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Nickel and Zinc Isotope Fractionation in Hyperaccumulating and Nonaccumulating Plants

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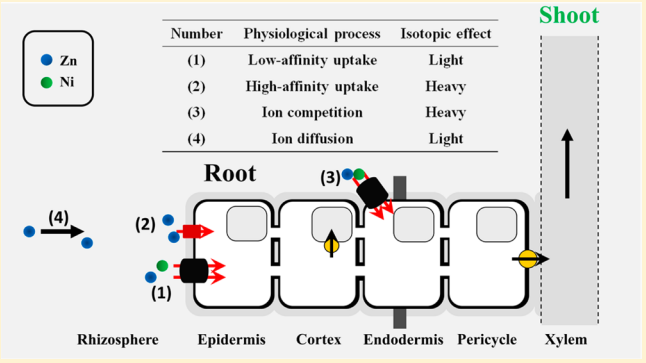
Supporting Information

ABSTRACT: Until now, there has been little data on the isotope fractionation of nickel (Ni) in higher plants and how this can be affected by plant Ni and zinc (Zn) homeostasis. A hydroponic cultivation was conducted to investigate the isotope fractionation of Ni and Zn during plant uptake and translocation processes. The nonaccumulator *Thlaspi arvense*, the Ni hyperaccumulator *Alyssum murale* and the Ni and Zn hyperaccumulator *Noccaea caerulescens* were grown in low (2 μM) and high (50 μM) Ni and Zn solutions. Results showed that plants were inclined to absorb light Ni isotopes, presumably due to the functioning of low-affinity transport systems across root cell membrane. The Ni isotope fractionation between plant and solution was greater in the hyperaccumulators grown in low Zn treatments ($\Delta^{60}Ni_{plant\text{-}solution}$ = −0.90 to −0.63‰) than that in the nonaccumulator *T. arvense* ($\Delta^{60}Ni_{plant\text{-}solution}$ = −0.21‰), thus indicating a greater permeability of the low-affinity transport system in hyperaccumulators. Light isotope enrichment of Zn was observed in most of the plants ($\Delta^{66}Zn_{plant\text{-}solution}$ = −0.23 to −0.10‰), but to a lesser extent than for Ni. The rapid uptake of Zn on the root surfaces caused concentration gradients, which induced ion diffusion in the rhizosphere and could result in light Zn isotope enrichment in the hyperaccumulator *N. caerulescens*. In high Zn treatment, Zn could compete with Ni during the uptake process, which reduced Ni concentration in plants and decreased the extent of Ni isotope fractionation ($\Delta^{60}Ni_{plant\text{-}solution}$ = −0.11 to −0.07‰), indicating that plants might take up Ni through a low-affinity transport system of Zn. We propose that isotope composition analysis for transition elements could become an empirical tool to study plant physiological processes.



INTRODUCTION

Nickel is the latest element to be listed as one of the essential mineral elements for higher plants.[1−3] Most plants have low Ni concentrations, normally ranging from 0.01 to 5 μg/g.[4] Nickel was first discovered as the central part of the active site of urease,[5] an enzyme that is widely distributed in higher plants,[6] which catalyzes urea hydrolysis. The activity of urease prevents urea accumulation, and contributes to the recycling of endogenous nitrogen for plant growth. As an irreplaceable metallic center in urease, Ni is essential for higher plants, even though it is usually required in ultramicro concentrations. For example, the Ni demand for the germination of barley grain is 90 ng/g.[3] In spite of the effect of urease activation, other physiological functions of Ni still remain obscure in higher plants.[1]

While most plants only contain less than 10 μg/g of Ni in their tissues, a particular group of plant species, termed Ni hyperaccumulators, have been discovered on Ni-rich soils. These plants are capable of accumulating more than 1000 μg/g of Ni in their shoots.[7] Instead of using root sequestration for metal detoxification, hyperaccumulators transfer most Ni to shoots, where it is stored and detoxified. Some authors have reported that these plants are capable of accumulating up to 30 000 μg/g Ni in their leaves.[8] Approximately 450 species of Ni hyperaccumulators have been identified around the world[9] and

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their unique trait has intrigued the idea of phytomining in nickelliferous soils.[10,11]

The physiological mechanisms involved in the Ni homeostasis in hyperaccumulators as well as ordinary plants (nonaccumulators) are far from being fully understood. Nickel absorption by soybean plants grown in various Ni concentrations fits Michaelis−Menten kinetics.[12] Aschmann et al.[13] measured the Michaelis−Menten constant (K_m) and maximum rate (V_{max}) of Ni uptake in oat plants, finding the K_m value for Ni to be 0.012 mM. Redjala et al.[14] also found that maize and *Leptoplax emarginata*, a Ni hyperaccumulator, have similar K_m values (0.08−0.10 mM) for the symplastic influx. These results indicate that Ni is transported through a low-affinity transport system. Until now, no high-affinity Ni transporter has been identified in higher plants. Moreover, little is known about how hyperaccumulators are able to take up Ni so efficiently. Ni is also found to compete with other cations during the absorption process. Copper (Cu)(II) and Zn(II) appear to strongly and competitively inhibit Ni(II) influx in soybean and barley, and calcium (Ca)(II) and magnesium(II) are noncompetitive inhibitors of Ni(II) influx.[12,15] For the Zn/Ni hyperaccumulator *Thlaspi pindicum*, which originates from a serpentine area, Ni absorption is inhibited by the addition of Zn in hydroponic culture solution.[16] This preference of Zn over Ni has also been observed in various populations of *Noccaea caerluscens*.[17] However, the physiological mechanisms underlying this phenomenon are still unclear.

Recent studies have suggested that the isotope fractionation in higher plants could be a consequence of the physiological processes involved in metal homeostasis. For the root uptake process, Weiss et al.[18] have proposed that carrier-mediated transport, or high-affinity transport, favors heavy isotopes, whereas low-affinity transport, e.g., ion channel and electrogenic pump, favors light isotopes. John et al.[19] demonstrated that a switch from high- to low-affinity transport would result in an isotopic shift from −0.2 to −0.8‰ for Zn uptake in marine diatoms. In addition, Guelke and von Blanckenburg[20] found a heavy isotope depletion of 1.6‰ in strategy I plants, for which Fe(III) is reduced to Fe(II) during root absorption, whereas the uptake of Fe(III)−siderophore complexes by strategy II plants, can result in 0.2‰ heavy isotope enrichment. For the shoot-root translocation process, Tang et al.[21] observed an enrichment of light Zn isotopes in shoots relative to roots, which might be attributable to the root sequestration and active xylem loading processes. The isotopes could further fractionate during the long distance transport. Moynier et al.[22] proposed that Zn isotope fractionation between stem and leaf could be caused by ion diffusion during xylem transport. Although many hypotheses have been put forward, the relationship between isotope fractionation and the underlying physiological mechanisms are still ambiguous.

Until now, most of the studies regarding isotope fractionation of micronutrients in higher plants have focused on Zn, Fe, and Cu. Little is known about Ni isotope fractionation in plants. The only study regarding Ni isotope fractionation in biotic samples found that Ni isotope fractionation does exist in microorganisms, and methanogens species with greater Ni requirement incorporated much lighter Ni isotopes than the nonmethanogens species.[23] In this study, we chose three model plant species, i.e. the nonaccumulator *Thlaspi arvense* L., the Ni hyperaccumulator *Alyssum murale* Waldst. & Kit., and the Ni and Zn hyperaccumulator *N. caerulescens* (J. & C. Presl) F. K. Mey to study their different Ni and Zn homeostasis mechanisms. The plants were exposed to low and high levels of Ni and Zn in hydroponics. Zinc was introduced into this experiment because the plant uptake and the isotope fractionation of this element are relatively well documented, and the comparison of its fractionation to that of Ni could help to interpret the latter. Moreover, we wished to assess the effect of Ni and Zn competition on the isotope fractionation of these elements. The objectives of this study are therefore (1) to give an overview of Ni isotope fractionation in higher plants, (2) to relate the isotope fractionation patterns to Ni uptake and translocation mechanisms of both hyperaccumulators and nonaccumulators, (3) to assess the consequence of Ni and Zn competition on isotope fractionation.

MATERIALS AND METHODS

Plant Cultivation and Harvest. Seeds of *T. arvense* (collected in Nancy, France), *A. murale* (collected in Pojska, Albania) and *N. caerulescens* (collected in Puy de Wolf, France) were sown on agar and germinated in the dark at 25 °C for 5 days. Then 24 seedlings of each species were transferred to 5 L nutrient solutions in a growth chamber for pretreatment. The solution for *T. arvense* contained the following nutrients (in μM): 1000 $Ca(NO_3)_2$, 1000 KNO_3, 500 $MgSO_4$, 100 KH_2PO_4, 50 KCl, 10 H_3BO_3, 1 $MnCl_2$, 0.2 $CuSO_4$, 0.2 Na_2MoO_4, 5 Fe(III)-EDTA, 2 $NiSO_4$ and 2 $ZnSO_4$. Two mM 2-morpholinoethanesulfonic acid (MES) was used to buffer the pH, which was adjusted to 5.8 by the addition of 1 M KOH. The nutrient solution used to cultivate *A. murale* and *N. caerulescens* was based on the previous one, with a lower Ca/Mg ratio, to mimic the soil conditions of serpentine areas where the plant seeds were collected; the Ca and Mg concentrations were 500 and 1000 μM, respectively. The growth conditions were 22/18 °C day/night temperatures, 70% relative humidity, 16 h photoperiod and 150 μmol s^{-1} m^{-2} light intensity.

After 14 days of pretreatment, the seedlings were transferred to 2 L containers and treated with low and high levels (2 and 50 μM) of Ni and Zn nutrient solutions. The treatments were (Ni/Zn sulfate in μM/μM): T.a. 2/2 (*T. arvense*), A.m. 50/2 (*A. murale*), A.m. 50/50 (*A. murale*), N.c. 2/2 (*N. caerulescens*), N.c. 50/2 (*N. caerulescens*), and N.c. 50/50 (*N. caerulescens*). To avoid iron deficiency, 20 μM instead of 5 μM of Fe(III)−EDTA was used in 50/2 and 50/50 treatments. Each treatment was replicated three times and each contained one plant. The solutions were renewed weekly during the first 2 weeks and then twice a week.

Plants were harvested after 12 days (for *T. arvense*) or 28 days (for *A. murale* and *N. caerulescens*) of treatment. Roots were soaked in 1 mM $LaCl_3$ and 0.05 M $CaCl_2$ solution for 15 min at 0 °C to remove the Ni and Zn adsorbed on the root surface.[18] The plants were washed by ultrapure water (Millipore, 18.2 MΩ cm), then separated into root, stem and leaf (for *T. arvense* and *A. murale*), or root and shoot (for *N. caerulescens*, which was at the rosette stage), and later dried at 70 °C for 3 days. The dry samples were ground to fine powders (0.5 mm sieve) for analysis.

Analytical Methods. All the harvested plant samples (from 3.4 to 95.8 mg) were placed in Teflon beakers and digested by 5 mL of concentrated HNO_3 on a hot plate. After digestion, the solutions were evaporated to dryness and the residues were dissolved by 1 mL of 0.1 M HNO_3. The Ni and Zn concentrations were determined by ICP-MS (PerkinElmer ICP-MS SCIEX Elan 6000 or Thermo X7). To evaluate blank

contribution, a procedural blank was introduced into each sample series. The average blank measured throughout the study was 35 ± 5 ng of Zn (n = 3), which is negligible compared to the Zn contents in samples (7−230 μg). Ni in the blanks was under the determination limit (<0.9 ng).

Ni and Zn purified fractions for isotope analyses were recovered simultaneously from the same aliquot of sample. The Zn purification method for the column chemistry was adapted from Cloquet et al.,[24] while Ni method from Quitte and Oberli[25] and Gueguen et al.[26] The detailed procedures were described in Figure S1 and its captions (Supporting Information (SI)).

Ni and Zn isotope measurements were carried out by MC-ICP-MS (Neptune Plus, Thermo Scientific) at CRPG-CNRS, University of Lorraine, France. Details on Zn isotope measurements are given in Tang et al.[21] Briefly, Zn samples were diluted to obtain the same signal as measured in 100 ng/g $Zn_{IRMM\ 3702}$ solution, and $Cu_{NIST\ 976}$ was added to both standard and samples for mass bias correction. In addition to Cu doping, standard-sample-standard correction was carried out to account for the difference between Cu and Zn behavior. The masses measured were ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{63}Cu, and ^{65}Cu. Mass dependent fractionation was verified for all samples. Throughout the study, $Zn_{JMC\ lyon}$ was regularly measured providing a $\delta^{66}Zn = -0.28 \pm 0.05‰$ (n = 27). Such a value is in agreement with the published values and can be used to recalculate all data against $Zn_{JMC\ lyon}$. Meanwhile, reference material BCR-482 (lichen) was digested and analyzed, having a $\delta^{66}Zn = -0.26‰ \pm 0.07$ (n = 3) (Aebischer, pers. comm.), which is in agreement with previous published data.[24]

For Ni isotope measurement, purified Ni was redissolved in 1 mL of 0.1 M HNO_3. Then the solution was diluted to 150 ng/g of Ni before being loaded via the Aridus II (Cetac) into the MC-ICP-MS in medium resolution mode. Spiked-standards NIST 986 and spiked-samples were run at similar concentrations sequentially as in the classic sample-standard bracketing method. The calibration of the double-spike was conducted following the calibration method provided by Rudge et al.[27] The whole analytical protocol was applied to two reference materials previously characterized, namely BHVO-2 (basalt) and SDO-1 (sedimentary rock). Several preparations and measurements of these reference materials showed that the values are in agreement with those published[26,28] ($\delta^{60}Ni = -0.01 \pm 0.05‰$ (2 SD, n = 11) for BHVO-2 and $\delta^{60}Ni = 0.54 \pm 0.05‰$ (2 SD, n = 11) for SDO-1). The external reproducibility (2 SD) of the method is thus 0.05‰.

Ni isotopic compositions are expressed in delta per mill (‰) relative to NIST SRM 986, while Zn composition is relative to IRMM 3702: $\delta^{60}Ni\ (‰) = [(^{60}Ni/^{58}Ni)_{sample}/(^{60}Ni/^{58}Ni)_{NIST\ 986} - 1] \times 1000$, $\delta^{66}Zn\ (‰) = [(^{66}Zn/^{64}Zn)_{sample}/(^{66}Zn/^{64}Zn)_{IRMM\ 3702} - 1] \times 1000$.

The average isotope compositions of the shoots of *T. arvense* and *A. murale*, and of the whole plants were calculated according to the following equations:

$$\delta^{60}Ni_{shoot} = \frac{\sum_i m_i c_i^{Ni} \delta^{60}Ni_i}{\sum m_i c_i^{Ni}} \text{ and}$$

$$\delta^{66}Zn_{shoot} = \frac{\sum_i m_i c_i^{Zn} \delta^{66}Zn_i}{\sum m_i c_i^{Zn}}$$

where m_i, c_i^{Ni} and c_i^{Zn} are the mass of plant part i (stem or leaf) and its concentrations of Ni and Zn, respectively;

$$\delta^{60}Ni_{plant} = \frac{\sum_j m_j c_j^{Ni} \delta^{60}Ni_j}{\sum m_j c_j^{Ni}} \text{ and}$$

$$\delta^{66}Zn_{plant} = \frac{\sum_j m_j c_j^{Zn} \delta^{66}Zn_j}{\sum m_j c_j^{Zn}}$$

where m_j, c_j^{Ni} and c_j^{Zn} are the mass of plant part j (root or shoot) and its concentrations of Ni and Zn, respectively.

Isotope fractionation between the two components A and B is expressed as $\Delta^{60}Ni_{A\text{-}B}$ and $\Delta^{66}Zn_{A\text{-}B}$, with $\Delta^{60}Ni_{A\text{-}B} = \delta^{60}Ni_A - \delta^{60}Ni_B$, $\Delta^{66}Zn_{A\text{-}B} = \delta^{66}Zn_A - \delta^{66}Zn_B$.

RESULTS

Plant Biomass and Ni and Zn Concentrations. All the plants grew healthily with the exception of A.m. 50/50, which presented retarded growth symptoms, probably due to Zn toxicity. This is clearly reflected in the plant biomass data (Figure 1a). It is noticeable that *N. carulescens* achieved similar biomasses in all the three treatments, indicating that the Ni and Zn levels used in this experiment had no significant effect on its growth.

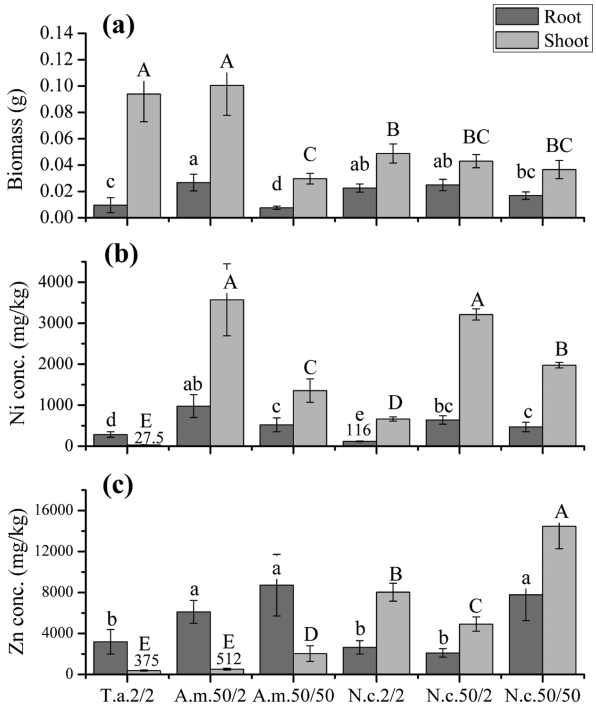

Figure 1. Biomass (a), Ni and Zn concentrations (b,c) of the nonaccumulator *T. arvense* (T.a.), the Ni hyperaccumulator *A. murale* (A.m.) and the Ni and Zn hyperaccumulator *N. caerulescens* (N.c.). The numbers in the treatment names (2/2, 50/2, and 50/50) represent Ni and Zn concentrations in nutrient solutions (in μM). Error bars show standard deviation (SD) of the three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's Test).

The Ni and Zn concentrations in plant organs showed clear species-specific patterns (Figure 1b,c). *T. arvense*, the non-hyperaccumulator, took up relatively small amounts of Ni and Zn, with a root-shoot translocation factor (shoot concentration/root concentration) of around 0.1 for both elements (SI, Table S1). *A. murale*, the Ni hyperaccumulator, presented

high Ni concentrations in shoots (3570 μg/g in A.m. 50/2 treatment), while most of the Zn was sequestrated in roots. *N. caerulescens* could hyperaccumulate both Zn and Ni in its shoots.

A competition effect between Ni and Zn in the uptake process was also observed. When Zn in solution increased from 2 to 50 μM, the Ni concentrations in shoots of *N. caerulescens* and *A. murale* dropped by 38% and 62%, respectively (Figure 1b; SI, Table S1). When Ni in solution increased from 2 to 50 μM, the Zn concentration in *N. caerulescens* shoots decreased on average by 39% (SI, Table S1).

Ni and Zn Isotopic Compositions. Figure 2 presents the Ni and Zn isotopic compositions (δ^{60}Ni and δ^{66}Zn in ‰) in

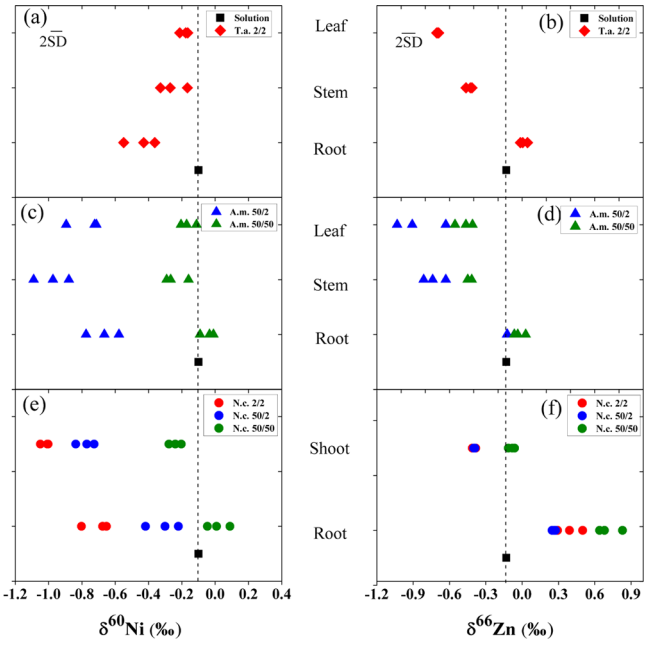

Figure 2. Ni (a,c,e) and Zn (b,d,f) isotope compositions (δ^{60}Ni and δ^{66}Zn in ‰) of plant organs and nutrient solutions (square). *T. arvense* (T.a.) (diamond) and *A. murale* (A.m.) (triangle) are separated into root, stem and leaf, while *N. caerulescens* (N.c.) (circle) is separated into root and shoot. The numbers in the treatment names (2/2, 50/2, and 50/50) represent Ni and Zn concentrations in nutrient solutions (in μM). Error bars show 2 SD of the measurements (0.05‰ for Ni and 0.07‰ for Zn).

plants and Figure 3a presents the extent of fractionation between plant and solution. All the plants were inclined to absorb light Ni isotopes, with $\Delta^{60}Ni_{plant\text{-}solution}$ values ranging from −0.90 to −0.21‰. It is noticeable that the hyperaccumulators had larger isotopic shift ($\Delta^{60}Ni_{plant\text{-}solution}$ = −0.90 to −0.63‰), in particular in low Ni treatment ($\Delta^{60}Ni_{plant\text{-}solution}$ = −0.90‰ in N.c. 2/2). Compared to Ni, however, Zn isotopes had a smaller shift with $\Delta^{66}Zn_{plant\text{-}solution}$ values of −0.23 to +0.20‰ (Figure 3a).

The competition between Ni and Zn also had an influence on the isotopic compositions of both *A. murale* and *N. caerulescens*. The Ni isotope fractionation in high Zn treatments ($\Delta^{60}Ni_{plant\text{-}solution}$ = −0.11 to −0.07‰) became less pronounced, in comparison with their corresponding low Zn treatments ($\Delta^{60}Ni_{plant\text{-}solution}$ = −0.73 to −0.63‰) (Figure 3a). This indicated that Zn had a great impact on Ni isotope fractionation during root absorption.

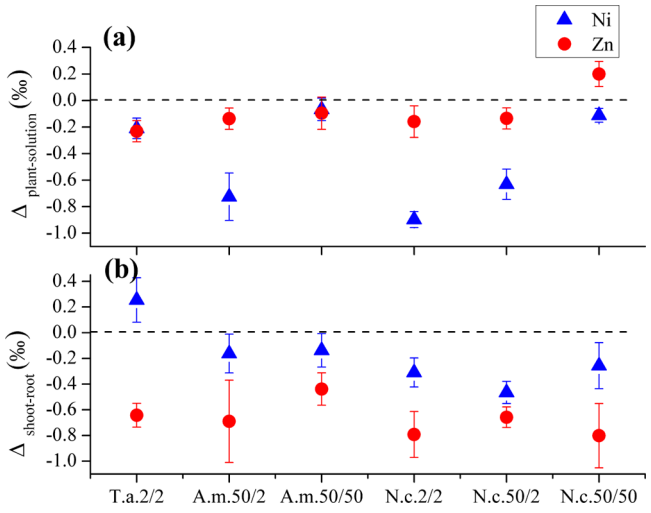

Figure 3. Ni (triangle) and Zn (circle) isotope fractionation between plant and solution (a) and between shoot and root (b). Error bars show 2 SD of the three replicates. The numbers in the treatment names (2/2, 50/2, and 50/50) represent Ni and Zn concentrations in nutrient solutions (in μM).

For Zn, shoots were enriched in light isotopes and the isotope fractionation between shoots and roots was relatively large ($\Delta^{66}Zn_{shoot\text{-}root}$ = −0.80 to −0.44‰). By contrast, heavy Ni isotopes were enriched in the shoots of *T. arvense* relative to the roots ($\Delta^{60}Ni_{shoot\text{-}root}$ = +0.25‰), while the hyperaccumulators *A. murale* and *N. caerulescens* still favored light Ni isotopes ($\Delta^{60}Ni_{shoot\text{-}root}$ = −0.47 to −0.14‰), but to a lesser extent relative to Zn (Figure 2 and 3b).

DISCUSSION

Isotope Fractionation between Plant and Media. Both kinetic and equilibrium fractionations could be triggered during the Ni and Zn uptake processes. For instance, ion speciation could cause equilibrium fractionation, as heavy isotopes are preferentially chelated by ligands, which results in light isotope enrichment in free ion pools.[18,29,30] Ni and Zn, as well as other mineral nutrients, are continuously absorbed by plants, and usually would not return to the media.[2] Thus, the plant absorption of Ni and Zn could result in kinetic fractionation. Three major processes which could influence the isotopic signature of a plant will be discussed in this section, that is, ion chelation in media, ion transport across root cell membrane and ion diffusion in the rhizospheric solution.

Plants usually take up trace elements in the form of free hydrated ions, for example, Zn(II) and Ni(II). However, many organic compounds existing in media could chelate large fractions of Zn and Ni, which could change the isotopic signatures of free ions left in media. Results showed that Zn bound to purified humic acid is heavier than free Zn(II) (Δ^{66}Zn = +0.24‰ ± 0.06), when pH > 6.[31] Therefore, increasing the concentration of organic ligands would result in a depletion of heavy metal isotopes in free ion pools, which would potentially affect the isotopic composition in plants. For instance, rice, lettuce, and tomato grown in the solution where more Zn was chelated (free Zn fraction = 0.03%), presented 0.09−0.21‰ greater negative isotopic shift than another treatment with higher Zn ion activity (free Zn fraction = 35%).[18]

In this study, organic ligands exuded by roots should have little interference on Ni and Zn speciation because plant

seedlings were grown in large quantities of solutions (2 L per plant), which were renewed once to twice a week. Thus, the concentrations of organic ligands potentially exuded by roots, were assumed to be low. Therefore, EDTA, which was introduced by the Fe salt, was the major organic ligand in the nutrient solutions. According to the GEOCHEM-EZ calculation, 80−89% of Zn remained as free hydrated ion in all the treatment solutions (SI, Table S2), which should theoretically represent the isotopic signature of the whole pool and had no significant impact on plant isotopic compositions. For Ni, around 55% of Ni was present as Ni(II) in high Ni treatments (Ni/Zn 50/2 and 50/50), whereas only 6.5% of Ni remained as Ni(II) and 93% was chelated by EDTA in low Ni treatment (Ni/Zn 2/2) (SI, Table S2). We are not able to provide the precise isotopic compositions of free Ni ion in low and high Ni treatments. However, it could be postulated that the small free ion pool in low Ni treatment should have larger negative isotopic shift than that in high Ni treatment, which might cause lighter isotope enrichment in plants. Indeed, our results conformed to this hypothesis. It is evident that *N. caerulescens* grown in low Ni treatment had the greatest negative isotopic shift ($\Delta^{60}Ni_{plant\text{-}solution}$ = −0.90‰ in Ni/Zn 2/2 treatment), whereas the isotopic shift became less pronounced in high Ni treatment ($\Delta^{60}Ni_{plant\text{-}solution}$ = −0.63‰ in Ni/Zn 50/2 treatment).

Metals are acquired by the plants via two pathways, i.e. an apoplastic and a symplastic route. It is presumable that Ni and Zn are taken up mainly through the symplastic pathway, and a purely apoplastic route for the entry into the xylem is of minor significance.[32,33] Thus, the uptake of Ni and Zn in plants is mainly controlled by the absorption of root cells and the isotopic signatures of Ni and Zn of the whole plant should represent the uptake mechanisms of root cell membrane.

Weiss et al.[18] proposed that high-affinity transport, e.g., ion carrier, should favor the heavy isotope, while low-affinity transport, e.g., ion channel and electrogenic pumps, should favor the light isotope. This is consistent with the fact that ion channels can move ions at rates of several millions per second, while carriers have much lower turnover rates, of hundreds to thousands per second.[34] Because of kinetic fractionation, ion channels should favor the internalization of light isotopes. In agreement with this, John et al.[19] found that a switch from a high- to a low-affinity transport pathway would lead to an isotopic composition shift from −0.2 to −0.8‰ ($\Delta^{66}Zn$) in marine diatoms.

The effect of high- and low-affinity transport on isotope fractionation could explain what is observed in our experiment. *T. arvense* and *A. murale*, the nonaccumulators of Zn, were isotopically light relative to the solution (−0.23 to −0.10‰) in all the treatments. In contrast, *N. caerulescens*, the Zn hyperaccumulator, was enriched in heavy isotopes in high Zn treatment ($\Delta^{66}Zn_{plant\text{-}solution}$ = +0.20‰). These divergent results suggest that different Zn transport systems are functioning in hyperaccumulators and nonaccumulators. Plants could switch from high- to low-affinity transport systems as the metal concentrations change from deficient to sufficient levels.[2] Usually, a high-affinity transport system only plays an important role at extremely low concentrations. It has been reported that in marine diatoms, nearly all the Zn is absorbed through a low-affinity transport system when solution Zn was more than 1 nM.[19] In bread wheat, 10 nM of Zn(II) is assumed to be the critical concentration between high- and low-affinity transport.[35] However, high-affinity transport could function in a wider range of concentrations in hyperaccumulators. The first high-affinity transporter gene, ZNT1, has been cloned from *N. caerulescens*.[36] This Zn transporter is expressed to very high levels in this hyperaccumulating plant, in both Zn-deficient and Zn-sufficient status. Whereas in the nonhyperaccumulator *T. arvense*, the transporter is expressed to very low levels in plants grown in Zn-sufficient solution (1 μM). In our case, 2 and 50 μM of Zn were used in the solution culture. Thus, for Zn, low-affinity uptake should take effect predominantly in the nonaccumulators *T. arvense* and *A. murale*, which resulted in light isotope enrichment. While both high- and low-affinity transport systems were functioning effectively in the hyperaccumulator *N. caerulescens*, which resulted in a final isotopic shift of +0.20‰ in high Zn treatment.

The Ni isotope fractionation pattern is different from that of Zn. All species presented light Ni isotope enrichment (Figure 2, 3a), which may reflect the functioning of low-affinity transport systems. This is corroborated with Aschmann et al.[13] and Redjala et al.,[14] who inferred that Ni is transported through a low-affinity transport system from uptake kinetic studies. Likewise, Assunção et al.[37] proposed that *N. caerulescens* seems to express low-affinity systems for Ni accumulation. The hyperaccumulators *A. murale* and *N. caerulescens* presented greater isotopic shifts than the nonaccumulator *T. arvense* in low Zn treatments (−0.90 to −0.63‰ vs −0.21‰), indicative of a greater permeability for the low-affinity transport systems in hyperaccumulators. This phenomenon is quite consistent with observations in microorganisms. Methanogens, one group of Archaea which have high Ni requirements, are isotopically light in Ni relative to the starting media ($\Delta^{60}Ni_{cells\text{-}starting\ medium}$ = −1.46 to −0.44‰); whereas for the archaeal hyperthermophile, *Pyrobaculum calidifontis*, whose Ni demand is much less, little fractionation could be observed.[23]

It is quite notable that Zn was able to reduce the Ni absorption by *A. murale* and *N. caerulescens*. This could be ascribed to Ni and Zn competition in root uptake process. In previous kinetic competition studies, results have also shown that Cu(II) and Zn(II) appear to inhibit Ni(II) influx strongly and competitively in soybean and barley.[12,15] Meanwhile, the extent of Ni isotope fractionation in the hyperaccumulators was also decreased in high Zn treatments (A.m. 50/50, N.c. 50/50), which indicates that Ni may share one of the transport systems with Zn. Thus, it could be speculated that high levels of Zn could compete with Ni, block the Ni transport pathway, and decrease the Ni internalization flow, resulting in not only a reduction of Ni uptake but also less isotope fractionation. Interestingly, *A. murale* and *N. caerulescens* had similar Ni isotope fractionation behaviors in both low Zn and high Zn treatments, thereby suggesting similar Ni transport systems may exist in these species.

Little is known about Ni uptake strategy in higher plants. From our observations along with previous studies, we propose that plants may take up Ni through a low-affinity transport system of Zn. In Ni hyperaccumulators, this transport system may be expressed in higher levels with a greater permeability for Ni.

The rapid absorption of ions by root symplast could cause a concentration gradient in the rhizosphere, and ion diffusion would then occur in the rhizospheric solution. The magnitude of diffusion zone depends on ion concentration in the bulk solution as well as on the assimilation rate of root cells. Degryse et al.[38] suggested that uptake of cadmium (Cd) and Zn by tomato and spinach in hydroponics could generate ion diffusion

zones in low concentrations (<1 μM), whereas Ni could not. It was estimated that the diffusive layer in the unstirred solution is 0.8 mm.[39] Luo et al.[40] compared Ni uptake by a hyperaccumulator *Thlaspi goesingense* and a nonhyperaccumulator *T. arvense*. The results suggested that Ni uptake is limited by diffusion only in the hyperaccumulator. From these studies, it can be concluded that a diffusion zone becomes more visible when ion concentrations in the bulk solution is lower, and/or ions are more efficiently absorbed by roots.

Ion diffusion could cause kinetic isotope fractionation, as light isotopes move faster than heavy ones. Rodushkin et al.[41] demonstrated that ion diffusion in solutions results in $^{66}Zn/^{64}Zn$ isotope ratios in excess of −0.3‰. John et al.[19] also attributed part of the reason for light Zn isotope enrichment in marine diatoms to ion diffusion when the organisms grew in low Zn concentrations.

In our case, 2 μM Ni or Zn in nutrient solution was considered sufficient for nonaccumulators, and no or little diffusive effect was expected to occur. However, hyperaccumulators grown in this concentration could create concentration gradients for ion diffusion. This could be seen in the evolution of Zn concentrations in nutrient solutions (SI, Figure S2). In low Zn treatments (N.c. 2/2 and N.c. 50/2), 40−50% of Zn in nutrient solutions were depleted in 3 or 4 days of cultivation, indicating a rapid uptake by the roots of *N. caerulescens* and probably the existence of an ion diffusion zone at the root surface. However, only 3−4% of Zn was removed from nutrient solutions in high Zn treatment (N.c. 50/50), suggesting only a slight concentration gradient should exist. The effect of ion diffusion could be also reflected in the isotope fractionation data of *N. caerulescens*. Light isotope enrichment was observed ($\Delta^{66}Zn_{plant\text{-}solution}$ = −0.16 to −0.13‰) in low Zn treatments, which is the combined effect of high- and low-affinity uptake and ion diffusion. However, when the Zn concentration in solutions increased to 50 μM (N.c. 50/50), where ion diffusion had little effect, the isotope fractionation became positive ($\Delta^{66}Zn_{plant\text{-}solution}$ = +0.20‰). Thus, ion diffusion might exist in the rhizosphere of *N. caerulescens*, which resulted in a negative isotopic shift to some extent.

Isotope Fractionation between Shoots and Roots. The isotope fractionation between shoots and roots are mainly influenced by root sequestration and xylem loading processes. Root sequestration, e.g., vacuolar compartmentation and apoplastic adsorption, usually results in heavy isotope enrichment.[18,42] Meanwhile, xylem loading process, at least for Zn, as discussed by Tang et al.,[21] should favor light isotopes. Thus, the combination of these two processes could result in light and heavy isotope enrichment in shoots and roots, respectively, which indeed was the result we obtained from both hyperaccumulators and nonaccumulator (Figure 2, 3b). Plotting $\Delta^{66}Zn_{shoot\text{-}plant}$ as a function of the Zn mass fraction in shoots (F_{shoot}) produced well-fitting logarithmic curve (R^2 = 0.909) (Figure 4b), indicating the root−shoot transport of Zn should be a kinetic reaction, which might be mainly controlled by xylem loading process. The fitting equation ($\Delta^{66}Zn_{shoot\text{-}plant}$ = 0.320 ln(F_{shoot}) − 0.060) yielded a fitting parameter of +0.32‰, which is quite consistent with previous studies.[21,43]

The shoot-root fractionation of Ni was less evident than that of Zn (Figure 3b), presumably due to the higher mobility of Ni in plants. By means of radioactive ^{63}Ni, Page et al.[44,45] found that Ni could be readily transferred from root to shoot via xylem, and its mobility is greater than that of Zn, manganese, cobalt and Cd. Therefore, Ni is more difficult to be

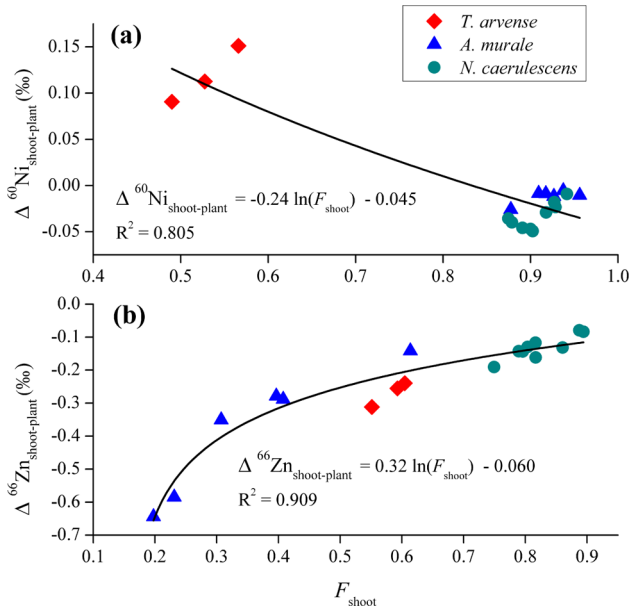

Figure 4. Ni (a) and Zn (b) isotope fractionation between shoot and whole plant ($\Delta^{60}Ni_{shoot\text{-}plant}$, $\Delta^{66}Zn_{shoot\text{-}plant}$), as a function of F_{shoot} in the three tested species. F_{shoot} is the ratio of Ni or Zn mass in the shoot to Ni or Zn mass in the whole plant.

sequestrated or bound in roots in comparison to Zn, which results in smaller shoot-root fractionation. This could also be certificated by the translocation factor (TF = shoot/root concentration) data of this study (SI, Table S3). Ni TFs were greater than Zn, indicating a higher mobility for Ni in these plant species. It is quite noticeable that heavy Ni isotopes enriched in the shoots of nonaccumulator *T. arvense*, which is in contrast with hyperaccumulators. The fitting equation of Ni ($\Delta^{60}Ni_{shoot\text{-}plant}$ = −0.24 ln(F_{shoot}) − 0.045, R^2 = 0.805) was distinct from that of Zn, with a fitting parameter of −0.24‰ (Figure 4a). Therefore, Ni translocation mechanism may be different from that of Zn, and different for nonaccumulating and hyperaccumulating plants. More data is needed to confirm this observation.

Implications for Ni and Zn Homeostasis in Plants. Many processes involved in the assimilation and transport of Ni and Zn could cause isotope fractionation, which makes it difficult to analyze and discuss. For instance, we separated shoots into stems and leaves for *A. murale* and *T. arvense*, and determined their Ni and Zn isotopic compositions. It is quite interesting to note that the Ni isotopic compositions in stems were usually heavier than that in leaves, whereas Zn was just the opposite (Figure 2a, b, c, d). The isotope fractionation between leaf and stem could result from various processes, including ion speciation in xylem flow, ion adsorption by stem apoplast and assimilation by stem symplast. However, our current knowledge on these processes does not enable us to propose ideal explanations to the metal fractionation between leaves and stems.

Therefore, we focused on two data sets in this study, i.e., the plant-solution and shoot-root fractionations. The former could provide useful information for tracing the root absorption process. For instance, light isotope enrichment could result from ion chelation and diffusion in bulk solutions, and low-affinity transport across root cell membrane, while heavy isotope enrichment could be explained by high-affinity

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transport. Meanwhile, shoot-root fractionation could be used to predict the root sequestration and xylem loading processes.

With an elaborate experimental design, i.e., separation of different Ni and Zn reservoirs in roots and shoot, the measurements of Ni and Zn isotopes could be used to estimate the extent of isotope fractionation caused by processes like Ni and Zn sequestration in different root fractions, and Ni and Zn xylem and phloem loading, etc. Future studies should further investigate the isotope fractionation from plant organ level to tissue and cellular levels, in order to clarify the contribution of each physiological process. Should further data be obtained in this manner, the link between isotope fractionation and physiological processes in plants could be established. Isotopic composition analysis could thereby become a useful complementary tool for the study of plant physiology.

■ ASSOCIATED CONTENT

Supporting Information

Table S1 includes biomass, Ni and Zn concentrations and Ni and Zn isotopic compositions of *T. arvense*, *A. murale* and *N. caerulescens*; Table S2 presents the speciation of Ni and Zn in nutrient solutions; Table S3 presents the translocation factors of Ni and Zn in all the plant species; Figure S1 describes the column chemistry for the purification of Zn and Ni. Figure S2 presents evolution of Zn concentrations in nutrient solutions of *N. caerulescens*. This material is available free of charge via the Internet at http://pubs.acs.org.

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