Potential role of NADPH-oxidase in early steps of lead-induced oxidative burst in *Vicia faba* roots

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Summary

The mechanism of oxidative burst induced by lead in *Vicia faba* excised roots was investigated by luminol-dependent chemiluminescence. Results showed that lead triggered a rapid and dose-dependent increase in chemiluminescence production. In this study, specific inhibitors of putative reactive oxygen species (ROS) sources were used to determine the mechanism of lead-induced ROS generation. This generation was sensitive to dephenylene iodonium (DPI), quinacrine and imidazole, some inhibitors of the NADPH-oxidase and not inhibited by other putative ROS sources inhibitors. Data reported in this work clearly demonstrated the pivotal role of NADPH-oxidase-like enzyme in early steps of lead-induced oxidative burst. To investigate the respective implication of calmodulin and protein kinase (PK) in lead-induced NADPH-oxidase activation, excised roots were treated with the calmodulin inhibitor W7 or with the PK inhibitor staurosporine. The chemiluminescence generation inhibition by these inhibitors illustrated the role of PK in lead-induced NADPH-oxidase activation and revealed a calmodulin-dependent step. Using the calcium entry blocker La^{3+} or different concentrations of calcium in the extracellular medium, our data highlighted the implication of Ca^{2+} channel in lead-induced oxidative burst.

Abbreviations: AO, amine oxidase; CaMKs, calmodulin-dependent protein kinases; CAT, catalase; CCaMKs, calcium-calmodulin-dependent protein kinases; CL, chemiluminescence; CNGC, cyclic nucleotide-gated channel; CuAO, copper-containing amine oxidase; DPI, dephenylene iodonium; PAO, polyamine oxidase; PK, protein kinase; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; *Vf*, *Vicia faba*

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Introduction

Lead is one of the most abundant heavy metals polluting the soil and environment. It originates from numerous sources and has become an environmental concern (Singh et al., 1997). Lead is toxic to many organs: it is a proven animal carcinogen (Johnson, 1998) and an inducer of many toxic symptoms in plants, such as decrease of growth due to interference with enzymes essential for normal metabolism and development, photosynthetic processes, water and mineral nutrient absorption and changes in cell ultrastructure (Singh et al., 1997). Lead is reported to produce reactive oxygen species (ROS) and to alter the activities of antioxidant enzymes (Malecka et al., 2001; Verma and Dubey, 2003). These ROS include superoxide anions ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), which are produced during membrane-linked electron transport activities as well as by a number of metabolic pathways (Shah et al., 2001).

The rapid production of ROS, or "oxidative burst", was originally identified in mammalian phagocytes and later demonstrated in plant cells (Low and Merida, 1996). It is believed that, in plants, putative sources of ROS during biotic or abiotic stresses are cell-wall-bound peroxidases (Kawano, 2003), xanthine oxidase, amine oxidases (AOs) in the apoplast (Allan and Fluhr, 1997) and plasma-membrane-bound NADPH-oxidases (Bolwell and Wojtaszek, 1997). The key enzyme that contributes to ROS formation in phagocytes is a plasma membrane-bound NADPH-dependent oxidase (Segal and Abo, 1993), the activation of which involves the assembly of at least three cytosolic proteins (*p67*^{phox}, *p47*^{phox} and *p40*^{phox}), two plasma membrane-associated ones (*gp91*^{phox} and *p22*^{phox}) and two small GTP-binding proteins (*p21*^{rac} and *Rap1A*). The activation of this complex induces the translocation and the association of the cytoplasmic proteins to the membrane-bound components. This process involves the phosphorylation of at least p47^{phox} by protein kinase C (PKC). Pharmacological approaches with dephenylene iodonium (DPI) demonstrate the involvement of a NADPH-oxidase-like enzyme in elicited cells of *Arabidopsis thaliana* (Desikan et al., 1996). Moreover, immunological studies using mammalian NADPH-oxidase subunit antibodies have reported the presence of subunits of this enzyme complex (Desikan et al., 1996; Razem and Bernards, 2003). Recently, Overmyer et al. (2003) identified respiratory burst oxidase homologues as plant homologues of the catalytic subunit of phagocyte NADPH-oxidase (*gp91*^{phox}) that were reported to be responsible for ROS production during biotic stress (Simon-Plas et al., 2002). Cadmium-induced oxidative stress is also thought to be mediated by an NADPH-oxidase-like enzyme (Garnier et al., 2006; Olmos et al., 2003). Garnier et al. (2006) suggested that cadmium enters cells via La^{3+}-sensitive transporters or calcium channels to generate this oxidative burst. Lead entry into plant cells also occurs, at least in part, through Ca^{2+}-permeable channels (Huang and Cunningham, 1996; Sunkar et al., 2000).

Although lead toxicity appears related to oxidative stress (Malecka et al., 2001; Verma and Dubey, 2003), the mechanism leading to ROS production remains unclear. An attempt was made in this study to determine the effect of lead on ROS production by chemiluminescence (CL), in lead-treated *Vicia faba* (*Vf*) roots. Specific inhibitors of putative ROS sources were used to determine the mechanism at the origin of lead-induced ROS generation and the role of NADPH-oxidase-like enzyme.

Material and methods

Plant material and growth

Broad beans (*Vf*, L.) were germinated on filter paper moistened with deionized water at 25 °C in the dark. After 7 d, plants were grown in a PVC tank (four plants per tank) containing 500 mL of aerated Hoagland solution. To keep the nutrient composition and pH constant, solutions were renewed daily. The culture systems were located in a growth chamber with day/night air temperatures of 24/22 °C and relative humidity of 70/75%. Plants were provided light 16 h a day. A 600 W Osram Nav-T Super High Pressure Sodium Lamp provided a minimum photosynthetical photon flux of 500 $\mu mol\, m^{-2}\, s^{-1}$ at the top of the plant.

Oxidative burst assay

Lead effects on ROS production were determined based on the rate of superoxide radical ($O_2^{\bullet -}$) formation detected via luminol-dependent CL. $O_2^{\bullet -}$ generate CL that is enhanced by the reaction with luminol (Forgue et al., 1991). CL was measured using a thermostatically controlled luminometer 1251 LKB (25 °C). CL response was expressed in mV.

For each sample, three segment roots (apex) of 1.5 cm, from 3-week-old plants, were rinsed with sterile water and cut in thin slices of 1 mm. *Vf* root segments were then kept in 20 mM Tris HCl medium (pH 8.2). Each assay (800 μL) contained excised plant roots and luminol (1.25 mM) in Tris HCl. For the study of lead capacity to induce CL of excised roots, three concentrations (5, 10 and 20 μM) of lead nitrate were tested. For each assay, lead nitrate was added after basal line stabilization,

directly on the luminometer chamber, and CL was recorded every 5 s.

To determinate the nature of ROS produced during lead-induced oxidative burst, superoxide dismutase (SOD, EC 1.15.1.1; 5 and 50 units) or catalase (CAT, EC 1.11.1.6; 50 and 150 units) were added to medium 10 min before lead treatment.

To investigate the potential source of ROS in lead-induced oxidative burst, the excised roots were respectively pre-incubated 10 min at 25 °C with the NADPH-oxidase inhibitors DPI (0.1 and 1 μM), imidazole (5 and 50 mM) and quinacrine (0.1 and 1 mM), with horseradish peroxidase inhibitor sodium azide (NaN_3, 1 and 10 mM), with xanthine oxidase inhibitor allopurinol (0.2 and 2 mM), with polyamine oxidase (PAO) inhibitor *N*-prenylagmatine (G3, 1 and 10 μM) or with copper-containing amine oxidase (CuAO) inhibitors 2-bromoethylamine (1 and 10 μM) and aminoguanidine (1 and 10 mM); excised roots were then exposed to lead (10 μM).

To analyse the role of protein kinases (PKs) and calmodulin in the NADPH-oxidase system, excised roots were pre-incubated 10 min at 25 °C with the PK inhibitor staurosporine (10 nM and 0.1 μM) or with the calmodulin antagonist W7 (0.2 and 2 mM) before lead treatment (10 μM).

In order to determine the putative lead entry in cells via calcium channels, excised roots were exposed to lead in the presence of $CaCl_2$ (1 or 10 μM) or were pre-incubated 10 min at 25 °C with the calcium channel antagonist $LaCl_3$ (4 and 40 μM) before lead exposure (10 μM).

Potential interference of lead nitrate, DPI, imidazole, quinacrine, NaN_3, allopurinol, G3, aminoguanidine, 2-bromoethylamine, staurosporine, W7 and $LaCl_3$ with the CL assay system (oxidation of luminol by ROS), was tested in the absence of roots using the ROS generating systems: H_2O_2-peroxidase and xanthine–xanthine oxidase systems according to the method previously described (Forgue et al., 1990). In addition, the lack of CL production by direct interaction between luminol and H_2O_2 was also evaluated.

Evaluation of calcium competition for lead entry into the roots

In order to test the antagonism of calcium on lead entry into the roots, lead uptake in *Vf* roots was analysed in the presence or absence of calcium in the culture medium. A stock solution of lead nitrate salt was prepared in Tris HCl medium (pH 8.2) just before the experiments. Three 1.5 cm segments from roots (apex) from 3-week-old plants were rinsed with sterile water and cut in thin slices of 1 mm. *Vf* root segments were put in Tris HCl medium and exposed to lead (10 μM). For investigations with calcium, $CaCl_2$ (1 and 10 μM) was added to medium 10 min before the start of the experiment. Root fragments were collected after 10 min or 1 h of lead exposure and rapidly washed in distilled water. Lead bound to the rhizoderm was removed as follows: fragments were shaken with 40 mL of 0.001 M HCl during 3 min, and then 360 μL of 1 M HCl were added to yield a final concentration of 0.01 M HCl. After shaking for another 5 min, root fragments were washed in distilled water, oven-dried at 80 °C for 48 h, and then dry weight.

Roots were mineralized in a 1:1 mixture of 65% HNO_3 and 30% H_2O_2 at 80 °C over 6 h. After filtration, lead concentrations were determined with an IRIS Intrepid II XDL ICP-OES. The accuracy of the analytical procedure was verified using a reference material: Virginia tobacco leaves (CTA-VTL-2, polish certified reference material; ICHTJ) with $22.1 \pm 1.2\ \mu g\ g^{-1}$ Pb.

Statistical analysis

Results were expressed as mean ± SE of five separate experiments. For each experiment, statistical analysis was performed using a one-way ANOVA and the multiple comparison method of Tukey (Keppel, 1973).

Results

Induction of oxidative burst by lead

Induction of *Vf* excised roots CL activity was measured in the presence of luminol as enhancer, for 120 s at 25 °C after lead treatment (Figure 1A). Lead stimulation was carried out under continuous CL recording. In the absence of lead, the time course of CL production remained constant at a low level (0.45 ± 0.05 mV). In the presence of lead, after a lag time of 15 ± 3 s, CL production increased rapidly and reached a maximum value after 90 ± 15 s of lead stimulation. The CL decreased slowly to the basal line after 350 ± 20 s (data not shown). Excised roots exposed to a lead concentration range from 5 to 20 μM, demonstrated a dose effect on the peak of CL (Figure 1B). The maximum effect was observed for 20 μM. In these conditions, regardless of lead concentration, CL production lag time after lead treatment remained constant (data not shown). Even if the maximum CL values were reached more rapidly for the higher concentrations of lead, results were not significantly different between 5 and 20 μM.

To analyse the nature of ROS produced during lead treatment, root fragments were co-treated with two antioxidant enzymes, the $O_2^{\bullet -}$ scavenger SOD or the H_2O_2 scavenger CAT. Results in Figure 2 show that CL production was dose dependently reduced by SOD (5 and 50 units). In contrast, lead-induced CL production was not modulated by CAT (50 and 150 units).

Potential source of ROS

To determine the role of NADPH-oxidase-like in lead-enhanced CL, excised roots were pre-incu-

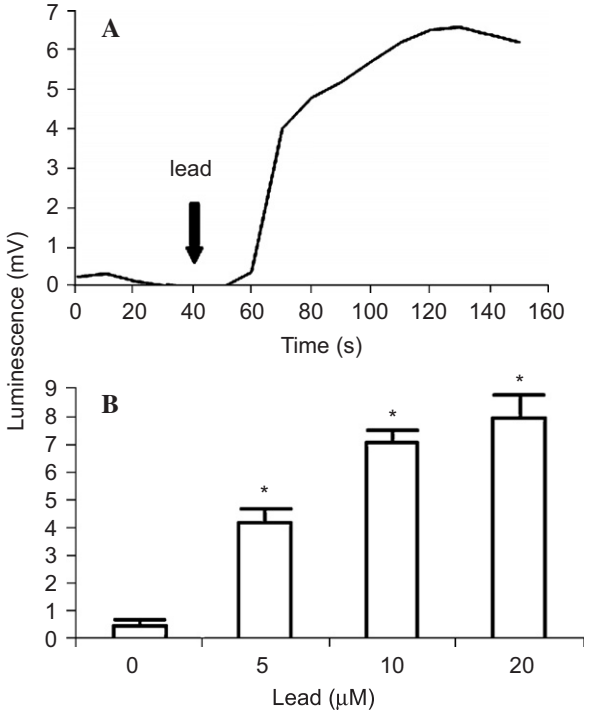

Figure 1. Oxidative metabolism of *Vicia faba* excised roots investigated by luminol-dependent chemiluminescence (CL). CL was measured using a thermostatically controlled luminometer 1251 LKB (25 °C). CL response was expressed in mV. (A) Excised roots were exposed to 10 μM of lead nitrate. The curve is representative of five different experiments with identical results. (B) Mean of CL peak value reached for 0, 5, 10 and 20 μM of lead nitrate treatment. *Values significantly different from control ($p < 0.01$).

bated with two concentrations of imidazole, quinacrine and DPI, 10 min before lead treatment. Results in Figures 3 and 4 show that CL production enhanced by lead (10 μM) was dose dependently inhibited by imidazole (5 and 50 mM) and low concentrations of DPI (0.1 and 1 μM). Results shown in Figure 3 also demonstrate that lead-induced ROS generation was inhibited by quinacrine (0.1 and 1 mM). The potential role of peroxidases in ROS production was investigated using the peroxidase inhibitor NaN_3. After 10 min of pre-incubation with NaN_3 (1 and 10 mM), results showed that this inhibitor had no effect on lead-dependent CL production (Figure 4). Similar results were found (Figure 5) using the xanthine oxidase inhibitor allopurinol (0.2 and 2 mM) and the PAO inhibitor G3 (1 and 10 μM).

In order to investigate the role of the CuAOs in lead-induced NADPH-oxidase-like activation, excised roots were incubated 10 min with the CuAO inhibitors aminoguanidine (1 and 10 mM) and 2-bromoethylamine (1 and 10 μM) before addition

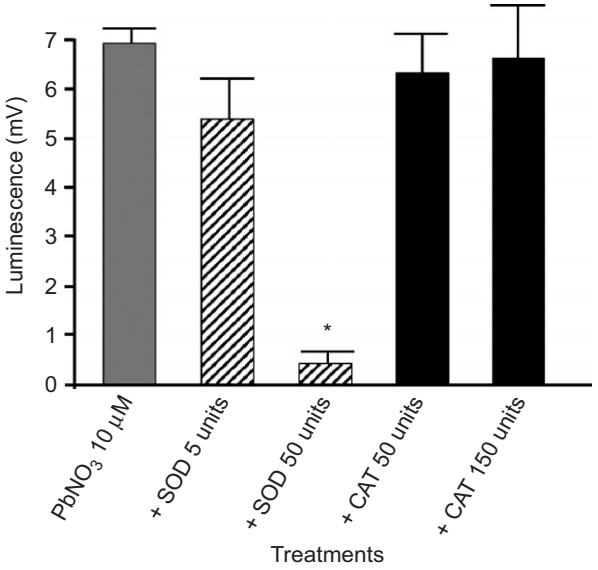

Figure 2. Nature of ROS produced during lead-induced oxidative burst, investigated by CL. Excised roots were pre-incubated 10 min with the $O_2^{\bullet -}$ scavenger SOD or the H_2O_2 scavenger CAT before lead exposure. Means of CL peak value ± SE of five experiments are reported. *Values significantly different from lead-treated roots ($p < 0.01$).

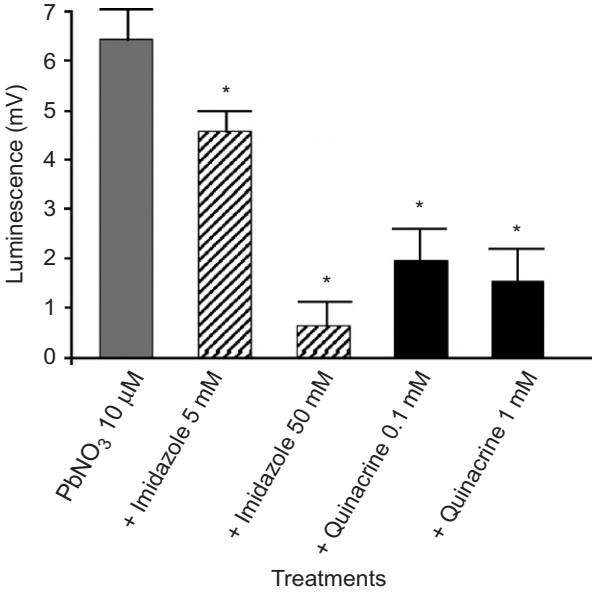

Figure 3. Potential role of NADPH-oxidase in lead-induced oxidative burst investigated by CL. Excised roots were pre-incubated 10 min with the NADPH-oxidase inhibitor imidazole and quinacrine before lead exposure. Means of CL peak value ± SE of five experiments are reported. *Values significantly different from lead-treated roots ($p < 0.01$).

of lead. The lead-induced ROS generation was not modulated by these inhibitors (Figure 6).

Results presented in Figure 7 show that CL production was dose dependently inhibited by the

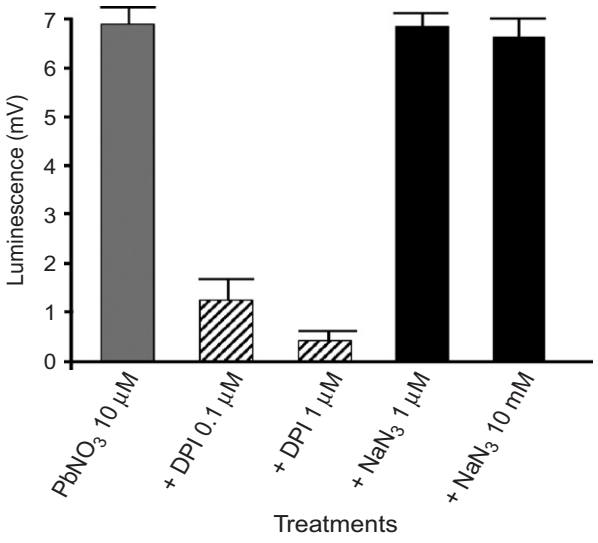

Figure 4. Potential role of NADPH-oxidase and peroxidases in lead-induced oxidative burst investigated by CL. Excised roots were pre-incubated 10 min with the NADPH-oxidase inhibitor DPI or with horseradish peroxidase inhibitor sodium azide (NaN_3) before lead exposure. Means of CL peak value $\pm$ SE of five experiments are reported. *Values significantly different from lead-treated roots ($p < 0.01$).

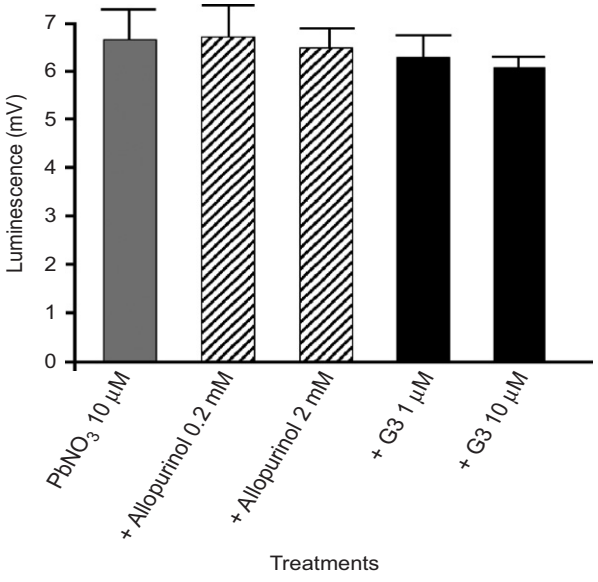

Figure 5. Potential role of xanthine oxidases and polyamine oxidases in lead-induced oxidative burst investigated by CL. Excised roots were pre-incubated 10 min with the xanthine oxidase inhibitor allopurinol or with the PAO inhibitor *N*-prenylagmatine (G3) before lead exposure. Means of CL peak value $\pm$ SE of five experiments are reported.

PK inhibitor staurosporine (10 nM and 0.1 µM). In the presence of the calmodulin inhibitor W7 (0.2 and 2 mM), a huge inhibition of CL production was observed (Figure 7).

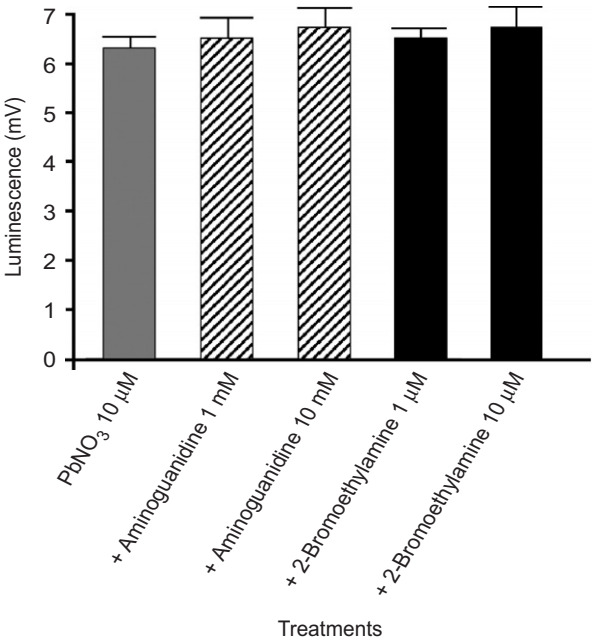

Figure 6. Potential role of copper-containing amine oxidases in lead-induced oxidative burst investigated by CL. Excised roots were pre-incubated 10 min with CuAO inhibitors 2-bromoethylamine or aminoguanidine before lead exposure. Means of CL peak value $\pm$ SE of five experiments are reported.

The role of the calcium channel in the lead-stimulated oxidative burst was investigated using the calcium entry blocker La^{3+}. In excised plant roots exposed to lead, La^{3+} (4 and 40 µM) strongly reduced ROS production (Figure 8), more than 90%. Similar results were obtained in presence of 10 µM $CaCl_2$ in the culture medium. Data also showed that calcium inhibition is dose dependent.

Lead content in roots

In order to investigate the calcium antagonism on lead entry into the roots, *Vf* root fragments were pre-incubated with $CaCl_2$ (1 and 10 µM). After 10 min of co-treatment, results showed that lead entry into roots was strongly reduced, by 93%, with 1 µM of $CaCl_2$ and by 90% with 10 µM $CaCl_2$ (Figure 9A). After 1 h of incubation, for all treatments, lead uptake was higher than that of 10 min-treated roots (Figure 9B). However, lead uptake inhibition by the presence of calcium in the medium culture was also strong. 1 and 10 µM $CaCl_2$ drastically reduced lead uptake by 95% and 91%, respectively. Differences observed between 1 and 10 µM $CaCl_2$ co-treatments were not significant.

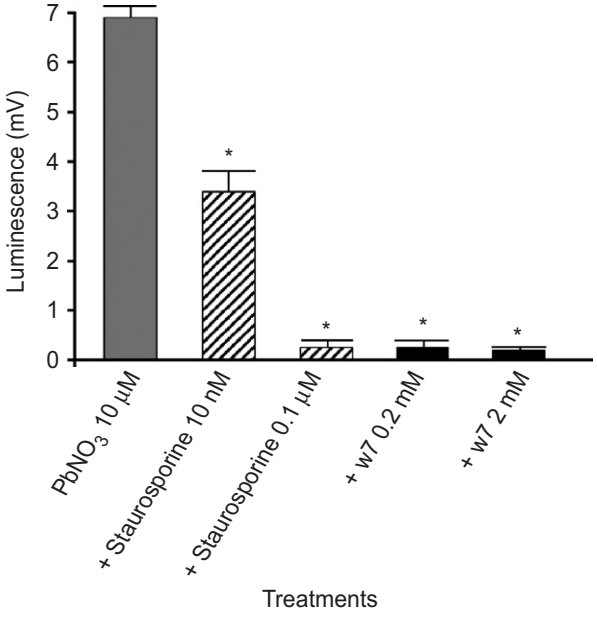

Figure 7. Potential role of protein kinases and calmodulin in lead-induced oxidative burst investigated by CL. Excised roots were pre-incubated 10 min with the PK inhibitor staurosporine or with the calmodulin antagonist W7 before lead exposure. Means of CL peak value ± SE of five experiments are reported. *Values significantly different from lead-treated roots ($p < 0.01$).

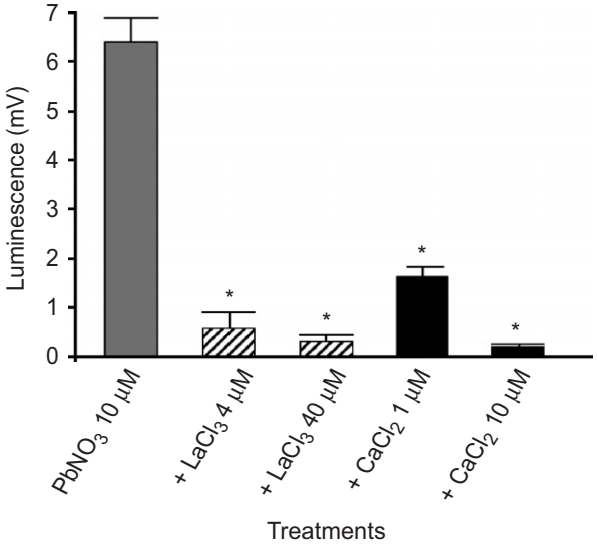

Figure 8. Implication of calcium channel in lead-induced oxidative burst investigated by CL. Excised roots were exposed to lead in presence of $CaCl_2$ or were pre-incubated 10 min with the calcium channel antagonist $LaCl_3$ before lead exposure. Means of CL peak value ± SE of five experiments are reported. *Values significantly different from lead-treated roots ($p < 0.01$).

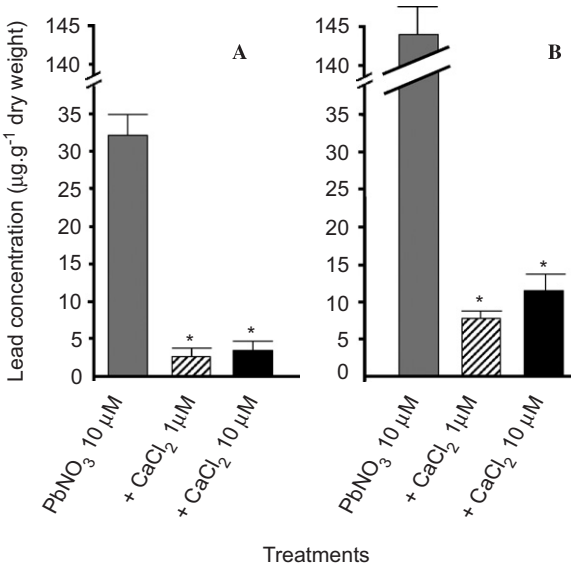

Figure 9. Calcium competition for Pb entry into roots investigated by ICP-OES. Excised roots were exposed to lead in presence of $CaCl_2$ 10 min (A) or 1 h (B). Means of lead concentration ± SE of five experiments are reported. *Values significantly different from lead-treated roots ($p < 0.01$).

Discussion

In this study, oxidative burst was investigated by luminol-dependent-CL. In excised *Vf* roots, lead triggered a rapid and dose-dependent increase in ROS production (Figure 1A and B). Results reported in Figure 2 suggested that, at least in first step, lead-induced oxidative burst is mediated by $O_2^{\bullet -}$ and not by H_2O_2. In plants, four biological systems are putative sources of ROS during biotic or abiotic stresses: plasma-membrane-bound NADPH-oxidases, cell-wall-bound peroxidases, AOs in the apoplast and xanthine oxidase. In this study, specific inhibitors of these putative ROS sources were used to determine the mechanism of lead-induced ROS generation. Our results (Figure 4) showed that ROS production by lead-exposed excised roots was sensitive to millimolar concentration of two widely used NADPH-oxidase inhibitors, imidazole and quinacrine (Auh and Murphy, 1995; Van Gestelen et al., 1997). Data also demonstrated that ROS production was extremely sensitive to micromolar concentration of DPI (Figure 4). This compound has been reported to inhibit the mammalian neutrophil NADPH-oxidase activity by binding itself to the two structural components of the protein (Doussière et al., 1999). These results underline the potential role of NADPH-oxidase-like enzyme in generating ROS in lead-treated *Vf* roots. Using DPI, some authors have suggested that cadmium-induced oxidative stress is

also mediated by an NADPH-oxidase-like enzyme (Garnier et al., 2006; Olmos et al., 2003). However, Frahry and Schopfer (1998) have shown that DPI can also inhibit peroxidase activity. In barley seedlings treated with Co, Cd, Cu or Al, Simonovicova et al. (2004) showed that hydrogen peroxide production is mediated by peroxidase. In this study (Figure 4), lead-induced ROS production was insensitive to millimolar concentration of NaN_3, a well-known peroxidase and cytochrome oxidase inhibitor (Keilin, 1936). These data demonstrated that peroxidase activation is not associated with lead-induced ROS generation. AOs are hydrogen peroxide-producing enzymes that play a role in defence responses to wounding and pathogen invasion (Rea et al., 2002; Yoda et al., 2006). In plants, two main types of AOs are known: the CuAO and the PAO. *In vitro* and *in vivo*, in maize mesocotyl segments, DPI has been reported to be a good PAO inhibitor (Cona et al., 2006). Moreover, PAO activity was modulated by cadmium and copper (Groppa et al., 2007). Cona et al. (2006) demonstrated that *N*-prenylagmatine (G3) is a powerful and selective inhibitor of PAO activity either *in vitro* or *in vivo*. Our data showed (Figure 5) that lead-induced oxidative burst was not modulated by this PAO inhibitor. Similar results (Figure 6) were found using the CuAO reversible inhibitor 2-bromoethylamine (Yu et al., 2001) and with the CuAO irreversible inhibitor aminoguanidine (Yu and Zuo, 1997). Our results demonstrated that AOs are not implicated in ROS generation in early steps of lead-induced oxidative stress. In addition, data demonstrated that ROS production was insensitive to allopurinol (Figure 5), a well-known inhibitor of the xanthine oxidase (Delgado et al., 1966). No work has yet underlined the specific role of xanthine oxidase during metal stress. Romero-Puertas et al. (1999) showed that cadmium had no effect on xanthine oxidase activity in cadmium-treated pea leaf peroxisomes. Moreover, metal ions could also inhibit bovine xanthine oxidase activity (Mondal and Mitra, 1996). According to the results obtained with DPI and the other inhibitors, data reported in this work clearly demonstrated the pivotal role of NADPH-oxidase-like enzyme in lead induction of the oxidative burst.

The inhibition of CL production in *Vf* root tissues treated by lead in presence of the PK inhibitor staurosporine revealed the role of PK enzyme in lead-induced NADPH-oxidase-like activation (Figure 7). In mammalian cells, PKC is involved in NADPH-oxidase activation. In PC12 cells, Jadhav et al. (2000) demonstrated that lead modulated PKC activity. However, there are few reports on the existence and functions of PKC homologues in plants. Although PKC-like enzyme activity has been shown in plants (Subramaniam et al., 1997), its corresponding gene in the *Arabidopsis* genome has not been identified. However, diacyl glycerol-dependent PKC homologous genes have been isolated from other plants, and showed to function in plant defense responses including production of defensive secondary metabolites (Chandok and Sopory, 1998; Subramaniam et al., 1997; Vasconsuelo et al., 2003, 2004). In this work, the complete inhibition of lead-induced ROS production by the calmodulin inhibitor W7 (Figure 7) suggested the role of calmodulin in NADPH-oxidase-like enzyme activation. In plants, calcium-calmodulin-dependent protein kinases (CCaMKs) and calmodulin-dependent protein kinases (CaMKs) are the only PKs with a calmodulin binding domain. Although CaMKs are well characterized from animals and yeast, only one putative representative is known in plants (Watillon et al., 1995). CCaMKs are also rare in plants and might be expressed in the tissues of only a few plants (Poovaiah et al., 1999). Moreover, their exact roles and functions in plants are not well known. Although this study underlined the importance of PK in NADPH-oxidase-like enzyme activation and revealed a calmodulin-dependent step in lead-induced ROS production, the link between PK and calmodulin is not clear.

Our results underlined the possible relationship between calcium channels and lead-induced oxidative burst in *Vf* roots. The calcium entry blocker La^{3+} was efficient in inhibiting CL production (Figure 8). La^{3+} may block voltage-operated channels by inhibiting not only ATPases, but also Ca^{2+}/nH^{+} antiporters (Bush, 1995). In plants, Ca^{2+} transport in isolated membrane vesicles has been attributed to the P-type-Ca^{2+}-ATPase (Bush, 1995). It has been observed that La^{3+} inhibited linear as well as saturable cadmium uptake kinetics. Some authors have suggested that La^{3+} may act by competing with cadmium for plasma membrane transporters (Cohen et al., 1998). Similarly, experiments with simultaneous application of cadmium and extra-cellular Ca^{2+} have shown a competitive response with the inhibition of oxidative burst by Ca^{2+} (Garnier et al., 2006; Olmos et al., 2003). In this work, the results obtained with simultaneous application of Ca^{2+} and lead showed the same competitive response and the total inhibition of lead-induced oxidative burst by extra-cellular Ca^{2+} (Figure 8). Results shown in Figures 9A and B strongly suggested that the inhibition of lead-induced oxidative burst by calcium could be due to the great inhibition of lead entry into the roots. Huang and Cunningham (1996) suggested that lead entry into plant cells occurs, at least in part,

through the Ca^{2+}-permeable channel. Some authors have found that cyclic nucleotide-gated channel (CNGC) may serve as entry pathways for lead in plant cells (Sunkar et al., 2000). These proteins are similar to the mammalian cyclic nucleotide-gated nonselective cation channels and have a calmodulin binding domain. Tobacco transgenic lines that over-expressed a CNGC isoform, NtCBP4, exhibited hypersensitivity to Pb^{2+}, associated with enhanced Pb^{2+} accumulation. In contrast, seedlings that expressed a truncated version of this protein, from which the C-terminal, with the calmodulin-binding domain was removed, showed improved tolerance to Pb^{2+}, with attenuated accumulation of this metal. Therefore, calmodulin could modulate lead uptake in plant cell roots. Our data, which highlighted the complete inhibition of lead-induced ROS production by the calmodulin inhibitor W7 (Figure 7), could confirm these results. Disruption of the *Arabidopsis* CNGC1 gene, which encodes a homologous protein to NtCBP4, reduced Pb^{2+} and Ca^{2+} uptake into plants (Ma et al., 2006). Thus, NtCBP4 and AtCNGC1 are probably Ca^{2+}-permeable channels providing a route for Pb^{2+} entry across the plasma membrane.

Conclusion

In summary, our results suggest that lead entry in root cells may occur via calcium-permeable channels. Data also demonstrated that the early step in lead-induced oxidative burst in *Vf* roots is strongly associated with NADPH-oxidase activation. This study underlined the importance of a PK in NADPH-oxidase-like enzyme activation and revealed a calmodulin-dependent step in lead-induced ROS production. However, NADPH-oxidase may not be the only source of ROS after hours of exposure. Garnier et al. (2006) highlighted that cadmium-induced oxidative burst in tobacco cells was composed of three waves of ROS. The first wave involved a transient NADPH-oxidase-dependent accumulation of ROS, followed by a second wave of $O_2^{\bullet -}$ production by mitochondria. The third wave consisted of fatty acid hydroperoxide accumulation. In this work, a transient NADPH-oxidase-dependent accumulation of ROS in lead-treated *Vf* roots was observed. In addition, Romanowska et al. (2002) observed that, at low concentrations, lead significantly stimulated respiratory rate in mitochondria. All these results suggest close mechanisms for lead and cadmium induction of ROS production. Further work will be necessary to determine other potential sources of ROS in lead-induced oxidative stress.

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