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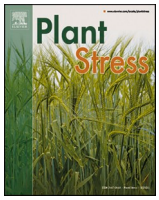

# Attenuation of mercury phytotoxicity with a high nutritional level of nitrate in alfalfa plants grown hydroponically

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ABSTRACT

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. Nitrate ($NO_3^-$) is the prevalent form to incorporate nitrogen (N) in higher plants, through its reduction to nitrite ($NO_2^-$) by the enzyme nitrate reductase (NR). 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. Several parameters of oxidative stress such as lipid peroxidation, chlorophyll content, biothiol concentration, and ascorbate peroxidase (APX) and glutathione reductase (GR) activities showed that HN plants were less affected by Hg. Nitrate reductase activity and $NO_3^-$ concentration were also altered under Hg stress, with lower impact in plants nourished with high $NO_3^-$. Our results highlight the importance of the $NO_3^-$ nutritional status to improve tolerance to toxic metals like Hg.

## 1. 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). Interruption of the production of Hg has imposed the necessity to impulse other economic activities, such as agriculture and farming, which are challenged by the accumulation of Hg in affected soils (Sierra et al., 2008). On other hand, the ability of plants to extract metals from the soil can be exploited to clean up Hg-polluted soils by using phytoremediation technologies (Wang et al., 2020).

The divalent cation ($Hg^{2+}$) is predominant in well aerated agricultural soils, amongst 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 growth inhibition, alteration of photosynthesis, mitochondrial respiration, and obstruction of water flow through the inhibition of plasma membrane aquaporins (Cargnelutti et al., 2006; Israr and Sahi, 2006; Patra and Sharma, 2000; 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)(EC 1.11.1.11) and glutathione reductase (GR)(EC 1.6.4.2), and redox metabolites like glutathione (GSH; gGlu-Cys-Gly) (Hasan et al., 2017; Khan et al., 2021; Sharma and Dietz, 2009), which also operate under Hg stress (Cargnelutti et al., 2006; Cho and Park, 2000; Elbaz et al., 2010). However, even under relative low Hg concentrations (1 to 3 μM), the activity of these antioxidant enzymes usually drops (Elbaz et al., 2010; Ortega-Villasante et al., 2007; Sobrino-Plata et al., 2009; Zhou et al., 2008). In particular, GR is extremely sensitive to Hg that accumulates in the roots of various 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

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to Hg depends highly on the levels of GSH and derived biothiols, including phytochelatins (PCs), a family of thiol-rich peptides with general $(gGlu\text{-}Cys)_n$-Gly-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)(EC 1.7.1.1), and then to $NH_4^+$ thanks to the plastidial enzyme nitrite reductase (NiR)(EC 1.7.1.4) (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), such as the addition of organic matter and NPK inorganic fertilizers containing $NO_3^-$ (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 (Chaffei et al., 2004; Gouia et al., 2000; Hernandez et al., 1996). Similar experiments showed that $NO_3^-$ and $K^+$ uptake, and NR activity decreased in pea plants treated with 50 μM Cd (Hernández et al., 1997), while $NO_3^-$ and $NH_4^+$ assimilation was also inhibited in Cd-exposed bean and tomato plants (Chaffei et al., 2004; Gouia et al., 2000). 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; Yang et al., 2020). Under N starvation, Cd caused stronger phytotoxic symptoms in *Hordeum vulgare*, in parallel to the up-regulation of phytochelatin synthase (PCS)(EC 2.3.2.15) and natural resistance-associated macrophage protein (NRAMP) metal transporters gene expression, with modest changes in the expression of oxidative stress related genes (Finkemeier et al., 2003). However, excessive $NO_3^-$ promoted enhanced Cd uptake without affecting the biomass at different nitrogen nutritional status (Yang et al., 2016).

In consequence, $NO_3^-$ nutritional status seems to modulate toxic metals tolerance in plants, as might occur with Hg. In this sense, we previously found that fertilization of alfalfa plants with $NO_3^-$ grown in a Hg-polluted soil improved tolerance to Hg (Carrasco-Gil et al., 2012). It should be noted that the information available about the interaction between $NO_3^-$ nutrition status and Hg phytotoxicity is very limited, which would be useful for designing improved phytoremediation procedures in Hg-polluted soils. Therefore, we aimed to characterise the physiological responses of alfalfa plants to Hg exposure with low and high $NO_3^-$ nutritional status in a semi-hydroponic system using perlite as inert substrate, to avoid physicochemical interferences on metal availability usually occurring when soils are used as 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.

## 2. Materials and methods

### 2.1. 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 × 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 (Sobrino-Plata et al., 2009). To mitigate unwanted cationic macronutrient and redox imbalances caused by nitrate salts such as $NaNO_3$ or $NH_4NO_3$ that supply excessive $Na^+$ and $NH_4^+$ to the nutrient solution, we prepared a mixture of nitrate solutions containing a K to Ca + Mg ratio to favour a sufficient K nutrient status (Domínguez-Valdivia et al., 2008; Raddatz et al., 2020). One of them with low $NO_3^-$ concentration (LN; 2 mM; comprised of 0.63 mM $KNO_3$, 0.9 mM $Ca(NO_3)_2$, and 0.47 mM $Mg(NO_3)_2$), whereas the other one contained high $NO_3^-$ concentration (HN; 12 mM; made with 3.8 mM $KNO_3$, 5.4 mM $Ca(NO_3)_2$, 2.8 mM $Mg(NO_3)_2$). Both nutrient solutions were supplemented with the rest of macronutrients [mM] (1.0 $KH_2PO_4$, 0.5 $MgSO_4$, 0.1 NaCl) and 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 $Mo_7O_{24}(NH_4)_6$), and pH was adjusted to 6.2. Plants grew for 12 days in a controlled environment chamber (16 h light (lamps of 120 $Wm^{-2}$)/8 h darkness) at 25/18 °C, respectively and relative humidity, 75% to produce sufficient plant biomass. Then, Hg (as $HgCl_2$) was supplied at 0, 6 and 30 μM concentrations for another 7 days. At harvest, shoots and roots were thoroughly washed in deionized $H_2O$, the fresh weight measured and stored at −80 °C until analysis.

### 2.2. 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 ($HNO_3$:$H_2O_2$,:$H_2O$ 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 μg $kg^{-1}$. Certified Reference Materials (CRM) were used to determine the accuracy of the measurements and validation.

### 2.3. Nitrogen and $NO_3^-$ concentration in plants

The nitrogen (N) in plant tissue was determined by Kjeldahl digestion, performed in a wet digestor system B-440 (Buchi, Switzerland). 50 mg of ground dry plant material were digested in 10 ml concentrated $H_2SO_4$ and 10 g solid $K_2SO_4$. The mixture was heated at 410 °C for 1.5 h, cooled for 30 min, and resulting $NH_4^+$ was distilled after the addition of 25 ml of 32% NaOH (w/v) using a K-355 distillation unit (Buchi). The concentration of $NH_3$ was calculated by back titration in 2% $H_3BO_3$ (v/v) buffer (pH 4.65) with 0.02 M HCl, following the KF Titrino Plus 870 (Metrohm, Switzerland) specifications. $NO_3^-$ was analysed in 100 mg ground dry plant material boiled in 1 mL deionized $H_2O$ for 5 min in a 25 mL glass test tube using a water bath. After cooling, the homogenate was centrifuged (14,000 x g) for 15 min at 4 °C, and 10 μL of the supernatant was used for $NO_3^-$ determination using the NECi Microplate Nitrate Test Kit (Ref. Code NTK-MPSR) according to the manufacturer's instructions (NECi, Lake Linden, MI, USA), and absorbance was measured at 540 nm with a TECAN Spectrafluor microplate reader (Männedorf, Switzerland).

### 2.4. Lipid peroxidation and chlorophyll analysis

Lipid peroxidation was estimated by measuring the concentration of malondialdehyde (MDA). Ground frozen tissue (0.1 g) was transferred to a screw-capped 1.5 ml Eppendorf tube and mixed with 1 ml reaction solution (15% (w/v) trichloroacetic acid, 0.37% (w/v) 2-thiobarbituric acid, 0.25 M HCl, and 0.01% (w/v) buthylated hydroxytoluene). After incubation at 90 °C for 30 min, samples were centrifuged at 12,000 x g for 10 min. The absorbence of clear supernatant was measured at 535 nm (UV-2401 PC spectrophotometer, Shimadzu, Tokyo, Japan), and the concentration of MDA was calculated using the 1.56 × $10^5$ $M^{-1}$ $cm^{-1}$ extinction coefficient (Rellán-Álvarez et al., 2006). For chlorophyll (Chl) determination, 0.05 g of frozen leaves were homogenized in 10 ml 80% (v/v) acetone, filtered through a paper filter, and absorbence read at 645 and 663 nm (UV-2401 PC spectrophotometer, Shimadzu). Total chlorophyll concentration was calculated according to Porra (Porra, 2002).

### 2.5. Analysis of biothiols

Frozen tissue (0.1 g) was ground with 300 µl of 0.25 N HCl, the homogenate centrifuged for 15 min at 12,000 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 × 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 Ellmań's reagent (5,5-dithio-bis (2 nitrobenzoic acid) at 412 nm absorbence (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, homoglutathione (hGSH), cysteine (Cys), homophytochelatin-2 ($\gamma$-(Glu-Cys)$_2$-Ala; hPC$_2$), and homophytochelatin-3 ($\gamma$-(Glu-Cys)$_3$-Ala; hPC$_3$).

### 2.6. Determination of nitrate reductase activity in vitro

The *in vitro* 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 of enzymatic extraction solution, respectively for shoot and root. This solution was freshly prepared by mixing 10 ml extraction buffer (30 mM 3-(N-morpholino) propanesulfonic acid buffer (pH 7.5), 5 mM $Na_2$-EDTA, 10 mM dithiothreitol, 10 mM ascorbic acid, 0.6% polyvinylpyrrolidone (PVP) (w/v), 10 µl 100 mM phenylmethylsulfonyl fluoride), and 1 ml protease inhibitors cocktail Sigma-Aldrich (P2714; St. Louis, MO, USA). After centrifugation (14,000 x g) for 15 min at 4 °C, the supernatant was kept in ice, and NR activity was measured by adding 0.1 ml of the enzymatic extract, 0.1 ml 100 mM $KNO_3$, 0.5 ml reaction buffer (100 mM K-phosphate buffer, 1 mM EDTA at pH 7.5) and 0.1 ml of 1 mg ml$^{-1}$ NADH. Formed $NO_2^-$ 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% (w/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 10,000 x g for 15 min, and absorbence read at 540 nm using a UV-2401 PC spectrophotometer (Shimadzu). Nitrite concentration was calculated using the extinction coefficient of 48.8 mM$^{-1}$·cm$^{-1}$ with the Lambert-Beer equation.

### 2.7. Glutathione reductase and ascorbate peroxidase

GR and 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 extraction solution (see above). After centrifugation (14,000 x 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 (Bio-Rad, 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, 0.2 mg ml$^{-1}$ thizolyl blue tetrazolium bromide, 0.2 mg ml$^{-1}$ 2, 6-dichlorophenol indophenol, 0.5 mM NADPH and 3.5 mM oxidised glutathione) (Sobrino-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 $H_2O_2$ in 50 mM Na-phosphate (pH 7.0), and revealed after incubation with 0.5 mM nitroblue tetrazolium and 10 mM tetramethylethylenediamine (TEMED) in 50 mM Na-phosphate (pH 7.8).

### 2.8. 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-NRT1.1 (AS122611), and α-NRT2.1 (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) in transfer buffer (48 mM Tris–HCl, 39 mM glycine, and 20% methanol at pH 8.3). Membranes were blocked with 1% bovine serum albumin in Tris-buffer-saline 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 1/10,000 for 2 h. Proteins were detected using LumiSensor™ Chemiluminescent HRP Substrate Kit (Ref. L00221V300, GenScript, USA).

### 2.9. Statistical analysis

Results were mean of at least three independent replicates. ANOVA statistical analysis was performed with post-hoc Duncan test with significant differences at $p<0.05$ using SPSS 25.0 (SPSS Inc., Chicago, USA). Enzymatic activities and Western-blot immunodetection images were obtained with a ChemiDoc™ XRS+ System (BioRad). Band intensities were measured to calculate fold-change relative to control samples, and the values shown in figures corresponded to relevant differences representative of three independent assays.

## 3. Results

### 3.1. Plant biomass, lipid peroxidation and biothiol concentration

Low $NO_3^-$ supply in plants not exposed to Hg had little impact on plant biomass and chlorophyll concentration in comparison with high $NO_3^-$ plants, with only a slight significant increase of lipid peroxidation in roots (Fig. 1). These results suggested that there were not strong symptoms of $NO_3^-$ deficiency under the experimental conditions used, which was one of the objectives to attain to avoid N-deficiency damages along with Hg stress. On the other hand, exposure of alfalfa seedlings to Hg led to minimal loss of shoots fresh weight, especially when $NO_3^-$ supplementation was low (LN plants) in comparison with those fed with high $NO_3^-$ (HN plants; Fig. 1A). Lipid peroxidation only changed significantly under Hg stress in roots, with constant higher values in LN plants than in HN plants, despite MDA concentration increased in a dose manner in the latter plants (Fig. 1B). Finally, 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). hGSH was the major biothiol in alfalfa, being its shoot concentration 2–3 times higher than in roots (Table 1). 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).

### 3.2. Mercury accumulation

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

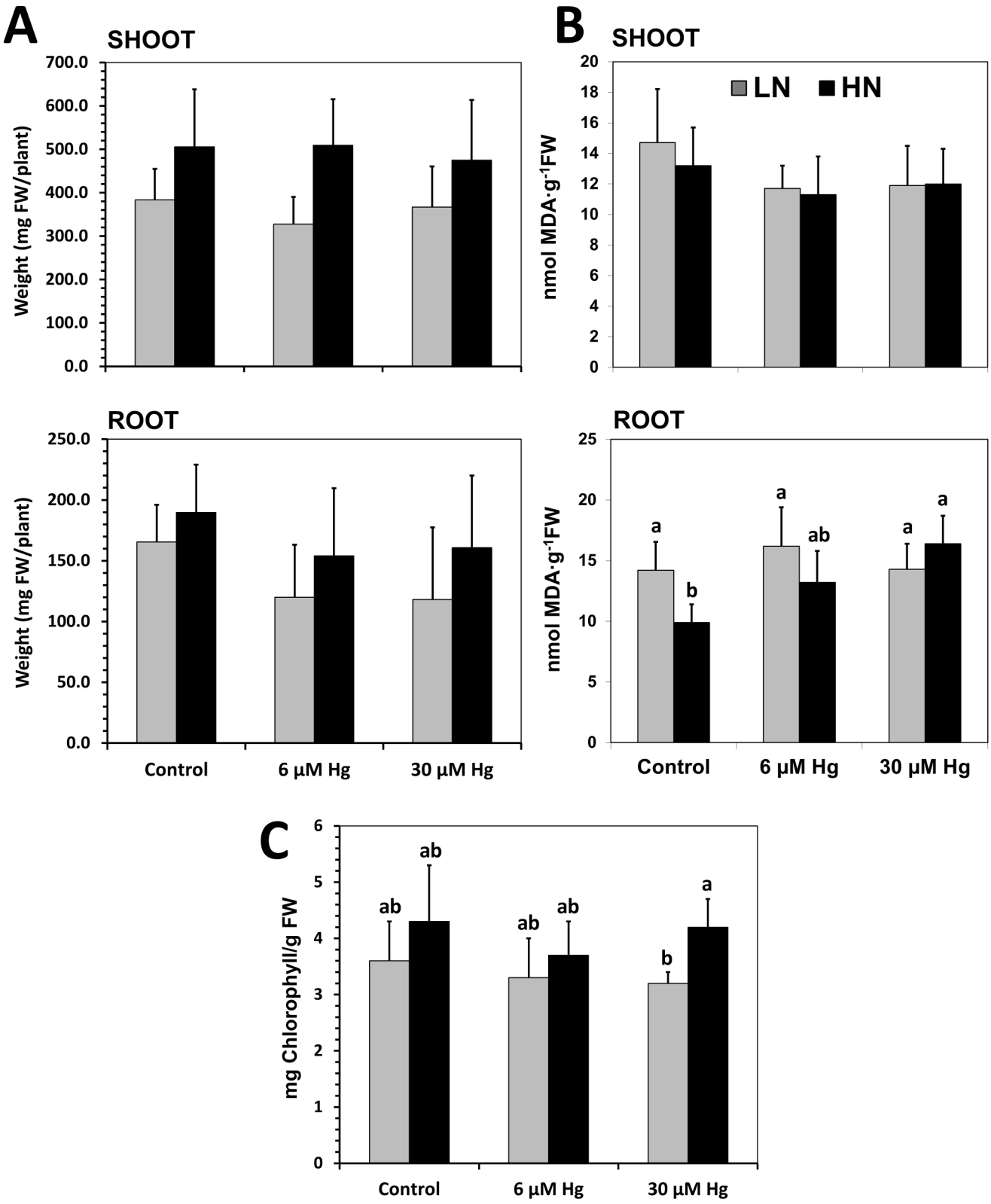


**Fig. 1.** Physiological parameters of stress to Hg in alfalfa. **A**) Fresh weight (mg plant$^{-1}$); **B**) lipid peroxidation as MDA subproduct concentration (nmol·g$^{-1}$ FW) in shoots or roots, and C) total chlorophyll (Chla + Chlb) concentration in shoots (mg·g$^{-1}$ FW) of *Medicago sativa* 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 three independent assays (± SD). Different letters denote significant differences between treatments at $p < 0.05$. Bars without letters indicate that there were not significant differences.

**Table 1**
Biothiols concentration (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 (± SD).

| Thiol | $NO_3^-$ | ROOT Control | 6 μM Hg | 30 μM Hg | SHOOT Control | 6 μM Hg | 30 μM Hg |
|---|---|---|---|---|---|---|---|
| Cys | **LN** | 21.6 ± 12.2[a] | 16.1 ± 3.1[a] | 25.0 ± 6.8[a] | nd | nd | nd |
| | **HN** | 17.4 ± 2.8[a] | 16.8 ± 4.5[a] | 25.5 ± 7.1[a] | nd | nd | nd |
| hGSH | **LN** | 64.3 ± 10.4[a] | 64.1 ± 18.3[a] | 93.7 ± 21.5[a,b] | 192.7 ± 63.5[a] | 178.6 ± 64.3[a] | 192.8 ± 61.4[a] |
| | **HN** | 69.4 ± 33.0[a] | 127.7 ± 44.1[b] | 102.6 ± 19.5[a,b] | 193.9 ± 38.9[a] | 205.1 ± 69.7[a] | 224.7 ± 92.5[a] |
| GSH | **LN** | 31.7 ± 15.7[a] | 29.0 ± 9.9[a] | 39.8 ± 9.7[a] | 27.9 ± 10.9[a] | 25.8 ± 9.5[a] | 18.9 ± 5.4[a] |
| | **HN** | 27.9 ± 12.2[a] | 29.5 ± 11.5[a] | 29.9 ± 9.9[a] | 41.3 ± 6.2[a] | 21.1 ± 7.2[a] | 18.8 ± 3.5[a] |

n.d: no detected.
Different letters denote significant differences between treatments at $p < 0.05$.

in shoot (Fig. 2). It should be noted that control shoot accumulated between 7 and 10 μg $g^{-1}$ 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.

### 3.3. Nitrogen nutrition parameters

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 had negligible effect on the concentration of $NO_3^-$ in plants fed with LN and in roots of HN plants, with a slight diminution in the shoot of HN plants (approximately 20%) when treated with 6 or 30 μM Hg (Fig. 3A). 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.

### 3.4. Antioxidant in gel enzymatic activities

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. Two GR bands of different electrophoretic mobility were observed, this is consistent with the presence of several isoforms in plants, such as cytosolic and plastidial GRs (Yannarelli et al., 2007). 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.

### 3.5. Protein Western-blot immunodetection

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 NRT1.1 and NRT2.1 by Western-blot immunodetection (Fig. 6). The degree of similarity between alfalfa and *Arabidopsis* NRT1.1 and NRT2.1, was analysed by Clustal Omega comparison of translated proteins (Sievers et al., 2011) (See Supplementary Material File). We found high identity of alfalfa NRT1.1 and NRT2.1 (64 and 78%, respectively) with the *Arabidopsis* proteins, implying that the bands detected in our experiment were genuine. 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 mM Hg. We could not detect the $NO_3^-$ transporters NRT2.1 and NRT1.1 in the shoot, implying that these transporters are meant to incorporate $NO_3^-$ from the substrate and/or its transfer to the shoot (Bajgain et al., 2018; Feng et al., 2013). $NO_3^-$ enhanced modestly the amount of NRT2.1 in control HN plants, and then reached values similar to control and Hg-treated LN plants. On the other hand, NRT1.1 was slightly lower in HN plants, independently of the Hg treatments. Regarding 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 electrophoretic mobility appeared in the shoot of plants exposed to 30 μM Hg of plants grown under LN and HN conditions.

## 4. Discussion

### 4.1. Influence of $NO_3^-$ supply on nitrogen nutrition parameters

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 leaves, in agreement with the previously described behaviour of alfalfa plants at a vegetative development stage (Arrese-Igor et al., 1991; Vance and Heichel, 1981). 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 addition, plants with limited supply of $NO_3^-$ in LN plants trend to accumulate less chlorophyll than plants fed with HN plants, although there were no statistical differences even under moderate Hg stress (only decreased in LN plants treated with 30 μM Hg), since chlorophyll concentration is a valid indicator of plants' N nutritional status (Evans, 1983; Houlès et al., 2007).

### 4.2. Impact of Hg on $NO_3^-$ assimilation

Several studies showed that Cd leads to overall diminution of N and/or $NO_3^-$ concentration in tomato (Chaffei et al., 2004), rice (Hassan et al., 2005), bean plants (Gouia et al., 2000) and *Arabidopsis* (Vazquez et al., 2020), and 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. Similarly, Hg exerted a negative effect on $NO_3^-$

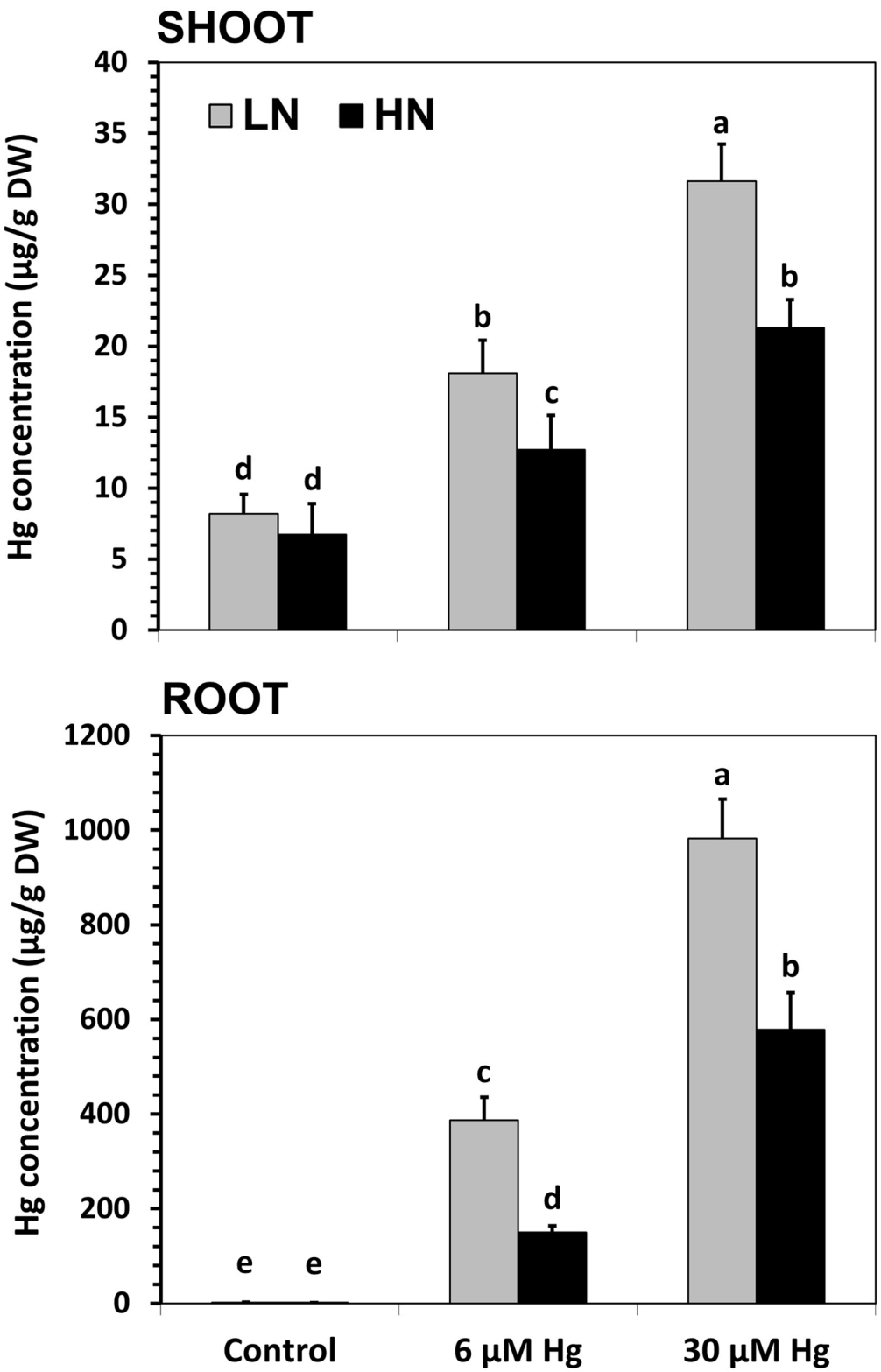


**Fig. 2.** Total Hg (µg·g$^{-1}$ DW) in shoot and root of *Medicago sativa* grown in low $NO_3^-$ (LN; 2 mM) and high $NO_3^-$ (HN; 12 mM) treated with 0 (control), 6 and 30 µM Hg for 7 days. Data are average of five independent replicates (± SD). Different letters denote significant differences between treatments at $p < 0.05$.

accumulation and N content in leaves, particularly in HN plants, which decreased mostly in shoots (Fig. 3). Remarkably, $NO_3^-$ and N levels did not change in LN plants in the presence of Hg, with only a significant diminution in N content in shoots of plants treated with 30 µM Hg. Analogous results appeared in shoots and roots of rice plants treated with As and Cd and grown with low and high $NO_3^-$ fertilization: Cadmium (100 µM) caused that shoots $NO_3^-$ concentration remained at control levels, while it augmented significantly in roots of rice plants subjected to $NO_3^-$ fertilization equivalent to the levels used in our work. However, $NO_3^-$ concentration in shoots and roots decreased in plant fed with high $NO_3^-$ (Yang et al., 2016). In the presence of As, $NO_3^-$ concentration augmented in roots of rice plants supplied with low N (50 µM),

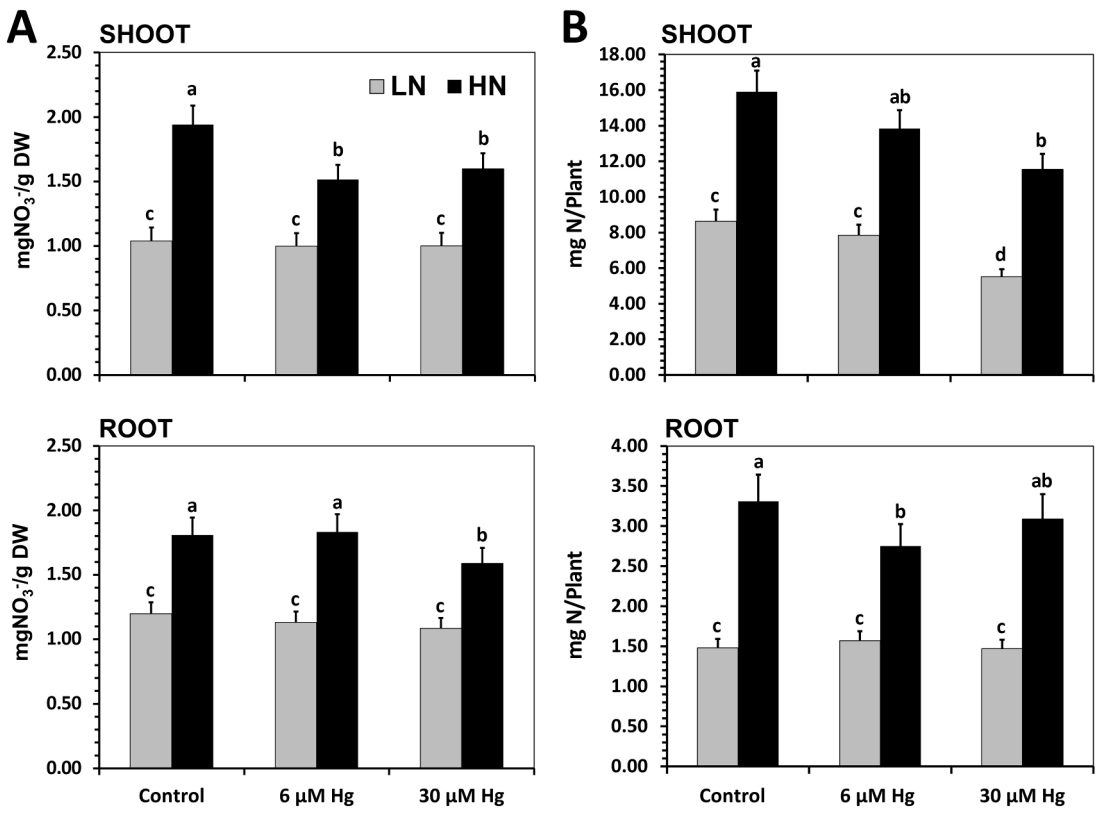


**Fig. 3.** **A**) Nitrate concentration (mg $NO_3^-$·$g^{-1}$ FW); and B) nitrogen accumulation (mg N·$plant^{-1}$) in shoot and root of *Medicago sativa* grown in low $NO_3^-$ (LN; 2 mM) and high $NO_3^-$ (HN; 12 mM) treated with 0 (control), 6 and 30 µM Hg for 7 days. Data are average of at least three independent assays (± SD). Different letters denote significant differences between treatments at $p < 0.05$.

but decreased under high N (1.4 mM) fertilization, whereas $NO_3^-$ concentration declined consistently in shoots of low and high $NO_3^-$ fertilization (Srivastava et al., 2019). It is know that the distribution of $NO_3^-$ between cytosol (metabolic) *versus* vacuole (storage) pools, along with uptake and assimilation partitioning between roots and shoors varies greatly depending on $NO_3^-$ concentration, physiological cues, particularly under stress (Krapp, 2015). Therefore, the combination of different levels of $NO_3^-$ nourishment and the exposure to toxic elements may have complex effects in the concentration of $NO_3^-$ and N in shoots and roots. In this respect, inhibition of $NO_3^-$ uptake by Cd was associated with a repression of the $NO_3^-$ transporter *NRT1.1* transcription, but Cd induced in parallel the expression of *NRT2.1* in *Arabidopsis* (Mao et al., 2014). Therefore, we tested possible changes in the levels of $NO_3^-$ transporters, as they are also known to modulate the xylem loading (Li et al., 2010) and the plasma membrane uptake (Guan et al., 2021) of Cd. However, in our experiments we did not observe meaningful changes in the amount of NRT2.1 or NRT1.1 in roots due to Hg in LN alfalfa plants, where high affinity transporters should operate, although they are subjected to complex post-translational regulation (Wirth et al., 2007). In addition, we only detected 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 [$^{15}$N] $NO_3^-$ tracer experiments (Cui et al., 2020).

Another factor contributing to the altered accumulation of $NO_3^-$ in LN and HN plants under Hg stress could be the observed changes in NR activity, key step in $NO_3^-$ assimilation (Fig. 4). 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). It was shown that NR activity was differently affected by metal stress, decreasing in *Phaseolus vulgaris* (Gouia et al., 2000), *Arabidopsis* (Mao et al., 2014), tomato (Chaffei et al., 2004), *Pisum sativum* (Hernández et al., 1997), and *Lepidium* (Gill et al., 2012) at doses above 10–100 µM Cd; but increasing significantly in *Panicum maximum* at high doses of Cd (1.0 mM) (de Sousa Leite and Monteiro, 2019), as was observed in *Silene cucubalus* Zn-tolerant populations treated with 0.4 mM Zn (Mathys, 1975). Therefore, NR seems affected depending on the metal treatments, experimental conditions and the organs tested. Interestingly, the increase in root 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). This unexpected induction of NR *in vitro* activity under moderate Hg stress may reflect the complex flow of $NO_3^-$ through different pools, which is established by xylem/phloem transport, cytosol/vacuole partitioning, and assimilation (Cui et al., 2020). However, that activation of NR activity was not accompanied by a substantial drop in $NO_3^-$ concentration. It is known that numerous metabolic pathways are activated under metal stress, including several antioxidant defences to counteract the oxidative stress triggered by toxic metals, as occurs in roots exposed to Hg (Sharma and Dietz, 2009). Recent experiments showed that NR, enzyme that also contributes to the synthesis of nitric oxide (NO) under stress, was induced by Cd in *Brassica juncea* roots. It was hypothesised that building up of NO concentration modulates the antioxidant response, and contributes to Cd tolerance (Khator et al., 2021). Thus, NR induction under moderate Hg stress could be explained in this context, but this functional implication must be addressed in future experiments.

### 4.3. *Influence of $NO_3^-$ supply on Hg accumulation*

The concentration of Hg increased in a dose dependant manner, being accumulated basically in the root, as was described previously

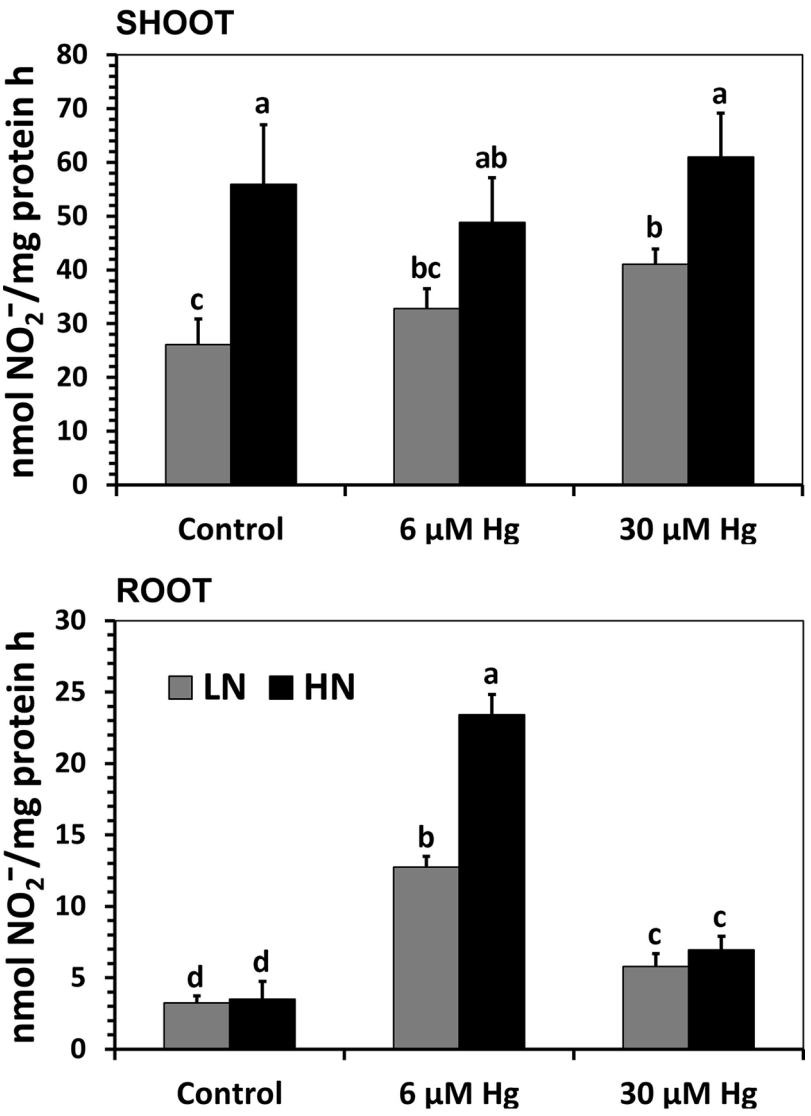


**Fig. 4.** Nitrate reductase activity (NR) (nmol $NO_2^-$ mg protein$^{-1}$ h$^{-1}$) in shoot and root of *Medicago sativa* grown in low $NO_3^-$ (LN; 2 mM) and high $NO_3^-$ (HN; 12 mM) treated with 0 (control), 6 and 30 µM of Hg for 7 days. Data are average of at least three independent replicates (± SD). Different letters denote significant differences between treatments at $p < 0.05$.

(Carrasco-Gil et al., 2011; Rellán-Álvarez et al., 2006; Sierra et al., 2008; Sobrino-Plata et al., 2009). 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* (Carrasco-Gil et al., 2013, 2011; Hall, 2002). 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 Zn or Cd in plants cultivated in metal-polluted soils (Barrutia et al., 2009; Gao et al., 2010; Wångstrand et al., 2007). Similarly, we showed previously that supply of $NO_3^-$ fertilizer promoted Hg accumulation of alfalfa plants grown in a Hg-polluted soil (Carrasco-Gil et al., 2012), in opposition with our current results where Hg accumulated to larger extent under LN (Fig. 2). In that experiment, alfalfa plants not fertilized with N and cultivated in that natural Hg-polluted soil, developed nodules for symbiotic nitrogen fixation; plants that had less biomass and eventually showed larger oxidative stress symptoms, which probably limited the ability to extract Hg (Carrasco-Gil et al., 2012). On the contrary, plants cultivated in hydroponic solutions or pots under controlled conditions also experienced that Cd-treated *Arabidopsis* (Vazquez et al., 2020), soybean (Konotop et al., 2012), wheat (Yotsova et al., 2020), and *Populus* (Chen et al., 2011) accumulated less metal with high N nutrition, possibly due to a metal dilution effect associated with biomass increment. Nevertheless, the nitrogen nutritional influence depends on genotypes, phenology status and growth conditions, leading to conflicting results (Bi et al., 2020; Sarwar et al., 2010; Yang et al., 2016). In this sense, our results cannot be explained solely in terms of a toxic metal dilution effect, as there were only significant differences in shoot biomass compared with HN plants. Excess $NO_3^-$ supply may alter the net metal influx and root-shoot translocation, as were observed by several studies with Cd-treated plants, in comparison with sufficient or deficient N nutrition. There are a number of mechanisms that may contribute for those differences, including regulation of metal transporters, metal speciation linked to metal-biothiol complexes, and adjustment of the cellular redox imbalance (Yang et al., 2020).

Therefore, one possible mechanism of tolerance 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),

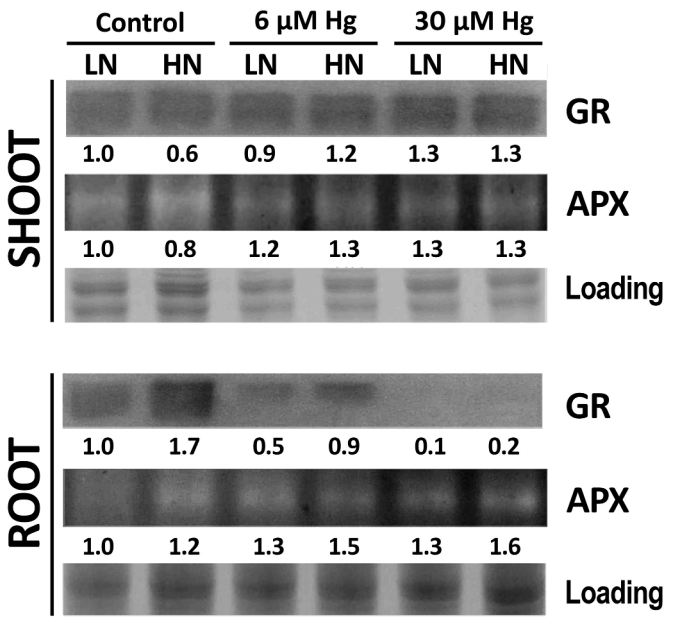


**Fig. 5.** Effect of mercury on *in gel* glutathione reductase (GR) and ascorbate peroxidase (APX) activity in shoot and root of *Medicago sativa* grown in low $NO_3^-$ (LN; 2 mM) and high $NO_3^-$ (HN; 12 mM) treated with 0 (control), 6 and 30 µM Hg for 7 days. Numbers below the band represent the relative fold-change against the LN control, normalized relative to the intensity of Coomassie blue protein staining (loading).

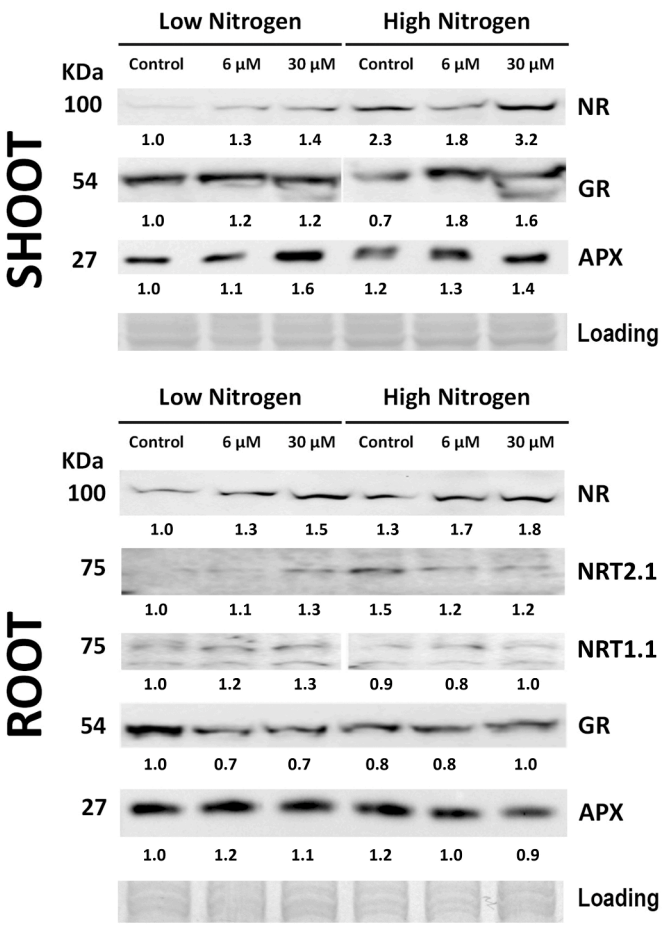


**Fig. 6.** Western-blot immunodetection using specific antibodies against nitrate reductase (NR), nitrate transporters (NRT2.1 and NRT1.1), glutathione reductase (GR), ascorbate peroxidise (APX) in protein extracts prepared from shoots and roots of *Medicago sativa* grown in low nitrogen (2 mM $NO_3^-$) and high nitrogen (12 mM $NO_3^-$), treated with 0 (control), 6 and 30 µM 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).

which determine the tolerance to metal-induced oxidative stress (Hernández et al., 2015). Similar pattern was reported in barley seedlings subjected to N-deficiency under Cd stress, which had significantly lower levels of GSH than N-fed seedlings (Finkemeier et al., 2003). 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., 2021, 2014a, 2014b, ). Stronger cellular damage caused by oxidative stress could, in turn, affect membrane integrity and permeability, altering metal compartmentation, which could explain the accumulation of toxic metals in more susceptible plants (Sharma and Dietz, 2009). On the other hand, 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).

### 4.4. *High $NO_3^-$ nutritional level attenuate Hg-induced oxidative stress*

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). In addition, immunodetection showed that there was a second GR band with higher electrophoretic mobility under 30 µM Hg stress in shoots of LN and HN alfalfa plants (Fig. 6). This is consistent with the known presence of several GR isoforms in plants (Anjum et al., 2012). The pattern of GR isoforms also changed in wheat plants treated with 100 µM for 7 to 21 days, with bands of high electrophoretic mobility appearing under metal stress (Yannarelli et al., 2007), implying that both differential transcription of GR genes encoding cytosolic and cytosolic isoforms, and/or post-translational protein modifications may occur under Hg stress.

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., 2021, 2014b, 2013, 2009). 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 activity in alfalfa plants grown in semi-hydroponic conditions, without changes in the amount of APX, probably owing to post-translational regulation mechanisms (Ortega-Villasante et al., 2007).

High supply of $NO_3^-$ prevented toxic effects caused by Hg, in the same manner that ameliorated damages observed by toxic metals in different plant species, as follows: 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). 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), when supplied with high levels of N fertilizers. *Matricaria chamomilla* plants well nourished with N also showed higher proportion of reduced GSH, improved APX activity and lower phenolic metabolism under Pb stress (Kováčik et al., 2020).

## 5. Conclusion

The $NO_3^-$ nutritional status of plants might be important to improve their tolerance to Hg, particularly at moderate levels of Hg (*i.e.* 6 μM), according to the stress parameters assessed. In addition, low $NO_3^-$ nutrition led to significant accumulation of Hg possibly because of stronger cellular damage and/or metal distribution in the plant tissues. However, more work is needed for a better understanding of the mechanisms of tolerance involved, where modulation of oxidative stress may be a relevant aspect. Future research, probably using isotopic N tracing and analysis of nitrogen reactive species, will improve our understanding of the mechanisms of tolerance to Hg, which in turn will help to optimise the use of plants in phytotechnologies to clean up metal polluted soils or stabilise them to avoid erosion and loss of fertility.

## Declaration of Competing Interest

The authors declare no competing interests.

## Data Availability

No data was used for the research described in the article.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2023.100131.

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