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BRIEF COMMUNICATION

Cadmium modulates NADPH oxidase activity and expression in sunflower leaves

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Abstract

The production of reactive oxygen species (ROS) and the ways by which ROS are generated are very important facts related to heavy metal toxicity in plants. In this work, superoxide anion ($O_2^{\bullet-}$) generation diminished in cadmium treated sunflower (*Helianthus annuus* L.) leaf discs, and this reduction was time and Cd-concentration dependent. In line with these findings, we observed that NADPH-dependent oxidase activity was significantly inhibited by 0.1 and 0.5 mM Cd^{2+} treatments and the expression of the NADPH oxidase putative gene related to $O_2^{\bullet-}$ synthesis in sunflower leaves was 83 % inhibited by 0.1 mM $CdCl_2$ and almost completely depleted by 0.5 mM $CdCl_2$.

Additional key words: *Helianthus annuus*, reactive oxygen species, superoxide anion.

Reactive oxygen species (ROS) participate in plant growth and development (Foreman *et al.* 2003) and in plant responses to biotic and abiotic stress (Potters *et al.* 2009). In the respiration, oxygen becomes reduced to H_2O involving different pathways. Some enzymatic systems reduce oxygen *via* tetravalent mechanisms [*e.g.* cytochrome *c* oxidase, alternative oxidase (AOX)], others operate in a stepwise manner where oxygen accepts electrons one by one, leading to the formation of a reactive intermediate molecule, the superoxide anion (Navrot *et al.* 2007). Reactive oxygen species generation has been reported in plant mitochondria (Zhang *et al.* 2009), chloroplasts (Asada 1994) and plasma membranes (Garnier *et al.* 2006). Although many enzymes such as cell wall peroxidases, amine oxidase, xanthine oxidase, oxalate oxidase, and flavin-containing oxidase are potential H_2O_2 sources, the NADPH oxidase complex, now represented by the NOXs family, is considered one of the most important sources of $O_2^{\bullet-}$ in the plant cell (Van Gestelen *et al.* 1997, Bolwell *et al.* 2002, Torres and Dangl 2005, Grant *et al.* 2000). It has been reported that the source of ROS generated in potato tubers and in tobacco plants is likely to be a plasma membrane NADPH-dependent oxidase (Razem and Bernards 2003, Simon-Plas *et al.* 2002).

Cadmium is a heavy metal toxic for humans and animals and contamination with this metal is a serious problem that leads to considerable losses in plant productivity (Gratao *et al.* 2008). The phytotoxic effects of Cd are probably a consequence of its interference with a number of metabolic processes associated with normal development of plants (Benavides *et al.* 2005, Daud *et al.* 2009, Gonçalves *et al.* 2009). Although information focused on the oxidative stress induced by Cd in plants has grown in the last few years, it is still difficult to draw general conclusions about the mechanism involved in its toxicity mainly due to the great variation observed in the responses of different plant species (Olmos *et al.* 2003, Gomes *et al.* 2006, John *et al.* 2007, Groppa *et al.* 2008). In contrast to other heavy metals, like Cu, cadmium does not act directly on the production of ROS *via* Fenton and/or Haber Weiss reactions (Benavides *et al.* 2005, Gratao *et al.* 2005), but the molecular basis underlying its mechanism of action is still poorly understood. In this work, we explored the effect of Cd on $O_2^{\bullet-}$ formation in sunflower leaf discs, focusing on the involvement of

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Abbreviations: DPI - diphenyl iodonium; NBT - nitroblue tetrazolium; PMSF - phenylmethylsulphonylfluoride; ROS - reactive oxygen species; SOD - superoxide dismutase.

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NADPH oxidase.

Sunflower (*Helianthus annuus* L.) seeds (provided by *Nidera*, Argentina) were surface sterilized and grown during 21 d at 26/20 °C (day and night), a 16-h photoperiod, irradiance of 175 µmol m^{-2} s^{-1} and relative humidity of 50 % in a growth chamber. Then, 10 mm-leaf discs from the first pair of leaves were obtained using a cork borer and incubated in flasks containing 25 cm^3 of 0.01, 0.1 or 0.5 mM $CdCl_2$ for 14 h in a rotary shaker under continuous irradiance. Controls (C) were incubated in distilled water. After incubation, discs were thoroughly washed with distilled water and used for analyses.

Superoxide accumulation in sunflower leaf tissues were monitored by the reduction of nitroblue tetrazolium (NBT) producing a blue formazan precipitate (Romero-Puertas *et al.* 2004). To verify that blue precipitate corresponded to superoxide anion 10 mM $MnCl_2$ and/or SOD (75 U cm^{-3}) were used. Subcellular fractionation was performed to measure NADPH oxidase activity in microsomes according to Shen *et al.* (2000). The pellet obtained after ultra-centrifugation at 140 000 *g* for 1 h was resuspended in 0.5 cm^3 of the reaction buffer and used as the microsomal fraction. NADPH-dependent $O_2^{\bullet-}$ generation was measured using the tetrazolium dye NBT as an electron acceptor, as described by Van Gestelen *et al.* (1997). NADPH oxidase activity in microsomes of control plants was also assayed "*in vitro*" with the addition to the reaction medium of: $CdCl_2$ (0.01, 0.1 and 0.5 mM), DPI (10 µM), NaN_3 (1 mM), NADH (100 µM) and $CaCl_2$ (5 mM). Diphenyl iodium (DPI) was used to inhibit NADPH oxidase-like enzyme activity and NaN_3 was used to block peroxidase activity.

Total RNA was extracted from leaf tissue using a modified *TRIzol* (*Invitrogen*, Carlsbad, CA, USA) procedure and the RNA was then treated with DNase I (Sambrook *et al.* 1989). It was converted to cDNA with random primers using the *RevertAid™ MMuLV* reverse transcriptase (*Fermentas*, USA). Primers for a putative sunflower NADPH oxidase (forward primer: 5'-CACCCGGAGATGACTACCTAAG-3' and reverse primer: 5'-GTAAGCCCACTAGCAACACGAC-3') were designed and optimized, using the bioinformatics program *Primer 3*, from a nucleotide sequences available in *DFCI Plant Gene Indices* which presents a certain similarity with TC280605 RbohAp108, GO 0016174 from *Arabidopsis thaliana* (*DCFI* database). PCR reactions were performed using a programmable minicycler (*Ivema T-18*, Argentina). Cycling conditions were: 94 °C for 5 min, then 40 cycles at 94 °C denaturing for 1 min, 51 °C annealing for 1 min, and 72 °C extension for 2 min, and then a final step of 72 °C for 10 min. Primers for *Helianthus annuus* 18S ribosomal cDNA (forward primer:, 5'- GGCTACCACATCCAAGGAA-3'; reverse primer: 5'- CTATTGGAGCTGGAATTACCG-3' were used as an internal control for the amount of RNA and RT efficiency. Samples were kept at 94 °C for 1 min and then subjected to thermocycling (19 cycles of 0.5 min at 94 °C, 1 min at 54°C, and 1 min at 72 °C, with a final extension at 72 °C for 7 min). Each PCR reaction was amplified using an optimized number of cycles to ensure the linearity requirement for semi-quantitative RT-PCR analysis. The PCR products were electro-phoresed through 2 % agarose and visualized with ethidium bromide. Gels were photographed with *FOTO/Analyst® Investigator/Eclipse* systems (*Fotodyne*, Hartland, USA), analyzed with *GelProAnalyzer* (*Media Cybernetics*, USA) software and data expressed as arbitrary units (assuming control value equal to 1), based on absolute integrated absorbance of each band.

All determinations were performed from three independent experiments with three measurements for each parameter in each experiment. One-way *ANOVA* and Tukey tests were performed to assess statistical significance between treatments, using $P \leq 0.05$ as significant.

In control leaf discs, an intense formazan staining, revealing a high $O_2^{\bullet-}$ production, was observed (Fig. 1). These precipitates were caused by superoxide anion, since they were significantly abolished in the presence of $MnCl_2$ (a $O_2^{\bullet-}$dismutating catalyst) or SOD.

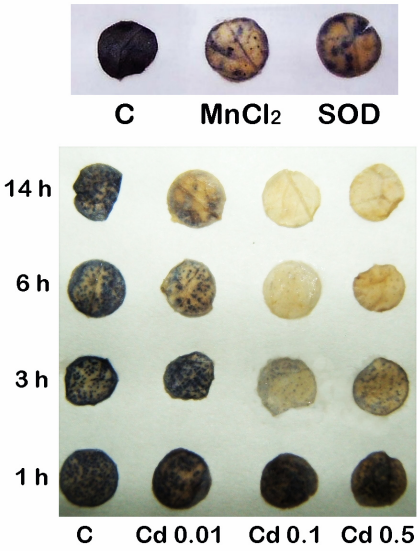

Fig. 1. Histochemical localization of superoxide anion in sunflower leaf discs. Leaf segments were treated as described in Materials and Methods. Treated-leaves discs were infiltrated with 0.01 % (m/v) NBT and then irradiated until appearance of blue spots characteristic of formazan precipitates.

In order to ascertain whether $O_2^{\bullet-}$ production was mediated by a NADPH-oxidase like enzyme, NADPH-dependent oxidase activity was measured "*in vitro*". The synthesis of $O_2^{\bullet-}$ using NADH as electron donor was very low and a similar result was obtained after exclusion of NADPH from the reaction medium (Table 1). In plants, two mechanisms have been proposed for ROS generation. First, the action of a multimeric NADPH-dependent superoxide synthase, that belongs to the NOX family of enzymes and uses NADPH to generate $O_2^{\bullet-}$ which is rapidly dismutated to H_2O_2 by SOD (Van Gestelen *et al.* 1997, Simon-Plas *et al.* 2002, Torres and Dangl 2005).

Alternatively, a pH-dependent cell wall peroxidase could be responsible for the reduction of O_2 to $O_2^{\bullet-}$ at the expense of an apoplastic reductant (Bolwell *et al.* 1999, Kawano 2003). Data presented here in the "*in vitro*" determinations corroborate the existence of a NADPH dependent oxidase activity in sunflower leaf discs (Table 1).

Table 1. "In vitro" and "in vivo" NADPH oxidase activity [nmol($O_2^{\bullet-}$) mg^{-1}(protein) min^{-1}]. Leaf segments were treated as described in Materials and Methods. Values are the means of two different experiments with three replicated measurements (* - significant differences at $P < 0.05$ according to Tukey´s multiple range test).

Treatments		NADPH oxidase
In vitro	control	29.05 ± 3.58
	+Cd 0.01 mM	39.06 ± 4.10*
	+Cd 0.10 mM	19.22 ± 3.26*
	+Cd 0.50 mM	15.95 ± 2.24*
	+DPI	0.62 ± 0.08*
	+NaN_3	17.49 ± 1.52*
	+NADH	6.83 ± 0.95*
	–NADPH	0.86 ± 0.06*
In vivo	control	19.12 ± 3.01
	+Cd 0.01 mM	25.33 ± 3.45
	+Cd 0.10 mM	13.89 ± 1.28*
	+Cd 0.50 mM	8.56 ± 1.16*
	+Cd 0.50 mM + Ca 5.0 mM	12.21 ± 1.44*

The NADPH oxidase inhibitor DPI has been reported to inhibit the mammalian neutrophil NADPH oxidase by binding itself to both structural components of the protein, a flavoprotein and *b*-type cytochrome (Doussiere *et al.* 1999). In the "*in vitro*" reaction using control samples, this compound inhibited NADPH-dependent $O_2^{\bullet-}$ production thus confirming that the enzyme involved in superoxide formation has a flavoprotein or a cytochrome in the active site (Doussiere *et al.* 1999) and are in agreement with the assumption that an enzyme from the NOX family is responsible for the $O_2^{\bullet-}$ production observed in microsomes of sunflower leaf discs (Table 1).

Cell wall peroxidases have also been suggested to be involved in the oxidative burst in plants (Bolwell *et al.* 2002). In the "*in vitro*" assay, azide addition resulted in an average of 40 % inhibition in the NADPH-dependent oxidase activity demonstrating that the involvement of peroxidases in sunflower ROS formation should not be discarded

Proteins from the NOX family were reported to be involved in Cd-induced oxidative stress in the cell (Olmos *et al.* 2003, Garnier *et al.* 2006, Heyno *et al.* 2008). In the "*in vitro*" determinations, 0.01 mM $CdCl_2$ produced an increase in the rate of NADPH oxidation. However, at higher concentrations, Cd induced a marked inhibition of the enzyme activity (34 % for 0.1 mM and 45 % for 0.5 mM $CdCl_2$, Table 1). In microsomes obtained from 0.1 mM and 0.5 mM Cd-treated leaf discs, $O_2^{\bullet-}$ generation was inhibited by 32 and 58 %, respectively, whereas 0.01 mM $CdCl_2$ concentration did not produce any effect (Table 1). Heyno *et al.* (2008) reported that Cd inhibited $O_2^{\bullet-}$ production in isolated plasma membranes from soybean and in cucumber roots, but stimulated $O_2^{\bullet-}$ formation in potato tuber mitochondria. Other studies reported that Cd enhanced $O_2^{\bullet-}$ and/or H_2O_2, but using different experimental systems, plants species and Cd concentrations, *i.e.* 14 d-old pea plants treated with 50 μM Cd^{2+} (Romero-Puertas *et al.* 2004, Rodriguez-Serrano *et al.* 2006) or tobacco bright yellow-2 cells with 3 mM Cd^{2+} (Garnier *et al.* 2006). These could be one of the reasons for the reported differences compared to our results.

When Cd^{2+} was used as stressor, formazan deposition was evidently reduced, and this reduction in formazan intensity was dependent on time and Cd-concentration (Fig. 1). Under 0.1 or 0.5 mM Cd^{2+}-treatment superoxide anion formation was completely abolished in correlation with Cd-induced inhibition of NADPH oxidase activity (Table 1). In a similar way, Rodriguez *et al.* (2007) demonstrated that the reduced apoplastic $O_2^{\bullet-}$ levels observed under salinity was associated with a direct effect of NaCl on the activity of a plasma membrane NADPH oxidase.

To test if this decreased activity should be associated with decreased mRNA levels of the putative sunflower NADPH oxidase, the expression of this gene was analyzed. The analysis of PCR products showed that gene expression (detected as a 230 pb fragment of the gene) was inhibited by 30, 73 and almost 91 % by 0.01, 0.1 and 0.5 mM $CdCl_2$, respectively (Fig. 2). This result correlated

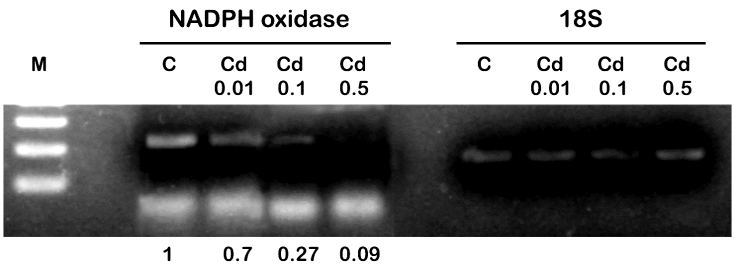

Fig. 2. NADPH oxidase transcript levels in sunflower leaf discs. RT-PCR for NADPH oxidase was performed as described in Materials and methods. Relative mRNA values were calculated as the ratio NADPHox/18S. The figure shows results typical of those obtained in three independent experiments.

to the depleted activity of the enzyme observed under Cd^{2+} stress. In *Arabidopsis thaliana* cell cultures treated with 75 μM Cd^{2+} an increased transcription of *RBOHF* gene was detected after 15 min of treatment, but a decreased expression was observed after 3 h (Horemans *et al.* 2007), denoting that the effect of the metal is time-dependent. To our knowledge, our report is the first report describing the expression of NADPH oxidase genes in sunflower leaves.

Given that Cd^{2+}, as a potent competitor for Ca^{2+}, may bind to the EF-hand motifs of the NOX and affect the enzyme activity (Rivetta *et al*. 1997), we tested the effect of 5 mM Ca^{2+} on the Cd^{2+}-induced inhibition of $O_2^{\bullet-}$ production since lower Ca^{2+} concentrations (0.1 and 1 mM $CaCl_2$) did not produce any effect (data not shown). Table 1 shows that Ca^{2+} competed efficiently with Cd^{2+}, reducing the effect of the metal on $O_2^{\bullet-}$ production to half of the effect observed without Ca^{2+}. A similar effect was observed by Heyno *et al.* (2008) working with soybean plasma membranes and using the same Ca^{2+} concentration. It is known that increased cytosolic Ca^{2+} induces NADPH oxidase activity resulting in the production of ROS, which induce diverse physiological responses (Kadota *et al*. 2004, Potocký *et al*. 2007). In our work, Cd-induced inhibition of NADPH oxidase activity and the concomitant decrease of ROS could be mediated by a competition between Cd and Ca for the Ca^{2+}-binding site in the NADPH oxidase complex. Cadmium is clearly able to displace essential metal cations from proteins generally leading to inhibition of enzyme activities (Nocentini 1987). In addition to this, because of similar ionic radii and identical charges, Cd^{2+} can affect Ca^{2+} homeostasis. In radish for example, Cd^{2+}competes with Ca^{2+} for specific ionic binding sites to inhibit calmodulin-dependent phosphodiesterase activity (Rivetta *et al*. 1997). Perfus-Barbeoch *et al*. (2002) suggested that, in *Arabidopsis thaliana*, Cd might permeate the guard cell plasma membrane through calcium channels whereas White (2000) suggested that Ca^{2+} channels were permeable to Cd in wheat roots. According to this, Cd could also block calcium channels, consequently inhibiting NADPH oxidase activity. This possibility has to be checked in future experiments

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