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Low doses of Pb affected *Lactuca sativa* photosynthetic performance

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Abstract

The effects of soil and water contamination by lead (Pb) and the consequences on plant growth and yield are of great concern worldwide. Limits of the Pb concentration in water have been established by governmental institutions but these differ from each other. In this study, *Lactuca sativa* (var. Reine de Mai) plants were exposed to low $Pb(NO_3)_2$ doses (0.05–20 mg L^{-1}), including the recommended limit values for irrigation water by the Food and Agriculture Organization (FAO). After 28 d of exposure, lettuce plants did not present visible morphological alterations or growth impairment, but CO_2 assimilation rate (P_N), photochemical quenching, and effective quantum efficiency of PSII were negatively affected, while intercellular CO_2 concentration, stomatal conductance, or transpiration rate were not influenced. Our results suggested that limitations on photosynthesis occurred from different reasons than due to the decrease of internal CO_2 availability, alterations of photophosphorylation, and/or electron transport rate. Thus, this lettuce cultivar showed photosynthetic susceptibility to low doses of Pb, even at lower concentrations than those maximal allowed for irrigation water by FAO. Furthermore, P_N seemed to be the most sensitive biomarker for evaluation of Pb susceptibility.

Additional key words: abiotic stress; chlorophyll fluorescence; gas exchange; phytotoxicity; toxic metal.

Introduction

Contamination of the environment by metals remains a matter of great concern worldwide. Lead (Pb) is one of the potential toxic metals (Ahmad *et al.* 2011) and is a persistent and multi-target pollutant (Shahid *et al.* 2014). Among Pb sources are mining, industry, metal plating and finishing operations, batteries, pesticides, fertilizers, and additives in pigments and gasoline (Ahmad *et al.* 2011). Soil and water contamination by Pb is real and human exposure to Pb above a baseline is common (Abadin *et al.* 2007). Atmospheric deposition is the main source of Pb in soils, which are an important sink for Pb (Abadin *et al.* 2007). Although Pb release from industry to aquatic systems is smaller than that to soil and air, Pb may be present in important concentrations in drinking and in irrigation water. For irrigation water in Canada, the maximum allowed dose is 0.2 mg(Pb) L^{-1}, whereas 5 mg(Pb) L^{-1} is the recommended dose by Food and Agriculture Organization (FAO). In soil, a natural content ranges from <10 to 30 µg(Pb) g^{-1}(soil), but in contaminated areas, it may reach 30–2,000 µg(Pb) g^{-1}, which is far higher than occurs naturally (Grant *et al.* 1986). Taking all into account, Pb contamination is a serious and actual environmental problem.

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Abbreviations: Chl – chlorophyll; C_i – intercellular CO_2 concentration; DM – dry mass; ETR – electron transport rate; F – steady-state fluorescence; F_0 – minimal fluorescence yield of the dark-adapted state; FAO – Food and Agriculture Organization; FM – fresh mass; F_m – maximal fluorescence yield of the dark-adapted state; F_m' – maximal fluorescence yield of the light-adapted state; F_v/F_m – maximum photochemical efficiency of PSII; g_s – stomatal conductance; LS – leaf succulence; NPQ – nonphotochemical quenching; P_N – net photosynthetic rate; q_P – photochemical quenching coefficient; TSS – total soluble sugar; WC – water content; Φ_{PSII} – actual photochemical efficiency of PSII.
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Lead is phytotoxic and may reduce crop yield (Pourrut *et al.* 2011). Pb binds strongly to a large number of molecules, such as amino acids, enzymes, DNA, and RNA, and may disrupt many metabolic pathways (Patra *et al.* 2004). Some symptoms (Patra *et al.* 2004) of Pb toxicity are a decline of plant growth, reduction of leaf area, alterations in photosynthesis, and nutrient uptake (Capelo *et al.* 2012, Feleafel and Mirdad 2012), induction of oxidative stress (Verma and Dubey 2003, Capelo *et al.* 2012, Kumar *et al.* 2012), genotoxicity (Shahid *et al.* 2011), and photosynthesis inhibition (Pourrut *et al.* 2011, Alkhatib *et al.* 2012). Plants can acquire and accumulate Pb and its uptake occurs *via* roots and/or foliage (Feleafel and Mirdad 2012). Lead uptake, accumulation, and effects on plants depend on many factors, such as pH, soil/solution composition, Pb concentration and speciation, exposure period, plant species and development stage, among others (Pourrut *et al.* 2011). In general, effects are more pronounced at higher concentrations and after long-term exposure (Patra *et al.* 2004, Capelo *et al.* 2012).

Lactuca sativa (lettuce) is one of the most commonly grown hydroponic vegetable and Pb presence in water may affect its growth and yield and can be a potential risk for consumers. In this work, we aimed to study the effect of environmentally relevant Pb concentrations in irrigation water on growth and photosynthesis of lettuce plants during a long-exposure period and, evaluate if those values are adequate for lettuce growth in hydroponics. For that, lettuce plants were grown hydroponically in the presence of low Pb concentrations, including the recommended values, and several characteristics were analyzed: growth, chlorophyll *a* fluorescence, gas exchange-related parameters, total soluble sugars, and pigment content.

Materials and methods

Plant material and hydroponic culture: *Lactuca sativa* (cultivar Reine de Mai) plants (two weeks old) purchased from a local greenhouse were washed and grown further for 28 d on adapted Hoagland's nutrient solution [57.52 mg ($NH_4H_2PO_4$) L^{-1}; 2.86 mg(H_3BO_3) L^{-1}; 656.4 mg [$Ca(NO_3)_2$] L^{-1}; 0.04 mg($CuSO_4 \cdot 5\ H_2O$) L^{-1}; 5.32 mg(Fe-tartrate) L^{-1}; 120.38 mg($MgSO_4$) L^{-1}; 1.81 mg($MnCl_2 \cdot 4\ H_2O$) L^{-1}; 0.016 mg(MoO_3) L^{-1}; 606.6 mg(KNO_3) L^{-1}; and 0.11 mg ($ZnSO_4 \cdot 7\ H_2O$) L^{-1}]. Nutritive solution was supplemented with different concentrations of $Pb(NO_3)_2$: 0, 0.05, 0.5, 5, 10, and 20 mg L^{-1}, corresponding to 0.031, 0.31, 3.1, 6.25, and 12.5 mg L^{-1} Pb. These concentrations were chosen in order to include the maximum recommended values for Pb in irrigation waters (0.2–5 mg L^{-1}). Plants were grown in a climate chamber at 24°C, under light intensity of 200 µmol(photon) m^{-2} s^{-1} with 75% humidity and 16/8-h photoperiod. The nutrient solution was continuously aerated and renewed every 3 d. The pH was maintained at 5.8 throughout the experiment. We carried out two different exposures and all measurements were performed at the end of the exposure period.

Growth and water content analysis: Six plants from each concentration were used to measure growth, water content (WC), and leaf succulence (LS). The shoot length and fresh mass (FM) were measured. The WC was calculated using the following formula: WC = [(FM – DM)/FM)] × 100 (Silva *et al.* 2010). The LS was calculated according to: LS = (FM – DM)/area, where DM is dry mass.

Gas exchange and chlorophyll (Chl) *a* fluorescence: Gas-exchange measurements were done with a portable photosynthesis system (*LCpro+*, *ADC*, Hoddesdon, UK) operating in an open mode. The youngest completely expanded leaf of each plant was measured under a saturating PPFD of 200 µmol m^{-2} s^{-1} provided by an external halogen lamp. Net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) were estimated. Measurements were performed under growth chamber conditions and atmospheric CO_2 concentration. Six plants were measured per treatment.

Steady-state modulated Chl fluorescence was measured on the same leaves as gas-exchange measurements with the help of a portable pulse amplitude modulation fluorometer (*Mini-PAM*, *Walz*, Effeltrich, Germany), as described in Jesus *et al*. (2015). Steady-state fluorescence (F) and maximal fluorescence (F_m') were measured in light-adapted leaves. Minimal fluorescence (F_0) was measured in 30 min dark-adapted leaves by applying a weak modulated light and maximal fluorescence (F_m) was measured after applying a 0.7-s saturating pulse of white light [> 1,500 µmol(photon) m^{-2} s^{-1}]. Definitions of fluorescence parameters maximal efficiency of PSII (F_v/F_m) and actual photochemical efficiency of PSII (Φ_{PSII}) were used as described by van Kooten and Snel (1990). Photochemical quenching was calculated as $q_P = (F_m' - F')/(F_m' - F_0')$ and nonphotochemical quenching as $NPQ = (F_m - F_m')/F_m'$. Electron transport rate (ETR) was calculated as a linear correlation of Φ_{PSII} ($ETR = \Phi_{PSII} \times PAR \times 0.5 \times 0.84$). Six plants per treatment were used for measurement of these parameters.

Pigment analysis and total soluble sugar quantification: Photosynthetic pigments (Chl *a*, Chl *b*) and carotenoids (Car) were measured using pure acetone/50 mM Tris buffer (80:20) according to Sims and Gamon (2002). The absorbance at 470, 537, 647, and 663 nm was determined with a *Genesys 10* spectrophotometer (*Thermo Fisher Scientific Inc.*, Waltham, USA).

Total soluble sugar (TSS) concentration was determined by using the anthrone method. TSS were quantified by a *Genesys 10* spectrophotometer (*Thermo Fisher Scientific Inc.*, Waltham, USA) as described by Irigoyen *et*

al. (1992) and Osaki *et al.* (1991). For pigments and carbohydrates quantifications, six replicates were used. Each replica consisted of leaf pools from 7–10 plants.

Statistical analysis: Values are given as mean ± standard error. The comparison between Pb concentrations and control was made using one-way analysis of variance (*ANOVA*) test. When the data were statistically different, *ANOVA* test was followed by a *Holm Sidak*'s comparison test ($p<0.05$). *Pearson*'s correlation for the tested end points was also performed using the *Sigma Stat* program, and correlations were considered significant for $p<0.05$.

Results

Growth and water content analysis: After 28 d of culture, Pb-treated and control plants presented similar survival rates (100%). Morphologically, no differences were observed between plants of the different groups (Fig. 1). Shoot (Fig. 2) and root (data not shown) growth was not significantly affected by Pb exposure, however, a decreasing trend in length of aerial portion was detected at the highest concentrations. Furthermore, a negative correlation ($R^2 = –0.42$; $p=0.01$) was obtained between Pb concentrations and shoot length. Lead exposure did not affect succulence or leaf water content (Fig. 2).

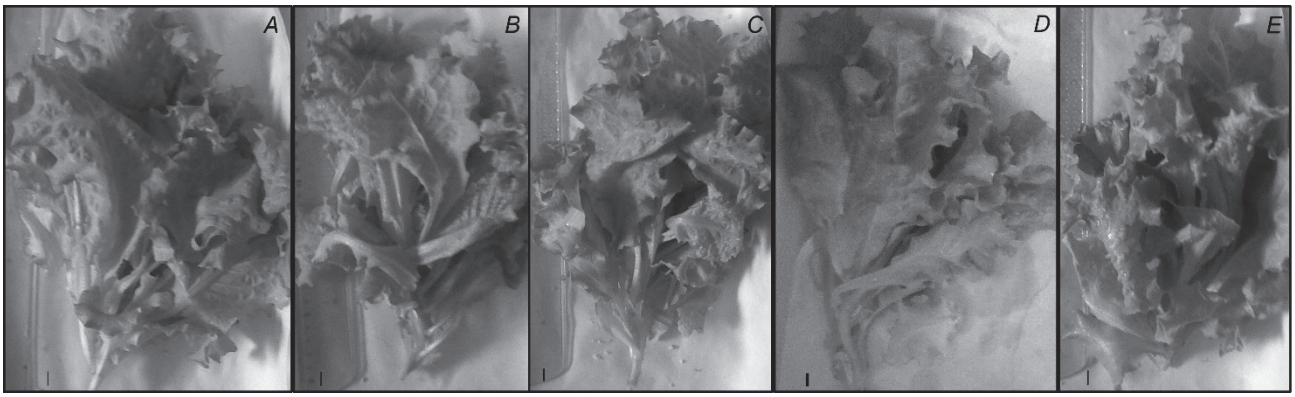

Fig. 1. Shoots of lettuce exposed to different $Pb(NO_3)_2$ concentrations: control (*A*); 0.05 mg L^{-1} (*B*); 0.5 mg L^{-1} (*C*); 5 mg L^{-1} (*D*); and 10 mg L^{-1} (*E*). *Bar* 1 cm.

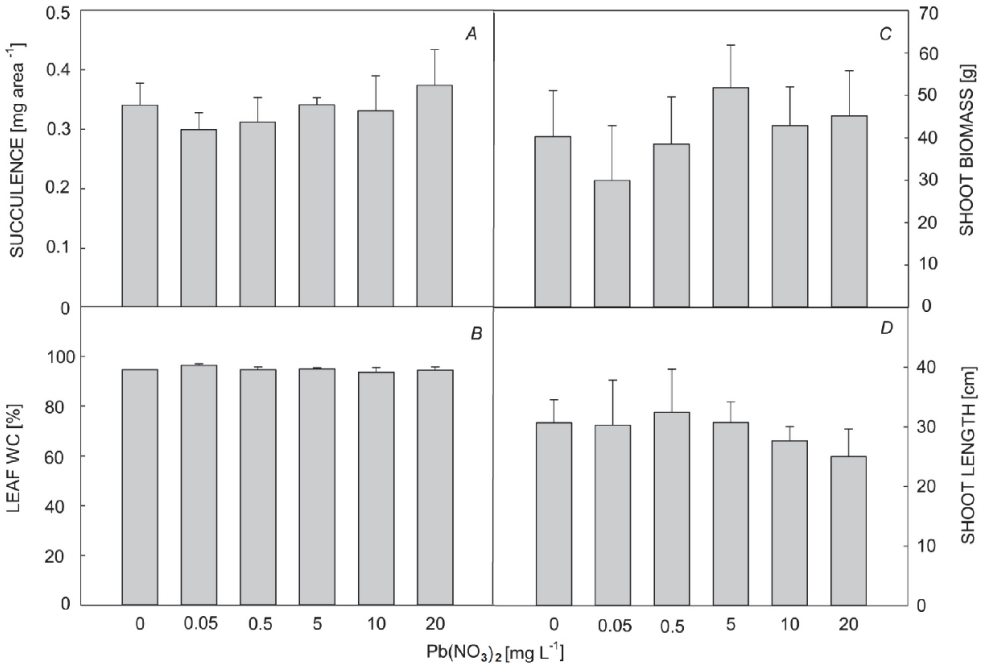

Fig. 2. Leaf succulence [mg $area^{-1}$] (*A*), water content (WC, *B*), shoot biomass (*C*), and length (*D*) of plants exposed during 28 d to different $Pb(NO_3)_2$ concentrations.

Gas exchange and Chl *a* fluorescence: Lead exposure affected plant photosynthetic status in different parameters: a decrease of P_N at all Pb concentrations [between 18.8% (10 mg L^{-1}) and 40.3% (0.5 mg L^{-1})] and increase of E at 10 mg($Pb(NO_3)_2$) L^{-1} (Fig. 3). Also, an increasing trend in C_i and of g_s was detected. A positive correlation was obtained between Pb concentration and C_i (R^2 = 0.4; p=0.005), E (R^2 = 0.5; p=0.0003), and g_s (R^2 = 0.3; p=0.02).

Concerning Chl *a* fluorescence, alterations were observed in Φ_{PSII}, decreasing in all concentrations, but mostly at 0.5, 10, and 20 mg($Pb(NO_3)_2$) L^{-1} (31.4 to 39.0%) (Fig. 4). F_0 increased at 0.05 and 0.5 mg L^{-1} and decreased at 5 mg L^{-1}. Some significant differences were detected in q_P values after Pb exposure with a decrease at 0.5 and 20 mg L^{-1}, but a decreasing trend was observed with the increase of the concentration. A negative correlation was obtained between Pb concentration and Φ_{PSII} (R^2 = –0.4; p=0.006) and between Pb and q_P (R^2 = –0.3; p=0.03). Pb exposure did not affect significantly the F_v/F_m ratio in lettuce plants and no significant differences were observed in NPQ values between plants exposed to Pb and control plants. Nevertheless, positive correlations were detected between Pb concentration and NPQ (R^2 = 0.4; p=0.005) and between q_P and NPQ (R^2 = 0.3; p=0.01). Since all the measurements were made under constant light intensity, ETR values (data not shown) presented the similar behavior as Φ_{PSII}.

Other significant correlations were obtained mainly between Φ_{PSII} and C_i (R^2 = –0.4; p=0.02), E (R^2 = –0.3; p=0.01), q_P (R^2 = 0.7; p=0.000002), P_N (R^2 = 0.4; p=0.04); between C_i and E (R^2 = 0.6; p=0.00004) and g_s (R^2 = 0.7; p=0.0000005), and between E and g_s (R^2 = 0.9; $p = 9 \times 10^{-17}$).

Pigments and total soluble sugar quantification: Pigment contents were not significantly affected by Pb exposure, but a decrease in Chl *a* and Car content was observed in leaves exposed to 0.05 and 0.5 mg L^{-1} (Fig. 5). A strong positive correlation was detected between Chl *a* and Car content (R^2 = 0.9; $p = 1 \times 10^{-12}$). The Chl *a/b* ratio was not affected in plants exposed to Pb.

Leaves of exposed plants did not present significant differences in TSS values when compared with control ones (Fig. 6). A negative correlation was found between Φ_{PSII} and TSS (R^2 = –0.4; p=0.04).

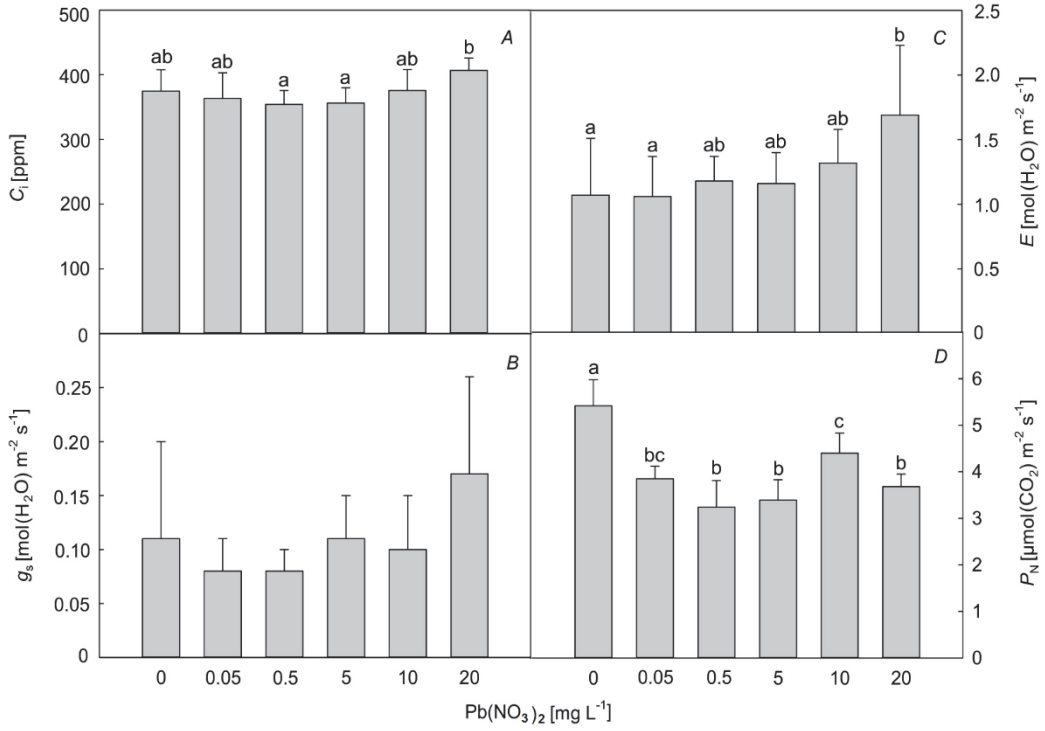

Fig. 3. Gas-exchange profile of plant leaves exposed to different $Pb(NO_3)_2$ concentrations. Values are given as mean ± standard error for each condition. Intercellular CO_2 concentration (C_i, *A*); stomatal conductance (g_s, *B*); transpiration rate (*E, C*); and net photosynthetic rate (P_N, *D*). *Different letters* correspond to significant differences between treatments.

Discussion

Lead toxic effects on plants have been intensely investigated and several works were published on this research field. Nevertheless, in most of the available studies, much higher Pb concentrations were used than

those found in the environment (Lamhamdi *et al.* 2011, Nautiyal and Sinha 2012, Rodriguez *et al.* 2014, Yan and Tam 2013). In this work, we restricted the Pb concentrations to more realistic values [0.03–12.5 mg(Pb) L^{-1}]. After the growth period (28 d) normally used in hydroponic systems, lettuce plants did not present visible morphological alterations or growth impairment at any of the Pb concentrations tested. Furthermore, the hydric status of the exposed leaves did not seem to be compromised by Pb (no alteration in leaf WC and succulence were detected), which is supported by the absence of significant differences in E and g_s at most of the concentrations. Most of the studies available reported negative effects on plant growth after high Pb dose, as in pigeon pea (exposed to 0.2–1 mM) (Nautiyal and Sinha 2012), tomato (exposed to 150–900 mg kg^{-1}) (Zhao *et al.* 2011), *Kandelia abovata* and *Acanthus ilicifolius* (exposed to 100–800 mg kg^{-1}) (Yan and Tam 2013), and wheat (exposed to 100–1,000 mg L^{-1}) (Lamhamdi *et al.* 2011). Contrary to our findings, in maize plants grown in sand and supplemented with low concentrations of Pb (0.01, 0.1, and 1 mg L^{-1}), plant growth decreased (Ahmad *et al.* 2011). Growth was also negatively affected in tobacco plants after exposure to 5–500 µM Pb (1–100 mg L^{-1}) (Alkhatib *et al.* 2012). Differently from the present work, the above authors did not determine or correct the pH value of the solutions, which may have contributed to a low pH value and a higher Pb toxicity (Igwe *et al.* 2005). Nevertheless, Pb effects on growth seem to be dependent on plant species and even on variety (Wierzbicka 1999). Other species exposed to relatively low doses of Pb and under controlled pH were also negatively affected: cotton (50 and 100 µM = 10 and 20 mg L^{-1}) (Bharwana *et al.* 2014), *Brassica napus* (100 and 400 µM = 20 and 80 mg L^{-1}) (Ali

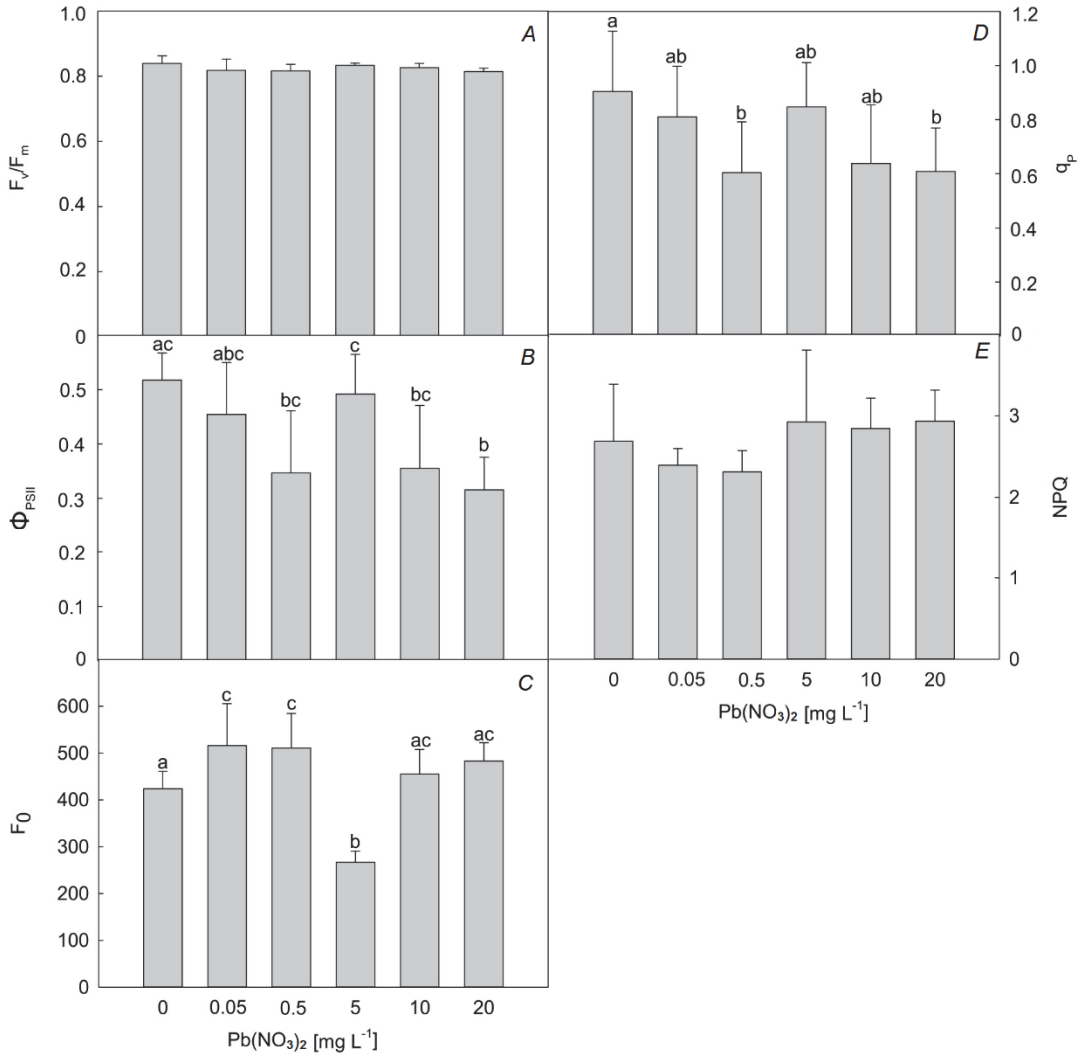

Fig. 4. Fluorescence parameters variations in leaves of plants exposed to different $Pb(NO_3)_2$ concentrations during 28 d. Values are given as mean ± standard error for each condition. Maximum quantum efficiency (F_v/F_m, *A*); total photochemical efficiency (Φ_{PSII}, *B*); minimal fluorescence (F_0, *C*); photochemical quenching (q_P, *D*); and nonphotochemical quenching (NPQ, *E*). *Different letters* correspond to significant differences between treatments.

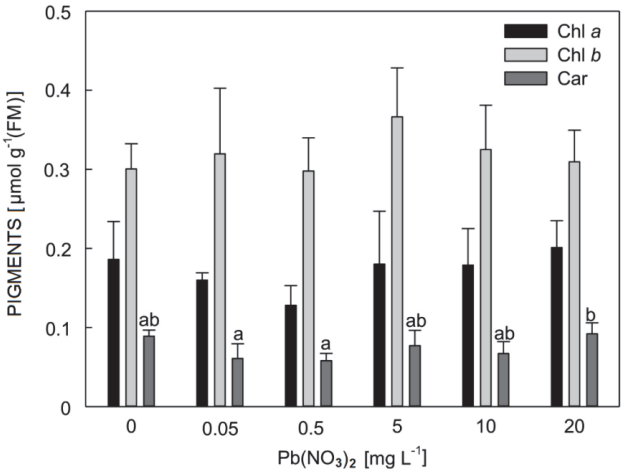

Fig. 5. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid (Car) contents in leaves of plants exposed to several $Pb(NO_3)_2$ concentrations after 28-d exposure. Values are means ± standard error. *Different letters* correspond to significant differences between treatments. FM – fresh mass.

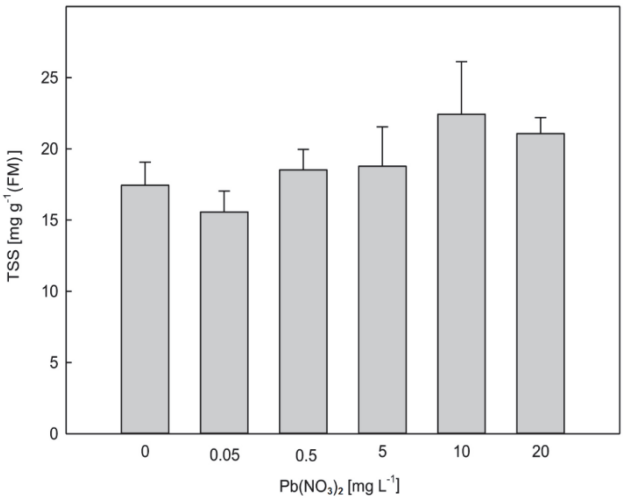

Fig. 6. Total soluble sugar (TSS) content in leaves exposed to different $Pb(NO_3)_2$ concentrations during 21 d. Values are means ± standard error. FM – fresh mass.

et al. 2014). In *L. sativa* plants, Lamb *et al.* (2010) and Capelo *et al.* (2012) reported susceptibility of plants to 0.1–500 µM (0.02–100 mg L^{-1}) and to 125 mg(Pb) L^{-1}, respectively, with root growth reduction. Contrary, after exposure to 12.5 mg(Pb) L^{-1} (15 d in soil), *L. sativa* (variety Raine Mai) plants did not show growth impairment (Capelo *et al.* 2012). Furthermore, the Raine Mai variety (the same used in the present work) did not accumulate detectable Pb concentrations (determined by induced coupled plasma spectroscopy) in roots nor in shoots when exposed to 12.5 mg(Pb) L^{-1} for 15 d (Capelo *et al.* 2012). Thus, the absence of significant accumulation of Pb may indicate that the observed effects in Capelo *et al.* (2012) and in the present work occurred due to indirect action.

Although the low doses of Pb analyzed here did not affect lettuce growth, photosynthesis was impaired. Several works reported deleterious effects of Pb on photosynthesis-related parameters (Kosobrukhov *et al.* 2004, Bibi and Hussain 2005, Kaznina *et al.* 2005, Rodriguez *et al.* 2014, Tian *et al.* 2014), but again, most of them used high doses of Pb. In the present work, Pb decreased P_N, q_P, and Φ_{PSII} at least at two Pb concentrations (0.5 and 20 mg L^{-1}). Of all photosynthesis-related parameters determined, P_N was the most affected. Furthermore, a negative correlation was obtained between Pb concentration and q_P and Φ_{PSII}, also suggesting some negative effects on the photophosphorylation. These alterations were not accompanied by stomatal limitations since no decreases in related parameters were observed (*e.g.*, C_i, E, g_s). Also, since a positive correlation was obtained between Pb concentration and NPQ and C_i, data suggest that the observed effects on photosynthetic parameters were not due to decrease of internal CO_2 availability. Similarly to our data, maize plants exposed to low doses of Pb also showed an impairment of P_N, while E and g_s were not affected (Ahmad *et al*. 2001). Those authors suggested that P_N reduction was due to other reasons than stomatal limitations (Ahmad *et al.* 2011), also supporting our conclusions for lettuce.

As stomata apparently were not involved, we hypothesize that Pb might indirectly affect the Calvin cycle, as already demonstrated for other crops (Rodriguez *et al.* 2015). Using pea exposed to higher doses of Pb, we have compared the sensitivities of light-dependent *vs.* light-independent photosynthetic reactions and found that the light-independent reactions, as Calvin cycle reactions, are sensitive to Pb (Rodriguez *et al.* 2015). This fact together with current data support that P_N reduction occurred due to a decrease of Calvin cycle-related enzymes, such as Rubisco. In fact, it was demonstrated for *P. sativum* (Parys *et al.* 1998, Rodriguez *et al.* 2014) and spinach (Xiao *et al.* 2008) where Pb exposure decreased Rubisco activity. A direct/indirect effect of Pb on photophosphorylation and on electron transport rate (ETR) with implications on Rubisco/Calvin cycle was proposed for spinach by Xiao *et al.* (2008). Similarly to our findings for Pb-exposed lettuce, cucumber plants exposed to other metals (Cu and Cd) showed a decrease in Φ_{PSII} but not of C_i or in F_v/F_m, and the authors suggested putative alterations on photophosphorylation (Burzynski and Klobus 2004). Futhermore, putative detrimental effects of Pb on photophosphorylation and/or ETR in lettuce were supported by the observed decreases in Φ_{PSII} and consequently of ETR.

No alterations were detected in pigment contents (Chl *a*, *b* and Car) or Chl *a*/*b*. Our data support similar findings in *Pisum sativum* (Rodriguez *et al.* 2014) and in rice (Zeng *et al.* 2006), where pigment contents were not affected or even increased. The lack of effects on Chl content (in these species and for the Pb doses tested) suggests no significant detrimental effects on the core complexes of PSII. However, other studies, mostly using

higher Pb doses, reported a decrease in Chl contents after Pb exposure, for example in *Plantago major* (Kosobrukhov *et al.* 2004), black gram (Bibi and Hussain 2005), barley and oat (Kaznina *et al.* 2005), and *Brassica rapa* (Cenkci *et al.* 2010).

The decrease of P_N did not significantly impair plant growth (biomass and length), despite it was observed a trend to decrease length, which negatively correlated with Pb concentration. Sugars are the principal end products of photosynthesis. They act as nutrient and metabolite signalling molecules and are used to maintain cell turgor (Couee *et al.* 2006). The Pb doses did not significantly affect TSS contents, which combined with the absence of detrimental effects on growth indicated that this cultivar possesses tolerance to Pb as already demonstrated for Cd (Monteiro *et al.* 2009).

It has been well demonstrated that Pb effects depend greatly on dose, conditions of exposure, and plant species/variety. Regarding lettuce (cv. Reine de Mai) we demonstrated that even concentrations below maximum admissible values of Pb in irrigation waters affected photosynthesis. In face of our results, more studies are required to evaluate if recommended values of Pb for irrigation water (*e.g.,* 5 mg L^{-1}) do not present toxicity to some crops. We also demonstrated that from the biomarkers used, P_N showed to be a sensitive parameter in order to evaluate plant susceptibility even to low doses of Pb. The use of physiological end points (such as P_N) is therefore a valuable tool that should complement the morphological characterization currently used in risk assessment studies.

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