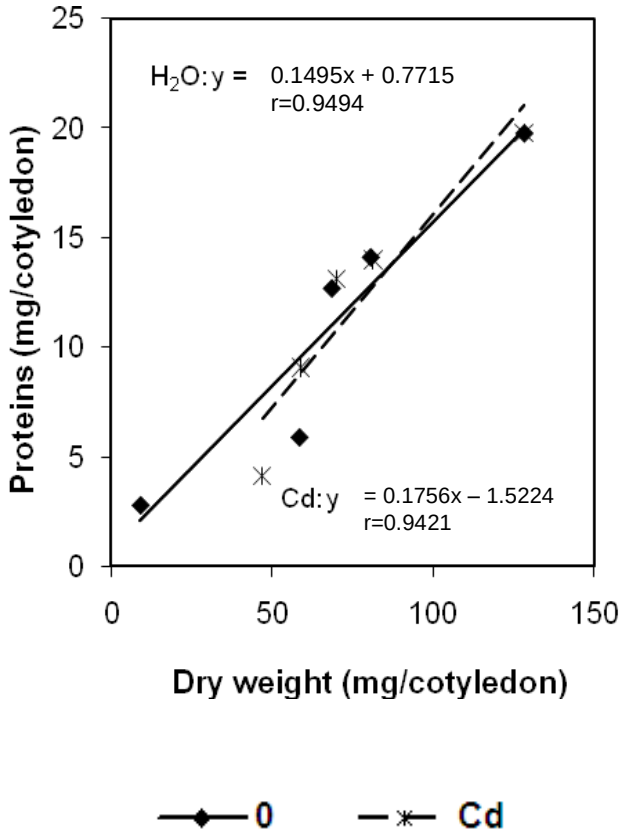

Figure 4. Correlation between proteins (globulin + albumin) content and dry weight in cotyledons of pea treated with H_2O (0) or 200 µM Cd for 0, 3, 6, 9 and 15 days. Values are the means of five measurements. Each measurement was performed with 80 germinating seeds. r: correlation coefficient.

interfering with the enzyme activities of the krebs cycle and electron transport chain (Smiri et al., 2009, 2010b) or by affecting mitochondrial redox systems (Smiri et al., 2010a), and failure in micronutrients and carbohydrates availability (Mihoub et al., 2005; Smiri et al., 2009; Sfaxi-Bousbih et al., 2010). Our findings suggest that nitrogen starvation should be correlated with the Cd-imposed inhibition in embryo growth.

The SDS-PAGE subunit proteins found in pea cotyledons under control conditions (Figures 2 and 3) were similar to those obtained by Martinez-Villaluenga et al. (2007); the main albumins fraction showed majority bands with molecular weights of 47.5, 40.5, 32.5 and 23.5 kDa, while the main globulins with molecular weights of 69, 45-50, 35-43, 20-35 kDa has been assigned as convicilin, non processed vicilin, acidic and alkaline subunits of legumin and processed vicilin, respectively (Casey and Domoney, 1999; Tzitzikas et al., 2006). Moreover, the hydrolysis patterns of major storage proteins (globulin and albumin) in germinated-pea seeds agree with the results previously reported by Basha and Beevers (1975). In fact, the maximum of albumin degradation occurred before the globulin one. The quantitative serological measurement revealed the same finding: first albumins (2-3 days), then legumin until day 7 to 8 after onset of the germination and finally vicilin (9-10 days) (Müntz et al., 1985). The exposure to Cd increases the contents of the globulin and albumin polypeptides, especially bands of 19 kDa and 17 kDa, respectively (Figures 2 and 3).

Possible inhibitory action of the heavy metal on protease activities may be suggested, since proteolytic breakdown of storage proteins takes place by combined actions of endo- and exopeptidases (Wilson, 1986; Shutov and Vaintraub, 1987; Bewley and Black, 1994; Müntz 1996).

During germination of pea seeds, the nitrogen content of the cotyledons decreases (Guardiola and Sutcliffe, 1971) and the accumulation of amino acids, which accompanies this depletion, indicates that alterations are caused by hydrolysis of the reserve proteins in the cotyledon followed by transport of the ultimate products

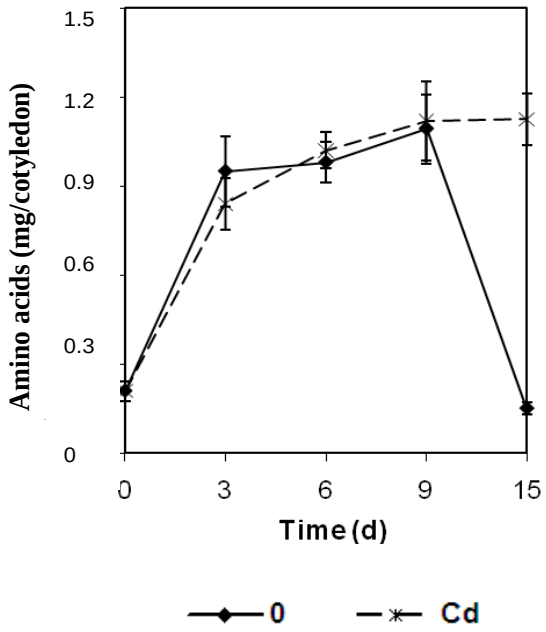

Figure 5. Amino acids content in cotyledons of pea treated with H_2O (0) or 200 µM Cd. Values are means of six measurements. Each measurement was performed in an extract obtained from several cotyledons. Error bars (SE) indicated when large enough to be shown.

to the growing embryonic axis (Müntz et al., 1985). After 9 days of Cd treatment, cotyledons retain the amino acids provided by proteolysis, suggesting that the transport mechanism must be restricted as previously suggested in other situations of heavy metal stress during the germination of legume seeds (Mihoub et al., 2005; Smiri et al., 2009; Rahoui et al., 2010).

Conclusions

The effects of Cd can be summarized in the limitation in proteins mobilization following categories: the alkaline legumin subunit (33 kDa), the small chain of vicilin (19 kDa) and the low molecular weight polypeptides (17-19 kDa) natives of the mature seed or products of degradation of higher molecular weight albumins.

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