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Rapid report

Isotopic discrimination of zinc in higher plants

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Summary

• The extent of isotopic discrimination of transition metals in biological processes is poorly understood but potentially has important applications in plant and biogeochemical studies.

• Using multicollector inductively coupled plasma (ICP) mass spectrometry, we measured isotopic fractionation of zinc (Zn) during uptake from nutrient solutions by rice (*Oryza sativa*), lettuce (*Lactuca sativa*) and tomato (*Lycopersicon esculentum*) plants.

• For all three species, the roots showed a similar extent of heavy Zn enrichment relative to the nutrient solution, probably reflecting preferential adsorption on external root surfaces. By contrast, a plant-species specific enrichment of the light Zn isotope occurred in the shoots, indicative of a biological, membrane-transport controlled uptake into plant cells. The extent of the fractionation in the shoots further depended on the Zn speciation in the nutrient solution.

• The observed isotopic depletion in heavy Zn from root to shoot (−0.13 to −0.26‰ per atomic mass unit) is equivalent to roughly a quarter of the total reported terrestrial variability of Zn isotopic compositions (*c.* 0.84‰ per atomic mass unit). Plant uptake therefore represents an important source of isotopic variation in biogeochemical cycling of Zn.

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Introduction

Stable isotope ratios are routinely used in studying the biogeochemical cycling of light elements such as carbon (C), oxygen (O), nitrogen (N) and sulphur (S) in the environment (Hoefs, 1987). Examples include studies of the mechanisms of photosynthesis and of nutrient uptake and translocation in plants (Taiz & Zeiger, 2002). However, equivalent methods have not been available for heavier elements with atomic masses above *c.* 40 atomic mass units (amu), such as zinc (Zn), due to instrumental limitations. But since the development of the multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS), in which a high-temperature plasma achieves high ionization and a multiple array of collectors allows simultaneous mass determinations, extremely precise isotope ratio measurements of heavier elements have become possible

(Halliday *et al.*, 1998). This has led to the discovery of significant isotope fractionation of transition elements in nature resulting from equilibrium and kinetic reactions in both biotic and abiotic processes, notably for iron (Fe) (Anbar *et al.*, 2000; Zhu *et al.*, 2002; Beard *et al.*, 2003) and for Zn and copper (Cu) (Maréchal *et al.*, 1999, 2000; Maréchal & Albarède, 2002; Zhu *et al.*, 2002). However, to date there have been no systematic studies with plants.

The objectives of this study were to assess if isotopic fractionation of Zn occurs during its uptake by higher plants and to explore possible mechanisms. We conducted experiments in hydroponic cultures with three plant species (one monocotyledon and two dicotyledons) and two nutrient solutions differing in Zn speciation.

Materials and Methods

Plant growth

Seeds of rice (*Oryza sativa* L.) cv. IR64 were obtained from the International Rice Research Institute (Manila, Philippines), and seeds of lettuce (*Lactuca sativa* L.) cv. 'Romana' and tomato (*Lycopersicon esculentum* L.) cv. 'Alicante' were obtained from Johnson Seeds (Newmarket, Suffolk, UK). All seeds were washed in running deionised water before being germinated in the dark on moistened filter paper for 5–10 d. The seedlings were then transferred to blackened polycarbonate pots containing 1 l of nutrient solution with five plants per pot.

Two nutrient solutions were used: (1) an ethylenediaminetetraacetic acid (EDTA) buffered solution prepared with equimolar proportions of EDTA and Fe; and (2) an *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDTA) + nitrolotriacetic acid (NTA) buffered solution which contained 70 μmol excess chelator over the sum of Fe, Zn, Cu and manganese (Mn). Nutrient stock solutions were prepared gravimetrically from analytical grade reagents, from which final nutrient solutions were prepared in deionised water. The pH of the nutrient solution was buffered at 6.0 with 2(*N*-morpholino)-ethanesulfonic acid (MES; 2 mM in the nutrient solutions), adjusted using analytical grade 1 M KOH. Zinc contaminants in the deionised water were insignificant compared with the contribution from the stock solutions. Table 1 shows the total concentrations, free ion activities and speciation data for both nutrient solutions.

Eight replicates for rice and four replicates each for lettuce and tomato were prepared for each nutrient solution. Plants were grown for 42 d under controlled conditions (16 h photoperiod with a white light intensity of 350 μmol photons m^{-2} s^{-1}; day : night temperatures 25 : 18°C; relative humidity 60–70%). For the first 21 d, nutrient solutions were changed weekly, after which they were changed every 3–4 d to avoid nutrient depletion. Water lost through transpiration was replaced daily. Nutrient solutions were aerated throughout the experiment. The nutrient solutions were left after the preparation for 1 d before use to ensure that the Zn-EDTA, Zn-HEDTA and Zn-NTA complexes in the nutrient solutions

Table 1 Total concentrations, free ion activities and speciation data for the HEDTA + NTA and EDTA nutrient solutions used in the hydroponic study. All data were calculated using GEOCHEM-PC (Parker *et al.*, 1995) and a fixed solution pH of 6.0. Charge balance calculations for the HEDTA + NTA and EDTA nutrient solutions give values of 2.8% and 3.8%, respectively, indicating that the model fits the concentration data reasonably well. Ionic strengths for the HDTA + NTA and EDTA solutions equal 0.0158 and 0.0155 M, respectively

	HEDTA + NTA nutrient solution		Proportion associated with indicated ligand in solution (%)							
Metal	Conc. (M)	Free ion activity	Free metal	SO_4^{2-}	Cl^-	PO_4^{3-}	NO_3^-	HEDTA	NTA	MES
Ca^{2+}	1.00E–03	4.91E–04	81.18	14.98	0.01	0.25	<0.01	0.08	2.54	0.95
Mg^{2+}	1.64E–03	8.48E–04	85.51	12.54	0.12	0.22	<0.01	<0.01	0.34	1.26
K^+	4.99E–03	4.34E–03	98.76	1.20	0.04	<0.01	<0.01	<0.01	<0.01	<0.01
Na^+	4.00E–07	3.49E–07	99.03	0.76	0.20	<0.01	<0.01	<0.01	<0.01	<0.01
Fe^{3+}	2.00E–04	8.72E–16	<0.01	<0.01	<0.01	<0.01	<0.01	85.17	14.83	<0.01
Mn^{2+}	2.00E–05	3.94E–06	32.63	6.02	0.20	<0.01	<0.01	47.91	12.85	0.38
Zn^{2+}	**2.00E–06**	**4.10E–10**	**0.03**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**78.82**	**21.14**	**<0.01**
	EDTA nutrient solution		Proportion associated with indicated ligand in solution (%)							
Metal	Conc. (M)	Free ion activity	Free metal	SO_4^{2-}	Cl^-	PO_4^{3-}	NO_3^-	EDTA	OH^-	MES
Ca^{2+}	1.00E–03	5.06E–04	83.48	15.37	0.01	0.15	<0.01	<0.01	<0.01	0.98
Mg^{2+}	1.64E–03	8.55E–04	85.92	12.57	0.11	0.13	<0.01	<0.01	<0.01	1.27
K^+	4.99E–03	4.34E–03	98.76	1.20	0.04	<0.01	<0.01	<0.01	<0.01	<0.01
Na^+	4.00E–07	3.50E–07	99.06	0.76	0.18	<0.01	<0.01	<0.01	<0.01	<0.01
Fe^{3+}	1.00E–04	3.19E–15	<0.01	<0.01	<0.01	40.95	<0.01	19.21	39.83	<0.01
Mn^{2+}	2.00E–05	1.01–05	82.95	15.27	0.46	<0.01	<0.01	0.34	<0.01	0.97
Zn^{2+}	**1.00E–06**	**2.14E–07**	**35.25**	**6.49**	**0.12**	**0.19**	**0.08**	**57.39**	**0.48**	**<0.01**

were in equilibrium (Price *et al.*, 1998). The total amount of Zn in the nutrient solution was always far in excess of the amount of Zn taken up by plants to avoid reservoir depletion effects.

Upon harvest, root and shoot fractions were separated, and all plant materials were washed in running deionised water to remove superficial nutrient solution. Root materials were submerged sequentially in two 1-l baths of ice-cold deionised water for 5 min each to remove surface-bound nutrient solution. In addition, roots of four of the eight replicate rice samples were submerged in a 1-l bath of 1 mM $LaCl_3$ +0.05 mM $CaCl_2$ for 10 min to remove apoplastically bound Zn (following Rengel, 1999).

Sample preparation

Oven-dried root and shoot specimens were ground using a porcelain pestle and mortar with liquid nitrogen to pass through a 0.5 mm^2 sieve. Approximately 0.3 g of material were then digested in 6 : 2 : 1 ml concentrated Aristar grade HNO_3:H_2O_2:H_2O (Merk, Darmstadt, Germany) using a closed-vessel MarsX microwave digestion system (CEM Corporation, North Carolina, USA) (Dolgopolova *et al.*, 2004). For the rice samples, 0.5 ml HF was included to break down biogenic silica.

Matrix components were separated from Zn prior to isotope analysis using an adapted anion exchange procedure previously described (Maréchal *et al.*, 1999) and yielded quantitative recoveries of Zn from the plant matrix (Mason, 2003). Recovered Zn fractions were evaporated to dryness and residual Cl^- and Br^- ions were driven off by re-evaporating in 10 µl ultra-pure conc. HNO_3. Samples were subsequently dissolved in 0.05% (v v^{-1}) ultra-pure HNO_3 and spiked with NIST-SRM 976 Cu. Sample and standard solutions were concentration-matched for Zn and Cu to within ±0.05 µg ml^{-1} prior to isotope analysis.

Procedural blanks for Zn were approx. 100 ng, reflecting absorption of Zn from the Aristar reagents used on to the ion-exchange column during matrix separation. Isotopic analyses of procedural blanks indicate their compositions to be identical within error to that of the Johnson Matthey Purontronic™ (Alfa Aesar, Karlsruhe, Germany) Zn standard used. The procedural blank contribution accounted for < 3% of the Zn analysed in all cases, and calculations showed that at this level the procedural zinc blank has had a negligible influence on the isotope measurements within the achieved analytical reproducibility.

Isotope analysis

All Zn isotope compositions were measured using the ThermoElemental Axiom MC-ICP-MS at the NERC Isotope Geosciences Laboratories, Keyworth, UK. Mass spectrometric and data processing procedures followed previously described methods (Mason *et al.*, 2004a,b). Samples were introduced using a low-uptake (100 µl min^{-1}) microconcentric nebuliser in combination with a water-cooled (10°C) cyclonic and impact-bead spray chamber set-up. Peak intensities of $^{63}Cu^+$, $^{64}Zn^+$, $^{65}Cu^+$, $^{66}Zn^+$, $^{67}Zn^+$ and $^{68}Zn^+$ were measured on Faraday detectors using a static collection protocol at a spectral resolution of M/ΔM = 400. Instrumental backgrounds and amplifier offsets were corrected using an on-peak acid blank subtraction procedure. Isobaric $^{64}Ni^+$ and Ba^{2+}-related interferences were corrected by monitoring secondary interference peaks at masses 62 and 67.5, respectively, using an off-line peak subtraction. Instrumental mass bias drift and sample-related non-spectral mass discrimination effects were accounted for using the approach of empirical external normalisation as previously described (Mason *et al.*, 2004b). Samples were randomised to avoid systematic errors. Measurement solutions were prepared in 0.05% (v v^{-1}) ultra-pure HNO_3 and matched to ensure approx. 4 V signals on ^{63}Cu and ^{64}Zn (typically at concentrations around 0.4 mg ml^{-1} for Cu and 1 mg ml^{-1} for Zn). Analyses comprised 200 5-s integrations. No statistical outliers were rejected during data reduction.

All Zn isotope data are expressed relative to our in-house standard (Johnson-Matthey Purontronic™ Batch NH 27040) using the conventional notation: $\delta^{66}Zn$ (‰) = $[\{(^{66}Zn/^{64}Zn)_{sample}/(^{66}Zn/^{64}Zn)_{standard}\} - 1] \times 1000$. Repeat measurements indicate that this standard is 0.044 ± 0.035‰ per atomic mass unit (pamu) (± 2 SD) isotopically heavier than the Johnson-Matthey Zn standard 3-0749 L (Maréchal *et al.*, 1999). Total analytical errors associated with the isotope measurements, estimated from the standard deviation of repeated standard analyses over 9 months, gave a combined uncertainty in $\delta^{66}Zn$ measurements of ±0.07‰ (± 2 SD). This is in agreement with six repeated analyses of standard seeds of rice IR34 from the International Rice Research Institute during the same period, which give an average $\delta^{66}Zn$ value of 0.631 ± 0.046‰ (± 2 SD).

Results

The isotopic variability of Zn in the three plant species is shown in Fig. 1. All three species showed a similar pattern of isotope discrimination in both nutrient solutions, with a small enrichment in heavy Zn (^{66}Zn) between the bulk nutrient solution and root of 0.04–0.09‰ pamu, followed by an isotopic depletion in heavy Zn from root to shoot of −0.13 to −0.26‰ pamu. These shifts are significant relative to the analytical reproducibility (±0.035‰ pamu (± 2 SD)) and provide the first direct evidence of Zn isotope discrimination in higher plants. In general, more than 85% of the total plant Zn (unless in rice) was distributed in the shoots (Fig. 2) so that overall relatively light Zn was withdrawn from the nutrient solutions. The magnitudes of the shifts were similar between the nutrient solutions for solution-to-root transfer, but for root-to-shoot transfer the shift was significantly greater in

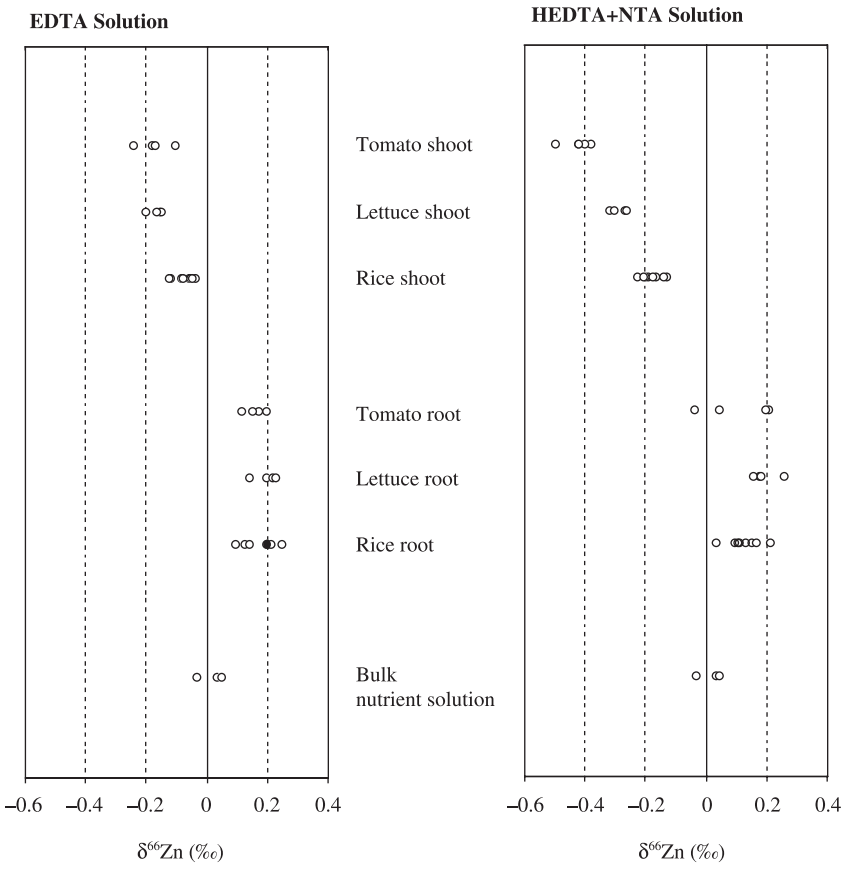

Fig. 1 Isotopic discrimination of zinc (Zn) in shoots and roots of rice (*Oryza sativa*), lettuce (*Lactuca sativa*) and tomato (*Lycopersicon esculentum*) grown in nutrient solutions containing either EDTA (left-hand panel) or excess HEDTA + NTA (right-hand panel) resulting in different concentrations of free Zn^{2+}. The error of determination is estimated to be ±0.07‰ (± 2 SD). The average isotopic composition of naturally occurring Zn is ^{64}Zn, 48.98%; ^{66}Zn, 27.81%; ^{67}Zn, 4.11%; ^{68}Zn, 18.57%; ^{70}Zn, 0.62%.

HEDTA + NTA than EDTA (−0.15 to −0.26‰ pamu compared with −0.13 to −0.18‰ pamu). The isotopic depletion within the shoots varied between the plant species, with tomato showing the greatest and rice the least depletion in heavy Zn (Fig. 1).

Discussion

The following five processes may contribute to the observed isotopic fractionation: (1) mobilisation of seed Zn reserves in the plants; (2) Zn speciation in the nutrient solution; (3) Zn uptake into roots; (4) Zn binding to root cell walls; and (5) Zn translocation from roots to shoots. We now discuss each of these in turn.

Mobilisation of seed Zn

Seed Zn reserves in the rice and lettuce were isotopically heavier than Zn in the bulk nutrient solution by 0.03 and 0.36‰ pamu, respectively. This heavy Zn could influence the isotopic composition of the roots and shoots if the seed reserves represented a significant proportion of the overall plant Zn budget. From the Zn contents of the seeds and whole plant (Fig. 2), however, we calculate that > 96.5% of the plant Zn budget originated from the nutrient solutions in all the plant species. Therefore seed reserves would not have significantly affected the Zn isotopic compositions of the roots and shoots.

Speciation in the nutrient solution

Because free Zn^{2+} is preferentially taken up over complexed Zn species in higher plants (Hacisalihoglu & Kochian, 2003), isotopic discrimination in the nutrient solutions during speciation will affect the isotopic composition of the plants. In the EDTA solution, 57% and 35% of the Zn is present as Zn-EDTA and free Zn^{2+}, respectively, and in the 'excess' HEDTA + NTA solution, > 99.9% of the Zn is chelated; only 0.03% is present as free Zn^{2+} (Table 1). Because of slight mass dependence of chemical bond strengths, isotopes of the same element are unequally distributed between different chemical species during equilibrium reactions, with heavy isotopes being preferentially partitioned into the complex with the strongest covalent character (Bigeleisen & Mayer, 1947). As EDTA, HEDTA and NTA form strong covalent bonds with Zn^{2+}, the chelated Zn fraction will approximate to the isotopic composition of the bulk nutrient solution, but the free Zn^{2+} fraction will be strongly enriched in isotopically light Zn (^{64}Zn). The roots will also participate in this equilibrium as a result of Zn binding to root cell walls and uptake into the roots. However, because there is more than an order of magnitude more

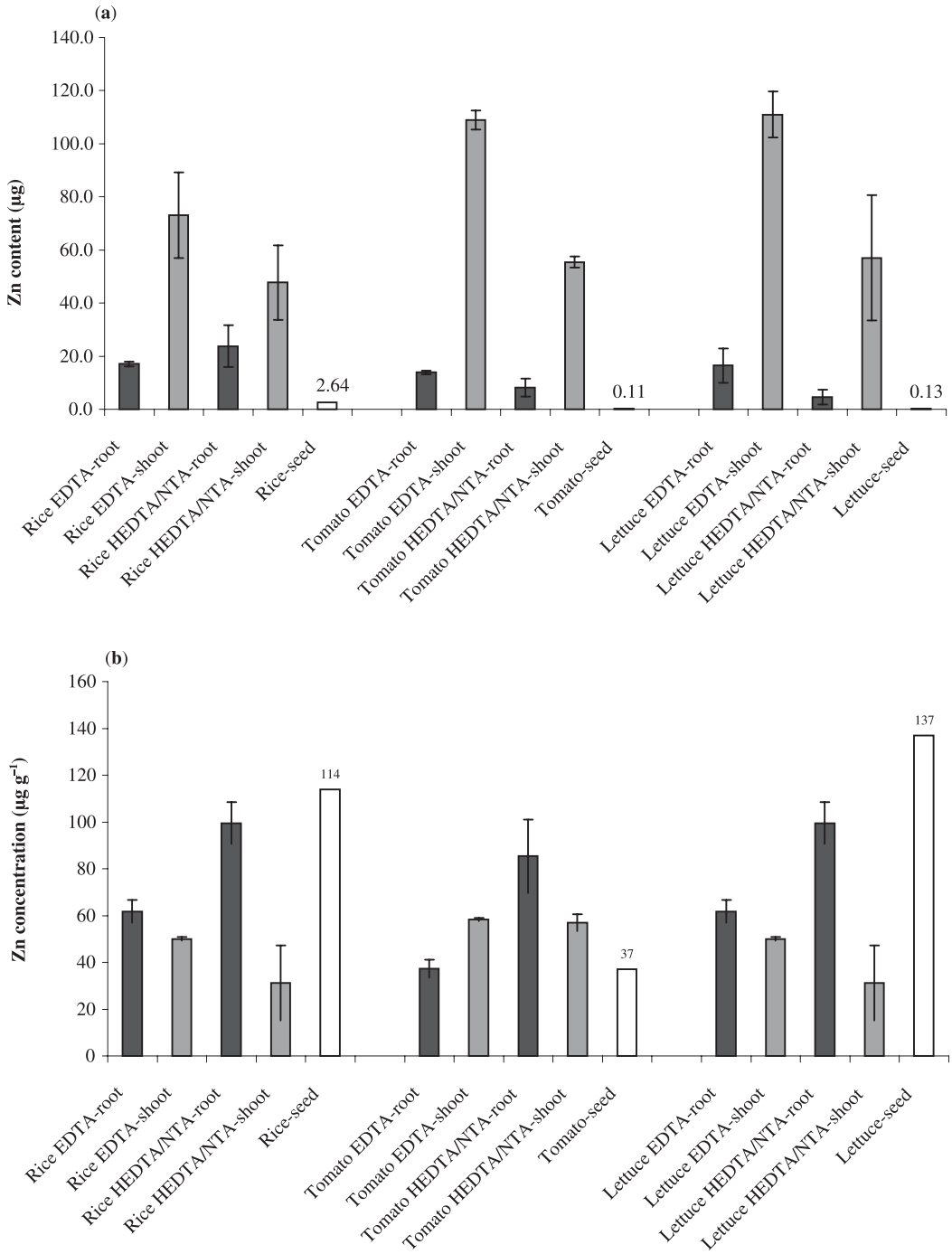

Fig. 2 Average root, shoot and seed zinc (Zn) contents (a) and concentration (b) of rice (*Oryza sativa*), lettuce (*Lactuca sativa*) and tomato (*Lycopersicon esculentum*) per experimental pot containing five plants after 42 d of growth. The error bars indicate ± 1 SD, $n = 4$ (unless for seeds, with $n = 1$).

Zn in the solution than in the roots, this effect will only be minor.

The enrichment of light Zn in the free Zn^{2+} pool will be greater in the HEDTA + NTA solution than in the EDTA solution. Consistent with this and preferential absorption of free Zn^{2+} by the roots, the plants in HEDTA + NTA solution show a larger negative isotopic shift than the plants in EDTA. Because in general much of the Zn in soil solutions is complexed with humic substances and other organic ligands, at least in uncontaminated soils (Tipping, 2002; Kirk, 2004), this result will apply in natural systems in general, though the degree of binding by the various naturally occurring ligands will be different.

Uptake by roots

The fact that the isotopic composition of the shoots varies between the plant species (Fig. 1) strongly suggests that biologically mediated isotopic discrimination occurs during uptake or translocation or both. The mechanisms controlling Zn transport across the root and other cell membranes are not well understood (Hacisalihoglu *et al.*, 2001; Hacisalihoglu & Kochian, 2003), but they are thought to be metabolically controlled and involve some combination of transfer along the electrochemical gradient via ion channels, carrier proteins or against the electrochemical gradient via electrogenic pumps. Carrier-mediated transport should favour the heavy isotope because it involves covalent binding to a carrier protein on the outer side of the membrane, with subsequent release on the inner side as a result of conformational changes in the carrier. However, transport through ion channels or via electrogenic pumps will favour the light isotope because of its greater diffusion coefficient. The observed net enrichment of the shoots and of the plants as a whole with the light isotope, and the differences between the plant species, therefore suggests that membrane transport is dominated by ion channels and electrogenic pumps rather than by carrier-mediated transport. However, it is not possible with the current data to separate these effects from the bias in favour of the light isotope caused by speciation in the external solution.

A further factor is that the preferential removal of heavy Zn during binding to root cell walls (next section) will tend to bias the labile Zn pool in the root apoplast in favour of light Zn, and hence will bias uptake into root cells and translocation to the shoots in favour of light Zn. However, this would neither explain the absence of differences in fractionation in the roots between the different nutrient solutions nor the differences between plant species. Also, from the root : shoot partitioning of Zn (Fig. 2), the flux to the shoots is far greater than that to compartments in the roots, and so the isotopic composition of the apoplast will be dominated by fractionation occurring in uptake into the plant rather than in retention on root cell walls.

Binding to root cell walls

Some proportion of the Zn in the root apoplast will become bound to the external surfaces of root cell walls in nonexchangeable forms due to covalent bonding to carbonyl and hydroxyl groups in the cell walls (Santa Maria & Cogliatti, 1988; Lasat *et al.*, 1996; Hart *et al.*, 1998). Reid *et al.* (1996) showed that more than 90% of cell-associated Zn is retained in cell walls and several per cent of this is in nonexchangeable forms. Because heavy Zn will be preferentially bound, this would explain the positive shift of the isotope ratio to heavier Zn in the total root digests. We found no change in isotopic composition of rice roots leached with $LaCl_3$ solution for 10 min; however, this regime would not have removed all the nonexchangeable Zn (Reid *et al.*, 1996).

The positive shift in the roots is similar in all three plant species regardless of solution composition and the different isotope distribution in the solutions. A possible explanation is that free and complexed Zn are adsorbed on the root walls in the proportions in which they occur in the solutions, so that any isotopic discrimination resulting from speciation in the solution is not apparent in the root digests. However, it is unlikely that complexed Zn is adsorbed to anything like the extent of Zn^{2+}, so we reject this explanation. An alternative explanation is that the isotopic composition of Zn in the root apoplast is enriched in the heavier isotope compared with the bulk solution as a result of preferential uptake of light Zn^{2+} into root cells and diffusion limitations in transfer of Zn^{2+} to the root surface. An unstirred layer of *c.* 10–100 μm thickness surrounds individual roots even in stirred solutions (Grignon & Sentenac, 1991). This is thin enough for the root surface to be regarded as a plane without serious error, hence the flux, F, of Zn across the unstirred layer is given by $F = D(C_\infty - C_0)/\delta x$, where D is the diffusion coefficient, δx is the layer thickness and $C_\infty - C_0$ is the concentration difference across the layer. A typical flux of Zn into plant roots is 10^{-14} mol cm^{-2} s^{-1} (Tinker & Nye, 2000, chapter 5), so if δx = 100 μm and $D = 10^{-5}$ cm^2 s^{-1}, then $C_\infty - C_0 = 10^{-8}$ M. This is small compared with the free Zn^{2+} concentration in the EDTA solution (10^{-7} M), but two orders of magnitude greater than that in the HEDTA + NTA solution (10^{-10} M). Buffering of free Zn^{2+} by dissociation of complexed Zn is slow compared with diffusion of Zn^{2+} across the unstirred layer (Stumm & Morgan, 1996, pp. 318–319), so would not greatly alter the above calculations. Hence the calculations indicate that the free Zn^{2+} concentration at the root surfaces in the HEDTA + NTA solution will be extremely small and that its isotopic composition will be highly sensitive to discriminatory processes. The greater the flux of light Zn^{2+} into the roots, the greater will be its depletion at the root surface, so the differences in isotopic discrimination between the different nutrient solutions will be offset, explaining the lack of differences in binding to roots. A test of this explanation would be the extent to which the isotopic discrimination depended on stirring of the solution and consequent changes in thickness of the unstirred layer.

Translocation from roots to shoots

The root : shoot partitioning of Zn in the three plant species indicates an efficient Zn transfer from root to shoot, with typically over 75% of the Zn being transferred to the shoot (Fig. 2). The shoots will therefore tend to inherit an isotopic composition similar to the average composition of Zn taken across the root cell membranes. There may be further discrimination where the Zn crosses cell membranes between the roots and shoots. Presumably this discrimination would be similar to that occurring in transfer across the root cell membranes.

The fact that isotopic fractionation in the shoots differs between the nutrient solutions, whereas that in the roots does

not, is evidence that at least two discriminatory processes are operating and that they affect the roots and shoots differently. The fractionation in the shoots and its dependence on the nutrient solution composition are consistent with the effects of solution speciation discussed earlier, possibly but not necessarily compounded by discrimination during membrane transport into and within the plants. The fractionation in the roots requires some process that is independent of the composition of the nutrient solution, such as binding to root cell walls coupled to diffusion limitations in light Zn uptake as discussed earlier.

Concluding remarks

The observed isotopic depletion in heavy Zn from root to shoot (−0.13 to −0.26‰ pamu) accounts for roughly a quarter of the total reported terrestrial variability of Zn isotopic compositions (0.84‰ pamu). This suggests that plant uptake is an important source of isotopic variation in biogeochemical cycling of Zn. However, the shift during plant Zn uptake is small compared with that produced in industrial processes (up to 5‰ pamu, Marcus & Zevenbergen, 1999; Mason *et al.*, 2004b), so the industrial isotopic discrimination is not likely to be overprinted during plant uptake. Hence discrimination in plant uptake will probably not compromise Zn stable isotopes as a tool for tracing environmental pollution, although it is possible that plant-induced fractionation is cumulative depending on mass balances and recycling of biologically incorporated Zn in soils. Our explanations for the observed fractionations are somewhat speculative and clearly need further investigation. Nonetheless, the effects shown indicate that isotopic fractionation could be a useful tool for studying Zn uptake and translocation in plants, without the need for excessively large and physiologically unrealistic Zn concentrations or artificially enriched isotopes.

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