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Research article

Pb low doses induced genotoxicity in *Lactuca sativa* plants

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ABSTRACT

Soil and water contamination by lead (Pb) remains a topic of great concern, particularly regarding crop production. The admissible Pb values in irrigation water in several countries range from ≈0.1 to ≈5 mg L^{-1}. In order to evaluate putative effects of Pb within legal doses on crops growth, we exposed *Lactuca sativa* seeds and seedlings to increasing doses of $Pb(NO_3)_2$ up to 20 mg L^{-1}. The OECD parameter seed germination and seedling/plant growth were not affected by any of the Pb-concentrations used. However, for doses higher than 5 mg L^{-1} significant DNA damage was detected: Comet assay detected DNA fragmentation at ≥ 5 mg L^{-1} and presence of micronuclei (MN) were detected for 20 mg L^{-1}. Also, cell cycle impairment was observed for doses as low as 0.05 mg L^{-1} and 0.5 mg L^{-1} (mostly G_2 arrest). Our data show that for the low doses of Pb used, the OECD endpoints were not able to detect toxicity, while more sensitive endpoints (related with DNA damage and mitotic/interphase disorders) identified genotoxic and cytostatic effects. Furthermore, the nature of the genotoxic effect was dependent on the concentration. Finally, we recommend that MN test and the comet assay should be included as sensitive endpoints in (eco)toxicological assays.

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1. Introduction

The presence of heavy metals in the environment occurs naturally or as a consequence of human actions. Since the industrial revolution, the levels of heavy metals as contaminants in the environment increased, often reaching values that became toxic for living organisms (Nagajyoti et al., 2010; Agency for Toxic Substances and Disease Registry, 2007). Soil, water and atmospheric contamination by lead (Pb) is a worldwide problem and humans are commonly exposed to Pb above naturally-occurring levels (Agency for Toxic Substances and Disease Registry, 2007). Being a toxic metal, Pb is a environmentaly persistent and multitarget pollutant (Shahid et al., 2013; Kumar and Kumari, 2015) and is known to have no function either in animals or plants. Furthermore, based on the frequency of occurrence, toxicity and human exposure potential, Pb ranks second after arsenic (Agency for Toxic Substances and Disease Registry, 2015). Therefore, Pb remediation is a matter of discussion and concern worldwide.

Most plant species are susceptible to Pb exposure with effects at morphological, physiological and/or biochemical levels (Pourrut et al., 2011b). There are, however, several plant species that are Pb hyper accumulators (accumulating ≥1000 ppm) and are recommended for soil/water remediation (Kumar and Kumari, 2015). Pb reaches plants mostly by soil (where it accumulates mostly due to atmospheric depositions) and by water. According to the Agency for Toxic Substances and Disease Registry (2015), industrial Pb release to aquatic systems contributes to the observed high levels of metal detected in drinking and irrigation water. Also, Pb concentration in surface water depends on contamination sources, water physicochemical properties and Pb content in soil. According to the US Environmental Protection Agency (EPA), Pb values in surface/ground water usually range from 5 to 30 μg L^{-1}, but higher levels (<890 μg L^{-1}) may be detected (EPA, 1986). Pb content in drinking/tap water is usually <5 μg L^{-1} but higher levels (100 μg L^{-1}) have

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also been found (World Health Organization, 2011). For irrigation water, the recommended values differ with country/organization: for example, Food and Agriculture Organization (FAO, http://www.fao.org/docrep/003/t0234e/t0234e06.htm) and EPA (2012) recommend 5 mg L^{-1}, whereas Korea, Israel, Cyprus, Greece and Italy recommend 0.1 mg L^{-1} (Jeong et al., 2016), and Canada allows a maximum of 0.2 mg L^{-1} (http://www.env.gov.bc.ca/wat/wq/BCguidelines/lead/lead.html).

Roots are the first organ of contact and the main system of entrance and accumulation of Pb in plants (Pourrut et al., 2011b), being therefore expected that roots are also the primary organ to evidence sensitivity to Pb. As the metal reacts with important biomolecules, a large number of metabolic pathways may be disrupted (Patra et al., 2004) leading to diverse phytotoxic symptoms. Nevertheless, Pb induced toxicity depends on the species, the plant stage of development and the exposure conditions, including Pb concentration and speciation, exposure period, pH, soil or mineral solution and organic composition (Patra et al., 2004; Lamb et al., 2010; Pourrut et al., 2011a; Capelo et al., 2012).

Pb can delay or even inhibit germination, as described for wheat (Yang et al., 2010; Lamhamdi et al., 2011; Wang et al., 2011) and rice (Mishra and Choudhuri, 1999), but the most commonly reported physiological effect concerns plant growth imbalances. Shoot and/or root growth inhibition was detected in *Vicia faba* (Wang et al., 2010a), *Sesbania grandiflora* (Malar et al., 2014), *Lactuca sativa* (Capelo et al., 2012), *Hordeum vulgare* and *Lycopersicon esculentum* (Cheyns et al., 2012), *Hordeum vulgare* (Varun et al., 2011), *Cynara cardunculus* (Burak Batir et al., 2016), *Triticum aestivum* (Lamhamdi et al., 2011) and *Pisum sativum* (Rodriguez et al., 2015). Nevertheless, most of these alterations were observed at moderate to extremely high Pb concentrations (>100 ppm). Under more environmentally relevant concentrations, Pb effects on plant growth (length and/or biomass) is species dependent (Lamb et al., 2010; Lamhamdi et al., 2011; Capelo et al., 2012; Ali et al., 2014; Bharwana et al., 2014; Rodriguez et al., 2015; Burak Batir et al., 2016; Silva et al., 2016b). Taking all together, it seems that parameters related with growth are not the most sensitive and adequate endpoints to assess Pb toxicity. Therefore, alternative and more sensitive biomarkers are required to accurately assess and predict Pb phytoxicity. Photosynthesis related endpoints have been proposed as sensitive biomarkers, such as CO_2 assimilation rate and ribulose bisphosphate carboxylase oxygenase activity (Rodriguez et al., 2015; Silva et al., 2016b). On the other hand, genotoxicity induced by Pb has been reported even under low concentrations (Pourrut et al., 2011a,b; Shahid et al., 2011; Burak Batir et al., 2016). Several parameters seem promising for the determination of Pb genotoxicity in plants, including micronucleus (MN) frequency, and the DNA damage assessed by the comet assay (Pourrut et al., 2011a; Shahid et al., 2011), since these techniques have demonstrated high sensitivity and are relatively easy to perform.

The aim of the present work was to analyze the effects of environmentally relevant Pb concentrations in irrigation water on growth, cell cycle dynamics and DNA stability of the economically important crop *L. sativa*. The cultivar Reine de Mai has demonstrated to be a reliable model for toxicological/ISO assays and we have previously demonstrated that plants of this cultivar accumulated Pb (67.3 mg gDW^{-1} in roots) when exposed to 125 mg L^{-1} Pb, whereas at low doses, as 12.5 mg L^{-1}, no significant alterations were detected between control and exposed roots (Capelo et al., 2012). *L. sativa* plants of the cultivar Reine de Mai were grown hydroponically in the presence of low Pb concentrations and several endpoints were used to assess Pb toxicity: germination, growth, micronuclei (MN) frequency, DNA damage (by comet assay), ploidy stability and cell cycle analyses. At the end we aim to propose the most sensitive biomarkers for assessing Pb phytotoxicity.

2. Material and methods

2.1. Germination

Lactuca sativa (cultivar Reine de Mai) seeds were surface disinfected with sodium hypochlorite 10% (v/v), rinsed in water and germinated for 7 days in Petri dishes containing filter paper (25 seeds per dish). Seeds were grown on modified Hoagland's nutrient solution with the following composition: 57.52 mg L^{-1} $NH_4H_2PO_4$; 2.86 mg L^{-1} H_3BO_3; 656.4 mg L^{-1} $Ca(NO3)_2$; 0.04 mg L^{-1} $CuSO_4.5H_2O$; 5.32 mg L^{-1} Fe-tartrate; 120.38 mg L^{-1} $MgSO_4$; 1.81 mg L^{-1} $MnCl_2.4H_2O$; 0.016 mg L^{-1} MoO_3; 606.6 mg L^{-1} KNO_3; 0.11 mg L^{-1} $ZnSO_4.7H_2O$. Different concentrations of Pb: 0, 0.05, 0.5, 5, 10 and 20 mg L^{-1} $Pb(NO_3)_2$ corresponding to 0.031, 0.31, 3.1, 6.25 and 12.5 mg L^{-1} Pb were used in the assay. To each dish, 9 mL of the nutritive solution (pH 5.8) supplemented with Pb were added, and germination took place in the dark at 24 °C. After 7 days, the germination rate, plantlet growth (root and aerial portion) and biomass were assessed. For germination rate determination 4 dishes were used and for growth evaluation three to six plantlets from four different dishes were used.

2.2. Hydroponic culture

Lactuca sativa (cultivar Reine de Mai) plants (2 weeks old) purchased from "Viveiros Litoral" (Aveiro, Portugal) were washed and grown for a further 28 days on modified Hoagland's nutrient solution described in 2.1 section. Nutritive solution was supplemented with different concentrations of $Pb(NO_3)_2$: 0, 0.05, 0.5, 5, 10, 20 mg L^{-1} (Pb concentrations described in 2.1 section). These concentrations were chosen in order to include the maximum recommended values for Pb in irrigation waters (EPA, 2012). Plants were grown in a climate chamber at 24 °C, under light intensity of 200 μmol m^{-2} s^{-1} with 70% humidity and 16/8 h photoperiod (Silva et al., 2016b). The nutrient solution was continuously aerated and renewed every 3 days. The pH was maintained at 5.8 throughout the assay. All measurements were performed at the end of the exposure period. After the exposure period, root length, root biomass and root water content (WC) were assessed. For that, were used six different plants from each treatment. The WC was calculated as follows: (fresh weight-dry weight)/fresh weight *100.

2.2.1. Cell cycle analysis

For flow cytometry (FCM) studies, nuclear suspensions of root apices (6 pools of 5–8 fresh apices from three different replicates) were obtained by chopping the roots in Woody Plant Buffer (Loureiro et al., 2007), and processed according to Silva et al. (2012). After addition of RNAse (e.g., Loureiro et al., 2007; Dolezel and Bartos, 2005) and 50 μL propidium iodide (PI), approximately 3000 nuclei were analyzed with a Coulter EPICS-XL flow cytometer (Coulter Electronics, USA), with an argon-ion laser (15 mW, 488 nm). The percentage of nuclei in each phase of the cell cycle (G_0/G_1, S and G_2 phases) were analyzed using the FlowJo software (Tree Star Inc., Ashland, Oregon, USA).

For mitotic phase analysis, root tips were fixed in Carnoy's solution (3:1 methanol: acetic acid) and stored at 4 °C. The apices were hydrolyzed in 1 N HCl at 70 °C, washed in water and stained with PI (Silva et al., 2016a). The mitotic index and the number of cells in each mitotic phase and in cytokinesis were determined with a fluorescence microscope Nikon Epics 80i (Nikon, Japan). For that, in each condition, 2–3 tips from three replicates were assessed and from each tip 1000 cells were analyzed. The percentage of cells in mitosis (in 1000 cells), corresponding to the mitotic index, and the

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