Supplementary Material

Stable isotopes of Cu and Zn in higher plants: Evidence for Cu reduction at the root surface and two conceptual models for isotopic fractionation processes

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1. Material and Methods

With respect to experiment 1, the plant growth experiments were conducted at the Institute of Arable Crop Research at Rothamsted Research, Harpenden, UK. The sample processing (acid digestion, ion exchange chromatography) and concentration measurements were conducted at Imperial College London, UK. The isotope measurements were conducted at the NERC Isotope Geosciences Laboratory, Keyworth, UK. With respect to experiment 2, the plant growth experiments, sample digestion and concentration measurements were conducted at INRA in Montpellier, France. The ion exchange chromatography and isotope measurements were conducted at the Institute de Physique du Globe de Paris, France.

1.1. Plant growth experiments

During experiment 1, sterile seeds of rice (*Oryza sativa* L.) cv IR64 were obtained from the International Rice Research Institute (Manila, Philippines). Seeds of lettuce (*Lactuca sativa* L.) cv 'Romana' and tomato (*Lycopersicon esculentum* L.) cv 'Alicante' were purchased from Johnson Seeds (Newmarket, Suffolk, UK). All seeds were washed in running deionised water before being germinated in the dark on moisten filter paper for 5 to 10 days. Seedling were then transferred to 1 dm^3 blackend polycarbonate growth tubes with five plants per tube. Two nutrient solutions were used in the study: (i) an Ethylenediaminetetraacetic acid (EDTA) buffered solution prepared with equal molar proportions of EDTA and iron, and (ii) a N-(2-Hydroxyethyl)ethylenediaminetriacetic acid (HEDTA) + Nitrolotriacetic acid (NTA) buffered solution with 70 μmol excess chelator. These solutions were selected to assess how differences in the speciation of Cu and Zn within the nutrient solutions influence isotopic discrimination during plant uptake. Nutrient stock solutions were prepared gravimetrically from analytical grade reagents, from which final nutrient solutions were prepared in demineralised water. 2(N-morpholino)-ethanesulfonic acid (MES) was used as a pH buffer, and the pH of the final nutrient solutions were adjusted using analytical grade 1 N HCl. Zinc and Cu contaminants in the demineralised water were insignificant compared to the contribution of Cu and Zn from the stock solutions. Eight replicates for rice, and four replicates for lettuce and tomato, were prepared for each nutrient solution. Plants were grown under controlled conditions for 42 days. For the first 21 days, nutrient solutions were changed on a weekly basis, after which nutrient solutions were changed every three to four days to avoid nutrient depletion. Water lost through transpiration was

replaced on a daily basis. Nutrient solutions were aerated throughout the experiment, and humidity was maintained at 60-70%. 16/8 h day/night cycle at 25/18ºC was used with a photon flux density of 350 μmol.m^{-2}.s^{-1} supplied by warm white fluorescent tubes. Upon harvest, root and shoot fractions were separated, and all plant materials were washed in running demineralised water to remove superficial nutrient solution. Root materials were submerged sequentially in two 1-litre baths of ice-cold deionised water for five minutes each to remove surface bound nutrient solution. Root and shoot materials were air-dried at 60°C for 48 hours, and dry yields determined.

During experiment 2, plants were grown in a growth chamber with the following conditions (day/night): 16/8 h, 25/20°C, 75/70 % of relative humidity and a photon flux of 450-480 μmol$m^{-2}s^{-1}$ in the range 400-700 nm. Seeds of durum wheat (*Triticum turgidum durum* L., cv 'Acalou') and tomato (*Lycopersicon esculentum* L. cv 'Saint Pierre') were surface sterilized with 6 % H_2O_2 and then germinated in Petri dishes containing a cellulose filter paper moistened with a germination solution containing 600 μmol.L^{-1} of $CaCl_2$ and 2 μmol.L^{-1} of H_3BO_3 for three days. Plant seedlings were transferred in four 35 l containers (two containers with tomato seeds and two containers with wheat seeds), with twenty groups of plants per container and 2 and 10 seedlings per group for durum wheat and tomato, respectively. Until the end of the first week of growth, the nutrient solution had the same composition (600 μmol.L^{-1} $CaCl_2$ and 2 μmol.L^{-1} H_3BO_3) and was then changed to a solution with the following concentrations (in μmol.L^{-1}): 2000 of KNO_3, 2000 of $Ca(NO_3)_2$, 1000 of $MgSO_4$, 500 of KH_2PO_4, 100 of NaFe(III)EDTA, 10 of H_3BO_3, 2 of $MnCl_2$, 1.6 of $ZnSO_4$, 1.3 of $CuCl_2$, 0.05 of Na_2MoO_4. This Fe-sufficient solution, denoted as (high Fe), was applied to all the containers during the second week of growth. During the third week of growth, this Fe-sufficient solution was used again for two containers (one container with tomato and one with wheat), while the other two containers were filled with a Fe-deficient nutrient solution, denoted as (low Fe), with the following lower Fe, Cu and Zn concentrations (in μmol.L^{-1}): 2 of NaFe(III)EDTA, 0.4 of $CuCl_2$, 0.62 of $ZnSO_4$ (Cornu J-Y. et al., 2007; Michaud A. M. et al., 2008). The lower concentrations of Cu and Zn in the Fe-deficient solutions enabled us to keep Cu^{2+} and Zn^{2+} activities similar to these in the (high Fe) solutions. We confirmed this with Visual MINTEQ model calculations. All nutrient solutions were continuously aerated and renewed every two days to avoid nutrient depletion. Upon harvest (3 weeks), five groups of plants among twenty were selected in each container. For both experiments, roots and shoots

were separated from each other and briefly rinsed with deionised water to eliminate the residual nutrient solution.

1.2. Acid digestion and Cu and Zn concentration measurements

Root and shoot specimens during experiment 1 were ground using a porcelain pestle and mortar with liquid nitrogen to pass through a 0.5 mm^2 sieve. Approximately 0.25 g of oven dried (60ºC for 24 hours) powder were digested in 6:2:1 ml conc. Aristar grade HNO_3:H_2O_2:H_2O using a high-temperature, high-pressure MarsX microwave digestion system (CEM Corporation, North Carolina, USA) (Dolgopolova et al., 2004). For the rice samples, 0.5 ml HF was included to breakdown biogenic silica. Digests were evaporated to dryness. Analyses of a variety of organic reference materials by ICP-AES indicated the digestion procedure was quantitative for Cu and Zn. Following acid digestion, samples were taken up in 2 ml 7 N HCl and volumetrically split with half being analysed for elemental composition and half being used for isotope analysis. Copper and Zn concentrations were measured using a Fison Instruments ARL 3508B ICP-AES. To check the accuracy and precision of the digestion procedure and concentration measurements, certified standard CRM 482 *Pseudovernia furfurea* (Lichens) and NIST 1547 (Peach Leaves) were included in the study. Recovery for Zn and Cu was 100±5% and the precision of all concentration measurements was within 5 %.

Shoots and roots during experiment 2 were oven-dried at 105 °C for 48 hours and weighted. Approximately 0.5 g of the dried samples were digested in a microwave oven (Milestone Ethos Touch Control) with conc. HNO_3 along with blanks and a maize reference material (*Zea mays* L., V463, Bureau InterProfessionnel d'Etudes Analytiques, France). Concentrations of Cu and Zn in the digests were determined by a XII Series ICP-MS from Thermo Fischer Scientific (detection limit around 50 $ng.L^{-1}$ and typical standard deviation of 2 %). Standard river water SLRS4 was run every 10 samples to check the accuracy and the stability of the ICP-MS.

1.3. Isotopic measurements for Experiment 1

Copper and Zn fractions were isolated from matrix components using ion exchange chromatography described in detail elsewhere (Chapman et al., 2006). In brief, 2 ml (4 cm bed height) columns of the macroporous anion exchange resin AG-MP-1 were pre-treated with 7 ml 7 N HCl. Samples were loaded onto the columns in 1 ml 7 N HCl, and the majority of matrix components

were eluted in 6 ml 7 N HCl. The Cu fractions were recovered in 24 ml 7 N HCl, following which Fe was washed from the columns with 10 ml 2N HCl. The Zn fraction was recovered in 10 ml of 0.5 N HNO_3 + 0.05 N HBr. Quantitative recoveries were routinely obtained, circumventing problems related to isotopic fractionation of Cu and Zn on the columns. Aristar grade reagents were used throughout. Copper and Zn elutes were evaporated to dryness, and residual traces of HCl and HBr were driven off by evaporating the samples for a second time in 0.1 µl ultra-pure concentrated HNO_3. Samples were taken up in 0.2 % (v/v) ultra-pure HNO_3 for isotope analysis.

Isotope measurements were made on a ThermoElemental Axiom MC-ICP-MS at the NERC Isotope Geosciences Laboratories (NIGL), Keyworth, UK. Full analytical details are given elsewhere (Mason et al., 2004a; Mason et al., 2004b). In brief, measurements were made using a static collection protocol at a spectral resolution of $M/\Delta M = 400$. Samples were introduced using a low-uptake (100 $\mu L.min^{-1}$) micro-concentric nebuliser in combination with a water-cooled (4°C) cyclonic and impact-bead spray chamber set-up. Instrumental backgrounds and amplifier offsets were corrected using an on-peak acid blank subtraction procedure. Isobaric Ni and double-charged Ba interferences were corrected by measuring secondary interference peaks at masses 62 and 67.5, respectively, and by applying an off-line peak subtraction. Instrumental mass bias drift and sample-related non-spectral mass discrimination effects were accounted for using the 'empirical external normalisation' approach, with Cu as an internal mass discrimination monitor during Zn isotope measurements and vice versa. Individual samples were run for 100, 5-second integrations, requiring 2.5 µg Zn and 1 µg Cu for optimal measurement precision. Samples were randomised to avoid systematic errors. All processed Cu fractions were analysed by ICP-AES to screen for Ti contamination, with Cu isotope data being discarded where the [Ti]/[Cu] ratio of the analyte solution exceeded 0.02.

Total procedural blanks for Cu and Zn were 10.9 ± 8.2 ng (±2σ) and 104 ± 41 ng (±2σ), respectively, based on four repeats. This represented 1% or less of the total Cu and Zn. In contrast to Zn, the Cu procedural blank was isotopically distinct relative to terrestrial signature towards isotopically light compositions. Therefore we tested potential blank effects by plotting $\delta^{65/63}Cu$ against the reciprocals of the quantity of Cu processed for each sample. This provided strong evidence for Cu isotopic variability within the nutrient solution-plant system. All $\delta^{65/63}Cu$ data were calculated relative to NIST-SRM 976 Cu certified isotopic standard, where $\delta^{65/63}Cu = 0.4456$ ±

0.0004 (Shields et al., 1964), and all $\delta^{66/64}$Zn data were calculated relative to an in-house Johnson Matthey Purotronic Zn metal (IMP Zn - batch no. NH 27040). Repeat measurements indicated IMP Zn was 0.044 ± 0.035 ‰ pamu (±2σ) isotopically heavier than an aliquot of the Johnson Matthey Zn standard (3-0749 L) used by Maréchal et al. (1999). A robust regression fitted to $\delta^{66/64}$Zn versus $\delta^{67/64}$Zn for the complete data set using IsoPlot software (Ludwig, 1982) yielded a gradient within error of 1.5, and an intercept within error of the origin, indicating that Zn isotopes exhibited mass-dependent behaviour. δ-values incorporating ^{68}Zn were not calculated due to unresolved difficulties associated with the measurement of ^{68}Zn using the 'empirical external normalisation' approach under wet plasma conditions. A detailled discussion is given elsewhere (Mason et al., 2004a; Mason et al., 2004b).

The accuracy of the $\delta^{66/64}$Zn and $\delta^{65/63}$Cu measurements during the period of the experiment was assessed measuring selected metal standards using a GVi *IsoProbe* MC-ICP-MS based at ICL using sample-standard bracketing (Mason et al., 2004b). The relative isotopic compositions of the Cu and Zn metal standards were in good agreement between the two instruments, being within error in all cases, and are reported elsewhere (Mason et al., 2004b). Furthermore, the δ-values for JMC Cu were within uncertainty to those reported by the Lyon group (Maréchal et al., 1999). No memory effects were noticed. Errors associated with individual analyses (95 % confidence interval) represent the combined internal precisions for sample and associated standard runs used in calculating each δ-value. Total analytical errors associated with the measurements have been estimated from the standard deviation of repeated standard analyses over a nine-month period. This gave a combined uncertainty on $\delta^{65/63}$Cu and on $\delta^{66/64}$Zn measurements of ±0.07 ‰ (±2σ). Repeated analyses of the rice standard IR34 over a year gave $\delta^{66/64}$Zn = 0.631 ± 0.046 ‰ (±2σ) for six replicates, and $\delta^{65/63}$Cu = 0.497 ± 0.065 ‰ (±2σ) for four replicates. These values were in agreement with the reproducibility of standard measurements.

1.4. Isotopic measurements for experiment 2

Copper and Zn were separated from other elements and from each other by ionic chromatography following the procedure published by (Borrok D. M. et al., 2008) using distilled HNO_3 and HCl acids. The procedures yielded quantitative recoveries of Cu and Zn. Recovered fractions were evaporated to dryness and residual Cl^- and Br^- ions were driven off by re-evaporating in 10 µl ul-

tra-pure conc. HNO_3. The total procedure blanks were below 14.1 ± 2.3 ng for Cu and 42.2±27.2 ng for Zn, based on six repeats, which represent 1% or less of entire metal quantity. Given their isotopic composition (below 1 ‰pamu), they will not affect the isotopic composition of samples within our uncertainties.

Copper and Zn isotopic compositions were determined using a ThermoFinnigan Neptune MC-ICP-MS. The mass discrimination correction procedure used is described elsewhere (Jouvin D. et al., 2009). Samples were introduced after addition of Zn (for Cu isotopes measurements) or Cu (for Zn isotopes measurements) as internal standard, with a Cu/Zn ratio of 2, leading to typical concentrations of 200 ppb for Zn and 100 ppb for Cu, in 0.05 M HNO_3 matrix. Intensities of 40 and 80 $Vppm^{-1}$ (at 10^{11} W amplification) were measured for ^{64}Zn and ^{65}Cu, respectively. The typical ^{64}Zn and ^{65}Cu blank during a measurement session was around 1 mV. All the data are expressed relative to the Cu and Zn in-house standard solutions (Chen J. et al., 2008) from Aesar, Germany (lot OC405617 and OC469583 for Cu and Zn respectively).

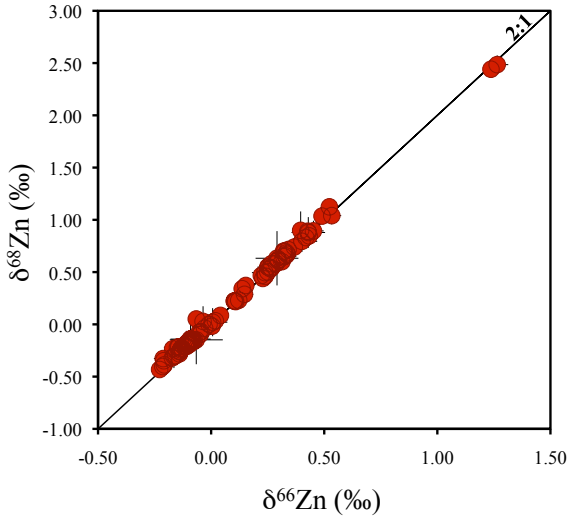

Figure S1: $\delta^{68}Zn$ vs. $\delta^{66}Zn$ plots of all reported isotopic data. The solid line represents the mass-dependant fractionation line. The external reproducibility on the measurements was ±0.05‰ (2σ) minimum.

1.5. Notation

The isotope ratios were expressed following the delta notation:

$$\delta^{66/64}Zn = \left(\frac{\left(\frac{^{66}Zn}{^{64}Zn} \right)_{sample}}{\left(\frac{^{66}Zn}{^{64}Zn} \right)_{standard}} - 1 \right) \times 1000$$

$$\delta^{65/63}Cu = \left(\frac{\left(\frac{^{65}Cu}{^{63}Cu} \right)_{sample}}{\left(\frac{^{65}Cu}{^{63}Cu} \right)_{standard}} - 1 \right) \times 1000$$

For better comparison with other studies, isotopic fractionation between two samples A and B are expressed using the Δ-notation,

$$\Delta^{x}Me_{A\text{-}B} = \delta^{x}Me_{A} - \delta^{x}Me_{B}.$$

, where Me is the metal (Cu or Zn), x stands for the isotope ratio, and A,B stand for the different reservoirs, e.g., root, solution and shoot

2. Possible effects of seed reserves on Zn and Cu isotopic fractionation

During the initial stages of growth, a plant seedling derives a significant proportion of nutrients (including the micronutrients Cu and Zn) from the seed. As the roots develop and seed reserves get depleted, the dominant source of nutrients switches to the growth media. Analysis of some seeds used in the first experiment indicates significant isotopic differences between the seeds and the growth medias used, particularly for lettuce seeds which are enriched in isotopically heavy Cu and Zn relative to the bulk nutrient solutions by +0.46 and +0.35 ‰ amu^{-1}, respectively. However, upon harvest, the proportion of Cu and Zn derived from seed reserves accounted for <3.5 % of the total Cu and Zn budget of all plant species studied**,** with seed reserves of rice being roughly an order of magnitude higher compared to other plant species (for both studies). Seed reserves

would thus not have significantly affected the Cu and Zn isotopic compositions of the roots and shoots.

3. Proportions of metal taken up by plants

The proportion of Cu and Zn taken up by plants during the experiments were calculated using the biomass, Cu and Zn concentration in plants and in the nutrient solution and the total volume of nutrient solution used during the experiments. By comparing this with the speciation in the solution, this allows us to evaluate the Cu and Zn species taken up by the plants.

		Wheat	Tomato		Lettuce	Rice	
			Exp 2	Exp 1		EDTA	HEDTA+NTA
Cu	Roots	70	45	25	15	30	45
	Shoots	30	55	75	85	70	55
Zn	Roots	30	25	15	10	25	25
	Shoots	70	75	85	90	75	75

Table S1: In experiment 1, no significant difference is observed for the two nutrient solutions (EDTA) and (NTA+HEDTA), except for Cu in rice, where less translocation is observed. In experiment 2, no effect of nutrient solution was observed. However, for tomato, a significant difference was observed between experiment 1 and 2. This surely reflects the different concentrations of Cu in the nutrient solution. Plants control the Cu translocated, leading to a maximum concentration 25 $\mu g.g^{-1}$ in the shoots, whatever the concentration in the roots. This is in line with a previous observations (Chaignon V. et al., 2002).

4. References

Borrok D. M., Nimick D. A., Wanty R. B., Ridley W. I., 2008. Isotopic variations of dissolved copper and zinc in stream waters affected by historical mining. Geochimica and Cosmochimica Acta, 72(2): 329-344.

Chaignon V., Bedin F., Hinsinger P., 2002. Copper bioavailability and rhizosphere pH changes as affected by nitrogen supply for tomato and oilseed rape cropped on an acidic and a calcareous soil. Plant and Soil, 243: 219-228.

Chapman, J., Mason, T.F.D., Weiss, D.J., Coles, B.J., Wilkinson, J.J., 2006. Chemical separation and isotopic variations of Cu and Zn from five geological reference material. Geostand. Geoanal. Res., 30(1): 5-16.

Chen J., Louvat P., Gaillardet J., Birck J-L., 2008. Direct separation of Zn from dilute aqueous solutions for isotope composition determination using multi-collector ICP-MS. Chemical Geology, 259: 120-130.

Cornu J-Y., Staunton S., Hinsinger P., 2007. Copper concentration in plants and in the rhizosphere as influenced by the iron status of tomato (Lycopersicon esculentum L.). Plant and Soil, 292: 63-77.

Dolgopolova, A., Weiss, D., Seltmann, R., Coles, B., 2004. Improved closed vessel microwave digestion method for lichens. Intern. J. Environ. Anal. Chem., 84(12): 889-899.

Jouvin D., Louvat P., Maréchal C. N., Juillot F., Benedetti M. F., 2009. Zn isotopic fractionation: why organic matters. Environmental Science and Technology, In revision.

Ludwig, K., 1982. A computer program to convert raw U-Th-Pb isotope ratios to blank-corrected isotope ratios and concentrations with associated error clculation. OF.-88-0557, U.S. Geol. Surv.

Maréchal, C.N., Télouk, P., Albarède, F., 1999. Precise analysis of copper and zinc isotopic compositions by plasma-source mass spectrometry. Chemical Geology, 156(1-4): 251-273.

Mason, T.F.D. et al., 2004a. High precision Cu and Zn isotope analysis by plasma source mass spectrometry: Part 1: Spectral interferences and their correction. J. Anal. At. Spectrom., 19(2): 209 - 217.

Mason, T.F.D. et al., 2004b. High precision Cu and Zn isotope analysis by plasma source mass spectrometry: Part 2: Correcting for mass bias discrimination effects. J. Anal. At. Spectrom., 19(2): 218-226.

Michaud A. M., Chappellaz C., Hinsinger P., 2008. Copper phytotoxicity affects root elongation and iron nutrition in durum wheat (*Triticum turgidum durum* L.). Plant and Soil, 310: 151-165.

Shields, W.R., Murphy, T.J., Garner, E.L., 1964. Absolute isotopic abundance ratio and the atomic weight of a reference sample of copper. Journal of Research of the National Bureau of Standards, 68A: 589.