

Comparative physiology of xylem nickel loading in the hyperaccumulator *Odontarrhena inflata* and a non-accumulator *Aurinia saxatilis*

Soraya Soleymanifar

Ali Akbar Ehsanpour

aliakbarehsanpour2@gmail.com

University of Isfahan

Rasoul Ghasemi

Sascha Heinrich

Julia Elisabeth Badow

Henk Schat

Ute Krämer

Research Article

Keywords: nickel hyperaccumulation, *Odontarrhena*, xylem loading, Fe deficiency, chelators

Posted Date: October 4th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4982560/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Plant and Soil on November 7th, 2024. See the published version at <https://doi.org/10.1007/s11104-024-07028-4>.

Abstract

Background and Aims

This study aimed to gain insight into the biochemical mechanisms of Ni movement in *Odontarrhena inflata* and *Aurinia saxatilis*.

Methods

We examined the effects of Ni exposure on the concentrations of histidine and nicotianamine in roots, shoots, and in xylem sap of *Odontarrhena inflata* (as Ni hyperaccumulator) and *Aurinia saxatilis* as a non-accumulator. Furthermore, we analysed the effects of Fe and Zn deficiency, and of an apoplastic pathway blocker, on the mobility of Ni.

Results

In plants unexposed to Ni, root His and shoot NA concentrations were higher in *O. inflata* than in *A. saxatilis*. Ni exposure caused an increase in His in the xylem sap of *O. inflata*. Ni exposure caused concentration-dependent increases in shoot His and in root NA concentrations, which were similar in both species for His and distinct between the two species for NA. Fe deficiency, followed by a short-term Fe luxury and Ni exposure, led to enhanced Ni uptake and Ni flux from the root to the shoot of *O. inflata*. By contrast, we observed decreased Ni loading into the xylem in *O. inflata* subjected to Zn deficiency. An apoplastic pathway blocker resulted in a decrease in root Ni levels by almost 20%, and in decreased shoot Ni concentrations only under high Ni exposure.

Conclusion

The processes enhanced in response to Fe deficiency can contribute to root uptake and xylem loading of Ni in the hyperaccumulator species. The contribution of apoplastic pathway to root-to-shoot Ni flux is negligible under natural ecological conditions.

Introduction

Soil contamination by nickel (Ni), a potentially toxic metal (PTM), is an increasing global concern due to an intensified use of Ni for stainless steel and battery production (Kumar et al. 2020; Albanese et al. 2015). Nickel mining and consequent environmental pollution left large areas (approximately more than 5 million locations) of metal-contaminated soils unsuitable for crop cultivation globally (He et al. 2015).

A small number of plant species named nickel hyperaccumulators accumulate extraordinarily high nickel concentrations of more than $1,000 \mu\text{g g}^{-1}$ dry biomass in their leaves (Baker et al. 2010; Reeves et al.

2018). Such concentrations are highly toxic for growth, photosynthesis and metal homeostasis of non-accumulator taxa (Seregin and Kozhevnikova 2006). Ni hyperaccumulators are mostly restricted to serpentine soils, which are naturally enriched with Ni, iron (Fe), cobalt (Co) and chromium (Cr), contain low organic matter and are deficient in essential elements such as nitrogen (N), phosphorus (P), and potassium (K) (Baker and Brooks 1989). These properties make serpentine soils uninhabitable for most plant species, and thus they host a number of endemic taxa, which can exhibit adaptive morphology, or physiology including hyperaccumulation of nickel or cobalt (Palm et al. 2014; Harrison and Rajakaruna 2011; Konečná et al. 2022).

A remarkable number of Ni hyperaccumulator species is known in the *Brassicaceae* family (83 species), with the maximum in the genus *Alyssum* (50 species, currently syn. *Odontarrhena*) (Baker and Brooks 1989). Metal hyperaccumulators have attracted considerable interest because of their potential use in phytoremediation or phytomining, plant-based technologies aimed at removing metal contaminants from soil by harvesting above-ground metal-enriched biomass (Kidd et al. 2009). Yet, efficient technological applications require an improved understanding of the molecular and physiological basis of metal uptake and transport mechanisms in metal hyperaccumulator plants.

To date, metal hyperaccumulation is thought to involve (1) a high capacity for root metal uptake, (2) attenuated metal sequestration in root vacuoles and the radial inward metal translocation to the stele, (3) efficient metal loading into the xylem and root-to-shoot translocation, followed by (4) metal sequestration in large quantities in above-ground biomass, predominantly in vacuoles, in innocuous chemical forms (Clemens 2001; Richau et al. 2009; Krämer 2010; Corso and de la Torre 2020; Merlot et al. 2018). After uptake into root cells of plants, cytosolic metal cations are thought to form complexes of with low-molecular-mass ligands, such as histidine (His), nicotianamine (NA) or organic acids, and this is thought to be critical for metal detoxification. In Ni hyperaccumulators, metal chelation by specific low-molecular-mass ligand molecules additionally prevents the vacuolar sequestration and thus the immobilization of Ni in the root, and thus contributes to high rates of Ni flux into the xylem (Clemens 2019, Richau et al. 2009, Kozhevnikova et al. 2014). A decisive role has been attributed to the free amino acid His in Ni hyperaccumulation in the genera *Odontarrhena* (Krämer et al. 1996) and *Noccaea* (formerly *Thlaspi*) of the *Brassicaceae* (Richau et al. 2009). His concentrations in the roots of the Ni hyperaccumulators *Alyssum lesbiacum*, *Alyssum serpyllifolium* and *N. caerulescens* are constitutively higher than in closely related non-accumulator species (Krämer et al. 1996; Kerkeb and Krämer 2003; Richau et al. 2009; Ingle et al. 2005). In these Ni hyperaccumulator species, Ni exposure results in a characteristic dose-dependent increase in the concentrations of histidine in the xylem sap, the “His response” (Krämer et al. 1996).

Another well-known metal chelator in plants is the non-proteinogenic amino acid nicotianamine (NA), which is synthesized from three molecules of S-adenosylmethionine by the enzyme nicotianamine synthase (NAS) (Noma et al. 1971; Shojima et al. 1989). Metal chelation by NA is universally important in plant Fe homeostasis, for example (Haydon and Cobbett 2007; Clemens 2019; Kobayashi et al. 2019). As a hexadentate chelator, NA can form stable complexes not only with Fe(II) and Fe(III), but also with the

divalent cations of Ni, Zn, Co, Cu, and Mn, for example (Mari et al. 2006; Curie et al. 2009; Callahan et al. 2006; Blindauer and Schmid 2010). Strongly elevated *NAS2* transcript levels were reported in roots of the Zn and Cd hyperaccumulator species *Arabidopsis halleri* (Weber et al. 2004; Deinlein et al. 2012) and *Noccaea caerulescens* (Van de Mortel et al. 2006). Knock-down of *AhNAS2* by RNA interference (RNAi) caused a nearly 80% decrease in root NA content compared to the wild type, which was associated with decreased Zn and Cd accumulation in leaves (Deinlein et al. 2012). In response to Ni exposure, roots of *N. caerulescens* accumulated NA, and a stable Ni–NA complex was identified in the xylem sap of this Zn/Cd/Ni hyperaccumulator species (Mari et al. 2006). Overexpression of *NAS* in *Arabidopsis* and tobacco plants conferred elevated Ni tolerance. Exposure to excess Ni resulted in an increase in the activity of *NAS* gene promoters of *Arabidopsis thaliana*, which supports a role of NA in Ni detoxification (Kim et al. 2005).

Energy-dependent uptake of Ni into the root symplasm followed Michaelis-Menten kinetics not only in Ni hyperaccumulators, including *Leptoplax emarginata* (Redjala et al. 2010), *Odontarrhena bracteata*, *O. inflata* (Mohseni et al. 2018) and *Thlaspi goesingense* (Puschenreiter et al. 2005), but also in non-accumulator plants such as *Vigna radiata* (Zhang et al. 2001). This implicates transmembrane transport proteins in the uptake of Ni from the soil solution. Ni uptake into root cells is hypothesized to involve poorly selective transport systems of other trace elements such as Zn, Fe, and Mn, notably members of the ZRT, IRT-like Protein (ZIP) family, sometimes also named ZNT proteins in plants (Nishida et al. 2015; Deng et al. 2014; Ghaderian et al. 2015; Halimaa et al. 2014). IRT1 (Iron-Regulated Transporter 1) was suggested to contribute to Ni uptake in *A. thaliana* at least under Fe deficiency (Nishida et al. 2012). In *N. caerulescens*, except for a few calamine, poorly Zn-hyperaccumulating accessions (La Calamine), Zn strongly inhibits, or almost completely blocks, Ni uptake into roots, whereas Zn uptake was unaffected by Ni in Monte Prinzera, a Ni-hyperaccumulating accession originating from a Ni-rich, so-called serpentine, soil (Assunção et al. 2001). In three populations of the Zn/Ni hyperaccumulator *Noccaea pindica*, applying equimolar concentrations of Ni and Zn (100 μM Ni + 100 μM Zn) resulted in a decreased Ni uptake (Taylor and Macnair 2006; Peer et al. 2006). It is presently thought that two uptake systems for Zn exist in *Noccaea*: a high-affinity Zn transporter with elevated metal specificity, and a second low-affinity transport system with a preference for Zn transport that can mediate the uptake of both Zn and Ni (Assunção et al. 2001; Deng et al. 2018).

Generally, the uptake of metals by plant roots can proceed via two different alternative pathways: the symplastic pathway, which is thought to predominate, and the apoplastic pathway (Marschner 1995). More recently, the coupled transcellular pathway was additionally brought forward as an intermediate pathway. In the symplastic pathway, epidermal root cells take up ions, followed by cell-to-cell transfer through plasmodesmata. After uptake into root epidermal cells, ions moving along the coupled transcellular pathway move from one cell to another through consecutive cellular efflux and re-uptake. Ions following the apoplastic pathway diffuse passively in solution within the apoplast of the root, and they are taken up into the endodermal cell layer because the Casparian Strip blocks further apoplastic movement towards the xylem. In regions where the Casparian Strip is either not present or not functional, it may be possible for ions to move fully apoplastically across the root by passive diffusion

and mass flow from the soil solution into the xylem vessels without ever entering any root cell (Burch-Smith and Zambryski 2012; Barberon and Geldner 2014).

In addition to enhanced rates of metal uptake by the root system, hyperaccumulation requires effective metal loading into the xylem for root-to-shoot metal translocation. Competition between Ni and Zn for root-to-shoot translocation was observed in Ni-hyperaccumulating accessions of *N. caerulescens* (Assunção et al. 2001). Moreover, Ni decreased the root-to-shoot translocation of Fe in *O. inflata* (two populations) and *O. bracteata* (Mohseni et al. 2018). Ferroportin1/Iron-Regulated 1 (IREG1) was implicated as a candidate protein acting in xylem loading of Ni of several phylogenetically distinct Ni hyperaccumulator species (Merlot et al. 2014; Sobczyk et al. 2017). *A. thaliana* IREG1 was previously proposed to act in root-to-shoot Co translocation and in xylem loading of Fe (Morrissey et al. 2009).

The goal of this study was to gain insights into the pathways and biochemical mechanisms of Ni movement in the hyperaccumulator species *Odontarrhena inflata* (Nyár.), which originates from serpentine soils of western Iran (Baneh population). Therefore, we comparatively examined the concentration profiles of the chelators His and NA in response to Ni treatments in *O. inflata* and the non-accumulator species *A. saxatilis*. Like other Ni-hyperaccumulating *Odontarrhena* species, *O. inflata* contains high levels of His in roots and mounts the well-known His response in the xylem. To investigate potential roles of processes related to Zn and Fe deficiency, which are the most likely to interact with root uptake or xylem loading of Ni, we examined the effects of Zn and Fe starvation on Ni concentrations in roots and in the xylem sap. These experiments support the involvement of Fe nutrition-related pathways in Ni hyperaccumulation. Finally, we tested the contribution of the root apoplastic pathway to Ni uptake and translocation by inducing the formation of precipitates in the root intercellular spaces to block the apoplastic pathway. The results suggest against a contribution of the apoplastic pathway to Ni hyperaccumulation in *O. inflata*.

Material and methods

Seeds of *Odontarrhena inflata* (genus *Odontarrhena* C.A.Mey, formerly considered part of the genus *Alyssum* L.) were harvested from a serpentine site of the Baneh region (35°14' N, 46°28' E) in western Iran (Ghaderian et al. 2007). Seeds named *Aurinia saxatilis* were purchased (Horti Tops-Tuinplus, Heerenveen, Netherlands) and were of the species *Aurinia saxatilis* (L.) Desv. in the tribe Alysseae but in a different genus (*Aurinia*) of the Brassicaceae. Seeds were surface-sterilized in 5% (w/v) NaOCl for 10 min, followed by five washes with ultrapure water (Milli-Q; Merck) for 5 min. Seeds were placed on ultrapure water solidified with 0.8% (w/v) agar Type M (Sigma) in square 120-mm polystyrene Petri dishes, and stratified at 4°C for 3 d in the dark in a cold room and subsequently allowed to germinate in a growth chamber (CLF Plant Climatics GmbH, Wertingen, Germany) for 7 d. Subsequently, seedlings were transferred to fresh square Petri dishes containing a modified 0.25x Hoagland solution consisting of 1.5 mM Ca(NO₃)₂, 0.28 mM KH₂PO₄, 0.75 mM MgSO₄, 1.25 mM KNO₃, 0.5 µM CuSO₄, 2.5 µM ZnSO₄, 5 µM MnSO₄, 25 µM H₃BO₃, 0.1 µM Na₂MoO₄, 50 µM KCl, 5 µM FeHBED (FeIII(N,N'-di-(2-hydroxybenzoyl)-ethylenediamine-N,N'-diacetic acid)), 3 mM MES (2-(N-morpholino) ethanesulfonic acid), pH 5.7,

additionally supplemented with 1% (w/v) sucrose and solidified with 0.8% (w/v) agar Type M (Merck KGaA, Darmstadt, Germany) and pre-cultivated for 7 d (Hoagland and Arnon, 1950). Seedlings were then transferred into hydroponic vessels filled with 400 ml 0.25x modified Hoagland solution. The hydroponic medium was aerated using aquarium pumps (12–15 bubbles of air per minute), and nutrient solutions were replaced by fresh solutions every 6 d. Growth chamber conditions were 16 h of white light at an intensity of $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for pre-cultivation in Petri dishes and $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for hydroponic culture (22°C) /8 h dark (20°C), with a constant relative humidity of 50%. In all experiments that included the collection of xylem sap, hydroponic cultivation was done in the glasshouse with 16 h light (22°C minimum to 35°C maximum)/8 h dark (19°C minimum to 28°C maximum), with constant relative humidity of 60% and with supplementary lighting provided during the day by metal halide lamps for maintaining a total light intensity of $350 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Experimental procedures

Experiment 1. To characterize the effect of Ni exposure on shoot Ni, His, and NA and root His and NA concentrations, plants were cultivated hydroponically for 3 w, and subsequently, hydroponic solutions were supplemented with NiSO_4 (0, 10, 30, 100, or 300 μM) for 8 d. At harvest, shoot and root systems were separated, rinsed in ultrapure (Milli-Q) water three times, blotted dry with tissue paper, weighed and transferred into 50-ml polypropylene screw-cap tubes, frozen in liquid nitrogen, and stored at -80°C until analysis. The pooled tissues from three plants cultivated in one culture vessel were considered one biological replicate. Three culture vessels were used for each treatment and species. Data are shown from one experiment representative of three independent experiments.

Experiment 2. To analyze the effect of short-term Ni treatments (0 and 100 $\mu\text{M NiSO}_4$) on Ni and His concentrations in xylem sap, plants were grown hydroponically under glasshouse conditions for 12 w, and then root systems were supplemented with 0 and 100 $\mu\text{M NiSO}_4$. Treatment solutions were exchanged with fresh solutions every 24 hours. After 48 h of treatment, shoots were cut off with a scalpel exactly beneath the lowest leaf, and flexible silicon tubes of appropriate diameters (10–15 μm) were fitted tightly onto the remaining hypocotyls of each root system to end inside 1.5-ml reaction vials. Xylem sap was collected as root pressure exudates for 8 h from 10 pm to 6 am. Roots were kept in the treatment solutions during the collection period. Xylem sap samples from 9 plants (corresponding to 3 culture vessels with three plants per vessel) were pooled per treatment and frozen at -20°C until analysis. Data are shown from one experiment representative of three independent experiments.

Experiment 3. To examine the effects of continuous Fe starvation and Fe starvation followed by Fe luxury on Ni and Fe concentrations in roots and concentrations of Ni, Fe, His and NA in xylem sap, plants were grown in hydroponics for 9 w, then transferred into Fe-deficient hydroponic solutions (without FeHBED) or kept in modified 0.25x Hoagland solution (control). Before the transfer into these treatment solutions, all roots were placed in a solution of 2 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM Na_2EDTA , 1 mM MES/KOH, pH 5.7, for 15 min, and then in a solution containing 2 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM MES/KOH, pH 5.7, for 5 min, to remove

apoplastically bound metal cations (50 ml for the root systems of the three plants per culture vessel). After 5 d, Fe-starved plants were transferred into a combination of continued Fe deficiency and Ni (nutrient solution without FeHBED and with 100 μM NiSO_4) or a combination of Fe luxury and Ni (nutrient solution with 50 μM FeHBED and 100 μM NiSO_4). Control plants were supplemented with 100 μM NiSO_4 . After 4 h, xylem sap was collected for 8 h, followed by pooling and storage, as described above (Experiment 2). Afterwards, root systems were desorbed in an ice-cold desorption solution containing 2 mM $\text{Ca}(\text{NO}_3)_2$, 10 mM Na_2EDTA , 1 mM MES/KOH, pH 5.7, for 10 min, followed by incubation in a solution containing 0.3 mM bathophenanthroline disulphonate and 5.7 mM sodium dithionite for 3 min (50 ml for the root systems of the three plants per culture vessel) (Cailliatte et al. 2010). Finally, roots were rinsed twice in ultrapure water for 1 min, respectively, blotted dry with tissue paper, weighed and oven-dried at 60°C for 3 d. The pooled xylem sap and roots from nine plants cultivated in three culture vessels were considered one biological replicate. Nine culture vessels were used for each treatment and species. Data are shown from one experiment representative of three independent experiments.

Experiment 4. To examine the effect of Zn deficiency on the concentrations of Ni, Zn, His and NA in roots and xylem sap, plants were cultivated in Zn-sufficient nutrient solution (2.5 μM ZnSO_4) for 9 w, and subsequently transferred to a nutrient solution without added ZnSO_4 for 2 w or kept in modified 0.25x Hoagland solution (control) after removal of apoplastically bound metal cations (see Experiment 3). Zn-starved plants were transferred into a combination of continued Zn deficiency and Ni (0 ZnSO_4 and 100 μM NiSO_4) or a combination of Zn luxury and Ni (15 μM ZnSO_4 and 100 μM NiSO_4) in the hydroponic nutrient solution. Control plants were supplemented with 100 μM NiSO_4 . Four h later, shoots were cut off and xylem sap was collected for 8 h, followed by pooling and storage as described above (see Experiment 2). Subsequently, roots were desorbed by incubating twice in ice-cold desorption solution containing 2 mM $\text{Ca}(\text{NO}_3)_2$, 10 mM Na_2EDTA , and 1 mM MES/KOH, pH 5.7, for 10 min (50 ml for the root systems of the three plants per culture vessel). Finally, roots were rinsed twice with ultrapure water for 1 min, respectively, blotted dry, weighed, flash frozen in liquid nitrogen and stored at - 80°C for subsequent analysis. The pooled xylem sap and roots from nine plants cultivated in three culture vessels were considered one biological replicate. Nine culture vessels were used for each treatment and species. Data are shown from one experiment representative of two independent experiments.

Experiment 5. To address the contribution of the apoplastic pathway to Ni entry into the xylem of *O. inflata*, shoot Ni accumulation was compared between plants pre-treated and not pre-treated with an apoplastic blocker and subsequently exposed to different concentrations of NiSO_4 (0, 30, 100, and 300 μM) in nutrient solution. The reaction between 1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ and 0.05 mM CuSO_4 leads to the formation of rusty-brown precipitates (crystals) of $\text{Cu}_2[\text{Fe}(\text{CN})_6]$ or $\text{Cu}[\text{CuFe}(\text{CN})_6]$. This reaction can also take place in the apoplastically accessible parts of root tissues, and the formed precipitates inhibit the apoplastic movement of water and likely of solutes (Ranathunge et al. 2005). In a preliminary experiment, we established 0.05 mM Cu to be sub-toxic and sufficient for crystal formation and block of apoplastic water movement in *O. inflata*. For this preliminary experiment, the biomass of control plants

and plants treated with a concentration range of CuSO_4 was monitored once every 15 minutes over a period of time (3 h). We selected the maximum Cu concentration tolerated by plants (i.e. not leading to a decrease in biomass indicating water loss through wilting).

Plants were grown hydroponically for 6 w, and subsequently root systems were incubated in 1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ overnight to allow for the gradual diffusion of this compound in the root apoplast. On the following morning, the solution was replaced with a sub-toxic concentration of 0.05 mM CuSO_4 for 1 h. Thereafter, plants were transferred into a modified 0.25x Hoagland hydroponic solution, supplemented with 0, 30, 100, or 300 μM NiSO_4 for 8 hours (the earliest time for Ni to reach the shoot of 6-week-old plants). Plants unexposed to apoplast blockage treatment and continuously kept in modified 0.25x Hoagland hydroponic solution until the start of the Ni treatments were considered as controls. After 8 hours of Ni exposure, shoots and roots were harvested. Roots were desorbed and further processed as described (see Experiment 4).

To examine the possible effects of either 1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ or 0.05 mM CuSO_4 alone on root-to-shoot Ni translocation, additional controls were conducted in which plants were exposed to these compounds individually, as control treatments A and B, in experimental conditions as described above. The pooled tissues from three plants cultivated in one culture vessel were considered one biological replicate. Three culture vessels were used for each treatment and species. Data shown are from one experiment representative of two independent experiments.

To examine the efficacy of apoplastic blockage, free-hand longitudinal sections were prepared of the root apex and at 4 cm distance from the root apex, and transverse sections at 4 and 6 cm distance from the root apex. Sections were viewed under a light microscope (SZX12, Olympus, Japan), and photographs taken using a Nikon Digital SLR camera.

Analytical procedures

Multi-element analysis

Oven-dried tissues were equilibrated at room temperature for 2 d. Oven-dried tissues and samples flash-frozen in liquid nitrogen were ground to a powder with an acid-washed (0.2 M HCl) mortar and pestle. Aliquots of powdered frozen material were freeze-dried overnight (Alpha 1–4 LDplus, Martin Christ, Osterode, Germany), and then equilibrated at room temperature for 2 days. Subsamples of 22 mg of powdered dry tissues were transferred into PFA microwave vessels (CEM GmbH, Kamp-Lintfort, Germany), and 3 ml 65% (w/w) nitric acid were added to each vessel and plant tissues were digested at 190°C for 15 min (MarsXpress, CEM GmbH, Kamp-Lintfort, Germany). After cooling to RT, samples were transferred into 15-ml polypropylene screw cap tubes and the final volume of each sample was adjusted to 10 ml with ultrapure water (Milli-Q, Merck). For multi-element quantification in xylem sap, 1.5 ml of 65% (w/w) nitric acid was added to aliquots of 100 μl of xylem sap and filled up to a final volume of 5 ml with ultrapure water. Multi-element analysis of digests of root and shoot tissues and of xylem sap samples was conducted using Inductively-Coupled Plasma Optical Emission Spectrometry (ICP-OES;

iCAPDuo 6500, Thermo Fisher Scientific, Dreieich, Germany). For quality control, digests of certified reference material (Virginia tobacco [*Nicotiana tabacum*] leaves, INCT-PVTL 6; Institute of Nuclear Chemistry and Technology, Poland) were analysed at the beginning and at the end of each set of *ca.* 50 samples. The composition of calibration standards and wavelengths used for analysis are shown in Table 1, Supplementary Data.

Quantification of His and NA

Sample preparation

Frozen plant tissues were ground in liquid nitrogen using a mortar and pestle. Per sample, 200 mg of tissue powder were put into a 1.5-ml polypropylene reaction vial, and 400 µl of 80% (v/v) ethanol in a 2.5 mM HEPES buffer, pH 7.5, were added per aliquot (Scheible et al. 1997). The resulting mixture was vortexed and incubated at 80°C and 1,000 rpm for 20 min in a heating block (Thermomixer Comfort, Eppendorf GmbH, Hamburg, Germany). Tissue extracts were then centrifuged at 20,800 g for 10 min, and the supernatants were collected in a fresh reaction vial. In two additional steps, the pellet was re-extracted as described above, first with 400 µl of 50% (v/v) ethanol in 2.5 mM HEPES buffer, pH 7.5, and then 200 µl of 80% (v/v) ethanol. Pooled supernatants were stored at -20°C until analysis.

For analysis, the samples were thawed and then centrifuged at 4°C and 17,530xg for 20 min. The clear supernatant was used for sample preparation. HPLC vials (Macherey and Nagel, 2 mL glass vials with 0.2 mL silanized micro inlay) were filled with 180 µL of a mixture of 50:50:0.1% (v/v/v) acetonitrile:H₂O:FA. Twenty µl of the clear supernatant were added, followed by vortexing for 3 s. Samples were transferred to the autosampler and kept at 10°C until analysis by LC-coupled MS^E-based quantification.

LC-coupled MS^E-based quantification of nicotianamine and histidine

Five µl of each sample was injected in an ACQUITY UPLC I-Class System (Waters, Milford, Massachusetts) equipped with an ACQUITY BEH Amide PREMIERE column (particle size 1.7 µm, column dimensions: 2.1 x 100 mm, Waters). A gradient with H₂O (**A**) and acetonitrile (**B**), each with 0.1% formic acid (FA), was used with a flow rate of 0.5 mL min⁻¹ (Table 1). The column temperature was 40°C.

Table 1
Liquid chromatography gradient

time [min]	%(v/v) acetonitrile in water (0.1% (v/v) FA)
0.0	90
5.0	90
6.0	75
7.0	75
10.0	65
11.0	20
13.0	20
13.5	90
16.5	90

Molecules were identified by comparison to data obtained from standards: *L*-Histidine, neutral mass 155.0695 Da, adduct + H⁺, collision cross section (CCS) 130.00 Å², retention time 7.65 min, fragments [m/z] 110.0709 and 93.0443. Nicotianamine, neutral mass 303.1430 Da, adduct + H⁺, CCS 164.00 Å², retention time 10.40 min, fragments [m/z] 185.0917 and 114.0550. Method parameters in UNIFI for identification: retention time tolerance 0.5 min, target match tolerance 6 ppm, fragment match tolerance 10 mDa, CCS tolerance 2%. The automated target selection was made based on the best fitting measurement data (least deviation from the given values for retention time, CCS and mass error).

L-Histidine and nicotianamine were quantified against calibration curves of 0.001 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, and 1 mM (for measurements of root and shoot tissues these concentrations corresponded to: 0.005 µmol·g⁻¹ FW, 0.05 µmol·g⁻¹ FW, 0.25 µmol·g⁻¹ FW, 0.5 µmol·g⁻¹ FW, 1 µmol·g⁻¹ FW, 2.5 µmol·g⁻¹ FW, 5 µmol·g⁻¹ FW) of the respective standard. Stock solutions of the standards were prepared in H₂O. The stock solutions were diluted to the final concentrations using 68% (v/v) ethanol containing 2 mM HEPES, pH 7.5, in order to maintain the pH as used for sample preparation. For measurements, 20 µL of each standard were mixed with 180 µL of a mixture of 50:50:0.1% (v/v/v) acetonitrile:H₂O:FA and vortexed for 5 s each. Calibration curves were prepared as technical triplicates.

Statistical analysis

All datasets were subjected to Analysis of Variance (two-way ANOVA). Multiple comparisons of means were performed using one-way ANOVA (Duncan's test; *P* < 0.05). Both one-way and two-way ANOVA analyses were carried out using SPSS software version 22, for Windows.

Results

With increasing Ni concentrations, a slight decrease in shoot Fe concentrations is characteristic of the Ni hyperaccumulator *O. inflata*

We comparatively analysed the effects of Ni exposure and their dose-dependence in the Ni hyperaccumulator *O. inflata* and the non-accumulator *A. saxatilis* after exposure to Ni for 8 d. In both species, we observed a dose-dependent increase in the shoot Ni concentrations, which were between 4.3 and 5.8-fold higher in *O. inflata* than in *A. saxatilis* at all the Ni treatment levels (Fig. 1a). We concluded that the two species respond differently to Ni exposure, with overall much higher shoot Ni accumulation in *O. inflata*.

Shoot Fe concentrations decreased with increasing Ni concentration supplied in the hydroponic solution, and thus also with shoot Ni concentrations, in hyperaccumulator *O. inflata* only, but not in *A. saxatilis* (Fig. 1b). In *O. inflata*, the shoot Fe concentrations were $1.44 \mu\text{mol Fe g}^{-1} \text{ DW}$ and $0.15 \mu\text{mol Ni g}^{-1} \text{ DW}$ in the controls without Ni supplementation, and $0.91 \mu\text{mol Fe g}^{-1} \text{ DW}$ and $91.8 \mu\text{mol Ni g}^{-1} \text{ DW}$ upon supplementation with $300 \mu\text{M Ni}$ in the hydroponic solution. The observed differences in shoot Fe concentrations were less than 0.6% of the differences in the shoot Ni concentrations. Unlike for Fe, no significant changes in shoot Zn concentrations were observed with increasing Ni treatments in either of the two species (Fig. 1b).

Constitutively elevated root His and shoot NA concentrations are characteristic of the Ni hyperaccumulator *O. inflata*

With increasing Ni concentrations in the hydroponic solutions, the concentration of free His increased significantly in shoots of *O. inflata* and in shoots and roots of *A. saxatilis* (except in *A. saxatilis* at $300 \mu\text{M Ni}$; Fig. 2). In plants grown in control hydroponic solutions unamended with Ni, the concentration of His was more than 5.4-fold and 1.5-fold higher in roots and shoots of *O. inflata*, respectively, than in the corresponding tissues of *A. saxatilis*.

The concentrations of NA in shoots were constitutively about 2-fold higher in *O. inflata* than in *A. saxatilis*, without any significant changes in response to Ni exposure in either one of the two species (Fig. 2). NA concentrations in roots of *O. inflata* were mostly unresponsive to Ni, but were increased after exposure to $300 \mu\text{M Ni}$ (NA: $0.51 \mu\text{mol g}^{-1} \text{ fresh biomass}$) in comparison to control plants (NA: $0.27 \mu\text{mol g}^{-1} \text{ fresh biomass}$). In *A. saxatilis*, only exposure to low concentrations of Ni (10 and $30 \mu\text{M}$) caused increases in root NA concentrations compared to control plants.

A “His response” but no “NA response” in the Ni hyperaccumulator *O. inflata*, with a characteristic Ni exposure-induced small decrease in xylem sap Fe concentrations

In order to analyse the responses of xylem sap composition to Ni exposure, we subjected 12-week-old plants to $100 \mu\text{M Ni}$ or no Ni (controls) in hydroponic solutions for 48 h before the onset of an 8-h xylem

sap collection period while Ni exposure continued. As expected, Ni exposure caused significant increases in the concentrations of Ni in the xylem sap of treated plants, compared to controls (0 Ni). The concentrations of Ni in the xylem sap of Ni-exposed *O. inflata* were significantly (approximately 3-fold) higher than in *A. saxatilis* (Fig. 3). Ni exposure of *O. inflata* also caused a significant increase in xylem sap His concentrations in comparison with control plants. By contrast, no statistically significant increase in xylem sap His concentration was observed in Ni-exposed *A. saxatilis* when compared to untreated controls. It is noteworthy that in *O. inflata*, the Ni concentration in the xylem sap was more than 9.6-fold higher than the His concentration. The concentration of NA in the xylem sap showed no significant increase in response to Ni exposure in either of the two plant species. We conclude that there was no “NA response” in *O. inflata* after Ni treatment for 48 h.

Compared with untreated control plants, the Fe concentration was decreased by 33% in response to Ni exposure in the xylem sap of *O. inflata*, but remained unchanged in *A. saxatilis* (Fig. 3c). Note that in the xylem sap of Ni-exposed *A. saxatilis*, the Fe concentrations were less than 20% of the Ni concentrations. There were no significant differences in xylem sap Zn concentrations between Ni-exposed and control plants or between species (Fig. 3c).

Effects of continuous Fe deficiency or Fe deficiency followed by short-term Fe luxury treatment on Ni concentrations in xylem sap and in roots

Next we asked whether processes and pathways involved in root uptake and xylem loading of Fe contribute to Ni hyperaccumulation. We cultivated plants in hydroponic solutions lacking Fe for 5 d to induce Fe deficiency responses, or continuously in standard hydroponic solutions as control. Subsequently, we supplemented hydroponic solutions of all plants with 100 μM NiSO_4 . For Fe-deficient plants, we combined Ni exposure either with continued Fe starvation, or with exposure to luxury Fe concentrations. Four h later, we began collecting xylem sap as root pressure exudate. In all treatments, the concentration of Ni in xylem sap of both species was considerably higher than Fe concentration, e.g. more than 14- and 8-fold higher Ni than Fe concentrations in Fe-deficient Ni hyperaccumulator and non-accumulator plants, respectively (Fig. 4a). In Fe-starved *O. inflata* Ni hyperaccumulator plants exposed to Ni in the presence of luxury Fe for 4 h, we observed remarkably increased, approximately 3-fold higher concentrations of Ni in the xylem sap, when compared to the continued Fe deficiency and the control treatments (Fig. 4a). By contrast, there was no statistically significant difference for the equivalent comparison in *A. saxatilis* (Fig. 4a). In *O. inflata*, but not in *A. saxatilis*, both the His and NA concentrations in the xylem sap were significantly higher in Fe-starved plants re-supplied with luxury Fe compared to continuously Fe- starved plants (Fig. 4b).

Interestingly, only in the Ni hyperaccumulator *O. inflata*, and not in *A. saxatilis*, xylem sap concentrations of another trace element, namely the micronutrient Mn (1.4-fold) but not of Mo, Zn, Cu, (and not of Cr, and Co, data not shown), as well as those of macronutrients Ca (1.3-fold), Mg (1.3-fold), K (1.5-fold) and S (1.7-fold) showed similar profiles to the one described above for Ni, but with much smaller quantitative differences (Fig. 4c, d and e).. These effects are unlikely to result from influx into the xylem *via*

apoplastic routes, because for all corresponding ions, including also those of Ni, the concentrations in the xylem sap were substantially higher than those supplied in the hydroponic solutions. Only for Fe, the concentrations in the xylem sap were similar to those in the hydroponic solutions.

Next we examined the composition of roots harvested after the end of xylem sap collection in order to gain additional information for the interpretation of our results on xylem sap composition. Compared to control plants of *O. inflata* that were continuously grown in normal Fe supply (5 μM FeHBED) before and during Ni exposure, the Ni and Cu concentrations were significantly higher in roots of Fe-deficient plants exposed to Ni (0 FeHBED and 100 μM NiSO_4), and the Ni, Fe and Zn concentrations were significantly higher in Fe-deficiency plants re-supplied with luxury Fe (50 μM Fe-HBED and 100 μM NiSO_4). In *A. saxatilis*, the latter was observed only for Fe, but not for Ni (Fig. 5a and b). Treatments did not affect the concentrations of any of the macronutrients in either hyperaccumulator or non-accumulator, except for K, which was significantly decreased in *O. inflata* plants exposed to a combination of continued Fe deficiency and Ni (0 Fe-HBED and 100 μM NiSO_4 , Fig. 5c and d).

Effects of continuous Zn deficiency or Zn deficiency followed by short-term Zn luxury treatment on Ni concentrations in xylem sap and in roots

To investigate the effects of plant Zn status on xylem sap and root composition, we transferred 9-week-old *O. inflata* and *A. saxatilis* into hydroponic media lacking added Zn for 2 w, followed by Ni treatment for 4 h in combination with either continued Zn deficiency or a luxury concentration of 15 μM Zn. Control plants were continuously cultivated in Zn-replete medium. In xylem sap of plants cultivated under Zn deficiency, we observed lower Ni concentrations than in xylem sap of control plants of *O. inflata*, with the strongest reduction to 45% of controls in the plants re-supplied with luxury Zn (Fig. 6a). This was paralleled by a decrease in xylem sap His and also NA concentrations (Fig. 6c). By contrast, plant Zn status had no effect on xylem sap Ni or NA concentrations in *A. saxatilis*, but it caused a small decrease in xylem sap His concentrations (Fig. 6a, c). Compared to control plants of both species that were continuously grown in normal Zn supply (2.5 μM ZnSO_4), the Zn concentration was significantly lower in the xylem sap of Zn-deficient plants exposed to Ni (0 ZnSO_4 and 100 μM NiSO_4). The Zn concentrations were higher in xylem sap of Zn-deficient plants re-supplied with luxury Zn (15 μM ZnSO_4 and 100 μM NiSO_4) than in that of plants continuously grown in Zn-deficient solutions (0 ZnSO_4 and 100 μM NiSO_4), as expected, but this effect was only statistically significant in *A. saxatilis* (Fig. 6b). In *O. inflata*, Zn deficiency caused a significant decrease in the Fe concentration in the xylem sap of Ni-exposed plants (0 ZnSO_4 and 100 μM NiSO_4) (Fig. 6b).

In roots of *A. saxatilis* grown continuously in Zn-deficient hydroponic solutions, we observed slightly higher root Ni concentrations than for plants grown continuously in Zn sufficiency (Fig. 7a). As expected, the root Zn concentrations were lower in plants cultivated under Zn deficiency than in control plants, with no significant recovery in plants re-supplied with a luxury Zn concentration by the time of harvest. The Zn status had no significant effects on the root Fe or His concentrations in either of the species. However, in both species, Zn deficiency resulted in a strong decrease in the root NA concentration (Fig. 7b).

The effects of a blocker of the apoplastic pathway on the Ni concentrations in roots and shoots

Finally, we tested the contribution of the apoplastic pathway to Ni hyperaccumulation in *O. inflata*. Sequential treatment of root systems with solutions of 1 mM $K_4[Fe(CN)_6]$ and $CuSO_4$ causes the formation of a precipitate in the root apoplast, which acts as an apoplastic blocker of water and presumably also ion movement along the apoplastic pathway across towards the stele (Ranathunge et al. 2005). After this pre-treatment, plants were supplemented with a range of Ni concentrations in fresh hydroponic solutions for 8 h, followed by harvest of roots and shoots for Ni quantification.

Roots treated with the apoplastic blocker contained 21–23% lower Ni levels than the roots of control plants supplemented with the respective Ni concentration in hydroponic solutions (Fig. 8a). The apoplastic blocker tended to cause a slight decrease in shoot Ni accumulation by approximately 9% on average when compared to controls, but the difference was not statistically significant (Fig. 9b) in plants exposed to 30 or 100 μM Ni. However, in hydroponic solutions supplemented with 300 μM Ni, the apoplastic blocker caused a substantial reduction in shoot Ni accumulation by about 40% compared to controls. Importantly, negative control pre-treatments with 1 mM $K_4[Fe(CN)_6]$ (overnight) alone or with $CuSO_4$ alone (for 1 h in the following morning) had no effect on either root or shoot Ni accumulation (Fig. 8a and b).

Discussion

Effect of Ni exposure on the concentration of elements and chelators in root and shoot

The Ni concentrations in the shoot of the hyperaccumulator species *O. inflata* were several-fold higher than those in the shoot of the non-accumulator *A. saxatilis* in all Ni treatments, thus validating the respective status of the two species. The shoot Zn concentration was not significantly affected by Ni treatments in either of the two species, but the concentration of Fe in the shoot decreased significantly with Ni exposure in *O. inflata*. Consequently, it is possible that the pathway of Ni uptake or root-to-shoot translocation is partly shared with Fe. Interestingly, upon Ni exposure of *O. inflata*, we also observed an about 33% decrease in xylem sap Fe concentrations, but not in Zn concentrations. This suggests that Ni may interfere with the uptake, the radial inward transport across the root, or the xylem loading of Fe. In agreement with our results, other researchers have reported that Ni can interfere with Fe homeostasis of Ni hyperaccumulators (Ghasemi et al. 2009; Mohseni et al. 2018).

Compared to *A. saxatilis*, the His concentration in the root of *O. inflata* were approximately 5.4-fold elevated, and they did not respond to the Ni levels in the hydroponic solution. This is in agreement with previous findings of constitutively elevated His levels in roots of *A. lesbiacum* and *A. serpyllifolium*, two other Ni hyperaccumulators from the genus *Alyssum*/*Odontarrhena* (Krämer et al. 1996; Kerkeb and Krämer 2003; Ingle et al. 2005).

It is noteworthy that in *A. lesbiacum* high free His content in roots might be due to the elevated expression levels of the genes encoding proteins of the His biosynthetic pathway. In transgenic lines of *A. thaliana* overexpressing these genes, free His concentrations and tolerance to Ni were enhanced, without changes in the quantity of other amino acids (Wycisk et al. 2004; Ingle et al. 2005). Moreover, supplying exogenous free His alongside Ni in a hydroponic solution increased Ni accumulation in the shoot of the non-accumulator *Alyssum montanum* (Krämer et al. 1996). An intrinsically high pool of free His in the root appears to be required also for Ni hyperaccumulation in *O. inflata*.

Regarding the role for free His in Ni hyperaccumulation, there are two, mutually non-exclusive, possibilities, i.e. 1) it enhances Ni tolerance through chelating free Ni ions in the cytoplasm, 2) it enhances the root-to-shoot-translocation of Ni. Regarding the second possibility, it is noteworthy that the root-to-shoot translocation of Ni and Zn has been observed to be stimulated by exogenously supplied L-His, via the nutrient solution, in many Brassicaceae species, both in Ni or Zn hyperaccumulators (Kozhevnikova et al. 2021; Soleymanifar et al. 2024) and non-accumulators (Seregin et al. 2022), probably because the chelation of Ni or Zn by L-His in the root cell cytoplasm seems to inhibit the uptake of Ni and Zn into root cell vacuoles in many species (A.D. Kozhevnikova, unpublished results), such as demonstrated for *N. caerulea* (Richau et al. 2009; Kozhevnikova et al. 2014), thus promoting these metals' symplastic radial transport to the root stele. Moreover, exposure to luxurious concentrations of Ni or, more rarely, Zn caused histidine accumulation, either in roots or in shoots, in many non-accumulator Brassicaceae, though the root His concentrations in most of the Zn/Ni hyperaccumulator Noccaeae and Odontarrhena species tested thus far) remained unaffected (A.D. Kozhevnikova, unpublished results), as in our study. In any case, the constitutive His concentrations in Ni/Zn hyperaccumulator plants growing in nutrient solutions without added Ni and with 2 μM Zn were higher (up to 25-fold) than in all of the non-accumulator reference species used thus far, though only consistently in the roots, not in the shoots (Krämer et al. 1996; Kerkeb and Krämer 2003; Ingle et al. 2005; A.D. Kozhevnikova, unpublished results), which clearly argues in favour of a role for His in Ni or Zn hyperaccumulation, and suggests that His primarily exerts its effect on Ni or Zn translocation through chelating these metals in the root cytoplasm, thus promoting their radial cytoplasmic transport to the root stele.

Our data did not provide evidence for a role of NA in root-to-shoot Ni translocation of *O. inflata*. Root NA levels were higher in *A. saxatilis* than in *O. inflata* throughout, and they only increased at the highest Ni exposure (300 μM NiSO_4) in *O. inflata*, but at low-to-intermediate Ni exposures (10 and 30 μM NiSO_4) in *A. saxatilis*. This may indicate a general role of NA in the formation of complexes with Ni (NiNA) and Ni detoxification in both species, given that *A. saxatilis* is likely to be less Ni-tolerant, as it does not originate from serpentine soils. Overall, our results are in agreement with the hypothesis that exposure to Ni or high Zn may interfere with Fe uptake or translocation, which might also explain the relatively high expression of NAS genes in Ni or Zn hyperaccumulators.

After Ni uptake and entry into the root symplast, organic acids rapidly engage in complex formation with Ni^{2+} cations, which suggests vacuolar Ni sequestration in the root epidermis and cortex of non-accumulators (Haydon and Cobbett 2007). Conversely, Ni chelation by His in the root symplast was

proposed to prevent its vacuolar sequestration in the cortex of Ni hyperaccumulators (Richau et al. 2009; Kozhevnikova et al. 2014), thus maintaining Ni mobility for the radial symplastic transport across the root towards the xylem.

In shoots, the concentration of His increased with increasing Ni levels in the hydroponic solution in both species, despite the much higher levels of Ni accumulated in leaves of *O. inflata*. Thus, His may contribute to the protection of both the hyperaccumulator and the non-hyperaccumulator plant from Ni toxicity, as was proposed based on subcellular localization and speciation studies in leaves of the Ni hyperaccumulator *N. goesingense* (Krämer et al. 2000). Shoot NA levels were higher in *O. inflata* than in *A. saxatilis*, with no significant response to Ni treatment. The role of NA in the shoot of *O. inflata* and possibly other Ni hyperaccumulators remains to be examined.

The parallel increase in both Ni and His concentrations in the xylem sap collected between 48 h and 56 h after the onset of exposure to 100 μ M Ni demonstrated that *O. inflata* exhibits a so-called “His response” that was absent in the non-accumulator *A. saxatilis*. This is similar to the “His response” observed for *A. lesbiacum*, which was absent in the non-accumulators *A. montanum* and *Brassica juncea* L. cv *Vitasso*. Respectively, after 8 d or 9 h of Ni exposure (Krämer et al. 1996; Kerkeb and Krämer 2003). We observed no “NA response”. Unfortunately, these responses have not been studied in *Noccaea* hyperaccumulators. However, it seems possible that the “His response” represents a common characteristic of all the Brassicaceae Ni/Zn hyperaccumulators, which is lacking in all of the related non-accumulators studied thus far (Kerkeb and Krämer 2003). If so, then the observed increase of the shoot His concentration in non-accumulators under Ni exposure (A.D. Kozhevnikova, unpublished results) must entirely result from a Ni-imposed stimulation of His synthesis in the shoot itself.

In *O. inflata*, we observed a pronounced increase in the Ni concentrations in roots and partially also in the xylem sap of plants pre-cultivated in Fe-deficient hydroponic solution for 5 d before Ni exposure, and even more so when Fe was re-supplied at a luxury concentration in combination with Ni for 4 h. This effect was not observed in the non-accumulator *A. saxatilis*. Our results suggest a possible role of Fe deficiency-inducible transporters with poor selectivity, for example IRT1, in root Ni uptake by *O. inflata*. Correspondingly, split-root experiments in *A. thaliana* suggested that physiologically Fe-deficient plants produce higher amounts of *IRT1* transcripts and protein levels when Fe is present in the hydroponic solution (Vert et al. 2003).

Cross-species comparative transcriptomics of Ni hyperaccumulators and non-accumulators across diverse families of dicotyledonous plants revealed an apparently convergent association of high transcript levels of *IREG/Ferroportin* transporter-encoding genes with Ni hyperaccumulation (García et al. 2021). The abilities of ZRT/IRT-like protein (ZIP) and *IREG/Ferroportin* transporter family members to mediate cellular Ni uptake and efflux from the cytosol, respectively, have been reported in *A. thaliana* (Nishida et al. 2011; Morrissey et al. 2009).

We also observed a significant increase in the concentrations of some other micro- and macronutrients (Mn, Ca, Mg, S, and K) in the xylem sap of *O. inflata* upon short-term Fe re-supply to Fe-deficient plants,

which was of a smaller magnitude than the increase in Ni concentration. Like Ni, Mn is also a substrate of IRT1. The other nutrients, or part of them, may experience enhanced mobility for entry into the stele as a result of Fe deficiency-dependent delay in the formation of the suberin permeability barrier surrounding the endodermis, based on work done in *A. thaliana* (Barberon et al. 2016). Yet the profile of nutrients affected in the xylem sap of *O. inflata* according to this study does not match well with that reported in *A. thaliana*.

Importantly, between these treatments there were no statistically significant differences in His and NA concentrations in the xylem sap of *O. inflata* or *A. saxatilis*. This may indicate a lack of competition for chelation of Ni and Fe by His, or alternatively result from the quantitatively minor Fe levels by comparison to Ni (ratio of 1:14), which will render it difficult to detect significant changes in His concentrations. According to the literature, citrate efflux into the xylem, but not His or even NA, is crucial for root-to-shoot translocation as Fe^{III}citrate complex (dominant Fe species) in the xylem sap (Rellán-Álvarez et al. 2010). His generally forms more stable complexes with Ni²⁺ (pK_S = 8.6) than with Fe²⁺ (pK_S = 5.9; Callahan et al. 2006; Blindauer and Schmid 2010).

Previous work in Ni-hyperaccumulating *Noccaea caerulescens* revealed that Zn interferes with Ni hyperaccumulation in this species. Zn inhibited Ni accumulation almost completely (80 to 90%) in a serpentine population of *N. caerulescens*, when equimolar concentrations of Zn and Ni were applied in the root medium (Assunção et al. 2001). According to root and xylem sap composition of Ni-exposed plants, we found no indication that this occurs in *O. inflata*, in agreement with earlier findings on *A. lesbiacum* (Krämer et al. 1996). Quantitatively very small (ca. 10%) increases in root Ni concentrations of Zn-deficient plants may suggest a very small degree of Ni uptake via Zn transporters. However, concentrations in roots are the net result of root uptake and export into the xylem, which complicates the interpretation of this small effect. Mohseni et al. (2018) concluded that in *O. bracteata*, Ni is not taken up by Zn transporters. When Ni was supplied at 50 or 500 µM in the root medium of *O. bracteata* in combination with Zn (0, 100, 200 and 300 µM) for 4 h, root Ni concentrations were unaffected by either the presence or the absence of Zn.

The Zn starvation experiments of this study are difficult to interpret in relation to a competition between Zn and Ni or an effect on root-to-shoot Ni translocation operating through root His levels, possibly because there are indirect effects or because several processes acted simultaneously. The variation in the His concentration in roots illustrated in Fig. 2 and Fig. 8B, might be due to the difference in plant ages and growth conditions (growth chamber and greenhouse).

The contribution of the root apoplastic pathway to Ni uptake and Ni accumulation in the shoot

There are four different possible pathways for ions from the soil solution into the stele, where ions are exported into xylem vessels from adjacent cells, including (1) the symplastic pathway, (2) the coupled trans-cellular pathway, (3) the apoplastic pathway, and (4) the fully apoplastic pathway (Burch-Smith and Zambryski 2012; Barberon and Geldner 2014). The apoplastic pathway blocker employed here is

expected to decrease the flux into the xylem for ions following all pathways except the symplastic pathway, and thus decrease accumulation in the shoot of all ions not following the symplastic pathway (1). The apoplastic blocker did not cause any significant decrease in shoot Ni concentrations upon exposure to 30 or 100 μM Ni. However, shoot Ni accumulation was decreased by about 40% in plants exposed to 300 μM Ni. This suggested that at the lower two Ni concentrations Ni entered the xylem vessels exclusively *via* the symplastic pathway. By contrast, our data suggested that at 300 μM Ni, the entry of a small proportion of Ni into xylem vessels involved Ni movement in the apoplast (pathways 2, 3 or 4). An about 20% decrease in root Ni concentrations at all three levels of Ni exposure (30, 100, and 300 μM) in the presence of the apoplastic pathway blocker may be taken to support a minor contribution of the trans-cellular (2) or of an apoplastic pathway (3, 4) to root Ni accumulation. Taken together, these results would suggest that at low-to-moderate levels of Ni in the hydroponic solution, only the pool of Ni taken up by roots through the symplastic pathway is available for subsequent xylem loading. However, caution must be applied in the interpretation of these results. The components used to form the apoplastic block might be toxic. Therefore, we included the two compounds used to form the apoplastic block as controls and demonstrated that individually, they did not affect Ni accumulation. Yet, when both compounds were combined, the formation of the copper ferrocyanides may still have altered root ion uptake and ion movement within the plant through additional uncharacterized effects. It is also unclear whether the apoplastic block formed was complete and inhibiting all apoplastic movement of ions in the root. An incomplete apoplastic block would lead to the underestimation of the contributions of pathways 2 to 4. The apoplastic block might form on the outer surface of the root epidermis and thus interfere with the proton gradient or ion uptake into root epidermal cells, i.e. with the symplastic pathway. This would lead to an overestimation of the contribution of pathways 2 and 3 to root Ni uptake. To quantify Ni concentrations in roots, it is essential to desorb apoplastically bound Ni ions. This procedure was not specifically established and validated for the present work, but instead conducted according to standard protocols. Finally, the apoplastic blocker might influence the efficacy of the desorption procedure. Therefore, these results are preliminary and will require in-depth follow-up studies.

In summary, the results of our study unambiguously point at a key role for histidine as a low-molecular-weight Ni chelator in Ni hyperaccumulator *Odontarrhena inflata*, in line with results obtained with other Brassicaceae Ni/Zn hyperaccumulators. Its role in hyperaccumulators may be two-fold, i.e. 1) enhancing the root-to-shoot translocation of Ni (or that of Zn, in Zn-hyperaccumulators), probably through decreasing their vacuolar retention in peripheral root cells, thus enhancing the radial symplastic transport across the root toward the root stele, and 2) enhancing the Ni (or Zn) tolerance, probably through chelating the metal ions in the cytoplasm of root and/or shoot cells. The capacity for His-mediated Ni or Zn translocation and/or tolerance, and the phenomenon of stimulation of the His biosynthesis upon exposure to excess Ni, though less commonly, Zn, seems to be wide-spread also among non-accumulator Brassicaceae (see above), although exogenous His supply is often insufficient to obtain hyperaccumulator-like translocation and shoot metal concentration phenotypes in non-accumulators (A.D. Kozhevnikova, unpublished results), suggesting that enhanced His concentrations in the roots are not sufficient for that. It is likely that an enhanced uptake capacity and/or xylem loading

capacity are additionally required. However, the only component trait of the Ni or Zn hyperaccumulation syndrome, which appeared to be unique for hyperaccumulators thus far, is the “His response”, i.e. a parallel increase of the Ni and His concentrations in the xylem sap upon Ni exposure, which has only been observed in Ni hyperaccumulator *Odontarrhena* species, but not in any of the non-accumulator reference species (Krämer et al. 1996; Krämer and Kerkeb 2003; this study). It is difficult to decide whether this “His response” is critical for Ni hyperaccumulation capacity, or not: *Noccaea* Ni hyperaccumulators have not been studied at this point, and only a few of the non-accumulators have been shown to lack the “His response” in the xylem thus far (see above). It is also conceivable that the “His response” merely represents a secondary adaptation in hyperaccumulators, to improve the efficiency of the use of His, which is, in terms of ATP expenditure, a costly amino acid after all, in the chelation of Ni, or at least, it is unlikely that His would significantly improve the Ni transport through the xylem itself (see above). Finally, it is also conceivable that the major difference between Ni or Zn hyperaccumulators and non-accumulators lies in the degree of inhibition of the vacuolar retention in roots of these metals. Unfortunately, there is information only for *N. caerulescens* and *Thlaspi arvense* at this point, that is, chelation by His strongly inhibits the transport of Ni and Zn across the root cell tonoplast in *N. caerulescens*, but barely or not in *T. arvense* (Richau et al. 2009; Kozhevnikova et al. 2014).

In any case, many component traits of at least the Ni or Zn hyperaccumulation syndrome, such as the capacity for His-mediated Ni or Zn translocation or tolerance, and a Ni- or Zn-induced stimulation of the His biosynthesis, are also existent among non-accumulator Brassicaceae, and several non-metallicolous, non-accumulator species show “hyperaccumulator-like” translocation rates for Zn at least (Mohtadi and Schat 2024), suggesting a degree of “pre-adaptation” for Ni or Zn hyperaccumulation within this family. This might explain the relatively high frequency of hyperaccumulators among Brassicaceae. Unfortunately, there is barely information for other families. Further studies are urgently required.

Conclusion

In conclusion, our study suggests that the pools of free His in roots and NA in shoots of *O. inflata* are larger than in *A. saxatilis*. We observed a “His response” in the xylem of *O. inflata*, with higher Ni concentrations than His concentrations. Root and xylem His profiles are thus highly similar to those published for other Ni hyperaccumulating species of the genus *Alyssum* (Krämer et al. 1996; Kerkeb and Krämer 2003). Only in the Ni hyperaccumulator, we observed a positive effect of physiological Fe deficiency, and even more so of Fe deficiency followed by Fe luxury, on Ni levels in root and xylem sap. This suggests that Fe deficiency-induced processes contribute to increased Ni uptake and root-to-shoot flux. We did not test here whether Ni competes with Fe, and our data suggest against a predominant competition of Fe with Ni. We observed reduced Ni loading into the xylem under Zn deficiency in *O. inflata*, regardless of a short-term supplementation with luxury Zn. This may be explained by a decrease in the level of chelating compounds in the roots of Zn-deficient plants. In *O. inflata*, an apoplastic blocker reduced root Ni accumulation by about 20%. Moreover, root uptake pathways involving apoplastic movement of Ni are unlikely to contribute to shoot Ni accumulation, because Ni flux involving these

pathways was negligible below 300 μM Ni, a concentration unlikely to be reached in the soil solution in nature.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Funding

This research project was supported by the Ruhr University Bochum Research School through the award of a Ph.D. Exchange Scholarship to Soraya Soleymanifar.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by [Soraya Soleymanifar], [Ali Akbar Ehsanpour], and [Ute Kramer]. The first draft of the manuscript was written by [Soraya Soleymanifar] and [Ute Kramer] did the very extensive corrections of the manuscript.

Acknowledgment

We gratefully acknowledge the Ministry of Science, Research and Technology of Iran (MSRT) and University of Isfahan for their support. Special thanks to the Ruhr University Bochum Research School for supporting this research project through the award of a Ph.D. Exchange Scholarship to SS. JEB gratefully acknowledges funding from the German Research Foundation and the German State of North Rhine-Westphalia for the mass spectrometer ("Forschungsgroßgeräte" nach Art. 91b GG, INST 213/961-1 FUGG).

Data availability statement

Data is available based on reasonable request.

References

1. Albanese S, Sadeghi M, Lima A, Cicchella D, Dinelli E, Valera P, Falconi M, Demetriades A, De Vivo B, Team TGP)2015(GEMAS: cobalt, Cr, Cu and Ni distribution in agricultural and grazing land soil of

- Europe. J Geochem Explor 154: 81–93. <https://doi.org/10.1016/j.gexplo.2015.01.004>
2. Assunção AGL, Martins PDC, De Folter S, Vooijs R, Schat H, Aarts MGM (2001) Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 24(2): 217–226. <https://doi.org/10.1111/j.1365-3040.2001.00666.x>
 3. Baker AJ, Brooks R (1989) Terrestrial higher plants which hyperaccumulate metallic elements. A review of their distribution, ecology and phytochemistry. Biorecovery 1(2):81–126
 4. Baker AJM, Ernst WHO, van Der Ent A, Malaisse F, Ginocchio R (2010) Metallophytes: the unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America. In: Batty LC, Hallberg KB (eds) Ecology of industrial pollution. Cambridge University Press, Cambridge, UK, pp 7–40. <https://doi.org/10.1017/CBO9780511805561.003>
 5. Barberon M, Geldner N (2014) Radial transport of nutrients: the plant root as a polarized epithelium. Plant Physiol 166(2):528–537. <https://doi.org/10.1104/pp.114.246124>
 6. Barberon M, Vermeer JEM, De Bellis D, Wang P, Naseer S, Andersen TG, Humbel BM, Nawrath C, Takano J, Salt DE, Geldner N (2016) Adaptation of root function by nutrient-induced plasticity of endodermal differentiation. Cell 164(3):447–459. <https://doi.org/10.1016/j.cell.2015.12.021>
 7. Blindauer CA, Schmid R (2010) Cytosolic metal handling in plants: determinants for zinc specificity in metal transporters and metallothioneins. Metallomics 2(8):510–529. <https://doi.org/10.1039/c004880a>
 8. Burch-Smith TM, Zambryski PC (2012) Plasmodesmata paradigm shift: regulation from without versus within. Annu Rev Plant Biol 63:239–260. <https://doi.org/10.1146/annurev-arplant-042811-105453>
 9. Cailliatte R, Schikora A, Briat JF, Mari S, Curie C (2010) High-affinity manganese uptake by the metal transporter NRAMP1 is essential for *Arabidopsis* growth in low manganese conditions. Plant Cell 22(3):904–917. <https://doi.org/10.1105/tpc.109.073023>
 10. Callahan DL, Baker AJ, Kolev SD, Wedd AG (2006) Metal ion ligands in hyperaccumulating plants. J Biol Inorg Chem 11(1):2–12. <https://doi.org/10.1007/s00775-005-0056-7>
 11. Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212(4):475–486. <https://doi.org/10.1007/s004250000458>
 12. Clemens S (2019) Metal ligands in micronutrient acquisition and homeostasis. Plant Cell Environ 42(10):2902–2912. <https://doi.org/10.1111/pce.13627>
 13. Corso M, de la Torre VSG (2020) Biomolecular approaches to understanding metal tolerance and hyperaccumulation in plants. Metallomics 12(6):840–859. <https://doi.org/10.1039/d0mt00043d>
 14. Curie C, Cassin G, Couch D, Divol F, Higuchi K, Le Jean M, Misson J, Schikora A, Czernic P, Mari S (2009) Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. Ann Botany 103(1):1–11. <https://doi.org/10.1093/aob/mcn207>
 15. Deinlein U, Weber M, Schmidt H, Rensch S, Trampczynska A, Hansen TH, Husted S, Schjoerring JK, Talke IN, Krämer U, Clemens S (2012) Elevated nicotianamine levels in *Arabidopsis halleri* roots play

- a key role in zinc hyperaccumulation. *Plant Cell* 24(2):708–723. <https://doi.org/10.1105/tpc.111.095000>
16. Deng THB, Cloquet C, Tang YT, Sterckeman T, Echevarria G, Estrade N, Morel JL, Qiu RL (2014) Nickel and zinc isotope fractionation in hyperaccumulating and nonaccumulating plants. *Environ Sci Technol* 48(20):11926–11933. <https://doi.org/10.1021/es5020955>
 17. Deng THB, van der Ent A, Tang YT, Sterckeman T, Echevarria G, Morel JL, Qiu RL (2018) Nickel hyperaccumulation mechanisms: a review on the current state of knowledge. *Plant Soil* 423(1):1–11. <https://doi.org/10.1007/s11104-017-3539-8>
 18. de la García VS, Majorel-Loulergue C, Rigai GJ, Alfonso-González D, Soubigou-Taconnat L, Pillon Y, Barreau L, Thomine S, Fogliani B, Burtet-Sarramegna V, Merlot S (2021) Wide cross-species RNA-Seq comparison reveals convergent molecular mechanisms involved in nickel hyperaccumulation across dicotyledons. *New Phytol* 229(2):994–1006. <https://doi.org/10.1111/nph.16775>
 19. Ghaderian SM, Mohtadi A, Rahiminejad R, Reeves RD, Baker AJM (2007) Hyperaccumulation of nickel by two *Alyssum* species from the serpentine soils of Iran. *Plant Soil* 293(1):91–97. <https://doi.org/10.1007/s11104-007-9221-9>
 20. Ghaderian SM, Ghasemi R, Hajihashemi F (2015) Interaction of nickel and manganese in uptake, translocation and accumulation by the nickel-hyperaccumulator plant, *Alyssum bracteatum* (Brassicaceae). *Aust J Bot* 63(2):47–55
 21. Ghasemi R, Ghaderian SM, Krämer U (2009) Interference of nickel with copper and iron homeostasis contributes to metal toxicity symptoms in the nickel hyperaccumulator plant *Odontarrhena inflata*. *New Phytol* 184(3):566–580. <https://doi.org/10.1111/j.1469-8137.2009.02993.x>
 22. Halimaa P, Lin YF, Ahonen VH, Blande D, Clemens S, Gyenesei A, Häikiö E, Kärenlampi SO, Laiho A, Aarts MG, Pursiheimo JP (2014) Gene expression differences between *Noccaea caerulea* ecotypes help to identify candidate genes for metal phytoremediation. *Environ Sci Technol* 48(6):3344–3353. <https://doi.org/10.1021/es4042995>
 23. Harrison S, Rajakaruna N (2011) *Serpentine: evolution and ecology of a model system*. University of California Press, Berkeley, CA, USA
 24. Haydon MJ, Cobbett CS (2007) Transporters of ligands for essential metal ions in plants. *New Phytol* 174(3):499–506. <https://doi.org/10.1111/j.1469-8137.2007.02051.x>
 25. He Z, Shentu J, Yang X, Baligar VC, Zhang T, Stoffella PJ (2015) Heavy metal contamination of soils: sources, indicators and assessment. *J Environ Indic* 9:17–18
 26. Hoagland DR, Arnon DI (1950) *The Water-Culture Method for Growing Plants without Soil*. California Agricultural Experiment Station, Circular, p 347
 27. Ingle RA, Mugford ST, Rees JD, Campbell MM, Smith JAC (2005) Constitutively high expression of the histidine biosynthetic pathway contributes to nickel tolerance in hyperaccumulator plants. *Plant Cell* 17(7):2089–2106. <https://doi.org/10.1105/tpc.104.030577>
 28. Kerkeb L, Krämer U (2003) The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. *Plant Physiol* 131(2):716–724. <https://doi.org/10.1104/pp102.010686>

29. Kidd P, Barceló J, Bernal MP, Navari-Izzo F, Poschenrieder C, Shilev S, Clemente R, Monterroso C (2009) Trace element behaviour at the root–soil interface: implications in phytoremediation. *Environ Exp Bot* 67(1):243–259. <https://doi.org/10.1016/j.envexpbot.2009.06.013>
30. Kim S, Takahashi M, Higuchi K, Tsunoda K, Nakanishi H, Yoshimura E, Mori S, Nishizawa NK (2005) Increased nicotianamine biosynthesis confers enhanced tolerance of high levels of metals, in particular nickel, to plants. *Plant Cell Physiol* 46(11):1809–1818. <https://doi.org/10.1093/pcp/pci196>
31. Kobayashi T, Nozoye T, Nishizawa NK (2019) Iron transport and its regulation in plants. *Free Radic Biol Med* 133:11–20. <https://doi.org/10.1016/j.freeradbiomed.2018.10.439>
32. Konečná V, Šustr M, Požárová D, Čertner M, Krejčová A, Tylová E, Kolář F (2022) Genomic basis and phenotypic manifestation of (non-) parallel serpentine adaptation in *Arabidopsis arenosa*. *Evolution* 76(10):2315–2331. <https://doi.org/10.1111/evo.14593>
33. Kozhevnikova AD, Seregin IV, Erlikh NT, Shevyreva TA, Andreev IM, Verweij R, Schat H (2014) Histidine-mediated xylem loading of zinc is a species-wide character in *Noccaea caerulea*. *New Phytol* 203(2):508–519. <https://doi.org/10.1111/nph.12816>
34. Kozhevnikova AD, Seregin IV, Schat H (2021) Translocation of Ni and Zn in *Odontarrhena corsica* and *Noccaea caerulea*: the effects of exogenous histidine and Ni/Zn interactions. *Plant Soil* 468:295–318. <https://doi.org/10.1007/s11104-021-05080-y>
35. Krämer U, Cotter-Howells JD, Charnock JM, Baker AJ, Smith JAC (1996) Free histidine as a metal chelator in plants that accumulate nickel. *Nature* 379(6566):635–638. <https://doi.org/10.1038/379635a0>
36. Krämer U, Pickering IJ, Prince RC, Raskin I, Salt DE (2000) Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. *Plant Physiol* 122:1343–1354. <https://doi.org/10.1104/pp.122.4.1343>
37. Krämer U (2010) Metal hyperaccumulation in plants. *Annu Rev Plant Biol* 61(1):517–534. <https://doi.org/10.1146/annurev-arplant-042809-112156>
38. Kumar A, Cabral-Pinto M, Kumar A, Kumar M, Dinis PA (2020) Estimation of risk to the eco-environment and human health of using heavy metals in the Uttarakhand Himalaya, India. *Environ Sci* 10(20):7078
39. Mohtadi A, Schat H (2024) A comparison of nickel and zinc uptake and translocation in three species of Brassicaceae: The Ni hyperaccumulator *Odontarrhena corsica* and two non-hyperaccumulators, *Aurinia saxatilis* and *Lobularia maritima*. *Ecol Res* 39(4):596–604. <https://doi.org/10.1111/1440-1703.12439>
40. Nishida S, Tsuzuki C, Kato A, Aisu A, Yoshida J, Mizuno T (2011) AtIRT1, the primary iron uptake transporter in the root, mediates excess nickel accumulation in *Arabidopsis thaliana*. *Plant Cell Physiol* 52(8):1433–1442. <https://doi.org/10.1093/pcp/pcr089>
41. Nishida S, Aisu A, Mizuno T (2012) Induction of IRT1 by the nickel-induced iron-deficient response in *Arabidopsis*. *Plant Signal Behav* 7(3):329–331. <https://doi.org/10.4161/psb.19263>

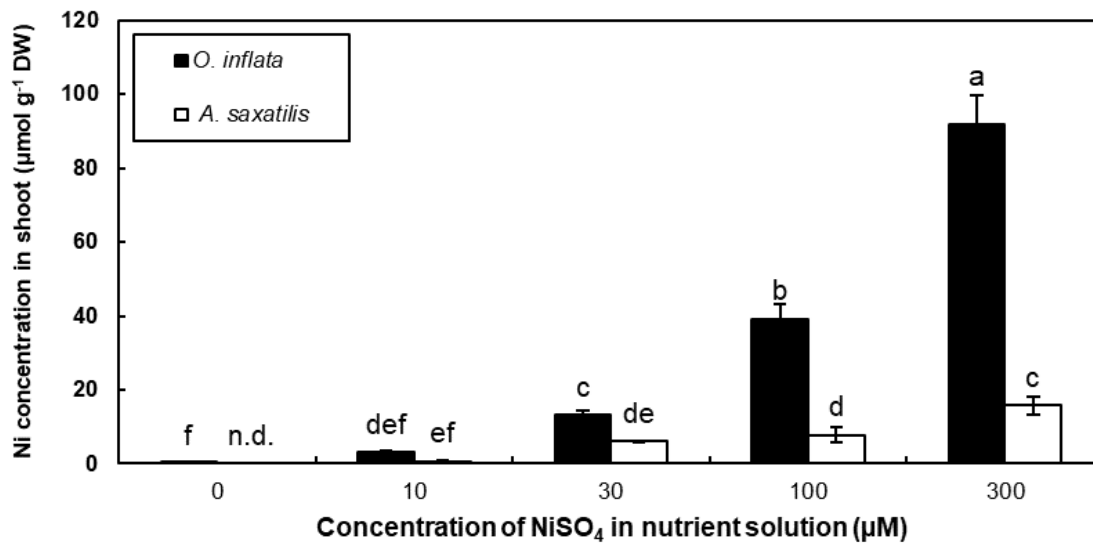
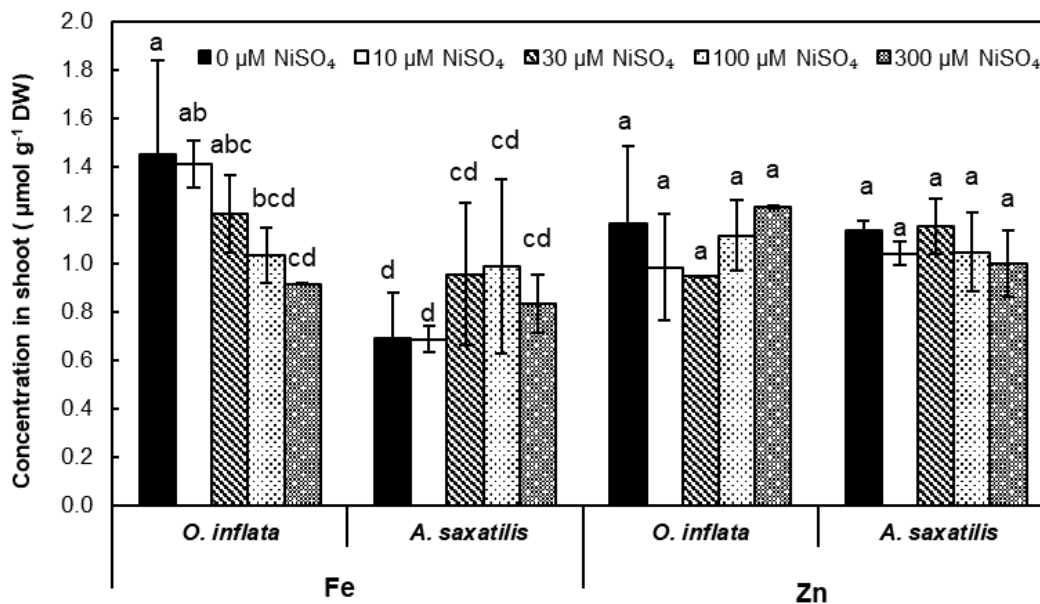
42. Nishida S, Kato A, Tsuzuki C, Yoshida J, Mizuno T (2015) Induction of nickel accumulation in response to zinc deficiency in *Arabidopsis thaliana*. *Int J Mol Sci* 16(5):9420–9430. <https://doi.org/10.3390/ijms16059420>
43. Noma M, Noguchi M, Tamaki E (1971) A new amino acid, nicotianamine, from tobacco leaves. *Tetrahedron Lett* 12:2017–2020. [https://doi.org/10.1016/S0040-4039\(01\)96769-3](https://doi.org/10.1016/S0040-4039(01)96769-3)
44. Mari S, Gendre D, Pianelli K, Ouerdane L, Lobinski R, Briat JF, Lebrun M, Czernic P (2006) Root-to-shoot long-distance circulation of nicotianamine and nicotianamine–nickel chelates in the metal hyperaccumulator *Thlaspi caerulescens*. *J Exp Bot* 57(15):4111–4122. <https://doi.org/10.1093/jxb/erl184>
45. Marschner H (1995) Mineral nutrition of higher plants. 2nd Edition, Academic Press, London
46. Merlot S, Hannibal L, Martins S, Martinelli L, Amir H, Lebrun M, Thomine S (2014) The metal transporter PglREG1 from the hyperaccumulator *Psychotria gabriellae* is a candidate gene for nickel tolerance and accumulation. *J Exp Bot* 65:1551–1564. <https://doi.org/10.1093/jxb/eru025>
47. Merlot S, Garcia de la Torre VS, Hanikenne M (2018) Physiology and molecular biology of trace element hyperaccumulation. In: van der Ent A, Baker AJM, Echevarria G, Simonnot MO, Morel JL (eds) *Agromining: farming for metals: extracting unconventional resources using plants*. Springer International Publishing, Cham, pp 155–181. https://doi.org/10.1007/978-3-319-61899-9_6
48. Mohseni R, Ghaderian SM, Ghasemi R, Schat H (2018) Differential effects of iron starvation and iron excess on nickel uptake kinetics in two Iranian nickel hyperaccumulators, *Odontarrhena bracteata* and *Odontarrhena inflata*. *Plant Soil* 428(1):153–162. <https://doi.org/10.1007/s11104-018-3666-x>
49. Morrissey J, Baxter IR, Lee J, Li L, Lahner B, Grotz N, Kaplan J, Salt DE, Guerinot ML (2009) The ferroportin metal efflux proteins function in iron and cobalt homeostasis in *Arabidopsis*. *Plant Cell* 21(10):3326–3338. <https://doi.org/10.1105/tpc.109.069401>
50. Palm ER, Van Volkenburgh E (2014) Physiological adaptations of plants to serpentine soils. In: Rajakaruna N, Boyd RS, Harris TB (eds) *Plant ecology and evolution in harsh environments*. Nova Science, New York, pp 129–148
51. Peer WA, Mahmoudian M, Freeman JL, Lahner B, Richards EL, Reeves RD, Murphy AS, Salt DE (2006) Assessment of plants from the Brassicaceae family as genetic models for the study of nickel and zinc hyperaccumulation. *New Phytol* 172(2):248–260. <https://doi.org/10.1111/j.1469-8137.2006.01820.x>
52. Puschenreiter M, Schnepf A, Millan IM, Fitz WJ, Horak O, Kle J, Schrefl T, Lombi E, Wenzel WW (2005) Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator *Thlaspi goesingense*. *Plant Soil* 271(1):205–218. <https://doi.org/10.1007/s11104-004-2387-5>
53. Ranathunge K, Steudle E, Lafitte R (2005) Blockage of apoplastic bypass-flow of water in rice roots by insoluble salt precipitates analogous to a Pfeffer cell. *Plant Cell Environ* 28(2):121–133. <https://doi.org/10.1111/j.1365-3040.2004.01245.x>
54. Reeves RD, Baker AJ, Jaffré T, Erskine PD, Echevarria G, van der Ent A (2018) A global database for plants that hyperaccumulate metal and metalloid trace elements. *New Phytol* 218(2):407–411.

<https://doi.org/10.1111/nph.14907>

55. Redjala T, Sterckeman T, Skiker S, Echevarria G (2010) Contribution of apoplast and symplast to short term nickel uptake by maize and *Leptoplax emarginata* roots. *Environ Exp Bot* 68(1):99–106. <https://doi.org/10.1016/j.envexpbot.2009.10.010>
56. Rellán-Álvarez R, Giner-Martínez-Sierra J, Orduna J, Orera I, Rodríguez-Castrillón JÁ, García-Alonso JI, Abadía J, Álvarez-Fernández A (2010) Identification of a tri-iron (III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. *Plant Cell Physiol* 51(1):91–102. <https://doi.org/10.1093/pcp/pcp170>
57. Richau KH, Kozhevnikova AD, Seregin IV, Vooijs R, Koevoets PL, Smith JAC, Ivanov VB, Schat H (2009) Chelation by histidine inhibits the vacuolar sequestration of nickel in roots of the hyperaccumulator *Thlaspi caerulescens*. *New Phytol* 183(1):106–116. <https://doi.org/10.1111/j.1469-8137.2009.02826.x>
58. Scheible WR, González-Fontes A, Morcuende R, Lauerer M, Geiger M, Glaab J, Gojon A, Schulze ED, Stitt M (1997) Tobacco mutants with a decreased number of functional nia genes compensate by modifying the diurnal regulation of transcription, post-translational modification and turnover of nitrate reductase. *Planta* 203(3):304–319. <https://doi.org/10.1007/s004250050196>
59. Seregin IV, Kozhevnikova AD (2006) Physiological role of nickel and its toxic effects on higher plants. *Russ J Plant Physiol* 53:257–277. <https://doi.org/10.1134/S1021443706020178>
60. Seregin IV, Kozhevnikova AD, Schat H (2022) Histidine-mediated nickel and zinc translocation in *Arabidopsis thaliana* and *Lepidium ruderales*. *Russ J Plant Physiol* 69(1). <https://doi.org/10.1134/S1021443722010186>
61. Shojima S, Nishizawa NK, Fushiya S, Nozoe S, Kumashiro T, Nagata T, Ohata T, Mori S (1989) Biosynthesis of nicotianamine in the suspension-cultured cells of tobacco (*Nicotiana megalosiphon*). *Biology Met* 2:142–145. <https://doi.org/10.1007/BF01142552>
62. Sobczyk MK, Smith JAC, Pollard AJ, Filatov DA (2017) Evolution of nickel hyperaccumulation and serpentine adaptation in the *Alyssum serpyllifolium* species complex. *Heredity* 118(1):31–41. [10.1038/hdy.2016.93](https://doi.org/10.1038/hdy.2016.93)
63. Soleymanifar S, Ehsanpour AA, Ghasemi R, Schat H (2024) The effect of L-histidine on nickel translocation and the activities of antioxidant enzymes in hyperaccumulator (*Odontarrhena inflata*) and non-accumulator (*Aurinia saxatilis*) plants. *Plant Soil* 495:411–428. <https://doi.org/10.1007/s11104-023-06340-9>
64. Taylor SI, Macnair MR (2006) Within and between population variation for zinc and nickel accumulation in two species of *Thlaspi*. (Brassicaceae) *New Phytologist* 169(3):505–514. <https://doi.org/10.1111/j.1469-8137.2005.01625.x>
65. Van de Mortel JE, Villanueva LA, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, van Ver Loren E, Koornneef M, Aarts MGM (2006) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and

- the related metal hyperaccumulator *Thlaspi caerulescens*. Plant Physiol 142(3):1127–1147.
<https://doi.org/10.1104/pp.106.082073>
66. Vert GA, Briat JF, Curie C (2003) Dual regulation of the *Arabidopsis* high-affinity root iron uptake system by local and long-distance signals. Plant Physiol 132(2):796–804.
<https://doi.org/10.1093/plphys/kiad677>
67. Weber M, Harada E, Vess C, Roepenack-Lahaye EV, Clemens S (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. Plant J 37(2):269–281. <https://doi.org/10.1046/j.1365-313X.2003.01960.x>
68. Wycisk K, Kim EJ, Schroeder JI, Krämer U (2004) Enhancing the first enzymatic step in the histidine biosynthesis pathway increases the free histidine pool and nickel tolerance in *Arabidopsis thaliana*. FEBS Lett 578:128–134. <https://doi.org/10.1016/j.febslet.2004.10.086>
69. Zhang Q, Smith AF, Sekimoto H, Reid RJ (2001) Effect of membrane surface charge on nickel uptake by purified mung bean root protoplasts. Planta 213(5):788–793.
<https://doi.org/10.1007/s004250100555>

Figures

a**b****Figure 1**

Effects of nickel (Ni) treatment on the concentrations of Ni, iron (Fe) and zinc (Zn) concentrations in shoots of *O. inflata* and *A. saxatilis*. Five-week-old plants cultivated hydroponically in a modified 0.25x Hoagland solution were supplemented with a range of Ni concentrations for 8 d. (a) Ni, (b) Fe and Zn concentrations in shoots. Bars represent arithmetic means $\pm$ SD, $n = 3$ pools of tissues from three plants cultivated in one culture vessel, with three culture vessels per treatment and species. Different

characters denote statistically significant differences ($P < 0.05$, based on ANOVA and Duncan's test). DW, dry biomass; n.d. below detection limit

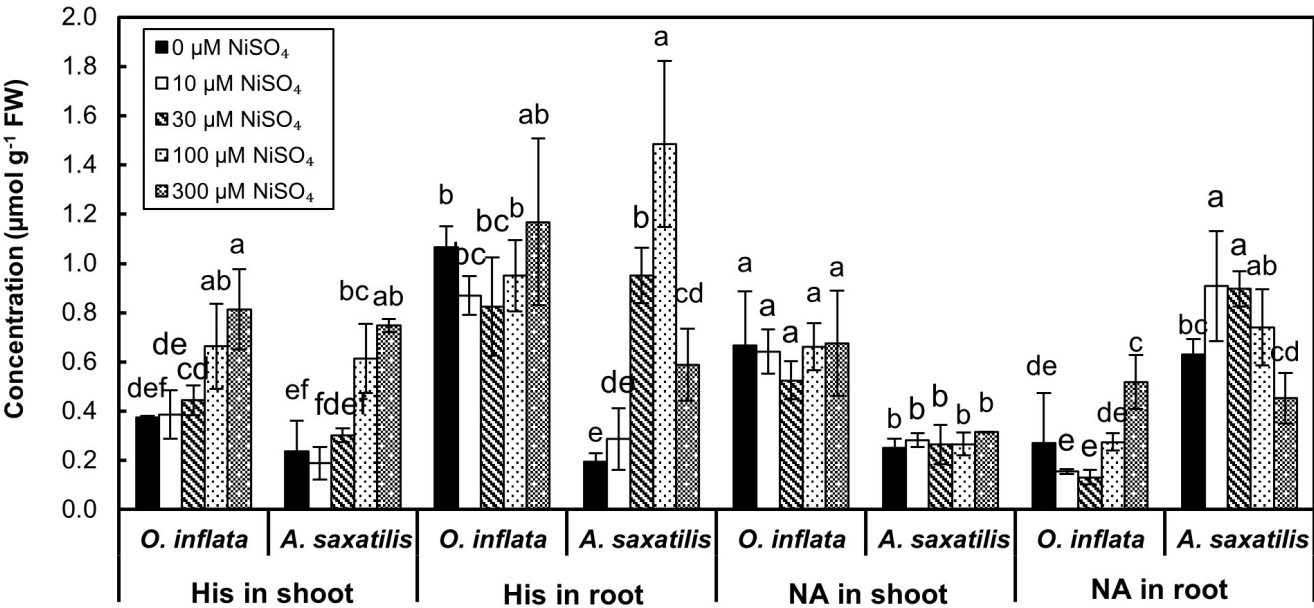


Figure 2

Effects of nickel (Ni) treatment on the concentrations of histidine (His) and nicotianamine (NA) in shoots and roots of *O. inflata* and *A. saxatilis*. Five-week-old plants were cultivated hydroponically in a modified 0.25x Hoagland solution supplemented with a range of Ni concentrations for 8 d (see Fig. 1). Bars represent arithmetic means $\pm$ SD, $n = 3$ pools of tissues from three plants cultivated in one culture vessel, with three culture vessels in each experiment per treatment and species. Different characters denote statistically significant differences ($P < 0.05$, based on ANOVA and Duncan's test). FW, fresh biomass

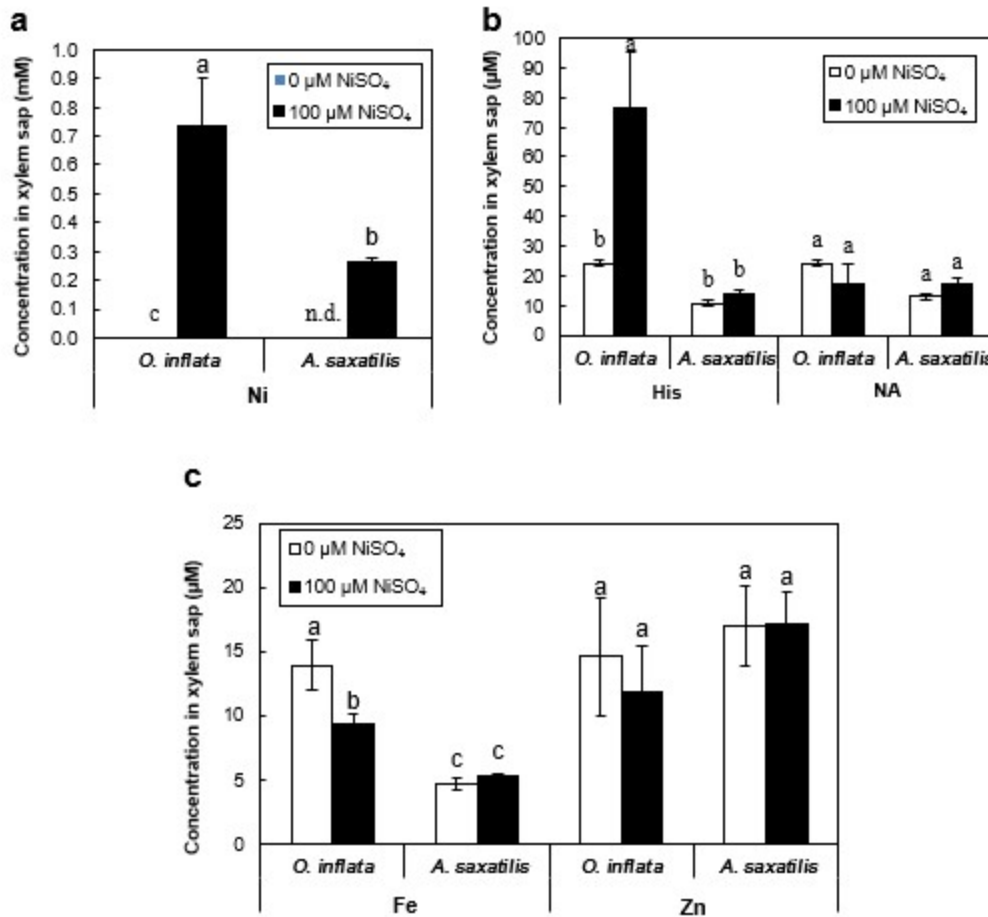


Figure 3

Effects of short-term Ni treatments on xylem sap composition. Shown are (a) concentrations of Ni, (b) His and NA, and (c) of Fe and Zn in xylem sap of *O. inflata* and *A. saxatilis*. Roots of 12-week-old plants were supplemented with 0 and 100 μM Ni for 48 h before the collection of xylem sap as root pressure exudate for a period of 8 h. Bars represent arithmetic means $\pm$ SD ($n = 3$, each replicate representing pooled xylem sap from nine plants cultivated in three culture vessels, with three replicate vessels per treatment and species). Different characters denote statistically significant differences between species and treatments ($P < 0.05$, ANOVA with Duncan's test)

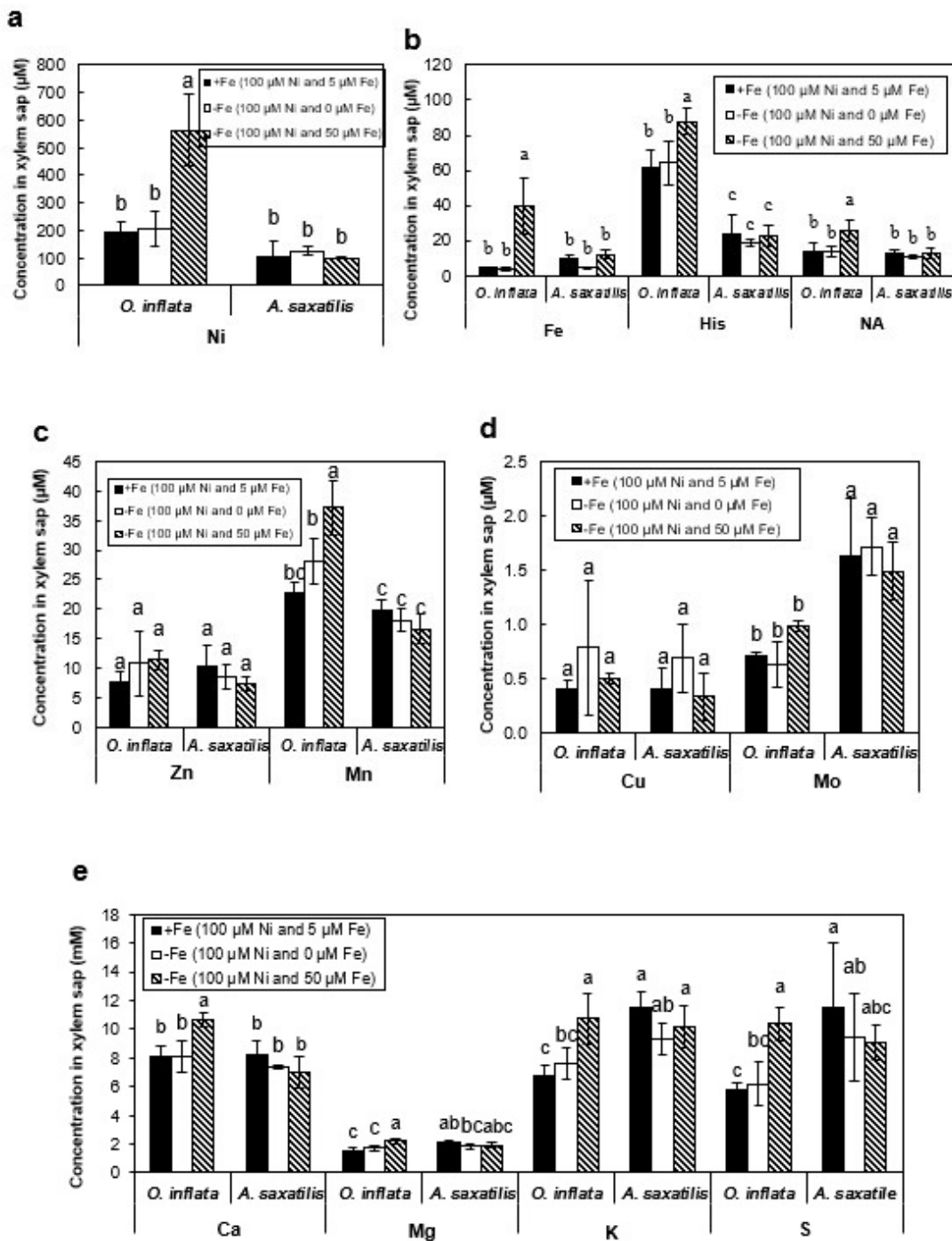


Figure 4

Effects of short-term Ni exposure under Fe sufficiency, Fe deficiency and Fe deficiency followed by short-term Fe luxury on the composition of xylem sap of *O. inflata* and *A. saxatilis*. Concentrations in xylem sap of (a) Ni, (b) Fe, His and NA, (c) Zn and manganese (Mn), (d) copper (Cu) and molybdenum (Mo) and (e) macronutrients calcium (Ca), magnesium (Mg), potassium (K), and sulfur (S). Prior to Ni exposure for 4 h, nine-week-old plants were Fe-starved for 5 d (- Fe, white and dashed bars), or continuously cultivated

in a hydroponic solution with normal Fe supply (+ Fe controls, 5 μM FeHBED, black bars). Subsequently, all plants were exposed to 100 μM Ni, for Fe-deficient plants in combination with either continued Fe deficiency (white bars), or Fe luxury (50 μM FeHBED, dashed bars). Shoots were detached and xylem sap was collected for a period of 8 h. Shown are means $\pm$ SE ($n = 3$ replicate xylem sap samples, each pooled from nine plants cultivated in three culture vessels, with three replicate culture vessels per species and treatment). For each compound, different characters reflect statistically significant differences ($P < 0.05$, ANOVA with Duncan's test)

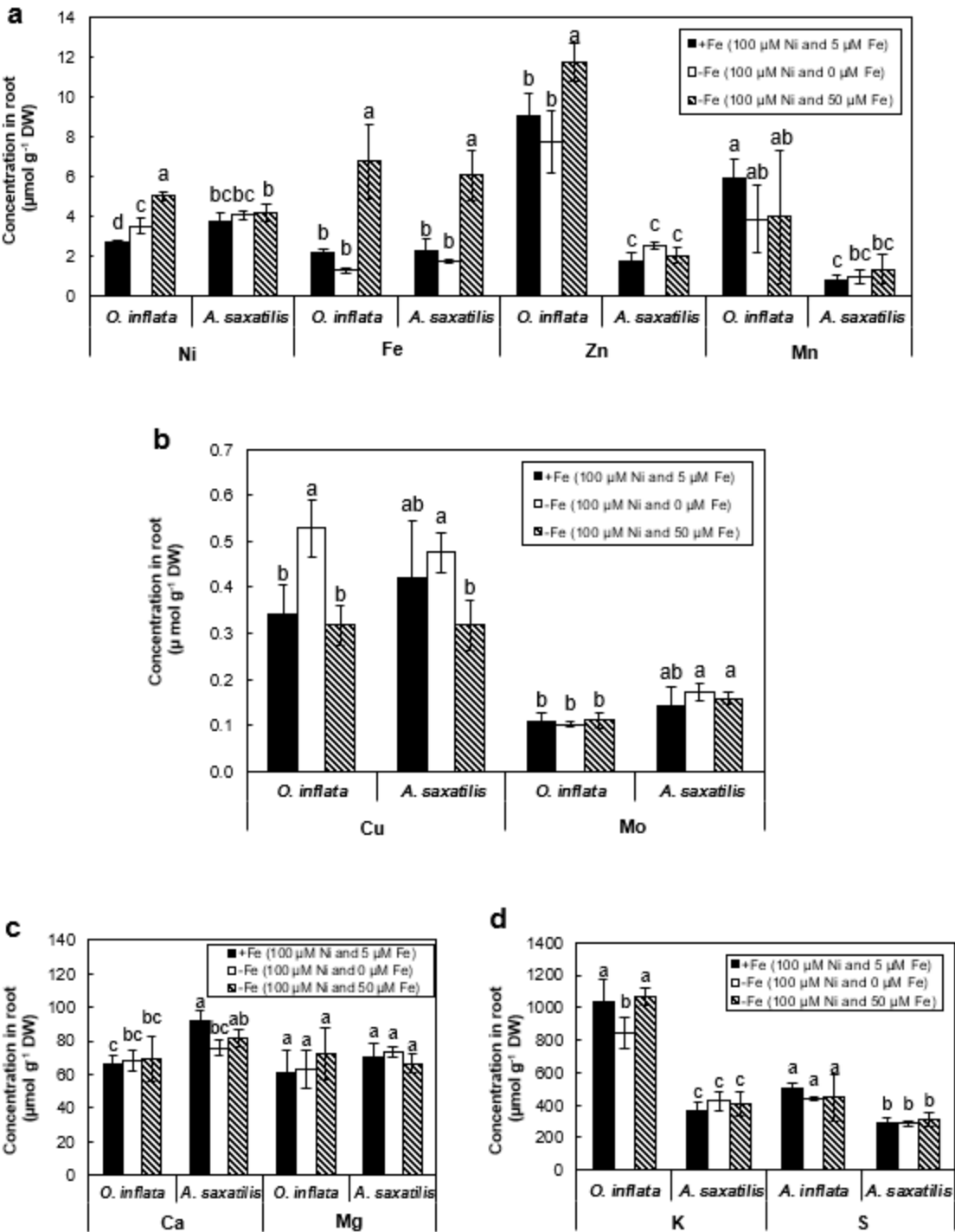


Figure 5

Effects of short-term Ni exposure under Fe sufficiency, Fe deficiency and Fe deficiency followed by short-term Fe luxury on the composition of roots of *O. inflata* and *A. saxatilis*. Concentrations in roots of (a) Ni, Fe, Zn and Mn, (b) Cu and Mo, and (c and d) Ca, Mg, K, and S. Nine-week-old plants were Fe-starved for 5 d prior to Ni exposure (- Fe, white and dashed bars), or continuously cultivated in a hydroponic solution with normal Fe supply (+ Fe controls, 5 μ M FeHED, black bars). Subsequently, all plants were exposed to 100 μ M Ni, for Fe-deficient plants in combination with either continued Fe deficiency (white bars), or Fe luxury (50 μ M FeHED, dashed bars) for 4 h. Roots were harvested at the end of the 8-h xylem sap collection period. Shown values are means $\pm$ SD (n = 3 root samples, each pooled from nine plants cultivated in three culture vessels, with three replicate culture vessels per species and treatment). For each compound, different characters reflect statistically significant differences (P < 0.05, ANOVA with Duncan's test)

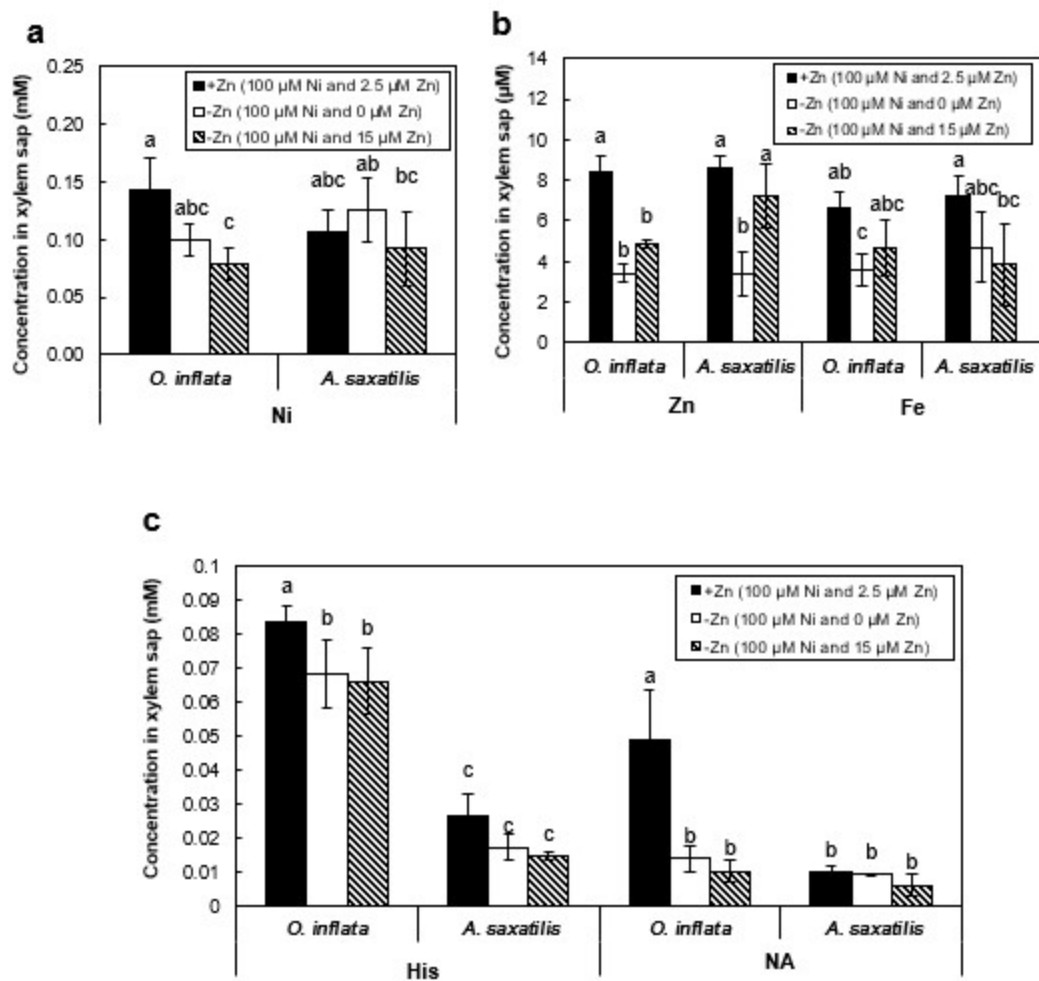


Figure 6

Effects of short-term Ni exposure under Zn sufficiency, Zn deficiency and Zn deficiency followed by short-term Zn luxury on the composition of xylem sap of *O. inflata* and *A. saxatilis*. Shown are (a) concentrations of Ni, (b) Zn, Fe, and (c) and His and NA in xylem sap. Nine-week-old plants were Zn-

starved for 2 w prior to Ni exposure (white and dashed bars) or continuously maintained in a nutrient solution with normal Zn supply (2.5 μM ZnSO_4 controls, black bars). Subsequently, all plants were exposed to 100 μM Ni, for Zn-deficient plants in combination with either continued Zn deficiency (white bars) or Zn luxury (15 μM ZnSO_4 , dashed bars) for 4 h. Shoots were detached and xylem sap was collected for a period of 8 h. Shown are means $\pm$ SE ($n = 3$ replicate xylem sap samples, each pooled from nine plants cultivated in a total of three culture vessels, with three replicate culture vessels per species and treatment). For each compound, different letters show statistically significant differences ($P < 0.05$) between treatments, based on ANOVA (Duncan's test)

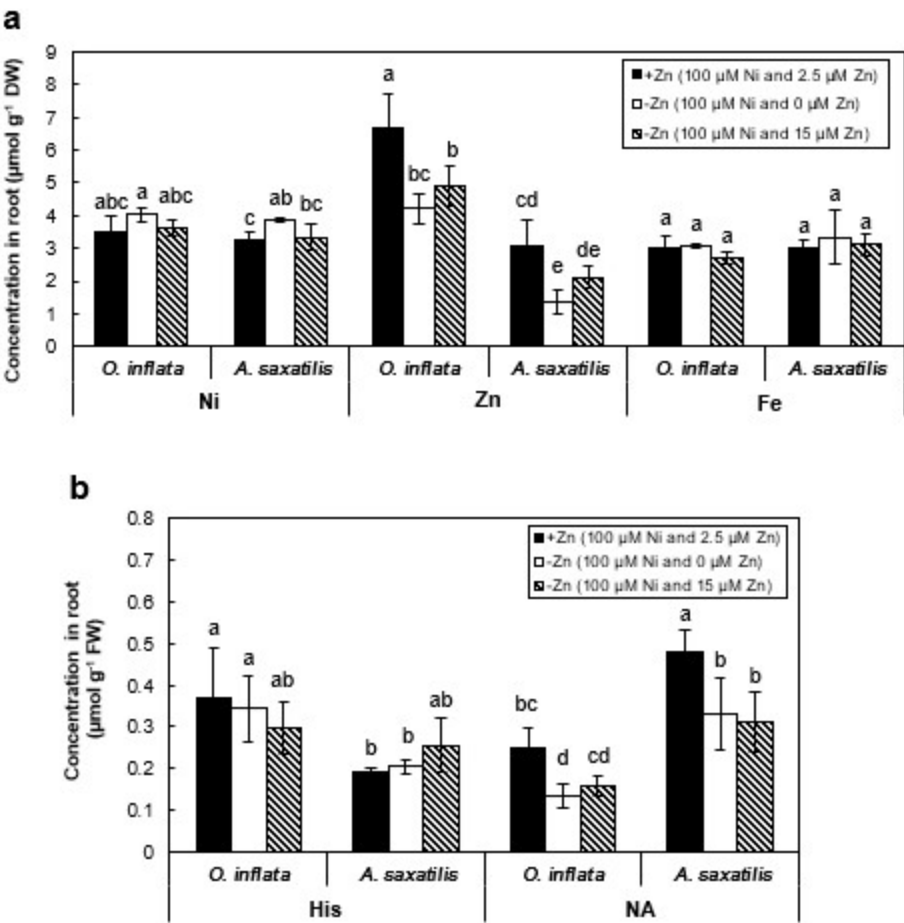


Figure 7

Effects of short-term Ni exposure under Zn sufficiency, Zn deficiency and Zn deficiency followed by short-term Zn luxury on the composition of roots of *O. inflata* and *A. saxatilis*. Shown are (a) concentrations of Ni, Zn and Fe, and (b) concentrations of His and NA in roots. Nine-week-old plants were Zn-starved for 2 w prior to Ni exposure (white and dashed bars) or continuously maintained in a nutrient solution with normal Zn supply (2.5 μM ZnSO_4 controls, black bars). Subsequently, all plants were exposed to 100 μM Ni, for Zn-deficient plants in combination with either continued Zn deficiency (white bars) or Zn luxury (15 μM ZnSO_4 , dashed bars) for 4 h. Roots were harvested at the end of the 8-h xylem sap collection period. Shown values are means $\pm$ SD ($n = 3$ root samples, each pooled from nine plants cultivated in three culture vessels, with three replicate culture vessels per species and treatment).

For each compound, different characters denote statistically significant differences ($P < 0.05$, based on ANOVA with Duncan's test)

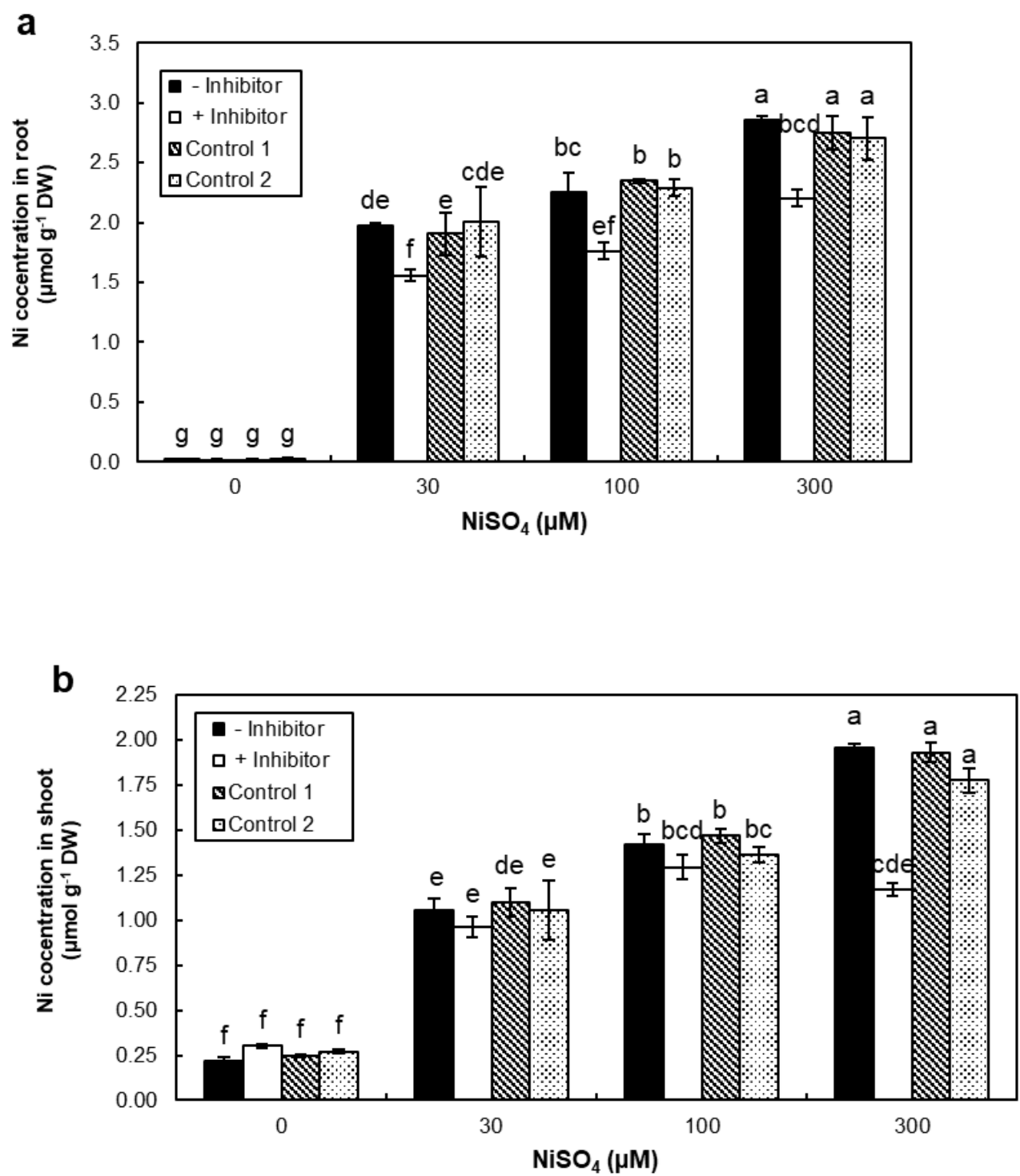


Figure 8

The effects of a blocker of the apoplastic pathway on Ni concentrations in roots and shoots. The concentrations of Ni in (a) root and (b) shoot of *O. inflata* plants without (black bars) and with pre-treatment with a blocker of the apoplastic pathway (1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ and 0.05 mM CuSO_4 ; white bars).

Two additional controls were included of 1 mM $K_4[Fe(CN)_6]$ (dashed bars, Control 1) and 0.05 mM $CuSO_4$ (dotted bars, Control 2), in order to assess possible effects of these two compounds individually. Eight-week-old plants were treated with 1 mM $K_4[Fe(CN)_6]$ overnight (10 h) and then exposed to 0.05 mM $CuSO_4$ for 1 h in the following morning. Afterwards, the plants were exposed to a range of Ni concentrations for 8 h, followed by harvest of roots and shoots. Shown are arithmetic means $\pm$ SD ($n = 3$ pools of tissues from three plants cultivated in one culture vessel, with three replicate culture vessels per treatment). Different characters denote statistically significant differences ($P < 0.05$, based on ANOVA with Duncan's test)

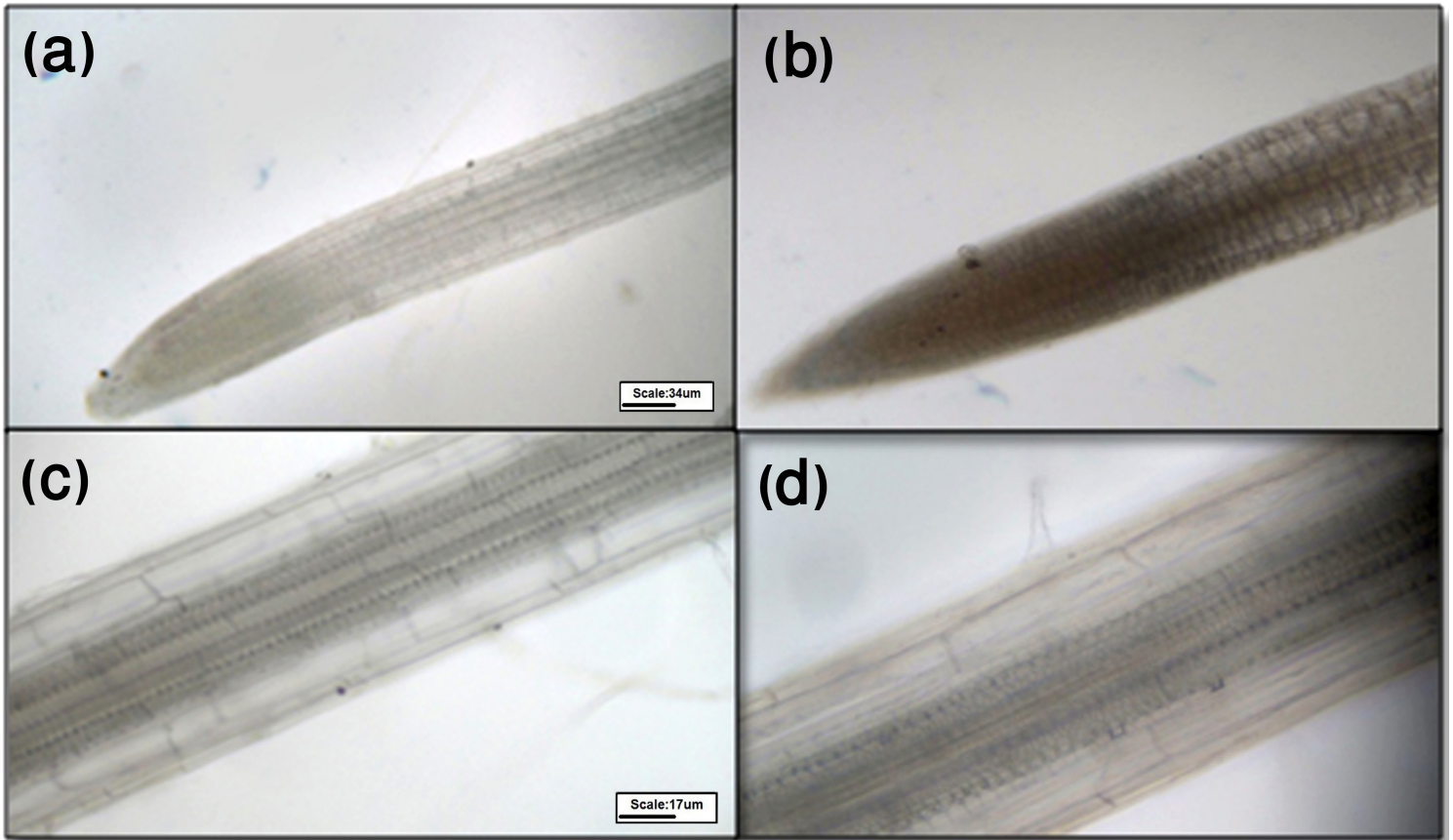


Figure 9

Microscopic images of roots of *O. inflata* exposed to compounds generating blockage of the apoplastic pathway of root ion uptake and unexposed controls. (a-d) Longitudinal view of the root apex (a and b; magnification 20x; scale bar 34 μ m) and root differentiation zone (c and d; magnification 40x; scale bar 17 μ m) of 8-week-old *O. inflata* plants. The root of control plants untreated with apoplastic blockage components (a, c). The root of plants after applying the apoplastic blocker and the formation of brown precipitates of copper ferrocyanide in the root apoplast (b, d)

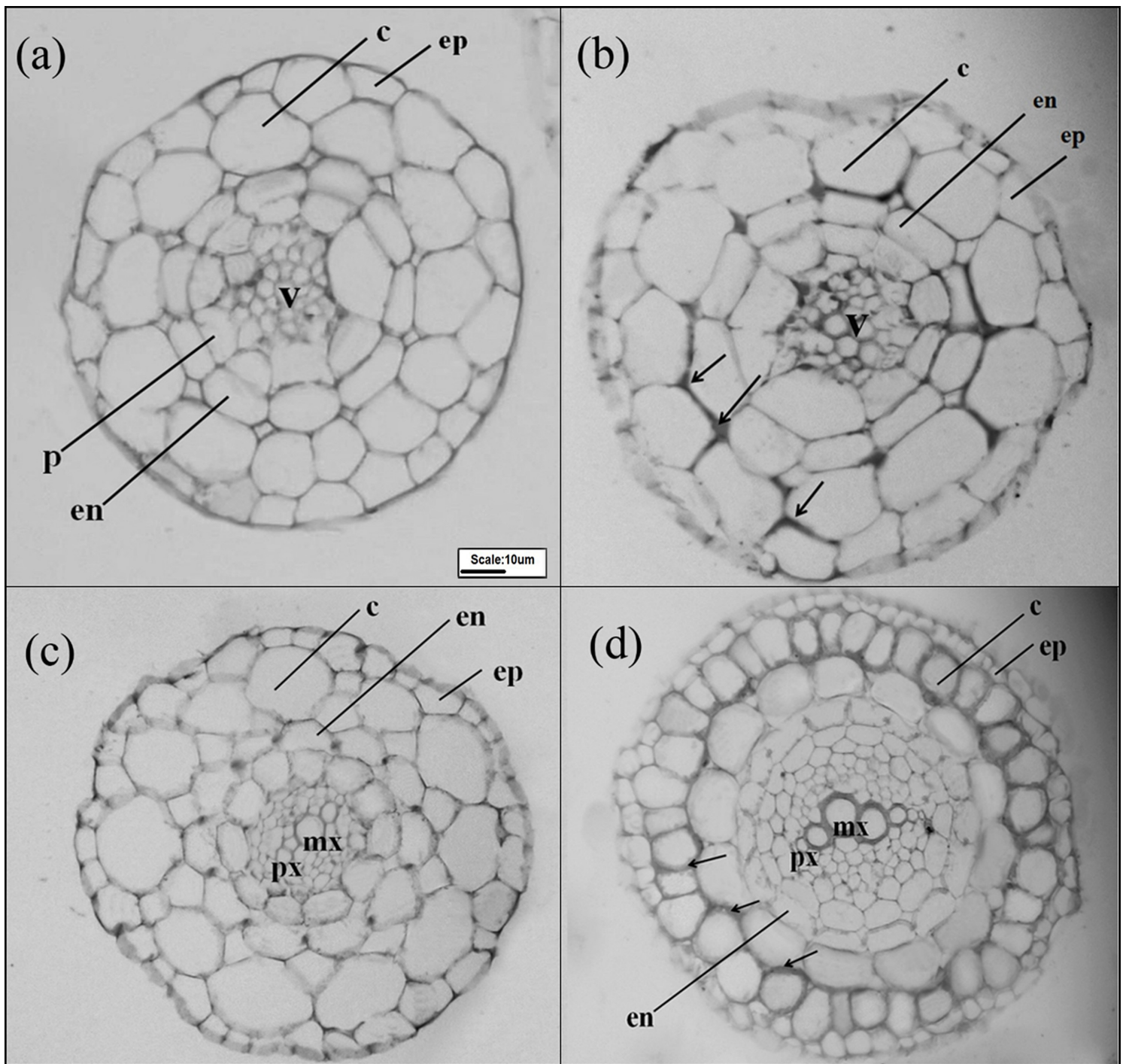


Figure 10

Microscopic images of transverse sections of roots of *O. inflata* exposed to compounds generating blockage of the apoplastic pathway of ion uptake and unexposed controls. (a-d) Free-hand cross-sections of *O. inflata* root segments, approximately 40 mm (a, b) and 60 mm (c, d) from the root apex, before (a and c, left) and after (b and d, right) the formation of $\text{Cu}_2[\text{Fe}(\text{CN})_6]$ or $\text{Cu}[\text{CuFe}(\text{CN})_6]$ salt crystals in the root apoplast. Epidermis – ep, cortex – c, endodermis – en, pericycle – p, vasculature – v, protoxylem – px, metaxylem – mx, with arrows pointing to apoplastic precipitates of copper ferrocyanides. Images were taken 1 h after supplying roots with CuSO_4 of plants pre-treated with 1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ overnight.

Magnification 60x; scale bar 10 μm (a, b, c and d). Sections of two replicate roots are shown for each control and treatment

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supportinginformation.docx](#)