Environmental and Experimental Botany 123 (2016) 1–12

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Characterisation of lead-induced stress molecular biomarkers in *Medicago sativa* plants

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ARTICLE INFO

Article history:
Received 12 June 2015
Received in revised form 16 October 2015
Accepted 19 October 2015
Available online 11 November 2015

Keywords:
Alfalfa
Lead
Oxidative stress
Antioxidant defenses
Phytochelatins
Small HSP

ABSTRACT

In this study, we investigated physiological changes and transcriptional responses in four-week-old hydroponically grown *Medicago sativa* seedlings exposed to $(PbNO_3)_2$ (0, 10 and 100 μM) for 2 and 7 days. Fresh weight and length were reduced in both shoots and roots after 7 days exposure. Lead accumulation was time and dose-dependent with stronger phytotoxic effects in roots than in shoots, with a relatively low amount of Pb translocated from roots to shoots. Lipid peroxidation augmented remarkably, suggesting the onset of oxidative damages, with increased glutathione reductase (GR), ascorbate peroxidase (APX) and superoxide dismutase (SOD) activities. This result was accompanied by a remarkable up-regulation of APX and SOD genes. In roots, the increase of SOD transcripts was concomitant to an enhanced SOD enzymatic activity in all Pb treatments. However, the 30-fold up-regulation occurred with a remarkable APX activity inhibition, which suggests that there might be post-transcriptional modifications able to regulate root APX. Root glutathione (GSH) and homoglutathione (hGSH) concentrations decreased in a dose dependent manner, while we could not detect the accumulation of phytochelatins (PC), albeit the related gene was up-regulated. The lack of PCs synthesis suggests a post-translational regulation of its enzymatic activity. Heat shock proteins (HSP70 and HSP17.7) were increased in alfalfa shoots, implying the triggering of cellular protection mechanism to cope with lead phytotoxicity. It is concluded that alfalfa plants mitigate the oxidative damage through induction of antioxidant enzymes, and the expression of chaperone proteins to alleviate Pb toxicity; metabolic changes that could be exploited as Pb-stress bioindicators.

1. Introduction

Anthropogenic contamination of soils by lead (Pb) is considered as a global environmental threat (Sharma and Dubey, 2005). Lead is becoming the most common contaminant trace element due to its high persistence in the environment (Brennan and Shelley, 1999; Islam et al., 2007) representing a risk of bio-accumulation through the food chain (Pourrut et al., 2011). Several human activities, such as petroleum fueled vehicles, mining, smelting, sewage sludge, and other industrial wastes, are the major sources of Pb (Fahr et al., 2013; Srivastava et al., 2007). An example of a heavily Pb-contaminated area is Jebal Ressass at the Northeast of Tunisia, where mining dumps contain large amounts of Pb, Zn and Cd, threatening the health of nearby village's population (Ghorbel et al., 2010, 2014). Lead has not recognized biological role in living organisms, and is toxic even at very low concentrations (Zhai et al., 2015). Besides being classified as a possible human carcinogen (Cornelis, 2005), lead is known to cause a serious disease called "lead poisoning", which provokes nervous system malfunction and causes irreversible damages (Labbe, 1990; Lidsky and Schneider, 2003). Additionally, the risk of Pb poisoning in the population of a contaminated area can be detected by its accumulation in children's hair, as occurred in Alcala de Henares (Spain) (Peña-Fernández et al., 2014).

Abbreviations: APX, ascorbate peroxidase; GI, % growth inhibition; GS, glutathione synthase; GSH, glutathione; hGSH, homoglutathione; GPx, glutathione peroxidase; GR, glutathione reductase; HSP, heat shock protein; hGSSG, oxidized homoglutathione; hPCs, homo-phytochelatins; PAGE, polyacrylamide gel electrophoresis; PCS, phytochelatin synthase; PCs, phytochelatins; ROS, reactive oxygen species; SOD, superoxide dismutase.

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http://dx.doi.org/10.1016/j.envexpbot.2015.10.005
0098-8472/

Plants may be also affected by Pb toxicity as is readily absorbed and accumulated if grown in Pb-polluted soils (Auguy et al., 2013). Plants exposed to Pb suffer the disruption of several metabolic processes, which leads to the decrease in biomass production (Malar et al., 2014), induction of leaf chlorosis (Kopittke et al., 2007), and depletion of photosynthesis rate (Gupta et al., 2013). Despite being an inactive redox metal, Pb is known to induce oxidative stress in several plants species such as *Vicia faba* (Shahid et al., 2014a), *Zygophyllum fabago* (López-Orenes et al., 2014) and *Triticum aestivum* (Lamhamdi et al., 2011). It is feasible that Pb affects the antioxidant defense mechanisms that lead to the disturbance of the redox cellular balance, which results in the accumulation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet}-$), hydrogen peroxide (H_2O_2), and hydroxylradical ($OH^{\bullet}$) (Shahid et al., 2014b). The function of the complex antioxidant system that maintains ROS levels under control in plant cells can be affected by the accumulation of toxic elements (Sharma and Dietz, 2009; Israr et al., 2011), which results in the oxidation of membrane lipids, proteins and/or nucleic acids, affecting ultimately the plant metabolism (Ortega-Villasante, 2005; Rellán-Álvarez et al., 2006). Toxic elements, such as Pb, promote alterations in the concentration of non-enzymatic antioxidants such as glutathione (GSH) and ascorbate, and modifications in the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione reductase (GR) and ascorbate peroxidise (APX) (Gupta et al., 2009). On the other hand, the synthesis of phytochelatins (PCs), non-protein thiols with known metal-binding capability, constitutes another defense mechanism triggered by toxic metals in order to minimize the induced cellular damages (Cobbett, 2000). Phytochelatins are synthesized from GSH and its homologous (i.e., homoglutathione, hGSH) by the enzyme phytochelatin synthase (PCS) (Grill et al., 1989). However, different degrees of PCs synthesis are observed when plants are exposed to several toxic metals (Sobrino-Plata et al., 2013), and in particular Pb is known as a rather poor inducer of PCs accumulation (Ha et al., 1999; Scheidegger et al., 2011).

Heavy metals alter the activity of different antioxidant enzymes and non-protein thiols patterns in a specific manner, as some previous works described in alfalfa (*Medicago sativa*) plants exposed to Cd, Hg and Cu (Sobrino-Plata et al., 2009; Hattab et al., 2013, 2014). In general, SOD enzymes were not affected by any of the metals, while APX activity increased after a short exposure to 30 μM Cd but decreased when cell damages were extensive (Ortega-Villasante et al., 2007). Interestingly, root GR activity is specifically inhibited by Hg (Sobrino-Plata et al., 2009), but is activated by Cd and As (Yannarelli et al., 2007; Sobrino-Plata et al., 2009, 2013). On the other hand, not all toxic metals induced the same alterations in the non-protein thiols profile, where Cd is a potent inducer of PCs synthesis whereas Hg led to a milder response (Sobrino-Plata et al., 2014a,b), albeit Hg–PC complexes are formed and contribute to Hg tolerance (Carrasco-Gil et al., 2011). Interestingly, non-toxic metals did not alter the non-protein thiols metabolism, such as Cu (Flores-Cáceres et al., 2015) or Zn (Castiglione et al., 2007). Several studies indicate that plants also accumulate PCs in response to Pb exposure (Ghelich and Zarinkamar, 2013; Ghelich et al., 2014; Gupta et al., 2013; Zhixin et al., 2007; López et al., 2005, 2007). However, several contradictory results indicate that PCs and the non-protein thiols metabolism play a secondary role in the tolerance of plants to Pb (Zhang et al., 2008; Gupta et al., 2010).

The aim of our study was to elucidate the role of non-protein thiols in the responses of alfalfa plants to sublethal Pb doses. We also characterized the behavior of several metal-sensitive antioxidant enzymes, which is fundamental for a better understanding of Pb detoxification mechanisms that would help to optimize plant selection for future phytostabilization programs in Pb contaminated soils.

2. Material and methods

2.1. Plant material and growth conditions and treatments

Sterilized seeds of alfalfa (*M.sativa* cv. Aragon) were germinated in aqueous 1.5% agarose for 48 h under dark at 28 °C. Homogenous germinated seeds were grown in a pure hydroponic system with continuous aeration (Carrasco-Gil et al., 2013). To avoid Pb precipitation with phosphate and other oxy anions, a half-strength modified Hoagland solution at pH 6.0 was used according to Sobrino-Plata et al. (2009). Solubility of Pb^{2+} ions in the nutrient solution was confirmed *in silico* using Visual MINTEQ3.0 chemical speciation software (Gustafsson, 2009). After 3 weeks of plant growth, $Pb(NO_3)_2$ was added to the nutrient solution (0, 10, 100 μM) and plants were harvested after 2 and 7 days of treatment with Pb. At harvesting, plants were rinsed with 20 mM Na_2EDTA to remove superficial Pb before separating shoots and roots, and biometric parameters were measured. Samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Biometric and phytotoxic stress parameters

In order to highlight the effect of Pb on growth parameters, the growth inhibition of plants was calculated relative to the control as follows:

$$\%\text{Growth inhibition(GI)} = \left(1 - \frac{\text{MT}}{\text{MC}}\right) \times 100$$

MT—mean of treated plants; MC—mean of control plants.

2.3. Stress indexes

Lipid peroxidation was analyzed according to Ortega-Villasante (2005), using TBARS « *Thiobarbituric acid reactive substances* » accumulation as indicator. Chlorophylls concentration was determined after extraction with 80% (v/v) acetone by measuring the absorbance at 663 and 645 nm as described by Sobrino-Plata et al. (2013).

2.4. Lead tissue concentration

Shoots and roots were dried at 60 °C for 72 h, then 100 mg of ground material was acid-digested in 1 mL of digestion solution (HNO_3:H_2O_2:H_2O, 0.3:0.2:0.5, v:v) under pressure (1.5 atm, 120 °C, 30 min; Presoclave-75 Selecta Autoclave, Barcelona, Spain). The Pb concentration was determined by atomic inductively coupled plasma-mass spectrometry (PerkinElmer SciexNexION 300, San Jose, CA, USA).

2.5. Antioxidants enzymes activities in gel

Protein extraction was prepared by grinding 0.5 g of frozen sample in 1 mL of freshly prepared extraction solution 30 mM MOPS at pH 7.5 mM Na_2-EDTA, 10 mM DTT, 10 mM ascorbic acid, 0.6% PVP, 10 μL 100 mM PMSF and 1 mL protease inhibitors cocktail (P2714, Sigma–Aldrich, St. Louis MO, USA) in a total volume of 10 mL MiliRO H_2O. The homogenized suspension was centrifuged at 14,000C × *g* for 15 min at 4 °C. The clear supernatant was separated in single use 100 μL aliquots stored at −80 °C. Protein concentration in the extracts was determined according to Bradford (1976), using the Protein Assay Reagent (BioRad, Hercules, CA, USA) and BSA as standard. The final loading of protein for activity assays was adjusted by Coomassie-blue staining after denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). For each enzymatic assay, equal