1 **TITLE**

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

3 zinc.

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24 **RUNNING HEAD**

25 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.

85   Multicollector Inductively-Coupled Plasma Mass-Spectrometry

86   qN, NPQ

87   non-photochemical quenching

88   qP

89   photochemical

90   ΦPSII

91   quantum yield of PSII photochemistry

92   ΦCO_2

93   quantum yield of CO_2 fixation

94   $\Delta^{66}Zn$

95   isotopic discrimination with respect to the growth medium

96   $\delta^{66}Zn$

97   isotopic signature

98   $\Delta\delta^{66}Zn_{i-j}$

99   isotopic fractionation between sections i and j,

100

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,

151 current research on plants is focused on Zn isotopic discrimination under normal

152 or Zn-deficient conditions. We still need to endeavour the use of isotopes to

153 recognise the activation of tolerance mechanisms in response to high levels of Zn,

154 e.g. extrusion, sequestration by metal-binding compounds or subcellular

155 compartmentation. The aim of this study was to demonstrate that the

156 physiological mechanisms of response to toxic levels of Zn are able to

157 discriminate between Zn isotopes.

158 *Phragmites australis* (Cav.) Trin. ex Steud. was chosen as model plant because it

159 is tolerant to toxic Zn concentrations, and responds accumulating excess Zn

160 mainly in the roots and restricting its uptake and transport to the shoots (Weis and

161 Weis, 2004). The specific objectives of this research were 1) to test whether the

162 exposure to toxic Zn levels causes any alteration in the Zn fractionation pattern of

163 *P. australis*, 2) to check the hypothesis proposed by Moynier et al. (2009) and

164 Viers et al. (2007) that there is a correlation between the height of leaves and the

165 Zn isotopic fractionation and 3) to examine the usefulness of the technique to

166 study the physiology of Zn toxicity.

167

168 **MATERIALS AND METHODS**

169 **Plant material**

170 *Phragmites australis* (Cav.) Trin. ex Steud plants were purchased from a local

171 nursery (Bioriza, Breda, Spain). Plants were root-washed in tap water to remove

172 the original peat-vermiculite substrate, weighed, and placed in a pure hydroponics

173 system in individual pots. The nutritive solution comprised: 130.25 mg L^{-1} NO_3^-,

174 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}

175 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,

176 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

177 was adjusted to 6.5. Plants were allowed to acclimate to hydroponics for 27 d,

178 until they recovered a vigorous growth, and then selected within a small range of

179 fresh weight of 161.2 ± 5.0g (FW ± SE; n = 16). There were two Zn treatments:

180 Control (3.2 μM Zn), where plants were grown in the same nutritive solution as

181 during acclimation, and Zn+ (2 mM Zn), where the nutritive solution was

182 amended with $ZnSO_4 \cdot 7H_2O$ (Sigma-Aldrich, 99% ACS reagent) to reach the

183 desired concentration. Eight plants per treatment were randomly distributed and

184 grown under glasshouse conditions for 40 d (29th April to 13th July 2009).

185 Previous research proved that this time span allows for enough Zn accumulation

186 and fractionation (Weiss et al., 2005). The temperature was 23.1 ± 0.3 ℃ (mean ±

187 SE), the relative humidity 53.6 ± 1.3 %, and the transmission of the greenhouse

188 covers 51 %. Nutritive solution was renewed every 3 to 4 d and deionised water

189 was added daily to compensate the loss due to evaporation and transpiration.

190 Plants were then thoroughly washed in tap water, bathed 30 min in ice-cold 1 mM

191 $LaCl_3$ and 0.05 mM $CaCl_2$ to remove adsorbed and apoplastically-bound Zn

192 (following Weiss et al. 2005), and rinsed in deionised water. The isotopic

193 composition of absorbed Zn depends on the physicochemical characteristics of the

194 solution and the adsorbent surface rather that on biologically regulated processes

195 (Gélabert et al., 2006), and will not be considered in this study. The isotopic

196 fractionation of Zn adsorbed on iron oxides or onto biological surfaces leads to

197 the enrichment of the heavy isotopes (Pokrovsky et al., 2005; Gélabert et al.,

198 2006; John et al., 2007). This approach was selected to allow for the comparison

199 between studies, even with species that show no metal plaques.

200 Plant height was recorded and eight samples were collected from each plant:

201 living roots (LR), dead roots (DR), rhizomes (RZ), low shoots (LS), low leaves

202 (LL), high shoot (HS), high leaves (HL) and youngest leaves (YL). Stems were

203 collected at distances from the root: between 5 to 12 cm for the low shoots and

204 between 20 to 27 cm for the high shoots. Leaves growing at these two different

205 height intervals were named low and high leaves, respectively. The three last

206 leaves of each stem were labelled as youngest leaves. Each two plants were

207 pooled together. Fresh samples were oven-dried at 60 ºC until constant weight,

208 and ground with a ball mill.

209

210 **Photosynthetic performance**

211 The chlorophyll content and fluorescence and the gas exchange of leaves was

212 measured 1 to 2 d before the end of the experiment. Chlorophyll content on leaf

213 area basis was obtained using a portable chlorophyll meter (SPAD-502 Minolta,

214 Illinois, USA), following Krugh et al. (1994). This device provides an indexed

215 relative chlorophyll content (IRCC) ranging from 0 to 99.9. Always the third

216 fully-developed leaves at 2.5 cm of the leaf base were measured on five

217 representative pre-bloom leaves per plant.

218 Photosynthetic gas exchange and chlorophyll fluorescence were determined in the

219 third last fully expanded leaf of each plant using a LI-COR 6400 Portable

220 Photosynthesis System (LI-COR Inc., Lincoln, NE, USA), with a saturating light

221 (photosynthetic photon flux density of 1200 µmol photons $m^{-2}s^{-1}$), 400 µmol mol^{-1}

222 of CO_2, and air temperature of 25.9 ± 0.1°C. Leaves were previously dark-adapted

223 for 30 min to measure maximum quantum yield (F_v/F_m). The same leaves were

224 then re-acclimated to environmental light to determine relative quantum yield

225 (F_v'/F_m'), quantum yield of PSII photochemistry (ΦPSII) (Gentyet al., 1989),

226 quantum yield of CO_2 fixation (ΦCO_2), electron transport rate (ETR, $\mu mol\ m^{-2}s^{-1}$),

227 photochemical (qP) and non-photochemical quenching (qN, NPQ), light-saturated

228 net CO_2 assimilation rate (A_s, $\mu mol\ CO_2\ m^{-2}s^{-1}$), stomatal conductance to water

229 (g_s, $mol\ H_2O\ m^{-2}s^{-1}$), intercellular CO_2 concentration (C_i, $\mu mol\ CO_2\ mol\ air^{-1}$), and

230 transpiration rate (E, $mmol\ H_2O\ m^{-2}s^{-1}$).

231

Zinc content

233 Plant samples were digested in two steps, first overnight at 90 ºC in $HNO_3:H_2O_2$

234 (1:1 v/v), then added 0.5 mL HF and digested 2 h at 90 ºC. Digests were

235 evaporated to dryness on a hotplate at 120 ˚C and the residues were re-dissolved

236 in 3 ml of 7 M HCl. Each solution was split into three aliquots: 1 ml for Zn

237 concentration measurements, 1 ml for Zn isotope analysis and 1 ml for archive.

238 The first aliquot was made up to 3.5 ml 1 M HCl prior to concentration

239 measurements on a Varian VISTA PRO (Palo Alto, USA) ICP-AES (Inductively

240 Coupled Plasma Atomic Emission Spectrometer), for which analytical errors were

241 0.4 to 5 % of the measured values. Per each 12 samples, a blank and a sample of

242 either olive leaves (*Olea europaea* L., BCR-62), aquatic plant (*Lagarosiphon*

243 *major* [Ridl.] Moss, BCR-60), light sandy soil (BCR-142R), or lichen (BCR-482)

244 certified reference material from the Community Bureau of Reference (BCR®)

245 were processed in the same way and analysed (Table 1). Digestions were carried

246 out in the clean laboratory of the Department of Earth Science and Engineering

247 (Imperial College of London). Zinc content determination was performed in the

248 research facilities of the Natural History Museum (London, UK).

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

250   (2004):

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

251

252   where $[Zn_p]$ is the Zn concentration of the plant sample ($\mu g \, g^{-1}$ DW), and $[Zn_s]$ is

253   the Zn concentration of the nutritive solution ($\mu g \, ml^{-1}$).

254

255   **Zinc isotopic signature**

256   Zinc isotopes were analysed on the second aliquot. An isotope spike enriched in

257   ^{64}Zn, ^{66}Zn and ^{67}Zn was added to the sample aliquot to achieve a total of 1000 ng

258   Zn and a spike:sample mass ratio of 1 (Arnold et al., 2010b). Zinc was separated

259   from the matrix using anion exchange chromatography as detailed in Arnold el al.

260   (2010a). Zinc fractions were re-dissolved in 0.1 M HNO_3 for the subsequent

261   isotope ratio analysis using a HR Nu Plasma MC-ICP-MS (Multi-Collector

262   Inductively Coupled Plasma Mass Spectrometer, Nu Instruments, Wrexham, UK).

263   Isotope ratios are reported in δ-notation:

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

264

265

266   where $(^{66}Zn/^{64}Zn)_{sample}$ is the isotope ratio of the sample and $(^{66}Zn/^{64}Zn)_{JMC \, Lyon}$ is

267   the isotope ratio of the standard reference solution used, i.e. JMC 3-0749L.

268   Accuracy of the isotope measurements was assessed by the analysis of two

269   in-house single element solutions (Romil Zn, London Zn) and two natural

270   standard reference materials (Ryegrass BCR-281 and Blend ore BCR-027). As

271   shown in Table 2, data from this study agree within error with previously

272   published values for the in-house standards, the rye grass BCR-281 and for the

273   blend ore BCR-027 (Mason et al., 2004; Chapman et al., 2006; Peel et al., 2008;

274   Arnold 2009).

275   Precision of the isotope measurements was estimated from replicate analysis of

276   the BCR-281 standard (see Table 2). The typical error (expressed as 2σ standard

277   deviation) was ± 0.12 ‰. Procedural blank contributions were around 4 ng of Zn.

278   All mineral acids were sub-boiled in a quartz still and diluted using 18 MΩ grade

279   Millipore system (Bedford, MA, USA).

280   To further assess the effect of the treatment on the distribution of isotopes across

281   plant sections, the fractionation between sections was calculated following

282   Moynier et al. (2009) as:

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

284

285   Where $\Delta\delta^{66}Zn_{i\text{-}j}$ is the fractionation between sections i and j, and $\delta^{66}Zn_i$ and

286   $\delta^{66}Zn_j$ are the isotopic signature of section i and j respectively. The discrimination

287   with respect to the growth medium was calculated according to the equation

288   (Farquhar, 1989):

289

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

291

292   Where $\delta^{66}Zn_{so}$ is the isotopic signature of the source, in this case the nutritive

293   solution, and $\delta^{66}Zn_p$ is the isotopic signature of the plant sample.

294

Statistical Methods

Two-way ANOVA was carried out to evaluate the effect of plant section, Zn treatment and their interaction with Zn concentration, and δ^{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 δ^{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

320 The Zn content of all plant sections increased with increasing Zn supply (Table 4).

321 The Zn concentration of plant samples was higher than that of the growth

322 solution, but the BCF was much reduced in Zn+ plants (Table 4). Plants grown at

323 different Zn concentrations differed in the distribution pattern of Zn (and

324 consequently BCF). In controls, living roots achieved the highest Zn levels,

325 whereas dead roots had the lowest. Oppositely, in Zn+ plants dead and living

326 roots achieved the highest levels, whereas leaves, shoots and rhizomes contained

327 little Zn in comparison. All Zn+ emerged sections had very similar Zn

328 concentration except high and youngest leaves, where it was lower.

329

330 **Zinc isotopes**

331 The δ^{66}Zn varied between plant sections (Fig. 1). In the control experiment, only

332 the youngest leaves were significantly different from the rest of plant sections,

333 showing a lighter isotopic signature. The shoots were slightly heavier than the

334 leaves. The Zn+ treatment altered the fractionation pattern (Fig. 1). Shoots of Zn+

335 plants were lighter than the leaves, whereas the root samples were heavier than the

336 shoots and the youngest leaves. However, only the rhizomes and the shoots were

337 significantly different among treatments, and much isotopically lighter in Zn+

338 plants. The isotope signature of youngest leaves was similar in both treatments,

339 although the shoots of Zn+ plants were shorter. There was no significant

340 difference detectable between low and high shoot or between low and high leaves

341 in any of the treatments either.

342 The $\Delta\delta^{66}$Zn$_{i-j}$ was calculated between adjacent sections and between the roots and

343 the youngest leaves or the high shoots (Table 5). The $\Delta\delta^{66}$Zn$_{leaves-shoots}$ as well as

344 $\Delta\delta^{66}$Zn$_{RZ-LR}$, and $\Delta\delta^{66}$Zn$_{HS-LR}$ were significantly affected by Zn+ treatment. In

15

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, $\Phi PSII$, 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', $\Phi PSII$ 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

395 nutrient uptake. The deficiency of N can lead to an indirect impairment of

396 photosynthetic apparatus, and limit A_s. This is consistent with the decay of total

397 chlorophyll content indicated by decreased IRCC figures. Decreased N content

398 has also been associated in literature with the inhibition of Rubisco and the dark

399 phase of photosynthesis (Ciompi et al., 1996).

400 The strong positive correlation of $\delta^{66}Zn_{HL}$ with and g_s, $\Phi PSII$, ΦCO_2, and negative

401 correlation with NPQ indicates that $\delta^{66}Zn_{HL}$ could be an interesting parameter to

402 assess the inhibition of photosynthesis due to the toxic effect of excess Zn.

403 Nevertheless, results must be taken with caution due to the low number of

404 replicates.

405

406 **Zn content**

407 *P. australis* has innate tolerance to Zn and other metals (Ye et al. 1997). The

408 lower BCF, the accumulation of Zn in roots, and the limitation to Zn export to the

409 green tissues compose an avoidance response that confers increased tolerance to

410 Zn excess (Denny and Wilkins, 1987; Maestri et al., 2010). The higher BCF of

411 dead roots in Zn+ is consistent with the use of root senescence to release Zn, an

412 excretion mechanism of tolerant plants (Duarte et al. 2010). The Zn levels

413 achieved by leaves and shoots are far of reaching the 1 % Zn in leaf dry matter

414 generally accepted as the threshold to reach Zn hyperaccumulation (Verbruggen et

415 al., 2009). The Zn concentrations of the different tissues (12-14 mg g^{-1} dw in

416 roots, 2-3 in shoots, and 0.5-3 in leaves) were comparable to those of Jiang and

417 Wang (2008) study (14 mg g^{-1} dw in roots, 0.95 shoots, 1.5 leaves), who used the

418 same species and Zn supply. The small discrepancies in the Zn content of shoots

419 and leaves can be easily explained, as we sampled at specific heights instead of

420 taking samples representative of the whole stem.

421

422 **Zinc isotopes**

423 *Mechanisms explaining the isotopic fractionation pattern under normal Zn supply*

424 Under Zn sufficient conditions, all the plant tissues except the youngest leaves are

425 enriched in the heavier isotopes compared to the nutrient solution. The δ^{66}Zn of

426 the youngest leaves is isotopically lighter than the rest of sections. Mature leaves

427 are slightly lighter isotopically than roots and shoots. These observations are in

428 line with field observations of Viers and co-workers (2007). They found that only

429 *Megaphrynium macrostachyum* (Benth.) Milne-Redh among the four species

430 analysed showed a significant fractionation between root and shoot. The most

431 negative δ^{66}Zn values measured along the plant were found in leaves (Viers et al.,

432 2007; Moynier et al., 2009). By contrast, different degrees of root-to-shoot

433 fractionation were described in crops like tomato, lettuce and rice under different

434 experimental conditions (Weiss et al., 2005; Arnold et al., 2010a). This suggests

435 that the mechanisms of Zn uptake and transport are highly species-specific and

436 conditioned by the physiological status of the plant. Here, we propose that the

437 isotopic distribution of controls comes from the combination of two processes: i)

438 the enrichment in heavy isotopes generated by Zn uptake in roots and ii) the

439 enrichment in light isotopes during the long-distance transport of free Zn ions in

440 the plant.

441 The observed pattern is consistent with the uptake of Zn by root cells facilitated

442 by transmembrane transporters, as previously suggested by Weiss et al. (2005).

443 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. We refuse this explanation because Zn is mostly taken up and transported as Zn^{2+} (Marschner, 1995). Other mechanisms favouring the heavy isotopes are in disagreement with the observations reported here, like adsorption onto 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 $\delta^{66}Zn_{root}$ would be more positive than the rest of the plant, in discrepancy with the present results. The protocol used to remove the root-adsorbed and apoplastically-bound Zn was thus apparently efficient. The obtained data ($\delta^{66}Zn_{root} = 0.18$ ‰) are in line with previous experiments (Weiss et al., 2005), where a similar root-desorption protocol was used for tomato, rice and lettuce ($\delta^{66}Zn_{root} = 0.15$, 0.15 and 0.2 ‰, respectively).

The youngest leaves of controls were more negative than the rest of the plant. The transport of Zn^{2+} 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 $\delta^{66}Zn$ 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 ($\Delta\delta^{66}Zn_{HL-LL} = -0.090$ ‰). 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

469   of controls were slightly lighter than the shoots at the same height, probably due

470   to the translocation of Zn from the shoot along the leaves. Thus, the present data

471   are consistent with an enrichment of lighter isotopes with distance from the root,

472   but this can only be assessed if there is enough separation between samples.

473

474   *The isotopic fractionation pattern reflects the tolerance response to high Zn*

475   *concentrations*

476   The protective mechanisms activated by plants under high-Zn stress disrupt the

477   Zn uptake, accumulation, distribution and transport routes, which translated into a

478   completely different fractionation pattern in this experiment.

479   There is little information about the regulation of ZIP transporters under excess

480   Zn in plants. However, experiments in yeast demonstrated that ZRT1 is

481   inactivated by high Zn supply (Gitan et al., 1998), limiting Zn influx into the cell.

482   The activity of the transporters is probably inhibited in Zn+ plants, as shown by

483   the decreased BCF. Thus, we consider that Zn uptake mediated by transporters is

484   not the cause for the enrichment in heavy isotopes of Zn+ roots.

485   Excess Zn is mainly accumulated in roots and localised in cell walls, intercellular

486   spaces and vacuoles (Heumann, 2002; Li et al., 2006; Jian and Wang, 2008). In

487   the present experiment, $\delta^{66}Zn$ was less negative in roots than in the rest of the

488   organs, and Zn translocation from root to shoot was lower in Zn+ plants. This

489   indicates that heavy Zn is effectively retained in roots, and the isotopically lighter

490   sap is transported to the above-ground tissues. The youngest leaves of Zn+ plants

491   have a $\delta^{66}Zn$ similar to controls, even if their shoots were shorter and Zn was

492   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

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 missing. Metallothioneins are cysteine-rich low molecular weight proteins found in plants, animals and fungi, and able to chelate Zn and many other metals. They are involved in Zn homeostasis and/or tolerance, but their exact function is yet unknown (Rodríguez-Llorente et al., 2010). Finally, phytate is a P storage molecule that can bind to Zn as a mechanism for Zn storage or immobilisation. Phytate-Zn complexes are found in roots (Van Steveninck et al., 1987 and 1993; Terzano et al., 2008), and in seeds (Otegui et al., 2002; Rodrigues-Filho et al., 2005), either compartmented 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 of the enrichment in heavy isotopes of Zn+ roots. The youngest leaves of Zn+ were more negative than the rest of leaves. Similarly to controls, the fractionation between low and high leaves was not statistically significant in this experiment ($\Delta\delta^{66}Zn_{HL-LL}$ = -0.011 ‰). The calculated $\Delta\delta^{66}Zn_{HL-LL}$ obtained from the linear regression of Zn+ leaves (Fig. 3) is of -0.090‰, very different from the observed but similar to that of controls. This evidences the restriction of long-distance transport under toxic Zn levels. Both linear regressions for controls and Zn+ have a very similar slope, but Zn+ plot is biased to the negative side. The youngest leaves of Zn+ have a $\delta^{66}Zn$ similar to controls, in spite of plants being shorter. The correlation between plant height and

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

543   be modified by Zn status.

544   The enrichment in heavy isotopes of the nutritive solutions with time is coherent

545   with plants taking up preferably Zn by bulk flow, favouring the light isotopes, and

546   with the higher biomass of above-ground tissues in this species (Ye et al., 1997).

547   Also the Zn+ solution was more enriched in heavy isotopes than the control

548   solution, as expected from the discrimination pattern observed for each treatment.

549

550   **CONCLUSIONS**

551   We have proved that the study of Zn isotopes has a great potential to investigate

552   the mechanisms of tolerance to Zn excess in plants. We have demonstrated that *P.*

553   *australis* is able to discriminate Zn isotopes, and that the magnitude and sign of

554   the resultant fractionation depends on Zn-status and organ. We have shown that

555   under Zn sufficient levels, roots and shoots are enriched in the heavier Zn isotopes

556   as compared to the source ($\delta^{66}Zn$ = 0.2 ‰) and the youngest leaves are

557   impoverished (-0.5 ‰), whilst under Zn excess roots are enriched in the heavy

558   isotopes (0.5 ‰) and the rest of the plant is isotopically lighter (up to -0.5 ‰). We

559   have exposed that Zn uptake by plants causes the enrichment in heavy isotopes of

560   the nutritive solutions, which was stronger in Zn+ treatment ($\Delta\delta^{66}Zn_{control}$ = 0.3

561   ‰, $\Delta\delta^{66}Zn_{Zn+}$ = 0.6 ‰). In conclusion, the tolerance response of *P. australis*

562   increased the range of Zn fractionation within the plant and with respect to the

563   environment.

564   An outline of the fractionation mechanisms compatible with the observed

565   response was also provided. The enrichment in heavy isotopes of the roots was

566   attributed to Zn uptake under Zn-sufficient conditions and to chelation and

567   compartmentation in Zn excess. The enrichment in light isotopes of shoots and

568   leaves is consistent with long-distance root-to-shoot transport, in accord with the

569   observations by Viers et al., (2007), and Moynier et al., (2009). Further research

570   needs to be conducted to confirm these hypotheses and establish what molecules

571   or processes are responsible for the described pattern.

572

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

Reference	Sample type	Zn content ($\mu g\ g^{-1}$)		
		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 $\pm$ 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 $\pm$ 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 δ^{66}Zn and some photosynthetic performance parameters. The δ^{66}Zn was measured in high leaves. Correlation coefficient (r) is indicated as significant at P<0.05 (*) or P<0.01 (**).

δ^{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 Control

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 Zn+

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

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