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Full Length Research Paper

Effect of cadmium on germination, growth, redox and oxidative properties in *Pisum sativum* seeds

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Pea seeds were treated with 5 mM $CdCl_2$ for 5 days. Experiments carried out in cotyledons and embryonic axes were performed to evaluate the redox and oxidative properties. Germination rate and embryonic axis growth were determined. After five days, Cd treatment caused 60% decrease in germination success, and 50% inhibition in embryo length. The reduction level [NADPH/ ($NADP^+$ + NADPH)] was used to define the redox status. The reduction level in Cd-treated mitochondrial and peroxisomal fractions was ~20 to 70% lower than that of the control (water-treated) from 120 h of exposure. Under normal conditions, the intracellular milieu is predominately reducing, but stress conditions can shift the redox balance toward an oxidizing milieu. NADPH oxidase is considered to be oxidative stress-related enzymes. NAD(P)H oxidase activities were strongly stimulated after Cd exposure. We suggest that alteration of redox and oxidative properties in both tissues of pea seeds due to treatment with $CdCl_2$ is highly responsible for decrease of germination success and inhibition of embryonic axes growth.

Key words: Cadmium, germination, oxidative stress, *Pisum sativum*, redox.

INTRODUCTION

Environmental stresses often lead to great yield losses under various agricultural production systems. Of diverse abiotic stresses, heavy metal is a pernicious problem affecting the productivity and quality of economically valuable crops (Wagner, 1993). Certain heavy metals, such as copper (Cu) or iron (Fe), can be toxic through their participation in Fenton-type reactions producing reactive oxygen species (ROS), which are known to be extremely harmful for living cells (Stochs and Bagchi, 1995). Cadmium (Cd^{2+}) is a non-redox metal unable to take part in this type of reaction. Nevertheless, it has been clearly demonstrated that Cd^{2+} induces changes in the antioxidant status in plants (Grataö et al., 2005). Cd^{2+} is regarded as a non-essential metal without any known physiological function. It is extremely toxic to plants and animals, has a long half-life and is extremely persistent in the environment (Wagner, 1993).

Moreover, in pea plants, long-term exposure to Cd^{2+} produces oxidative stress in roots, as a result of disturbances in enzymatic and no enzymatic antioxidant defenses, bringing about an increase in ROS accumulation (Rodríguez-Serrano et al., 2006). Garnier et al. (2006) demonstrated that Cd^{2+} induces a transient increase in cytosolic Ca^{2+} concentration that appears to regulate the extracellular NADPH-oxidase dependent generation of hydrogen peroxide (H_2O_2). In this way, transcriptome analysis of the antioxidative enzymes in leaves of pea plants grown with Cd^{2+} and treated with some modulators of the signal transduction cascade suggested that at least Ca^{2+} channels and H_2O_2 were involved in some steps between the Cd^{2+} signal and transcript expression of some antioxidant enzymes. This indicated the existence of cross-talk between these elements and ROS metabolism during Cd^{2+} stress (Romero-Puertas et al., 2007). Moreover, in roots and leaves of pea plants Cd^{2+} produced a significant inhibition of growth, as well as a reduction in the transpiration and photosynthesis rate, chlorophyll content of leaves, and an

alteration in the nutrient status (Sandalio et al., 2001; Romero-Puertas et al., 2004).

Usually, experimental studies of heavy metals impact on adult plants have applied low concentrations (Woolhouse, 1983; Ernst, 1998). Nevertheless, high pollutant doses have been used in seed germination assays (Chugh and Sawhney, 1996; Rahoui et al., 2008, 2010), although the germination is considered as a sensitive process as compared to other stages of plant development (Ernst, 1998). This is explained, at least in part, by the fact that seed coats may be impermeable to heavy metals. This can avoid an over-accumulation of contaminant during the vital heterotrophic regime of germinating seed. Consequently, the behaviour of seed germination is not regarded according to heavy metal doses in the seed surrounding medium, but considered with respect to the real accumulation and the compartmentation of pollutant at cellular and subcellular levels (Woolhouse, 1983; Ernst, 1998; Rahoui et al., 2010). Delay in germination can be associated with disorders in the event chain of germinative metabolism which is a highly complex multistage process, but one of underlying metabolic activities following imbibition of the seed is the resumption of respiration.

Studies on the mitochondrial biogenesis during germination have focused on seed storage tissues in the post-germination period. In pea cotyledons, this has been suggested as a result of the activation of preformed mitochondria through the import of existing polypeptides in the cytoplasm (Nawa and Asahi, 1971; Sato and Asahi, 1975). Functional mitochondria capable of adenosine triphosphate (ATP) synthesis have been isolated from dry sunflower seeds (Attucci et al., 1991), suggesting that the energy for mitochondrial metabolism may be supplied by oxidative phosphorylation. As well as a general increase in mitochondrial protein in the post-germination phase, there is also a differential regulation of mitochondrial metabolism in lipid storing seeds, to support the conversion of storage lipid to sugars for export to the growing embryo (Ehrenshaft and Brambl, 1990; Hill et al., 1992). All these processes require energy and thus depend on the competence and stability of mitochondria (Prasad et al., 1995). Abiotic stresses have pronounced and complex effects on mitochondrial properties, in terms of both their function and their biogenesis. The response of mitochondrial respiration is likely to be controlled by the adenylates and the substrate supplies (Atkin and Tjoelker, 2003).

Nicotinamide adenine dinucleotide phosphate (NADP) play vital roles in signalling via the generation and scavenging of reactive oxygen species (Berger et al., 2004; Noctor et al., 2006; Hunt et al., 2007) and in systems controlling adaptation to environmental stresses such as UV irradiation, salinity, heat shock and drought (Mittler et al., 2004; Chai et al., 2006). The plant mitochondrial inner membrane contains a branched electron transport pathway that includes multiple enzymes for the oxidation of both cytosolic and matrix NAD(P)H (Agius et al., 1998; Moore et al., 2003). When plants are grown under stressful environments, the reduced form of cytosolic NADP is produced in the pentose phosphate pathway by glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) and plays critical roles in the generation of ROS and in the production of anti-oxidants as ROS scavengers (Murata et al., 2001; Tamoi et al., 2005). In addition to the classical complexes, all plant mitochondria possess alternative enzymes that confer on them the capacity to oxidize external NAD(P)H or internal NAD(P)H independently of complex I and to transfer electrons directly from reduced ubiquinone to oxygen via an alternative oxidase (Douce and Neuburger, 1989; Rasmusson et al., 2004).

This work aimed to examine the impact of imbibition with Cd solution on germination rate and the behaviour of some enzyme capacities involved in the redox regulation in the mitochondrial and peroxisomal fractions of cotyledons and embryos of germinating pea seeds.

MATERIALS AND METHODS

Seeds of pea (*Pisum sativum* L. cv. douce province) were disinfected with 2% of sodium hypochlorite for 10 min and then rinsed thoroughly and soaked in distilled water at 4°C for 30 min to obtain an initial stage. Seeds were germinated at 25°C in the dark for 5 days over two sheets of filter paper moistened with distilled water or aqueous solution of 5 mM $CdCl_2$. Germinated seeds were recorded until the maximum germination of control (H_2O) was obtained. Germination rate was calculated as a percentage of the control and germinating seeds were sampled for the assays. At harvest, the coat was removed and the embryonic axes and cotyledons were weighed and stored in liquid nitrogen until analysis.

Mitochondria, from germinating pea seeds were isolated by the procedure of Smiri et al. (2009). Cotyledons and embryonic axes were ground in a mortar and pestle with sand and the following medium (w/v = 1/5): 50 mM Tris- HCl (pH 8.0), 0.4 M saccharose, 1 mM EDTA, 5 mM ascorbic acid and 1 mM $MgCl_2$. The homogenate was squeezed through double cheesecloth, centrifuged at 3,000 g for 20 min. Mitochondria from supernatant were sedimented by centrifuging it at 20,000 g for 30 min. The supernatant obtained was carefully decanted and designated as “peroxisomal fraction”. The pellet was re-suspended in the homogenising media (w/v = 1/0.5) 50 mM Tris- HCl (pH 8.0), 0.4 M saccharose and referred to as “mitochondrial fraction” (Smiri et al., 2010a). Coenzyme extraction and concentration determination was processed as previously described (Smiri et al., 2010a). For NAD^+ and $NADP^+$, 0.2 N HCl was used to homogenize the burst mitochondria and the peroxisomal fractions of cotyledons and embryonic axes (w/v = 1/10); for NADH and NADPH, 0.2 N NaOH were used. Each homogenate was heated in a boiling water bath for 5 min, cooled in an ice bath, then centrifuged at 10,000 g at 4°C for 10 min. The supernatant solutions were transferred to separate tubes and kept on ice for coenzyme assay (Zhao et al., 1987). Enzyme cycling assays were employed with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazoliumbromide (MTT) as the terminal electron acceptor (Matsumura and Miyachi, 1980). The rate of reduction of MTT (measured at 570 nm) was directly proportional to the concentration of coenzyme. Standard curves were prepared for each coenzyme in each experiment.

NADPH oxidase activity was determined in an assay mixture

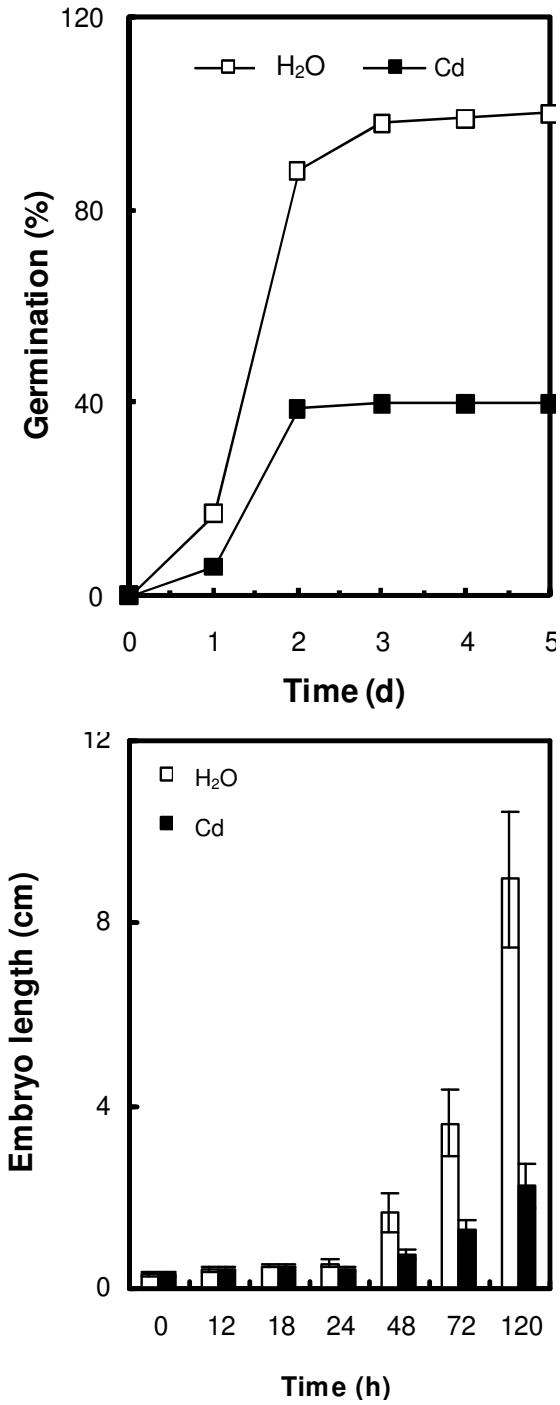

Figure 1. Germination rate and embryonic axis length of pea seeds after imbibition with H_2O or 5 mM $CdCl_2$. Each experiment was carried out with 80 germinating seeds.

containing 100 mM sodium acetate (pH 6.5), 1 mM $MnCl_2$, 0.5 mM p-coumaric acid, 0.2 mM NADPH and enzyme extract. The reaction was monitored by following the decrease in absorbance at 340 nm, with extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ (Ishida et al., 1987). Each treatment consisted of six replicates and each experiment was carried out at least twice at different times. All data were statistically analyzed using one-way ANOVA. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The most critical stages in the life cycle of higher plants are seed germination and seedling establishment (Bewley, 1997). Germination starts with the uptake of water by the quiescent dry seed and terminates with the elongation of the embryonic axis (Bewley and Black, 1994). The decreased percentage of germination was observed after 24 h and after 5 days, it reached a 60% decrease from the control. Analysis of embryo length showed significant differences between seeds germinating on water and Cd. The inhibitory effect of the metal was increased in a time-dependent manner (Figure 1). When the medium surrounding the seed was contaminated with Cd, delays in germination were often observed (Rahoui et al., 2008, 2010). This can be associated with several disorders in the event chain of germinative metabolism.

Especially, seed germination and subsequent embryo growth are important stages of the plant life and highly sensitive to surrounding medium fluctuations, because the germinating seed is the first interface of material exchange between plant development cycle and environment (Ernst, 1998). The interactions of Cd with crucial physiological functions in adult plants have been widely investigated (Wagner, 1993), and drastic reduction of biomass production and nutritional quality have been observed in crops grown on soils contaminated with this non-essential element (Ernst, 1998). Cd^{2+}, with no reported biological function except one occasion as a cofactor for carbonic anhydrase in marine diatom (Xu et al., 2008), disturbs the cellular metabolic process by producing excessive ROS leading to oxidative stress. ROS is known to react with proteins, nucleic acids and lipids causing deleterious effects on various cellular processes (Møller et al., 2007). Its high affinity for sulfhydryl and oxygen containing groups results in blocking the essential functional groups of biomolecules (Noctor et al., 2006).

Consequently, it inhibits the uptake and transport of many macro/micronutrients and thus, induces the nutrient deficiencies. Considerable effort has been devoted to understanding the molecular basis of resistance to heavy metal, and it has been revealed that plants react to abiotic stresses by the alterations in metabolic rates, protein turnover, osmolytes, membrane function, and gene expression (Møller, 2001). All these processes require energy and thus depend on the competence and the stability of mitochondria (Prasad et al., 1995). Abiotic stresses have pronounced and complex effects on mitochondrial properties in terms of both their function and their biogenesis. Germinating seeds must adapt their metabolic and developmental programmes to the prevailing

Table 1. Effect of Cd on NADP balance in germinating pea seeds.

Time(h)			0	12	18	24	48	72	120
Plant part	**Fractions**	**Treatments**							
			$NADP^+$ Balance:[$NADP^+$ /($NADP^+$ + NAD^+)]						
C	P	H_2O	48±5	33±3	24±2	14±1	15±1	10±1	9±1
		Cd	48±5	41±1	22±2	24±1*	24±1*	24±1*	26±2*
	M	H_2O	20±1	15±1	15±3	16±4	14±1	14±1	14±2
		Cd	20±1	11±1	11±1	12±1*	12±1*	13±1*	21±1*
E	P	H_2O	8±1	4±1	5±1	3±1	1±1	1±1	1±1
		Cd	8±1	4±1	4±1	3±1	1±1	1±1	2±1
	M	H_2O	64±6	59±1	43±3	40±1	35±3	35±2	32±3
		Cd	64±6	59±1	47±4	39±1	33±1	35±1	33±1
			NADPH balance:[NADPH /(NADPH + NADH)]						
C	P	H_2O	28±1	17±1	19±1	22±1	17±1	14±1	14±1
		Cd	28±1	18±1	18±1	17±1	15±1	15±1	15±1
	M	H_2O	64±3	50±3	46±1	45±3	51±4	50±1	44±1
		Cd	64±3	46±1	40±5	47±2	46±3*	38±1*	36±1*
E	P	H_2O	21±1	18±1	16±1	16±1	14±1	21±1	21±1
		Cd	21±1	19±1	14±1	10±1*	9±1*	11±1*	12±1*
	M	H_2O	36±1	39±1	41±1	46±1	42±1	47±2	44±1
		Cd	36±1*	53±1*	61±1*	71±1*	77±2*	62±2*	54±1*

$NADP^+$ balance was evaluated as the percentage of $NADP^+$ from total coenzyme ($NADP^+$ + NAD^+). NADPH balance was evaluated as the percentage of NADPH from total coenzyme (NADPH + NADH). Data are the means ± S.E. of 6 individual measurements. Each measurement was performed in an extract obtained from several germinating seeds. Significant differences between treated and control were determined using one-way ANOVA. *P<0.05. C, cotyledons; E, embryonic axis; M, mitochondria; P, peroxisomal fraction.

environmental conditions to become photoautotrophic before their nutrient reserves become exhausted. A germinating seed relies almost exclusively on its reserves to supply metabolites for respiration. To operate in stressful conditions, plant mitochondria might have evolved distinctive features designed to increase their metabolic flexibility and stress tolerance, which is clearly illustrated by both the complexity of the mitochondrial proteome and by its dynamic response to environmental stress, and the response of mitochondrial respiration is likely to be controlled by the substrate supplies (Atkin and Tjoelker, 2003). In the present study, the NADP reduction levels and NADPH oxidase activity was determined to evaluate their possible role in combating the cadmium toxicity.

The $NADP^+$ balance [$NADP^+$/($NADP^+$+NAD^+)] in Cd-treated mitochondrial and peroxisomal fractions of cotyledons was ~50 to 190% higher than that of the control (water-treated) from 120 h of exposure, and did not change in the embryonic axis. The NADPH balance [NADPH/(NADPH+NADH)] in Cd-treated mitochondria of cotyledons was ~20% lower than that of the control (water-treated) from 120 h of exposure and ~20% higher than that of the control in embryonic axis. It was ~20% lower than that of the control (water-treated) peroxisomal fractions in the embryonic axis (Table 1). The reduction level [NADPH/($NADP^+$ + NADPH)] in Cd-treated mitochondrial and peroxisomal fractions of both tissues was ~20 to 70% lower than that of the control (water-treated) from 120 h of exposure (Figure 2).

In a previous work, we have demonstrated that Cd interferes with the enzyme activities of the Krebs cycle and electron transport chain in germinating pea seeds (Smiri et al., 2009, 2010c). Thus, the decline in NAD(P)-dependent respiratory rates in pea mitochondria may be due to the disorder in metabolism of coenzymes (Figure 2). Moller and Lin (1986) have been suggested that, the reduction level is modulated by factors such as enzyme kinetics, respiratory state and the concentration of pyridine nucleotides. In fact, the decrease in nicotinamide adenine dinucleotide (NAD) and NADP reduction level observed in this paper or in the previous (Smiri et al., 2010b) may be due to the mitochondrial dysfunction, and the response of mitochondrial respiration is controlled by NAD and NADP levels in pea seeds treated with $CdCl_2$. The respiratory chain of plant mitochondria contains three NAD(P)H dehydrogenases on the matrix surface of the inner membrane. The activities of these dehydrogenases are dependent on the concentration and reduction levels of NAD and NADP in the matrix (Agius et al., 2001). NADP has a multitude of potential roles (Møller and Rasmusson, 1998; Agius et al., 2001). One such role is

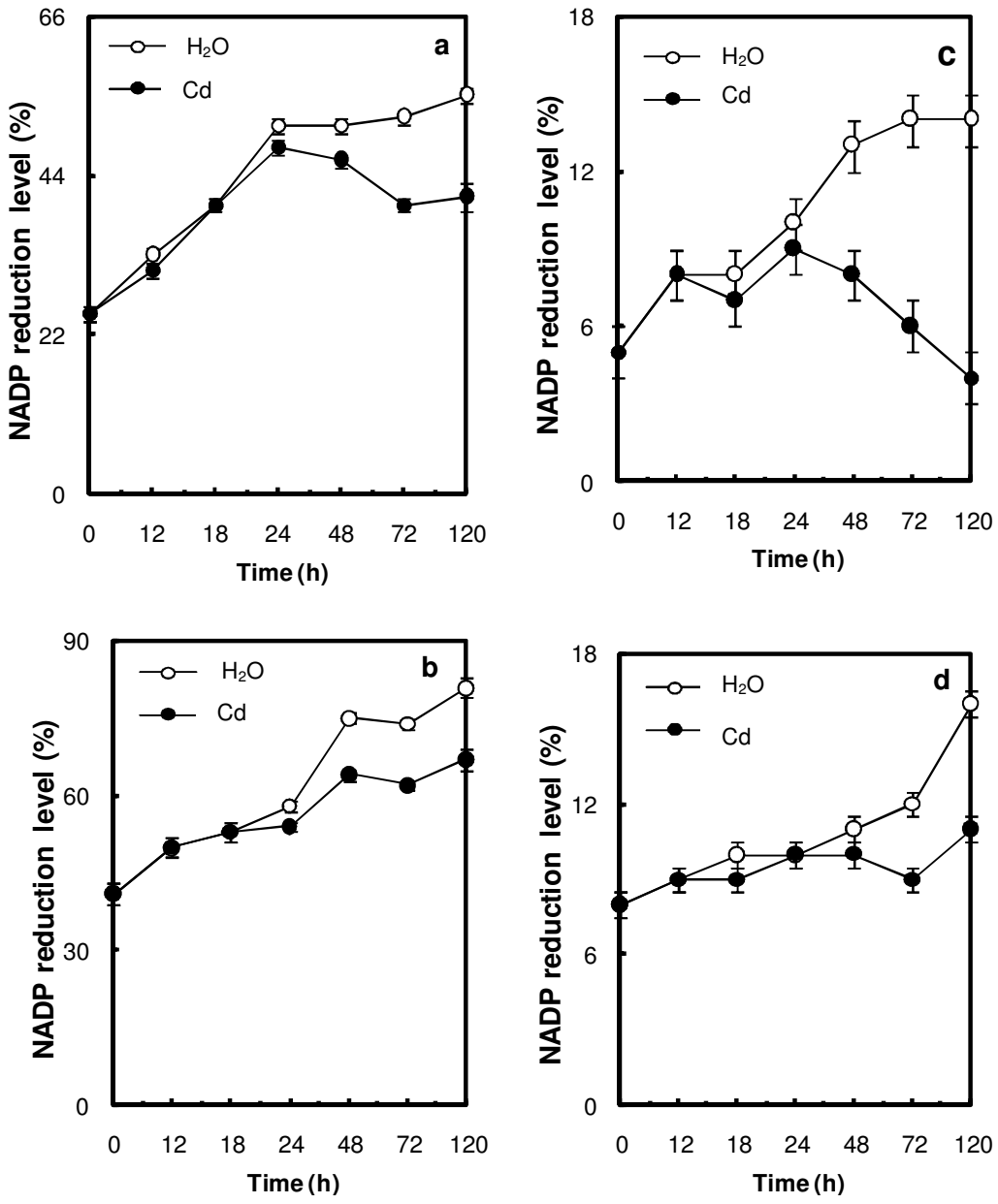

Figure 2. NADP reduction level (evaluated as the percentage of NADPH from total coenzymes (NADPH + $NADP^{+}$) in peroxisomal fraction (a, cotyledons; b, embryonic axis) and mitochondria (c, cotyledons; d, embryonic axis) of pea seeds during germination after imbibition with H_2O or 5 mM $CdCl_2$. Values are the averages of 6 individual measurements (± SE). Each measurement was performed in an extract obtained from several germinating seeds.

contributing to electron transport through the activity of an NADPH dehydrogenase facing the matrix (Rasmusson and Møller, 1991; Melo et al., 1996; Agius et al., 1998). In addition, mitochondrial alternative pathways are expected to provide additional flexibility to mitochondrial metabolism in the situations of stress (Douce and Neuburger, 1989; Rasmusson et al., 2004). When mitochondria respiration capacity decreases, secondary pentose phosphate pathway can be activated to compensate for the failure in energy supply (Smiri et al., 2009). Reducing power can be supplied via secondary NAD(P)H-recycling dehydrogenase activities, such as G6PDH and 6PGDH (Smiri et al., 2009, 2010c).

The NADPH oxidase activity in the mitochondrial and peroxisomal fractions of cotyledons and embryos imbibed with water for five days was ~50 to 75% lower than that of dry (0 h) tissues. Activity in Cd-treated mitochondria and peroxisomal fractions was ~170 to 250% higher than that

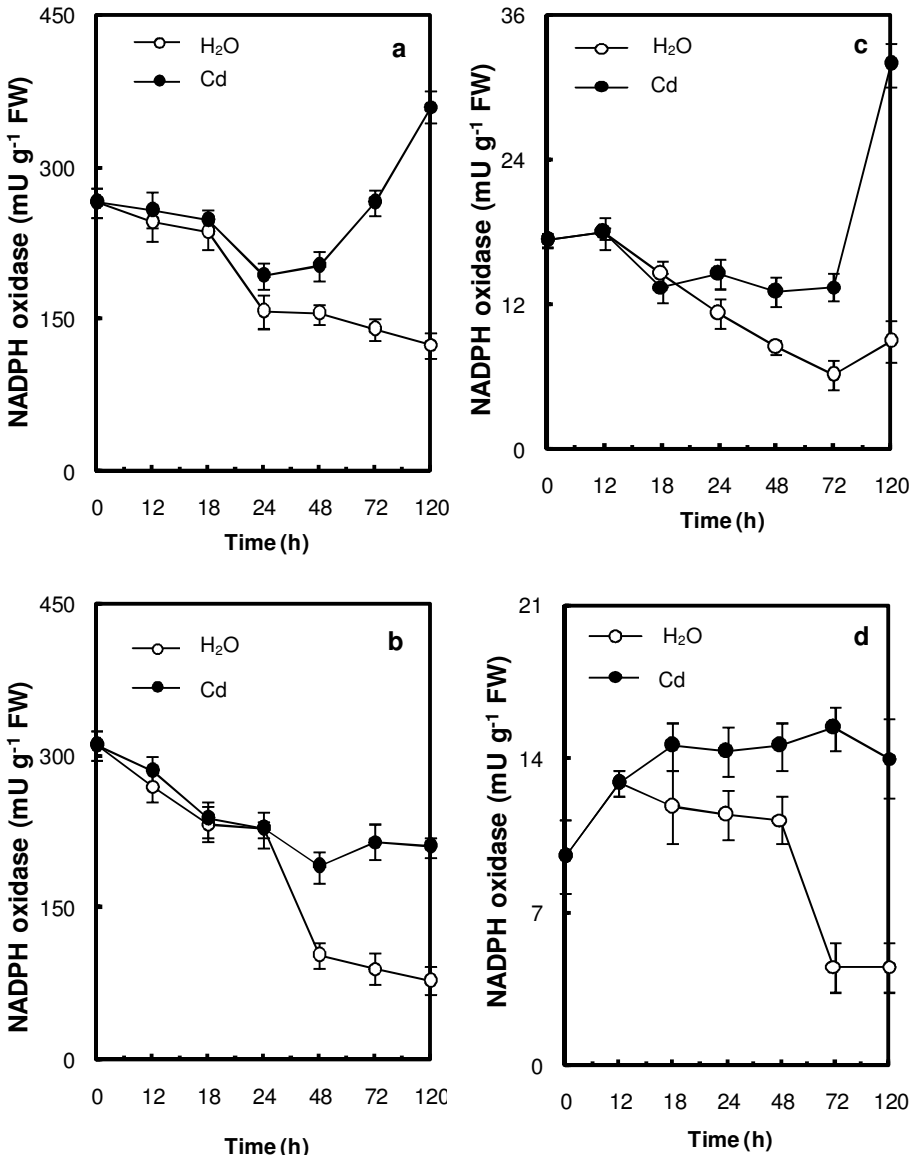

Figure 3. NADPH oxidase activity in peroxisomal fraction (a, cotyledons; b, embryonic axis) and mitochondria (c, cotyledons; d, embryonic axis) of pea seeds during germination after imbibition with H_2O or 5 mM $CdCl_2$. Values are the averages of 6 individual measurements (± SE). Each measurement was performed in an extract obtained from several germinating seeds.

of control (water-treated) from 5 days of exposure (Figure 3). NADPH oxidase transfers electrons from NADPH to O_2 to form superoxide radical ($O_2^{\cdot-}$), followed by the dismutation of $O_2^{\cdot-}$ to H_2O_2. Cd caused a significant consumption of reduced nicotinamide, as evidenced by the increase in $NADP^+$ balance (Table 1), probably by the stimulation of enzymatic oxidation via NADPH oxidase activities (Figure 3). In adult pea, plant responds to Cd toxicity by increasing the activity of antioxidative enzymes (Chaoui et al., 2004; Rodríguez-Serrano et al., 2006). In plant cells, the production of ROS such as $O_2^{\cdot-}$, H_2O_2 and the hydroxyl radical takes place in chloroplasts, mitochondria, peroxisomes, the plasma membrane and the apoplastic space (Navrot et al., 2006).

All biologically relevant macromolecules, that is nucleic acids, membrane lipids and proteins, are susceptible to damage by ROS. Thus, a number of studies have documented the production of ROS during the germination of various species (Bailly, 2004), and the production of reactive oxygen species by germinating seeds, has been regarded as a cause of stress, that might affect the success of germination. We suggest that alteration of redox and oxidative properties in both tissues of pea seeds due to treatment with $CdCl_2$ is highly

responsible for the decrease of germination success.

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