RESEARCH PAPER

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

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Abstract

Stable isotope signatures of Zn have shown great promise in elucidating changes in uptake and translocation mechanisms of this metal in plants during environmental changes. Here this potential was tested 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 (δ66ZnMC Lyon = 0.2‰) while the youngest leaves were isotopically light (–0.5‰). During Zn excess, roots were still isotopically heavier (δ66Zn = 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.

Key words: Isotope fractionation, MC-ICP-MS, metallomics, metals, nutrition, Phragmites australis, reed.

Introduction

Increasing Zn environmental pollution has originated from several anthropogenic sources (Popovic et al., 2001; Konstantinou and Albanis, 2004; Mathur et al., 2005; Pruvo et al., 2006; Kong and White, 2010). Zn is a micro-nutrient 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, a multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS) appears to be 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). MC-ICP-MS has allowed the research on stable isotopes to be extended to heavier elements such as Zn, opening up a field of new possibilities. The
study of the isotopic fractionation of essential elements such as 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.

Zn has four stable isotopes, $^{64}_{1}Zn$, $^{66}_{1}Zn$, $^{67}_{1}Zn$, and $^{68}_{1}Zn$. 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, such as adsorption to a surface or the formation of covariant bounds, favour the accumulation of the heavier isotopes in the reaction product, whereas kinetic processes such as diffusion-mediated transport discriminate against the heavy isotopes (Criss, 1999; Rodushkin et al., 2004). Weiss et al. (2006) 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 the diatom surface, and that desorbed cells were impoverished in $^{66}_{1}Zn$. 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 $^{66}_{1}Zn_{\text{leaves}}$ 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. The use of isotopes is still needed to recognize the activation of tolerance mechanisms in response to high levels of Zn, for example 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 by 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 (i) to test whether the exposure to toxic Zn levels causes any alteration in the Zn fractionation pattern of P. australis; (ii) 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 (iii) 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$$^{+}$, 28.5 mg l$^{-1}$ PO$_4$$^{3—}$, 35.5 mg l$^{-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$_2$O$_3$, 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 were selected within a small range of fresh weight of 161.2±5.0 g (FW±SE; n=16). There were two Zn treatments: control (3.2 mM 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$_4$.7H$_2$O (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 April to 13 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 °C (mean±SE), the relative humidity 53.6±1.3%, and the transmission of the greenhouse covers 51%. Nutritive solution was renewed every 3–4 d and deionized water was added daily to compensate the loss due to evaporation and transpiration.

Plants were then thoroughly washed in tap water, bathed for 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 deionized water. The isotopic composition of adsorbed Zn depends on the physicochemical characteristics of the solution and the adsorbent surface rather than 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 cm and 12 cm for the low shoots and between 20 cm and 27 cm for the high shoots. Leaves growing at these two different height intervals were named low and high leaves, respectively. The last three leaves of each stem were labelled as the youngest leaves. Pairs of plants were pooled together. Fresh samples were oven-dried at 60 °C until constant weight, and ground with a ball mill.

Photosynthetic performance

The chlorophyll content and fluorescence and the gas exchange of leaves was measured 1–2 d before the end of the experiment.
Chlorophyll content on a leaf area basis was obtained using a portable chlorophyll meter (SPAD-502 Minolta, Illinois, USA), following Krug 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 from 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 CO₂, and an air temperature of 25.9±0.1 °C. Leaves were previously dark adapted for 30 min to measure the maximum quantum yield (Fₚ/Fₚₜₐₚ). The same leaves were then re-acclimated to environmental light to determine the relative quantum yield (Fₚ′/Fₚₜₐₚ), quantum yield of photosystem II photochemistry (FPSII) (Genty et al., 1989), quantum yield of CO₂ fixation (φCO₂), electron transport rate (ETR, μmol m⁻² s⁻¹), photochemical (qP) and non-photochemical quenching (qN, NPO), light-saturated net CO₂ assimilation rate (Aᵣ, μmol CO₂ m⁻² s⁻¹), stomatal conductance to water (gs, mmol m⁻² s⁻¹), intercellular CO₂ concentration (Ci, μmol CO₂ mol air⁻¹), and transpiration rate (E, mmol H₂O m⁻² s⁻¹).

Zn content
Plant samples were digested in two steps, first overnight at 90 °C in HNO₃:H₂O₂ (1:1 v/v), then 0.5 ml of hydrofluoric acid was added and they were digested for 2 h at 90 °C. Digests were evaporated to dryness on a hotplate at 120 °C and the residues were redissolved in 3 ml of 7 M HCl. Each solution was split into three aliquots: 1 ml for Zn concentration measurements, 1 ml for Zn isotopic analysis, and 1 ml for archive. The first aliquot was made up to 3.5 ml of 1 M HCl prior to concentration measurements on a Varian VISTA PRO (Palo Alto, CA, USA) ICP-AES (inductively coupled plasma atomic emission spectrometer), for which analytical errors were 0.4–5% of the measured values. Per each solution used—that is, JMC 3-0749L. Accuracy of the isotope measurements was assessed by the analysis of two in-house single element solutions (Romil Zn and London Zn) and two natural standard reference materials (rye grass 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., 2008; Peel et al., 2008; Arnold, 2009).

The 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 ~4 ng of Zn. All mineral acids were sub-boiled in a quartz still and diluted using a 18 MW grade Millipore system (Bedford, MA, USA).

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

\[ \Delta \delta^{66}Zn_{ij} = \delta^{66}Zn_i - \delta^{66}Zn_j \]

where \( \Delta \delta^{66}Zn_{ij} \) 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 et al., 1989):

Table 2. Isotopic signature of the standards used in this study

| 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) | –0.01±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 1. Zinc content of the standards used in ICP-AES analyses

| Sample type | Zn content (μg g⁻¹) | % Recovery |
|-------------|---------------------|------------|
| Certified   | Measured            |            |
| 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 |
\[
\Delta^{66}\text{Zn} = \frac{\delta^{66}\text{Zn}_{\text{root}} - \delta^{66}\text{Zn}_{\text{leaves}}}{1 + \delta^{66}\text{Zn}_{\text{leaves}}} \tag{3}
\]

where \(\delta^{66}\text{Zn}_{\text{root}}\) is the isotopic signature of the root, in this case the nutritive solution, and \(\delta^{66}\text{Zn}_{\text{leaves}}\) is the isotopic signature of the plant sample.

**Statistical methods**

Two-way analysis of variance (ANOVA) was carried out to evaluate the effect of plant section, Zn treatment, and their interaction with Zn concentration, and \(\Delta^{66}\text{Zn}\). Logarithmic transformation was performed when data did not meet the assumption of equal variances. To determine which groups were significantly different from each other, the post-hoc test that best separated the groups, either Student–Neuman–Keuls or Duncan, was selected. Student’s \(t\)-test was chosen for mean comparisons between treatments for the photosynthetic parameters (\(A_s\), \(g_s\), \(F_{v}/F_{m}\), \(F_{v}'/F_{m}'\), \(\Phi\text{PSII}\), \(\Phi\text{CO}_2\), \(\text{ETR}\), \(q\text{P}\), \(q\text{N}\), \(\text{NPQ}\), and \(\text{E}\)) and Zn isotopic fractionation between sections (\(\Delta^{66}\text{Zn}_{\text{leaves}}\)). Pearson’s correlation was employed to test whether there was a linear relationship between \(\delta^{66}\text{Zn}\) 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 to 50% in Zn+ plants with no changes in \(C_i\) occurred. In the dark-adapted leaves of both treatments \(F_v/F_m\) remained stable. In contrast, \(F_v/F_m\), \(\Phi\text{PSII}\), \(\Phi\text{CO}_2\), \(q\text{P}\), and \(\text{ETR}\) showed a clear decrease in Zn+ light-adapted leaves. This was accompanied by an increase in \(q\text{N}\) and \(\text{NPQ}\).

**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. In contrast, 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 for the high leaves and youngest leaves, where it was lower.

**Zn isotopes**

The \(\delta^{66}\text{Zn}\) varied between plant sections (Fig. 1). In the control experiment, only the youngest leaves were significantly different from the rest of the 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 isotopically much lighter in Zn+ plants. The isotope signature of the youngest leaves was similar in both treatments, although the shoots of Zn+ plants were shorter. There was no significant difference detectable between low

| Parameter | Control | Zn+ | \(t\) |
|-----------|---------|-----|------|
| Plant height (cm) | 106±4 | 79±3 | 5.8** |
| IRC | 38.0±1.2 | 32.6±1.1 | 4.0** |
| \(A_s\) (μmol CO₂ m⁻² s⁻¹) | 14±3 | 7±1.3 | 2.7* |
| \(g_s\) (μmol H₂O m⁻² s⁻¹) | 0.18±0.05 | 0.08±0.01 | 2.4* |
| \(C_i\) (μmol CO₂ mol⁻¹ 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** |
| \(\Phi\text{PSII}\) | 0.24±0.02 | 0.15±0.02 | 3.7** |
| \(\Phi\text{CO}_2\) | 0.014±0.002 | 0.008±0.001 | 2.8* |
| \(q\text{P}\) | 0.52±0.02 | 0.39±0.04 | 2.6* |
| \(q\text{N}\) | 0.81±0.02 | 0.88±0.01 | -4.1** |
| NPQ | 2043±12 | 2652±141 | -3.1* |
| ETR (μmol m⁻² s⁻¹) | 122±9 | 75±9 | 3.7** |
| \(E\) (μmol H₂O m⁻² s⁻¹) | 3.9±0.8 | 1.9±0.3 | 2.6* |

\(A_s\), light-saturated net CO₂ assimilation rate; \(C_i\); intercellular CO₂ concentration; \(E\), transpiration rate; \(\text{ETR}\), electron transport rate; \(F_v/F_m\), maximum quantum yield; \(F_v'/F_m'\), relative quantum yield; \(g_s\), stomatal conductance to water; IRC, index of relative chlorophyll content; \(q\text{N}\), NPQ, non-photochemical quenching; \(q\text{P}\), photosynthetic quenching; \(\Phi\text{CO}_2\), quantum yield of CO₂ fixation; \(\Phi\text{PSII}\), quantum yield of PSII photochemistry.

Table 4. Concentration of Zn achieved in different plant sections

| Plant section | Zn content (mg g⁻¹) | BCF |
|---------------|---------------------|-----|
| Controls | Zn+ | Controls | Zn+ |
| Roots | | | |
| Living | 0.09±0.04 | 12±6 | 960±167 | 93±19 |
| Dead | 0.02±0.01 | 14±7 | 268±27 | 105±27 |
| Rhizomes | 0.02±0.01 | 2.7±1.4 | 274±46 | 21±5 |
| Shoots | | | |
| Low | 0.04±0.02 | 3±2 | 433±46 | 25±3 |
| High | 0.06±0.03 | 2.3±1.1 | 640±40 | 17±2 |
| Leaves | | | |
| Low | 0.05±0.03 | 4±2 | 583±79 | 27±5 |
| High | 0.04±0.02 | 1.1±0.6 | 490±48 | 8.7±0.4 |
| Youngest | 0.04±0.02 | 0.5±0.2 | 389±8 | 3.7±0.2 |

BCF, bioconcentration factor.
and high shoot or between low and high leaves in any of the treatments either.

The $\Delta^{66}$Zn$_{i,j}$ was calculated between adjacent sections and between the roots and the youngest leaves or the high shoots (Table 5). The $\Delta^{66}$Zn$_{\text{leaves-shoots}}$ as well as $\Delta^{66}$Zn$_{\text{RZ-LR}}$ and $\Delta^{66}$Zn$_{\text{HS-LR}}$ were significantly affected by Zn+ treatment. In contrast, high Zn did not modify the $\Delta^{66}$Zn between stem or leaf samples collected at different heights. Also there was no influence of the treatments on the fractionation between the roots and the youngest leaves. In agreement with the previous results, the controls discriminated against the light isotope except in the youngest leaves, whereas the Zn+ discriminated in favour of the lighter isotope except in the roots (Fig. 2). Both treatments differed significantly in the $\Delta^{66}$Zn of rhizomes, shoots, and leaves. Plants caused the enrichment in heavy isotopes of the nutritive solution, which was more evident in high Zn solutions.

**Table 5.** Fractionation between plant sections

| Sample | $\Delta^{66}$Zn$_{i,j}$ (‰) | Zn+ | t (df) |
|--------|-----------------|------|-------|
| 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)* |
| LS-HS | -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-LL | -0.64±0.05 | -1.0±0.2 | 2.73 (4) |
| HS-LL | 0.02±0.08 | -1.0±0.2 | 4.57 (4)* |

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

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

Table 5. Fractionation between plant sections

Fractionation was calculated as $\Delta^{66}$Zn$_{i,j}$ = $\delta^{66}$Zn$_{j}$ - $\delta^{66}$Zn$_{i}$. Data represent means ±SE (n=3). The t-test value (t) is indicated as significant at $P < 0.05$ (*) or $P < 0.01$ (**).

Plant height showed a strong and positive linear correlation with $\delta^{66}$Zn of high shoot ($r=0.972$, $P=0.001$) and low shoot ($r=0.929$, $P=0.007$), and a weaker one with rhizome ($r=0.813$, $P=0.049$). The correlations of plant height with the rest of plant sections were not significant. The gas exchange and chlorophyll fluorescence were correlated with $\delta^{66}$Zn of high leaves where measurements were performed in both control and Zn+ plants. The relationships found to be significant are shown in Table 6. The results are consistent with the effect of the treatments on the photosynthetic performance parameters seen above. High values of $\delta^{66}$Zn in high leaves (as shown by controls) were associated with a higher $g_s$, $\Phi_{\text{PSII}}$, and $\Phi_{\text{CO}_2}$, and with a lower NPQ.

Finally, the concentration of Zn showed a negative linear correlation with $\delta^{66}$Zn, which was strong in the sections low shoots ($r=-0.964$, $P=0.002$) and high shoots ($r=-0.971$, $P=0.001$), and weaker in high leaves ($r=-0.828$, $P=0.042$).

**Discussion**

**Photosynthetic performance and growth**

The results showed 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 to 50%, indicating a strong inhibition of stomatal opening. 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 $C_4$. 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, did $F_v/F_m$, $\Phi$PSII, and $\Phi$CO$_2$ show a clear decrease in Zn+ plants. This was accompanied by a reduction in 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 $\Phi$CO$_2$. 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, the oxygen-evolving complex, the plastoquinone pool, PSI, and Rubisco (Prasad, 2004). Many of these effects could cause the observed decreased photosynthetic performance. In addition, stomatal closure could reduce nutrient uptake. Deficiency of N can lead to an indirect impairment of the photosynthetic apparatus, and limit $A_s$. This is consistent with the decrease in total chlorophyll content indicated by decreased IRCC figures. Decreased N content has also been associated in the 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 $g_s$, $\Phi$PSII, and $\Phi$CO$_2$, and the 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, the results must be treated with caution due to the low number of replicates.

**Zn content**

Phragmites 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 comprise 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 from 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 mg g$^{-1}$ DW in shoots, and 0.5–3 mg g$^{-1}$ DW in leaves) were comparable with those found in the study of Jiang and Wang (2008) (14 mg g$^{-1}$ DW in roots, 0.95 mg g$^{-1}$ DW in shoots, and 1.5 mg g$^{-1}$ DW in leaves), using the same species and Zn supply. The small discrepancies in the Zn content of shoots and leaves can be easily explained, as in the present study sampling was done at specific heights instead of taking samples representative of the whole stem.

**Zn 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 with the nutrient solution. The $\delta^{66}$Zn of the youngest leaves is isotopically lighter than the rest of the 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
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(Benth.) Milne-Redh among the four species analysed showed a significant fractionation between root and shoot. The most negative δ66Zn values measured throughout the plant were found in leaves (Viers et al., 2007; Moynier et al., 2009). In contrast, different degrees of root to shoot fractionation were described in crops such as 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, it is proposed 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 on the plasmatic membrane and facilitate Zn uptake (Grotz and Guerinot, 2006). Alternatively, the chelation of Zn by ligands and its subsequent transport in the complexed form could cause the observed enrichment in the heavier isotopes. This explanation can be rejected because Zn is mostly taken up and transported as Zn2+ (Marschner, 1995). Other mechanisms favouring the heavy isotopes are in disagreement with the observations reported here, such as adsorption onto the root surface, binding to the cell walls, and compartmentation in cell organelles. All of them imply the retention of the heavier isotopes in the roots, preventing its transport to other reservoirs. The δ66Zn_root would be more positive than the rest of the plant, in disagreement with the present results. The protocol used to remove the root-adsorbed and apoplastic bound Zn was thus apparently efficient. The obtained data (δ66Zn_root = 0.18‰) are in line with previous experiments (Weiss et al., 2005), where a similar root desorption protocol was used for tomato, rice, and lettuce (δ66Zn_root = 0.15, 0.15, and 0.2‰, respectively).

The youngest leaves of controls were more negative than the rest of the plant. The transport of Zn2+ along the shoot has been suggested as the cause for the enrichment in light isotopes of shoots and leaves with height (Moynier et al., 2009), in agreement with the present results. There was a positive correlation between plant height and δ66Zn of transporting tissues, as previously suggested by Viers et al. (2007). The correlation was stronger as the samples were higher (high shoots > low shoots > rhizomes). The fractionation between low and high leaves was not statistically significant in this experiment (Δδ66ZnHL–LL = −0.090‰). However, the results are consistent with the small distance that separates the samples. The fractionation per distance was of −0.005‰ cm−1, very similar to −0.006‰ cm−1 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, it is considered that Zn uptake mediated by transporters is not the cause of the enrichment in heavy isotopes of Zn+ roots.

Excess Zn is mainly accumulated in roots and localized in cell walls, intercellular spaces, and vacuoles (Heumann, 2002; Li et al., 2006; Jian and Wang, 2008). In the present experiment, δ66Zn 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 δ66Zn similar to that of controls, even if their shoots were shorter and Zn was transported a shorter distance (106.0 ± 3.5 cm for controls, 78.6 ± 3.1 for Zn+, means ± SE, n = 4). In the opinion of the authors, this is because the xylem sap of Zn+ plants had a more negative δ66Zn from the root than that of controls.

When examined in detail, all the known mechanisms for Zn sequestration in roots are likely to select the heavy isotopes. Zinc probably forms covalent bounds with carboxyl and hydroxyl groups of pectin and with hydroxyl groups of cellulose in the cell walls (Straczek et al., 2008), and precipitates with insoluble phosphates or silicon in the apoplast (Heumann and zur Nieden, 2001; Straczek et al., 2008). In the cell, Zn binds to various ligands and is stored in subcellular organelles to keep Zn2+ low in the cytosol. Zinc is transferred into the vacuoles by metal tolerance proteins (MTPs) localized to the tonoplast (Blaudez et al., 2003; Dräger et al., 2004; Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006; Gustin et al., 2009). The tonoplast transporter AtZIP1 is also involved in Zn sequestration, probably by transporting either organic Zn ligands or Zn-ligand complexes into the vacuole (Haydon and Cobbett, 2007b). Different authors expect Zn to be chelated in the vacuoles by various ligands such as organic acids (OAs), proteins, and phytate (Van Steveninck et al., 1987; Salt et al., 1999; Tennstedt et al., 2009). The best candidates for Zn ligands in the vacuole are OAs such as citrate and malate, which are the most abundant metal ligands in plants and accumulate mainly in the vacuoles, as do excess metals. In agreement with this, the optimal stability of OA–metal complexes is achieved at vacuolar pH (Haydon and Cobbett,
Besides, metal-binding peptides and proteins have been described to chelate Zn. Phytochelatins (PCs) are glutathione oligomers synthesized in response to metals, that chelate and detoxify Cd and As (Jabeen et al., 2009). Recent advances established that Zn promotes the synthesis of PCs, which are essential for Zn detoxification and contribute to Zn accumulation (Tennstedt et al., 2009). Cd complexed with PCs is pumped and sequestered into the vacuole (Salt et al., 1995; Cobbett and Goldsbrough, 2002). It is probable that PC–Zn complexes follow the same route, but direct evidence is lacking. Metallothioneins are cysteine-rich low molecular weight proteins found in plants, animals, and fungi, and are able to chelate Zn and many other metals. They are involved in Zn homeostasis and/or tolerance, but their exact function is as yet unknown (Rodriguez-Llorente et al., 2010). Finally, phytoate is a P storage molecule that can bind to Zn as a mechanism for Zn storage or immobilization. Phytate–Zn complexes are found in roots (Van Steveninck et al., 1987, 1993; Terzano et al., 2008) and in seeds (Otegui et al., 2002; Rodrigues-Filho et al., 2005), either compartmentalized in the vacuoles or forming insoluble precipitates.

All three processes, Zn binding to cell walls, precipitation in intercellular spaces, and sequestration in the vacuole, are mass dependent and thus expected to favour the heavy isotope. It is difficult from the present design to tell which process was chiefly responsible for the enrichment in heavy isotopes of Zn+ roots. The youngest leaves of Zn+ were more negative than the rest of the leaves. Similarly to controls, the fractionation between low and high leaves was not statistically significant in this experiment (\( \Delta^{66}\text{Zn}_{\text{HL-LL}} = -0.011\%\)). The calculated \( \Delta^{66}\text{Zn}_{\text{HL-LL}} \) obtained from the linear regression of Zn+ leaves (Fig. 3) is of -0.090\%, very different from that observed but similar to that of controls. This provides evidence for the restriction of long-distance transport under toxic Zn levels. Both linear regressions for controls and Zn+ have a very similar slope, but the Zn+ plot is biased to the negative side. The youngest leaves of Zn+ have a \( \delta^{66}\text{Zn} \) similar to controls, in spite of the 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 the Zn status.

The enrichment in heavy isotopes of the nutritive solutions with time is in agreement with plants taking up Zn preferentially 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

It has been proved that the study of Zn isotopes has great potential for investigation of the mechanisms of tolerance to Zn excess in plants. It was 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. It was shown that under Zn-sufficient levels, roots and shoots are enriched in the heavier Zn isotopes as compared with the source (\( \delta^{66}\text{Zn} = -0.2\% \)), 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\%). It has also been shown that Zn uptake by plants causes the enrichment in heavy isotopes of the nutritive solutions, which was stronger in Zn+ treatment (\( \Delta^{66}\text{Zn}_{\text{control}} = 0.3\% \), \( \Delta^{66}\text{Zn}_{\text{Zn+}} = 0.6\% \)). 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 agreement 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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