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Influence of nitrate fertilization on Hg uptake and oxidative stress parameters in alfalfa plants cultivated in a Hg-polluted soil

Sandra Carrasco-Gil[a,b], María Estebaranz-Yubero[b], David Medel-Cuesta[b], Rocío Millán[a,1], Luis E. Hernández[b,*,1]

[a] Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Avd. Complutense, 22, 28040 Madrid, Spain
[b] Laboratory of Plant Physiology, Department of Biology, Universidad Autónoma de Madrid, 28049 Madrid, Spain

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ABSTRACT

The mineral nutrition status of plants might affect the accumulation of heavy metals, which in turn could be reflected in altered plant sensitivity. Very little information is available about the effect of the nitrogen nutritional status on mercury (Hg) toxicity. We compared physiological stress parameters sensitive to Hg in alfalfa (*Medicago sativa*) plants grown in Hg-polluted soils that were amended with two different fertilizers: without NO_3^- (PK) or with NO_3^- (NPK). Several parameters of oxidative stress, such as antioxidative enzymatic activities, lipid peroxidation, and chlorophyll content were analysed. The study was carried out in a green house with soil from an agricultural land plot located in Almadén (Spain), which contained an average Hg concentration of 12.4 mg kg^{-1}, most of it found mainly as cinnabar (HgS). Our results suggest that nitrogen supply prevents oxidative stress in roots, but may improve root development and increase the uptake of Hg from the soil above safety consumption limits. This work highlights the importance of proper nitrogen fertilization towards future phytoremediation applications with alfalfa plants.

1. Introduction

Mercury poses a serious threat to the environment and to human health, and as a result of more strict environmental policies its ore extraction, metal processing and trade are under severe restrictions in the European Union (European Commission, 2008). Amalgamation of gold with Hg in mining, chlor-alkali industry, dye production, elaboration of lamps and electronic equipment are examples of uses of Hg responsible of severe environmental problems in many regions of the World (UNEP, 2011). The accumulation of Hg in several ecosystems is aggravated by its long-term persistence, which led to dramatic human health problems as occurred in Minamata Bay (Japan), caused by the bioaccumulation and biomagnification of Hg in the food chain (Dushenkov et al., 1997). Soils are the sink of Hg in polluted urban and agricultural areas, accumulation that may result in structure degradation, crop yield reduction, and poor quality of agricultural products (Long et al., 2002).

The accumulation of Hg in plants is influenced greatly by the Hg available in soils. Mercury is absorbed by the root system and is translocated to the shoot (Patra et al., 2004), although most portion of Hg absorbed remain immobilised in roots as has been observed by a number of studies (Rellán-Álvarez et al., 2006; Sobrino-Plata et al., 2009; Carrasco-Gil et al., 2011). Nevertheless, Hg can accumulate in the aerial parts of the plants by several ways: (i) translocation from the roots once Hg is taken up from the soil normally as Hg^{2+}, (ii) via the stomata from the atmosphere as volatile Hg^0 (or even as Hg^{2+}), (iii) through adsorption of particulate Hg deposited on leaves or stem surfaces (Lindberg et al., 1979; Frescholtz and Gustin, 2004).

It is thought that the high toxicity of Hg is mainly due to the high affinity for sulfhydryl groups (–SH) of proteins and other S-containing molecules (i.e. biothiols). Once Hg binds proteins, their tertiary and quaternary structures are disturbed, losing their function (Clarkson, 1997). Several visual symptoms may develop under Hg stress: stunned seedling growth, reduction in rhizosphere expansion, decrease in the stem length, or diminution in chlorophyll content (Patra and Sharma, 2000). The induction of oxidative stress is one of the several phytotoxic effects of Hg that occur rapidly in plants exposed to Hg. Accumulation of moderate to high levels of Hg in the plants may stimulate the production of reactive oxygen species (ROS), which leads to damage of proteins and membrane lipids (Rellán-Álvarez et al., 2006; Ortega-Villasante et al., 2005). In addition, the onset of oxidative stress may produce the alteration in the concentration of glutathione (GSH), an important antioxidant

* Corresponding author. Tel.: +34 914978191; fax: +34 914978344.
E-mail address: luise.hernandez@uam.es (L.E. Hernández).
[1] R.M. and L.E.H. contributed equally to this work.

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that forms part of the ascorbate–glutathione ROS scavenging pathway (Mittler, 2002). The toxic action of Hg compounds may also be related to an alteration of antioxidant enzyme activities such as superoxide dismutase (SOD), ascorbate peroxidise (APX), and glutathione reductase (GR; Ortega-Villasante et al., 2007; Zhou et al., 2007). Of these stress parameters, we have studied in particular the responses of GR, enzyme very sensitive to Hg accumulation in alfalfa roots (Sobrino-Plata et al., 2009). In addition, GSH play a second important role in metal detoxification: is the precursor of phytochelains, a group of Cys rich peptides capable of form complexes with several metal cations (Cobbett and Goldsbrough, 2002). In particular, alfalfa plants accumulate homoglutathione (hGSH), a GSH homologous biothiol that occurs in certain legume plants (Matamoros et al., 1999); in consequence alfalfa synthesizes also homophytochelatins (hPCs) under metal stress (Sobrino-Plata et al., 2009).

The present study was performed using soil from Almadén area (Ciudad Real, Spain), which constitutes the largest and most unusual natural concentration of Hg in the World, where mining activities had been carried out for centuries (Higueras et al., 2003). According to Higueras et al. (2000), the major proportion of Hg existing in Almadén originates from cinnabar (HgS) bed rock weathering and erosion, anthropogenic dispersion from abandoned mineral dumps and metal extraction and refining. In spite of the low solubility of HgS, Hg may be mobilised and absorbed by plants and animals, as some studies have shown. Millán et al. (2006) found that there was a high correlation between available Hg in soil and Hg accumulation in plants sampled in ten different locations in the Almadén mining area. Due to changes in land usage after the banning of mining activities in Almadén, several crop plants are being introduced to develop new economic activities based on agriculture in polluted soils. Consequently, it is important to evaluate the potential risk for human health and the environment considering the uptake and distribution of Hg in suitable crops (Berzas-Nevado et al., 2003; Higueras et al., 2003). With this aim, some studies were performed growing plants under greenhouse conditions in pots containing polluted soil from Almadén sites (Sierra et al., 2008a,b, 2009), and showed that Hg may accumulate in the harvestable parts of crops and represent a risk for consumers, as occurred with *Vicia sativa*.

The use of fertilizers and/or soil amendments is sometimes necessary to establish crops or to revegetate a degraded area, but these treatments may influence metal bioavailability and phytoextraction processes (Cunningham and Ow, 1996). The mineral nutrition status influence greatly the capability of plants to accumulate toxic heavy metals like Cd, which has been studied recently in a number of crops to minimize the metal concentration (see review by Sarwar et al., 2010). Nitrogen (N) is very limiting for plant biomass production among other macronutrients, and is commonly assimilated as nitrate (NO_3^-). Soils are usually low in N availability and most crops would require certain degree of N fertilization (Mengel and Kirby, 2001). On the other hand, heavy metals like Cd may disturb N assimilation by inhibiting the activities of the enzymes involved in this process, and affecting the uptake of NO_3^- (Hernández et al., 1997; Chaffei et al., 2004). Therefore studying the relation between N status and heavy metals toxicity is required. In addition, it is known that nitrogen fertilization alter soil solution equilibrium (Alloway, 1995), and root morphological parameters (Barber, 1995), which might increase Hg availability and potentially augment Hg-plant uptake. To our knowledge, there are only few reports focused on the interactive effect of nitrogen fertilizers with Cd accumulation in plants. Interestingly, Cd concentration in durum wheat grains augmented remarkably in soils fertilized with nitrogen (Gao et al., 2010). Similar results were found in Cd concentration of winter wheat grains, which augmented in parallel with N availability in soil (Wångstrand et al., 2007). In consequence, it is relevant the understanding of how nitrogen fertilization modify the ability of crops to uptake and translocate Hg, information that is nowadays scarce.

Alfalfa is a leguminous plant that has the ability to assimilate atmospheric nitrogen (N_2) thanks to the association with symbiotic N_2-fixing bacteria (i.e. *Sinorhizobium meliloti*). Therefore, these plants are normally able to obtain their own nitrogen requirements, and are able to grow in poor soils (Garau et al., 2005). In fact, these plants are used to improve soil fertility, and decreases fertilization needs (Zahran, 1999). In the present work, we compared physiological stress parameters sensitive to Hg in alfalfa plants grown in Hg-polluted soils obtained from Almadén, that were amended with two different fertilizers: without NO_3^- (PK) or with NO_3^- (NPK). Mercury accumulation and physiological stress parameters, like chlorophyll concentration, non-protein thiols content, lipid peroxidation level and activity of GR and APX antioxidant enzymes, were assessed in plants subjected to two different nitrogen nutritional statuses.

2. Material and methods

2.1. Physical and chemical soil analysis

The soil for this study was collected from "Castilseras", a site where agricultural activities have been carried out. This plot is in the area of influence of "El Entredicho" mine located in Almadén (Ciudad Real, Spain). The soil was sampled in ten points along the plot collecting every sample from the first 30 cm. The soil was air dried and sieved to 2 mm. The texture of the soil was determined by granulometric analysis according to Sierra et al. (2009). The soil pH was measured with a pH-meter Orion 525a in 2:1 distilled water:soil (v/v). Electrical conductivity was determined in 2:1 (v/v) extraction ratio of soil and distilled water using a Crison-MICRO CM 2200 conductivity/temperature meter. Organic matter content and the CEC (using ammonium acetate) were measured according to Sierra et al. (2008b). Total C/H/N was analysed using an Elemental Analyzer CHN-600 Leco (St. Joseph, Michigan, MI, USA) based on a dry combustion method. Available macronutrients were extracted with the procedure described by Soltanpour and Schwab (1977) and analysed using inductively coupled plasma-mass spectroscopy (ICP-MS).

2.2. Experimental design, plants and growth conditions

The experiment was performed under greenhouse conditions. The soil sieved to 2 mm was mixed with perlite and sand in equal proportions (v/v) and placed in 48 pots with a total volume of 3 L. And then, they were watered with 0.5 L of water to initiate the drainage. Half of the plants (24 pots) were fertilized with a NPK mixture (30:110:100; adequate NO_3^-), where nitrogen was added as NH_4NO_3. A second batch (24 pots) was only fertilized with a PK mixture (110:100, poor NO_3^-). Phosphate and potassium were also added as K_2HPO_4 and KH_2PO_4 forms. Following the agronomic practices in Almadén area, plants were sown in April. Two weeks before sowing, the pots were ground fertilized with phosphate and potassium doses. Nitrogen was applied at the moment that alfalfa seedlings were sown to avoid excess drainage due to the high solubility of NH_4NO_3.

Alfalfa (*Medicago sativa* cv. Aragon) seedlings were surface sterilized for 5 min in 5% (v/v) commercial bleach. After rinsing several times with distilled water, seeds were soaked overnight at 4 °C. Fifteen homogeneous selected seedlings were transferred to each pot where they were germinated. Periodic irrigations with tap water were performed according to the water requirements of the culture.

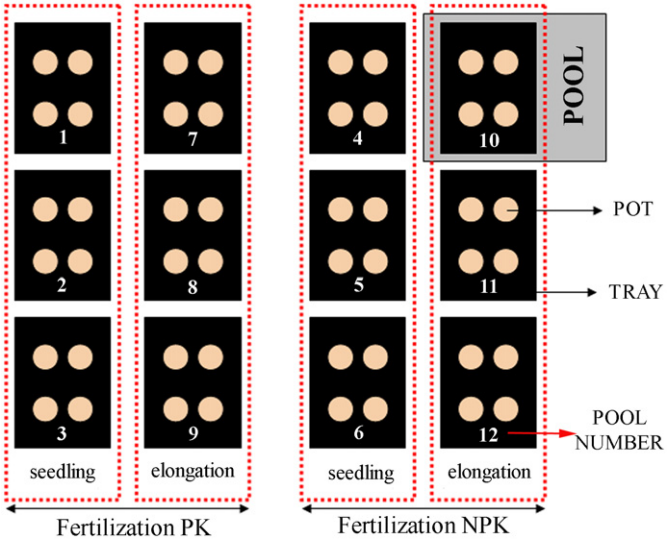

Fig. 1. Pots distribution in the greenhouse with respect to the fertilization applied and the growth stages sampled.

2.3. Sampling

Sampling was performed at two different growth stages: seedling (three weeks after sowing) and elongation (seven weeks after sowing), which was close to the first "cut in green" for forage uses. Soil and plants were collected in 12 pools formed by the mixing of four pots (see Fig. 1). Plants were divided in shoot and root and placed into beakers and rinsed several times with 10 mM Na_2 EDTA solution to remove superficial Hg. Then length and fresh weight of roots and shoots were measured and stored at −80 °C or air dried until analysis. The soils were air dried and stored at room temperature until analysis.

2.4. Mercury determination

Solid samples of soil and plants (roots and shoot) were air dried and ground with mortar and pestle. Available Hg was extracted following the procedure described by Soltanpour and Schwab (1977). Hg concentration was measured using an 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: SRM 2709 (San Joaquin agricultural soil, 1.40 ± 0.08 mg kg^{-1} of Hg), BCR-CRM 62 (olive leaves, 0.28 ± 0.02 mg kg^{-1} of Hg), BCR-CRM 150 (spiked skim milk powder, 0.0094 ± 0.0017 mg kg^{-1} of Hg). The experimental value determined for 5 measurements of each BCR was: 1.34 ± 0.05 mg kg^{-1}, 0.30 ± 0.01 mg kg^{-1}, and 0.0102 ± 0.0009 mg kg^{-1}, respectively.

To evaluate the alfalfa capacity to uptake the Hg from the soil, bioaccumulation factor (BAF) was calculated as an index of Hg accumulation in plants. According to Tu and Ma (2002), the BAF factor is the ratio between the total Hg concentration in shoot or root and Hg available in soil. To study the mobility of Hg inside the plant the ratio between Hg concentration in shoot and Hg concentration in root was calculated according to Baker (1981).

2.5. Nitrogen in plants

The nitrogen in plant tissue was determined by Kjeldahl digestion, which converts organic N (proteins and nucleic acids) to inorganic ammonium (NH_4^+) for its posterior determination. The digestion was performed into the wet digestor system B-440 (Büchi, Flawil, 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 1:30 h, and then cooled for 30 min. NH_4^+ was distilled after the addition of 25 ml of a 32% NaOH (v/w) solution in a K-355 distillation unit (Büchi, Flawil, Switzerland). The concentration of NH_4^+ released in the resulting alkaline mixture was calculated by back titration of a 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 (Metrohm, Zofingen, Switzerland).

2.6. Metal stress indexes

Lipid peroxidation was estimated by the formation of malondialdehyde, a by-product of lipid peroxidation that reacts with thiobarbituric acid (TBA). The resulting chromophore absorbs at 535 nm, and the concentration was calculated directly from the extinction coefficient of 1.563105 M cm^{-1}. Ground frozen tissue (0.1 g) was transferred to a screw-capped 1.5 ml Eppendorf tube, and homogenized following addition of 1 ml of TCA–TBA–HCl reagent (15% (w/v) trichloroacetic acid (TCA), 0.37% (w/v) 2-TBA, 0.25 M HCl, and 0.01% butylated hydroxytoluene). After homogenization, samples were incubated at 90 °C for 30 min in a hot block, then chilled in ice, and centrifuged at 12 000 g for 10 min. Absorbance was measured in a UV-2401 PC spectrophotometer (Shimadzu Corporation, Japan) at 535 nm and 600 nm, the last one to correct the non-specific turbidity.

Chlorophylls were extracted from 0.05 g of frozen leaves with 10 ml 80% (v/v) acetone using a mortar and pestle. Homogenates were filter and absorbance was measured in a UV-2401 PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 645 and 663 nm. Total chlorophyll concentrations were calculated according to the formula described by Arnon (Porra, 2002):

Total chlorophyll (mg g^{-1} FW) = chlorophyll a + chlorophyll b, where:

$$\text{Chl}a\left(\frac{\text{mg}}{\text{gFW}}\right) = [(12.7 \times A_{663}) - (2.69 \times A_{645})]\frac{\mu\text{g}}{\text{mL}} \times \frac{10\,\text{mL} \times \text{mg}}{0.05\,\text{gFW} \times 10^3\,\mu\text{g}}$$

$$\text{Chl}b\left(\frac{\text{mg}}{\text{gFW}}\right) = [(22.90 \times A_{645}) - (4.68 \times A_{663})]\frac{\mu\text{g}}{\text{mL}} \times \frac{10\,\text{mL} \times \text{mg}}{0.05\,\text{gFW} \times 10^3\,\mu\text{g}}$$

2.7. Glutathione reductase and ascorbate peroxidase

Glutathione and ascorbate peroxidise 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, freshly prepared by mixing 10 ml extraction buffer (30 mM 3-(N-morpholino)propanesulfonic acid at pH 7.5, 5 mM Na_2-EDTA, 10 mM dithiothreitol, 10 mM ascorbic acid, 0.6% polyvinylpyrrolidone, 10 μl 100 mM phenylmethylsulfonyl fluoride and 1 ml protease inhibitors cocktail-P2714 (Sigma-Aldrich, St. Louis, MO, USA)). After centrifugation (14,000 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 analysis was 15 μg and 10 μg of seedling and elongation shoot extract, and 5 μg and 3 μg. of seedling and

elongation root extract, respectively. Protein loading for APX detection was 30 μg of seedling and elongation shoot extract and 7 μg of seedling and elongation root extract. GR activity was revealed with the procedure developed by Kaplan and Beutler (1968), with minor modifications. Gel slabs were incubated in GR staining solution (250 mM Tris–HCl buffer at pH 7.5, supplemented with 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; GSSG). Bands corresponding to diaphorase activity (higher electrophoretic mobility than GRs) were identified by incubating a second gel in a staining solution without GSSG.

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 buffer at pH 7.0. The APX activity was detected with 0.5 mM nitroblue tetrazolium and 10 mM TEMED in 50 mM phosphate buffered at pH 7.8. An EDAS 290 Digital Imaging System (Kodak, Rochester, NY, USA) was used to take the gel pictures that were processed with Kodak 1D Image Analysis Software (version 3.6). Regions of interest (ROIs) were selected with the same surface and pixel intensity was adjusted to the background.

2.8. Preparation of non-protein thiol standard solutions

Biothiol stock standard solutions containing 50 mM of glutathione, homoglutathione, cysteine (Cys), N-acetyl cysteine (N-AcCys), and 2 mM of γ-(Glu-Cys)$_2$-Ala (hPC_2), and γ-(Glu-Cys)$_3$-Ala (hPC_3) were prepared in analytical-grade type I water (Milli-Q Synthesis, Millipore). Aliquots of the stock solutions were immediately frozen in liquid N_2, lyophilized and stored at −80 °C. Standards of 0.5 mM GSH, Cys and N-AcCys and standards of 0.1 mM hPC_2 and hPC_3 were injected in the HPLC to set the retention times.

2.9. Analysis on non-protein thiols

Non-protein thiols were analysed by High Performance Liquid Chromatography (HPLC) following the procedure described by Ortega-Villasante et al. (2005). 0.1 g of frozen tissue was ground in liquid N_2 and 15 μl of 5 mM N-acetyl cysteine (N-AcCys) was spiked as internal standard prior homogenization with 300 μl of a extraction buffer (0.1 M HCl, 1 M EDTA, 5% metaphosphoric acid, 1% polyvinylpolypyrrolidone) to quantify the thiols. The homogenate was centrifuged twice for 15 min at 12 000 g and 4 °C in Eppendorf tubes. The clear supernatant was transferred to a boron–silica glass injection vial. Separation and detection of the thiols was carried out using the method described by Sobrino-Plata et al. (2009). Extracts (100 μl) were injected in a Mediterranea Sea18 column (5 μm, 250 mm × 4.6 mm; Teknokroma, Spain), using an Agilent 1200 HPLC system (Santa Clara, CA, USA). The mobile phase was built using two eluents: A (dH_2O:acetonitrile (v/v) in 98:2 ratio plus 0.01% TFA) and, B (dH_2O:acetonitrile (v/v) in 2:98 ratio plus 0.01% TFA). The gradient program, as for % solvent B, was: 2 min, 0%; 25 min; 25%; 26 min, 50%; 30 min, 50%; 35 min, 0%; 45 min, 0% Thiols were detected after post-column derivatization with Ellman's reagent (1.8 mM 5,5-dithio-bis, 2 nitrobenzoic acid in 300 mM K-phosphate, 15 mM EDTA at pH 7.0), in a thermostatic 1.8 ml reactor at 38 °C. The derivative compound, 5-mercapto-2-nitrobenzoate, had and absorbation maximum at 412 nm. To identify the retention time of non-protein thiols of extract tissue before quantification, commercial biothiol standards (Cys, GSH, hGSH, hPC_2 and hPC_3) were run previously. Data were processed with the Agilent Chemstation software.

2.10. Statistical analysis

Statistical analysis was performed with the software package SPSS for Windows (version 15.0), by using an ANOVA with Tukey test when the signification in Levene test was >0.05 or Welch with Games–Howell test when the signification in Levene test was <0.05. Results were mean of at least three independent replicates ± standard deviation, with significant differences between treatments at $p < 0.05$.

3. Results

3.1. Soil

The physical and chemical parameters of the soil collected from Almadén had a loamy sand texture according to the USDA (Supplemental Table 1). This soil had pH 6.5 and a very low electric conductivity (EC), so it was classified as moderately acidic and not saline (Boul et al., 2003). Organic matter content, C/N ratio, total nitrogen and cation exchange capacity (CEC) parameters were low for common agricultural soils. The content of Mg^{2+}, Na^+ and K^+ were slightly low, but Ca^{2+} concentration was adequate for the nutritional needs of most crops (Tisdale et al., 1985). The total Hg concentration in the soil was 12.48 mg kg^{-1}DW in average, being 0.007 mg kg^{-1} DW the concentration of available Hg.

The total Hg concentration of the substrate placed in each pot was within the range of 2.7–4.0 mg kg^{-1} DW, whereas available Hg was within the range of 2.2–6.6 μg kg^{-1} DW, representing less than 0.2% of the total Hg (Table 1). After the experiment, NPK and PK did not alter both levels of Hg, remaining stable at the seedling and plant elongation sampling periods (data not shown).

3.2. Mercury concentration and distribution in alfalfa plants

According to the data showed in Table 2, the concentration of Hg in roots was 5–7 times higher than in shoots at the seedling stage. This difference was attenuated in alfalfa at the elongation stage, being the concentration in roots over 2.5-fold higher than in shoots. Mercury concentration decreased in roots and shoots during the elongation stage, possibly caused by a dilution effect associated to the augment in plant biomass. It should be noted that nitrogen fertilization affected only the concentration of Hg in roots at the seedling stage, being significantly higher with NPK fertilization. To highlight the mobility of Hg inside the plant, the ratio between Hg concentration in shoots and roots was calculated, being in all cases below 0.5, confirming that Hg was mainly accumulated in roots (Table 2).

The bioaccumulation factor was used as an index of Hg accumulation in plants to evaluate the capacity of alfalfa to uptake Hg from the soil. BAF was calculated as a ratio between the concentration of Hg in each organ (root and shoot) and the concentration of Hg available in the soil (Table 2). As already stated, alfalfa plants showed a remarkable preference to accumulating Hg in roots, since its BAF values were in the range of 2–7 times higher than in shoots depending on the stage of growth. With respect to fertilization, BAF values were much higher in alfalfa plants grown with the NPK fertilization, value that decreased at the elongation stage.

3.3. Biometric parameters

Plants did not show external symptoms of toxicity due to Hg exposure (i.e. chlorosis or darkened roots; Fig. 2C). However, NO_3^- fertilization affected the length of plants at the seedling stage: plants grown with NPK were significantly larger than plants grown with PK fertilization. However, at the elongation stage, the growth of PK treated plants reached almost the size of NPK treated plants, when differences became not significant (Fig. 2A and C). A similar trend was observed in the fresh weight of shoots and roots, which augmented in NPK-fertilized plants at the seedling stage,

Table 1
Total Hg (mg kg^{-1} DW), available Hg (μg kg^{-1} DW) and available Hg (%) in the soil of the pots treated with a PK fertilizer (without NO_3^-) or a NPK fertilizer (with NO_3^-) after harvesting the alfalfa plants at different stage of growth (seedling and elongation). Data are average of three independent replicates (±SD).

Stage of growth	Fertilization	Total Hg (mg kg^{-1} DW)	Available Hg (μg kg^{-1} DW)	Available Hg (%)
Seedling	PK	3.1 ± 0.5	2.6 ± 0.6	0.08
	NPK	4.0 ± 0.3	2.2 ± 0.1	0.06
Elongation	PK	2.7 ± 0.8	6.6 ± 1.2	0.24
	NPK	3.3 ± 0.2	2.4 ± 0.1	0.07

Table 2
Total Hg (mg kg^{-1} DW) in root and shoot, ratio ([Hg_{shoot}]/[Hg_{root}]), and bioaccumulation factor BAF ([Hg organ]/[Hg soil available]) of alfalfa plants harvested at seedling and elongation growth stages, fertilized with NO_3^- (NPK) or without NO_3^- (PK). Data are average of three independent replicates (±SD), and different letters denote significant differences at $p < 0.05$.

Growth stage	Treatment	Hg (mg kg^{-1} DW)		Ratio	BAF	
		Root	Shoot	Shoot/root	Shoot/soil	Root/soil
Seedling	PK	1.28 ± 0.41^a	0.24 ± 0.02^a	0.19	92.3	492.3
	NPK	2.12 ± 0.37^b	0.31 ± 0.04^a	0.14	140.9	963.6
Elongation	PK	0.28 ± 0.09^c	0.12 ± 0.02^b	0.43	18.9	42.4
	NPK	0.36 ± 0.03^c	0.12 ± 0.04^b	0.33	50.0	150.0

although differences were only statistically significant when shoots were compared (Fig. 2B and C).

3.4. Nitrogen content in alfalfa plants

At the seedling stage, plants fertilized with NPK accumulated a significant higher amount of NH_4^+ per plant than plants fertilized only with PK (Fig. 3A). However, at the elongation stage there were no differences in the amount of NH_4^+ per plant. Roots sampled at the elongation stage were completely nodulated, with pinkish nodules that possibly harbour an effective symbiotic nitrogen-fixation metabolism. Apparently, the soils collected at Almadén had enough *S. meliloti* inoculum to allow the establishment of symbiotic N_2-fixation, which would provide sufficient organic N to maintain the requirements of alfalfa plants in the absence of an N fertilizer.

3.5. Oxidative stress parameters

The nitrogen fertilization did not affect significantly the concentration of chlorophyll in alfalfa plants, although there was a slight diminution in chlorophyll content at the seedling stage with the

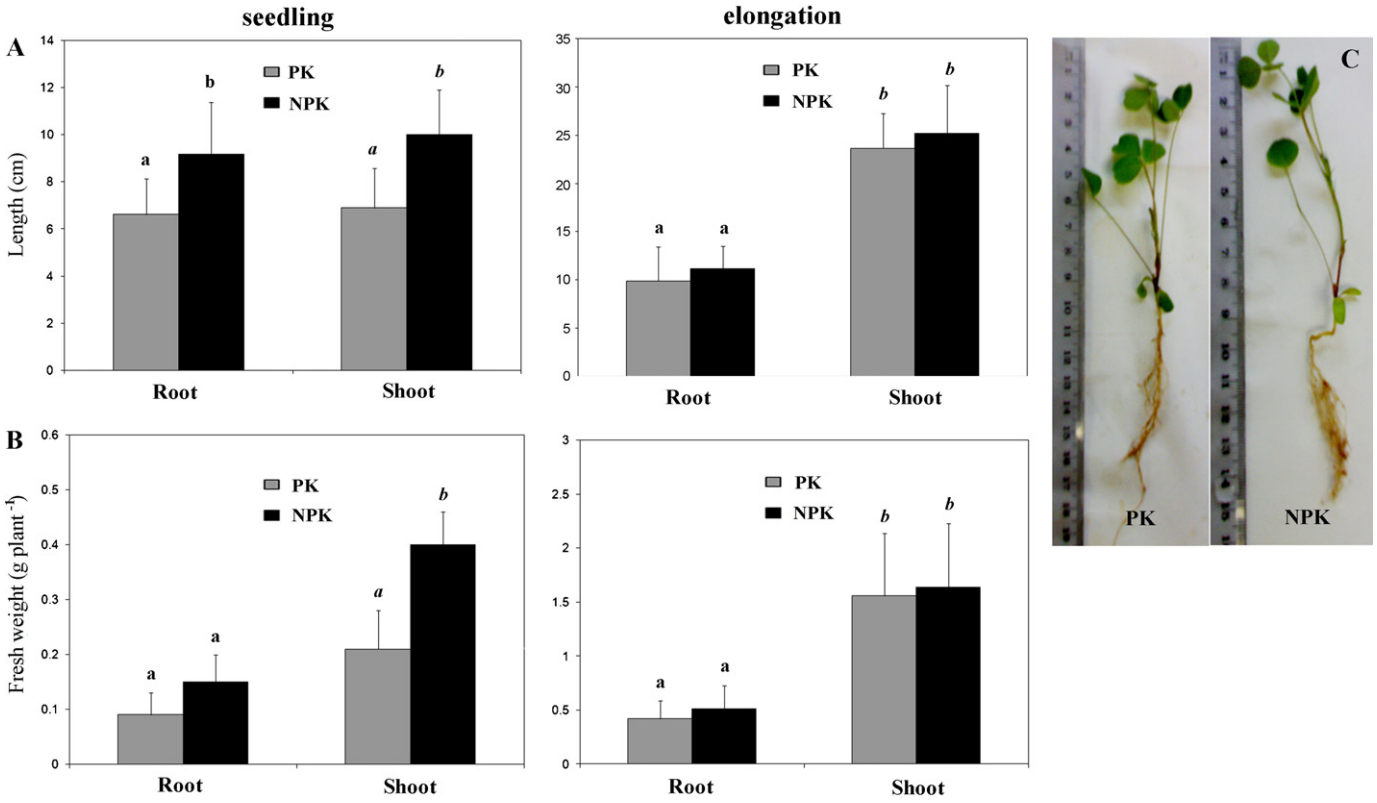

Fig. 2. Length (cm plant^{-1}) (**A**) and fresh weight (g plant^{-1}) (**B**) of roots and shoots of alfalfa plants harvested at seedling and elongation growth stages, fertilized with NO_3^- (NPK) or without NO_3^- (PK). (**C**) Detail of alfalfa plants collected at the seedling stage. Data are average of three independent replicates (±SD), and different letters denote significant differences at $p < 0.05$.

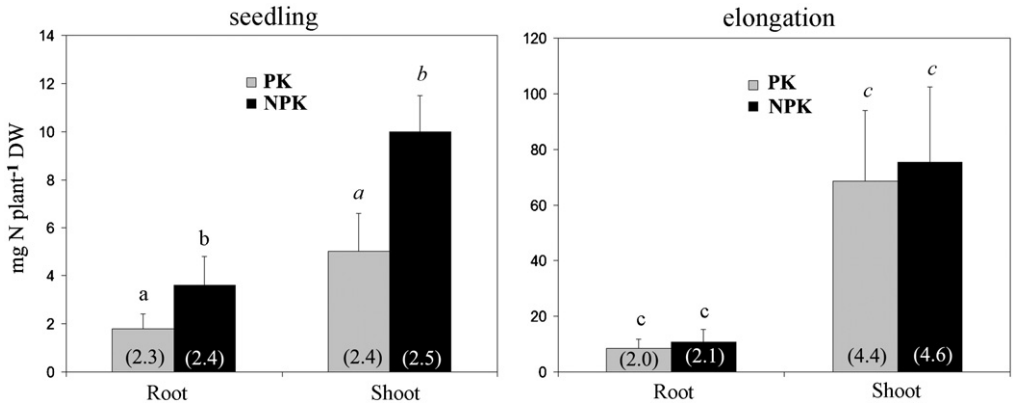

Fig. 3. Nitrogen content (mg NH_4^+ $plant^{-1}$) and nitrogen percentage (%; between parenthesis) in root and shoot of *Medicago sativa* harvested at seedling and elongation growth stages, fertilized with NO_3^- (NPK) or without NO_3^- (PK). Data are average of three independent replicates (±SD), and different letters denote significant differences at $p < 0.05$.

PK treatment (Fig. 4). Malondialdehyde (MDA) content was determined as an index of lipid peroxidation in alfalfa roots (Fig. 5), being this level higher in plants fertilized with PK at the seedling stage. However, the differences in MDA concentration between plants treated with or without NO_3^- were lower at the elongation stage, indicating that the oxidative stress decreased under PK fertilization when plant biomass augmented.

The activity of APX and GR antioxidant enzymes was determined by in gel staining after non-denaturing polyacrylamide gel electrophoresis. Under the experimental conditions used, shoot GR and APX activities were not affected by NO_3^- fertilization at both growth stages (Fig. 6A). However, GR and APX activity in roots increased remarkably in plants that were fertilized without NO_3^- (PK) compared with those fertilized with NO_3^- (NPK; Fig. 6B). Apparently, these differences were also attenuated in plants with higher biomass, since APX and GR activities of PK-treated plants decreased at the elongation stage compared with the seedling stage (Fig. 6B).

3.6. Analysis of non-protein thiols

The non-protein thiol content was analysed in shoots and roots of alfalfa seedling considering the different nitrogen nutritional status of the plants, as it is know the effect of adequate nitrogen fertilization in the assimilation and metabolisation of sulphur containing metabolites in plants (Kopriva and Rennenberg, 2004). At

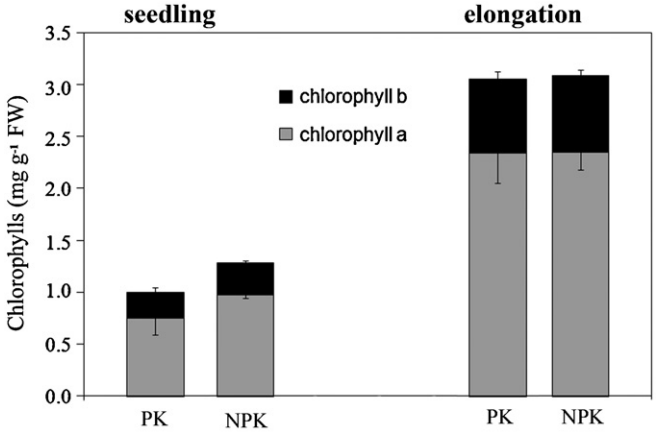

Fig. 4. Chlorophyll concentration (mg g^{-1} FW) in leaves of *Medicago sativa* harvested at seedling and elongation growth stages, fertilized with NO_3^- (NPK) or without NO_3^- (PK). Data are average of three independent replicates (±SD). There were not significant differences between treatments at $p < 0.05$.

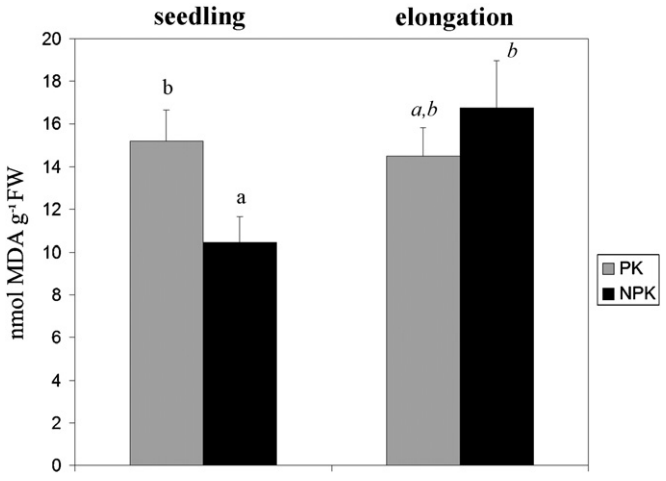

Fig. 5. Malondialdehyde content (nmol MDA g^{-1} FW) in *Medicago sativa* roots harvested at seedling and elongation growth stages, fertilized with NO_3^- (NPK) or without NO_3^- (PK). Data are average of three independent replicates (±SD), and different letters denote significant differences at $p < 0.05$.

the seedling growth stage, shoot concentration of hGSH in plants treated with NPK was 384 ± 85 nmol g^{-1} FW, while in plants treated with PK was 347 ± 33 nmol g^{-1} FW. Therefore, it was slightly higher in plants fertilized with NO_3^-, although not significantly different ($p < 0.05$). hGSH content in roots was three times lower (100 nmol g^{-1} FW), and no differences were observed between NPK and PK fertilized plants. At the elongation stage, there were no differences between treatments, and the concentration was of the same order as in plants at the seedling stage (data not shown). We could not detect accumulation of PCs or hPCs, at any growth stage in the plants fertilized with NPK or PK.

4. Discussion

The physical and chemical characteristic of the soil obtained from Castilseras (Almadén, Spain) revealed that, despite its low C/N ratio, low CEC and poor nutrient content (in particular N), was adequate to sustain a crop of alfalfa (Walworth and Sumner, 1990). It is feasible that nutrients were depleted by previous agricultural activities, as cereals were cultivated for several seasons in the collected soil. It is well known that cereal crops exhaust the nutrients of soils, and supply of fertilizers and amendments are required for maintain proper crop yield in subsequent seasons (Mengel and Kirby, 2001). Therefore, the low N background found in the collected

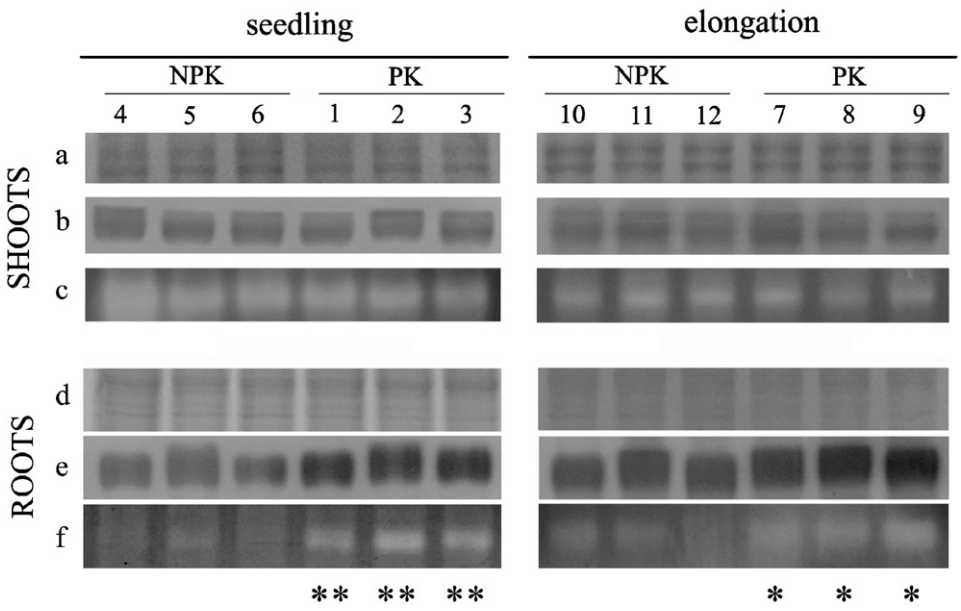

Fig. 6. Glutathione reductase (GR) and ascorbate peroxidise (APX) in gel activity in shoots and roots of *Medicago sativa* plants harvested at seedling and elongation growth stages, fertilized with NO_3^- (NPK) or without NO_3^- (PK). Numbers correspond to the pools sampled (see Fig. 1). (a and d) Coomassie-blue general staining of proteins to show equivalent loading of protein (10–30 μg); (b and e) GR activity; and (c and f) APX activity. More asterisks (*) represents larger differences in bands intensity between treatments.

soil (0.11%) explains the beneficial effect of NH_4NO_3 fertilization in alfalfa biomass observed at the seedling growth stage (Fig. 2).

The total Hg content of the original soil used in this study was twenty five times higher than is usually found in natural soils, where the levels ranges from 0.01 to 0.5 mg kg^{-1} DW (Alloway, 1995). Taking into account that the study area belongs to a region rich in Hg deposits and with intense and prolonged mining activities, the value of 12.48 mg kg^{-1} DW should be considered normal, even moderate as there are plots in the Almadén area with concentrations up to 34,000 mg kg^{-1} DW (Gray et al., 2004; Bernaus et al., 2006). The concentration of available Hg in the initial soil was a 0.06% of the total Hg, in agreement with the level found previously in other soils from Almadén (Moreno-Jiménez et al., 2006; Sierra et al., 2008b). Therefore, despite the high total Hg concentration of the soil, only the 0.2% was available for the plants. The range of total Hg concentration and available Hg measured in the substrate used for our experiments was three times lower than the total and available Hg in the initial soil. This was due to the dilution of the original soil with perlite and sand in equal proportions to avoid clogging and compaction, in agreement with experiments carried out under similar conditions (Sierra et al., 2008a).

Mercury accumulated to a higher extent in roots, as found in many wild and crop plants (Patra et al., 2004). In all cases, the ratio between shoots and roots Hg concentration was less than 0.5. Therefore, according to Baker (1981) alfalfa presents typical excluder behaviour; as this ratio was lower than 1. The addition of nitrogen fertilizer augmented significantly the concentration of Hg in alfalfa roots at the seedling stage. The BAF parameter highlighted the increased effectiveness of NPK-fertilized plants to remove Hg from the soil. This result was somehow surprising, as increased N supply would decrease heavy metal concentration due to a dilution effect (Sarwar et al., 2010). Indeed, in plants grown in hydroponics where plants have all nutrients available there was a dilution in the concentration of Cd in wheat plants (Landberg and Greger, 2003). However, other teams that studied the effects of N nutritional status on plants grown in soils observed that fertilization with NO_3^- had a positive effect in increasing the concentration of Cd in roots and plant aerial parts (Mitchell et al., 2000; Schwartz et al., 2003; Xie et al., 2009). Gao et al. (2010) evaluated the effect of nitrogen fertilizers on Cd concentration in the grain of durum wheat under field conditions, and found also that the supply of nitrogen fertilizer increased Cd uptake and accumulation in different parts of the plant, such as in the grain. Similarly, Barrutia et al. (2009) found that *Rumex* spp. accessions grown in a metalliferous soil were capable of accumulating higher concentrations of Zn and Cd with a NPK fertilization.

Several hypotheses have been drawn to explain the increase in Cd concentration in plants well fertilized with N. One possible explanation could be the effect of some N fertilizers on soil pH, as may occur with NH_4^+, which acidifies soil pH by the extrusion of H^+ and increases metal solubility (Zaccheo et al., 2006). However, the biomass of plants grown exclusively with NH_4^+ augmented less than in plants fertilized mainly with NO_3^-, and in turn accumulated less Cd (Schwartz et al., 2003; Xie et al., 2009). This effect could be explained by an improved root metabolism in plants treated with NO_3^-, as this nitrogen form is less harmful to root cells than NH_4^+; which is toxic when is applied alone (Babourina et al., 2007). Another possibility is the stronger exudation of substances in the rhizosphere that occurs in plants fertilized with NO_3^-, thought to increase the uptake of other nutrients and metals from the soil (Mench and Martin, 1991). Finally, well-nourished plants have improved development of root architecture, which allows better extraction of nutrients and water from the soil (Lynch, 1995). In particular, NO_3^- availability induces lateral root elongation permitting in turn a higher capacity to uptake more nutrients (Mantelin and Touraine, 2004). Indeed, a higher root density caused by N fertilization correlated well with As and Hg uptake in Zorro fescue plants grown in a polluted soil (Heeraman et al., 2001). To sum up, probably due to a combination of factors, it is feasible that under adequate nutritional status plants would be able to absorb more Hg from the soil.

The differences in biomass yield and Hg accumulation between plants fertilized with or without NO_3^- decreased at the elongation stage (were in most cases not significant). This could be explained by the appearance of nodules in alfalfa roots, formed by the symbiotic interaction with an endogenous *S. meliloti* existing in the collected soil from Almadén. Consequently, N was not a

limiting factor in PK-treated plants. This was indeed detected by the amount of NH_4^+ measured in the plants: At the seedling stage NPK-treated plants showed a significantly higher content of organic N, differences that were attenuated in the elongation stage.

Alfalfa is commonly cropped as forage for cattle, being harvested between the elongation and early flowering stages when contains the optimum protein content (Broderick, 1985). Regarding forage use, the accepted maximum Hg concentration relative to the feeding material must be less that 0.1 mg kg^{-1} (EC Directive, 2002). According to this legislation, the low level of Hg accumulation found in shoots (0.12 mg kg^{-1} DW) at the elongation stage was just above the legal limit, so precautions should be imposed to guarantee a safe consumption of edible parts of the plants.

The concentration of biothiols was studied in alfalfa plants subjected to NPK and PK fertilizers, as those metabolites are relevant for the tolerance of plants to Hg (Carrasco-Gil et al., 2011). Previous experiments, with maize cultivated in a pure hydroponic system (Rellán-Álvarez et al., 2006) and alfalfa plants grown in a semi-hydroponic system (Sobrino-Plata et al., 2009), revealed that Hg was capable of inducing the synthesis of PCs and hPCs. However, this response was attained in plants that accumulated approximately 1000-fold the concentration measured in our experiments, carried out with alfalfa plants grown in a polluted soil fertilized with NPK or PK. This relatively low concentration of Hg in alfalfa roots would not be sufficient to synthesise PCs or hPCs. It should be quoted here that the tolerance mechanisms described in plants cultivated in hydroponic experiments may not work exactly the same way as in plants grown in soils, where the plant developmental pattern is different and metal availability is subjected to unpredictable factors (Sobrino-Plata et al., 2009; Redjala et al., 2011). Additionally, it is feasible that we might have been incapable of detecting minor concentrations of biothiols with the current analytical techniques available. Hydroponic experiments are utterly necessary to characterise the metabolic responses of plants to Hg, as some state-of-the-art analytical techniques are not sensitive enough to detect Hg-interacting cellular components when the concentration of metal is low (Carrasco-Gil et al., 2011). It is expected that when more powerful analytical procedures would be available, we could have a better understanding of Hg tolerance mechanisms in plants grown in the field.

With respect to the oxidative stress parameters measured in NPK- and PK-fertilized plants, lipid peroxidation augmented in the roots of plants grown in the absence of NO_3^- at the seedling growth stage (Fig. 5). In agreement with this observation, the activity of the antioxidant enzymes APX and GR was remarkably higher in the roots of PK-fertilized plants at the seedling stage (Fig. 6). When the level of N was recovered in PK-treated plants thanks to N_2-symbiotic fixation at the elongation stage (Fig. 3), both MDA concentration and APX and GR enzymatic activities were more similar to NPK-treated plants. Our data were completely in agreement with the induction of oxidative stress in N-starved tobacco plants (Rubio-Wilhelmi et al., 2011): the depletion of NO_3^- led to lipid peroxidation and higher antioxidant enzymatic activity. In particular, there was a significant increase in GR activity, which occurred in parallel with a diminution in oxidised GSH concentration. In addition, it has been described that in the early stages of *Rhizobium* bacteria infection and nodule formation, the leguminous roots experienced a cellular redox imbalance. In such conditions, the antioxidant defence system was triggered, resulting in the overexpression of genes coding antioxidant enzymes, the modulation of their enzymatic activity and the accumulation of antioxidant metabolites (Gogorcena et al., 1997; Pauli et al., 2006). Therefore, alfalfa plants depleted of N (PK-treated) suffered oxidative stress, which was only alleviated when a functional symbiotic N-fixation was established.

5. Conclusions

Fertilization with NO_3^- augmented plant biomass at the earliest developmental stages, but thanks to symbiotic N_2-fixation plants without NO_3^- could also obtain sufficient N for an adequate normal biomass production. The supply of NO_3^- also prevented oxidative stress in roots and may improve root metabolism and development, but this was followed by an undesirable increase in Hg-uptake from the soil. A proper N-management should be considered in case plants being cropped in Hg-polluted soils, as the metal can accumulate in edible parts of the plants above safety consumption limits. Future experiments should be aimed to study the dynamics of Hg in plants grown in soils with different levels of NO_3^- and/or types of NO_3^- fertilizers (i.e. different counter-ions), where the specific growing conditions might lead to increased uptake of the toxic metal.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envexpbot.2011.08.013.

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