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Evidence for the mechanisms of zinc uptake by rice using isotope fractionation

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ABSTRACT

In an earlier study, we found that rice (*Oryza sativa*) grown in nutrient solution well-supplied with Zn preferentially took up light ^{64}Zn over ^{66}Zn, probably as a result of kinetic fractionation in membrane transport processes. Here, we measure isotope fractionation by rice in a submerged Zn-deficient soil with and without Zn fertilizer. We grew the same genotype as in the nutrient solution study plus low-Zn tolerant and intolerant lines from a recombinant inbred population. In contrast to the nutrient solution, in soil with Zn fertilizer we found little or heavy isotopic enrichment in the plants relative to plant-available Zn in the soil, and in soil without Zn fertilizer we found consistently heavy enrichment, particularly in the low-Zn tolerant line. These observations are only explicable by complexation of Zn by a complexing agent released from the roots and uptake of the complexed Zn by specific root transporters. We show with a mathematical model that, for realistic rates of secretion of the phytosiderophore deoxymugineic acid (DMA) by rice, and realistic parameters for the Zn-solubilizing effect of DMA in soil, solubilization and uptake by this mechanism is necessary and sufficient to account for the measured Zn uptake and the differences between genotypes.

Key-words: DMA; isotope fractionation; phytosiderophore; rice; solubilization; stable isotopes; zinc.

INTRODUCTION

The development of the multi-collector inductively coupled plasma mass spectrometer (MC-ICPMS) has enabled measurements of natural-abundance isotope fractionations in heavier elements in natural systems in the way that is routinely done for light elements such as C, O, N and S (Weiss *et al.* 2008). Heavy isotope systems are, therefore, now available to study biogeochemical processes controlling element cycling in the natural environment. We are particularly interested in their potential for studying metal uptake by plants, and the importance of rhizosphere processes in metal solubilization and uptake. To date, such complex root–soil interactions have only been studied with indirect measurements in solution cultures or other artificial laboratory systems, underpinned by mathematical modelling. The lack of direct techniques for measuring interactions in intact plants under natural conditions, without artificial manipulations of the system, has hampered progress. As we will show, isotope fractionation at natural abundance has much to offer in this.

The particular application we consider is the uptake of Zn by rice growing in submerged soils and differences in uptake efficiency between rice genotypes. This is an important practical problem because of the importance of Zn deficiency in submerged soils due to their biogeochemistry (Kirk 2004), and because of current efforts to breed rice for high micronutrient contents (Graham, Welch & Bouis 2007; Wissuwa *et al.* 2008). There is indirect evidence that rhizosphere processes are involved, including the observation that genotype rankings in nutrient solution culture give poor predictions of performance in the field (Wissuwa, Ismail & Yanagihara 2006), and observed interactions between uptake efficiency and planting density (Hoffland, Wei & Wissuwa 2006). However the mechanisms are not understood.

In grass species as a whole it is well established that efficient Fe acquisition involves secretion of phytosiderophores (low molecular weight, non-protein amino acids that form soluble complexes with Fe(III) and other micronutrients in soil) and absorption of Fe(III) – phytosiderophore complexes by roots (Marschner 1995). It has been suggested that phytosiderophores are also involved in Zn uptake by grasses (Reid *et al.* 1996; von Wirén, Marschner & Romheld 1996), but this is yet to be established unequivocally (Hacisalihoglu & Kochian 2003; Suzuki *et al.* 2008). In rice, because Fe(II) is far more soluble in anaerobic submerged soils than is Fe(III) in aerobic soils, and rice roots take up free Fe(II) ions directly (Ishimaru *et al.* 2006; Cheng *et al.* 2007), Fe deficiency is relatively rare (Marschner 1995). Rates of release of phytosiderophores from rice

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roots are correspondingly small relative to other grasses (Takagi 1993). Nonetheless, Inoue *et al.* (2009) recently showed that rice roots possess transporters of complexes of the phytosiderophore DMA with Fe(III), and that this was essential for Fe uptake by seedlings in aerobic soil. The possibility of enhanced Zn uptake by release of phytosiderophores is, therefore, a possible explanation for the field observations on rice Zn efficiency.

In an earlier paper (Weiss *et al.* 2005), we reported isotopic fractionation during Zn absorption and assimilation by rice, tomato and lettuce grown in solution cultures. Plant shoots were enriched in ^{64}Zn relative to ^{66}Zn, consistent with a kinetically based bias in favour of light ^{64}Zn in transport of free Zn^{2+} across cell membranes. By contrast, root Zn was isotopically heavier than Zn in the external solution, probably reflecting preferential adsorption of heavy Zn onto root surfaces and plaque deposits (Gélabert *et al.* 2006; Balistrieri *et al.* 2008; Juillot *et al.* 2008). In soil-grown trees and various herbaceous species, Viers *et al.* (2007) and Moynier *et al.* (2008) have found light Zn isotope bias in the plant leaves relative to the soil, though not in roots or stems. The increasing fractionation with distance up the plant suggests translocation processes favour the light Zn isotope, consistent with fractionation during diffusion or transmembrane transport or both. By contrast Guelke & von Blanckenburg (2007) found light isotope fractionation in Fe uptake by plants with 'Strategy I' type Fe nutrition, but heavy fractionation in 'Strategy II' type plants. They attributed this to uptake of free Fe^{2+} by the Strategy I plants following enzymatic Fe(III) reduction in the rhizosphere, but uptake of a phytosiderophore-Fe(III) complex by the Strategy II plants following phytosiderophore secretion from the roots and heavy isotopic fractionation in formation of the complex.

The aim of this study was to investigate Zn isotopic fractionation in soil-grown rice, extending our earlier study in solution culture (Weiss *et al.* 2005). The specific objectives were to: (1) study fractionation in the genotype IR64 used in the solution culture study but now grown in soil; (2) study fractionation in low-Zn tolerant and intolerant lines from a recombinant inbred population; (3) interpret the results using a mathematical model of root-soil interactions; and (4) derive a preliminary conceptual model of the processes leading to Zn isotope fractionation in soil–plant systems.

MATERIALS AND METHODS

Field experiments

Three field experiments were made: the first two to study differences in isotope fractionation between genotypes, with and without Zn fertilizer, and the third to study a genotype by planting density interaction. In the first experiment, we used the same genotype as Weiss *et al.* (2005), IR64, which is considered moderately tolerant of low-Zn soils (Quijano-Guerta *et al.* 2002). In the subsequent experiments, we used two lines from a population used to identify quantitative trait loci associated with tolerance to Zn-deficient soil (Wissuwa *et al.* 2006): one was the intolerant parent of the mapping population (IR74) and the other a tolerant recombinant inbred line (RIL46). RIL46 resembles IR74 more closely than donor parent *Jalmagna*, both genetically and in appearance under non-stressed conditions, but it shows several-fold greater growth and Zn uptake than IR74 under Zn-deficiency in the field.

Table 1. Properties of the experimental soil

Aerobic pH (1:1 H_2O)	7.8
Anaerobic pH	7.0
$CaCO_3$ (g kg^{-1})	32
Organic C (g kg^{-1})	47
CEC (mol_c kg^{-1})	0.43
Clay (g kg^{-1})	30
Available Zn (0.05 M HCl) (mg kg^{-1})	0.1
Total Zn (mg kg^{-1})	60
Total Fe (g kg^{-1})	34

The field experiments were made in plots at the International Rice Research Institute, Los Baños, Philippines during the dry seasons of 2002, 2006 and 2007. The plots contain a Zn-deficient soil from Tiaong, Quezon, Philippines. The soil is a perennially wet, montmorillonitic, calcareous Hydraquent (relevant properties in Table 1). It was submerged by irrigation 3 weeks before transplanting and kept submerged throughout the experiments. Half the plots were fertilized with Zn by mixing powdered $ZnSO_4$ (15 kg Zn ha^{-1}) into the soil before transplanting. The other plots received no Zn fertilizer. All plots received the standard recommended dose of NPK as a compound fertilizer (14-14-14) at a rate of 136 kg ha^{-1}.

Seeds of the rice genotypes were germinated and raised for 20 d in seedling trays. They were then transplanted into the plots in rows at 20 cm spacing within and between rows, with four rows per genotype and 20 single plants per row. There were four replications. Four weeks after transplanting four plants per replicate were harvested for tissue analyses, and an additional four plants per replicate were harvested seven weeks after transplanting for isotope analyses. The plants were harvested by gently pulling them intact from the loose submerged soil. They were scored for leaf bronzing as described by Wissuwa *et al.* (2006). Roots and shoots were repeatedly washed under running water to remove any adhering soil. Numbers of new and old roots per plant were counted, new roots being white without laterals. Root and shoot materials were oven dried at 70 °C for 4 d and their dry weights recorded. They were then ground to a fine powder using a vibrating sample mill (T1-100, Heiko Seisakusho, Tokyo, Japan). Digests of 0.5 g subsamples in H_2SO_4–H_2O_2 were made as described by Wissuwa *et al.* (2006) and analysed for tissue Fe and Zn concentrations by ICP-AES. Samples were shipped to the UK for isotope analyses, as was the air-dried soil.

In the third field experiment, the effect of planting density was assessed by growing the plants as previously mentioned, but with 1, 5 or 10 plants per hill (a hill is a bundle of rice seedlings planted together). Each

experimental unit consisted of four rows of single plants with 20 cm spacing within and between rows and two 5- or 10-plant hills each at the head of single-plant rows.

Isotope analysis

The air-dried soil and oven-dried plant materials were ground to pass a 0.5 mm sieve. To measure total soil Zn, soil samples (0.1 g) were digested in conc. HNO_3-$HClO_4$-HF mixtures on a hot block. To measure plant-available Zn in the soil, samples (0.3 g) were combined with 30 cm^3 of 0.1 M HCl and the resulting suspension stirred continuously for 48 h at 25 °C. To measure Zn in plant tissues, samples (0.35 g) were digested in conc. HNO_3-H_2O_2-HF mixtures on a microwave accelerated reaction system (MARS-X, CEM Corporation, Matthews, NC, USA) as described by Weiss *et al.* (2007). All digests and extracts were evaporated to dryness, the residue dissolved in ~1 mL 7 M HCl plus 0.05 mL 9 M H_2O_2, the resulting solutions refluxed, then dried and re-dissolved in 2.5 mL 7 M HCl containing 0.001% H_2O_2. The resulting solutions were split into three parts: 1 mL for isotope analyses, 0.5 mL for analysis of Zn concentration by ICP-AES and 1 mL for archiving.

For isotope analyses, matrix components were separated from Zn using an anion exchange procedure previously described (Weiss *et al.* 2007). Zinc was recovered from the resin in 12 mL 0.1 M HCl, the solution dried, re-dissolved in 0.2 mL 15.4 M HNO_3 and evaporated to drive off chlorine ions. Finally, 1 mL 0.1 M HNO_3 was added, followed by refluxing for >2 h to ensure complete dissolution. The solution was then ready to be analysed by MC-ICPMS. The procedural Zn blank of the ion exchange chromatography was ~20 ng in the clean laboratory at Imperial and ~40 ng in the microwave laboratory at the Natural History Museum; all the plant and soil digests contained >5 μg Zn, so the blank contributions were negligible.

The isotope ratios were measured on an IsoProbe MC-ICPMS using previously described protocols (Peel *et al.* 2008). We report all data using the conventional notation

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

where subscript standard indicates our in-house standard, Imperial Zn. The Imperial Zn has an isotopic composition relative to the widely used standard for inter laboratory comparison Lyon Zn (Johnson Matthey Zn solution batch JMC 3-0749L) of

$$\delta^{66}\mathrm{Zn}_{\mathrm{JMC3-0749L}} - \delta^{66}\mathrm{Zn}_{\mathrm{IMP}} = -0.09 \pm 0.05‰ \text{ (2 s.d., } n = 12)$$

The isotope ratios measured by external normalization could be plotted in three isotope space illustrating the absence of isobaric interferences. All points lay within error (0.15‰ on δ^{68}Zn and 0.07‰ on δ^{66}Zn) on a linear regression line of gradient 0.501. Theoretical mass dependent fractionation lines over the given spread of isotope ratios approximate to linearity with gradients 0.507 and 0.515 for kinetic and equilibrium fractionation, respectively. Data points lie within error of both theoretical fractionation lines.

Model of Zn solubilization and uptake

List of symbols

X, Y	concentrations of X and Y in the whole soil
X_L, Y_L	concentrations of X and Y in the soil solution
b_X, b_Y	buffer powers of X and Y, defined as $(\partial X/\partial X_L)_Y$, $(\partial Y/\partial Y_L)_X$
λ, ν	interaction coefficients $(\partial X_L/\partial Y_L)_X$, $(\partial Y_L/\partial X_L)_Y$
D_{LX}, D_{LY}	diffusion coefficients of X and Y in free solution
θ	volume fraction of soil water
f	diffusion impedance factor
D_X, D_Y	$D_{LX}\theta f/b_X$, $D_{LY}\theta f/b_Y$
r	radial distance
a	radius of root
x	radius of root's zone of influence
L_V	root length density.

The model (developed by Kirk 1999 after Nye 1983) describes the coupled diffusion of two interacting solutes X (e.g. Zn) and Y (e.g. a phytosiderophore) in the soil near a cylindrical root that simultaneously absorbs X and releases Y, the reaction of Y with the soil increasing the concentration of X in the soil solution. The model allows for: (1) diffusion of Y away from the root and its reaction with the soil solubilizing X; (2) diffusion of solubilized X towards the root, where it is taken up, as well as away from it; and (3) 'reflection' of Y and solubilized X at the boundary between the zones of influence of neighbouring roots.

The equations for the diffusion and interaction of X and Y in the soil around an individual root are:

$$\begin{aligned} \frac{\partial}{\partial t}(X_L - \lambda Y_L) &= D_X(1-\nu\lambda)\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial X_L}{\partial r}\right) \\ \frac{\partial}{\partial t}(Y_L - \nu X_L) &= D_Y(1-\nu\lambda)\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial Y_L}{\partial r}\right) \end{aligned} \quad (1)$$

The root system is treated as a regular parallel array and each root is assigned a zone of influence of radius x such that the entire soil volume is divided equally between the roots. Hence:

$$x = 1/\sqrt{\pi L_V} \quad (2)$$

where L_V is the total root length density. The boundary conditions for solving Eqn 1 for an individual root are (a) at the root surface, $r = a$

$$\begin{aligned} D_{LX}\theta f dX_L/dr &= -\alpha X_L \\ D_{LY}\theta f dY_L/dr &= F_Y \end{aligned} \quad (3)$$

where α = root absorbing power for X and F_Y = efflux of Y from the root; and (b) at $r = x$

$$D_{LX}\theta f d X_L/dr = 0$$
$$D_{LY}\theta f d Y_L/dr = 0 \quad (4)$$

These equations are solved numerically as described by Kirk (1999).

Nye (1984) gives the following approximate solution of Eqn 1 for an isolated root (i.e. $x \to \infty$) at whose surface the concentrations of X and Y are abruptly changed and then held constant, and when the diffusion of Y is not significantly affected by the diffusion of X (i.e. $v \to 0$):

$$\frac{M_X}{M_Y} \approx -\lambda \frac{b_X}{b_Y}\left(\frac{1}{1+\sqrt{D_Y/D_X}}\right)\left(\frac{1}{1+\left(\sqrt{\pi D_Y t}/4a\right)}\right) \quad (5)$$

where $-M_X/M_Y$ is the additional amount of X taken up as a result of solubilization by Y per unit secretion of Y. Eqn 5 shows that if $D_Y >> D_X$, or if $\sqrt{D_Y t} >> a$, then $M_X/M_Y \to 0$. This is because any X solubilized by Y at a distance far from the root surface is more likely to spread outwards than inwards, so the recovery of solubilized X by the root is small.

Nye (1984) defines the 'solubilizing effect' of Y on X as $(-\partial X/\partial Y)_{X_L}$; i.e. the amount of X that needs to be removed from the soil for a given uniform addition of Y in order to leave the concentration of X in the soil solution unchanged, and he shows that this is equal to $-\lambda b_X/b_Y$.

Parameter values

The following parameter values are realistic for the conditions of the field experiments and solubilization of Zn by the phytosiderophore deoxymugineic acid (DMA). Component X is Zn and component Y is DMA. The concentration of available Zn buffering Zn in solution in the experimental soil without Zn fertilizer is approx. 2 μmol kg^{-1} ($\equiv$ 0.1 mg kg^{-1}, Table 1), and, based on the solubility of 'soil-Zn' at the pH of the experimental soil when flooded (pH 7) the concentration of Zn in the soil solution (X_L) is of the order of 0.01 μM (McBride 1994). So for the Zn buffer power in –Zn soil, $\overline{b_X} = (\Delta X/\Delta X_L)_{Y=0} \cong 2/0.01 \cong 200$. Substituting $D_{LX} = 7 \times 10^{-6}$ cm^2 s^{-1} and the experimental values $\theta = 0.7$, $f = 0.5$ gives $D_X \approx 1.2 \times 10^{-8}$ cm^2 s^{-1}.

Hiradate & Inoue (2000) measured mugineic acid sorption by a wide range of soils, and found that although >50% of added DMA was sorbed in soils with pH < 6.5, it was largely non-adsorbed in soils with pH > 7.5, and in neutral soils, the median amount sorbed was 5% (at soil : solution ratio 1:25). From this, we estimate $b_Y = 1.25$ for our experimental soil. Substituting $D_{LY} = 7 \times 10^{-6}$ cm^2 s^{-1} and the values for θ and f gives $D_Y \approx 2.0 \times 10^{-6}$ cm^2 s^{-1}.

The Zn–DMA interaction coefficients λ and v are estimated as follows. Scharpenseel *et al.* (1983) equilibrated anaerobic samples of our experimental soil (from the Tiaong plots) with amounts of ^{65}Zn in shaken suspensions with and without DTPA (which is a satisfactory analogue of DMA – Hiradate & Inoue (2000), and measured ^{65}Zn in solution after 48 h. With addition of 1–10 μg ^{65}Zn-labelled Zn g^{-1} soil, addition of 0.05 M DTPA increased the activity of ^{65}Zn in the equilibrium solution by more than 5×10^4-fold. The following calculations of Zn complexation by DMA show that this increase in Zn solubility is realistic. From the equilibrium stability constants of DMA, the concentration of complexed Zn(II) is

$$[\mathrm{ZnDMA^-}] = K_f[\mathrm{Zn^{2+}}]\frac{[\mathrm{DMA_{tot}}]}{1+[\mathrm{H^+}]/K_{a3}+[\mathrm{H^+}]^2/(K_{a2}K_{a3})+[\mathrm{H^+}]^3/(K_{a1}K_{a2}K_{a3})}$$

where $K_{a1,2,3}$ are the H^+ dissociation constants for the last carboxylate group and two amino groups of DMA, and K_f is the formation constant for $ZnDMA^-$. $pK_{a1,2,3} = 3.19$, 8.25, 10.00 and $pK_f = -12.82$ ($T = 25$ °C, $I = 0.1$ M; Murakami *et al.* (1989). Therefore at pH = 7.0 (the pH of the anaerobic experimental soil), $[ZnDMA^-]/[Zn^{2+}] = 3.52 \times 10^7 \times [DMA_{tot}]$, and for $[DMA_{tot}] = 0.05$ M, $[ZnDMA^-]/[Zn^{2+}] = 1.76 \times 10^6$. So the above 5×10^4 fold increase in [Zn(II)] with 0.05 M DMA is well within the theoretical limit. We have $\lambda = (\Delta X_L/\Delta Y_L)_X$. Therefore if $X_{L0} = 1 \times 10^{-8}$ M, and $X_L/X_{L0} = 5 \times 10^4$ at $Y_L = 0.05$ M, then $\lambda = 5 \times 10^4 \times 1 \times 10^{-8}/0.05 = 0.01$. Because the amount of DMA reacting with the soil is much larger than the amount of Zn being removed by the plants, the diffusion of Zn will have little influence on the diffusion of DMA, so $v = 0$.

Rates of DMA release from seedling roots in solution culture systems range from 100 to 500 pmol g^{-1} root FW s^{-1} during the 4–6 h secretion period in Fe-deficient barley and wheat (Tolay *et al.* 2001; Reichman & Parker 2007; Suzuki *et al.* 2006), but are at least an order of magnitude smaller than this in rice (Takagi 1993; Suzuki *et al.* 2008). For an average root radius $a = 0.01$ cm, 10 pmol g^{-1} root FW s^{-1} is equivalent to $F_Y = 0.05$ pmol cm^{-2} s^{-1}.

Realistic values for the root geometry parameters for rice in flooded soil are $a = 0.01$ cm and $L_V = 0.5$ to 50 cm cm^{-3} (Morita & Yamazaki 1993). We assign the root Zn absorbing power a sufficiently large value that uptake is insensitive to it: $\alpha = 1.5 \times 10^{-2}$ cm s^{-1}.

The amount of Zn taken up by the root system of one plant after a particular time (μg plant^{-1}) is found from

$$\text{Uptake} = A_X \sum (2\pi a \alpha X_{La} L_V * V \Delta t)$$

where A_X is the atomic mass of Zn (= 65 μg mol^{-1}), L_V* is the root length density of the individual plant, V is the rooting volume per plant and the sum is taken over all time steps. For plants at 20 cm spacing within and between rows, and 5 cm depth, $V = 2000$ cm^3. NB to find the radius of the zone of influence of the root, x, with Eqn 2, it is the combined root length density of all the plants in a hill that is to be used.

Note that the model does not allow for mass flow of solution towards the root in the transpiration stream, which might be expected to increase the inflow of the phytosiderophore-Zn complex. The fractional increase in

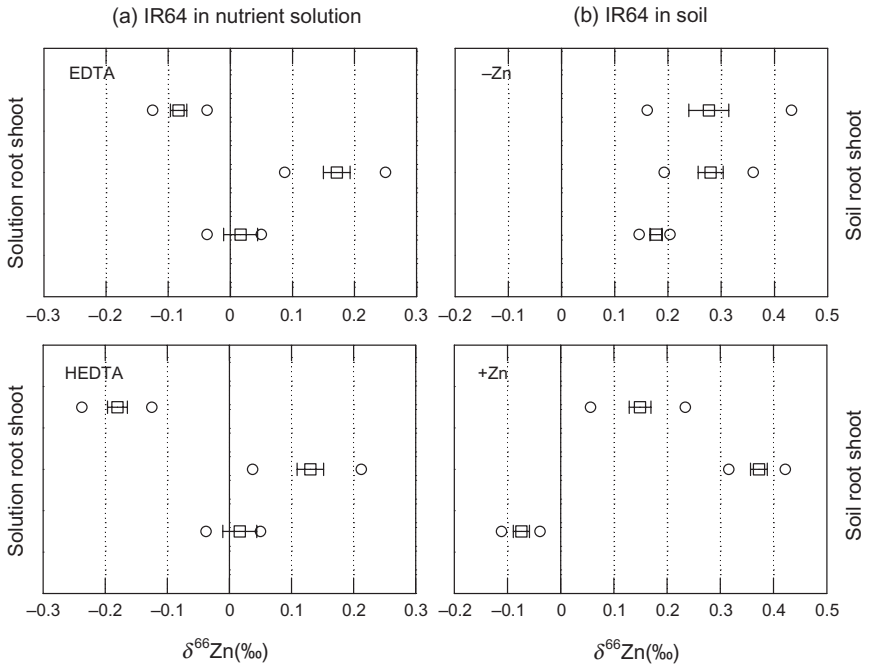

Figure 1. Zinc isotopic fractionation in rice (IR64) grown in (a) nutrient solution (data from Weiss *et al.* 2005), for indicated solutions); and (b) strongly Zn-deficient submerged soil with and without Zn fertilizer. Negative δ^{66}Zn values indicate enrichment in light Zn relative a standard, and *vice versa*. Data are means ± SE ($n \geq 4$) and extremes (open circles).

inflow resulting from mass flow is approx. $av/(0.5D_L\theta f)$ where v is the water flux (Roose & Kirk 2009). With these parameter values and $av = 1 \times 10^{-7}$ cm^2 s^{-1}, which corresponds to fast transpiration, the inflow would be only 8% larger.

RESULTS

Isotope fractionation

Figure 1 compares isotope fractionations in genotype IR64 grown in nutrient solution (LHS; data from Weiss *et al.* 2005) with those in IR64 grown in soil in the first field experiment (RHS; data from this study). In nutrient solution culture we found a positive fractionation in root material compared with the solution, which we attributed to sorption of Zn^{2+} on external root surfaces (Weiss *et al.* 2005); but we found a negative fractionation in the shoots of $\Delta^{66}Zn_{solution\text{-}shoot} = -0.10$‰ in EDTA solution and –0.20‰ in HEDTA solution (Table 2). By contrast, the results in Fig. 1 for in IR64 grown in soil show a positive fractionation in the shoots relative to plant-available Zn in the soil of $\Delta^{66}Zn_{soil\text{-}shoot} = 0.10$‰ in the –Zn soil and 0.22‰ in the + Zn soil (Table 2). The δ^{66}Zn values of plant-available Zn in the soil (measured in a 0.1 M HCl extract – Materials and methods) were little different from those of whole soil digests: the respective δ^{66}Zn values were 0.18 ± 0.01 and 0.25 ± 0.00‰ in the –Zn soil, and –0.07 ± 0.02 and –0.13 ± 0.02‰ in the +Zn soil.

Figure 2 shows the fractionation results for soil-grown IR74 and RIL46 in the second field experiment with and without Zn fertilizer. In the Zn fertilized soil, mean shoot δ^{66}Zn values were not significantly different from that of plant-available Zn in the soil (Table 2). But in the –Zn soil, there was a heavy isotopic enrichment in the shoots of RIL46. There was also a heavy enrichment in IR74, comparable to that in unfertilized IR64 in the first experiment, but it was not statistically significant. The heavy enrichment in RIL46 was more than twice that in IR74 and the difference was statistically significant.

External medium	Genotype and Zn status	$\Delta^{66}Zn_{ext\text{-}shoot}$ (‰)	Significance[b]
Nutrient solution[a]	IR64 +Zn EDTA	–0.10	+
	IR64 +Zn HEDTA	–0.20	+
Soil (1st expt)	IR64 +Zn	+0.22	+
	IR64 –Zn	+0.10	+
Soil (2nd expt)	IR74 +Zn	–0.05	–
	IR74 –Zn	+0.08	–
Soil (2nd expt)	RIL46 +Zn	+0.06	–
	RIL46 –Zn	+0.21	+

Table 2. Summary of isotope fractionation results in Figs 1 and 2. $\Delta^{66}Zn_{ext\text{-}shoot} = \delta^{66}Zn_{ext} - \delta^{66}Zn_{shoot}$ where $\delta^{66}Zn_{ext}$ and $\delta^{66}Zn_{shoot}$ are the isotopic ratios of the external medium (nutrient solution or soil extract) and plant shoots relative to the standard, respectively

[a]data from Weiss *et al.* (2005).
[b]+ indicates significant ($P \leq 0.05$) by a t test for unequal sample sizes with equal variance.

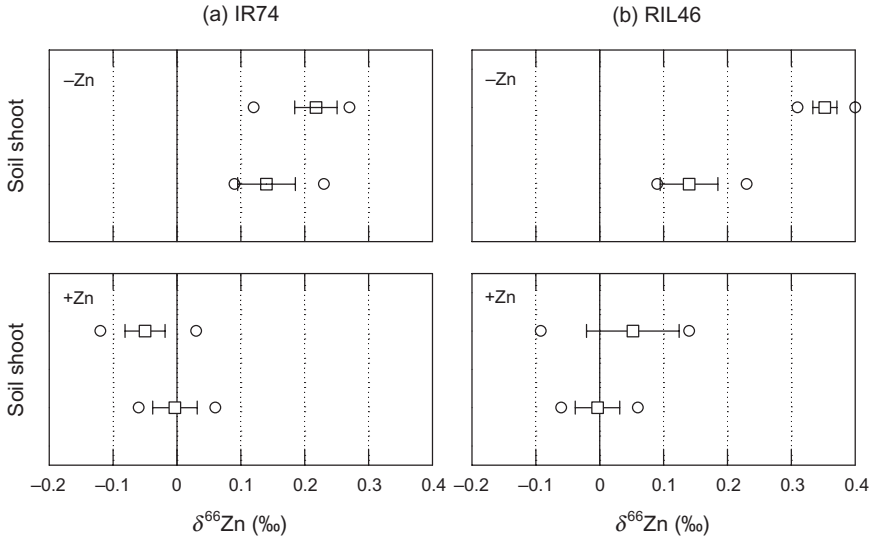

Figure 2. Zinc isotopic fractionation in shoots of rice grown in Zn-deficient submerged soil with and without Zn fertilizer: (a) low-Zn intolerant genotype IR74; and (b) low-Zn tolerant genotype RIL46. Data are means ± SE ($n \geq 4$) and extremes (open circles).

The isotopic compositions of the –Zn and +Zn soils in the second experiment (δ^{66}Zn of plant-available pool = 0.14 ± 0.05‰ and 0.00 ± 0.03‰, respectively) were similar to those in the first experiment. The isotopic composition of the –Zn soil was within the range of values reported for basalt (0.11 to 0.23‰; Chapman *et al.* (2006), consistent with the volcanic origin of the experimental soil. The +Zn soil had a lighter isotopic composition, consistent with the combined isotopic compositions of the Zn fertilizer (δ^{66}Zn = –0.23 ± 0.03‰) and –Zn soil.

Plant growth

Table 3 shows plant growth and Zn uptake in IR64 in the first field experiment. Shoot Zn concentrations were 29 μg g^{-1} in nutrient solution and 34.5 μg g^{-1} in the Zn-fertilized soil, but only 12.5 μg g^{-1} in the soil-grown plants without Zn fertilizer, which is below the threshold for deficiency (10–15 μg g^{-1} in the vegetative growth stages (Dobermann & Fairhurst 2000).

Table 4 shows plant growth and Zn uptake in the two genotypes in the second field experiment. In general growth and uptake were better than in the first experiment – such year to year variation is typical of these experimental plots in the vegetative growth stages (Wissuwa, unpublished observations). In both genotypes, growth and Zn uptake were decreased in the –Zn soil compared with the +Zn, but to a smaller extent in the low Zn tolerant genotype (RIL46) than the intolerant (IR74). Shoot Zn concentrations were below the threshold for deficiency in the –Zn soil in both genotypes, and there were clear visual symptoms of deficiency. In –Zn soil RIL46 produced 40% more dry weight and 45% more shoot Zn uptake than IR74, but growth and Zn uptake were similar in +Zn soil. Root growth of IR74 was more strongly depressed under Zn deficiency than that of RIL46, particularly growth of new adventitious roots.

Table 3. Performance of rice genotype IR64 in the first field experiment with (+Zn) and without (–Zn) Zn fertilizer. Plants were harvested 5 weeks after transplanting. Data are means of four replicates

	–Zn	+Zn	HSD
Shoot dry weight (g plant^{-1})	0.47	1.54	[a]
Root dry weight (g plant^{-1})	0.16	0.16	ns
Shoot Zn concentration (μg g^{-1})	12.5	34.5	[a]
Root Zn concentration (μg g^{-1})	37.2	142.5	[a]
Shoot Fe concentration (μg g^{-1})	487	256	[a]
Root Fe concentration (μg g^{-1})	22535	9370	[a]

[a]significant at 1% level.
HSD, honestly significant difference.

Shoot Fe concentrations were well above thresholds for deficiency [50–100 μg g^{-1} (Dobermann & Fairhurst 2000)] in all the genotypes in both Zn treatments in both experiments (Tables 3 and 4). Root Fe concentrations were far larger than shoot concentrations, and more so in the –Zn soil, but there were no differences between the genotypes. Greater root than shoot Fe concentrations were probably due to Fe oxide coatings on root surfaces [formed by oxidation of Fe(II) by O_2 released from the roots], which were only partly removed in the root washings.

Table 5 shows the effects of planting density on shoot growth and Zn content in the –Zn soil in the third field experiment. Increasing plant density from 1 to 5 plants hill^{-1} increased shoot growth and Zn content per plant approximately two-fold in RIL46 and four-fold in IR74. Growth and Zn contents were similar in the two genotypes at 10 plants hill^{-1}.

Model of solubilization and uptake

Figure 3 shows the calculated effects of the phytosiderophore DMA on Zn uptake, with the Zn-solubilizing effect of DMA and other soil parameters derived from independent measurements on the –Zn soil (parameter values). The

Table 4. Performance of rice genotypes IR74 and RIL46 in the second field experiment with and without Zn fertilizer. The genotypes are from a recombinant inbred population: RIL46 is tolerant of low Zn soils and IR74 intolerant. Plants were harvested 4 weeks after transplanting. Data are means of four replicates

	–Zn			+Zn			–Zn/+Zn	
	RIL46	IR74	HSD	RIL46	IR74	HSD	RIL46	IR74
Shoot dry weight (g plant^{-1})	1.1	0.8	[a]	2.7	3.0	ns	0.41	0.27
Root dry weight (g plant^{-1})	0.3	0.2	[b]	0.7	0.6	ns	0.43	0.33
Number of roots (plant^{-1})	158.0	97.8	[b]	190.7	188.8	ns	0.83	0.52
Number of new roots (plant^{-1})	53.7	28.7	[b]	105.6	112.1	ns	0.51	0.26
Max root length (cm)	15.0	16.3	ns	18.3	18.2	ns	0.82	0.90
Shoot Zn concentration (μg g^{-1})	14.8	14.0	ns	24.9	30.6	ns	0.59	0.46
Root Zn concentration (μg g^{-1})	32.7	36.3	ns	138.7	147.8	ns	0.26	0.25
Shoot Fe concentration (μg g^{-1})	1123	1189	ns	1093.1	1241.3	ns	1.03	0.96
Root Fe concentration (μg g^{-1})	27053	30247	ns	18327	17151	ns	1.48	1.76
Shoot Zn content (μg plant^{-1})	26.4	17.5	[b]	171.4	189.4	ns	0.15	0.09
Leaf bronzing score	0.3	2.1	[b]	0.2	0.4	ns		
Plant mortality (%)	2.8	8.3	ns	4.8	9.6	ns		

[a,b]significant at 5%, 1% level.
HSD, honestly significant difference between genotypes.

results indicate that measured rates of DMA secretion reported for rice in the literature are sufficient to significantly increase Zn uptake and to explain the difference between the genotypes. The effect of planting density measured in the third field experiment is also explained. Without solubilization, i.e. no secretion of DMA from the roots, Zn uptake increases linearly with root length density. But with solubilization, uptake increases non-linearly at a given rate of DMA secretion. Further, at greater planting density (Fig. 3b), the effect of solubilization is greatly increased. This can be understood from the concentration-distance profiles of Zn and DMA around the roots shown in Fig. 4. For non-solubilizing roots, increasing plant density means the zones of Zn depletion around individual roots tend to overlap faster, so Zn uptake per plant tends to decrease. However, for solubilizing roots, increasing plant density means DMA tends to accumulate in the soil between neighbouring roots and neighbouring roots increasingly benefit from Zn solubilized by each other; hence there is a greater recovery of solubilized Zn by the plants, and a greater net uptake per plant. The effects of DMA secretion and planting density are smaller in Zn-fertilized soil (results not shown) because there is less depletion of soil Zn and the roots are therefore less-dependent on solubilization.

Figure 5 shows the additional Zn taken by roots per unit DMA released. This ratio is independent of the rate of DMA secretion but increases with root length density as shown. For comparison, the ratio for an isolated root calculated with Eqn 5 for $t = 3$ d is only 0.001, showing the importance of recovery by neighbouring roots. Figure 5 also shows the effect of the soil water status as it affects the cross-sectional area for diffusion of Zn and DMA and the tortuosity of the diffusion path, with all other parameter values unchanged. In drier soil, the spread of solubilized Zn away from the solubilizing root surface is far smaller, so recovery by neighbouring roots is less. The effects of rooting density on Zn recovery are correspondingly smaller (note that differences in Zn solubility with water status were not simulated).

Table 5. Effect of planting density on shoot dry weight and shoot Zn content of genotypes RIL46 and IR74 in the third field experiment. Plants were harvested 7 weeks after transplanting. Data are means of four replicates

		Density	Genotype		
		(plants hill^{-1})	RIL46	IR74	HSD
Shoot dry weight (g plant^{-1})	+Zn	1	6.4	5.9	ns
	–Zn	1	2.5	0.7	[b]
	–Zn	5	4.8	2.8	[b]
	–Zn	10	3.9	3.2	ns
Shoot Zn content (μg plant^{-1})	+Zn	1	256.1	234.8	[a]
	–Zn	1	29.3	7.8	[b]
	–Zn	5	62.1	36.3	[b]
	–Zn	10	58.9	51.1	ns

[a,b]significant at 5%, 1% level.
HSD, honestly significant difference between genotypes.

DISCUSSION

We have found a heavy isotopic enrichment in the shoots of rice grown in soil relative to the isotope composition of the soil, in contrast to our earlier results for rice in solution culture where we found a light isotopic enrichment in the plants relative to the external solution. We have also found that the heavy enrichment in the soil-grown plants was significantly larger in the Zn-efficient genotype RIL46 than in inefficient IR74. In the following sections, we discuss these results in the light of what is known about stable

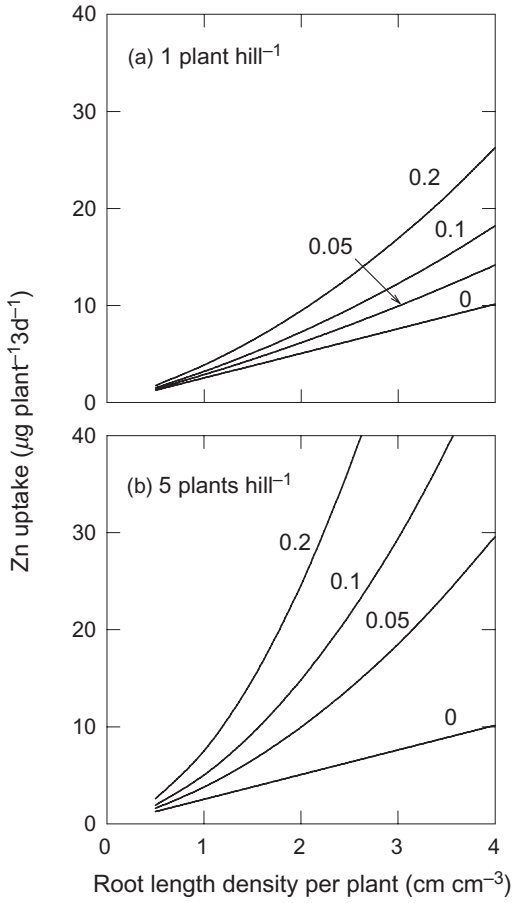

Figure 3. The calculated effect of DMA secretion on Zn uptake per plant at different planting densities. Numbers on curves are rates of DMA secretion (pmol cm^{-2} s^{-1} for 4 h per day). Total root length density (with which mean inter-root distance is calculated) = plants per hill × root length density per plant. (A hill is a bundle of seedlings planted together.) Time = 3 d. Other parameter values are given in the text.

isotope fractionation in natural systems. We show that the observed heavy enrichment in the soil-grown plants is consistent with Zn solubilization and uptake by a phytosiderophore released from the plant roots, but not with other potential fractionation processes in the soil–root–shoot pathway. We then discuss how the plant growth and modelling results support this conclusion.

Isotope fractionation

The theory of stable isotope fractionation is well established (Criss 1999; Hoefs 2004). Processes under kinetic control produce a light isotopic enrichment in the reaction products or flux sinks because bonds with the light isotope are broken faster. However, in processes at equilibrium, when rates of forward and backward reactions leading to isotope redistribution are equal, the reaction products are enriched in the heavy isotope because of the smaller vibrational energy in bonds with the heavier isotope. A main driver of equilibrium isotopic fractionation in non-traditional stable isotope systems is speciation (Maréchal & Albarède 2002; Zhu *et al.* 2002; Schauble 2004). Heavy fractionation during speciation has been demonstrated for many metals in aqueous solution. For example, Matthews, Zhu & O'Nions (2001) found experimentally that the $Fe(bipy)_3^{2+}$ complex was 10‰ heavier than the free Fe^{2+} ion. Similarly, Black *et al.* (2007) demonstrated using electronic structure calculations that free Mg^{2+} is 3‰ lighter than Mg bound to Chl-a and 2.5‰ lighter than Mg bound to Chl-b.

Consistent with these physicochemical constraints, we attributed the light isotopic bias in Zn uptake by plants in Zn-sufficient solution culture in our earlier study to kinetic fractionation during membrane transport (Weiss *et al.* 2005). Further, we found that the light isotope bias was greater in plants grown in HEDTA solution than in EDTA solution, which we attributed to the greater proportion of complexed Zn in the HEDTA solution (99.9% versus 57% in the EDTA) leading to a greater light enrichment in the free Zn^{2+} taken up. The absence of this light bias in our

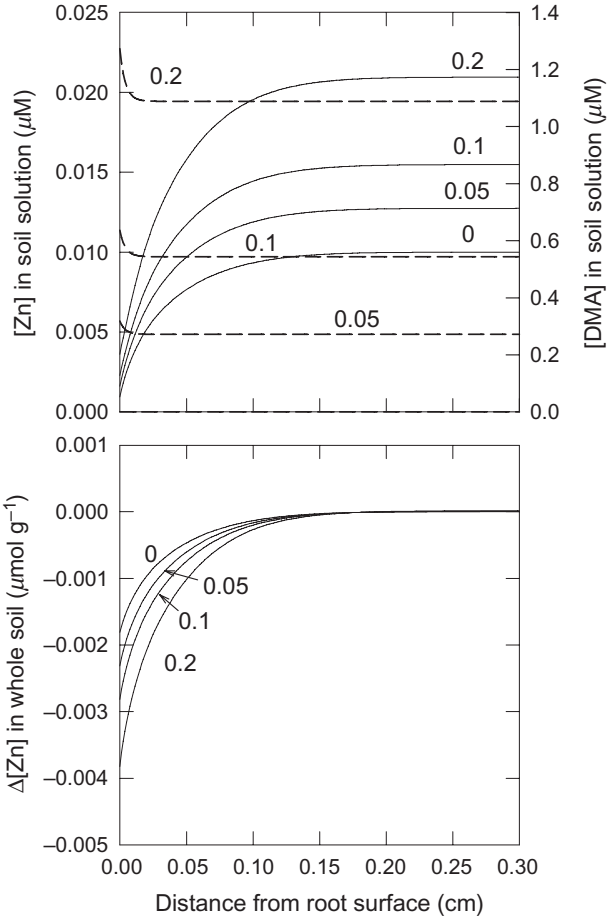

Figure 4. Calculated concentration profiles in the rhizosphere soil. Above: Zn in solution (solid lines) and DMA in solution (broken lines); below: change in Zn in whole soil. Numbers on curves are rates of DMA secretion (pmol cm^{-2} s^{-1} for 4 h per day). Planting density = 1 plant per hill root length density per plant = 2 cm cm^{-3}. Other parameter values as in Fig. 3.

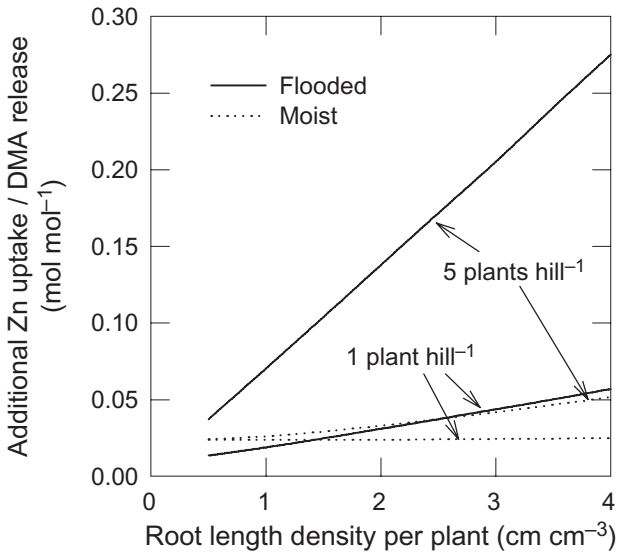

Figure 5. Calculated additional Zn uptake by roots per unit DMA released for two planting densities and soil water statuses. Flooded soil $\theta = 0.7$, $f = 0.5$; moist soil $\theta = 0.2$, $f = 0.1$. All other parameter values as in Fig. 3.

results with soil grown plants, and the significant heavy bias in the Zn-efficient line RIL46, therefore require an alternative explanation. We propose this is the formation and uptake of a phytosiderophore-Zn complex as a result of phytosiderophore secretion from the roots, as suggested by Guelke & von Blanckenburg (2007) to explain heavy Fe enrichment in grasses. This mechanism – i.e. phytosiderophore secretion – is supported by our modelling results, and it is the only mechanism that can account for the heavy isotope bias and the observed differences in fractionation between the genotypes and with soil Zn status, as we will show.

Two processes peculiar to the rice rhizosphere in submerged soils, which might cause fractionation of Zn isotopes, need to be considered. First, oxidation of Fe(II) by O_2 released from roots to overcome low redox conditions in the soil, leading to accumulation of Fe(III) oxides at and near root surfaces; and second, acidification as a result of H^+ generated in Fe(II) oxidation

$$4Fe^{2+} + O_2 + 10H_2O \rightleftharpoons 4Fe(OH)_3 + 8H^+$$

and H^+ release from the root to balance excess intake of cations over anions, the main form of plant-available N in submerged soil being NH_4^+ (Begg *et al.* 1994). These changes affect the solubility of soil Zn (Kirk & Bajita 1995) and may also cause changes in isotope fractionation.

Metal adsorption on mineral surfaces and organic matter at equilibrium results in a heavy isotope fractionation in the adsorbed phase (Pokrovsky, Viers & Freydier 2005; Juillot *et al.* 2008; Jouvin *et al.* 2009). Therefore, Zn adsorbed on Fe oxides in the rhizosphere or on root surfaces should be enriched in the heavy isotope, as we found in the nutrient solution study (Weiss *et al.* 2005). However, this enrichment will not persist into the plants because the adsorbed Zn needs to be re-solubilized before being taken up. By contrast, with solubilization by a phyosiderophore and absorption of the Zn-phyosiderophore complex by the plants, any heavy enrichment in the adsorbed phase would be conserved or reinforced and transferred to the plant. Note, therefore, that the presence of Fe(III) oxides on the roots and adsorption of heavy Zn on them may bias the isotope signature of the roots, but it does not affect the isotope signature of the shoots.

Note also that if free Zn^{2+} ions were taken up following dissociation of the Zn-phytosiderophore complex at the root surface, there would be kinetic isotopic fractionation, favouring overall negative isotope discrimination. This is what we found for uptake of free Zn^{2+} from solution cultures containing complexing agents that were not absorbed by the roots (Weiss *et al.* 2005). The heavy enrichment in the soil-grown plants therefore suggests that the Zn-phytosiderophore complex is absorbed by the roots. It is possible that fractionation in favour of the heavy isotope occurs within the plants as a result of chelation by organic anions. However, we reject this explanation because it is contrary to the negative fractionation in the shoots in nutrient solution culture.

The procedure used to extract Zn from the soil for isotope analyses (extraction in 0.1 M HCl) is a standard measure of Zn available to rice plants (Dobermann & Fairhurst 2000), and we expect it therefore gives a good measure of the isotopic signature of Zn available to the plant. Zinc trapped in very insoluble mineral phases is not released by this method. The question arises: could the plants have access to such mineral phases, so explaining the heavy enrichment in RIL46 under deficient conditions? This is very unlikely. The Zn content per plant of RIL46 under Zn deficient conditions was 1.5 times that of IR74 (Table 2). If the difference in isotopic abundance between the genotypes was solely due to extraction from a separate mineral phase, then this separate phase would need an isotopic composition of δ^{66}Zn ~0.53‰ (by mass balance calculation). Our soil extract had an isotopic composition of only ~0.16‰, and the possibility of such heterogeneity in the soil is slight, especially considering the δ^{66}Zn of the extract was comparable to the likely source rock (volcanic basalt) and more positive than for the bulk soil by ~0.12‰.

Plant growth

The better growth and Zn uptake of genotype RIL46 compared with IR74 in the –Zn soil and the absence of differences between the genotypes in the +Zn soil are consistent with previous findings with genotypes of the same population in this soil (Wissuwa *et al.* 2006). Our third field experiment in the following year confirmed the higher tolerance of RIL46 compared with IR74 and suggested that a root density effect partly explained the observed genotypic differences. Increasing plant density from 1 to 5 plants per hill improved Zn uptake in both genotypes but it took a further

increase to 10 plants per hill to raise to Zn uptake of IR74 to the same level as RIL46.

What could be the nature of this root density effect and the mechanisms behind the genotype differences? Various candidate mechanisms are plausible. More efficient use of Zn within the plant may result in more roots or root surface for a given Zn uptake, resulting in a positive feedback loop with further Zn uptake. This may well explain part of the genotype differences. However, it cannot explain the planting density effect: at greater density, the pool of plant-available Zn in the soil will tend to be depleted faster, tending to decrease uptake per plant, not increase it. The planting density effect is therefore good evidence for some form of root-induced solubilization mechanism and greater recovery of solubilized Zn at greater root density. At least two types of solubilization mechanism might be involved. One – unique to rice in submerged soils – is release of O_2 from the roots, with the soil Zn being solubilized in the accompanying transformations of Fe and acidification (Kirk & Bajita 1995). However, we have shown this is not consistent with our isotope fractionation results. Another is release of phytosiderophores, which is consistent with the isotope fractionation as discussed above.

Phytosiderophore secretion and Zn solubilization

The model results show that rates of secretion of the phytosiderophore DMA of the magnitude reported for rice in nutrient solution (parameter values) are sufficient to account for the measured Zn uptakes by the field-grown genotypes given the other model parameter values, including the interaction with planting density. It is not obvious how rates of phytosiderophore secretion in solution culture relate to those in soils, where root morphology and other variables are very different. We suppose the greater proportion of short fine laterals and, therefore, of root tips in soil-grown rice compared with solution culture (Morita & Yamazaki 1993), would favour greater rates of secretion.

We note that Suzuki *et al.* (2008) found that Fe deficiency induced greater DMA secretion by rice in nutrient solution, but Zn deficiency did not although endogenous DMA in the shoots increased. They concluded that DMA in Zn-deficient rice had a role in the distribution of Zn within the plant rather than in Zn uptake from the soil. However, in their studies Suzuki *et al.* used the genotype Nipponbare from the *japonica* sub-species, whereas the genotypes in our experiments are all *indicas* and may well have different Zn responses.

The model shows that because DMA and Zn complexed with DMA are only weakly sorbed by the soil, they tend to spread away from the excreting root, and recovery of the solubilized Zn by the plant consequently depends on interception by neighbouring roots. There is, therefore, a strong interaction between solubilization and rooting density. If the additional Zn taken up promotes growth of new roots, there will be a positive feedback loop, and so small differences in solubilization can have large effects. This phenomenon is discussed by Wissuwa (2003) for the case of phosphate acquisition.

The reported rates of phytosiderophore secretion by rice are small compared with those of other grasses, but nonetheless apparently they are effective in increasing Zn uptake. We offer the following explanations. First, the plant requirement for Zn in rice and other grasses is 5–10-fold smaller than the requirement for Fe (Dobermann & Fairhurst 2000), which is the main driver of phytosiderophore secretion in other grasses, and Fe deficiency is relatively rare in rice (Introduction). Secondly, the recovery of Zn-DMA by neighbouring roots in submerged soil is several-fold greater than that of Fe in moist soil as a result of greater rates of diffusion (our modelling results). Together, these factors can account for one to two orders of magnitude smaller rates of DMA secretion being effective in rice.

Conceptual model of Zn isotope fractionations in plant-soil systems

In Fig. 6, we give a preliminary conceptual model of the dominant isotope fractionation processes in the transfer of Zn from the soil solid via the soil solution to root surfaces and root cells. The following fractionations are possible (numbered as in the figure):

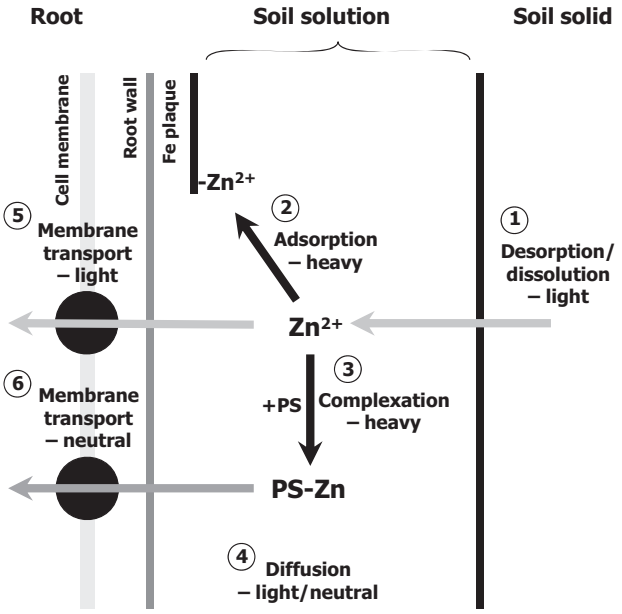

Figure 6. Summary of potential isotopic fractionations during transfer of Zn from the soil solid to absorbing roots. Arrows indicate flows of Zn (unidirectional or at equilibrium). Light, heavy = light, heavy isotope enrichment in reaction product relative to the reacting Zn. PS = phytosiderophore. Numbers refer to points in text: (1) desorption or dissolution from the soil solid; (2) adsorption onto root surfaces or iron oxide plaque; (3) complexation by PS; (4) diffusion though the soil solution; (5) uptake of free Zn^{2+} into roots; (6) uptake of the PS-Zn complex.

1. Desorption or dissolution of Zn from the soil solid tends to produce a light bias in Zn^{2+} in the soil solution compared with the plant-available Zn in the soil solid.
2. Adsorption of Zn^{2+} onto root surfaces or iron oxide plaque tends to produce a heavy bias in the adsorbed Zn and a corresponding light bias in the Zn^{2+} remaining in solution.
3. Complexation of Zn^{2+} by phytosiderophore PS produces a heavy bias in the PS-Zn complex relative to the free Zn^{2+} in solution.
4. Diffusion though the soil solution tends to produce a light bias in the diffusate, but this will be small because of the small distances involved (a few mm), and smaller yet for PS-Zn than free Zn^{2+} because of the smaller mass differences.
5. Uptake of free Zn^{2+} produces a light bias in the roots.
6. Uptake of the PS-Zn complex produces no additional bias, so the heavy bias in the complex will be transferred to the root.

The overall isotopic composition of Zn in the plant will be defined by the relative amounts of free and complexed Zn taken up by the plant. This in turn depends on the soil Zn status and differences between species and genotype. In our experiments, rice genotype differences control the ratio of Zn^{2+} to phytosiderophore-Zn taken up, and we propose that this is the mechanism of Zn-uptake efficiency in rice.

CONCLUSIONS

1. The observed negative isotopic fractionation in Zn-sufficient rice in nutrient solution culture is consistent with kinetic fractionation during uptake and translocation of free Zn^{2+}, whereas the positive fractionation in rice grown in soil can only be explained by secretion of a phytosiderophore from the roots and uptake of a Zn-phytosiderophore complex.
2. The greater positive fractionation in the low-Zn tolerant genotype RIL46 than its intolerant parent IR74 would be explained by greater secretion of the putative phytosiderophore by RIL46 under Zn deficiency.
3. The above mechanism is supported by initial modelling results using available data, and by the observed effect of planting density on Zn uptake by the genotypes.
4. The above conclusions and our conceptual fractionation model clearly warrant further investigation. Nonetheless the potential of isotope fractionation at natural abundance as a tool for studying metal fate and behaviour in plant – soil systems *in vivo* is clear and very promising.

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