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Evidence of a new Hg-tolerant ecotype of *Rumex induratus* from Almadén (Ciudad Real, Spain)

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Abstract

Mercury tolerance in wild vascular plants has hardly been studied and a tolerant ecotype is not known. In order to confirm the tolerance to Hg of *Rumex induratus* naturally growing in the biggest Hg mine in the world (Almadén population), the population was compared in a hydroponic experiment with another population from a non Hg-contaminated area (Colmenar). The plants were exposed to different doses of Hg and a dose of As to establish whether the tolerance to Hg coincides with tolerance to other trace elements. Plants from Colmenar reached up to 1322 μg Hg g^{-1} in roots and 65 μg Hg g^{-1} in shoots and showed a significant decrease of biomass due to Hg exposure, while Almadén accumulated only 812 μg Hg g^{-1} and 56 μg Hg g^{-1} respectively. The Almadén population showed a higher tolerance to intense exposure to Hg, but not to As. Plants from Almadén exposed to Hg showed higher capacity to synthesize thiols in the root and to control oxidative stress and Zn starvation. Our findings suggest that *Rumex induratus* could be used to enhance understanding of the mechanisms of Hg tolerance in plants.

Keywords: mercury phytostabilisation, tolerance, oxidative stress, As uptake, Zn uptake

1. Introduction

Mercury concentration in soils has increased due to human activities, some places therefore show levels far beyond background level of <0.15 mg Hg kg^{-1} (Patra and Sharma 2000). Almadén (Ciudad Real, Spain) has the largest Hg mine in the world, where levels of up to 10,000 mg Hg kg^{-1} have been reported (Millán et al. 2006; Millán et al. 2011). Similarly, common levels of Hg in plants range from 0.001 to 0.1 μg g^{-1} (Veiga et al. 1994), but levels greater than 10 μg g^{-1} have been detected in plants in Almadén (Millán et al. 2006). In such a Hg-contaminated site, plants have to compensate for the high levels of Hg by using physiological mechanisms similar to the response to other trace elements (Patra et al. 2004). In the case of other metal(loid)s, the studies of populations with different tolerance have helped to understand the physiological mechanisms implicated in metal tolerance: populations of *Thlaspi caerulescens* for Cd and Zn (Lombi et al. 2002), *Arabidopsis* spp. for Cu (Murphy and Taiz 1995), *Alyssum* spp. for Ni (Broadhurst et al. 2004, Marmiroli et al. 2004), *Silene* spp. for Zn, Cd, Cu and As (Verkleij et al. 2001; Arnetoli et al. 2008) and *Holcus lanatus* for Cu and As (Hartley-Whitaker et al. 2001). However, there is no report about different ecotypes from a plant species with different tolerance to Hg. Recently, the only papers reporting plant cultivars with Hg tolerance have used rice or Indian mustard (Yu et al. 2011; Shiyab et al. 2009), but these cultivars were not growing naturally in Hg-enriched environments nor do we know about any potential of these particular plants for Hg soil phytoremediation. The identification of tolerant ecotypes among metallophyte populations and the understanding of physiological mechanisms of tolerance is a key research question in order to be able to implement phytoremediation of Hg-polluted soils (Verbruggen et al. 2009). Toxic effects of Hg in plants have been studied less

intensively than other elements (e.g. As, Ni, Cd), but some concepts are already well established: Hg at high doses induces oxidative stress; depletes chlorophylls; alters water fluxes; mineral nutrition; and plant development (Moreno-Jiménez et al. 2007, 2009).

Previously, a population of *Rumex induratus* from Almadén (Almadén ecotype) was identified as a potential phytoremediator of Hg-degraded soils where thiol and malondialdehyde (MDA) concentrations were related to Hg toxicity (Moreno-Jiménez et al. 2007). Therefore, the main objectives of this study, under controlled laboratory conditions, arefirstly to determine the tolerance of *Rumex induratus* to Hg after the plant´s long-term exposure in Almadén and secondly, to investigate its potential adaptation to high Hg concentrations, comparing its properties with that of a population which has no record of Hg exposure. Additionally, As phytotoxicity (Moreno-Jiménez et al. 2012) and the potential induced co-tolerance to As was also tested.

2. Materials and methods

2.1. Seed collection

Two populations of *Rumex induratus* Boiss & Reuter (Polygonaceae) were selected from areas with different historical Hg exposure. The first population was collected from a mine tailing in the Almadén district (38º43'37"N, 4º40'36"W, Ciudad Real, SW Spain), where Hg concentrations of up to 1000 mg kg^{-1} were reported (Moreno-Jiménez et al. 2006). The other population was obtained from an agricultural plot in Colmenar Viejo (40º42'35"N, 3º46'04"W, Madrid, C Spain) with no previous Hg contamination reported. Mature seeds from both populations were collected and stored at 4ºC with silica gel until germination.

2.2. Plant culture

Plant seeds were germinated in perlite moistened with distilled water and 1.5 mM $CaSO_4$ in darkness for 4 days at 28 ºC. When the seeds were germinated, plants were maintained at 28 ºC (16 h light, 8 h darkness) in distilled water for the first week and then control nutrient solution for the next three weeks to the substrate. The nutrient solution had the following composition (pH ≈ 6.5): 1.5 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 0.75 mM K_2SO_4, 53.76 μM Fe-EDDHA, 27.3 μM $MnSO_4$, 0.32 μM $CuSO_4$, 0.77 μM $ZnSO_4$, 46.25 μM H_3BO_3, 0.016 μM $(NH_4)_6Mo_7O_{24}$.After four weeks of growth, three to four seedlings were transferred to each plant pot (1.5 L). For three weeks all the plants were grown with control nutrient solution. For the final four weeks Hg treatments wereadded as $HgCl_2$ in doses of 0, 5 and 50 μM Hg, whilst only one arsenic dose of 50 μM was supplied as NaH_2AsO_4. Doses of Hg and As were chosen according to the results from previous hydroponic experiments (Moreno-Jiménez et al. 2007 and Moreno-Jiménez et al. 2009). Each treatment was replicated four times. Nutrient solutions were replaced at 7-day intervals throughout the experiment. The experiment was carried out in a growth chamber under the following environmental conditions: night/day (8h/16h) T 20–25 ºC and relative humidity of 60–40%.

Plant exposure to Hg and As in treatments was assessed, showing a recovery higher than 70% with respect to the theoretical dose. Hg and As speciation in nutrient solutions was modeled using the software Visual MINTEQ version 2.53 and the predominant chemical species were predicted to be Hg^{2+} (more than 95% of mercury) and $H_2AsO_4^-$ (more than 75% of the total arsenic) based on the composition of the experimental solution.

2.3. Sample processing and analysis

At the end of the experiment, plants were harvested and divided into root, stem and leaves. All plant material was thoroughly washed with tap water followed by a subsequent rinse in distilled water for 2 min. Total fresh weight of each tissue was determined, then a representative 7 g aliquot was oven-dried to constant weight at 30ºC for 7 days prior to preparation for elemental analysis. The remaining sample was frozen in liquid N_2 and stored at –70 ºC for future use.

Dry plant material was acid digested using 4 mL of mili-Q water, 1.3 mL of HNO_3 and 0.9 mL of H_2O_2, added to 0.2 mg dry weight (DW) sample. Material was digested under pressure at 1.5 kPas and 125 ºC (Lozano-Rodríguez et al. 1995). Digests were filtered, then diluted to 15 mL with mili-Q water prior to As, Hg and Zn determination by ICP-MS (SIdI UAM, with ISO 9001). Certified reference material (CTA-VL2, tobacco leaves, 0.97 μg As g^{-1}; and BCR 62, olive leaves, 0.28 μg Hg g^{-1}) was used as a quality control of the method and found to contain 0.94 ± 0.06 μg As g^{-1} and 0.27 ± 0.01 μg Hg g^{-1}.

Acid soluble thiols were extracted and determined following Jocelyn (1987): 0.1 g fresh weight (FW) was extracted with 0.4 mL of NaOH (0.1 M) + $NaBH_4$ (25 mg mL^{-1}) and 0.2 mL of distilled water, then centrifuged at 11,000 g for 5 min. The resulting supernatant (0.5 mL) was diluted with 0.2 mL HCl (35%) and centrifuged at 11,000 g for 5 min. Then 0.5 mL of DTNB 300 μM in phosphate buffer 0.5 M (pH = 7.5) were added to 0.5 mL of supernatant and heated at 30 ºC for 2 min. Absorbance was determined at 412 nm. For correlation, GSH standards (0, 10, 20, 50, 100 and 150 nmol GSH) were used to fix the calibration curve.

Lipid peroxidation in plant tissues was based on an estimate of malondialdehyde (MDA) concentration, as described by Heath and Packer (1968). A 0.1g FW aliquot of tissue was extracted with 1 mL of colorimetric reactive TCA (15%) – TBA (0.37%) –

HCl (0.25 M), heated at 90 ºC for 30 min and then cooled. After centrifugation at 11,000 g for 10 min, the absorbance of supernatant was measured at 532 nm and 600 nm. The extinction coefficient was $1.56 \cdot 10^5$ M^{-1} cm^{-1}.

2.4. Statistical analysis and data processing

Data were processed with Microsoft Excel2007 and SPSS 15.0. Two-way ANOVA was used for mean comparison, with ecotype, Hg treatment and their interaction as factors. The influence of Hg on plant biomass or MDA concentration was expressed as the relative increase (in percentage) induced by each Hg dose with respect to the same parameter in control plants of each ecotype. To determine the relationship between thiols and Hg concentration in tissues, Hg concentration was transformed to a fresh basis using the percentage of water.

Results

Plant biomass, both in roots and in shoots, was reduced in both populations of *R. induratus* by the exposure to Hg in the nutrient solution (Figures 1a,c, Supplementary Material for statistics). Although both populations showed only a slight decrease of biomass caused by 5 μM Hg (<20% of reduction), in the case of 50 μM Hg results differed for each population: while the Almadén ecotype experienced a moderate reduction of growth (slightly higher than 20%), the Colmenar population showed a sharp growth inhibition, up to 72%.. The Hg concentrations in the shoots and roots of *R. induratus* were in accordance with the level of exposure (Figures 1b,d, Supplementary Material for statistics). Slight differences were detected between ecotypes, the most noticeable a higher Hg concentration in roots of the Colmenar population.

In contrast to Hg results, Table SM2 (Supplementary Material) shows that 50 μM As resulted in a higher growth reduction in the Almadén ecotype, whilst the Colmenar population did not suffer evident losses of biomass.

To test the damages induced by Hg in the two populations of *R. induratus*, MDA was used as an indicator of oxidative stress, and significant differences were observed (Supplementary Material). In both populations the concentration of MDA increased after a high exposure to Hg (50 μM), both in roots and in shoots (Figures 2a,c). This increase was only slight in the Almadén ecotype (about 9-14%), but was very noticeable in the Colmenar population (60-158%), suggesting that Hg caused significant damage to the tissues of the latter.

Thiols concentration was another parameter selected to study differences in the response to Hg. In both populations the roots of *R. induratus* showed increasing concentration of thiols at higher exposures to Hg, but leaves did not show any clear tendency (Figures 2b and 2d). For roots, the Almadén ecotype showed a higher correlation between concentration of Hg and of thiols (0.83) than the population of Colmenar (only 0.20), indicating that Almadén ecotype has a more effective response to Hg.

Zinc concentration significantly varied between populations and in response to the level of Hg exposure (Figure 3). For the control treatment, *R. induratus* from Colmenar had the highest Zn concentration, but it decreased sharply in both roots and shoots upon Hg exposure, decreasing Zn levels up to 72% in shoots and 61% in roots. The population from Almadén showed more constant plant Zn concentration after exposure to Hg in the nutrient solution.

Discussion

The Almadén population showed a higher tolerance to Hg, suggesting that the long-term exposure to Hg in the habitat has allowed the plant to develop protective physiological traits. Hg inhibits plant growth at high exposures (Cho and Park 2000; Moreno-Jiménez et al. 2007, 2009; Wang and Greger 2004), and the growth of the Colmenar population decreased by 70%, whilst the Almadén ecotype showed only a moderate inhibition (about 20%). Similarly, the Colmenar population also experienced higher oxidative stress. Oxidative stress is the result of the presence of Hg at high concentrations, when antioxidant systems are saturated causing damage at the cellular level (Ortega-Villasante et al. 2005; Sobrino-Plata et al. 2009). The Almadén ecotype showed a better antioxidant response than the Colmenar one. The concentration of –SH groups in cytosol plays an important role to maintain cell homeostasis because GSSH can alleviate oxidative stress (Rellán-Álvarez et al. 2006) and also thiol enriched biomolecules (GSSH, phytochelatins) can complex Hg and reduce its activity (Krupp et al. 2008; Carrasco-Gil et al. 2011). Roots of *R. induratus* responded to Hg by synthesizing –SH. The Almadén ecotype also had higher ability to synthesise thiols, indicating a better protective response. However, in leaves, this mechanism does not seem to be as effective as in roots, in agreement with previous findings (Moreno-Jiménez et al. 2007). This may suggest thiol groups play a role in Hg detoxification in roots, but as both populations show to an increase of thiols, this mechanism is not enough to explain the large differences in Hg-tolerance between the two ecotypes of *R. induratus*. This lack of response of thiols in shoots may be explained if the pool of –SH in the aboveground tissues is enough to maintain the cell homeostasis and alleviate toxicity despite the accumulation of Hg. Calgaroto et al. (2011) have suggested recently that Zn may alleviate Hg toxicity, probably protecting plant tissue against oxidative stress. In our study, the population of Colmenar showed a severe decrease of Zn levels

in shoot and root, even at lower levels of Hg. However, the population of Almadén showed only very slight depletions of Zn upon exposure to high levels of Hg, suggesting again higher resistance than the population of Colmenar. Our work reports for the first time an ecotype of *R. induratus* with different tolerance to Hg, but this fact is not associated to a higher resistance to As. So the detoxification mechanisms in the Almadén ecotype are mainly effective for Hg. This opens the possibility of further research to investigate the plant mechanisms associated to Hg-tolerance: root uptake, Hg compartimentalization (cell wall, vacuole), Hg complexation and speciation (PCs, methylmercury), genes involved (AFLP, QTLs), and maintenance of cell homeostasis (antioxidant enzymes). For instance, AFLP fingerprinting has been used to compare populations of *Onosma echioides* from different locations (Mengoni et al., 2006), the role of certain plant alleles in Hg-tolerance have been reported in some crops (Shiyab et al. 2009; Yu et al. 2011) and PCs have been shown to play a primary role in Hg-detoxification after short exposure (Carrasco-Gil et al. 2011), but these hypotheses should be validated in natural Hg-tolerant ecotypes.

Conclusion

The development of our knowledge for other trace elements (As, Ni, Cd, Zn) has been a step ahead, but as a result of our study we suggest to use similar models to explore Hg-tolerance mechanisms in plants. Historical exposure to Hg in Almadén mine district has promoted a tolerant ecotype of *R. induratus*, less sensitive to growth inhibition and oxidative stress upon Hg presence in the nutrient solution. The tolerant ecotype from Almadén demonstrated useful traits to make it a candidate for remediation of Hg-polluted soils.

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Figure Captions

Figure 1. Biomass (g FW) and mercury concentration (μg g^{-1} DW) in two populations of *Rumex induratus* (Almadén and Colmenar) exposed to different doses of Hg during 1 month. a) and b) shoot; c) and d) root. Numbers in the figures are the percentages of relative reduction of biomass with respect to control plants, mean ± SE (*n*=4). Statistical analyses are shown in Table SM1 (supplementary material).

Figure 2. Malondialdehyde (MDA) concentration (nmol MDA g^{-1} FW) and correlation between thiol concentration (nmol –SH g^{-1} FW) and Hg concentration (nmol Hg g^{-1} FW) in two populations of *R. induratus* (Almadén and Colmenar) exposed to increasing doses of Hg. a) and b) Shoot; c) and d) Root, mean + SE, *n*=4. The numbers in the figures on the left are percentage of relative increase of MDA with respect to the control plants. The right hand figures: ***$P < 0.001$; **$P < 0.01$; n.s. non significant. Further statistical analyses are shown in Table SM1 (supplementary material).

Figure 3. Zinc concentration (mg Zn g^{-1} DW) in two populations of *Rumex induratus* (Almadén and Colmenar) exposed to different doses of Hg during 1 month. a) Shoot and b) Root. The numbers on top of the columns are the percentage of depletion (-) or increase(+) of Zn in Hg treatments, with respect to the levels in control plants, mean ± SE (n=4).. Statistical analyses are shown in Table SM1 (supplementary material).

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Fig 1.

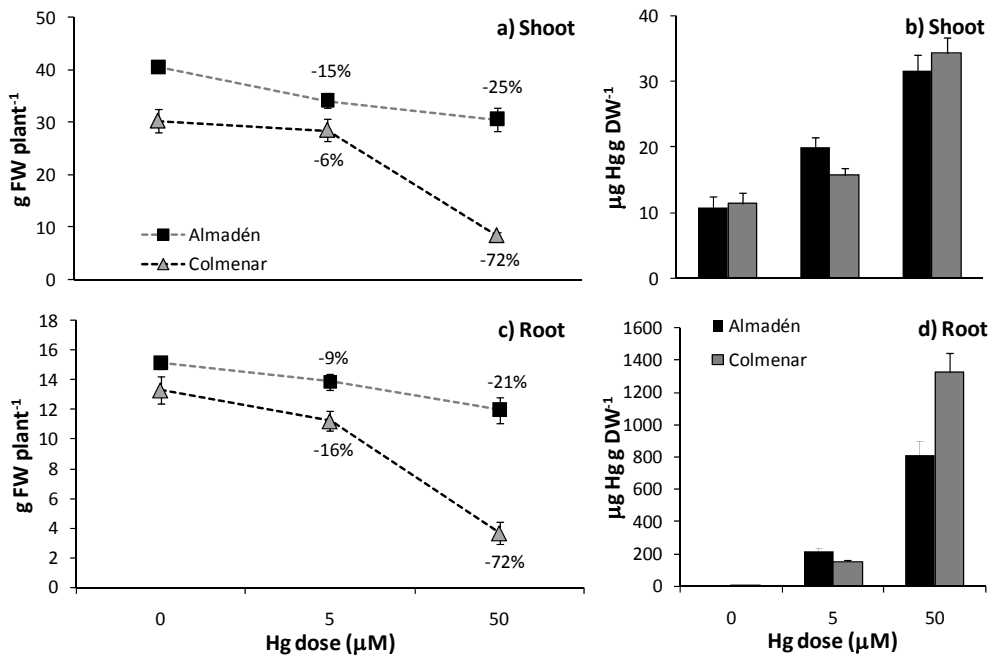

a) Shoot
g FW plant-1
-15%
-25%
-6%
-72%
Almadén
Colmenar
b) Shoot
µg Hg g DW-1
c) Root
g FW plant-1
-9%
-21%
-16%
-72%
d) Root
µg Hg g DW-1
Almadén
Colmenar
Hg dose (µM)
Hg dose (µM)

Fig 2.

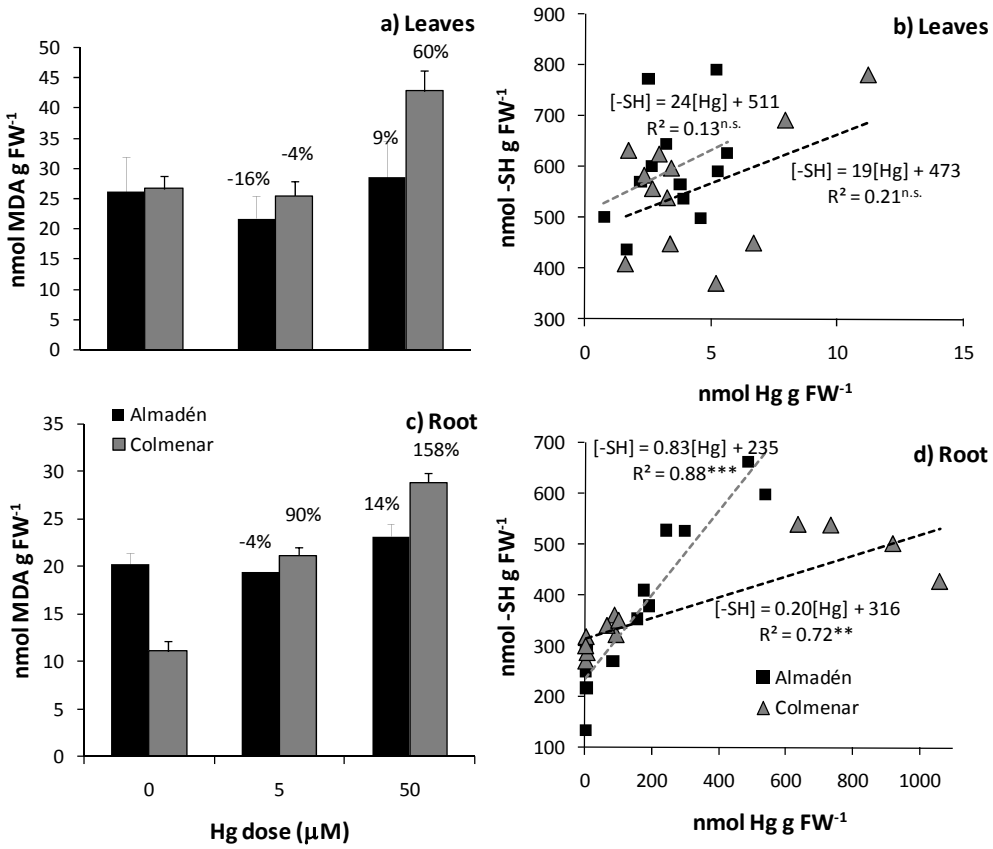
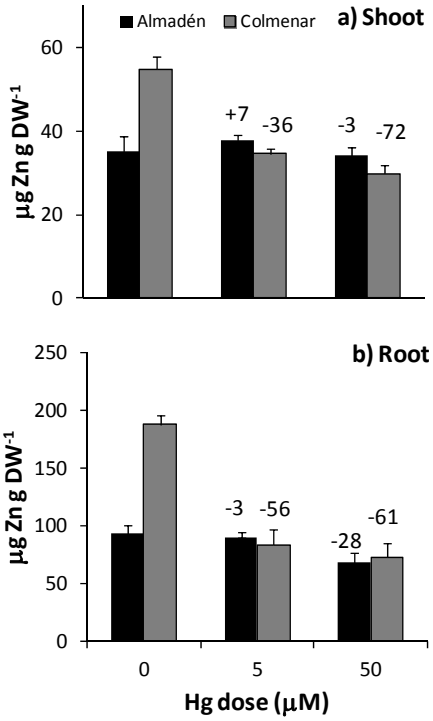

a) Leaves
nmol MDA g FW-1
-16%
-4%
9%
60%
b) Leaves
nmol -SH g FW-1
[-SH] = 24[Hg] + 511
R² = 0.13n.s.
[-SH] = 19[Hg] + 473
R² = 0.21n.s.
nmol Hg g FW-1
Almadén
Colmenar
c) Root
nmol MDA g FW-1
-4%
90%
14%
158%
Hg dose (µM)
d) Root
nmol -SH g FW-1
[-SH] = 0.83[Hg] + 235
R² = 0.88***
[-SH] = 0.20[Hg] + 316
R² = 0.72**
Almadén
Colmenar
nmol Hg g FW-1

Fig. 3


Almadén
Colmenar
a) Shoot
µg Zn g DW-1
60
40
20
0
+7
-36
-3
-72
b) Root
250
200
150
100
50
0
-3
-56
-28
-61
0
5
50
Hg dose (µM)