NAD pattern and NADH oxidase activity in pea (*Pisum sativum* L.) under cadmium toxicity

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Abstract Seeds of pea (*Pisum sativum* L.) were germinated for 5 days by soaking in distilled water or 5 mM cadmium chloride. Compared to the control, cadmium (Cd) caused a reduction in percent germination and embryo growth. Pyridine nucleotide coenzyme concentrations were determined in cotyledons and embryonic axis. Nicotinamide adenine dinucleotide (NADH) oxidase activity was examined. Cd treatment caused a restriction in levels of reduced coenzyme form in the mitochondria and the post-mitochondrial fraction of cotyledons, and embryonic axis. The oxidized coenzyme form has been accumulated by Cd-treated mitochondria of both tissues. It was also found that NADH oxidase activity was stimulated. The relationship between coenzyme levels, seed germination, pea growth, and Cd stress has been reported.

Keywords Cadmium · Germination · NAD · Pea

Introduction

In the life cycle of higher plants, seeds must complete the crucial task of protecting the embryo and driving it toward the establishment of a new generation. In germinating seeds, mitochondria provide most of the cellular adenosine

triphosphate (ATP) because germination is a heterotrophic process that is powered by the utilization of energy stored during seed development. It involves the mobilization of storage reserves and the initiation of growth and metabolic activity within the embryo. The generation of ATP underpins this process is concomitant with increases in respiration rate, accompany the earliest stages of germination following imbibition of the seed (Bewley and Black 1994). It has been suggested that in some plant species, fully functional mitochondria are present in the dry seed (Attucci et al. 1991), whereas in others, ATP synthesis is a product of fermentative pathways during the earliest stages of imbibitions (Raymond et al. 1985). It has been shown that mitochondria in dry seeds rapidly became active during imbibition, relying on nicotinamide adenine dinucleotide phosphate-oxidase (NADH) as substrates (Petit et al. 1990). In view of the fact that respiration is the driving force that powers germination, a high level of mitochondrial stress tolerance certainly contributes to the vigor of germinating seeds in the environment (Logan et al. 2001). The mechanisms involved in the extreme resistance of seeds to desiccation might provide the germinating seed with cross-tolerance to other abiotic stresses, such as temperature (Stupnikova et al. 2006).

Mitochondria in the seeds are especially resistant under aerobic conditions. The resumption of mitochondrial respiration after the imbibitions decreases the alternative or secondary activities of energy production via the decline of fermentation and the pentose phosphate pathway (Smiri et al. 2009; 2010). Stupnikova et al. (2006) have been demonstrated that pea seed mitochondria are endowed with a remarkable tolerance to extreme physiological temperatures. On another hand, nicotinamide adenine dinucleotide (NAD) plays vital roles in signaling via the generation and scavenging of reactive oxygen species (Berger et al. 2004)

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and in systems controlling adaptation to environmental stresses (Noctor et al. 2006), and represent an essential metabolites for numerous redox reactions in living organisms (Møller and Rasmusson 1998). Recent advances in the characterization of the *Arabidopsis* NAD biosynthetic genes have shed light on the significance of NAD biosynthesis in various developmental processes, including seed germination (Hunt et al. 2007). The production of reactive oxygen species (ROS) by germinating seeds has often been regarded as a cause of stress that might affect the success of germination. Therefore, antioxidant compounds and enzymes have been considered as being of particular importance for the completion of germination.

Cadmium (Cd) is considered one of the major pollutants in the current environment. It is a growing concern that the concentration of toxic metals including Cd is rapidly increasing in soil due to several reasons such as mining, urban traffic, metal-working industries, mineral fertilizer, heating systems, waste incinerators, and other sources (Sanita di Toppi and Gabbreilli 1999). This metal enters agricultural and is then transferred to the food chain (Wagner 1993). Although Cd is highly toxic to plants, it can be taken up easily by roots and interrupts a wide range of biological processes, including enzyme activity (Chugh and Sawhney 1996), photosynthesis (Clijsters and Van Assche 1985), respiration, and transpiration (Barcelo and Poschenrieder 1990), which can ultimately bring death. The rate of respiration of pea seeds, measured by oxygen uptake, was markedly impeded by Cd and the phase of rapid and development of respiration activity was almost completely suppressed of the presence of higher concentrations of Cd (Chugh and Sawhney 1996).

The exposure to toxic metals severely restricts plant growth (Woolhouse 1983). This is taken to be a consequence, in large part, of metal-imposed suppression of one or more of the numerous metal-sensitive structural and/or metabolic target sites. Amongst the most common ones are various enzymes where in several metal ions can bind to –SH groups, thereby altering the protein conformation and in turn, reducing their activity (Van Assche and Clijsters 1990). Metal ions with strong redox properties such as Cu could directly affect the membrane integrity and functions by owing to their capability to initiate the peroxidation of

the membrane lipid components. In addition, several other mechanisms have been proposed to explain the different phytotoxic effects of metals. In view of a wide array of the physiological and biochemical effects imposed by metal ions in plant systems, it is conceivable that metals would rely on multiple mechanisms for the induction of their toxic effects. These mechanisms could well be an effect—as well as metal specific. Cd inhibits the uptake and the transport of many macro/micronutrients and disturbs the cellular metabolic process by producing excessive ROS, leading to oxidative stress (Chaoui et al. 2004). ROS is known to react with proteins, nucleic acids and lipids causing deleterious effects on various cellular processes (Noctor et al. 2006). The central role played by coenzymes in metabolism and especially in its regulation suggests that the nucleotide pattern provides an index to the nature and the extent of the predominant metabolic processes. Accordingly, the aim of the present investigation has been to define the coenzyme patterns of seeds with a view to obtaining information relating to the effect of Cd during germination of pea seeds. The regulation of NADH oxidase activity imbalance was determined to evaluate their possible role in combating the cadmium toxicity.

Materials and methods

Plant material and growing conditions

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 in order 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 chloride salt of 5 mM cadmium chloride. Germinated seeds were recorded until obtaining the maximum of germination of control (H₂O). 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 or dried after 8 days at 70°C for dry weight determination.

Table 1 Germination success (%) and embryo axis length of *Pisum sativum* seeds after imbibition with H₂O (0) or 5 mM Cd

Each experiment was carried out with 80 seeds. Values with at least one the same letter are not significantly different at $P<0.05$	Germination rate (%)	0	17a	88d	98h	99h	100h
		Cd	6b	39e	40e	40e	40e
	Embryo length (cm)	0	0.5±0.05c	1.6±0.05f	3.6±0.42i	6.8±0.73k	8.9±1.49m
		Cd	0.4±0.05c	0.7±0.05g	1.2±0.12j	1.7±0.22l	2.2±0.47n

Each experiment was carried out with 80 seeds. Values with at least one the same letter are not significantly different at $P<0.05$

Table 2 Cd accumulation in pea seeds

		Cd ($\mu\text{g g}^{-1}$ DW)					
Time (h)		12	18	24	48	72	120
Plant Part	Fractions						
C	P	16 $\pm$ 2a	55 $\pm$ 2d	404 $\pm$ 3f	465 $\pm$ 5i	778 $\pm$ 4k	1,174 $\pm$ 5n
	M	1.2 $\pm$ 0.2b	1.6 $\pm$ 0.2b	4 $\pm$ 0.5g	5 $\pm$ 0.2g	6 $\pm$ 0.5g	16 $\pm$ 0.5a
E	P	35 $\pm$ 3c	74 $\pm$ 2e	340 $\pm$ 5h	738 $\pm$ 11j	1,655 $\pm$ 18l	2,647 $\pm$ 2o
	M	2 $\pm$ 0.2b	3 $\pm$ 0.2b	4 $\pm$ 0.3g	20 $\pm$ 0.6a	40 $\pm$ 0.5m	50 $\pm$ 0.4d

Data are the means ($\pm$ SE) of six replicates. Values with at least one the same letter are not significantly different at $P<0.05$. *C* cotyledons, *E* embryonic axes, *M* mitochondria, *P* peroxisomal fraction

Plant sampling

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 $3,000\times g$ for 20 min. Mitochondria from supernatant were

Fig. 1 Content of NAD^+ in post-mitochondrial 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 Cd. Data are the means ($\pm$ SE) of six replicates. Values with at least one the same letter are not significantly different at $P<0.05$

sedimented by centrifuging it at $20,000\times g$ for 30 min. The supernatant obtained was carefully decanted and designated as “post mitochondrial fraction”. The pellet was re-suspended in the homogenizing media ($w/v=1/0.5$) 50 mM Tris-HCl (pH 8.0), 0.4 M saccharose and referred to as “mitochondrial fraction” (Nawa and Asahi 1971; Bansal et al. 2002). Both fractions are used for NAD determination and NADH oxidase assay. All procedure was carried out at about 4°C .

Cadmium analysis

After wet digestion of dried mitochondria and post-mitochondrial fraction, the concentration of Cd was determined by atomic absorption spectrophotometer (Perkin Elmer) using Sigma Diagnostic Standards.

Estimation of the NADH and NAD^{+} pools

For nicotinamide adenine dinucleotide (NAD^{+}), 0.2 N HCl were used to homogenize the burst mitochondria and the post-mitochondrial fraction of cotyledons and embryonic axes ($w/v=1/10$); for NADH, 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 $10,000\times g$ 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-2 H tetrazoliumbromide (MTT) as the terminal electron acceptor. Procedures for these assays were described (Saher et al. 2005). A cycling mixtube of MTT, phenazine ethosulfate catalyst, ethanol and alcohol dehydrogenase was used to determine NAD^{+} and NADH. The rate of

Fig. 2 Content of NADH in post-mitochondrial 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 Cd. Data are the means ($\pm\text{SE}$) of six replicates. Values with at least one the same letter are not significantly different at $P<0.05$

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.

Determination of the NADH oxidase activity

NADH oxidase activity was determined in an assay mixture containing 100 mM sodium acetate (pH 6.5), 1 mM MnCl_2 , 0.5 mM p-coumaric acid, 0.2 mM NADH and enzyme extract. The reaction was monitored by following the decrease in absorbance at 340 nm, with extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ (Ishida et al. 1987).

Statistical analysis

Each treatment consisted of six replicates, and each experiment was carried out at least twice at different times.

All data were statistically analyzed using two-way ANOVA, and the means were separated using Newman–Keuls multiple-range test (Statistica 8, StatSoft Co., USA). Differences were considered significant at $P < 0.05$.

Results

The results presented in Table 1 show that 5 mM Cd treatment significantly affects pea seed germination. During the first day after the beginning of imbibition with metal solution, a marked decrease in seed germination success was observed; and after 5 days, it reached 60% from the control. The analysis of embryo length showed significant differences between seeds germinating on water and Cd. The inhibitory effect of metal increased in a time-dependent manner. After 5 days, embryo length reached a 75% of

Fig. 3 NAD^+ (P/M) ratio (a cotyledons, b embryonic axis) and NADH (P/M) ratio (c cotyledons, d embryonic axis) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Data are the means ($\pm \text{SE}$) of six replicates. Values with at least one the same letter are not significantly different at $P < 0.05$

diminishing from the control. The concentrations of Cd increased approximately 73–75 fold over 120 h in the post-mitochondrial fractions of cotyledons and embryos. In mitochondria, cadmium concentrations increased approximately 16–25 folds (Table 2).

From the observed changes in the concentrations of NAD during germination (Figs. 1 and 2), the NAD^+ and NADH levels in mitochondria and post-mitochondrial fractions of cotyledons and embryonic axis are seen to undergo a significant increase during H_2O imbibition. NAD^+ content increased approximately threefold over 120 h in the post-mitochondrial fractions of cotyledons and embryos grown in control conditions but stayed at a constant value in the presence of Cd (Fig. 1a, b). The NAD^+ content in Cd-treated mitochondria was ~200–300% higher than that of control (water treated) one from 5 days

of exposure (Fig. 1c, d). The NADH content increased approximately four- to ninefold over 120 h in mitochondria and post-mitochondrial fractions of cotyledons and embryos grown in control conditions. The NADH content in Cd-treated tissues was ~40–70% lower than that of the control (water treated) from 5 days of exposure (Fig. 2).

On the one hand, the NAD^+ (P/M) ratio in Cd-treated cotyledons and embryos was ~90% lower than that of the control (water treated) tissues from 5 days of exposure (Fig. 3a, b). The NADH (P/M) ratio in Cd-treated embryos was ~136% higher than that of the control (water treated) embryos from 120 h of exposure (Fig. 3d) and did not change in Cd-treated cotyledons (Fig. 3c).

On the other hand, the NAD^+/NADH ratio in Cd-treated post-mitochondrial fractions of cotyledons and embryos was ~30–50% lower than that of the control (water treated)

Fig. 4 NAD^+/NADH ratio in post-mitochondrial 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 Cd. Data are the means ($\pm\text{SE}$) of six replicates. Values with at least one the same letter are not significantly different at $P < 0.05$

fractions from 5 days of exposure (Fig. 4a, b). The NAD^+/NADH ratio in Cd-treated mitochondria was approximately ninefold higher than that of the control (water treated) mitochondria from 120 h of exposure (Fig. 4c, d).

As a result, the reduction percentage [$\text{NADH}/(\text{NADH}+\text{NAD}^+)$] in Cd-treated post-mitochondrial fractions was ~30–40% higher than that of the control (water treated) from 120 h of exposure (Fig. 5a, b). The reduction percentage in Cd-treated mitochondria was ~30–60% lower than that of the control (water treated) mitochondria from 120 h of exposure (Fig. 5c, d).

In addition, the cotyledons/embryonic axis ratio for both oxidized and reduced coenzymes in Cd-treated seeds was ~100–300% higher than that of control (water treated) from 5 days of exposure (Fig. 6).

Fig. 5 NAD reduction (%) in post-mitochondrial 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 Cd. Data are the means ($\pm\text{SE}$) of six replicates. Values with at least one the same letter are not significantly different at $P<0.05$

Finally, NADH oxidase transfers electrons from NADH to O_2 to form superoxide radical ($\text{O}_2^{\cdot-}$), followed by dismutation of $\text{O}_2^{\cdot-}$ to H_2O_2 . The NADH oxidase activity in the post-mitochondrial fractions of cotyledons and embryos imbibed with water for 5 days was ~70–90% lower than that of dry (0 h) tissues (Fig. 7a, b). Activity in Cd-treated mitochondria and post-mitochondrial fractions was ~50–750% higher than that of control (water treated) from 5 days of exposure (Fig. 7).

Discussion

Germination rate and embryonic axis growth were adversely affected by cadmium: at 5 days after the start of imbibition,

Fig. 6 Cotyledons/embryonic axis ratio of NAD⁺ (a) and NADH (b) of pea seeds during germination after imbibition with H₂O or 5 mM Cd. Data are the means ($\pm$ SE) of six replicates. Values with at least one the same letter are not significantly different at $P < 0.05$

the diminutions were 60% and 75% from controls, respectively (Table 1). Experimental studies of heavy metals impact on adult plants have often applied low concentrations (Ernst 1998; Rodríguez-Serrano et al. 2009). Nevertheless, high pollutant doses have been used in seed germination assays (Chugh and Sawhney 1996; Mihoub et al. 2005; Rahoui et al. 2008), 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 behavior 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 compartmentation of pollutant at cellular and subcellular levels (Ernst 1998; Mihoub et al. 2005; Rahoui et al. 2008; Smiri et al. 2009). According to recent studies, pointing to mitochondrial dysfunction as the main player in Cd intoxication (Cannino et al. 2009), we demonstrated that mitochondria can be considered a target of Cd. This assumption may be corroborated by an effective accumulation of Cd in mitochondria (Table 2).

Under stress conditions, key metabolic processes of germination can be depressed (Chugh and Sawhney 1996; Mihoub et al. 2005; Rahoui et al. 2008). In the present research, the influence of Cd on the coenzyme patterns at embryo level in seeds of pea is emphasized. Changes in nicotinamide nucleotide levels throughout seed growth are shown in Figs. 1 and 2. The levels show a number of significant features in relation to seed germination. Of the two nicotinamide forms, NAD⁺ was the most abundant

(Figs. 1 and 2). It increased from 16 nmol mg⁻¹ P/seed in dry seeds to 38 nmol mg⁻¹ P/seed at 5 days of germination. The concentrations of NADH increased during imbibition from 1.5 nmol mg⁻¹ P/seed in dry seeds to 7 nmol mg⁻¹ P/seed at 5 days. The high increase in NAD content at germination may represent a reducing equivalent available for the rapid and extensive increase in respiratory activity that accompanies germination. These high NAD concentrations are consistent with the rapid rise in ATP level noted for germinating seeds following imbibition (Moreland et al. 1974; Obendorf and Marcus 1974).

Alteration of coenzyme patterns can be due to the disorder in respiratory activity in mitochondria and post-mitochondrial fraction under Cd-stress condition (Figs. 1 and 2). In previous work, activities of malate- and succinate-dehydrogenases and NADH- and succinate-cytochrome c reductases were rapidly inhibited in Cd-treated mitochondria (Smiri et al. 2009; 2010). However, in post-mitochondrial fraction, Cd stimulated the NADPH-generating enzyme activities of the pentose phosphate pathway, glucose-6-phosphate- and 6-phosphogluconate-dehydrogenases, as well as enzyme activity of fermentation, alcohol dehydrogenase, with concomitant inhibition in the capacity of enzyme inactivator in germinating pea seeds (Smiri et al. 2009; 2010). When mitochondria respiration capacity decreases, secondary pentose phosphate pathway and alternative alcoholic fermentation can be activated to compensate for the failure in energy supply (Conley et al. 1999; Kato-Noguchi 2002; Shetty et al. 2002). The NAD-consuming events are traditionally well known to play a part in biotic and abiotic stress responses. Reduced NAD consumption may influence plant metabolism. Thus, the disturbance of NAD homeostasis shows effects in the

Fig. 7 NADH oxidase activity in post-mitochondrial fraction (**a** cotyledons, **b** embryonic axis) and mitochondria (**c** cotyledons, **d** embryonic axis) of pea seeds during germination after imbibition with H₂O or 5 mM Cd. Data are the means ($\pm$ SE) of six replicates. Values with at least one the same letter are not significantly different at $P < 0.05$

response to stresses. Plant alcohol dehydrogenase (ADH) reduces acetaldehyde to ethanol under anaerobic conditions and results in the oxidation of NADH to form NAD⁺ for recycling into the glycolytic pathway. The ameliorated statute of reducing equivalent could be decisive for energy metabolism in Cd-poisoned germinating seeds. It has been suggested (a) that the tolerance to heavy metal toxicity is enormously dependent on the availability of reduced cell metabolites, such as NADH (Léon et al. 2002), and (b) that the pentose phosphate pathway is implicated in responses to environmental stress (Kato-Noguchi 2002; Shetty et al. 2002). Moreover, the mitochondrial NAD appears to play a fundamental role in controlling redox status in animals and plants subjected to abiotic stress (Kasimova et al. 2006;

Schwarzländer et al. 2009). Nevertheless, cytosol/mitochondrion exchanges should be considered as an important aspect of the redox state of mitochondria in Cd treated plants. During the recent years, the existence of a regulatory network that modulates cytosol/mitochondrion exchanges has been argued (Krömer and Heldt 1991; Igamberdiev and Gardeström 2003). Engagement of NADH dehydrogenases would allow efficient generation of ATP from NADH (Igamberdiev and Gardeström 2003). In addition to the classical complexes, all plant mitochondria possess alternative enzymes that confer on them the capacity to oxidize external or internal NADH independently of complex I and to transfer electrons directly from reduced ubiquinone to oxygen via an alternative oxidase (Douce and Neuburger

1989). Alternative pathways are expected to provide additional flexibility to mitochondrial metabolism in situations of stress (Rasmusson et al. 2004).

On another hand, Cd appears to obstruct coenzyme mobilization from source (cotyledons) to sink (embryonic axes). The embryonic axes suffer from a restriction in coenzymes resulting from the failure in mobilization process as shown by the evolution of the cotyledons/embryonic axes ratio (Fig. 6) in the absence and in the presence of metal. The mobilization process in embryo is modulated by several parameters, such as hydrolase activities, transport, respiratory catabolism, and neosynthesis (Monerri et al. 1986). Moreover, the highest activity of NADH oxidase was observed in dry seeds (Fig. 7). NADH oxidase activity decreased during the germination period in both embryo axes and cotyledons. The observed changes in NADH oxidase activity in embryo axes and cotyledons appear to be more closely related to metabolic and developmental processes which are associated with preparation for germination. Earlier studies showed that the antioxidative system of embryos was very active in the first hours after germination, and decreased gradually after wards (Tommasi et al. 2001). The role of the NADH oxidase in Cd-induced oxidative stress was investigated in cotyledons and embryos of pea seeds. Treatment with metal resulted in significant increase in the activity of NADH oxidase. Similar results have been obtained with other tissues in adult pea (Chaoui et al. 2004). We cannot exclude the possible role of the oxidase activity in the coenzyme depletion (Fig. 7). The particularly high activity of NADH oxidase may be the cause of the decrease in the coenzyme pools in Cd-poisoned tissues of the germinating pea seeds.

The relationship between coenzyme levels and seed germination has also been reported. We have pointed out that Cd stress interfered with the coenzyme patterns and the NADH oxidase activity. The observation of these physiological disorders provides a new insight that would also lead to a better overall understanding of the molecular basis of heavy metal responses of the seeds at the germination stage.

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