TITLE

Zinc isotopic fractionation in *Phragmites australis* in response to toxic levels of zinc.

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Isotopic fractionation in plants under Zn excess.

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ABSTRACT

Stable isotope signature of Zn have shown great promise in elucidating changes in uptake and translocation mechanisms of this metal in plants during environmental changes. Here we tested this potential by investigating the effect of high Zn concentrations on the isotopic fractionation patterns of *Phragmites australis* (Cav.) Trin. ex Steud. Plants were grown for 40 d in a nutritive solution containing 3.2 µM (sufficient) or 2 mM (toxic) Zn. The Zn isotopic composition of roots, rhizomes, shoots and leaves was analysed. Stems and leaves were sampled at different heights to evaluate the effect of long-distance transport on Zn fractionation.

During Zn sufficiency, roots, rhizomes and shoots were isotopically heavy ($\delta^{66}Zn_{JMC\text{-}Lyon}$ = 0.2‰) while the youngest leaves were isotopically light (-0.5 ‰). During Zn excess, roots were still isotopically heavier ($\delta^{66}Zn$ = 0.5 ‰) and the rest of the plant was isotopically light (up to -0.5 ‰). The enrichment of heavy isotopes at the roots was attributed to Zn uptake mediated by transporter proteins under Zn-sufficient conditions and to chelation and compartmentation in Zn excess. The isotopically lighter Zn in shoots and leaves is consistent with long-distance root-to-shoot transport. The tolerance response of *P. australis* increased the range of Zn fractionation within the plant and with respect to the environment.

KEYWORDS

Metals; isotope fractionation; MC-ICP-MS; metallomics; nutrition; *Phragmites australis*; reed.

ABBREVIATIONS

A_s

light-saturated net CO_2 assimilation rate

BCF

bioconcentration factor

C_i

intercellular CO_2 concentration

DR

dead roots, HL, high leaves, HS, high shoot, LL, low leaves, LS, low shoots, LR, living roots, RZ, rhizomes, YL, youngest leaves.

E

transpiration rate

ETR

electron transport rate

F_v/F_m

maximum quantum yield

F_v'/F_m'

relative quantum yield

g_s

stomatal conductance

ICP-AES

Inductively Coupled Plasma Atomic Emission Spectrometer

IRCC

Index of Relative Chlorophyll Content

MC-ICP-MS

MC-ICP-MS	Multicollector Inductively-Coupled Plasma Mass-Spectrometry
qN, NPQ	non-photochemical quenching
qP	photochemical
ΦPSII	quantum yield of PSII photochemistry
ΦCO_2	quantum yield of CO_2 fixation
$\Delta^{66}Zn$	isotopic discrimination with respect to the growth medium
$\delta^{66}Zn$	isotopic signature
$\Delta\delta^{66}Zn_{i-j}$	isotopic fractionation between sections i and j,

INTRODUCTION

Increasing Zn environmental pollution is originated from several anthropogenic sources (Popovic et al., 2001; Konstantinou and Albanis, 2004; Mathur et al., 2005; Pruvot et al., 2006; Kong and White, 2010). Zinc is a micronutrient essential for plants at trace levels, but high concentrations can be toxic (Marschner, 1995). Toxicity symptoms in plants include stunting, chlorosis, induced Fe deficiency, leaf folding, and stem splitting (Rosen et al., 1978; Davis and Parker, 1993).

In spite of the increasing concern about Zn pollution, the mechanisms of Zn uptake, transport and tolerance remain poorly understood. In this scenario, the Multicollector Inductively-Coupled Plasma Mass-Spectrometry (MC-ICP-MS) appears as a valuable tool to explore plant metallomics (von Blanckenburg et al., 2009). Plants discriminate the stable isotopes of a variety of elements, i.e. C, N, O and S, a capacity that has been widely utilized to investigate the physiology and responses of plants to the environment (Monaghan et al., 1999; Yun and Ro, 2008; Cabrera-Bosquet, et al., 2009). The MC-ICP-MS has allowed to extend the research on stable isotopes to heavier elements like Zn, opening a field of new possibilities. The study of the isotopic fractionation of essential elements like Cu, Fe and Zn can make a substantial contribution to developing plant metallomics, by helping to unravel the mechanisms of uptake, distribution and compartmentation of metabolically relevant metals.

Zinc has four stable isotopes, ^{64}Zn, ^{66}Zn, ^{67}Zn and ^{68}Zn. Their average relative abundances in naturally occurring Zn are 48.98 %, 27.81 %, 4.11 % and 18.57 %, respectively (Rosman and Taylor, 1998). Processes at equilibrium, like adsorption to a surface or the formation of covalent bounds, favour the accumulation of the

heavier isotopes in the reaction product, whereas kinetic processes like diffusion-mediated transport discriminate against the heavy isotope (Criss, 1999; Rodushkin et al., 2004). Weiss et al. (2005) performed the first analyses of Zn isotopes in plants, and found that shoots were isotopically lighter with respect to roots, and roots isotopically heavier with respect to solution. They attributed these effects to root-to-shoot passive transport, cell wall binding of heavy Zn or preferential diffusion of light Zn into root cells. Gélabert et al. (2006) reported the enrichment in heavy isotopes of Zn adsorbed to diatoms with respect to solution. John et al. (2007) showed that this was removed by washing the Zn adsorbed onto diatom surface, and that desorbed cells were impoverished in ^{66}Zn. The magnitude of fractionation changed with increasing Zn supply from -0.2 ‰ to -0.8 ‰, corresponding to the switch from high to low-affinity Zn transport into the cell. Viers et al (2007) studied several plant species in a pristine watershed, and found a significant fractionation between species and between plant organs of the same species, which they ascribed to root uptake from soil and translocation within the plants. The leaves of the tallest species had the most negative isotopic signatures, and they hypothesized a correlation between the length of the plants and the extent of Zn fractionation. This was confirmed by Moynier et al. (2009), who described lower $\delta^{66}Zn_{leaves}$ in bamboo than in lentils. Bamboo leaves were also enriched in light isotopes as a function of the distance from the root. Finally, Arnold et al. (2010a) found that rice shoots were isotopically heavier in Zn deficiency, due to Zn uptake mediated by phytosiderophores.

These findings suggest that isotopes can be used: (i) to detect physiological responses to environmental changes (i.e. different amounts of available Zn), and (ii) to identify potential changes in uptake or transport mechanisms. However,

current research on plants is focused on Zn isotopic discrimination under normal or Zn-deficient conditions. We still need to endeavour the use of isotopes to recognise the activation of tolerance mechanisms in response to high levels of Zn, e.g. extrusion, sequestration by metal-binding compounds or subcellular compartmentation. The aim of this study was to demonstrate that the physiological mechanisms of response to toxic levels of Zn are able to discriminate between Zn isotopes.

Phragmites australis (Cav.) Trin. ex Steud. was chosen as model plant because it is tolerant to toxic Zn concentrations, and responds accumulating excess Zn mainly in the roots and restricting its uptake and transport to the shoots (Weis and Weis, 2004). The specific objectives of this research were 1) to test whether the exposure to toxic Zn levels causes any alteration in the Zn fractionation pattern of *P. australis*, 2) to check the hypothesis proposed by Moynier et al. (2009) and Viers et al. (2007) that there is a correlation between the height of leaves and the Zn isotopic fractionation and 3) to examine the usefulness of the technique to study the physiology of Zn toxicity.

MATERIALS AND METHODS

Plant material

Phragmites australis (Cav.) Trin. ex Steud plants were purchased from a local nursery (Bioriza, Breda, Spain). Plants were root-washed in tap water to remove the original peat-vermiculite substrate, weighed, and placed in a pure hydroponics system in individual pots. The nutritive solution comprised: 130.25 mg L^{-1} NO_3^-, 5.5 mg L^{-1} NH_4^+, 28.5 mg L^{-1} PO_4^{2-}, 35.5 mg L^{-1} K^+, 24.5 mg L^{-1} Ca^{2+}, 4 mg L^{-1} Mg^{2+}, 14.25 mg L^{-1} SO_4^{2-}, 0.325 mg L^{-1} Fe, 0.240 mg L^{-1} Mn, 0.09 mg L^{-1} Zn,

0.030 mg L^{-1} B, 0.090 mg L^{-1} Cu, 0.028 mg L^{-1} Mo, and 0.005 mg L^{-1} Co. The pH was adjusted to 6.5. Plants were allowed to acclimate to hydroponics for 27 d, until they recovered a vigorous growth, and then selected within a small range of fresh weight of 161.2 ± 5.0g (FW ± SE; n = 16). There were two Zn treatments: Control (3.2 μM Zn), where plants were grown in the same nutritive solution as during acclimation, and Zn+ (2 mM Zn), where the nutritive solution was amended with $ZnSO_4 \cdot 7H_2O$ (Sigma-Aldrich, 99% ACS reagent) to reach the desired concentration. Eight plants per treatment were randomly distributed and grown under glasshouse conditions for 40 d (29^{th} April to 13^{th} July 2009). Previous research proved that this time span allows for enough Zn accumulation and fractionation (Weiss et al., 2005). The temperature was 23.1 ± 0.3 ºC (mean ± SE), the relative humidity 53.6 ± 1.3 %, and the transmission of the greenhouse covers 51 %. Nutritive solution was renewed every 3 to 4 d and deionised water was added daily to compensate the loss due to evaporation and transpiration.

Plants were then thoroughly washed in tap water, bathed 30 min in ice-cold 1 mM $LaCl_3$ and 0.05 mM $CaCl_2$ to remove adsorbed and apoplastically-bound Zn (following Weiss et al. 2005), and rinsed in deionised water. The isotopic composition of absorbed Zn depends on the physicochemical characteristics of the solution and the adsorbent surface rather that on biologically regulated processes (Gélabert et al., 2006), and will not be considered in this study. The isotopic fractionation of Zn adsorbed on iron oxides or onto biological surfaces leads to the enrichment of the heavy isotopes (Pokrovsky et al., 2005; Gélabert et al., 2006; John et al., 2007). This approach was selected to allow for the comparison between studies, even with species that show no metal plaques.

Plant height was recorded and eight samples were collected from each plant: living roots (LR), dead roots (DR), rhizomes (RZ), low shoots (LS), low leaves (LL), high shoot (HS), high leaves (HL) and youngest leaves (YL). Stems were collected at distances from the root: between 5 to 12 cm for the low shoots and between 20 to 27 cm for the high shoots. Leaves growing at these two different height intervals were named low and high leaves, respectively. The three last leaves of each stem were labelled as youngest leaves. Each two plants were pooled together. Fresh samples were oven-dried at 60 ºC until constant weight, and ground with a ball mill.

Photosynthetic performance

The chlorophyll content and fluorescence and the gas exchange of leaves was measured 1 to 2 d before the end of the experiment. Chlorophyll content on leaf area basis was obtained using a portable chlorophyll meter (SPAD-502 Minolta, Illinois, USA), following Krugh et al. (1994). This device provides an indexed relative chlorophyll content (IRCC) ranging from 0 to 99.9. Always the third fully-developed leaves at 2.5 cm of the leaf base were measured on five representative pre-bloom leaves per plant.

Photosynthetic gas exchange and chlorophyll fluorescence were determined in the third last fully expanded leaf of each plant using a LI-COR 6400 Portable Photosynthesis System (LI-COR Inc., Lincoln, NE, USA), with a saturating light (photosynthetic photon flux density of 1200 μmol photons $m^{-2}s^{-1}$), 400 μmol mol^{-1} of CO_2, and air temperature of 25.9 ± 0.1°C. Leaves were previously dark-adapted for 30 min to measure maximum quantum yield (F_v/F_m). The same leaves were then re-acclimated to environmental light to determine relative quantum yield

(F_v'/F_m'), quantum yield of PSII photochemistry (ΦPSII) (Gentyet al., 1989), quantum yield of CO_2 fixation (ΦCO_2), electron transport rate (ETR, μmol $m^{-2}s^{-1}$), photochemical (qP) and non-photochemical quenching (qN, NPQ), light-saturated net CO_2 assimilation rate (A_s, μmol CO_2 $m^{-2}s^{-1}$), stomatal conductance to water (g_s, mol H_2O $m^{-2}s^{-1}$), intercellular CO_2 concentration (C_i, μmol CO_2 mol air^{-1}), and transpiration rate (E, mmol H_2O $m^{-2}s^{-1}$).

Zinc content

Plant samples were digested in two steps, first overnight at 90 ºC in HNO_3:H_2O_2 (1:1 v/v), then added 0.5 mL HF and digested 2 h at 90 ºC. Digests were evaporated to dryness on a hotplate at 120 ˚C and the residues were re-dissolved in 3 ml of 7 M HCl. Each solution was split into three aliquots: 1 ml for Zn concentration measurements, 1 ml for Zn isotope analysis and 1 ml for archive. The first aliquot was made up to 3.5 ml 1 M HCl prior to concentration measurements on a Varian VISTA PRO (Palo Alto, USA) ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometer), for which analytical errors were 0.4 to 5 % of the measured values. Per each 12 samples, a blank and a sample of either olive leaves (*Olea europaea* L., BCR-62), aquatic plant (*Lagarosiphon major* [Ridl.] Moss, BCR-60), light sandy soil (BCR-142R), or lichen (BCR-482) certified reference material from the Community Bureau of Reference (BCR®) were processed in the same way and analysed (Table 1). Digestions were carried out in the clean laboratory of the Department of Earth Science and Engineering (Imperial College of London). Zinc content determination was performed in the research facilities of the Natural History Museum (London, UK).

The bioconcentration factor for Zn (BCF) was calculated following Ali et al. (2004):

$$BCF = \frac{[Zn_p]}{[Zn_s]}$$

where $[Zn_p]$ is the Zn concentration of the plant sample (µg g^{-1} DW), and $[Zn_s]$ is the Zn concentration of the nutritive solution (µg ml^{-1}).

Zinc isotopic signature

Zinc isotopes were analysed on the second aliquot. An isotope spike enriched in ^{64}Zn, ^{66}Zn and ^{67}Zn was added to the sample aliquot to achieve a total of 1000 ng Zn and a spike:sample mass ratio of 1 (Arnold et al., 2010b). Zinc was separated from the matrix using anion exchange chromatography as detailed in Arnold el al. (2010a). Zinc fractions were re-dissolved in 0.1 M HNO_3 for the subsequent isotope ratio analysis using a HR Nu Plasma MC-ICP-MS (Multi-Collector Inductively Coupled Plasma Mass Spectrometer, Nu Instruments, Wrexham, UK). Isotope ratios are reported in δ-notation:

$$\delta^{66}Zn_{JMCLyon} = \left[\frac{\left(\frac{^{66}Zn}{^{64}Zn} \right)_{sample}}{\left(\frac{^{66}Zn}{^{64}Zn} \right)_{JMCLyon}} - 1 \right] \times 10^3$$

where $(^{66}Zn/^{64}Zn)_{sample}$ is the isotope ratio of the sample and $(^{66}Zn/^{64}Zn)_{JMC\ Lyon}$ is the isotope ratio of the standard reference solution used, i.e. JMC 3-0749L. Accuracy of the isotope measurements was assessed by the analysis of two in-house single element solutions (Romil Zn, London Zn) and two natural standard reference materials (Ryegrass BCR-281 and Blend ore BCR-027). As

shown in Table 2, data from this study agree within error with previously published values for the in-house standards, the rye grass BCR-281 and for the blend ore BCR-027 (Mason et al., 2004; Chapman et al., 2006; Peel et al., 2008; Arnold 2009).

Precision of the isotope measurements was estimated from replicate analysis of the BCR-281 standard (see Table 2). The typical error (expressed as 2σ standard deviation) was ± 0.12 ‰. Procedural blank contributions were around 4 ng of Zn. All mineral acids were sub-boiled in a quartz still and diluted using 18 MΩ grade Millipore system (Bedford, MA, USA).

To further assess the effect of the treatment on the distribution of isotopes across plant sections, the fractionation between sections was calculated following Moynier et al. (2009) as:

$$\Delta\delta^{66}Zn_{i\text{-}j} = \delta^{66}Zn_i - \delta^{66}Zn_j$$

Where $\Delta\delta^{66}Zn_{i\text{-}j}$ is the fractionation between sections i and j, and $\delta^{66}Zn_i$ and $\delta^{66}Zn_j$ are the isotopic signature of section i and j respectively. The discrimination with respect to the growth medium was calculated according to the equation (Farquhar, 1989):

$$\Delta^{66}Zn = \frac{\left(\delta^{66}Zn_{so} - \delta^{66}Zn_p\right)}{\left(1 + \frac{\delta^{66}Zn_p}{10^3}\right)}$$

Where $\delta^{66}Zn_{so}$ is the isotopic signature of the source, in this case the nutritive solution, and $\delta^{66}Zn_p$ is the isotopic signature of the plant sample.

Statistical Methods

Two-way ANOVA was carried out to evaluate the effect of plant section, Zn treatment and their interaction with Zn concentration, and $\delta^{66}Zn$. Logarithmic transformation was performed when data did not meet the assumption of equal variances. To determine which groups were significantly different from each other we selected the post-hoc test that best separated the groups, either Student-Neuman-Keules or Duncan. Student's t-test was chosen for mean comparisons between treatments for the photosynthetic parameters (A_s, g_s, C_i, F_v/F_m, F_v'/F_m', ΦPSII, ΦCO_2, ETR, qP, qN, NPQ, E) and Zn isotopic fractionation between sections ($\Delta \delta^{66}Zn_{i-j}$). Pearson's correlation was employed to test whether there was a linear relationship between $\delta^{66}Zn$ and photosynthetic performance. Statistical analyses were done with the software SPSS (Statistical Package for the Social Sciences) 2005 v14.0 for Windows. Sigma Plot software 2006 (v10.0) was used for graphic edition.

RESULTS

Photosynthetic performance and growth

There was a substantial reduction of plant height and chlorophyll content due to Zn exposure (Table 3). The A_s, g_s and E decreased a 50 % in Zn+ plants while no changes in C_i occurred. In the dark-adapted leaves of both treatments F_v/F_m remained stable. In contrast, F_v'/F_m', ΦPSII, ΦCO_2, qP and ETR showed a clear decrease in Zn+ light-adapted leaves. This was accompanied by an increase of qN and NPQ.

Zn content

The Zn content of all plant sections increased with increasing Zn supply (Table 4). The Zn concentration of plant samples was higher than that of the growth solution, but the BCF was much reduced in Zn+ plants (Table 4). Plants grown at different Zn concentrations differed in the distribution pattern of Zn (and consequently BCF). In controls, living roots achieved the highest Zn levels, whereas dead roots had the lowest. Oppositely, in Zn+ plants dead and living roots achieved the highest levels, whereas leaves, shoots and rhizomes contained little Zn in comparison. All Zn+ emerged sections had very similar Zn concentration except high and youngest leaves, where it was lower.

Zinc isotopes

The $\delta^{66}Zn$ varied between plant sections (Fig. 1). In the control experiment, only the youngest leaves were significantly different from the rest of plant sections, showing a lighter isotopic signature. The shoots were slightly heavier than the leaves. The Zn+ treatment altered the fractionation pattern (Fig. 1). Shoots of Zn+ plants were lighter than the leaves, whereas the root samples were heavier than the shoots and the youngest leaves. However, only the rhizomes and the shoots were significantly different among treatments, and much isotopically lighter in Zn+ plants. The isotope signature of youngest leaves was similar in both treatments, although the shoots of Zn+ plants were shorter. There was no significant difference detectable between low and high shoot or between low and high leaves in any of the treatments either.

The $\Delta\delta^{66}Zn_{i\text{-}j}$ was calculated between adjacent sections and between the roots and the youngest leaves or the high shoots (Table 5). The $\Delta\delta^{66}Zn_{leaves\text{-}shoots}$ as well as $\Delta\delta^{66}Zn_{RZ\text{-}LR}$, and $\Delta\delta^{66}Zn_{HS\text{-}LR}$ were significantly affected by Zn+ treatment. In

345 contrast, high Zn did not modify the $\Delta\delta^{66}Zn$ between stem or leaf samples
346 collected at different heights. Also there was no influence of the treatments on the
347 fractionation between the roots and the youngest leaves. In agreement with the
348 previous results, the controls discriminated against the light isotope except in
349 youngest leaves, whereas the Zn+ discriminated in favour of the lighter isotope
350 except in roots (Fig. 2). Both treatments differed significantly in the $\Delta^{66}Zn$ of
351 rhizomes, shoots and leaves. Plants caused the enrichment in heavy isotopes of the
352 nutritive solution, which was more evident in high Zn solutions.
353
354 **Correlation between $\delta^{66}Zn$ and plant height or photosynthetic parameters**
355 Plant height showed a strong and positive linear correlation with $\delta^{66}Zn$ of high
356 shoot ($r = 0.972$, Sig. = 0.001) and low shoot ($r = 0.929$, Sig. = 0.007), and a
357 weaker one with rhizome ($r = 0.813$, Sig. = 0.049). The correlations of plant
358 height with the rest of plant sections were not significant. The gas exchange and
359 chlorophyll fluorescence were correlated with $\delta^{66}Zn$ of high leaves where
360 measurements were performed, belonging to control and Zn+ plants. The
361 relationships found significant are shown in Table 6. The results are consistent
362 with the effect of the treatments on the photosynthetic performance parameters
363 seen above. High values of $\delta^{66}Zn$ in high leaves (as shown by controls) were
364 associated with a higher g_s, ΦPSII, ΦCO_2, and with a lower NPQ.
365 Finally, the concentration of Zn showed a negative linear correlation with $\delta^{66}Zn$,
366 which was strong in the sections low shoots ($r = -0.964$, Sig. = 0.002) and high
367 shoots ($r = -0.971$, Sig. = 0.001), and weaker in high leaves ($r = -0.828$, Sig. =
368 0.042).
369

DISCUSSION

Photosynthetic performance and growth

The results evidenced a clear toxic effect of Zn+ treatment on *P. australis*: growth, photosynthesis and gas exchange were impaired. Thus the Zn+ fractionation data are representative of Zn-stressed plants. Chlorophyll fluorescence and gas exchange data are examined to discuss the possible causes of the A_s decrement. In Zn+ plants, g_s and E decreased a 50 % indicating a strong inhibition of stomata aperture. A limited gas exchange can affect A_s by restricting the uptake of both C from the atmosphere and nutrients from the growth solution. In the present experiment, Zn+ plants did not show a reduction of C_i. Hence CO_2 availability was not the limiting factor for A_s in Zn+ plants, because the C demand for assimilation was lower. Nevertheless, chlorophyll fluorescence was unchanged in dark-adapted leaves, where F_v/F_m remained stable, showing that PSII was functional. Only when leaves were transferred to the light, F_v'/F_m', ΦPSII and ΦCO_2 showed a clear decrease in Zn+ plants. This was accompanied by a reduction on qP and ETR, and an increase in qN and NPQ. All these data put together suggest that whereas PSII remained mainly unaffected, Zn impaired the efficiency of electron transport downstream, causing PSII to become easily saturated by light. This explains the slow C assimilation and the decrease of ΦCO_2. Therefore, the present data suggest that the inhibition of transpiration was not the direct cause of the reduced C fixation. Zinc has been reported to inhibit or damage almost every point of the photosynthetic apparatus, i.e. chlorophyll synthesis, PSII, oxygen evolving complex, plastoquinone pool, PSI and Rubisco (Prasad, 2004). Many of these effects could cause the observed decreased photosynthetic performance. In addition, stomatal closure could reduce the

nutrient uptake. The deficiency of N can lead to an indirect impairment of photosynthetic apparatus, and limit A_s. This is consistent with the decay of total chlorophyll content indicated by decreased IRCC figures. Decreased N content has also been associated in literature with the inhibition of Rubisco and the dark phase of photosynthesis (Ciompi et al., 1996).

The strong positive correlation of $\delta^{66}Zn_{HL}$ with and g_s, ΦPSII, ΦCO_2, and negative correlation with NPQ indicates that $\delta^{66}Zn_{HL}$ could be an interesting parameter to assess the inhibition of photosynthesis due to the toxic effect of excess Zn. Nevertheless, results must be taken with caution due to the low number of replicates.

Zn content

P. australis has innate tolerance to Zn and other metals (Ye et al. 1997). The lower BCF, the accumulation of Zn in roots, and the limitation to Zn export to the green tissues compose an avoidance response that confers increased tolerance to Zn excess (Denny and Wilkins, 1987; Maestri et al., 2010). The higher BCF of dead roots in Zn+ is consistent with the use of root senescence to release Zn, an excretion mechanism of tolerant plants (Duarte et al. 2010). The Zn levels achieved by leaves and shoots are far of reaching the 1 % Zn in leaf dry matter generally accepted as the threshold to reach Zn hyperaccumulation (Verbruggen et al., 2009). The Zn concentrations of the different tissues (12-14 mg g^{-1} dw in roots, 2-3 in shoots, and 0.5-3 in leaves) were comparable to those of Jiang and Wang (2008) study (14 mg g^{-1} dw in roots, 0.95 shoots, 1.5 leaves), who used the same species and Zn supply. The small discrepancies in the Zn content of shoots

and leaves can be easily explained, as we sampled at specific heights instead of taking samples representative of the whole stem.

Zinc isotopes

Mechanisms explaining the isotopic fractionation pattern under normal Zn supply

Under Zn sufficient conditions, all the plant tissues except the youngest leaves are enriched in the heavier isotopes compared to the nutrient solution. The δ^{66}Zn of the youngest leaves is isotopically lighter than the rest of sections. Mature leaves are slightly lighter isotopically than roots and shoots. These observations are in line with field observations of Viers and co-workers (2007). They found that only *Megaphrynium macrostachyum* (Benth.) Milne-Redh among the four species analysed showed a significant fractionation between root and shoot. The most negative δ^{66}Zn values measured along the plant were found in leaves (Viers et al., 2007; Moynier et al., 2009). By contrast, different degrees of root-to-shoot fractionation were described in crops like tomato, lettuce and rice under different experimental conditions (Weiss et al., 2005; Arnold et al., 2010a). This suggests that the mechanisms of Zn uptake and transport are highly species-specific and conditioned by the physiological status of the plant. Here, we propose that the isotopic distribution of controls comes from the combination of two processes: i) the enrichment in heavy isotopes generated by Zn uptake in roots and ii) the enrichment in light isotopes during the long-distance transport of free Zn ions in the plant.

The observed pattern is consistent with the uptake of Zn by root cells facilitated by transmembrane transporters, as previously suggested by Weiss et al. (2005). Various members of the ZIP family of proteins (Zinc-Iron Permeases) are located

444 on the plasmatic membrane and facilitate Zn uptake (Grotz and Guerinot, 2006).
445 Alternatively, the chelation of Zn by ligands and its subsequent transport in the
446 complexed form could cause the observed enrichment in the heavier isotopes. We
447 refuse this explanation because Zn is mostly taken up and transported as Zn^{2+}
448 (Marschner, 1995). Other mechanisms favouring the heavy isotopes are in
449 disagreement with the observations reported here, like adsorption onto root
450 surface, binding to the cell walls, and compartmentation in cell organelles. All of
451 them imply the retention of the heavier isotopes in the roots, preventing its
452 transport to other reservoirs. The $\delta^{66}Zn_{root}$ would be more positive than the rest of
453 the plant, in discrepancy with the present results. The protocol used to remove the
454 root-adsorbed and apoplastically-bound Zn was thus apparently efficient. The
455 obtained data ($\delta^{66}Zn_{root}$ = 0.18 ‰) are in line with previous experiments (Weiss et
456 al., 2005), where a similar root-desorption protocol was used for tomato, rice and
457 lettuce ($\delta^{66}Zn_{root}$ = 0.15, 0.15 and 0.2 ‰, respectively).
458 The youngest leaves of controls were more negative than the rest of the plant. The
459 transport of Zn^{2+} along the shoot has been suggested as the cause for the
460 enrichment in light isotopes of shoots and leaves with height (Moynier et al.,
461 2009), in agreement with the present results. There was a positive correlation
462 between plant height and $\delta^{66}Zn$ of transporting tissues, as previously suggested by
463 Viers et al. (2007). The correlation was stronger as the samples were higher (high
464 shoots>low shoots>rhizomes). The fractionation between low and high leaves was
465 not statistically significant in this experiment ($\Delta\delta^{66}Zn_{HL-LL}$ = -0.090 ‰). However,
466 the results are consistent with the small distance that separates the samples. The
467 fractionation per distance was of –0.005 ‰ cm^{-1}, very similar to –0.006 ‰ cm^{-1}
468 calculated from Moynier et al. (2009) in bamboo. In the same direction, the leaves

of controls were slightly lighter than the shoots at the same height, probably due to the translocation of Zn from the shoot along the leaves. Thus, the present data are consistent with an enrichment of lighter isotopes with distance from the root, but this can only be assessed if there is enough separation between samples.

The isotopic fractionation pattern reflects the tolerance response to high Zn concentrations

The protective mechanisms activated by plants under high-Zn stress disrupt the Zn uptake, accumulation, distribution and transport routes, which translated into a completely different fractionation pattern in this experiment.

There is little information about the regulation of ZIP transporters under excess Zn in plants. However, experiments in yeast demonstrated that ZRT1 is inactivated by high Zn supply (Gitan et al., 1998), limiting Zn influx into the cell. The activity of the transporters is probably inhibited in Zn+ plants, as shown by the decreased BCF. Thus, we consider that Zn uptake mediated by transporters is not the cause for the enrichment in heavy isotopes of Zn+ roots.

Excess Zn is mainly accumulated in roots and localised in cell walls, intercellular spaces and vacuoles (Heumann, 2002; Li et al., 2006; Jian and Wang, 2008). In the present experiment, δ^{66}Zn was less negative in roots than in the rest of the organs, and Zn translocation from root to shoot was lower in Zn+ plants. This indicates that heavy Zn is effectively retained in roots, and the isotopically lighter sap is transported to the above-ground tissues. The youngest leaves of Zn+ plants have a δ^{66}Zn similar to controls, even if their shoots were shorter and Zn was transported a smaller distance (106.0 ± 3.5 cm for controls, 78.6 ± 3.1 for Zn+,

493 means ± SE, n = 4). In opinion of the authors, this is because the xylem sap of
494 Zn+ plants had from the root a more negative $\delta^{66}Zn$ than that of controls.
495 When examined in detail, all the known mechanisms for Zn sequestration in roots
496 are likely to select the heavy isotopes. Zinc probably forms covalent bounds with
497 carboxyl and hydroxyl groups of pectin and with hydroxyl groups of cellulose in
498 the cell walls (Straczek et al., 2008), and precipitates with insoluble phosphates or
499 silicon in the apoplast (Neumann and zur Nieden, 2001; Straczek et al., 2008). In
500 the cell, Zn binds to various ligands and is stored in subcellular organelles to keep
501 Zn^{2+} low in the cytosol. Zinc is transferred into the vacuoles by metal tolerance
502 proteins (MTP) localised to the tonoplast (Blaudez et al., 2003; Dräger et al.,
503 2004; Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006; Gustin et al.,
504 2009). The tonoplast transporter AtZIF1 is also involved in Zn sequestration,
505 probably by transporting either organic Zn ligands or Zn-ligand complexes into
506 the vacuole (Haydon and Cobbett, 2007b). Different authors expect Zn to be
507 chelated in the vacuoles by various ligands like organic acids (OA), proteins, and
508 phytate (Van Steveninck et al., 1987; Salt et al., 1999; Tennstedt et al., 2009). The
509 best candidates for Zn ligands in the vacuole are OA like citrate and malate, which
510 are the most abundant metal ligands in plants and accumulate mainly in the
511 vacuoles, the same as excess metals. In agreement, the optimal stability of
512 OA-metal complexes is achieved at vacuolar pH (Haydon and Cobett, 2007a).
513 Besides, metal-binding peptides and proteins have been described to chelate Zn.
514 Phytochelatins are glutathione oligomers synthesised in response to metals, that
515 chelate and detoxify Cd and As (Jabeen et al., 2009). Recent advances established
516 that Zn promotes the synthesis of PCs, which are essential for Zn detoxification
517 and contribute to Zn accumulation (Tennstedt et al., 2009). Cadmium complexed

518 with PCs is pumped and sequestered into the vacuole (Salt et al., 1995; Cobbett
519 and Goldsbrough, 2002). It is probable that PC-Zn complexes follow the same
520 route, but direct evidence is missing. Metallothioneins are cysteine-rich low
521 molecular weight proteins found in plants, animals and fungi, and able to chelate
522 Zn and many other metals. They are involved in Zn homeostasis and/or tolerance,
523 but their exact function is yet unknown (Rodríguez-Llorente et al., 2010). Finally,
524 phytate is a P storage molecule that can bind to Zn as a mechanism for Zn storage
525 or immobilisation. Phytate-Zn complexes are found in roots (Van Steveninck et
526 al., 1987 and 1993; Terzano et al., 2008), and in seeds (Otegui et al., 2002;
527 Rodrigues-Filho et al., 2005), either compartmented in the vacuoles or forming
528 insoluble precipitates.
529 All three processes, Zn binding to cell walls, precipitation in intercellular spaces
530 and sequestration in the vacuole are mass-dependent and thus expected to favour
531 the heavy isotope. It is difficult from the present design to tell which process was
532 chiefly responsible of the enrichment in heavy isotopes of Zn+ roots. The
533 youngest leaves of Zn+ were more negative than the rest of leaves. Similarly to
534 controls, the fractionation between low and high leaves was not statistically
535 significant in this experiment ($\Delta\delta^{66}Zn_{HL\text{-}LL}$ = -0.011 ‰). The calculated
536 $\Delta\delta^{66}Zn_{HL\text{-}LL}$ obtained from the linear regression of Zn+ leaves (Fig. 3) is of
537 -0.090‰, very different from the observed but similar to that of controls. This
538 evidences the restriction of long-distance transport under toxic Zn levels. Both
539 linear regressions for controls and Zn+ have a very similar slope, but Zn+ plot is
540 biased to the negative side. The youngest leaves of Zn+ have a $\delta^{66}Zn$ similar to
541 controls, in spite of plants being shorter. The correlation between plant height and

the intensity of Zn fractionation in leaves proposed by Viers et al. (2007) can thus be modified by Zn status.

The enrichment in heavy isotopes of the nutritive solutions with time is coherent with plants taking up preferably Zn by bulk flow, favouring the light isotopes, and with the higher biomass of above-ground tissues in this species (Ye et al., 1997). Also the Zn+ solution was more enriched in heavy isotopes than the control solution, as expected from the discrimination pattern observed for each treatment.

CONCLUSIONS

We have proved that the study of Zn isotopes has a great potential to investigate the mechanisms of tolerance to Zn excess in plants. We have demonstrated that *P. australis* is able to discriminate Zn isotopes, and that the magnitude and sign of the resultant fractionation depends on Zn-status and organ. We have shown that under Zn sufficient levels, roots and shoots are enriched in the heavier Zn isotopes as compared to the source ($\delta^{66}Zn$ = 0.2 ‰) and the youngest leaves are impoverished (-0.5 ‰), whilst under Zn excess roots are enriched in the heavy isotopes (0.5 ‰) and the rest of the plant is isotopically lighter (up to -0.5 ‰). We have exposed that Zn uptake by plants causes the enrichment in heavy isotopes of the nutritive solutions, which was stronger in Zn+ treatment ($\Delta\delta^{66}Zn_{control}$ = 0.3 ‰, $\Delta\delta^{66}Zn_{Zn+}$ = 0.6 ‰). In conclusion, the tolerance response of *P. australis* increased the range of Zn fractionation within the plant and with respect to the environment.

An outline of the fractionation mechanisms compatible with the observed response was also provided. The enrichment in heavy isotopes of the roots was attributed to Zn uptake under Zn-sufficient conditions and to chelation and

compartmentation in Zn excess. The enrichment in light isotopes of shoots and leaves is consistent with long-distance root-to-shoot transport, in accord with the observations by Viers et al., (2007), and Moynier et al., (2009). Further research needs to be conducted to confirm these hypotheses and establish what molecules or processes are responsible for the described pattern.

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Table 1. Zinc content of the standards used in ICP-AES analyses. Data are represented as means ± SE.

		Zn content (µg g^{-1})		
Reference	**Sample type**	**Certified**	**Measured**	**% Recovery**
BCR-142R	Light sandy soil	93 ± 3	91 ± 14	98
BCR-482	Lichen	101 ± 2	91 ± 9	91
BCR-60	*Lagarosiphon major* (Ridl.) Moss	313 ± 8	309 ± 13	98
BCR-62	*Olea europaea* L.	16.0 ± 0.7	13 ± 3	82

Table 2. Isotopic signature of the standards used in this study. Samples were double-spiked and analysed by MC-ICP-MS (see "Materials and Methods"), using the standard reference solution JMC 3-0749L. Data are compared with literature, $\delta^{66}Zn$ expressed in ‰ and displayed as means ± 2SD.

Reference material	Publication	$\delta^{66}Zn_{JMC\ Lyon}$	n
BCR-027 (Blend Ore)	Chapman et al. (2006)	0.33 ± 0.07	8
	Arnold (2009)	0.23 ± 0.06	4
	This study	0.34 ± 0.08	9
BCR-281 (Rye Grass)	Arnold (2009)	0.38 ± 0.09	7
	This study	0.5 ± 0.1	5
Romil	Mason et al. (2004)	-9.01 ± 0.08	6
	Weiss et al. (2007)	-8.98 ± 0.07	unknown
	Arnold (2009)	-9.0 ± 0.1	unknown
	This study	-9.1 ± 0.1	12
London	Arnold (2009)	0.08 ± 0.04	10
	This study	0.10 ± 0.06	9

Table 3. Effect of Zn levels on plant growth and photosynthetic traits of *Phragmites australis*. Plants were grown in 3.2 μM (Controls) or 2 mM Zn (Zn+). Data represent means ± SE, where n = 8 for plant height and IRCC (df = 14), and n = 6 for the rest of parameters (df = 10). The variable g_s was log-transformed. T-test value (t) is indicated as significant at P<0.05 (*) or P<0.01 (**)†.

Parameter	Control	Zn+	t
Plant height (cm)	106 ± 4	79 ± 3	5.8**
IRCC	38.0 ± 1.2	32.6 ± 1.1	4.0**
A_s (μmol CO_2 $m^{-2}s^{-1}$)	14 ± 3	7.0 ± 1.3	2.7*
g_s (mol H_2O $m^{-2}s^{-1}$)	0.18 ± 0.05	0.08 ± 0.01	2.4*
C_i (μmol CO_2 mol air^{-1})	246 ± 7	246 ± 11	0.0
F_v/F_m	0.80 ± 0.01	0.79 ± 0.01	0.8
F_v'/F_m'	0.46 ± 0.02	0.37 ± 0.01	4.2**
ΦPSII	0.24 ± 0.02	0.15 ± 0.02	3.7**
ΦCO_2	0.014 ± 0.002	0.008 ± 0.001	2.8*
qP	0.52 ± 0.02	0.39 ± 0.04	2.6*
qN	0.81 ± 0.02	0.88 ± 0.01	-4.1**
NPQ	2043 ± 127	2652 ± 141	-3.1*
ETR (μmol $m^{-2}s^{-1}$)	122 ± 9	75 ± 9	3.7**
E (mmol H_2O $m^{-2}s^{-1}$)	3.9 ± 0.8	1.9 ± 0.3	2.6*

† SE: standard error, df: degrees of freedom, IRCC: index of relative chlorophyll content, A_s: light-saturated net CO_2 assimilation rate, g_s: stomatal conductance to water, C_i: intercellular CO_2 concentration, F_v/F_m: maximum quantum yield, Fv'/Fm': relative quantum yield, ΦPSII: quantum yield of PSII photochemistry, ΦCO_2: quantum yield of CO_2 fixation, qP: photochemical quenching, qN and

NPQ: non-photochemical quenching, ETR: electron transport rate, E: transpiration rate.

Table 4. Concentration of Zn achieved in different plant sections. Plants were grown in 3.2 μM (Controls) or 2 mM Zn (Zn+). Data represent means ± SE[†] (n = 4). The effect of plant section, Zn treatment and their interaction was significant (P<0.001) according to two-way ANOVA (results not shown). Different letters indicate different groups according to Duncan post-hoc test on the log-transformed variables.

Plant section		Zn content (mg g^{-1})		BCF	
		Controls	Zn+	Controls	Zn+
Roots					
	Living	0.09 ± 0.04^{d}	12 ± 6^{h}	960 ± 167^{l}	93 ± 19^{p}
	Dead	0.02 ± 0.01^{a}	14 ± 7^{h}	268 ± 27^{i}	105 ± 27^{n}
Rhizomes		0.02 ± 0.01^{a}	2.7 ± 1.4^{g}	274 ± 46^{i}	21 ± 5^{m}
Shoots					
	Low	0.04 ± 0.02^{b}	3 ± 2^{g}	433 ± 46^{j}	25 ± 3^{o}
	High	0.06 ± 0.03^{c}	2.3 ± 1.1^{g}	640 ± 40^{k}	17.2 ± 1.3^{p}
Leaves					
	Low	0.05 ± 0.03^{bc}	4 ± 2^{g}	583 ± 79^{jk}	27 ± 5^{o}
	High	0.04 ± 0.02^{bc}	1.1 ± 0.6^{f}	490 ± 48^{jk}	8.7 ± 0.4^{o}
	Youngest	0.04 ± 0.02^{b}	0.5 ± 0.2^{e}	389 ± 8^{j}	3.7 ± 0.2^{o}

[†] SE: standard error, BCF: bioconcentration factor

Table 5. Fractionation between plant sections. Fractionation was calculated as $\Delta\delta^{66}Zn_{i-j} = \delta^{66}Zn_j - \delta^{66}Zn_i$. Data represent means ± SE† (n = 3). T-test value (t) is indicated as significant at P<0.05 (*) or P<0.01 (**).

	$\Delta\delta^{66}Zn_{i-j}$		
Sample	**Control**	**Zn+**	**t(df)**
DR-LR	0.02 ± 0.03	-0. 5 ± 0.2	1.91(2.1)
RZ-DR	0.03 ± 0.01	-0.3 ± 0.2	2.03(4)
LS-RZ	0.05 ± 0.04	-0.2 ± 0.1	1.40(4)
LL-LS	-0.2 ± 0.1	0.19 ± 0.07	-3.15(4)*
HS-LL	0.2 ± 0.1	-0.30 ± 0.01	3.50(4)*
HL-HS	-0.25 ± 0.05	0.29 ± 0.08	-5.68(4)**
YL-HL	-0.41 ± 0.05	-0.28 ± 0.03	-2.27(4)
RZ-LR	0.05 ± 0.04	-0.7 ± 0.3	3.11(4)*
HS-LS	-0.08 ± 0.06	-0.11 ± 0.07	0.33(4)
HL-LL	-0.09 ± 0.08	-0.01 ± 0.08	-0.68(4)
YL-LR	-0.64 ± 0.05	-1.0 ± 0.1	2.73(4)
HS-LR	0.02 ± 0.08	-1.0 ± 0.2	4.57(4)*

† SE: standard error, df: degrees of freedom, LR: living roots, DR: dead roots, RZ: rhizomes, LS: low shoots, LL: low leaves, HS: high shoots, HL: high leaves, YL: youngest leaves.

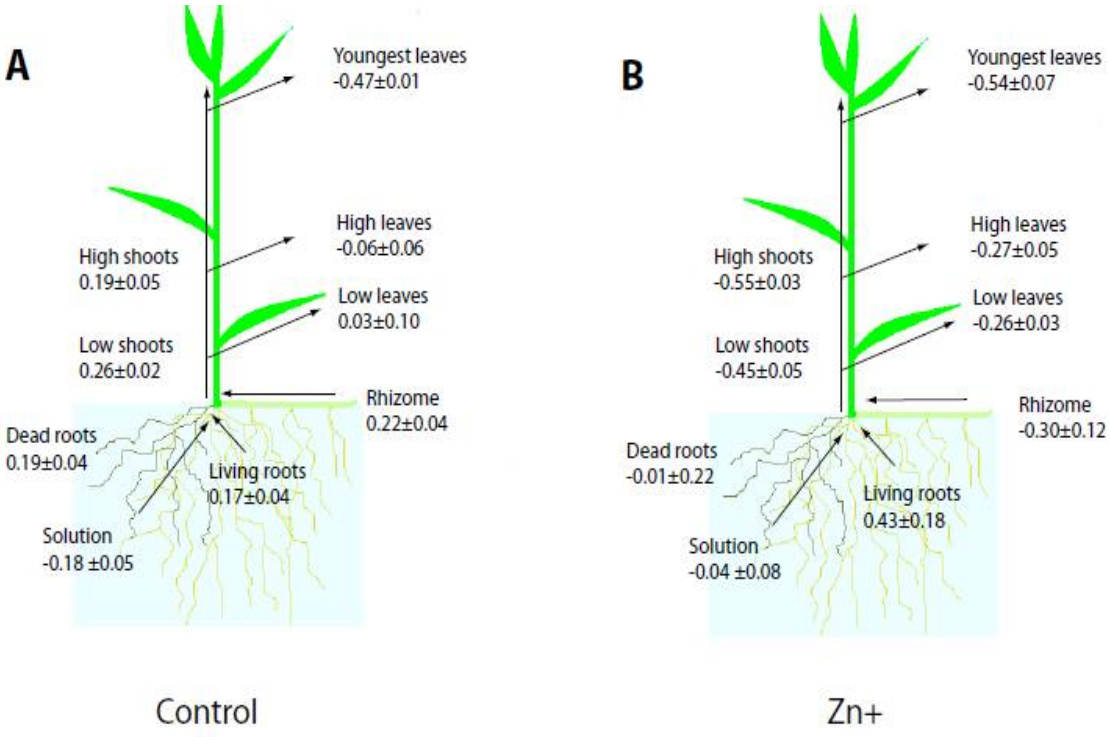
Table 6. Pearson's Correlation between $\delta^{66}Zn$ and some photosynthetic performance parameters. The $\delta^{66}Zn$ was measured in high leaves. Correlation coefficient (r) is indicated as significant at P<0.05 (*) or P<0.01 (**).

$\delta^{66}Zn$	g_s †	F_v'/F_m'	ΦCO_2	qN	NPQ
r	0.921(*)	0.921(*)	0.883(*)	-0.944(*)	-0.974(**)
Sig. (bilateral)	0.027	0.026	0.047	0.016	0.005
n	5	5	5	5	5

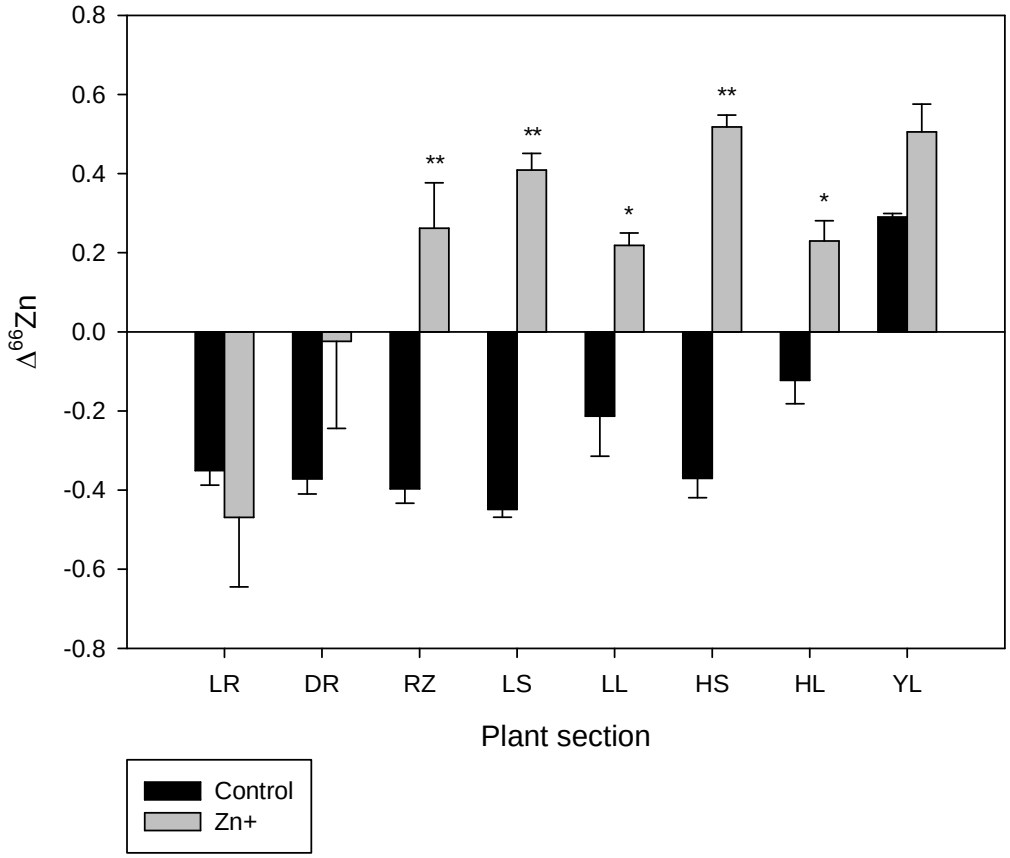
† F_v'/F_m': relative quantum yield, ΦCO_2: the quantum yield of CO_2 fixation, qN and NPQ: non-photochemical quenching, g_s: stomatal conductance to water.

Fig. 1. Isotopic signature of the studied plant sections compared to solutions. Plants were supplied with 3.2 µM (Control, A) or 2mM Zn (Zn+, B). Data represent means ± SE (n = 3), $\delta^{66}Zn$ is expressed in ‰.

Fig. 2. Isotopic discrimination of the studied plant sections with respect to nutritive solutions. Plants were supplied with 3.2 µM (Control) or 2 mM (Zn+) Zn. Data represent means ± SE (n = 3). The $\Delta^{66}Zn$ is expressed in ‰ and was calculated as $\Delta^{66}Zn = (\delta^{66}Zn_{source}-\delta^{66}Zn_{sample})/(1+\delta^{66}Zn_{sample}/10^3)$. T-test value (t) is indicated as significant at $P<0.05$ (*) or $P<0.01$ (**).LR: living roots, DR: dead roots, RZ: rhizomes, LS: low shoots, LL: low leaves, HS: high shoots, HL: high leaves, YL: youngest leaves.


A
Youngest leaves
-0.47±0.01
High leaves
-0.06±0.06
High shoots
0.19±0.05
Low leaves
0.03±0.10
Low shoots
0.26±0.02
Rhizome
0.22±0.04
Dead roots
0.19±0.04
Living roots
0.17±0.04
Solution
-0.18 ±0.05
B
Youngest leaves
-0.54±0.07
High leaves
-0.27±0.05
High shoots
-0.55±0.03
Low leaves
-0.26±0.03
Low shoots
-0.45±0.05
Rhizome
-0.30±0.12
Dead roots
-0.01±0.22
Living roots
0.43±0.18
Solution
-0.04 ±0.08

Control Zn+


0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Δ66Zn
**
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*
**
*
LR
DR
RZ
LS
LL
HS
HL
YL
Plant section
Control
Zn+