Environmental and Experimental Botany 78 (2012) 18–24

Contents lists available at SciVerse ScienceDirect

Environmental and Experimental Botany

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

Lead accumulation and synthesis of non-protein thiolic peptides in selected clones of *Melilotus alba* and *Melilotus officinalis*

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ARTICLE INFO

Article history:
Received 21 October 2011
Accepted 13 December 2011

Keywords:
Heavy metal
Hyperaccumulation
Homophytochelatins
Lead
Phytoremediation
Phytochelatins

ABSTRACT

The accumulation of Pb, its effect on plant growth, and the synthesis of non-protein thiolic peptides were evaluated in clone MA-X of *Melilotus alba* and clone MO-A of *Melilotus officinalis* cultured in a greenhouse in artificially-polluted substrate with 200 and 1000 mg Pb kg^{-1}. At 90 days of Pb exposure root growth was not affected, except at 1000 mg Pb kg^{-1} in MA-X. At this concentration hyperaccumulation value was exceeded in both clones, reaching more than 4800 mg Pb kg^{-1} dry wt. in shoots of MA-X and more than 3400 mg Pb kg^{-1} dry wt. in shoots of MO-A. Phytochelatins (PCs) and homophytochelatins (hPCs) were found in all cases for both species. Clone MA-X showed an increase in constitutive hPCs content in shoots whereas PC_2, PC_3 and hPC_3 were synthesized in roots after 90 days of culture in 1000 mg Pb kg^{-1}. On the other hand, MO-A synthesized a new PC (PC_4) in shoots while in roots only hPC_2 was found. Our results suggest that clones MA-X and MO-A show high Pb accumulation ability and that this capability can be related to the synthesis of PCs and hPCs.

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1. Introduction

Lead is a hazardous heavy metal pollutant that cannot be chemically degraded or biodegraded by microorganisms, and so tends to accumulate in soils (Zaier et al., 2010). Nowadays, there are three types of major sources of Pb contamination in soils: industrial activities such as mining and smelting processes, agricultural activities such as application of insecticides, and urban activities such as municipal sewage sludges and the use of lead in petrols, paints, and other materials (Shen et al., 2002). As conventional cleanup technology is generally too costly, increasing attention has been given to the development of a plant-based technology (phytoremediation) to extract heavy metal from contaminated soils (Chaney et al., 1997). Phytoremediation is based on the use of tolerant plants that accumulate high amounts of specific metals in their shoots and might reduce their concentration in contaminated soils to environmentally acceptable levels (Raskin et al., 1994). Thus, much effort has been made to identify hyperaccumulator species and the mechanisms of heavy metal accumulation in plants.

One of the major adaptive processes in plant tolerance is the prevention of high metal concentrations in the cytoplasm, by chelating metals through peptides in vacoules (Vázquez et al., 1994). These peptides are metallothioneins and phytochelatins (PCs), PCs being the most important in plants (Pal and Rai, 2010). These are cysteine-rich polypeptides with the general structure $(\gamma$-glutamylcysteine$)_n$-glycine, where $n = 2–11$. Similar peptides with β-alanine instead of glycine as the terminal amino acid have been identified in Fabaceae as homophytochelatins (hPCs) (Grill et al., 1986; Klapheck, 1988) and are the main form of chelating compounds in leguminous plants (Oven et al., 2002). However, these mechanisms of heavy metal accumulation are not yet fully understood and few studies have focused on Pb. This metal is not considered a strong inducer of phytochelatins in plant organisms, but Pawlik-Skowronska (2000) showed that Pb ions that enter algal cells immediately activate the synthesis of PCs.

On the other hand, there is now considerable debate on the role of PCs in metal detoxification. Several studies (Howden et al., 1995; Rauser, 1995; Haag-Kerwer et al., 1999; Srivastava et al., 2004; Martínez et al., 2006; Andra et al., 2009) showed that PCs form the main response to heavy metal accumulation in higher plants and play an important role in detoxification by chelating metals into a less toxic form. However, other authors have recently observed that in most hyperaccumulators, the synthesis of PCs is not correlated with metal accumulation (Ebbs et al., 2002; Gadapati and Macfie, 2006; Verbruggen et al., 2009). So, their role in hyperaccumulation is not clear and may vary with the species and metal studied.

Understanding the biochemical and physiological processes controlling accumulation will be crucial in order to design

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doi:10.1016/j.envexpbot.2011.12.016

practical and effective Pb phytoremediation projects. For this, it will be of great importance to study the ability of certain plants to tolerate and store high concentrations of Pb in their tissues.

In previous work (Fernández et al., 2010) we identified different Pb-polluted areas in Asturias (Spain) and chose the native species *Melilotus alba* as a plant that showed good growth and high Pb accumulation at these sites. We subsequently selected the best individual accumulator (MA-X) by comparing plant growth and metal accumulation during growth *in vitro* in the presence of Pb, and cloned this individual. We also found another related species *Melilotus officinalis* that grew in non-polluted soils, and tested its Pb accumulation ability, identified the best individual Pb accumulator (MO-A) and cloned it. Since such studies were performed in *in vitro* culture and the conclusions could differ from those obtained in soil, the aim of the present article was to study the ability of the MA-X clone from a Pb-polluted site and the MO-A clone from a non-polluted area to extract and accumulate Pb in greenhouse in artificially polluted soils and, due to the existing controversy, we also wanted to determine whether thiolic compounds such as PCs and hPCs are involved in Pb detoxification in these species.

2. Materials and methods

2.1. Culture of the best accumulator clones of Melilotus alba and Melilotus officinalis on Pb-polluted substrate

Seeds of *M. alba* Medik. and *M. officinalis* Mill. collected from plants growing on polluted and non-polluted soils respectively, were surface-sterilized and germinated under *in vitro* conditions (Fernández et al., 2010). Each germinated seed grew into a seedling and explants of each seedling constituted a clone. The Pb accumulation ability of these clones was tested after *in vitro* culturing for 40 days with 200 mg Pb kg^{-1}, and the best accumulator clone from each species was selected: MA-X for *M. alba* and MO-A for *M. officinalis* (Fernández et al., 2010).

The MA-X and MO-A clones were acclimated to greenhouse conditions by replacing the caps of the vessels with sterile ventilated ones and placing them in the culture chamber. Then the vessels were relocated to a mist tunnel at 25 °C and 80% humidity (progressively diminishing to 60%), and after one month the plants were transferred to 10 cm diameter pots filled with peat:vermiculite (1:1). One month later, these plants were separated into three groups and watered with distilled water or a solution of 200 mg Pb kg^{-1} or 1000 mg Pb kg^{-1}. Lead was added as $Pb(NO_3)_2$ together with EDTA in equimolar concentration.

After 90 days measurements were made of shoot and root length, number of leaves, and fresh and dry weights of shoots and roots.

2.2. Lead accumulation of clones MA-X and MO-A cultured on Pb-polluted substrate

At 48 h, 40 and 90 days, samples of shoots and roots were collected for further analysis by inductively coupled plasma–mass spectrometry (ICP–MS). Shoots and roots were separated, washed with water, soaked for 10 min in $CaCl_2$ (5 mM), rinsed twice with doubly de-ionized water (Milli-Q 185 Plus System, 18 MΩ cm^{-1}) for 5 min and oven-dried at 70 °C for 48 h. After powdering, 100 mg of plant material was digested with HNO_3 in a microwave oven as described previously (Fernández et al., 2008) in order to liquidize the samples completely (Montaser, 1998). After cooling, two consecutive dilutions with doubly de-ionized water were performed, first a 1:20 dilution and then a 1:10 dilution in which a solution of 10 μg Rh kg^{-1} concentrated HNO_3 was added as an internal standard. Control plants were used as reference samples. Blanks, in which the same procedure was followed but without adding any plant material, were also assayed. The samples were finally analysed for Pb by ICP–MS using a HP-7500c instrument connected to a personal computer.

Shoot-to-root translocation (*S*/*R*) was calculated (Baker, 1981):

$$\frac{S}{R} = \frac{\text{mg Pb kg}^{-1}\text{ dry wt. in shoots}}{\text{mg Pb kg}^{-1}\text{ dry wt. in roots}}$$

2.3. Analysis of non-protein thiolic peptides in clones of MA-X and MO-A

At 48 h, 40 and 90 days samples of shoots and roots were collected and washed as in Section 2.2. Shoots and leaves were analysed together and separated from roots. In all cases, tissues were powdered with N_2 and stored at −80 °C until extraction.

The extraction procedure was carried out in a cold chamber (4 °C) following the method described by Rauser (1991) with slight modifications. Five hundred milligram of powdered fresh material was mixed with 1% polyvinylpolypyrrolidone and 0.1 N HCl at a ratio 1:1.5 (w/v). The mixture was shaken for 30 s, sonicated for 5 min and, after shaking again for another 30 s it was centrifuged at 15,000 × *g* and 4 °C for 15 min. The supernatant was collected with a syringe and filtered through a Millex-HV (0.45 μm diameter) filter. Finally, it was distributed in 150 μL aliquots in vials and immediately injected into a high-performance liquid chromatograph (HPLC). Alternatively the vials were stored for a maximum of 1 h at −20 °C until analysis. HPLC was carried out using a Waters 600 chromatograph and the derivatized thiols were detected using a Waters 996 Photodiode-Array Detector according to Ortega-Villasante et al. (2005). The sample (100 μL) was injected into a Kromasil 100 C18 5 μm (250 mm × 4.6 mm) (Scharlau) column and eluted with solvent A (acetonitrile:H_2O, 2:98 (v/v) to which 0.05% trifluoroacetic acid (TFA) was added) and solvent B (acetonitrile:H_2O, 98:2 (v/v) also with 0.05% TFA). Samples were separated using a linear gradient (0–25% in 25 min and 25–50% in 5 min) of solvent B at 1.5 mL min^{-1} flow for 30 min. A post-column derivatization was carried out in a reaction loop (1 mL) with Ellman's reagent at 0.5 mL min^{-1} flow (Ellman, 1959), and the absorbance was measured at 412 nm. For identification of the resulting peaks, standards of hPC_2, hPC_3 and hPC_4 were obtained from Anaspec Inc. (Anaspec Inc., San Jose, CA, USA) and a mixture of PCs (PC_2, PC_3 and PC_4) was kindly donated by Prof. Meinhart H. Zenk (Friederich et al., 1998). The quantitative changes were analysed using the integration areas at 412 nm of absorbance converted into nmol and quantified in terms of GSH equivalents.

2.4. Statistical analysis

Data were analysed using *SPSS18* and tested for homogeneity of variance and for normal distribution. A one-way ANOVA was used for growth data and metal uptake experiments complemented by the least significant difference (LSD). The HPLC results of the thiolic compounds are shown as the mean ± standard error of three replicates each. Differences between 0 and 200 mg Pb kg^{-1} were analysed using the Student's *t*-test, and differences among the 0, 200 and 1000 mg Pb kg^{-1} treatments were analysed *via* a one-way ANOVA complemented by the LSD. Correlations between concentrations of thiolic compounds and concentrations of Pb in shoots and roots were determined by linear regression analysis. The level of significance was 0.05 in all cases.

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