Attenuation of Mercury Phytotoxicity with a High Nutritional Level of Nitrate in Alfalfa Plants Grown Hydroponically

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Abstract

Background and aims Mercury (Hg) is one of the most dangerous pollutant heavy metals to the environment, which causes several toxic effects in plants upon accumulation, such as induction of oxidative stress. Nitrogen (N) is one of the most limiting macronutrient for plants, being mostly assimilated as NO_3^- which is reduced to NO_2^- by the enzyme nitrate reductase (NR), prior its reduction to NH_4^+ .

Methods We studied the physiological alterations caused by Hg (0, 6 and 30 μM) in alfalfa plants grown at two different levels of NO_3^- : low, (2 mM; LN), and high (12 mM; HN) for one week using a semi-hydroponic culture system.

Results Several parameters of oxidative stress such as lipid peroxidation, chlorophyll content, biotiol concentration and, ascorbate peroxidase (APX) and glutathione reductase (GR) activity showed that HN plants were less affected by Hg. Nitrate reductase activity and NO_3^- concentration was altered under Hg stress, with lower impact in plants nourished with high NO_3^- .

Conclusions Our results highlight the importance of the nitrogen nutritional status to improve tolerance to toxic metals like Hg.

Introduction

Mercury is one of the most hazardous pollutants released to the environment by diverse anthropogenic activities like mining, as occurs in the Almadén district (Ciudad Real, Spain), where weathered litter and metallurgy wastes are spread in soils and sediments (Esbrí et al. 2010). Cessation of the production of Hg has imposed the necessity to impulse other economic activities, such as agriculture and farming, which are undermined by the accumulation of Hg in local soils (Sierra et al. 2008). On other hand, the ability of plants to extract metals from the soil can be exploited to clean up polluted soils by using phytoremediation technologies. In this sense, intense research is conducted to understand tolerance mechanism in plants that might help to optimise such promising clean techniques (Clemens et al. 2002).

The divalent cation (Hg^{2+}) is predominant in well aerated agricultural soils, among other Hg chemical forms (Han et al. 2006). Exposure of plants to Hg^{2+} lead to numerous physiological disorders and injuries (Zhou et al. 2007), including drastic growth inhibition, alteration of photosynthesis, mitochondrial respiration, and obstruction of water flow through the inhibition of plasma membrane aquaporins (Patra and Sharma 2000; Cargnelutti et al. 2006; Israr and Sahi 2006; Shiyab et al. 2009). One of the earliest symptoms of Hg toxicity is the induction of oxidative stress, resulting in oxidation of membrane lipids and severe alteration of membrane integrity (Ortega-Villasante et al. 2005), causing cell poisoning and death (Ortega-Villasante et al. 2007). To counteract this cellular redox imbalance, plants possess antioxidant defences including enzymes like ascorbate peroxidase (APX) and glutathione reductase (GR), and redox metabolites like glutathione (GSH; $\gamma\text{Glu-Cys-Gly}$) (Sharma and Dietz 2009), which also operate

under Hg stress (Cho and Park 2000; Cargnelutti et al. 2006; Elbaz et al. 2010). However, even under relative low Hg concentrations (1 to 3 μM), the activity of these antioxidant enzymes usually drops (Ortega-Villasante et al., 2007; Zhou et al., 2007; Sobrino-Plata et al., 2009; Elbaz et al., 2010). In particular, GR is extremely sensitive to Hg accumulated in the roots of several plant species (Sobrino-Plata et al. 2009, 2013), and has been proposed as specific biomarker of Hg phytotoxicity. This was confirmed in *Arabidopsis* mutant plants with limited cellular concentration of GSH, which showed severe Hg toxic symptoms and a drastic GR activity inhibition (Sobrino-Plata et al. 2021). Therefore, tolerance to Hg highly depends on the levels of GSH and derived biothiols, including phytochelatins (PCs), a family of thiol-rich peptides with general $(\gamma\text{Glu-Cys})_n\text{-Gly(-Ala)}$ structure (where n varies between the range 2–5) (Serrano et al. 2015).

Nitrogen (N) is a very limiting macronutrient, normally assimilated as nitrate (NO_3^-). The assimilation of NO_3^- comprises its reduction in a two-step process, firstly it is reduced to NO_2^- by the cytosolic enzyme nitrate reductase (NR), and then to NH_4^+ thanks to the plastidial enzyme nitrite reductase (NiR) (Yoneyama and Suzuki 2019). NH_4^+ is then incorporated to organic acids to form amino acids, through a series of amination and transamination reactions in a complex assimilatory pathway where NR is the limiting step (Campbell 1999). Heavy metal polluted areas are commonly waste lands with low N availability, so nitrogen fertilizers must be applied to improve the biomass yield of plants cultivated for phytoremediation purposes in those areas (Wong 2003). On the other hand, the addition of organic matter and NPK inorganic fertilizers containing NO_3^- is a common agricultural practice (Barrutia et al. 2009). Therefore, it is important to study the relation between NO_3^- nutritional status and heavy metals toxicity. Toxic heavy metals may disturb N metabolic system, inhibiting NR activity as observed under Cd stress (Hernandez et al. 1996; Gouia et al. 2000; Chaffei et al. 2004). Indeed, pea plants treated with 50 μM Cd suffered a severe diminution in NO_3^- assimilation, affecting NO_3^- and K uptake and NO_3^- reduction by NR (Hernández et al. 1997). Similar results were obtained in bean and tomato plants exposed to Cd, where the assimilation of NO_3^- and NH_4^+ was compromised by metal stress (Gouia et al. 2000; Chaffei et al. 2004). On the other hand, recent studies tested the effects of N supply on Cd uptake in plants, showing the beneficial effect of N fertilization (Gao et al. 2010; Hussain et al. 2020). Under N starvation, there were stronger Cd phytotoxic symptoms in *Hordeum vulgare*, in parallel to the up-regulation of phytochelatin synthase (PCS) and NRAMP metal transporters gene expression, with modest changes in the expression of oxidative stress related genes (Finkemeier et al. 2003).

In consequence, N nutritional status seems to modulate toxic metals tolerance in plants (Hussain et al. 2020). It should be noted that the information available about the interaction between NO_3^- nutrition status and Hg phytotoxicity is almost null, and would be useful for designing improved phytoremediation procedures in Hg-polluted soils. Therefore, we aimed to characterise the responses of alfalfa plants with low and high NO_3^- nutritional status to Hg exposure in a semi-hydroponic system using perlite as inert substrate. These experiments will help to understand the effect of NO_3^- fertilization on Hg tolerance, by analysing Hg distribution in plant, oxidative stress and N assimilation parameters.

Materials And Methods

Plant material, growth conditions and treatments

Alfalfa (*Medicago sativa* cv. Aragon) seedlings were surface sterilized for 5 min in 5% (v/v) commercial bleach. After rinsing several times with sterile water, seeds were soaked overnight at 4°C and germinated on 1.5% (w/v) agar in square Petri dishes (10 x 10 mm), in complete darkness for 48 h at 28°C. Homogeneous selected seedlings were transferred to a semi-hydroponic system using a perlite inert substrate in plastic trays submerged in two modified Hoagland nutrient solutions (Ortega-Villasante et al. 2005): One of them with low NO_3^- concentration (LN; 2 mM) (macronutrients [mM]: 1.0 KH_2PO_4 , 0.5 MgSO_4 , 0.1 NaCl, 0.9 $\text{Ca}(\text{NO}_3)_2$, 0.63 KNO_3 , 0.47 $\text{Mg}(\text{NO}_3)_2$) and the other one with high NO_3^- concentration (HN; 12 mM) (macronutrients [mM]: 1.0 KH_2PO_4 , 0.5 MgSO_4 , 0.1 NaCl, 5.4 $\text{Ca}(\text{NO}_3)_2$, 3.8 KNO_3 , 2.8 $\text{Mg}(\text{NO}_3)_2$); both supplemented with micronutrients [μM]: 45.0 Fe (EDDHA), 18.0 MnSO_4 , 3.0 ZnSO_4 , 6.0 CuSO_4 , 23.5 H_3BO_3 , 2.0 $\text{Mo}_7\text{O}_{24}(\text{NH}_4)_6$). The plants grew for 12 days in a controlled environment chamber (16 h light (lamps of 120 W m^{-2})/8 h darkness) at 25/18°C respectively and relative humidity, 75%. 0, 6 and 30 μM Hg was then supplied as HgCl_2 , and plants were collected after 7 days. Samples were thoroughly washed in deionized H_2O , fresh weight measured and stored at -80°C until analysis.

Mercury analysis

Solid samples of roots and shoots were air dried and ground with mortar and pestle. Dried plant material (100 mg) was acid digested in 2 mL of the digestion mixture ($\text{HNO}_3:\text{H}_2\text{O}_2:\text{H}_2\text{O}$, 0.6:0.4:1 v:v) in an autoclave (Presoclave-75 Selecta, Barcelona, Spain) at 120°C and 1.5 atm for 30 min (Ortega-Villasante et al. 2007). Mercury concentration was measured by atomic absorption spectrophotometry using the Advanced Mercury Analyser 254 Leco (St. Joseph, Michigan, MI, USA) with a detection limit of $0.5 \mu\text{g kg}^{-1}$. Certified Reference Materials (CRM) were used to determine the accuracy of the measurements and validation.

Nitrogen in plants

The nitrogen (N) in plant tissue was determined by Kjeldahl digestion, performed in a wet digester system B-440 (Buchi, Switzerland). Dry plant material was ground to powder using a mortar and pestle, and 0.05 g was transferred to the digestion tubes together with 10 ml of 98% H_2SO_4 (v/v) and 10 g of K_2SO_4 . The solution was heated at 410 °C for 1.5 h, and cooled for 30 min. NH_4^+ was distilled after the addition of 25 ml of 32% NaOH (v/w) in a K-355 distillation unit (Buchi, Switzerland). The concentration of NH_3 released in the resulting alkaline mixture was calculated by back titration of 2% H_3BO_3 (v/v) buffer adjusted to pH 4.65 with 0.02 M HCl, following the specifications of a KF Titrino Plus 870 equipment (Metrohm, Switzerland).

Lipid peroxidation and chlorophyll analysis

Lipid peroxidation was estimated by measuring the concentration of the by-product malondialdehyde, which reacts with thiobarbituric acid. The resulting chromophore absorbs at 535 nm, and the concentration was calculated directly from the extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Rellán-Álvarez et al. 2006). Ground frozen tissue (0.1 g) was transferred to a screw-capped 1.5 ml Eppendorf tube, and homogenized in 1 ml of TCA–TBA–HCl reagent (15% (w/v) trichloroacetic acid (TCA), 0.37% (w/v) 2-thiobarbituric acid (TBA), 0.25 M HCl, and 0.01% buthylated hydroxytoluene). Samples were incubated at 90 °C for 30 min, chilled in ice, and centrifuged at 12000 g for 10 min. Absorbance was measured in a UV-2401 PC spectrophotometer (Shimadzu, Tokyo, Japan). For chlorophyll (Chl) determination, 0.05 g of frozen leaves were homogenized with 10 ml 80% (v/v) acetone using a mortar and pestle. Homogenates were filtered through a paper filter and absorbance was measured in a UV-2401 PC spectrophotometer (Shimadzu) at 645 and 663 nm. Total chlorophyll concentration was calculated according to the procedure of Porra (2002).

Analysis of biothiols

0.1 g of frozen tissue was ground with 300 µl of 0.25 N HCl, the homogenate centrifuged for 15 min at 12000 g and 4°C, and the clear supernatant was transferred to a boron-silica glass injection vial. 100 µl were injected in a Mediterranea Sea18 column (5 µm, 250 x 4.6 mm; Teknokroma, Spain), and biothiols were eluted using an Agilent 1200 HPLC system (Santa Clara, CA, USA) with the elution program and detection procedure with Ellman's reagent (5,5-dithio-bis (2 nitrobenzoic acid) at 412 nm absorbance as described by Ortega-Villasante et al. (2005). Biothiol concentration was calculated relative to the acetyl cysteine (N-AcCys) internal standard (25 nmol). Several biothiol standards were prepared in analytical-grade type I water (Milli-Q Synthesis, Millipore), diluted to 0.1 mM and injected alone or as internal standards for retention time peak identification, of the following compounds: GSH, homogluthathione (hGSH), cysteine (Cys), homophytochelatin-2 (γ -(Glu-Cys)₂-Ala; hPC₂), and homophytochelatin-3 (γ -(Glu-Cys)₃-Ala; hPC₃).

Determination of nitrate reductase activity *in vitro*

The *in vitro* nitrate reductase (NR) activity was analysed following the procedure described by Ramón et al. (1989). Intact frozen tissue (0.5 g) was homogenised in 1 ml or 0.5 ml enzymatic Extraction Solution respectively for shoot and root, freshly prepared by mixing 10 ml extraction buffer (30 mM 3-(N-morpholino) propanesulfonic acid (MOPS) at pH 7.5, 5 mM Na₂-EDTA, 10 mM DTT, 10 mM ascorbic acid, 0.6% PVP, 10 µl 100 mM PMSF) and 1 ml protease inhibitors cocktail Sigma-Aldrich (P2714; St. Louis, MO, USA). After centrifugation (14000 x g) for 15 min at 4°C, the supernatant was kept in ice until NR activity assay, measured by adding 0.1 ml of enzymatic extract, 0.1 ml 100 mM KNO₃, 0.5 ml reaction buffer (100 mM KH₂PO₄/K₂HPO₄, 1 mM EDTA, pH 7.5) and 0.1 ml of 1 mg ml⁻¹ NADH. Formed NO₂⁻ was analysed after 15 min incubation at 28°C, and the reaction was stopped by the addition of 2 ml of freshly prepared colorimetric reagent (1% (v/v) sulphanilamide in 3 M HCl, and 0.02% (w/v) N-(1-naphthyl) ethylenediamide dihydrochloride mixed in a 1:1 ratio). Samples were centrifuged at 10000 g for 15 min, and absorbance read at 540 nm using a UV-2401 PC spectrophotometer (Shimadzu).

Glutathione reductase and ascorbate peroxidase

Glutathione reductase (GR) and ascorbate peroxidase (APX) activities were determined *in gel* after separation of protein extracts by non-denaturing electrophoresis in 10% polyacrylamide gels. Extracts were prepared from 0.5 g of intact frozen samples in 1 ml enzymatic Extraction Solution (see above). After centrifugation (14000 g) for 15 min at 4°C, the supernatant was stored as single use 100–200 µl aliquots at 80°C. Protein concentration in the extracts was preliminarily determined with the BioRad Protein Assay reagent (BioRad, Hercules, CA, USA), and the final loading for activity staining was adjusted after denaturing gel electrophoresis and Coomassie-blue staining (Laemmli 1970). Protein loading for GR and APX analysis was 15 µg and 5 µg of shoot and root extracts, respectively. Gel slabs were incubated in GR staining solution (250 mM Tris-HCl buffer at pH 7.5, supplemented with 0.2 mg ml⁻¹ thiazolyl blue tetrazolium bromide, 0.2 mg ml⁻¹ 2,6-dichlorophenol indophenol, 0.5 mM NADPH and 3.5 mM oxidised glutathione (Sobrinho-Plata et al. 2009). APX was detected as described by Jiménez et al. (1998). Gel slabs were incubated for 20 min with 2 mM ascorbate and 2 mM H₂O₂ in 50 mM Na-phosphate buffer at pH 7.0, and APX were revealed after incubation with 0.5 mM nitroblue tetrazolium (NBT) and 10 mM TEMED in 50 mM phosphate buffered at pH 7.8.

Protein immunodetection

Proteins of interest were immunodetected after denaturing gel electrophoresis and Western-blotting (Laemmli 1970), using α-cytosolic APX (AS06180), α-GR (AS06181), α-NR (AS08310), α-NTR1.1 (AS122611), and α-NTR2.1 (AS122612) (Agrisera, Vännäs, Sweden). After electrophoresis (20 µg total protein), proteins were blotted onto a nitrocellulose membrane (BioTraceNT Pall Corporation, East Hills, NY, USA) using a semi-dry procedure (Trans Blot SD Semi-Dry Electrophoretic Transfer Cell, BioRad), after incubation in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 1.3 mM SDS, and 20% methanol at pH 8.3). Membranes were blocked with 1% bovine serum albumin in Tris-buffer-saline (TBS), and incubated overnight at 4°C with the primary antibodies diluted 1/1000. Then, membranes were incubated with the secondary antibody (α-rabbit IgG goat:horseradish peroxidase, Ref. A00160, GenScript, Piscataway, NJ, USA) diluted 1/10000 for 2 h. Proteins were detected by incubating with LumiSensor™ Chemiluminescent HRP Substrate Kit (Ref. L00221V300, GenScript).

Statistical analysis

Results were mean of at least three independent replicates ± standard deviation, with significant differences between treatments at $p < 0.05$. ANOVA statistical analysis was performed with post-hoc Duncan test using SPSS 17.0 (SPSS Inc., Chicago, USA). For image analysis of *in gel* enzymatic activity and Western-blot immunodetection, images were obtained with a ChemiDoc™ XRS + System (BioRad). Band intensity were measured and relative fold-change to control samples was calculated. Only relevant differences of gels representative of three independent assays are shown.

Results

Toxicity caused by Hg was evaluated via determination of lipid peroxidation, biometric parameters and chlorophyll concentration (Fig. 1). In shoots, neither the NO_3^- supply nor Hg exposure produced significant changes in fresh weigh. However, Hg exposure produced reduced root biomass, especially when NO_3^- supplementation was low (LN plants, Fig. 1A). In control plants, low NO_3^- supply increased lipid peroxidation compared to high NO_3^- especially significant in roots (Fig. 1B). Despite Hg treatments, roots of HN plants showed similar lipid peroxidation values, while MDA content clearly increased in a metal concentration manner (Fig. 1B). However, plants of LN root lipid peroxidation remained at higher despite Hg treatments (Fig. 1B). Chlorophyll concentration was not affected by Hg exposure in HN plants and only decreased slightly in plants exposed 30 μM Hg and nourished with low NO_3^- (Fig. 1C).

The concentration of biothiols was determined to assess the effects of NO_3^- nutrition on the accumulation of GSH, hGSH, PCs and hPCs (Carrasco-Gil et al. 2011). As shown in Table 1, hGSH was the major biothiol in alfalfa being its shoot concentration 2–3 times higher than in roots. Significant differences in hGSH values were the only found between LN and HN in roots of plants exposed to 6 μM but not 30 μM Hg, suggesting that NO_3^- concentration produced minimal changes in the biothiols content. PCs, hPCs, and cysteine (Cys, in shoots), were not detected in our experimental conditions, probably due to analytical drawbacks (see discussion).

Table 1

Non-protein thiols content (nmol g⁻¹ FW) in shoot and root of *Medicago sativa* grown in low NO_3^- (LN; 2 mM) and high NO_3^- (HN; 12 mM) treated with 0 (control), 6 and 30 μM Hg for 7 days. Concentration of each thiol peptide was calculated equivalent to the internal standard of N-Ac-Cys. Data are average of three independent replicates ($\pm$ SD).

		ROOT			SHOOT		
Thiol	NO_3^-	Control	6 μM Hg	30 μM Hg	Control	6 μM Hg	30 μM Hg
Cys	LN	21.6 $\pm$ 12.2	16.1 $\pm$ 3.1	25.0 $\pm$ 6.8	nd	nd	nd
	HN	17.4 $\pm$ 2.8	16.8 $\pm$ 4.5	25.5 $\pm$ 7.1	nd	nd	nd
hGSH	LN	64.3 $\pm$ 10.4 ^a	64.1 $\pm$ 18.3 ^a	93.7 $\pm$ 21.5 ^{a,b}	192.7 $\pm$ 63.5	178.6 $\pm$ 64.3	192.8 $\pm$ 61.4
	HN	69.4 $\pm$ 33.0 ^a	127.7 $\pm$ 44.1 ^b	102.6 $\pm$ 19.5 ^{a,b}	193.9 $\pm$ 38.9	205.1 $\pm$ 69.7	224.7 $\pm$ 92.5
GSH	LN	31.7 $\pm$ 15.7	29.0 $\pm$ 9.9	39.8 $\pm$ 9.7	27.9 $\pm$ 10.9	25.8 $\pm$ 9.5	18.9 $\pm$ 5.4
	HN	27.9 $\pm$ 12.2	29.5 $\pm$ 11.5	29.9 $\pm$ 9.9	41.3 $\pm$ 6.2	21.1 $\pm$ 7.2	18.8 $\pm$ 3.5
n.d: no detected							
Different letters denote significant differences between treatments at $p < 0.05$.							

Mercury treatments caused significant Hg accumulation in shoots and roots, increasing concomitantly with Hg doses in the nutrient solution. Root Hg concentration was between 10 and 30 times higher than in shoot (Fig. 2). It should be noted that control shoot accumulated between 7 and 10 $\mu\text{g g}^{-1}$ of Hg (two to three times lower than plants exposed to 6 μM Hg), probably caused by growth media splashing and/or volatilisation over the 7 d of metal treatment. Low NO_3^- supply caused increased Hg accumulation both in roots and in shoots, indicating that nitrate nutritional status affects Hg accumulation in plants.

To characterise the effect of Hg on N nutrition, we analysed the concentration of NO_3^- and total content of N (Fig. 3). As expected, NO_3^- concentration was higher in HN than in LN plants, both in shoots and roots (Fig. 3A). Exposure to Hg decreased the concentration of NO_3^- in shoot of HN plants treated with Hg, and only in roots of plants exposed to 30 μM Hg. Similarly, N content was also higher in plants grown with HN than those nourished with LN. In the presence of 6 μM Hg, the amount of N per plant varied slightly, and decreased significantly in shoot of alfalfa treated with 30 μM Hg, particularly in LN plants (Fig. 3B). However, total N concentration in roots showed minimal (HN) or no (LN) changes in response to Hg treatments, with inconsistent diminution in plants grown with HN and 6 μM Hg, whereas values were not significantly different in plants exposed to 30 μM Hg..

NR activity reflected a similar behaviour as NO_3^- and N total concentration in plants, being higher in HN than LN plants (Fig. 4). In overall, shoot NR activity was three-times greater than in root, indicating that shoots assimilate the largest proportion of NO_3^- in alfalfa. Shoot NR activity was modestly affected by Hg in HN plants, increasing significantly only in LN plants exposed to 30 μM Hg. However, some remarkable changes were detected in roots: NR activity augmented in plants exposed to 6 μM Hg, especially if nourished with high NO_3^- (Fig. 4). Finally, root NR activity decreased almost to control values with 30 μM Hg, showing a typical hormetic response in both, HN and LN plants.

Due to the impact of Hg on the redox balance of plants, we analysed antioxidant APX and GR activities. APX activity augmented slightly in shoots of alfalfa plants exposed to Hg, independently of the level of NO_3^- (Fig. 5). Interestingly, root APX activity was higher in HN plants than LN ones, which increased concomitantly with Hg exposure; trend that was also found in LN plants but to a lower extent. On the other hand, shoot GR increased slightly under Hg stress, particularly in plants exposed to 30 μM Hg, independently of the nitrogen nutritional status. Conversely, root GR activity of HN plants decreased with 6 μM Hg until it was completely inhibited with 30 μM Hg. The inhibition of GR activity was stronger in plants grown with low NO_3^- exposed to 6 μM Hg, and was almost completely inhibited by 30 μM Hg.

Due to the changes observed in the enzymatic activities, we studied possible alterations in the accumulation of NR, GR, APX and, additionally, the nitrate transporters NTR1.1 and NTR2.1 by Western-blot immunodetection (Fig. 6). Plants nourished with HN showed higher levels of NR than LN ones, especially in the shoots. NR expression also augmented under exposure to Hg, with its highest accumulation in plants treated with 30 μM Hg. We could not detect the nitrate transporters NTR2.1 and

NTR1.1 in the shoot, implying that these transporters are meant to incorporate nitrate from the substrate and/or its transfer to the shoot (Feng et al. 2013; Bajgain et al. 2018). NO_3^- enhanced modestly the amount of NTR2.1 in control HN plants, and then reached values similar to control and Hg-treated LN plants. On the other hand, NTR1.1 was slightly lower in HN plants, independently of the Hg treatments. With regard to APX and GR redox enzymes, their amount did not change significantly in roots, but augmented in the root of LN and HN plants in the presence of Hg. Interestingly, A second band of GR with higher mobility appeared in the shoot of plants exposed to 30 μM Hg.

Discussion

The higher concentration of total N and NR activity in shoots compared to roots (c.a. 5-times) indicate that alfalfa plants assimilate NO_3^- mostly in the shoot, in agreement with the behaviour of alfalfa plants at a vegetative development stage previously described (Arrese-Igor et al. 1991; Vance et al. 2009). HN treatment resulted in the concomitant increase in NO_3^- concentration in roots and shoots, along with remarkably high NR activity and enzyme levels in shoots, compared with LN supplied plants. Such changes were expected, since NR regulation depends on the amount of available NO_3^- (Campbell 1999; Krapp 2015). In fact, the limited amount of NO_3^- in LN plants was reflected in lower chlorophyll content in plants grown with restricted NO_3^- . Indeed the large content of N observed in HN plants matches prevent chlorosis as it is known that chlorophyll concentration is a good N nutritional status indicator (Evans 1983; Houlès et al. 2007).

Under Hg exposure, the concentration of NO_3^- and the amount of N decreased remarkably in shoots of HN plants, whereas only 30 μM Hg led to a significant diminution of N content under LN supply. Other toxic metals also decreased the levels of N and NO_3^- supplied at similar doses and intervals as those used here, although little information is available at different levels of N nutrition: N and NO_3^- concentration decreased in Cd-treated tomato (Chaffei et al. 2004) and rice (Hassan et al. 2005). In addition, NO_3^- uptake and accumulation was severely compromised in bean plants (Gouia et al. 2000) and *Arabidopsis* (Mao et al. 2014; Vazquez et al. 2020). Moreover, Hernández et al. (1997) found a rapid (24 h) impairment of NO_3^- uptake and assimilation in pea plants exposed to 10 to 50 μM Cd, reflected in a remarkable inhibition of NR.

It has been shown that NR activity was differently affected by metal stress, decreasing in *Phaseolus vulgaris* (Gouia et al. 2000), *Arabidopsis* (Mao et al. 2014) and tomato leaves (Chaffei et al. 2004) at doses above 10–100 μM Cd, and in *Pisum sativum* leaves after 24 to 72 h of exposure to 50 μM Cd (Hernández et al. 1997). Additionally, Chugh et al. (1992) reported that NR activity in *Pisum sativum* leaves was affected by Cd concentrations above 2.5 mM, and augmented significantly in leaves of *Panicum maximum* at similar levels of Cd (de Sousa Leite and Monteiro 2019). Similar increase of NR activity was observed in *Silene cucubalus* Zn-tolerant populations treated with 0.4 mM Zn (Mathys 1975). In our case, NR activity augmented remarkably in the root of plants exposed to 6 μM Hg with high NO_3^- ,

activation that also occurred in LN plants but to a lesser degree. However, NR activity dropped under acute stress, in plants treated with 30 μM Hg possibly as a consequence of cell poisoning (Van Assche and Clijsters 1990). On the other hand, NR activity was less affected in shoots, where changes were less pronounced, but increased significantly in the presence of 30 μM Hg. Interestingly, the increase in NR activity was accompanied with an accumulation of NR enzyme, as we found by immunodetection, in agreement with the regulation of NR activity by *de novo* expression of the enzyme (Campbell 1999).

In consequence, Hg could lead to a remarkable unbalance of NO_3^- in the plant, causing a higher proportion of NO_3^- assimilation in the root under moderate stress. We tested possible changes in the levels of NO_3^- transporters, as they are known to modulate the tolerance to Cd by controlling its xylem loading (Li et al. 2010). In addition, Cd apparently inhibits NTR1.1 and subsequently blocks NO_3^- uptake (Mao et al. 2014), whereas also the plasma membrane NTR2.1 NO_3^- transporter seems to attenuate Cd uptake (Guan et al. 2021). However, in our experiments we did not observe meaningful changes in the amount of NTR2.1 or NRT1.1 due to Hg in LN alfalfa plants, where high affinity transporters should operate, although their regulation are complex and subjected to post-translational modifications (Wirth et al. 2007). In addition, we could only detect a minimal decrease of NRT2.1 in HN under Hg stress, which may contribute to the diminution observed in NO_3^- uptake. Nevertheless, more profound and detailed future experiments must be performed to confirm these results, probably in combination with $[^{15}\text{N}]\text{NO}_3^-$ tracer experiments.

The concentration of Hg increased in a dose dependent manner, being accumulated basically in the root, as was described previously (Rellán-Álvarez et al. 2006; Sierra et al. 2008; Sobrino-Plata et al. 2009; Carrasco-Gil et al. 2011). Alfalfa plant is prone to restrict the movement of Hg to shoot by Hg immobilization in root, showing typical excluder behaviour (Briat and Lebrun 1999). Mercury is a rather immobile element that binds strongly with different components at the cell wall and biological membranes, mainly associated with proteins and carbohydrates in alfalfa and *Marrubium vulgare* (Hall 2002; Carrasco-Gil et al. 2011, 2013). Control plants grown with low and high NO_3^- accumulated a detectable level of Hg in shoot, probably due to the potential volatilization of Hg from the nutrient solution and deposition in leaves (Suszcynsky and Shann 1995).

Usually, supply of nitrogen fertilization results in higher accumulation of Hg, Zn or Cd in plants cultivated in metal-polluted soils (Wångstrand et al. 2007; Barrutia et al. 2009; Gao et al. 2010; Carrasco-Gil et al. 2012). However, plants cultivated in hydroponic solutions or pots under controlled conditions experienced a metal dilution effect due to biomass increase, as was observed in Cd-treated *Arabidopsis* (Vazquez et al. 2020), soybean (Konotop et al. 2012), wheat (Yotsova et al. 2020), and *Populus* (Chen et al. 2011). Nevertheless, nitrogen nutritional influence depends on genotypes, phenology status and growth conditions, leading to conflicting results (Sarwar et al. 2010; Yang et al. 2016; Bi et al. 2020). For example, high N nutritional status increased root but slightly decreased shoot Cd concentrations in two rice genotypes (Du et al. 2009). We found enhanced accumulation of Hg under LN, which cannot be explained solely in terms of a toxic metal dilution effect, as there were only significant differences in shoot biomass

compared with high NO_3^- grown plants. Therefore, some tolerance mechanisms should promote a decrease in Hg uptake in high NO_3^- treated alfalfa, probably linked to improved Hg stress tolerance.

One possible mechanism could be the larger accumulation of hGSH in the roots of high NO_3^- plants exposed to 6 μM Hg, almost two-fold increase compared to low NO_3^- plants (Table 1), which determine the tolerance to metal-induced oxidative stress (Hernández et al. 2015). However, under acute stress in plants treated with 30 μM Hg values were similar at both NO_3^- nutritional status. hGSH and GSH are fundamental antioxidant biothiols required for metal tolerance: plants depleted of GSH or hGSH were more susceptible to oxidative damage induced by Hg and Cd stress (Sobrino-Plata et al. 2014a, b, 2021). hGSH and GSH are also the building blocks of PCs, biothiols that are able to form very stable complexes with Hg (Carrasco-Gil et al. 2011). Unfortunately, although we tried to analyse free PCs in our samples, we were unable to detect any of them in the different treatments performed. Hg-PC or Hg-hPC complexes are extremely durable even when subjected to strong acidic extraction procedures (Sobrino-Plata et al. 2021). It is feasible that during the relatively long exposure time used in our experiments (one week) Hg bound and blocked thiol groups of PCs/hPCs preventing their reaction with Ellman's reagent and, therefore, their detection (Iglesia-Turiño et al. 2006).

Accumulation of Hg to high levels in roots led to significant induction of oxidative stress, whereas shoots were much less affected, matching previous observations (Ortega-Villasante et al. 2005). Apparently, antioxidant enzymes like GR and APX had higher activity in shoots, corresponding with concomitant accumulation detected by immunoblots, irrespective of the NO_3^- nutritional status, and resembling the responses found in alfalfa with similar doses of Hg (Sobrino-Plata et al. 2009). Interestingly, roots of plants grown with low NO_3^- suffered stronger GR activity inhibition when treated with 6 μM Hg than plants supplied with high NO_3^- , without meaningful changes of enzyme amount. Similar behaviour occurred in alfalfa grown in a Hg-polluted soils under greenhouse conditions, where plants nourished with nitrogen (NH_4NO_3) were more tolerant and showed higher GR activity than plants only fertilized with P and K (Carrasco-Gil et al. 2012). In this sense, our group observed repeatedly that GR activity is an specific bioindicator of Hg toxicity in plant cells, which is inhibited to higher degree whenever plants are more susceptible to Hg (Sobrino-Plata et al. 2009, 2013, 2014b, 2021). However, under high Hg concentration (30 μM) both NO_3^- nutritional levels showed similar degree of damage, implying that above a certain threshold of Hg accumulation, the influence of low NO_3^- nutrition was overridden. Finally, APX activity increased in response to Hg, but independently to NO_3^- nutritional status in shoots, while it increased to a higher degree in HN alfalfa plants than in LN ones. These results were basically in agreement with those described by Sobrino-Plata et al. (2009), who found a modest induction of APX in alfalfa plants grown in semi-hydroponic conditions. Induction of APX occurs under moderate Hg-stress, without changes in the amount of APX, probably owing to post-translational regulation mechanisms (Ortega-Villasante et al. 2007).

In conclusion, high supply of NO_3^- prevented toxic effects caused by Hg, in the same manner that ameliorated damages observed mainly by Cd in different plant species. APX and GR activity increased (Chen et al. 2011), while lower lipid peroxidation and enhanced peroxidase and GR activity were described in *Populus* plants exposed to Cd (Bi et al. 2020). Similarly, wheat plants experienced lower oxidation stress and had improved photosynthetic efficiency in the presence of Cd (Yotsova et al. 2020), whereas there was strong induction of guaiacol peroxidase in Cd-exposed *Arabidopsis* (Vazquez et al. 2020). Finally, *Matricaria chamomilla* plants showed higher proportion of reduced GSH, improved APX activity and lower phenolic metabolism in Pb-stressed (Kováčik et al. 2020). Therefore, the NO_3^- nutritional status of plants might be important to improve their tolerance to Hg, in particular at moderate levels of Hg (6 μM). However, more work is needed for a better understanding of the mechanisms of tolerance involved. Such research will help to optimise the use of plants in phytotechnologies to clean up polluted soils or stabilise them to avoid erosion and loss of fertility.

Declarations

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Figures

Figure 1

Physiological parameters of stress to Hg in alfalfa. A) Fresh weight (mg plant⁻¹), B) lipid peroxidation as MDA subproduct concentration (nmol·g⁻¹ FW), and C) total chlorophyll (Chla + Chlb) concentration (mg·g⁻¹ FW) in shoots or roots of *Medicago sativa* grown in low NO₃⁻ (LN, 2 mM) and high NO₃⁻ (HN, 12 mM) treated with 0 (control), 6 and 30 μ M Hg for 7 days. Data are average of three independent

assays ($\pm$ SD). Different letters denote significant differences between treatments at $p < 0.05$. (Regular letter for LN and italics for HN).

Figure 2

Total Hg ($\mu\text{g}\cdot\text{g}^{-1}$ DW) in shoot and root of *Medicago sativa* grown in low NO_3^- (LN, 2 mM) and high NO_3^- (HN, 12 mM) treated with 0 (control), 6 and 30 μM Hg for 7 days. Data are average of five

independent replicates ($\pm$ SD). Different letters denote significant differences between treatments at $p < 0.05$. (Regular letter for LN and italics for HN).

Figure 3

A) Nitrate concentration (mg NO₃⁻·g⁻¹ FW), and B) nitrogen accumulation (mg NH₄⁺ plant⁻¹) in shoot and root of *Medicago sativa* grown in low NO₃⁻ (LN, 2 mM) and high NO₃⁻ (HN, 12 mM) treated with 0 (control), 6 and 30 μM Hg for 7 days. Data are average of at least three independent assays ($\pm$ SD). Different letters denote significant differences between treatments at $p < 0.05$. (Regular letter for LN and italics for HN).

Figure 4

Nitrate reductase activity (NR) (nmol NO₂⁻ g⁻¹ FW h⁻¹) in shoot and root of *Medicago sativa* grown in low NO₃⁻ (LN, 2 mM) and high NO₃⁻ (HN, 12 mM) treated with 0 (control), 6 and 30 μM of Hg for 7 days. Data are average of at least three independent replicates (± SD). Different letters denote significant differences between treatments at p<0.05. (Regular letter for LN and italics for HN).

Figure 5

Effect of mercury on in gel glutathione reductase (GR) and ascorbate peroxidase (APX) activity in shoot and root of *Medicago sativa* grown in low NO_3^- (LN, 2 mM) and high NO_3^- (HN, 12 mM) treated with 0 (control), 6 and 30 μM Hg for 7 days. Numbers below the band represent the relative fold-change against the LN control, normalized relative to the intensity of Coomassie blue protein staining (loading).

SHOOT

ROOT

Figure 6

Western-blot immunodetection using specific antibodies against nitrate reductase (NR), nitrate transporters (NTR2.1 and NTR1.1), glutathione reductase (GR), ascorbate peroxidase (APX) in protein extracts prepared from shoots and roots of *Medicago sativa* grown in low nitrogen (2 mM NO₃⁻) and high nitrogen (12 mM NO₃⁻), treated with 0 (control), 6 and 30 μ M Hg for 7 days. Numbers below the

bands represent the relative fold-change against the LN control, normalized relative to the intensity of Coomassie blue protein staining (loading).