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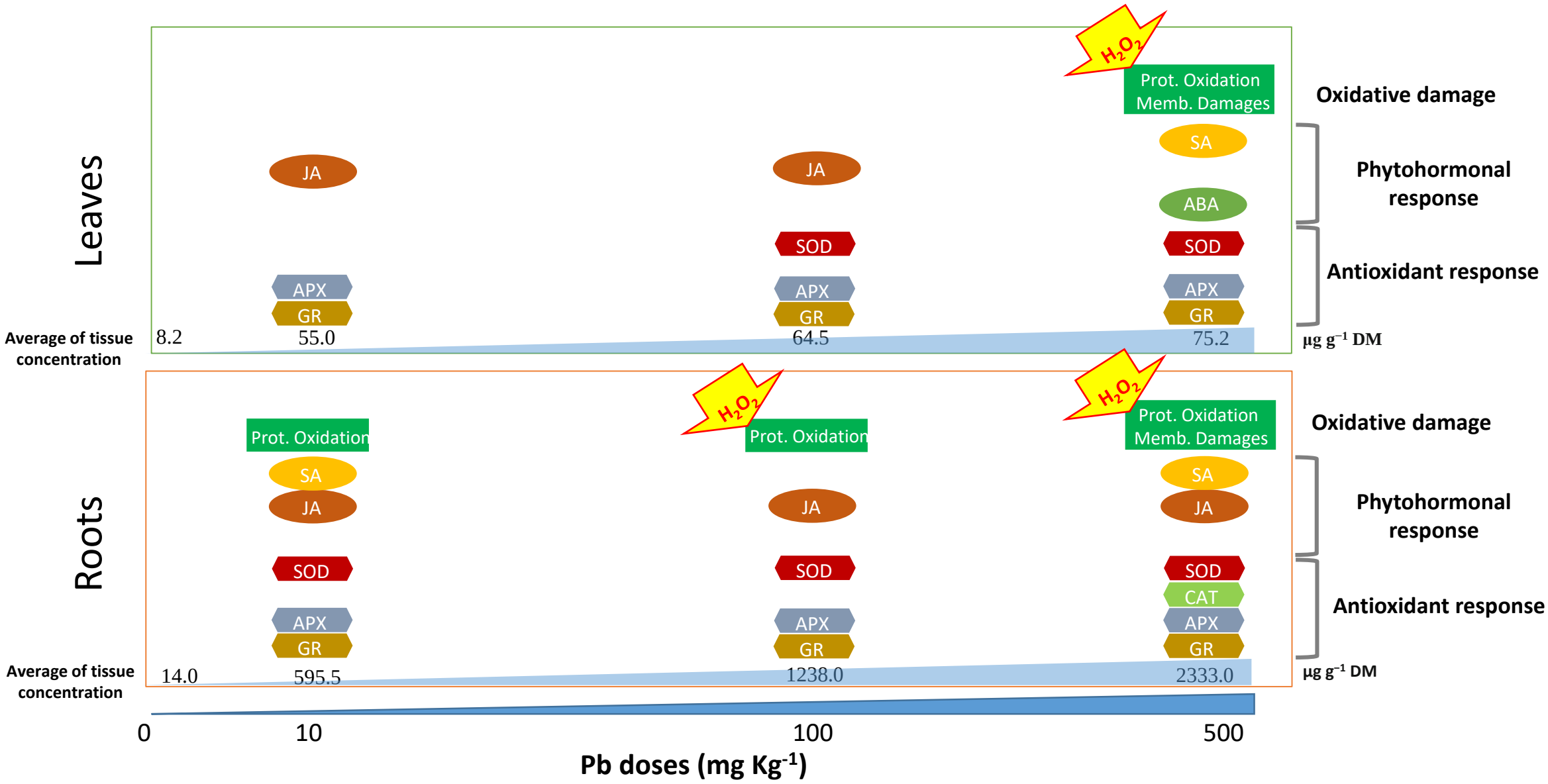
Leaves
Roots
H_2O_2
Prot. Oxidation
Memb. Damages
Oxidative damage
SA
JA
ABA
Phytohormonal response
SOD
CAT
APX
GR
Antioxidant response
Average of tissue concentration
8.2
55.0
64.5
75.2
14.0
595.5
1238.0
2333.0
µg g⁻¹ DM
0
10
100
500
Pb doses (mg Kg-1)

Lead induces oxidative stress in *Pisum sativum* plants and changes the levels of phytohormones with antioxidant role

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Abstract

The interaction of lead (Pb) with plant hormonal balance and oxidative stress remains under discussion. To evaluate how Pb induces oxidative stress, and modulates the antioxidant enzymes and the phytohormones pool, four-week old *Pisum sativum* plants were exposed during 28 days to 10, 100 and 500 mg kg^{-1} Pb in soil. In comparison to leaves, roots showed higher Pb accumulation, oxidative damages and changes in phytohormone pools. Contrarily to leaves, where glutathione reductase (GR) and ascorbate peroxidase (APX) activities were more stimulated than catalase (CAT) and superoxide dismutase (SOD), roots showed a stimulation of SOD, GR and APX in all doses, and of CAT in the highest dose. While protein oxidation occurred in roots even at lower Pb-doses, lipid peroxidation and membrane permeability also occurred but at 500 mg Kg^{-1} and in both organs, accompanied by increases of H_2O_2. Jasmonic acid (JA) responded in both organs even at lowest Pb-doses, while salicylic acid (SA) and abscisic acid (ABA, only in leaves), increased particularly at the concentration of 500 mg Pb kg^{-1}. In conclusion, and compared with leaves, roots showed oxidative damage even at 10 mg Pb Kg^{-1}, being proteins a first oxidative-target, although there is a stimulation of the antioxidant enzymes. Also, JA is mobilized prior to oxidative stress changes are detected, and may play a protective role (activating antioxidant enzymes), while the mobilization of SA is

particularly relevant in cells expressing oxidative damage. Other hormones, like indolacetic acid and ABA may have a low protective role against Pb toxicity.

Keywords: antioxidant enzymes; lipid peroxidation; *Pisum sativum*; phytohormonal response; protein oxidation; reactive oxygen species.

Abbreviations: Abscisic acid, ABA; ascorbate peroxidase, APX; catalase, CAT; cell membrane permeability, CMP; glutathione reductase, GR; hydrogen peroxide, H_2O_2; indolacetic acid, IAA; jasmonic acid, JA; malondialdehyde, MDA; salicylic acid, SA; superoxide dismutase, SOD.

1. Introduction

According to the U.S. Agency for Toxic Substances and Disease Registry (ATSDR, 2017), lead (Pb) persists the second priority hazardous substance. Soil contamination with Pb is mostly due to anthropogenic sources, such as metallurgical wastes, fertilizers, pesticides, Pb enriched sewage sludge and waste waters (Kumar et al., 2012). Although Pb is not an essential element for the plant metabolism, once inside the plant cell it induces a wide range of adverse effects including changes in water and mineral status, photosynthesis (chloroplast structure, pigments, enzymes, and light dependent/independent reactions of photosynthesis) and redox homeostasis (Kaur, 2014; Tripathi et al., 2016; Alamri et al., 2018; Zhou et al., 2018). The redox homeostasis is lost when the generation of reactive oxygen species (ROS) reaches levels that cells are unable to neutralize, and ultimately uncontrolled oxidation of lipids, nucleic acids and proteins occurs. Lead induction of oxidative stress has been documented for example in *Arabidopsis thaliana* (Corpas and Barroso, 2016), *Spinacia oleracea* (Khan et al., 2016), *Amaranthus viridis* and *Portulaca oleracea* (Javed et al., 2017), *Triticum aestivum* (Tripathi et al., 2016; Alamri et al., 2018), and in the metalliferous species *Zygophyllum fabago* (López-Orenes et al., 2017).

Multiple sites/organelles in the cell may be sources and targets of excessive ROS, and both enzymatic and non-enzymatic (e.g. phenolic compounds) antioxidant batteries may work in coordination to maintain a redox balance by neutralizing ROS (Singh et al., 2016; Corpas and Barroso, 2017; Alamri et al., 2018). Moreover, ROS are important signals in the secondary metabolism, for example promoting phytochelatin synthesis, which are major S-rich thiolate peptides crucial in the regulation of metal/metalloid homeostasis (Rodrigo et al., 2016). ROS also mediate hormonal signalling networks [e.g. abscisic acid (ABA), indoleacetic acid (IAA), salicylic acid (SA) and jasmonic acid (JA)] (Xia et al., 2015). This network allows plants to regulate developmental processes, including adaptive responses to

environmental challenges (Singh et al., 2016). Specific responses of phytohormones are usually interdependent in both synergistic and antagonistic ways (Verhage et al., 2010). Phytohormones are described to enhance plant stress defence through the stimulation of the antioxidant system, such as promoting the increase of the antioxidant enzymes and metabolites (e.g. metabolites containing thiols), which in turn helps to control oxidative stress (Piotrowska et al., 2009; Shing et al., 2016; Shukla et al., 2017). However, how metals interfere with this complex network linking hormonal signalling and oxidative stress remains under debate (reviewed by Singh et al., 2016; Bücker-Neto et al., 2017).

Metals like Cd, Ni, Zn and Al increased ABA levels in plants (Bücker-Neto et al., 2017). The JA–amino acid conjugate (jasmonyl-isoleucine), which responds to multiple biotic/abiotic factors, is connected with the phytotoxicity of heavy metals. For example, jasmonyl-isoleucine influenced Cu and Cd toxicity in *A. thaliana* plants (Maksymiec et al., 2005). However, like ABA little is known about the relation of JA with the excess of Pb. Salicylic acid, besides its protective role against pathogens, alleviated the toxicity of Cd and of Pb in maize (Popova et al., 2012; Elhassan et al., 2016). Finally, IAA regulates both cell division and elongation, and is involved in the adaptation of plants to drought, salinity and stress induced by metals (Park, 2007), including Zn (Fässler et al., 2010). Similarly to other hormones, how Pb regulates IAA pools remains to be addressed.

The objective of this work was to evaluate how Pb modulates the pools of phytohormones (IAA, ABA, SA and JA) and if these variations may correlate with changes in ROS and/or oxidative stress. For that we exposed young plants of *P. sativum* to Pb (up to 500 mg kg^{-1} soil) during 28 days. Oxidative damages, antioxidant enzymes, ROS species (hydrogen peroxide) and the profile of IAA, ABA, SA and JA were evaluated. The crop *P. sativum* is an important model species in metal toxicology, having a fast growth and being amenable to genetic manipulation and reproduction studies (Rodriguez et al., 2013, 2015). Moreover, the cultivars 'Kwestor', 'Little Marvel', 'Perfection', 'Corne de Bélier' and 'Alderman' have been pinpointed as candidate models in soil decontamination programs (Piechalak et al., 2003; Rodriguez et al., 2011, 2012, 2013).

2. Material and Methods

2.1. Plant culture conditions and exposure to Pb

Pea seeds (*P. sativum* L., cv 'Corne de Bélier', IPSO BP 301, 26401 Crest, France) were hydrated for 48h and then sown in black round pots (12 x 12 cm) containing 300g of a mixture of peat and perlite (4:1, dry weight). Plants (one per pot) were grown in a climate chamber at 24 ºC ± 2 ºC, under a photosynthetic light intensity of 250 µmol m^{-2} s^{-1} and a photoperiod of 16 h/8 h (light/dark). Plants with four-weeks were randomly divided for four groups, and each

group was randomly attributed to a specific treatment (4 treatments, 15 plants per treatment). Plants were exposed to the Pb treatments during 28 days. Lead chloride ($PbCl_2$ - Sigma-Aldrich, St. Louis, MO, USA) was used as source of Pb and was dissolved in a Hoagland's solution (1:10). Twice a week, plant pots (with a peat:perlite mixture) were irrigated with 100 mL of solutions with respectively 0 (control), 20, 200 and 1,000 mg L^{-1} to which corresponded the real concentrations of 0, 19.8, 201.9, 998.7 and 1988.4 mg L^{-1} (determined by inductively coupled plasma atomic emission spectroscopy, Jobin Yvon, JY70Plus, Longjumeau Cedex, France). With these irrigations the final content of Pb in the peat:perlite soil matrix was of 0, 10, 100 and 500 mg of $PbCl_2$ kg^{-1} (Rodriguez et al., 2015).

After treatments, plant height (roots and shoots) was assessed and fresh leaf samples were collected for cell membrane permeability quantification. For Pb quantification leaves were collected and dried, while for phytohormones, antioxidant enzymes, lipid peroxidation and protein oxidation assays, leaves were immediately collected, frozen in liquid nitrogen and stored at -80C. For phytohormone and Pb quantification, leaves and roots from three plants per treatment were used (n = 3, three plants, and for each plant, three technical-replicates were performed), while for the other parameters leaves and roots were collected from six to eight plants per treatment (n = 6 – 8, six to eight plants, one/two technical-replicates per plant).

2.2. Pb content in leaves and roots

At the end of the experiment, roots were immersed in a solution of 0.5mM $CaSO_4$ to remove Pb^{2+} adsorbed to the tissue surface for 10 min and after that rinsed in distilled water (López-Orenes et al., 2017). Then, fresh roots and leaves were dried at 60ºC until constant weight, followed by an incineration at 530ºC during 8h. Ashes were treated with 1mL HCl at 37% (Sigma-Aldricht, USA) and 5 drops of Milli-Q Water, and the mixture heated to boiling. Another 1 mL of HCl was added and the suspension heated again. Ten mL of HCl at 10% (v/v) was added and the mixture was filtered (2 μm filter). The samples were analysed for elemental determination by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Yvon, JY70Plus, Longjumeau Cedex, France).

2.3. Analyses of hormones

The content of the hormones, abscisic acid (ABA), salicylic acid (SA), indoloacetic acid (IAA) and jasmonic acid (JA) were quantified in pea leaves and roots. Frozen powdered leaf tissues (400 mg) were extracted in ultrapure water (8 mL) according to Durgbanshi et al. (2005). Before extraction, samples were spiked with deuterated standards of each hormone. Samples were centrifuged at 13,000 g for 10 min, the supernatant pH was adjusted with 30% acetic acid to 3.0 and partitioned twice against diethyl-ether. The organic layer was evaporated in a vacuum at

119 room temperature, and the dry pellet was resuspended with 1 mL of a water:methanol (9:1) solution. After filtration
120 (0.22 µm cellulose acetate filter), 20 µL of the resulting solution was injected in an HPLC system (Waters Alliance
121 2690 system, Waters Corp., Milford, USA). Hormones were detected according to their specific transitions using a
122 multiresidue mass spectrometric method (Quattro LC Triple Quadrupole, Micromass, Manchester, UK; for
123 chromatographic and mass spectrometry details see Durgbanshi et al., 2005).
124
125 2.4. Antioxidant enzyme activity, soluble protein and H_2O_2 contents
126 Leaves and roots frozen powder (0.5 g) were extracted with 5 mL of an extraction buffer containing 2.5 mL of 200
127 mM potassium phosphate buffer (pH 7.5), 1.25 mL of 2 mM ethylenediaminatetraecetate disodium (Na_2EDTA), 50
128 mg of polyvinylpyrrolidone (1%, m/v), 0.85 mg of phenylmethylsulfonyl fluoride (1mM), 10 µL of Triton X-100
129 (0.2%, v/v) and 1.5 mg of ditiothreitol (2 mM) (Araujo et al., 2016). After centrifugation (13,000 g for 15 min at
130 4ºC), the supernatant (extract) was used to quantify the activities of SOD (EC1.15.1.1), CAT (EC 1.11.1.6), GR (EC
131 1.6.4.2) and APX (EC1.11.1.11). Additionally, in the same extract the concentration of soluble proteins was
132 quantified using the Bradford method (Total Protein Kit, Micro Sigma, Germany).
133 Superoxide dismutase activity was determined according to Agarwal et al. (2005). The reaction mixture (1.5 mL)
134 contained 75 µL of 1 M potassium phosphate buffer (pH 7.8), 2.25 µL of Milli-Q Water, 195 µL of 0.1 M
135 methionine, 15 µL of 0.01 M Na_2EDTA, 150 µL of 0.5 M Na_2CO_3, 9.75 µL of 0.01 M nitro blue tetrazolium (NBT)
136 and 50 µL of the extract. Riboflavin (3 µL from 1 mM) was added last and the reaction was started by illuminating
137 (with a fluorescent lamp of 15 W) the tubes for 15 min. The reaction was stopped by switching off the light. A non-
138 irradiated reaction mixture was running in parallel (blank). Additionally, a reaction mixture without the extract
139 (control) was also prepared and illuminated as described above. The absorbance at 560 nm was determined using a
140 Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). One unit (U) of SOD activity was
141 defined as the amount of enzyme that inhibits the NBT photochemical reduction by 50% per minute.
142 Catalase activity was quantified according to Beers and Sizer (1952). In a centrifuge tube, 200 µL of 1 M potassium
143 phosphate buffer (pH 7.0), 50 µL of the extract and 1350 µL of Milli-Q Water were added. To start the reaction, 400
144 µL of 60 mM H_2O_2 was added and after 5 min the reaction was stopped with 4 mL of titanium reagent (150 mL of
145 H_2SO_4 + 1g of TiO_2 + 10 g of K_2SO_4). After centrifugation at 10,000 g for 10 min at 4ºC the decrease of absorbance
146 at 415 nm was recorded in a spectrophotometer (Genesys 10 - Thermo Fisher Scientific Inc., Waltham, USA). Two
147 controls were performed using the same procedure but without extract. Also, a blank without H_2O_2 was prepared.
148 The enzyme activity was determined from a CAT standard curve (R^2=0.91). Glutathione reductase activity was

assayed according to Sgherri et al. (1994) by mixing 10 µL of extract with 350 µL of 0.2 M potassium phosphate buffer (pH 7.5), 250 µL of 2 mM Na_2EDTA, 50 µL of 30 mM $MgCl_2$, 100 µL of 2.5 mM GSSG and 240 µL of Milli-Q Water. The reaction was initiated by the addition of 250 µL of 2 mM NADPH. The decrease in absorbance at 340 nm was measured at 25ºC with a spectrophotometer (Genesys 10 - Thermo Fisher Scientific Inc., Waltham, USA) and the enzyme activity calculated using the molar extinction coefficient 6.22 mM^{-1} cm^{-1}. Ascorbate peroxidase activity was assayed according to the method of Nakano and Asada (1981). The reaction mixture contained 75 µL of 0.1 M potassium phosphate buffer (pH 7.5), 15 µL of 1 mM of ascorbic acid, 15 µL of 0.5 mM Na_2EDTA, 50 µL of extract and 1342 µL of Milli-Q Water. The reaction was started by adding 3 µL of 0.05 M H_2O_2 and the decrease in ascorbate at 290 nm was measured using a Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA).

For the quantification of H_2O_2, 0.1 g of frozen leaf and root powder were mixed separately with 1 mL of trichloroacetic acid (TCA) (5%, w/v) and 0.15 g of activated charcoal (López-Orenes et al., 2017). After centrifugation (10,000 g for 20 min, 4ºC), the pH of the supernatant was adjusted to 8.4 with NH_3 (17M) and the extracts were divided in two aliquots. Catalase (8 µg) was added to one aliquot and 1 mL of the colorimetric reagent [10 mg of 4-aminoantipyrine + 10 mg of phenol + 5 mg of peroxidase (150 U/mg) in 50 mL of 100 mM acetic acid buffer at pH 5.6] was added to both aliquots. After incubation (10 min at 30ºC), the absorbance was read at 505 nm (Genesys 10 spectrophotometer - Thermo Fisher Scientific Inc., Waltham, USA), and H_2O_2 was quantified against a H_2O_2 standard curve ($R^2 = 0.99$).

2.5. Cell membrane permeability (CMP) and lipid peroxidation (MDA)

Electrolyte leakage was used to quantify CMP according to Lutts et al. (1996). Leaf and root samples were immersed in de-ionized water (1mL /10 mg of tissue) and after incubation overnight at 25ºC on a rotary shaker, the electrical conductivity of the solution (Lt) was measured. Samples were then autoclaved (20 min at 120ºC) and the conductivity was once again determined (L0). The electrolyte leakage was calculated as Lt/ L0 (%).

Lipid peroxidation was obtained by measuring malondialdehyde (MDA) formation according to Dhindsa et al. (1981). Leaves and roots frozen powder (0.1g) were grounded separately with 1 mL of 0.1% trichloroacetic acid (TCA, w/v). After centrifugation at 10,000 g (10 min at 4ºC), 1 mL of the supernatant was mixed with 4 mL of 20% TCA (w/v) with 0.5% of thiobarbituric acid (w/v) and incubated for 30 min at 95ºC. Extracts were immediately cooled on ice, samples were centrifuged (10,000 g for 10 min at 4ºC) and the supernatant was read at 600 and 532 nm using a Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). MDA content was

calculated by subtracting the nonspecific absorption (600 nm) from the absorption at 532 nm using an absorbance coefficient of extinction, 155 mM^{-1} cm^{-1}.

2.6. Protein Oxidation

Proteins were extracted with the same extraction buffer described above for the antioxidant enzymes (Monteiro et al., 2012). However, the extraction buffer also contained 1% (w/v) of sulphate streptomycin. The mixture was incubated for 20 min to eliminate nucleic acids and then centrifuged (5,000 g for 10 min at 4ºC). The supernatant (250 µL) was mixed with 250 µL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl. A tube without DNPH, but with the same volume of supernatant and HCl, was used to quantify protein in the samples (blank). Both tubes, with and without DNPH, were incubated for 15 min in dark at room temperature. A solution of 50% TCA (125 µL) was added and incubated at −20 °C, for at least 15 min. After centrifugation (9,000 g for 15 min at 4ºC), the pellets were washed three times with 900 µL of cold ethanol/ethyl acetate (1:1, v/v). After another centrifugation at 9,000 g for 2 min the pellet was resuspended with 1 mL of 6 M guanidine-HCl. The absorbance of the tubes contained DNPH were read at 370 nm, while those without DNPH were read at 280 nm. Carbonyl content was then calculated using a standard curve (R^2=0.99) from bovine serum albumin (Sigma, USA).

2.7. Statistics

All determinations were obtained with randomly chosen plants. A total of fifteen plants were used per Pb treatment. Of these, for phytohormone and Pb quantification, leaves and roots from three plants per treatment were used (n = 3), while for the other parameters leaves and roots were collected from six to eight plants per treatment (n = 6 – 8). Data were analysed by one-way analysis of variance (ANOVA) combined with the post-hoc Holm-Sidak multiple comparison test (significant level: 0.05). The statistical program, Sigma Stat (Windows, version 3.1) was used. Data are presented as mean ± standard deviation (SD). Principal component analysis (PCA) was performed using the "CANOCO for Windows" programme v4.02 (ter Braak and Smilauer, 1999).

3. Results

3.1. Pb content, plant survival rate and growth

Lead accumulation in roots and shoots significantly increased with the increase of Pb content in the medium, with maximal accumulation values observed at 500 mg kg^{-1} (Table 1). Most of the metal was accumulated in roots (Table 1). Lead exposure did not induce plant death (100% survival rate). Also, leaves from all conditions had no

symptoms of necrosis nor chlorosis. Whilst no differences were observed in roots' length in comparison to controls, plants exposed to 500 mg Pb kg^{-1} had a shorter ($P<0.05$) aerial part (reduction of 21%; Table 1).

3.2. Hormone level

Hormone levels showed different profiles in leaves and roots. ABA and SA levels increased significantly (49% and 40%, respectively) in leaves exposed to 500 mg Pb kg^{-1} compared to control plants (Table 1). In roots, ABA content were not affected ($P>0.05$) by Pb, while the SA content was significantly higher in 10 and 500 mg Pb kg^{-1} compared to control plants (increased 61% and 70%, respectively; Table 1). No significant differences were observed between IAA levels in control and Pb-exposed plants. The levels of this hormone in roots were below the detection levels (Table 1). Leaves exposed to 10 and 100 mg Pb kg^{-1} and roots exposed to all Pb content showed levels of JA higher ($P<0.05$) than controls (increase of 75% and 71% in leaves, respectively; and increase of 70%, 47% and 52% in roots, respectively; Table 1).

3.3. Cell membrane permeability, lipid and protein oxidation

Cell membrane permeability and the levels of MDA increased significantly only in leaves of plants exposed to the highest Pb concentration (Figs. 1 A and C). Similar increases were observed in roots for concentrations of 100 and 500 mg Pb kg^{-1} (Figs. 1 B and C). Protein oxidation only increased in leaves of plants exposed to 500 mg Pb kg^{-1} compared to controls (Fig. 1E). However, in all Pb-exposed roots the levels of carbonyl contents increased ($P\leq0.05$, Fig. 1F).

3.4. Antioxidant enzyme activities and H_2O_2 content

Leaves of plants exposed to 100 and 500 mg Pb kg^{-1} showed a SOD activity significantly higher than control and plants exposed to 10 mg Pb kg^{-1} (Fig. 2A). However, in roots SOD activity was already significantly higher upon the exposure to the lowest Pb concentration (10 mg kg^{-1}) (Fig. 2B). Concerning the activity of CAT, a significant increase was observed only in roots of plants exposed to 500 mg Pb kg^{-1} (Figs. 2C and D). The APX and GR activities were significantly higher in the leaves and roots of plants exposed to Pb compared to control plants (Figs. 2 E-H). The levels of H_2O_2 increased significantly only in leaves of plants exposed to the highest Pb concentration, while in roots this increase ($P<0.05$) was observed for both 100 and 500 mg Pb kg^{-1}, compared to control plants (Figs. 2 I and J).

3.5. PCA analysis

The multivariate data analysis approach was applied to check if the parameters related to growth, oxidative stress and hormones were involved in specific responses of pea plants (leaves and roots) for each Pb concentration (Figs. 3A and B). PCA ordination revealed a clear separation between control and Pb treatments. In leaves and roots, control plants were grouped together (in the upper left and bottom left quadrant, respectively) suggesting a homogeneity in the physiology and biochemistry of plants under control conditions. Concerning the Pb conditions, a clear separation between scores of the low and medium Pb concentrations (10 and 100 mg kg^{-1}), and the highest Pb concentration (500 mg kg^{-1}) is notorious.

In leaves, sample scores for the Pb concentration of 10 and 100 mg kg^{-1} were all located on the lower quadrant, further from the control scores, mostly because of the high levels of JA and the activity of the enzymes GR and APX (see arrow direction in Fig. 3A). Sample scores for the highest Pb concentration were located on the right quadrant, further from the other Pb concentration scores, most because of high levels of SA, H_2O_2, MDA, CMP and carbonyl (Fig. 3A). In roots, sample scores for the 10 and 100 mg kg^{-1} Pb concentrations were located on the upper quadrants (left and right), further from the control scores, mostly because of high GR activity, JA and carbonyl contents (Fig. 3B). Sample scores for 500 mg Pb kg^{-1} were mainly located on the right quadrant, further from the control, 10 and 100 mg Pb kg^{-1} scores, most because of the high activity of SOD and CAT, and content of MDA, CMP, H_2O_2 and SA (Fig. 3B).

4. Discussion

Lead accumulation in pea roots and leaves increased according to the increases of exogenous Pb concentrations, and preferably in roots. This low mobility to the aerial parts was also demonstrated by Kumar et al. (2012) and López-Orenes et al. (2017) supporting a strategy to avoid toxicity in above-ground parts. However, Pb low translocation to the shoot can be due to other reasons including Pb immobilization by negatively charged pectines within the cell wall, accumulation in plasma, precipitation of insoluble lead salts in intercellular spaces, and sequestration in the vacuoles of root cells (Pourout et al., 2011). Nevertheless, the accumulated Pb was sufficient to induce oxidative stress in both leaves and roots, in a dose- and organ-dependent manner.

For leaves, PCA (Fig. 3 A) showed that GR and APX are grouped together suggesting that these enzymes are correlated and varied together. Moreover, their proximity with 10 and 100 mg Kg^{-1} Pb scores (Fig. 3 A) confirm the important protective role of GR and APX for plants exposed to these Pb concentrations. For instance, leaf H_2O_2 contents at the Pb concentrations of 10 and 100 mg Kg^{-1} were maintained at the levels of control plants, probably

due to the coordinated action of GR and APX, efficiently avoiding high oxidative damages. In turn, CMP, H_2O_2, MDA and carbonyl, are also grouped together, but near the scores of 500 mg Pb Kg^{-1} (Fig. 3 A), indicating that this Pb concentration induced higher oxidative damages. So, for the highest Pb concentration, it seems that the plant antioxidant battery was not able to deal with the excess of ROS formed (H_2O_2) and high lipid and protein oxidative damages occurred together with an increased membrane degradation.

A different pattern of Pb modulation of the oxidative stress was observed in roots. Protein oxidation occurred already at the lowest Pb dose, while lipid peroxidation and membrane permeability were mostly increased at 500 mg Pb Kg^{-1}. This profile confirms that the accumulated Pb in roots at 10 mg kg^{-1} was sufficient to exceed the root detoxification capacity. Also, PCA (Fig. 3 B) showed that MDA, CMP and H_2O_2 are grouping together close to 500 mg Pb Kg^{-1} scores, indicating that this concentration was more harmful for the plants.

Membrane lipid peroxidation is commonly used as a sensitive biomarker of the deleterious effects of metals. Wang et al. (2008, 2010) reported for *Vicia faba* seedlings an enhancement of both MDA and carbonyl groups, after Pb (up to 2000 mg Pb Kg^{-1}) exposure. In the present work, being that protein peroxidation showed to be more sensitive to lowest Pb doses than MDA, the more widespread use of this parameter as a sensitive endpoint in further ecotoxicological assays deserves further attention. The increased CMP in pea plants is linked to lipid and protein degradation/oxidation, being a primary marker of loss of cell viability, which is also consistent with the tendency for lower plant height in plants exposed to the highest Pb concentration. The growth inhibition as result of oxidative stress induced by Pb has also been reported in several species (Piechalak et al., 2003; Rodriguez et al., 2015; López-Orenes et al., 2017), corroborating our data.

The antioxidant enzymatic battery play an important role in internal Pb detoxification, and several studies in other species showed that SOD, CAT, APX and GR activities increased with Pb accumulation in cell tissues (Fatma and Ahmad, 2005; Wang et al., 2008, 2010; Pourrut et al., 2013; Singh et al., 2016). However, the antioxidant enzymes in Pb-exposed pea plants (particularly in roots) were insufficient to counteract the damages produced by ROS. Ouzounidou et al. (1997) reported that CAT inactivation can be associated with the binding of Pb to the enzyme thiol group. On the other hand, other alternative enzymes may be considered in antioxidative protection, namely the chalcone synthase and phenylalanine ammonia-lyase, as proposed by Singh et al. (2016).

After being perceived by the receptors, metals initiate signalling cascades involving changes in cAMP, pH, and phytohormones. These are located downstream to the ROS signal, but also ROS molecules act as secondary messengers in many hormone signalling pathways (Das et al., 2015). Our data show an involvement of Pb on ABA, JA and SA response, but not in IAA. Studies on the effect of metals on IAA levels are scarce and contradictory

(Kohli et al., 2017). For example, contrarily to our findings, Sun et al. (2010) found a stimulation of this hormone by Al, while Choudhary et al. (2010) reported a decrease of IAA in radish plants exposed to Cu and Cr^{6+}.

Abscisic acid is one of the most studied hormones (Bücker-Neto et al., 2017) and its involvement as a metal-stress signalling molecule was demonstrated for Cu and Cd (Monni et al., 2001) and Ni (Kanwar et al., 2012). In the case of Pb, the multivariate analyses for pea roots (Fig. 3 B) showed that ABA is not linked with Pb exposure (arrow direction is contrary to Pb scores), having the opposite behaviour in leaves (Fig. 3 A) where ABA is near 500 mg Pb Kg^{-1} scores, which suggests its mobilization possible as a stress signalling molecule. Since ABA is usually related with stomatal opening, the increase observed here for leaves may also justify our previous findings that Pb exerts severe effects on photosynthesis promoting stomatal closure (transpiration and stomatal conductance reduction; Rodriguez et al., 2015).

Jasmonic acid seems to have an organ-independent response as it is stimulated in both roots and leaves. However, PCA for the leaves (Fig. 3 A) shows that JA in near the scores of 10 and 100 mg Pb Kg^{-1} and is positively correlated with GR and APX, suggesting that the protective role of JA at these Pb concentrations may imply the stimulation of these antioxidant enzymes (Bücker-Neto et al., 2017), thus preventing the increase of H_2O_2. This JA stress preventive role was less efficient in roots (probably due to the much higher accumulation of Pb), resulting in protein oxidation even at the lowest Pb dose. Similar to our findings, metal stress is reported to stimulate endogenous jasmonates levels in *A. thaliana*, *Phaseolus coccineus*, *Oryza sativa*, *Capsicum frutescens* and *Kandelia obovate* (Koeduka et al., 2005; Maksymiec et al., 2005; Yan and Tam et al., 2013), inducing protective effects, namely enhancing the antioxidant enzymes, reducing the membrane damages and H_2O_2 contents (Keramat et al., 2009). Piotrowska et al. (2009) reported that 0.1 μM JA supplementation activated CAT and APX, as well as ascorbate and glutathione, in the aquatic plant *Wolffia arrhizal* exposed to Pb, thus preventing oxidative destruction of cellular components induced by this metal. Similarly, higher levels of JA (1μM) supplementation stimulated the antioxidant machinery of *Glycine max* and protected the DNA synthesis of total proteins (Sirhindi et al., 2015). We suggest here that JA is highly involved in the increase of tolerance to Pb. Nevertheless, the reason why this hormone achieved the highest content in the lowest Pb dose deserves further studies.

In the case of the hormone SA, PCA (Fig. 3) for leaves and roots clearly shows its position close to the 500 mg Pb Kg^{-1} scores, suggesting that this hormone is preferentially stimulated by high Pb concentrations. Moreover, SA is the only hormone that correlates positively with both oxidative disorders and antioxidant enzymes (MDA, H_2O_2, CMP and SOD are grouping together with SA, Fig. 3) in leaves and roots. These data suggest that SA also stimulates the antioxidant enzymes, as demonstrated by Kholi et al (2017), but contrarily to JA, its action is more

relevant when plants are under a high oxidative pressure (Rao et al., 2000). Singh et al. (2016) even proposed that high levels of SA can promote metal tolerance through the stimulation of the antioxidant enzymes conducted putatively by ROS signalling, but it can also mediate cell death through oxidative stress signalling. This dual role of this hormone can also be evidenced in the global effects of Pb at 10 mg kg^{-1} and 500 mg kg^{-1}.

5. Conclusions

Figure 4 represents a general overview of the main responses of *P. sativum* to Pb concentrations. In conclusion, we demonstrate that Pb-induced oxidative stress and hormonal changes differ between roots and leaves, probably due to the much higher accumulation of Pb in roots. Root cells are under oxidative pressure even at the lowest Pb-dose, despite the stimulation of GR, APX and SOD. Differently, protein oxidation is only evident in leaves exposed at 500 mg Pb Kg^{-1}, and APX and GR seem to be sufficient to counteract any oxidative stress at the lowest dose. Interestingly, JA may have a broader protective role, responding in general to all Pb concentrations, but acting more intensively when cells are under lower oxidative stress, suggesting a preventive antioxidant action (activating some antioxidant enzymes). Contrarily, SA antioxidant role is more relevant when plants already show an accumulation of H_2O_2, and lipid/protein peroxidation and membrane degradation. Thus we suggest that SA may be involved in the activation of other antioxidant defence mechanisms when cells are already suffering oxidative damages.

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Author contributions

MC Dias performed the experiments and did the measurements. MCDias, CSantos and NMariz-Ponte interpreted the results and wrote the manuscript.

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Table

Table 1. Quantification of Pb in different organs of *P. sativum*. Pb content (µg g^{-1} DM), plant height (cm) and hormone content (ng g^{-1} FW) in control and Pb exposed pea plants. Values are means ± SD (n = 3-6). For each line, different letters indicate significant differences between treatments at a significant level equal to 0.05.

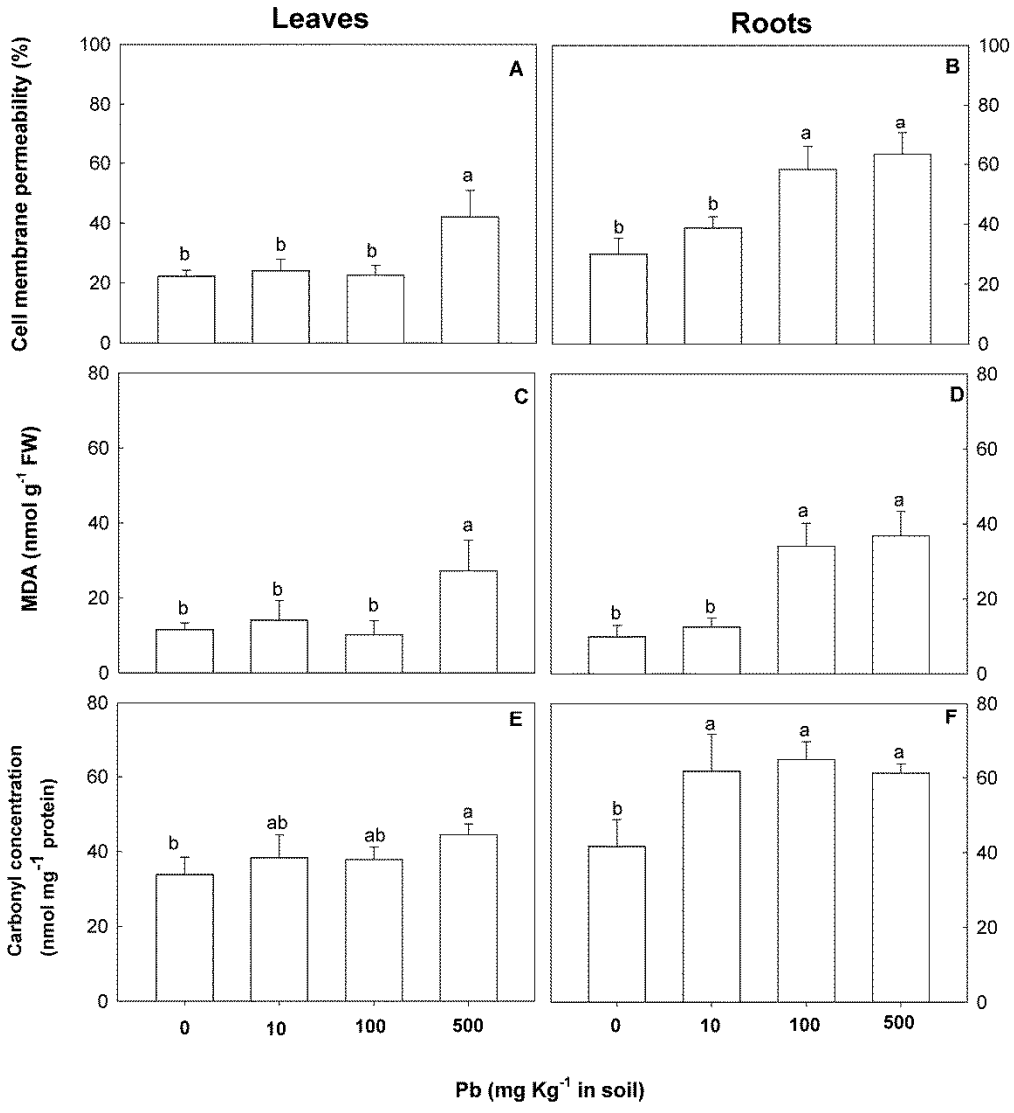
		0 mg Pb Kg^{-1}	**10 mg Pb Kg^{-1}**	**100 mg Pb Kg^{-1}**	**500 mg Pb Kg^{-1}**
Plant height	Shoots	65.4±3.6^{a}	63.2±3.573^{a}	61.2±2.6^{a}	51.7±4.6^{b}
	Roots	20.3±3.9^{a}	21.1±5.6^{a}	19.4±5.6^{a}	13.4±2.3^{a}
Pb	Leaves	8.2±1.4^{c}	55.0±8.0^{b}	64.5±10.1^{ab}	75.2±15.6^{a}
	Roots	14.0±8.1^{d}	595.5±57.9^{c}	1238.0±278.8^{b}	2333.0±335.6^{a}
ABA	Leaves	28.7±1.5^{b}	29.5±1.9^{b}	31.5±4.9^{b}	56.1±4.9^{a}
	Roots	3.0±0.1^{a}	2.0±1.2^{a}	1.7±0.3^{a}	2.3±0.4^{a}
SA	Leaves	7.4±2.5^{b}	8.5±0.8^{ab}	10.8±0.5^{ab}	12.4±1.1^{a}
	Roots	2.9±1.1^{c}	7.5±2.5^{ab}	4.74±1.2^{bc}	9.8±2.1^{a}
IAA	Leaves	9.7±3.6^{a}	4.8±3.2^{a}	6.3±2.1^{a}	10.3±2.8^{a}
	Roots	nd	nd	nd	nd
JA	Leaves	37.9±9.3^{b}	153.0±50.1^{a}	132.8±59.8^{a}	82.3±18.3^{ab}
	Roots	76.0±4.8^{c}	257.1±18.1^{a}	144.6±16.1^{b}	159.0±23.2^{b}

(ABA: abscisic acid; IAA: indolacetic acid; JA: jasmonic acid; nd: not detectable; SA: salicylic acid)

523

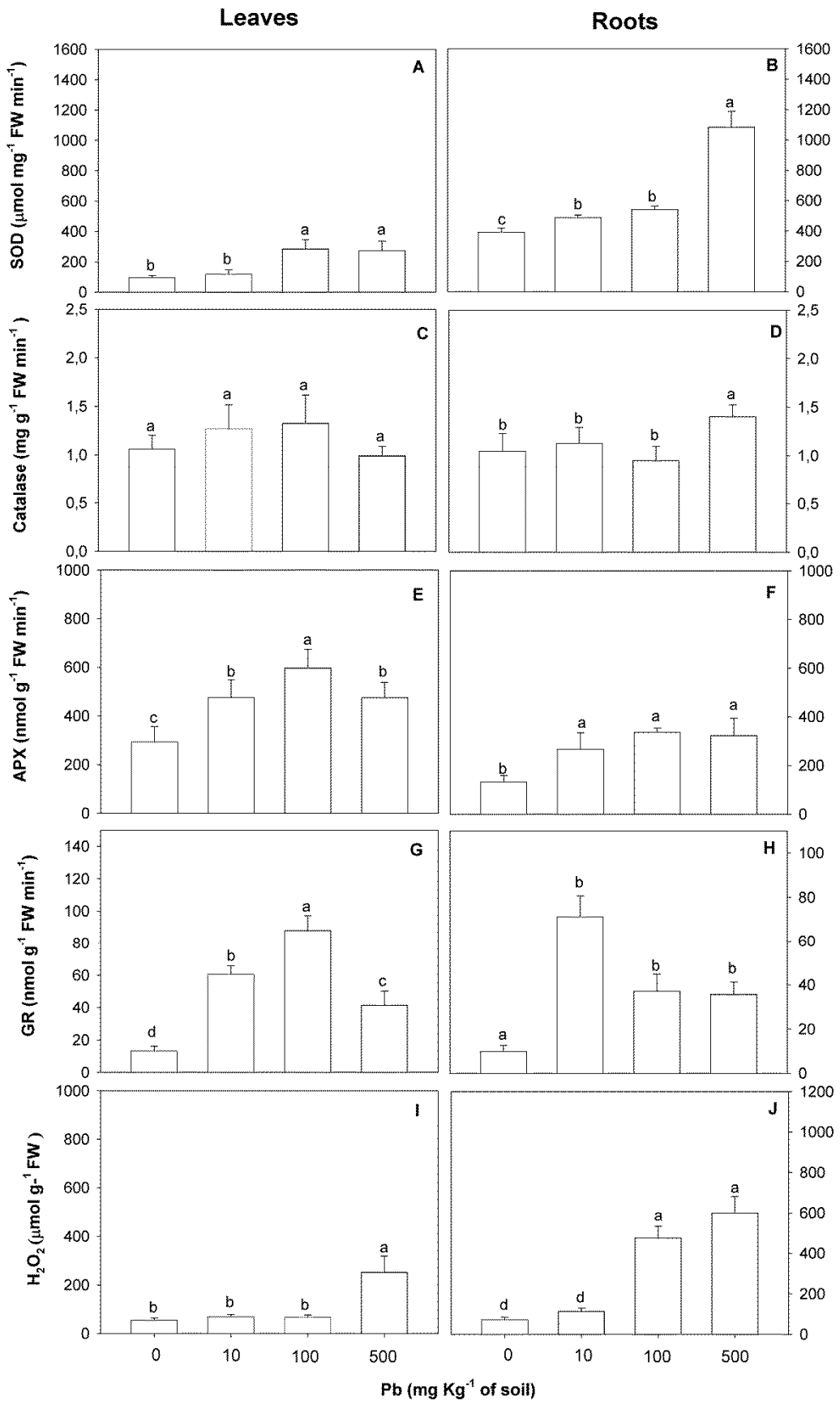
524

525 **Figures**


Leaves
Roots
A
B
C
D
E
F
Cell membrane permeability (%)
MDA (nmol g-1 FW)
Carbonyl concentration (nmol mg-1 protein)
Pb (mg Kg-1 in soil)
0
10
100
500

526

527 **Figure 1.** Evaluation of the leaves/roots damages. Effect of Pb exposure on cell membrane permeability, MDA
528 content and carbonyl concentration in leaves (A, C and E) and roots (B, D and F). Values are means ± SD (n = 6-8).
529 Different small letters indicate significant differences between treatments at a significant level equal to 0.05.
530


Leaves
Roots
A
B
C
D
E
F
G
H
I
J
SOD (µmol mg-1 FW min-1)
Catalase (mg g-1 FW min-1)
APX (nmol g-1 FW min-1)
GR (nmol g-1 FW min-1)
H2O2 (µmol g-1 FW)
Pb (mg Kg-1 of soil)
0
10
100
500

531

Figure 2. Enzymatic antioxidant activities and ROS concentration under Pb exposure. Effect of Pb accumulation on the activity of SOD, Catalase, APX and GR and on the content of H_2O_2 in lettuce leaves (A, C, E, G and I, respectively) and roots (B, D, F, H, and J, respectively). Values are means ± SD (*n* = 6-8). Different small letters indicate significant differences between treatments at a significant level equal to 0.05.

Figure 3 (in attach in a separate file)

Figure 3. Principle component analysis (PCA) plot (x – first component, y – second component) of the height, oxidative stress and hormone data of *P. sativum* leaves (A) and roots (B) during the experimental setup. PC1 explained 47.8% and 57.6% of the variance in leaves and roots, respectively, while PC2 explains only 26.1% and 22.7% of the variance in leaves and roots. Different circles depict sample scores while arrows show gradients resulting from the oxidative stress and phytohormone profiles.

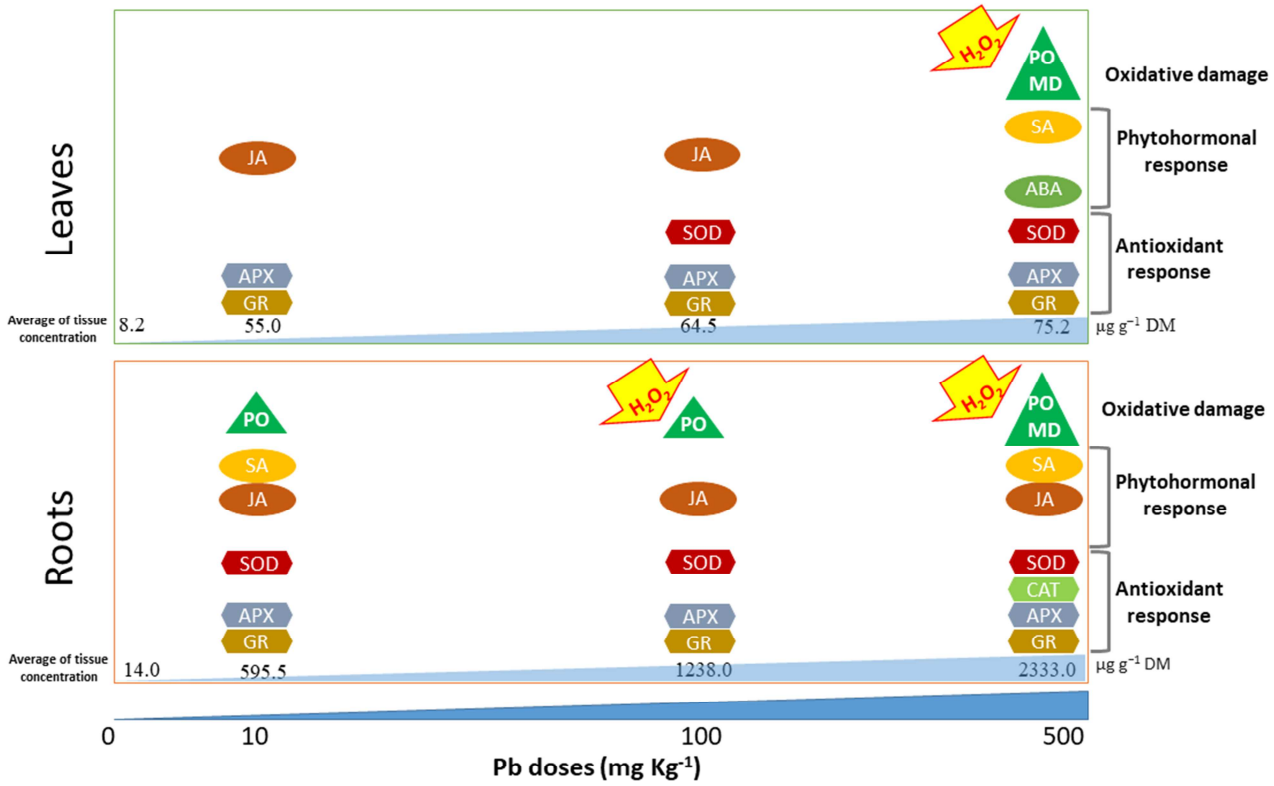
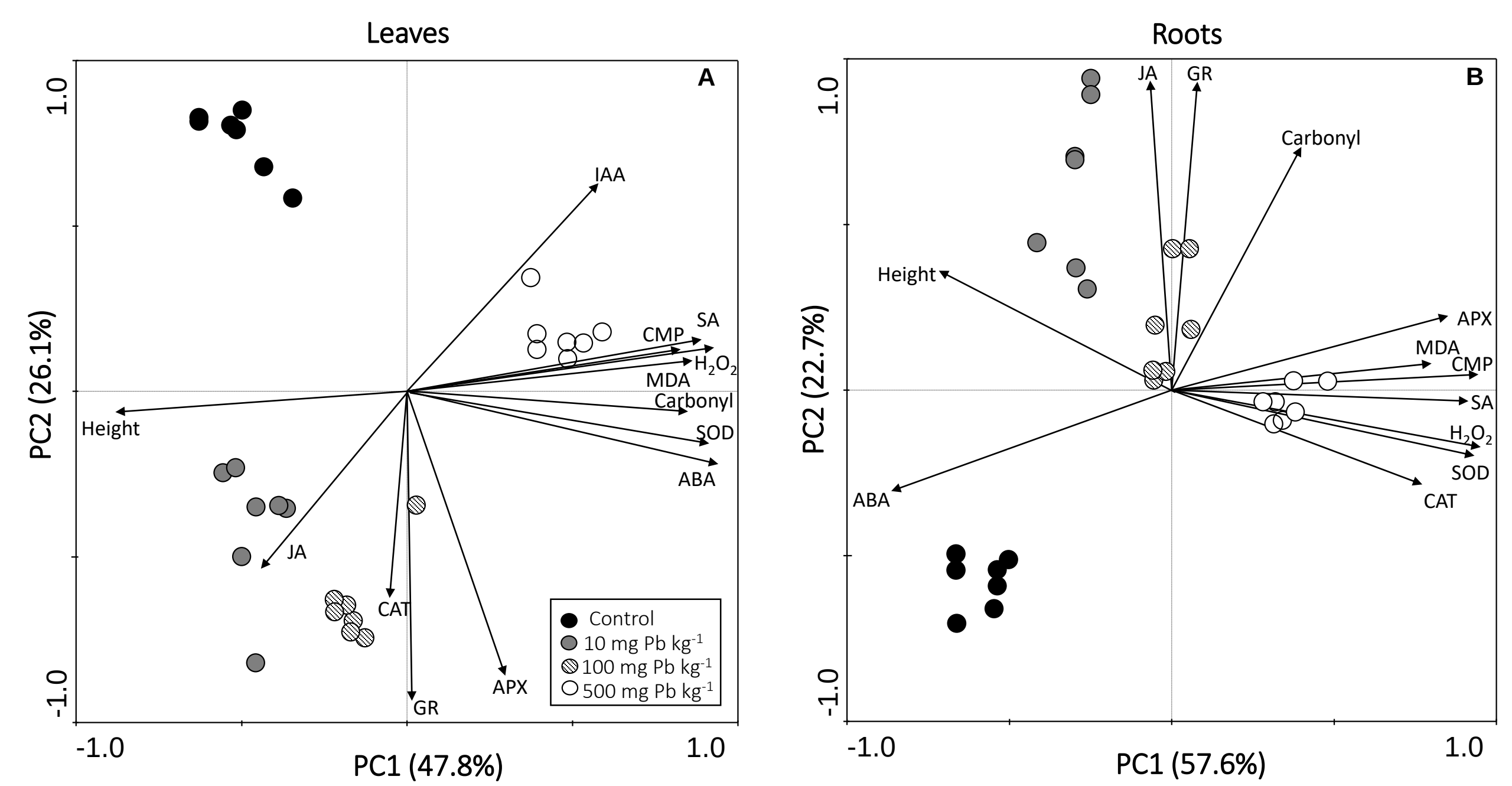


Figure 4. General overview of the main responses of *P. sativum* plants (roots and leaves) to increasing Pb doses. Each parameter represent increases ($P<0.05$) in the respective Pb treatment, and in relation to the control plants. ABA - abscisic acid; APX - ascorbate peroxidase; CAT - catalase; GR - glutathione reductase; H_2O_2 – hydrogen peroxide; JA - jasmonic acid; MD – lipid and membrane damages (malondialdehyde and cell membrane permeability); PO – protein oxidation; SA - salicylic acid; SOD - superoxide dismutase.


Leaves
A
1.0
-1.0
PC2 (26.1%)
-1.0
1.0
PC1 (47.8%)
IAA
SA
CMP
H2O2
MDA
Carbonyl
SOD
ABA
Height
JA
CAT
GR
APX
Control
10 mg Pb kg-1
100 mg Pb kg-1
500 mg Pb kg-1
Roots
B
1.0
-1.0
PC2 (22.7%)
-1.0
1.0
PC1 (57.6%)
JA
GR
Carbonyl
Height
APX
MDA
CMP
SA
H2O2
SOD
CAT
ABA

Highlights

- Oxidative stress and hormonal changes in response to Pb are more evident in roots
- JA responds to low/moderate Pb-doses, while SA and ABA respond to higher doses
- JA correlates with antioxidant enzymes, having a preventive role against ROS damage
- SA antioxidant role is more relevant when plants show some oxidative damages

Author contributions

MC Dias performed the experiments and did the measurements. MCDias, CSantos and NMariz-Ponte interpreted the results and wrote the manuscript.