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Title: Kinetics of mercury uptake by oilseed rape and white lupin. Influence of Mn and Cu.

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Abstract

Mercury influx in oilseed rape and white lupin was studied using short time influx experiments. The effect of Cu and Mn in Hg influx was also tested. Plants were grown for two weeks and then roots were incubated with increasing Hg concentrations (0 - 50 μM $HgCl_2$), both at 20ºC and ice-cold temperature. An active, saturable component in Hg uptake was found in oilseed rape and white lupin, with K_m and V_{max} values in the range of low affinity transporters for essential micronutrients. A reduction in Hg uptake was observed in the presence of Mn for oilseed rape, suggesting that Hg influx is mediated by a Mn transporter. No effects of Cu on Hg influx were observed for any of the two plant species, suggesting a different transport system for Hg and Cu in roots of oilseed rape and white lupin.

Keywords

Copper; Kinetic parameters; Manganese; Mercury; Oilseed rape (*Brassica napus* L.) White lupin (*Lupinus albus* L.)

Introduction

Elevated concentrations of both essential and nonessential heavy metals in the soil pose a serious environmental problem. Inorganic Hg is one of the environmental pollutants recognised as a highly toxic compound, which is derived from both natural sources and human activities like mining of gold, silver and Hg itself, Cu and Zn mining and smelting or coal burning (Kabata-Pendias and Pendias 2000). Plants are able to take up Hg from the soil or nutrient solution but, generally, absorbed Hg is slightly translocated to shoots because of the low mobility of this heavy metal in plants (Suszcynsky and Shann 1995).

Mercury accumulation has been studied in several plant species, like pea and spearmint (Beauford et al. 1977), spring wheat, sugar beet, oil-seed rape, white clover and willow (Greger et al. 2005), rice (Du et al. 2005), tomato (Cho and Park 2000), alfalfa (Ortega-Villasante et al. 2005), lentil, chickpea (Rodríguez et al. 2007) and common vetch (Sierra et al. 2008). In plants, the average Hg concentration ranges from 0.005 to 0.2 mg kg^{-1}, but it can be much higher in those grown on Hg-contaminated soils (Higueras et al. 2003). The toxicity of Hg results from its effects on the permeability of cell membranes, reactivity with –SH groups of proteins and capability to bind to ATP and thus influence its activity (Patra and Sharma 2000).

The determination of the kinetics of metal uptake is a necessary preliminary step for modelling soil-plant transfer in nutrient-poor contaminated environments (Nowack et al., 2006). Nevertheless, kinetic parameters of Hg^{2+} uptake in higher plants have scarcely been reported. Esteban et al. (2008) reported the contribution of an active uptake mechanism in white lupin, but reaching external Hg concentrations in the influx experiments until 1000 μM. Most of those concentrations are too high to be found as available Hg in contaminated environments, so the

validity of this mechanism of Hg uptake in more realistic conditions should be tested. Oilseed rape is a promising crop due to its potential as an energetic crop. To the best of our knowledge, no reports have been found in the literature about Hg uptake by oilseed rape, but it could be considered in reclamation of low contaminated soils, as an economic alternative to the use of those soils.

Previous studies with the cyanobacterium *Nostoc calcicola* Bréb. (Pandey and Singh 1993) have shown the antagonism of Cu^{2+} in the absorption of Hg^{2+}. This suggested that Hg uptake was taking place through essential nutrient membrane transporters, as is also probably the case in plant roots. According to their chemical properties, two divalent micronutrients (Cu^{2+} and Mn^{2+}) were selected to test their influence on Hg uptake. So, the objectives of this work were to evaluate the kinetic parameters of Hg uptake in oilseed rape and white lupin and to test the effect of Mn and Cu supply on Hg uptake in those plant species.

Materials and methods

Plant growth

Seeds of white lupin (*Lupinus albus* L.) cv. Marta and oilseed rape (*Brassica napus* L.) cv. Es Hidromel were surface-sterilised in 10% v/v sodium hypochlorite for 15 min, rinsed thoroughly with deionised water and germinated in darkness at $28^{o}C$ for 3 days on water-moistened filter paper. Seedlings were grown for two weeks in 3 L containers with continuously aerated nutrient solution (Esteban et al. 2008), under controlled environmental conditions (night/day T 20/25°C, photoperiod 11/13 h, relative humidity of 60/40 %, photon flux density of 520 µmol m^{-2} s^{-1}).

Influx experiments

Experimental design used for the influx experiments was similar to the one described by Esteban et al. (2008). Three replicate samples of whole roots (excised at the node) were used. Roots were incubated in aerated solution containing 0.5 mM $CaCl_2$, 2 mM MES (2-[N-Morpholino]ethanesulphonic acid) and 0, 1, 2, 5, 10, 20 and 50 µM Hg ($HgCl_2$) at pH 6.0 for 20 min both at 20 ºC and ice-cold (< 4 ºC) conditions. Afterwards, roots were rinsed in a fresh ice-cold solution containing 5 mM $CaCl_2$ and 5 mM MES at pH 6.0 for 2 min and incubated in a fresh ice-cold nutrient solution of the same composition during 10 min for desorbing out no-uptaken Hg. Roots were blotted dry and their fresh weight (FW) was determined before analysis.

To study the interaction of Hg with other metals (Cu and Mn), uptake solutions were prepared as described above, but containing 0, 2, 5, 10 and 50 µM of Mn and Cu respectively and a fixed Hg concentration of 50 µM. Influx experiments were run out as described above.

Mercury, Mn and Cu analysis

Whole fresh roots were digested with a mixture containing $HNO_3:H_2O_2:H_2O$ (3:2:10, v:v:v) for 30 min at 125 ºC under 1.5 kPa of pressure (Esteban et al. 2008). Hg, Cu and Mn concentrations were determined by ICP-MS (Elan 6000, Perkin–Elmer Saex).

Calculations and statistical analyses

Free ion activity (Hg^{2+}, Cu^{2+}, Mn^{2+}) have been calculated for each uptake solution using Visual MINTEQ v. 3.0 (Gustafsson, 2012). Michaelis-Menten functions were fitted to the activity-dependent influx data using SigmaPlot 10.0 software to calculate the kinetic parameters. Values in text and figures are means ± standard errors (SE) of three independent replicates.

Results and discussion

Hg^{2+} uptake

Activity dependent uptake kinetics for Hg^{2+} influx was significantly affected by temperature. In white lupin at 20ºC, Hg^{2+} influx was graphically resolved into a saturable (hyperbolic) component (Fig. 1a) when considering data until 20 µM Hg (free ion activity: 1.7 10^{-13} M), in order to calculate kinetic parameters in the range of a minimal contribution of a low affinity uptake system. Only a non-saturable linear component was best fitted for the ice-cold Hg influx. Values obtained for both adjustments are shown in Table 1. A significant reduction of Hg influx was observed in the ice-cold experiments, where metabolic energy-dependent uptake processes should be minimal. So, the ice-cold influx could be attributed to apoplastic adsorption of Hg that remained bound to the cell walls after desorption, while Hg influx at 20ºC should include an active component, suggesting the existence of a root transporter that can be used for Hg uptake into root cells. For divalent cations, such as Hg^{2+}, it is very difficult to completely remove metals adsorbed by the cell walls without causing significant efflux of the ions from the symplasm (Reid et al. 1996; Cohen et al. 1998). Costa and Morel (1993) showed that around 25% of the [109]Cd uptake by the roots of *Lupinus albus* L. was due to an apoplastically bound fraction, even after a 2 h desorption. Our results have been obtained after a 15 min desorption time in 5mM $CaCl_2$, to ensure desorption of most of the Hg^{2+} bound in the

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apoplast. Similar desorption times and Ca concentrations have been used for Zn^{2+} and Cd^{2+} (Lombi et al. 2001; Zhao et al. 2002). This desorption procedure was developed by Lasat et al. (1996) for Zn^{2+} and was reported to remove 80% of the Zn adsorbed on the cell walls of *Thlaspi caerulescens* roots.

When subtracting influx data obtained for 20ºC and ice-cold experiments, the contribution of the saturable component will only remain. These data were best fitted to a hyperbola with K_m and V_{max} values for the saturable component of $1.07 \ 10^{-13} \pm 5.93 \ 10^{-14}$ M and 418.7 ± 118.4 nmol Hg g^{-1} FW h^{-1} ($r^2 = 0.943$). Similar experimental approaches were previously used in influx experiments for Fe (Allnutt and Bonner 1987), Zn and Cd (Zhao et al. 2002) and Cu (Bravin et al. 2010). Our results are in agreement with those of Esteban et al. (2008), as an active component in Hg uptake is also shown at the lower external Hg concentration used in this experiment, but significantly lower K_m and V_{max} values were obtained. Despite that, influx data corresponding to the same external concentrations are very similar, suggesting that although those authors were unable to obtain a better fit for their data, probably two uptake mechanisms could be taking place, and the present study complete the information at lower external Hg concentrations, as well as provide data expressed in activity units.

In contrast to white lupin, oilseed rape showed a hyperbolic fit for both temperatures (Fig. 1b). This fact has also been observed for Cd (Cataldo et al. 1983; Zhao et al. 2002), Zn (Bowen 1987; Hacisalihoglu et al. 2001) or Cu (Bravin et al. 2010) in other plant species. A significant reduction of Hg influx was also observed in the ice-cold experiments, where metabolic energy-dependent uptake processes should be minimal. When subtracting influx data obtained for 20ºC and ice-cold experiments, the contribution of the saturable component will only remain. These data were best fitted to a hyperbola, with K_m and V_{max} values for the saturable component of $1.32 \ 10^{-13} \pm 2.32 \ 10^{-14}$ M and 979.3 ± 71.96 nmol Hg g^{-1} FW h^{-1} ($r^2 = 0.989$). No previous reports are available, to the best of our knowledge, of kinetics of Hg uptake in oilseed rape plants.

So, an active component on Hg influx was found for both plant species. Oilseed rape has a higher Hg absorption capacity than lupine (higher V_{max}) but a similar affinity of the transporter for Hg^{2+}. Both have similar V_{max} values to those of a low affinity Mn transporter in barley (Pedas et al. 2005), although K_m values are around 9 times lower. Both K_m and V_{max} values are in the same range, although slightly higher, than those corresponding to a low affinity Zn transporter described for wheat (Hacisalihoglu et al. 2001).

Hg^{2+} uptake as a function of external Cu^{2+} and Mn^{2+} concentrations

Competitive interactions at the uptake level have been reported in several plant species between toxic and essential ions: arsenate/phosphate in velvetgrass (Meharg et al. 1994) and

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white lupin (Esteban et al. 2003), Ca, Mg and Mn (Maas et al. 1969) or Zn and Cu (Bowen, 1987). As both Mn^{2+} and Cu^{2+} have similar chemical properties to Hg^{2+}, those elements were tested in Hg influx experiments. Mn influx in roots of white lupine (Fig. 2a) increased with increasing doses of Mn in the uptake solution until 10 μM (free Mn^{2+} activity $8.4 \cdot 10^{-6}$ M), although it shows a decrease for the highest concentration (50 μM, free Mn^{2+} activity $4.2 \cdot 10^{-5}$ M). This decrease was only observed at 20ºC, but not at ice-cold temperature (data not shown). No clear influence of Mn on Hg uptake by roots was observed (Fig. 2a), so these results suggest that Mn and Hg do not compete for the same transport system in roots. In contrast, a decrease in Hg uptake with increasing Mn concentrations was observed in oilseed rape roots (Fig.2b). No increase was observed in Mn influx (Fig 2b), indicating that Mn could compete at the uptake level with Hg in this plant.

White lupine showed an increase in the rate of Cu influx with Cu concentration, but no effect of Cu on Hg influx (Fig 2c) was observed. Oilseed rape showed a similar effect (Fig 2d). According to these results, Cu and Hg do not compete at the uptake level in any of the plant species studied.

The first identified plant member of the ZIP family of metal transporters, AtIRT1 from *Arabidopsis thaliana* is able to transport a wide range of trace elements, including Fe^{2+}, Mn^{2+}, Zn^{2+} and a competition effect in those elements uptake is observed also for Cd^{2+} and Co^{2+}, suggesting that those two elements could also be transported by IRT1 (Korshunova et al. 1999). Several members of the ZIP family from other plant species have also been reported to transport Mn (Pedas et al. 2008), so Hg^{2+} transport in oilseed rape could potentially be carried out by a Mn transporter belonging to this family, as a competition effect is observed between both elements in the influx experiments. The lack of a similar effect in white lupin could be related to the high Mn content usually found in this legume, having a protective role against Cd^{2+} toxicity (Zornoza et al. 2010). White lupin has previously shown some differences with other plants in down-regulation of arsenate by phosphate (Esteban et al. 2003) attributed to the special characteristics at the uptake level for P and Fe adquisition of this plant species. As the expression of IRT1 is regulated by Fe availability (Korshunova et al. 1999), a similar effect to that on phosphate could be taking place.

According to Burkhead et al. (2009), Cu uptake seems to be carried out by a member of the COPT-family transporters and/or by ZIP2, a member of the ZIP family transporters. Our results agree with this report, as if Hg influx is mediated by a different family of transporters, no effect of external Cu levels should be observed on Hg uptake in any of the two plant species studied.

Acknowledgements

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Figure captions

Fig 1 Mercury influx for increasing mercury activities in excised roots of white lupin (a) and oilseed rape (b) at 20 ºC (closed symbols) and ice-cold temperature (open symbols). Results are mean ±SE of three replications. Only circles where used for obtaining kinetic parameters.

Fig 2 Effect of increasing Mn (a,b) and Cu (c,d) activities in Hg, Mn and Cu influx in excised roots of white lupin (a, c) and oilseed rape (b,d). Results are mean ±SE of three replications.
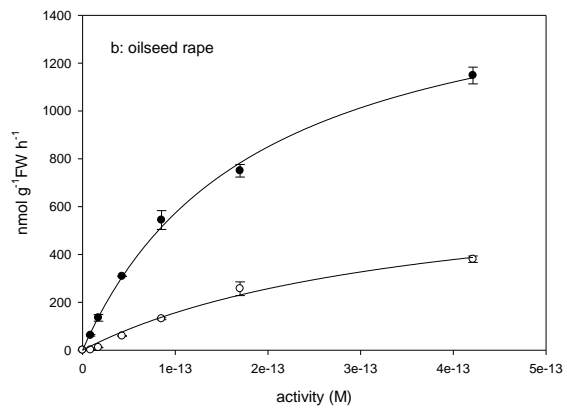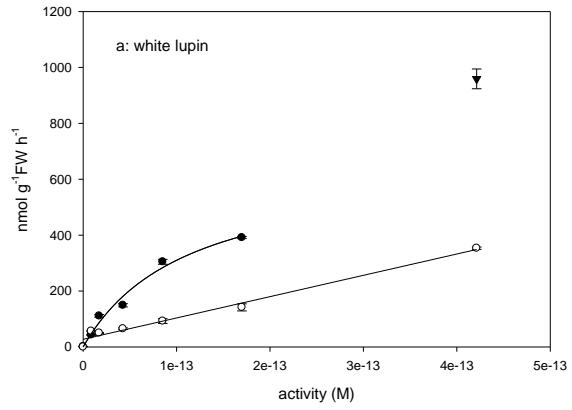
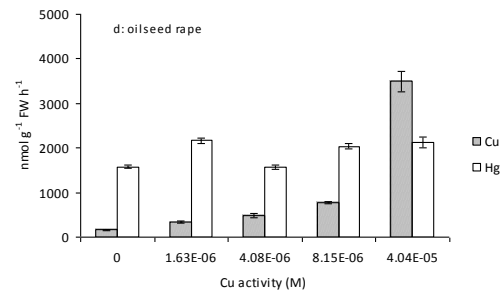Figure 1
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Figure 2
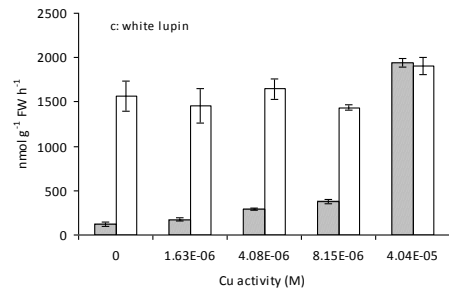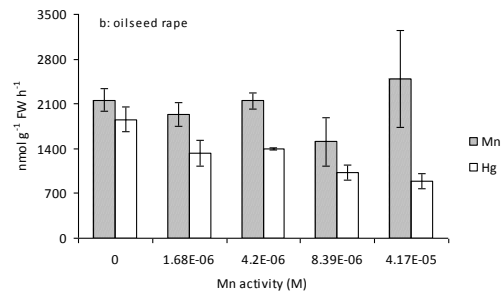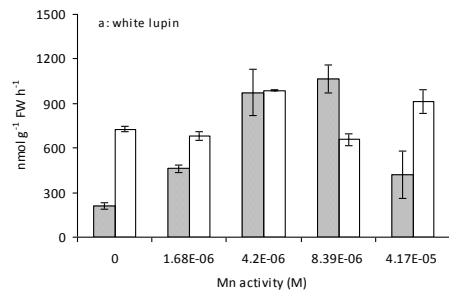
Table 1. Kinetic parameters (K_m in M, V_{max} in nmol g^{-1} FW h^{-1}) for Hg uptake in white lupin and oilseed rape at 20ºC and ice-cold conditions.

	White lupin		Oilseed rape	
T	20º C	ice-cold	20º C	ice-cold
K_m	$1.12\ 10^{-13} \pm 3.59\ 10^{-14}$		$1.87\ 10^{-13} \pm 1.56\ 10^{-14}$	$3.61\ 10^{-13} \pm 1.04\ 10^{-13}$
$V_{máx}$	658.5 ± 109.3		1644.8 ± 63.8	720.6 ± 118.3
R^2	0.982		0.998	0.987
P	<0.0001		<0.0001	<0.0001
a		27.42 ± 8.64		
b		$7.63\ 10^{14} \pm 4.92\ 10^{13}$		
R^2		0.979		
P		<0.0001		