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PHYTOTOXICITY OF LEAD ON *LACTUCA SATIVA*: EFFECTS ON GROWTH, MINERAL NUTRITION, PHOTOSYNTHETIC ACTIVITY AND OXIDANT METABOLISM

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ABSTRACT

Pb (lead) contamination in water and soils is increasing and its accumulation in plants may have serious deleterious consequences for environment and human health. Lead effects on plants still remain unclear and, therefore, a selection of Pb sensitive parameters used as biomarkers is required for improvement within the scope of Ecological Risk Assessment. In this study *Lactuca sativa* L. plants were exposed to Pb to contaminated irrigation solutions (12.5 mg l^{-1} and 125 mg l^{-1}) for 15 days. Orthogonal analyses (time *vs.* Pb concentration *vs.* organ) were carried out after one, seven and fifteen days to elucidate about the effects of this element in plants. During exposure, plant growth, nutritional imbalance, water content, soluble protein content, lipid peroxidation (malondyaldehyde accumulation) and photosynthesis (chlorophyll contents and PSII fluorescence) were assessed in order to find the most adequated parameters for further studies in under realistic environmental conditions.

Morphological and growth parameters showed decreases of root and shoot growth and alterations in root branching pattern. Biochemical parameters showed nutritional imbalance mostly concerning Pb, K (potassium) and Fe (iron) contents, changes in chlorophyll content and chlorophyll *a:b* ratio and malondyaldehyde accumulation. Therefore, such biochemical parameters may be used/ recommended as assessment parameters complementary to morphologic ones (*e.g.* shoot and root lengths) both in toxicological tests and further studies under realistic environmental conditions.

KEYWORDS: biochemical parameters, lettuce, nutritional imbalances, oxidative stress, Pb toxicity, soil contamination

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1. INTRODUCTION

Natural distribution and biogeochemical cycling of elements can condition soil chemistry and are strongly influenced by human activities [1]. Among water and soil components, heavy metals are not naturally removed or degraded and progressively accumulate [2]. Heavy metal accumulation in soil and water – even in trace concentrations – can therefore lead to their biomagnification through food chains.

Apart from natural weathering processes, Pb environmental contamination originates from different sources like mining and smelting activities, Pb containing paints, gasoline and explosives, as well as from the disposal of Pb enriched municipal sewage sludge [3]. Despite regulatory measures adopted in many countries to limit Pb environmental input, it still remains among the serious and global environmental and human hazards, and it is not likely that Pb levels will decrease in the near future (e.g. [4]). On the other hand, Pb contaminated soils (e.g. up to 100 g Pb kg^{-1}, e.g. [5]) can cause an accentuated decline in productivity of crops thereby posing a serious problem for agriculture.

According to the Scientific Committee on Toxicology and Environment, the evaluation of adverse effects of contaminants (including metals like Pb) on environments includes, among few other parameters, the analysis of plant biomass production and the analysis of the accumulation of the toxic contaminant in food items [6, 7].

Vascular plants are extremely sensitive to water and soil metal contamination.Therefore, bioassays using vascular plants proved to be useful and highly versatile tools in evaluating the effects of such contaminants in soil and to plan remediation processes. However, in already standardised media, the majority of test procedures are mostly focused on non specific evaluations, such as seedling survival and plant growth [8, 9]. Nevertheless, these two parameters are just a consequence of primary (and more sensitive) responses at biochemical and cytological levels that, eventually, culminate with plant senescence, reduction of growth or death. For example, the use of biochemical

parameters to assess plant responses and premature senescence induction, together with growth analyses in plants exposed to metal-contaminated soils–showed that the biomarkers used were parameters more or as sensitive as morphological features [7].

The induction of plant premature senescence caused by exposure to contamined irrigation water and/or soil is well documented and may be quantified by: a) *changes in growth parameters* [*e.g.* FW (fresh weight), DW (dry weight), root and shoot length] [1]; b) *changes in water content and nutritional imbalances* [10, 11]; c) *modification of antioxidant enzymes*, d) *changes in soluble protein content and/or profile and lipid peroxidation* (e.g. [12]); e) *changes in photosynthesis* such as in chlorophyll contents [13-15] and by f) *changes in fluorescence parameters* (*e.g.* [17, 19]).

Till now, and for Pb, the relations between these parameters and Pb concentration in *Lactuca sativa* L. remain speculative and require confirmation so that a model attempting to describe some of the changes induced by Pb in plants may be developed. Besides enabling the selection of adequate biomarkers in toxicological assessments, this model may lead to a significant improvement within the scope of ERA (Ecological Risk Assessment). In the present study, *Lactuca sativa* L. - a crop species very important in human diet and which is also recommended as a standard species for toxicity testing in ISO tests [8, 9] - was exposed to Pb contaminated soils. Parameters like plant growth, nutritional imbalances (mineral analysis), water and soluble protein contents, lipid peroxidation (malondyaldehyde accumulation) and photosynthesis (chlorophyll contents and fluorescence parameters) were evaluated after Pb exposure. In addition, we selected some biochemical parameters that while being correlated with Pb external concentration and time exposure seem the most adequate for further studies dealing with realistic environmental conditions.

2. MATERIAL AND METHODS

2.1. Plant material and growth conditions

Lettuce (*Lactuca sativa L.* cv. Reine de Mai) seeds were grown in pots containing the substrate currenly used in greenhouses of lettuce production for consume (i.e. perlite, Viveiros Litoral®, Aveiro, Portugal). The substrate was watered with Hoagland's nutrient solution (pH=5.8). Three groups of seedlings (n=30 for each group) were grown at 22±2°C, with a light intensity of 480 µmol m^{-2} s^{-1} (Osram 36 W lamps) and a photoperiod of 12 h light) [18]. After 5 weeks, two groups of seedlings were exposed to lead treatments by watering, every 48 h, the pots with 50 ml of Hoagland solution contaminated with $Pb(NO_3)_2$ at the concentrations of, respectively, 12.5 and 125 mg of Pb^{2+} l^{-1}. The third group of seedlings was used as the control group and was grown in similar conditions as Pb treated plants, but the pots were irrigated with Hoagland solution without $Pb(NO_3)_2$. At days 1 and 15, growth rates and water content determination (n=4), mineral analysis (n=3), soluble protein content (n=4), chlorophyll concentration and fluorescence parameters (n=4) and malondyaldehyde (MDA) accumulation (n=4) were measured.

2.2. Growth rates and water content determination

Shoots and roots were collected and their length measured. Organs were weighed immediately after collection (fresh weight, FW), and dried at 70 ºC until weight stabilisation (dry weight, DW). The hydric content was calculated by the difference between FW and DW. Osmolality was determined by analysing samples of plants using an automatic osmometer Knauer (Berlin, Germany) as described by Brito et al. [16].

2.3. Mineral analysis

Nutrient content was determined in washed roots and in shoots [18]. Tissues were dried at 70ºC until weight stabilisation and were then mineralised to determine the content of Pb, B, Ca, Cu, Fe, K, Mg, Mn, P, and Zn elements [12]. Elemental contents were determined by Induced Coupled Plasma Spectroscopy (ICPS) using a Jobin Ivon JY70 Plus according to procedures described by Azevedo et al. [12]. Results were averaged for four individuals for each treatment.

2.4. Soluble protein content

Tissue samples (0.5 g) were homogenized at 4°C in 1 ml of potassium phosphate buffer 0.05 M (pH=7.8) containing 0.1 mM ethylenediamine tetraacetic acid, 5 mM cysteine, 1 % (w/v) polyvinyl pyrrolidone, and 0.2 % Triton X-100 [1]. Homogenates were filtered and centrifuged at 8000 *g* for 15 min, at 4°C. The supernatant was dialysed at 4°C for 24 h against 1 l of 10 mM potassium-phosphate buffer (pH=7.8). The dialysed samples were centrifuged at 2500 *g* for 10 min, at 4°C and used for soluble protein quantification [19]. Soluble proteins were determined following procedures described by Bradford [20] and using a "total Protein Kit, Micro" (Sigma).

2.5. Chlorophyll concentration and fluorescence parameters

Chlorophyll *a* and *b* contents of leaves were determined following procedures described by Arnon [21]. For the determination of fluorescence parameters, plants were stored in the dark for adaptation for 20 min in a growth chamber at 22±2°C. Then, the Photosystem II fluorescence was monitored on young leaves using a Plant Efficiency Analyser (Hansatech Instruments Ltd., UK) by illuminating leaves with a peak wavelength of 650 nm and a saturating light intensity of 3000 µmol m^{-2} s^{-1}. The basal non-variable chlorophyll fluorescence (F_0) and the maximal fluorescence induction (F_m) were determined. The maximum quantum yield of PSII [$(F_m-F_0)/F_m$] was estimated [13].

2.6. Determination of MDA concentration

Lipid peroxidation was determined by MDA content [22]. Briefly, 0.25 g of tissue samples were homogenized in 5 ml trichloroacetic acid 0.1% (v/v) and centrifuged at 10 000 *g* for 10 min. The supernatant was collected and 1 ml was mixed with 4 ml of an aqueous solution with 20% trichloroacetic acid and 0.5% (v/v) thiobarbituric acid (TBA). The mixture was heated at 95°C (30 min), quickly cooled and centrifuged at 10 000 *g* for 10 min. The supernatant was used to determine MDA concentration at 532 nm and 600 nm. The level of lipid peroxidation was expressed as nmol g_{FW}^{-1} of MDA formed, using an extinction coefficient, at 532 nm, of 155 mM^{-1} cm^{-1}.

2.7. Statistical analysis

Collected endpoint data were compared using One Way ANOVA and Kruskal-Wallis One way ANOVA tests. One Way ANOVA was used for data that either were normally distributed or could become so through adequate transformation while Kruskal-Wallis one way ANOVA was used, when data were not normally distributed [23]. When there were significant differences the Dunnett's or Dunn's methods were run to identify them. After required normalization, correlations between some parameters were determined using the Pearson Correlation test. All statistical procedures were performed using Sigma Stat package for Windows, version 3.1, SPSS Inc. USA.

3. RESULTS

3.1. Plant growth bioassays

Shoot and root FW and DW significantly decreased in plants exposed to 125 mg l^{-1} Pb, while there was no significant change on these two parameters in plants treated with 12.5 mg l^{-1} Pb when compared with the control group (Figs. 1a, 1b, 2a, 2b). The toxic effect of Pb was evident also from the decrease of shoot and root lengths (Figs. 3a, 3b, 4). After- 15 days, a more significant reduced length was observed in plants grown in 125 mg l^{-1} (26.00±0.57 cm) compared to those exposed to 12.5 mg l^{-1} (35.50±2.25 cm) or to control plants (30.60±0.83 cm) (Dunnett's Method, $p<0.05$). These results show an hormesis pattern for the lower Pb dose (12.5 mg l^{-1}) by increasing shoots' growth than in the higher dose (125 mg l^{-1}) or control ($p<0.05$).

Also, only plants exposed to 125 mg l^{-1} showed visible symptoms of diffuse chlorosis in leaves (see Figure 4). Roots rapidly responded to Pb exposure, through a reduction in growth length and modification of branching pattern, since development and extension of the main root was much more affected than lateral roots**.**

3.2. Water content determination

After 15 days of treatment, hydric content of 125 mg l^{-1} Pb treated shoots decreased compared to controls (from 98.55 ±0.83% in controls to 95.93±0.57%) (average ± std. error) (Dunnett's Method, p=<0.05) (Fig. 5a). By the same time, the hydric content of roots also decreased significantly from 90.97 (±2.74% std. error) in control, to 89.05 (±2.07% std. error) in 125 mg l^{-1} Pb treated plants (Dunnett's Method, p=<0.05) (Fig. 5b).

On the other hand, osmolality presented significant differences in the first day between the control group and the group treated with Pb l^{-1}, with a decrease of osmolality (Fig. 6). For the same plant groups, after 15 days of treatment, osmolality values apparently increased, although not significantly (Fig. 6).

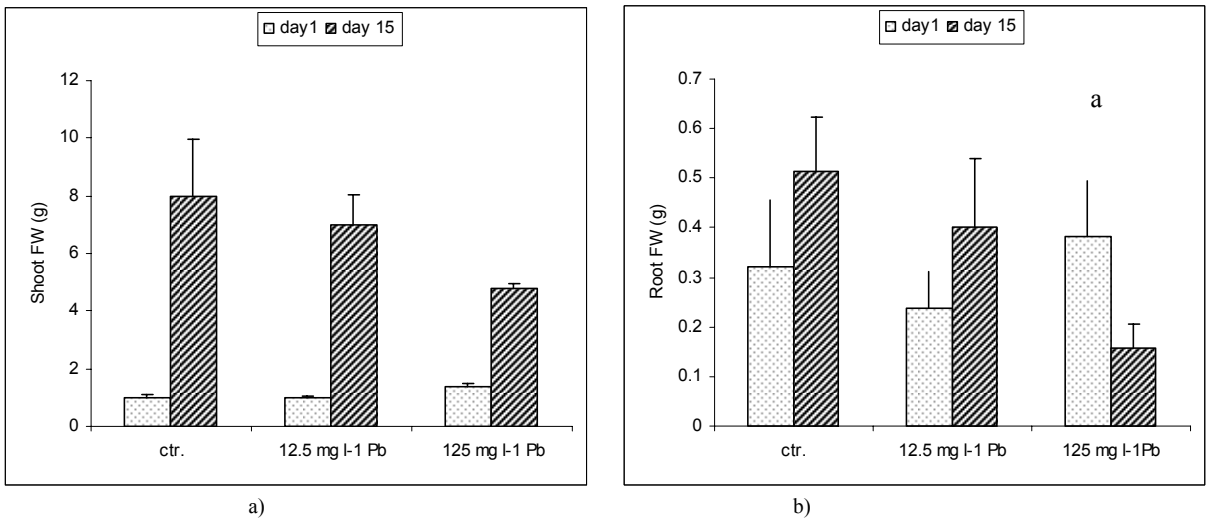
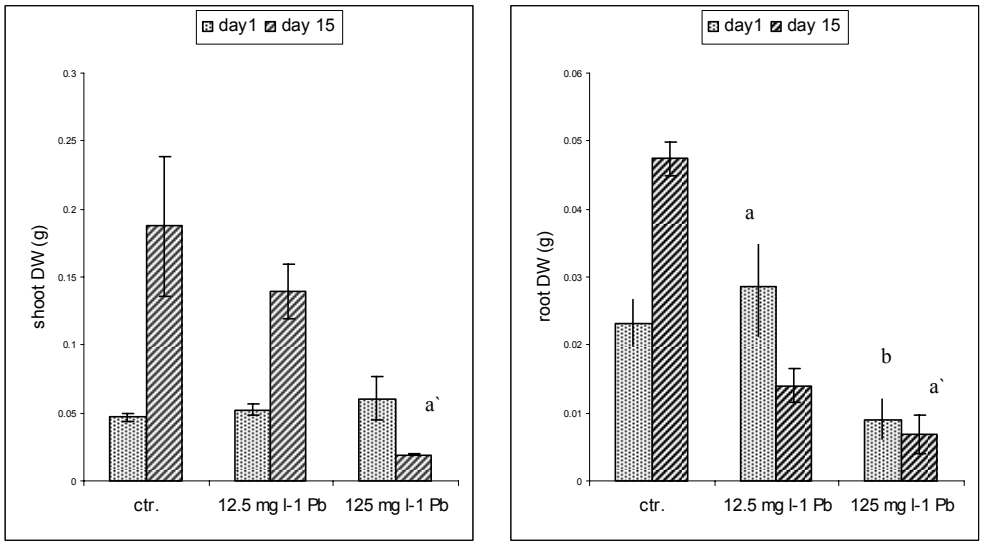
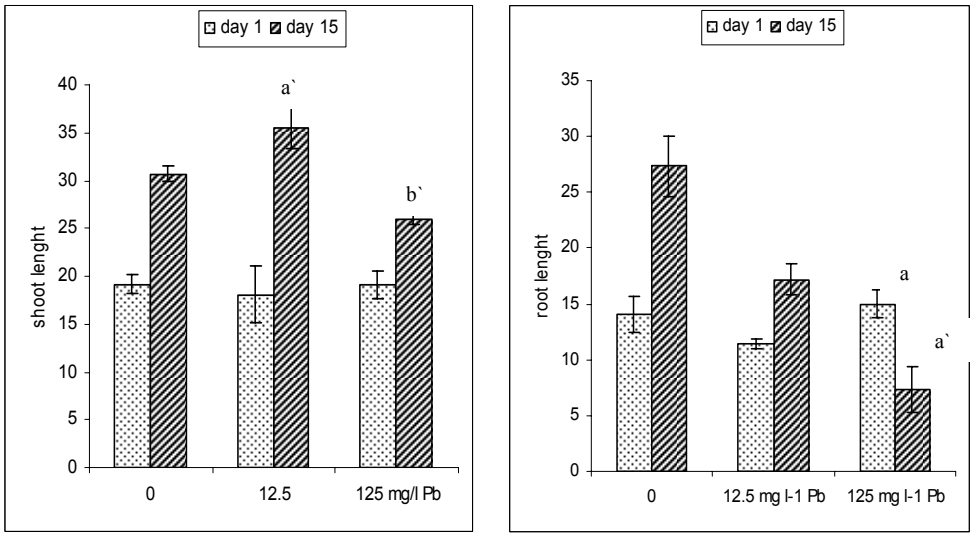


FIGURE 1 - a) Fresh weight (FW) of *Lactuca sativa* shoots after 1 and 15 days of lead exposure. b) Fresh weight (FW) of *Lactuca sativa* roots after 1 and 15 days of lead exposure. Symbol (a) indicate significantly different means between control and stressed root for day 1.



a b

FIGURE 2 – a) Dry weight (DW) of *Lactuca sativa* shoots after 1 and 15 days. Symbol (a`) indicate significantly different means between control and stressed shoot for day 15. b) Dry weight (DW) of *Lactuca sativa* roots after 1 and 15 days. Symbol (a) and (b) indicate significantly different means between control and stressed shoot for day 1. Symbol (a`) indicate significantly different means between control and stressed shoot for day 15.



a b

FIGURE 3 – a) Length growth parameter of *Lactuca sativa* shoot after 1 and 15 days. Symbols (a`) and (b`) indicate significantly different means between control and stressed shoot for day 15. b) Length growth parameter of *Lactuca sativa* root after 1 and 15 days. Symbol (a) indicate significantly different means between control and stressed root for day 1. Symbol (a`) indicate significantly different means between control and stressed root for day 15.

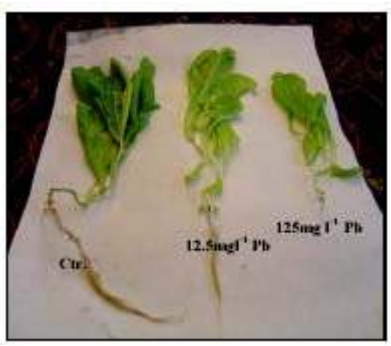
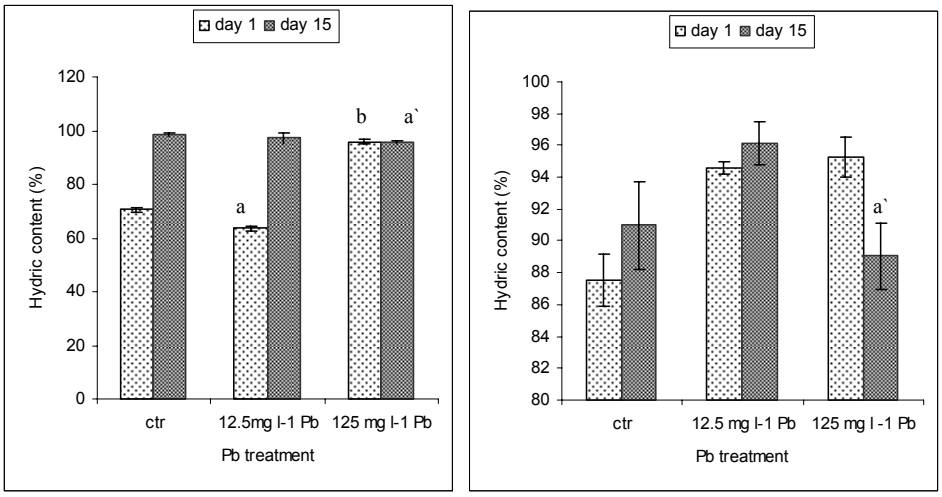


FIGURE 4 - Effects of lead stress on length shoots and color of leaves (chlorosis) of *Lactuca sativa*. The darker color of the plant (left) corresponds to a darker green colour of this plant when compared to Pb treated ones (center and right)



a b

FIGURE 5a - Effects of lead stress on shoot hydric content after 1 and 15 days. Symbols (a) and (b) indicate significantly different means between control and stressed shoots after 1 day. Symbol (a`) indicate significantly different means between control and stressed shoot for day 15. FIGURE 5b - Effects of lead stress on root hydric content after 1 and 15 days. Symbols (a`) indicate significantly different means between control and 125 mg l^{-1} stressed roots after 15 days.

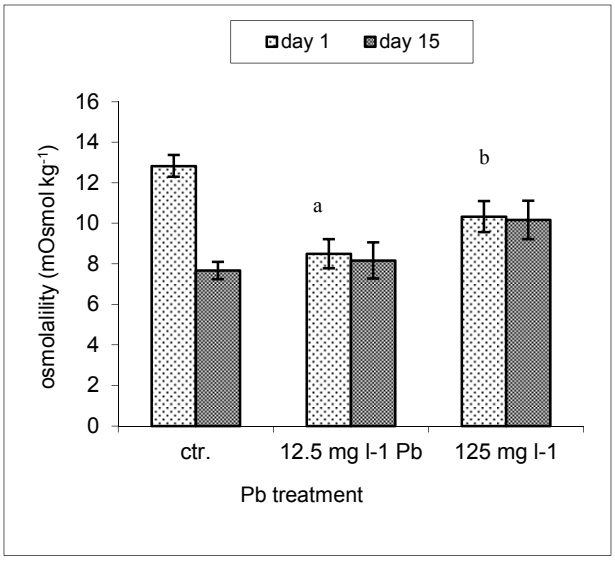


FIGURE 6 - Effects of lead stress on osmolality (mOsmol Kg^{-1}). Symbols (a) and (b) indicate significantly different means between control and stressed plants after 1 day.

3.3. Mineral analysis in plants

a) Lead content in lettuce

Pb contents in lettuce roots increased significantly with increasing external lead concentration increased, especially after 15 days of exposure (Kruskal-Wallis one-way ANOVA, H = 9.346, df=2, $p<0.05$; Dunn's method, $p< 0.05$) (Table 1a). In shoots, Pb contents followed the same trend observed in roots, though less Pb was accumulated (Dunnett's Method, p=<0.05) (Table 1b). In fact, Pb taken up by lettuce was accumulated mainly in the roots, once the 125 mg l^{-1} treated roots presented a mean concentration of 67.27 mg g_{DW}^{-1}. In shoots exposed to the same treatment the content of Pb was only 0.19±0.02 mg Pb g_{DW}^{-1} at the end of 15 days. This is a 348-fold difference between the Pb concentrations on roots *vs* shoots, observed at 125 mg Pb l^{-1} (Table 1a, 1b).

During the same period, the amount of Pb retained in roots of the 12.5 mg l^{-1} Pb group was only 11.7% of the total Pb taken by roots of the 125 mg l^{-1} Pb group.

b) Macro and micro-nutrients content in lettuce

Analysis of plant mineral composition showed that P, Mg and Ca and also Cu and Zn contents did not undergo significant changes in relation to control levels after 15 days of exposure (Table 1a, 1b). However, after the same period, the K content of the 125 mg l^{-1} Pb treated group

significantly decreased in roots (Dunnett's method, P< 0.05) and shoots (One way ANOVA, Dunnett's method, p<0.05) compared with the control.

Also at day 15, a similar pattern was observed for Fe content as it exhibited a significant decrease in roots exposed to 125 mg l^{-1} Pb compared with the control (Table 1a, One way ANOVA, Dunnett's method, p = 0.014). Besides, there was also a significant correlation between Fe root content and shoot length (Pearson correlation, p = 0.047).

3.4. Soluble protein content, chlorophyll content and fluorescence parameters

In the group exposed to 125 mg l^{-1} Pb, soluble protein content increase, although not significantly (Table 2).

Concerning chlorophyll (Chl) content, Chl *b* and total Chl contents (Chl *a*+*b*) in leaves were significantly lower on the first day, in both Pb treated groups (Dunnett's method, p<0.05), Table 2. In the same day, Chl *a* and Chl *a*:*b* ratio decreased in the 125mg l^{-1} Pb treated group

TABLE 1a - Effect of lead stress on nutrient contents (mg g_{DW}^{-1}) in *L. sativa* roots. Values are expressed as mean ± std. error; (a) indicates significantly different means between control and stressed individuals (n=3)

	Day 1			Day 15		
Roots nutrients	**Ctr.**	**12.5 mg l^{-1} Pb**	**125 mg l^{-1} Pb**	**Ctr.**	**12.5 mg l^{-1} Pb**	**125 mg l^{-1} Pb**
Pb	**0,173**±0,161	**4,654**±3,090	**0,313**±0,0	**0.087**±0.021	**7,342**±1,587	**67.267**±38.58 **a**
P	**4,785**±0,425	**11,574**±1,948	**9,867±0,0**	**11.769**±1.481	**12,183**±0,483	**10.056**±2.352
Mg	**3,530**±1,068	**7,719**±0,757	**7,500**±0,0	**1.736**±0.364	**2,764**±0,797	**1.531**±0.461
Ca	**14,692**±4,592	**37,897**±2,591	**30,133**±0,0	**11.407**±1.107	**16,286**±1,871	**8.716**±2.397
K	**22,230**±3,367	**50,668**±9,031	**51,000**±0, 0	**42.056**±3.795	**43,013**±2,236	**37.904**±5.534 **a**
Zn	**0,322**±0,025	**0,837**±0,175	**0,530**±0,000	**0.447**±0.054	**0,389**±0,110	**0.198**±0.028
Fe	0,340±0,094	**1,275**±0,247	**1,420**±0,000	**4.187**±0.420	**2,312**±0,377	**1.386±**0.205 **a**
Mn	**0,393**±0,155	**0,887**±0,511	**0,160**±0,000	**0.859**±0.252	**1,000**±0,334	**0.282**±0.071
Cu	**0,128**±0,0012	**0,308**±0,138	**0,080**±0,000	**0.292**±0.082	**0,459**±0,115	**0.157**±0.054

TABLE 1b - Effect of lead stress on nutrient contents (mg g_{DW}^{-1}) in *Lactuca sativa* shoots. Values are expressed as mean ± std. error; (a) indicates significant differences between control and stressed individuals. (n = 3)

	Day 1			Day 15		
Shoot nutrients	**Ctr.**	**12.5 mg l^{-1} Pb**	**125mg l^{-1} Pb**	**Ctr.**	**12.5 mg l^{-1} Pb**	**125mg l^{-1} Pb**
Pb	**0,0**±0,0	**0,0**±0,0	**0,152**±0,034	**0.0**±0.0	**0.032**±0.018	**0.193**±0.017 **a**
P	**9,665**±0,737	**8,005**±0,276	**16,417**±11,066	**8.906**±0.846	**3.768**±2.095	**3.736**±1.317
Mg	**7,625**±0,526	**7,69**±0,526	**16,213**±9,097	**5.324**±0.736	**2.844**±1.580	**4.256**±1.692
Ca	**19,575**±1,510	**17,917**±1,609	**38,206**±21,183	**16.027**±2.508	**7.453**±3.989	**12.434**±5.454
K	**68,283**±4,412	**64,101**±4,595	**131,26**±71,978	**45.157**±6.657	**21.657**±12.000	**33.772**±15.253 **a**
Zn	**0,313**±0,035	**0,245**±0,033	**0,539**±0,298	**0.157**±0.017	**0.087**±0.047	**0.12**±0.060
Fe	**0,363**±0,068	**0,369**±0,019	**0,717**±0,440	**2.296**±1.755	**2.453**±1.170	**1.229**±1.142 **a**
Mn	**0,541**±0,084	**0,287**±0,042	**0,643**±0,250	**0.242**±0.033	**0.293**±0.140	**0.186**±0.109
Cu	**0,037**±0,003	**0,028**±0,003	**0,072**±0,044	**0.039**±0.006	**0.018**±0.009	**0.018**±0.008

TABLE 2 - Effects of lead stress on soluble proteins, chlorophyll *b*, chl *a*:*b* ratio and fast kinetic fluorescence parameters in shoots. Values are expressed as mean ± std. error; (a) indicates significant differences between control and stressed individuals (n=4).

		Day 1			Day 15		
Shoot parameters		ctr	12.5 mg l^{-1} Pb	125 mg l^{-1} Pb	ctr	12.5 mg l^{-1} Pb	125 mg l^{-1} Pb
Soluble Protein content	Soluble proteins	188.143±25.99	291.883±21.60	194.503±55.14	318.09±42.88	426.02±115.59	269.16±19.4
Chlorophyll and ratio content	Chla	314.44±20.38	288.93±37.52	231.21±14.32	146.19±5.102	141.77±11.298	120.60±10.210
	Chl *b*	118.51±8.75	184.927±19.01	95.99±10.21 **a**	58.30±4.43	62.28±2.87	65.15±3.95
	Chl *a* + *b*	432.97±10.92	473.86±44.3	327.205±28.67 **a**	204.50±7.069	204.05±12.54	185.75±13.79
	Ch *a*:*b* ratio	2.66±0.44	1.59±0.208	2.54±0.07	2.55±0.007	2.28±0.168	1.85±0.220 **a**
Fluorescence parameters	*Fo*	665.25±20.38	660.0±33.63	594.5±36.59	570.0±33.63	689.25±31.74	671.75±47.35
	Fm	3038.75±50.31	3565.75±128.15	3320±72.34 **a**	3051±100.47	3616±29.55	3352±60.52
	Fv	2373.0±42.18	2905.75±138.29	2725.75±96.7 **a**	2323±53.04	2925±162	2680±80.33
	Fv/Fm	0.78±0.005	0.813±0.011	0.820±0.008	0.763±0.008	0.808±0.009	0.798±0.015

TABLE 3 - Effect of lead stress on MDA production in roots and shoots. Values are expressed as mean ± std. error; (a) indicates significant differences between control and stressed individuals (n=4).

MDA content	Roots						Shoots					
	Day 1			Day 15			Day 1			Day 15		
	ctr	12.5 mgl^{-1} Pb	125 mgl^{-1} Pb	ctr	12.5 mgl^{-1} Pb	125 mg l^{-1} Pb	ctr	12.5mg l^{-1}Pb	125mg l^{-1} Pb	ctr	12.5 mgl^{-1} Pb	125 mgl^{-1} Pb
MDA (nmol/ g Fw)	0.61±0.01	0.6±0.01	0.74±0.0	0.89±0.04	0.52±0.01	0.88±0.01	0.68±0.048	0.5±0.09	0.58±0.005 **a**	0.6±0.005	2.13±0.01 **a**	2.28±0.03 **a**

when compared with the control, although this effect was not significant.

After 15 days of exposure, leaves from 125 mg l^{-1} Pb treated plants showed decreases of Chl *a* and Chl *b* contents (though not significantly) and a significant reduction of Chl *a*:*b* ratio (Dunnett's method, $p<0.05$). These changes in chlorophyll contents and ratio were supported by observed leaf chlorosis, 15 days after exposure (Fig. 4).

In the analysis of fast kinetic fluorescence parameters, basal fluorescence (F_0) increased after 15 days in the 125mg l^{-1} Pb treated group although not significantly. On the other hand, maximal and variable fluorescence (F_m and F_v, respectively) manifested significant differences, on the first day, between the 12.5 mg l^{-1} Pb treated group and the control, (Dunnett's method, $p<0.05$) (Table 2).

3.5. Lipid peroxidation

During oxidation, unsaturated fatty acids are stepwise converted into various small hydrocarbon fragments such as MDA. As membranes are highly rich in lipids, MDA is often also used as a valuable metabolite indicator of oxidative degeneration of membranes.

A considerable variation on lipid peroxidation, expressed as MDA content (nmol g_{FW}^{-1}), was observed in both Pb treated groups (12.5 and 125 mg l^{-1}) compared to control. In detail, in lettuce shoots, a significant increase of MDA content was observed in Pb treatments at the end of 15 days of exposure (Kruskal-Wallis One-way ANOVA, $H=15.42$, $df=2$; Dunn's method, $p<0.05$), (Table 3).

4. DISCUSSION

4.1. Plant growth parameters

Plant growth inhibition parameters are often used to evaluate toxicity effects of heavy metals and other chemicals found in the environment and ERA recommends the evaluation of seed germination and root elongation [8, 24, 25]. However, such evaluation parameters take long, are unspecific and do not provide information on biochemical action and physiological interactions.

In the tests carried out on this work, roots responded to Pb, through reduction in growth rate and change in branching pattern. Several studies have previously reported an inhibition of root growth at 0.2072 to 2072 mg Pb l^{-1} concentration or at a soil Pb content above 10 mg kg^{-1} (*e.g.* [26]). As reported by Eun et al. [27], in roots exposed to metals the resulting growth inhibition appears to be caused by cell division inhibition in root tips.

According to the results shown in this paper, 12.5 and 125 mg l^{-1} Pb treatments strongly affected fresh weight, dry weight and shoot and root lengths. In addition, these results suggest an hormesis pattern for the low dose of Pb (12.5 mg l^{-1}) by increasing shoot growth than higher doses (125 mg l^{-1}) or control ($p<0.05$). This putative hormesis is presently being confirmed with more Pb concentrations. Hormesis effects have been also detected in other heavy metals (e.g. [1]), and may suggest that at low doses, this species has defence mechanisms of using this metal in growth processes, but further studies on this effect are being conducted. Another issue of interest is that if these metal-accumulating plants showing hormesis effect (having higher growth rates than control) may pose health problems due to metal trophic transfer.

4.2. Water content

Water content decreased when plants were exposed to high Pb concentrations (125 mg l^{-1} Pb) in the 15th day. Besides, osmolality increased, together with a decrease in osmotic potential, in most Pb treated plants, mainly at day seven (data not show) suggesting that this is a crucial time for the plant to perform osmoregulation. Nevertheless no correlation between the osmolality and osmotic potential was found for these Pb treated plants. It has already been reported that Pb may decrease the water potential [28]. On the otherwise, water content decrease may be related to growth retardation that probably results in a decrease of leaf area [29]. Breckle [26] also reported that high Pb concentrations may alter stomata morphology and function, which may constrain transpiration, photosynthesis and respiration. Poschenrieder and Barceló [29] add that plants exposed to very low metal concentrations can show higher transpiration rates or stomatal conductance than otherwise non-exposed plants. This increase in transpiration (with presumed increases in nutritional translocation) may be one of the possible causes of the hormesis effect stated above (shoot growth increase for 12.5 mg l^{-1}Pb than for 125 mg l^{-1} Pb treated plants), and this hypothesis is presently being tested. In fact, low Pb concentrations (specifically, the inner Pb concentration) may be inhibitory to root growth and cause alterations in assimilate partitioning, leading to decreased osmotic potential turgor maintenance in leaves and consequently alteration in transpiration rates.

4.3. Lead content in lettuce organs

The results for the Pb content in roots (detected after 7 days, data not show) and shoots (detected after 15 days) followed the trends of movement and partioning typically described for metals inside a plant [30]. At day 15, approximately 90% of the Pb was taken up in the roots (the remaining Pb was transported into the shoots) wich is consistent data for other species. For example, Seregin and Ivanov [30] also reported higher Pb contents in roots than in shoots of *Lactuca sativa* L.

The exclusion of heavy metals from shoots is very common in plants called excluders and based on the results of this research, lettuce may be considered a Pb excluder. Several authors (*e.g.* [28]) reported that the limited Pb transport from the roots to other organs is due to the barrier of root endodermis. Therefore, once Pb is detected in shoots (as occurred at day 15 in Pb exposed lettuce plants), it may mean that the normal barriers (mainly endodermis) present in the root was surpassed. It was also found for plants exposed to Al, that endodermis differentiation was affected by metal [11].

4.4. Macro and micro-nutrients content in lettuce

Lettuce exposed to Pb showed positive correlation between the FW and K content of roots and of shoots, which might indicate that K^+ in roots influence shoot growth (by *e.g.* influencing osmotic and hydric potential). Maathuis and Amtmann [31] reported several examples of altered K^+ metabolism in plants, mostly focusing on the soil/root interface (since most knowledge regarding the molecular mechanisms of K^+ and transport deals with its uptake into roots). The same feature was found for Fe content that decreased in roots as Pb concentration increased.

These findings may be explained by two mechanisms: ion transport can be affected by 1) the metal ion radii size and/or 2) on the disorder that Pb can cause on cellular metabolism leading to changes in membrane enzyme activities and/or in membrane structure and, consequently, in ion compartimentation. The efflux of K^+ from roots, apparently due to the extreme sensitivity of K^+-ATPase and SH-groups of cell membrane proteins to Pb, is an example of the latter mechanism [30].

In turn, the decrease of Fe content in this research is another typical example of cell metabolic disorder produced by heavy metals (*e.g.* [32]). The same authors found a metal-induced decrease in Fe (III) reductase for cucumber roots. Seregin and Ivanov [30] also indicated that Fe deficiency in cucumber roots was induced by Pb, but did not clarify putative causes for this correlation.

All these nutritional data content changes support that Pb effects in plant mineral nutrient are an important aspect which still needs detailed investigation.

4.5. Soluble protein content, chlorophyll content and fluorescence parameters

The increase of soluble protein content after 7 days of exposure of 125 mg Pb l^{-1} (data not show) probably corresponds to the membrane bound proteins detachment as demonstrated by Hashimoto et al. [33] assuming that most of insoluble proteins were membrane bound proteins, supporting the loss of membrane integrity proposed above.

The lower chlorophyll contents were a typical effect of Pb treatments and in particular, chlorophyll *b* was more affected than chlorophyll *a* in 125 mg l^{-1} Pb treated shoots. Furthermore, leaf chlorosis was seen after 15 days of exposure together with a decrease of chl *a*:*b* ratio.

These results may be explained by inhibition of chlorophyll-synthesing enzymes more than by nutrients deficiency [30]. In fact, modifications of membrane composition or structure, or inhibition of protochlorophyll reductase and inhibition of aminolevulinic acid synthesis can occur (e.g., [15]). Santos [15] also stated that chlorophyll and carotenoid degradation are often observed in response to high concentrations of various heavy metals. Metal effects on chlorophyll and photosynthetic structures were also described for aquatic organisms as *Chlorella* sp. [34]. In the analysis of fast kinetic fluorescence parameters, basal fluorescence (F_0) apparently increased although not significantly after 15 days between the control group and 125 mg l^{-1} Pb treated shoots. This may indicate low or no effect of Pb on the photochemical efficiency and/or in the open centers stage.

On the other hand, in the first day maximal and variable fluorescence (F_m and F_v, respectively) manifested significant differences between the control group and the 125 mg l^{-1} Pb treated shoots. These results indicate that negative effects of Pb in PSII transferrance may be observed within short periods (hours) and that this negative effect may be recovers (at least for lettuce within the concentrations tested) with time, as for longer periods no significant effects were found with respect to control. Effects of Pb in PSII transferrance were also reported by Sharma and Dubey [28].

4.6. Lipid peroxidation

Pb exposure led to an increased MDA content in shoots after 15 days. Other Pb treatments enhanced MDA, which is an index of lipid peroxidation and, therefore, of oxidative stress. Similar increases of lipid peroxidation by Cd treatment have been reported regarding lettuce [1] and *Helianthus annuus* [10].

This increase of peroxidation, indirectly, suggests changes in fatty acid composition of membranes. In fact, membrane injury is related to an increase of lipid peroxidation, due to the action of lipoxygenase or of highly toxic free radicals causing senescence [35; 36]. Lipoxigenase activity is increased upon exposure to heavy metals oxidising polyunsaturated lipids and generating AOS namely hydroperoxides and oxi-free radicals that damage lipids among other compounds and it was reported the effects of lipid oxidation on cell membrane integrity damage (*e.g.* [1]). Consequently, the peroxidation of cell membranes severelly affects its functionality and integrity and can produce irreversible damage to the cell functioning.

Furthermore, Jones and Kochian [37] argued that several metal ions are known to cause peroxidation of both plasma membrane and chloroplast membrane lipids (specifically thylakoid membranes).

5. CONCLUSION

In this research, limited translocation of Pb from root to leaves occurred in lower external Pb concentrations (probably due to combined endodermis barrier and changes in root branching patterns), but increased in plants exposed to higher Pb concentrations (where endodermis barrier may be broken). Pb accelerated the development of senescence characteristics in lettuce cells and these changes were accompanied by changes in classical growth parameters (biomass, root and organ length). Also nutritional imbalances (e.g. K, Fe) and lipid peroxidation (MDA accumulation) were observed in Pb treated plant. Less significant were the effects on photosynthetic apparatus, though chl*a*/chl*b* ratio decreased and PSII photochemical efficiency was affected within short term exposure.

These biochemical biomarkers could be used in a complementary approach with traditional assays in toxicological tests and, further, improve information concerning plant performance aiming at a reliable extrapolation to realistic environmental conditions.

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