

Cadmium modulates NADPH oxidase activity and expression in sunflower leaves

M.D. GROPPA¹*, M.P. IANUZZO¹, E.P. ROSALES¹, S.C. VÁZQUEZ² and M.P. BENAVIDES¹

Cátedra de Química Biológica Vegetal, Departamento de Química Biológica¹ and Cátedra de Microbiología Industrial y Biotecnología, Departamento de Microbiología, Inmunología y Biotecnología², Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina

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^-) 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 Cd²⁺ treatments and the expression of the NADPH oxidase putative gene related to O_2^- synthesis in sunflower leaves was 83 % inhibited by 0.1 mM CdCl₂ and almost completely depleted by 0.5 mM CdCl₂.

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 H₂O 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 H₂O₂ sources, the NADPH oxidase complex, now represented by the NOXs family, is considered one of the most important sources of O_2^- 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^- formation in sunflower leaf discs, focusing on the involvement of

Received 28 December 2009, accepted 25 January 2011.

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

Acknowledgements: This work was supported by the University of Buenos Aires (Project B017). M.D. Groppa, S. Vázquez and M.P. Benavides are also researchers of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

* Corresponding author; fax: (+54) 11 4508-3645, e-mail: mgroppa@ffyb.uba.ar

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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 CdCl₂ 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 MnCl₂ 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 O₂^{·-} 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: CdCl₂ (0.01, 0.1 and 0.5 mM), DPI (10 μM), NaN₃ (1 mM), NADH (100 μM) and CaCl₂ (5 mM). Diphenyl iodium (DPI) was used to inhibit NADPH oxidase-like enzyme activity and NaN₃ 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'-GTAAGCCCCTAGCAACACGAC-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'-GGCTACCATCCAGGAA-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 O₂^{·-} production, was observed (Fig. 1). These precipitates were caused by superoxide anion, since they were significantly abolished in the presence of MnCl₂ (a O₂^{·-}-dismutating catalyst) or SOD.

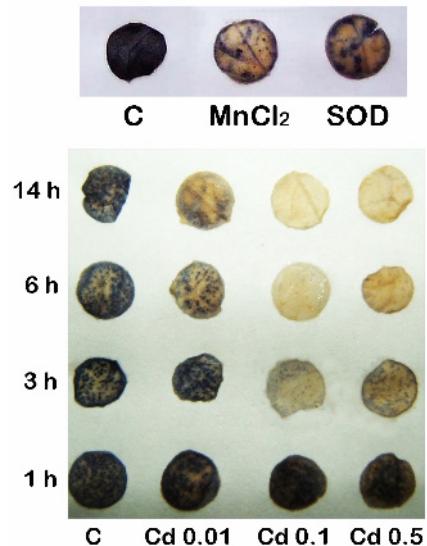


Fig. 1. Histochemical localization of superoxide anion in sunflower leaf discs. Leaf segments were treated as described in Materials and Methods. Treated-leaves discs were infiltrated with 0.01 % (m/v) NBT and then irradiated until appearance of blue spots characteristic of formazan precipitates.

In order to ascertain whether O₂^{·-} production was mediated by a NADPH-oxidase like enzyme, NADPH-dependent oxidase activity was measured "in vitro". The synthesis of O₂^{·-} 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 O₂^{·-} which is rapidly dismutated to H₂O₂ 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^- 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 [$\text{nmol}(O_2^-) \text{ mg}^{-1}(\text{protein}) \text{ min}^{-1}$]. 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
<i>In vitro</i>	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*
<i>In vivo</i>	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^- 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^- 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 CdCl₂ 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 CdCl₂, Table 1). In microsomes obtained from 0.1 mM and 0.5 mM Cd-treated leaf discs, O_2^- generation was inhibited by 32 and 58 %, respectively, whereas 0.01 mM CdCl₂ concentration did not produce any effect (Table 1). Heyno *et al.* (2008) reported that Cd inhibited O_2^- production in isolated plasma membranes from soybean and in cucumber roots, but stimulated O_2^- formation in potato tuber mitochondria. Other studies reported that Cd enhanced O_2^- and/or H₂O₂, but using different experimental systems, plants species and Cd concentrations, *i.e.* 14 d-old pea plants treated with 50 μ M Cd²⁺ (Romero-Puertas *et al.* 2004, Rodriguez-Serrano *et al.* 2006) or tobacco bright yellow-2 cells with 3 mM Cd²⁺ (Garnier *et al.* 2006). These could be one of the reasons for the reported differences compared to our results.

When Cd²⁺ 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 Cd²⁺-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^- 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 CdCl₂, respectively (Fig. 2). This result correlated

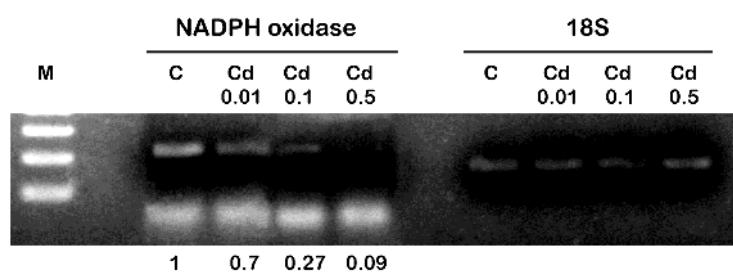


Fig. 2. NADPH oxidase transcript levels in sunflower leaf discs. RT-PCR for NADPH oxidase was performed as described in Materials and methods. Relative mRNA values were calculated as the ratio NADPHox/18S. The figure shows results typical of those obtained in three independent experiments.

to the depleted activity of the enzyme observed under Cd²⁺ stress. In *Arabidopsis thaliana* cell cultures treated with 75 µM Cd²⁺ 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 Cd²⁺, as a potent competitor for Ca²⁺, 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 Ca²⁺ on the Cd²⁺-induced inhibition of O₂^{·-} production since lower Ca²⁺ concentrations (0.1 and 1 mM CaCl₂) did not produce any effect (data not shown). Table 1 shows that Ca²⁺ competed efficiently with Cd²⁺, reducing the effect of the metal on O₂^{·-} production to half of the effect observed without Ca²⁺. A similar effect was observed by Heyno *et al.* (2008) working with soybean plasma membranes and using the same Ca²⁺ concentration. It is known that increased cytosolic Ca²⁺ 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 Ca²⁺-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, Cd²⁺ can affect Ca²⁺ homeostasis. In radish for example, Cd²⁺ competes with Ca²⁺ 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 Ca²⁺ 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

References

Asada, K.: Production and action of active oxygen species in photosynthetic tissues. - In: Foyer, C.H., Mullineaux, P.M. (ed.): Causes in Photooxidative Stress and Amelioration of Defense System in Plants. Pp. 77-103. CRC Press, Boca Raton 1994.

Benavides, M.P., Gallego, S.M., Tomaro, M.L.: Cadmium toxicity in plants. - *Braz. J. Plant Physiol.* **17**: 21-34, 2005.

Bolwell, G.P., Bindschedler, L.V., Blee, K.A., Butt, V.S., Davies, D.R., Gardner, S.L., Gerrish, C., Minibayeva, F.: The apoplastic oxidative burst in response to biotic stress in plants: a three component system. - *J. exp. Bot.* **53**: 1367-1376, 2002.

Bolwell, G.P., Blee, K.A., Butt, V.S., Davies, D.R., Gardner, S.L., Gerrish, C., Minibayeba, F., Rowntree, E.G., Wojtaszek, P.: Recent advances in understanding the origin of the apoplastic oxidative burst in plant cells. - *Free Radical Res.* **31** (Suppl.): S137-S145, 1999.

Daud, M.K., Variath, M.T., Ali, S., Najeeb, U., Jamil, M., Hayat, Y., Dawood, M., Khand, M.I., Zaffar, M., Cheema, S.A., Tong, X.H., Zhu, S.: Cadmium-induced ultra-morphological and physiological changes in leaves of two transgenic cotton cultivars and their wild relative. - *J. Hazard. Materials* **168**: 614-625, 2009.

Doussiere, J., Gaillard, J., Vignais, P.V.: The heme component of the neutrophil NADPH oxidase complex is a target for arylidonium compounds. - *Biochemistry* **38**: 3694-3703, 1999.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D.G., Davies, J.M., Dolan, L.: Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. - *Nature* **422**: 442-446, 2003.

Garnier, L., Simon-Plas, F., Thuleau, P., Agnel, J.P., Blein, J.P., Ranjeva, R., Montillet, J.L.: Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. - *Plant Cell Environ.* **29**: 1956-1969, 2006.

Gomes, R.A., Jr., Moldes, C.A., Delite, F.S., Pompeu, G.B., Gratao, P.L., Mazzafera, P., Lea, P.J., Azevedo, R.A.: Antioxidant metabolism of coffee cell suspension cultures in response to cadmium. - *Chemosphere* **65**: 1330-1337, 2006.

Gonçalves, J.F., Antes, F.G., Maldaner, J., Pereira, L.B., Tabaldi, L.A., Rauber, R., Rossato, L.V., Bisognin, D.A., Dressler, V.L., De Moraes Flores, E.M., Nicoloso, F.T.: Cadmium and mineral nutrient accumulation in potato plantlets grown under cadmium stress in two different experimental culture conditions. - *Plant Physiol. Biochem.* **47**: 814-821, 2009.

Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A., Mansfield, J.: The *RPM1* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. - *Plant J.* **23**: 441-450, 2000.

Gratao, P.L., Polle, A., Lea, P.J., Azevedo, R.A.: Making the life of heavy metal-stressed plants a little easier. - *Funct. Plant Biol.* **32**: 481-494, 2005.

Gratao, P.L., Pompeu, G.B., Capaldi, F.R., Vitorello, V.A., Lea, P.J., Azevedo, R.A.: Antioxidant response of *Nicotiana tabacum* cv. Bright Yellow 2 cells to cadmium and nickel stress. - *Plant Cell Tissue Organ Cult.* **94**: 73-83, 2008.

Groppa, M.D., Rosales, E.P., Iannone, M.F., Benavides, M.P.: Nitric oxide, polyamines and Cd-induced phytotoxicity in wheat roots. - *Phytochemistry* **69**: 2609-2615, 2008.

Heyno, E., Klose, C., Krieger-Liszakay, A.: Origin of cadmium-induced reactive oxygen species production: mitochondrial electron transfer versus plasma membrane NADPH oxidase. - *New Phytol.* **179**: 687-699, 2008.

Horemans, N., Raeymaekers, T., Van Beek, K., Nowocin, A., Blust, R., Broos, K., Cuypers, A., Vangronsveld, J., Guisez, Y.: Dehydroascorbate uptake is impaired in the early response of *Arabidopsis* plant cell cultures to cadmium. - *J.*

exp. Bot. **58**: 4307-4317, 2007.

John, R., Ahmad, P., Gadgil, K., Sharma, S.: Antioxidative response of *Lemna polyrrhiza* L. to cadmium stress. - J. environ. Biol. **28**: 583-589, 2007.

Kadota, Y., Goh, T., Tomatsu, H., Tamauchi, R., Higashi, K., Muto, S., Kuchitsu, K.: Cryptogein-induced initial events in tobacco BY-2 cells: pharmacological characterization of molecular relationship among cytosolic Ca^{2+} transients, anion efflux and production of reactive oxygen species. - Plant Cell Physiol. **45**: 160-170, 2004.

Kawano, T.: Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. - Plant Cell Rep. **21**: 829-837, 2003.

Navrot, N., Rouhier, N., Gelhaye, E., Jacquot, J.P.: Reactive oxygen species generation and antioxidant systems in plant mitochondria. - Physiol. Plant. **129**: 185-195, 2007.

Nocentini, S.: Inhibition of DNA replication and repair by cadmium in mammalian cells. Protective interaction of zinc. - Nucl. Acids Res. **26**: 4211-4225, 1987.

Olmos, E., Martínez-Solano, J.R., Piqueras, A., Hellín, E.: Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line). - J. exp. Bot. **381**: 291-301, 2003.

Perfus-Barbeoch, L., Leonhardt, N., Vavasseur, A., Forestier, C.: Heavy metal toxicity: cadmium permeates through calcine channels and disturbs the plant water status. - Plant J. **32**: 539-548, 2002.

Potocký, M., Jones, M.A., Bezdová, R., Smirnoff, N., Žářský, V.: Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. - New Phytol. **174**: 742-751, 2007.

Potters, G., Pasternak, T.P., Guisez, Y., Jansen, M.A.K.: Different stresses, similar morphogenic responses: integrating a plethora of pathways. - Plant Cell Environ. **32**: 158-169, 2009.

Razem, F.A., Bernards, M.A.: Reactive oxygen species production in association with suberization: evidence for an NADPH-dependent oxidase. - J. exp. Bot. **54**: 935-941, 2003.

Rivetta, A., Negrini, N., Cocucci, M.: Involvement of Cd^{2+} toxicity during the early phases of radish (*Raphanus sativus* L.) seed germination. - Plant Cell Environ. **20**: 600-608, 1997.

Rodríguez, A.A., Lascano, H.R., Bustos, D., Taleisnik, E.: Salinity-induced decrease in NADPH oxidase activity in the maize leaf blade elongation zone. - J. Plant Physiol. **164**: 223-230, 2007.

Rodríguez-Serrano, M., Romero-Puertas, M.C., Zabalza, A., Corpas, F.J., Gómez, M., Del Río, L.A., Sandalio, L.M.: Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation *in vivo*. - Plant Cell Environ. **29**: 1532-1544, 2006.

Romero-Puertas, M.C., Rodríguez-Serrano, M., Corpas, F.J., Gómez, M., Del Río, L.A., Sandalio, L.M.: Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. - Plant Cell Environ. **27**: 1122-1134, 2004.

Sambrook, J., Fritsch, E.F., Maniatis, T. (ed.): Molecular Cloning - a Laboratory Manual. 2nd Edition. - Cold Spring Harbour Laboratory Press, Cold Spring Harbour - New York 1989.

Shen, W., Nada, K., Tachibana, S.: Involvement of polyamines in the chilling tolerance of cucumber cultivars. - Plant Physiol. **124**: 431-440, 2000.

Simon-Plas, F., Elmayan, T., Blein, J.P.: The plasma membrane oxidase Ntrboh D is responsible for AOS production in elicited tobacco cells. - Plant J. **31**: 137-147, 2002.

Torres, M.A., Dangl, J.L.: Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. - Curr. Opin. Plant Biol. **8**: 397-403, 2005.

Van Gestelen, P., Asard, H., Caubergs, R.J.: Solubilization and separation of a plant plasma membrane NADPH- O_2^- synthase from other NAD(P)H oxidoreductases. - Plant Physiol. **115**: 543-550, 1997.

White, P.: Calcium channels in higher plants. - Biochem. biophys. Acta **1465**: 171-189, 2000.

Zhang, H., Fang, Q., Zhang, Z., Wang, Y., Zheng, X.: The role of respiratory burst oxidase homologues in elicitor-induced stomatal closure and hypersensitive response in *Nicotiana benthamiana*. - J. exp. Bot. **60**: 3109-3122, 2009.