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RESEARCH PAPER

Effects of zinc toxicity on sugar beet (*Beta vulgaris* L.) plants grown in hydroponics

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ABSTRACT

The effects of high Zn concentration were investigated in sugar beet (*Beta vulgaris* L.) plants grown in a controlled environment in hydroponics. High concentrations of Zn sulphate in the nutrient solution (50, 100 and 300 μm) decreased root and shoot fresh and dry mass, and increased root/shoot ratios, when compared to control conditions (1.2 μm Zn). Plants grown with excess Zn had inward-rolled leaf edges and a damaged and brownish root system, with short lateral roots. High Zn decreased N, Mg, K and Mn concentrations in all plant parts, whereas P and Ca concentrations increased, but only in shoots. Leaves of plants treated with 50 and 100 μm Zn developed symptoms of Fe deficiency, including decreases in Fe, chlorophyll and carotenoid concentrations, increases in carotenoid/chlorophyll and chlorophyll *a*/*b* ratios and de-epoxidation of violaxanthin cycle pigments. Plants grown with 300 μm Zn had decreased photosystem II efficiency and further growth decreases but did not have leaf Fe deficiency symptoms. Leaf Zn concentrations of plants grown with excess Zn were high but fairly constant (230–260 $\mu g \cdot g^{-1}$ dry weight), whereas total Zn uptake per plant decreased markedly with high Zn supply. These data indicate that sugar beet could be a good model to investigate Zn homeostasis mechanisms in plants, but is not an efficient species for Zn phytoremediation.

INTRODUCTION

Zinc is essential for cell physiological processes, and in most living organisms it is the second most abundant transition metal after Fe. Zinc has no redox activity but plays structural and/or catalytic roles in many processes, and is the only metal present in all enzyme classes (Vallee & Auld 1990; Barak & Helmke 1993). Zinc is also essential for plants, and Zn deficiency is a common problem in plants grown in high pH, calcareous soils (as it also found with Fe) (Casona *et al.* 1991; Cakmak *et al.* 1996), whereas in low pH soils Zn availability is generally high (Foy *et al.* 1978; Chaney 1993). When present at high concentrations, Zn can be toxic, and plants affected may show symptoms similar to those found in other heavy metal toxicities, such as those of Cd or Pb (Foy *et al.* 1978). In most cases, excess Zn generates reactive oxygen species and/or displaces other metals from active sites in proteins. Zinc toxicity also induces chlorosis in young leaves, and this has been suggested to result from a Zn-induced Fe or Mg deficiency, based on the fact that the three metals have similar ion *radii* (Marschner 1995). Other common Zn toxicity effects include decreases in tissue water content and changes in the P and Mg concentrations in plant tissues (Marschner 1995).

The mechanisms controlling Zn homeostasis in plants are still not fully known (Hacisalihoglu *et al.* 2004; Broadley *et al.* 2007; Kramer *et al.* 2007). Plant roots acquire Zn predominantly as the divalent ion, and the metal is then distributed throughout the whole plant in a complex series of processes. Several families of plant metal transporters have been identified in recent years (Kramer *et al.* 2007), with at least three being involved in Zn transport through membranes: ZIP (IRT-like proteins) (Grotz *et al.* 1998; Wintz *et al.* 2003), CDF (Cation Diffusion Facilitator proteins) (Blaudez *et al.* 2003; Kim *et al.* 2004; Kobae *et al.* 2004; Kramer 2005) and P_{1B}-type ATPases (HMAs, metal transporting ATPases) (Hussain *et al.* 2004; Papoyan & Kochian 2004; Verret *et al.* 2004; Mills *et al.* 2005). The roles these transporters play in Zn

uptake, efflux, compartmentalisation, storage and detoxification have been partially characterised (Kramer *et al.* 2007). After uptake, Zn can be transported in the xylem where it is chelated by different small molecules (Haydon & Cobbett 2007), including organic acids such as malate and citrate (White *et al.* 1981; Broadley *et al.* 2007), His (Salt *et al.* 1999; Kupper *et al.* 2004) and nicotianamine (von Wirén *et al.* 1999; Callahan *et al.* 2006). Under high Zn supply, a large part of the Zn in the cell is also chelated by organic acids such as malate and citrate (Salt *et al.* 1999; Sarret *et al.* 2002; Kupper *et al.* 2004), amino acids such as His and NA (Callahan *et al.* 2006), phytate (Rauser 1999) and metallothioneins (Kawashima *et al.* 1992; Rauser 1999; Papoyan & Kochian 2004), and is most likely stored in vacuoles.

Zinc release to the environment may be associated with biotic or natural atmospheric processes, with the ratio of Zn emissions arising from human activities to those coming from natural causes being above 20 (Friedland 1990). Human activities releasing Zn to the environment include fossil fuel combustion and the use of sewage sludge, manure and lime. In contaminated and acid soils some crops may suffer Zn toxicity, and species which have a high Zn uptake capacity, such as spinach and beet, could be more sensitive to its excess (Chaney 1993; Broadley *et al.* 2007). Bioaccumulation of trace metals in plant tissues may present a health risk to wildlife and potentially to humans (Singh & Agrawal 2007).

The objective of the present study was to investigate the effects of high concentrations of Zn in the nutrient solution on growth, photosynthetic characteristics and nutrient composition of different parts of the model plant sugar beet (*Beta vulgaris* L.). The aim was to establish a basis for studies of the mechanisms of heavy metal transport in this model plant.

MATERIALS AND METHODS

Plant material

Sugar beet (*Beta vulgaris* L. cv Orbis) was grown in a growth chamber with a photosynthetic photon flux density (PPFD) of 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, measured with a LiCor sensor placed horizontally at maximum plant height, 80% relative humidity and a 16 h at 23 °C/8 h at 18 °C light/dark regime. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for an additional 2-week period in half-strength Hoagland nutrient solution (Terry 1980) with 45 μM Fe(III)-EDTA, and then transplanted to 20-l plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution with 45 μM Fe(III)-EDTA and different concentrations of Zn. A concentration of 1.2 μM $ZnSO_4$ was used as a control, and the excess Zn treatments were 50, 100 and 300 μM $ZnSO_4$. Plants were used for measurements 9–10 days after imposing the high Zn treatments. Young, fully expanded leaves were chosen for all photosynthetic measurements.

Chemical speciation

In silico estimations of the concentrations of Zn ionic species in the different nutrient solutions were carried out with MINTEQA2 for Windows (Version 1.50, Allison Geoscience Consultants, Flowery Branch, GA and HydroGeoLogic, Inc., Hermdon, VA, USA).

Growth parameters

Plants were divided in three fractions at day 10: shoots (leaf blades + petioles), main root and fine absorption roots. Fresh (FW) and dry weights (DW) of each fraction, root/shoot ratios and water content (WC) were also determined.

Mineral nutrient analysis

All plant tissues were washed with pure water. Samples were dried in an oven at 60 °C for 76 h until constant weight. Samples were then dry-ashed and dissolved in HNO_3 and HCl following the AOAC procedure (Association of Official Analytical Chemists, Washington DC, USA). Calcium (after La addition), Mg, Fe, Mn, Cu and Zn were determined by FAAS, K by FES and P spectrophotometrically by the molybdate-vanadate method (Abadía *et al.* 1985; Igartua *et al.* 2000). Nitrogen was determined with a NA2100 Nitrogen Analyzer (ThermoQuest, Milan, Italy).

Photosynthetic pigment analysis

The leaf concentration of Chl was estimated on a leaf area basis in attached leaves using a SPAD portable apparatus (Konica Minolta Co., Osaka, Japan). For calibration, leaf disks were taken at day 10 after treatment, first analysed with the SPAD apparatus, then frozen in liquid N_2, pigments extracted with 100% acetone in the presence of Na ascorbate and the extracts analysed spectrophotometrically (Abadía & Abadía 1993). Photosynthetic pigments were also quantified by HPLC (Larbi *et al.* 2004). In these experiments, leaf sampling was carried out in leaves illuminated for 3–4 h.

Gas exchange measurements

Nine days after treatments were imposed in the growth chamber, measurements were made on attached leaves using a portable gas exchange system (CIRAS-1, PP Systems, Herts, UK), using a PLC broadleaf cuvette in closed circuit mode. Transpiration rates (E), stomatal conductance (g_S), net photosynthetic rate (P_N) and substomatal CO_2 concentrations (C_i) were measured and calculated. Experiments were made at ambient CO_2 concentration, 130–170 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD, and at the temperature and relative humidity prevailing in the growth chamber. All measurements were taken in leaves illuminated for 3–4 h.

Modulated chlorophyll fluorescence analysis

Modulated Chl fluorescence measurements were made in attached leaves, 9 days after treatments were imposed in the growth chamber, using a PAM 2000 apparatus (H. Walz, Effeltrich, Germany). F_o was measured by switching on the modulated light at 0.6 kHz; PPFD was below 0.1 μmol photons $m^{-2}{\cdot}s^{-1}$ at the leaf surface. F_m was measured at 20 kHz with a 1-s pulse of 6,000 μmol photons $m^{-2}{\cdot}s^{-1}$ of white light. The experimental protocol for the analysis of Chl fluorescence quenching followed Morales *et al.* (2000) and references therein. F_o and F_o' were measured in the presence of FR light (7 μmol photons $m^{-2}{\cdot}s^{-1}$) to fully oxidise the PSII acceptor side (Belkhodja *et al.* 1998; Morales *et al.* 1998; Logan *et al.* 2007). Dark-adapted, maximum potential PSII efficiency was calculated as F_v/F_m, where F_v is $F_m - F_o$ (Morales *et al.* 1991; Abadía *et al.* 1999). Actual (Φ_{PSII}) and intrinsic (Φ_{exc}) PSII efficiency were calculated as $(F_m' - F_s)/F_m'$ and F_v'/F_m', respectively. Photochemical quenching (qP) was calculated as $(F_m' - F_s)/F_v'$. Non-photochemical quenching (NPQ) was calculated as $(F_m/F_m') - 1$. Experiments were carried out at ambient CO_2 concentration, 200–250 μmol·$m^{-2}{\cdot}s^{-1}$ PPFD, and at the temperature and relative humidity prevailing in the growth chamber. All measurements were taken in leaves illuminated for 3–4 h.

Iron reductase activity measurements

Ferric chelate reductase (FC-R) activity was measured as described by Gogorcena *et al.* (2000) by following the formation of the Fe(II)-$BPDS_3$ complex from Fe(III)-EDTA. Root FC-R activity was determined in intact plants 2–3 h after light onset and at 4, 7 and 10 d after adding excess Zn to the nutrient solution. Plants were placed in 1-l buckets, in a solution containing 1 mM MES, pH 5.5, 100 μM BPDS and 100 μM Fe(III)-EDTA in MilliQ water. The reaction was stopped after 30 min by removing the plant from the bucket, and absorbance readings of the solution at 535 nm were taken using 1 ml aliquots after centrifugation at 10,000*g* for 1 min. Controls were also measured in the absence of plants to correct for non-enzymatic Fe reduction.

Statistical analysis

For statistical analysis, an LSD Bonferroni test was used for comparison of means on all data sets. The test used the SPSS software (v.14, SPSS Inc, Chicago, IL, USA).

RESULTS

Chemical speciation

In the 50, 100 and 300 μM $ZnSO_4$ treatments, the major Zn chemical species in solution was free Zn(II), accounting for 91–93% of total Zn (Table 1). Approximately 6% of total Zn was in the form $ZnSO_4$(aq) in the three high Zn treatments, whereas the concentration of the Zn(II)-EDTA complex was predicted to be very low, accounting for only 1.9, 1.4 and 0.2% of total Zn in the solutions containing 50, 100 and 300 μM Zn, respectively. In control solutions with 1.2 μM total Zn, approximately 86, 8 and 6% of total Zn was predicted to occur as Zn(II), Zn(II)-EDTA and $ZnSO_4$(aq), respectively (Table 1).

Table 1. Major Zn chemical species in the nutrient solutions, as a percentage of the total Zn present, estimated *in silico* with the software MINTEQA2.

total Zn concentration (μM)	Zn^{2+} (%)	Zn[EDTA] (%)	$ZnSO_4$(aq) (%)
1.2 (control)	86.0	7.5	5.8
50	91.2	1.9	6.0
100	91.7	1.4	6.0
300	92.9	0.2	6.0

Effects of excess Zn on growth

Sugar beet shoot and root fresh and dry mass decreased progressively when the Zn concentration in the nutrient solution increased (Fig. 1). Decreases were significant when considering whole plants (data not shown), shoots, main roots and fine roots (Fig. 1). Also, plants treated with high Zn contained less water than control plants (Fig. 1). A significant decrease in the root/shoot ratio was only found with the 300 μM Zn treatment (data not shown). Zinc excess also decreased the number of leaves and leaf area, leaf margins were distorted or wrinkled and leaves were rolled inwards and showed chlorosis symptoms (see changes in leaf concentrations of photosynthetic pigments below).

Effects of excess Zn on plant nutrient concentrations

Zinc toxicity altered the plant concentrations of several nutrients (Fig. 2). Nitrogen and K concentrations decreased progressively with Zn excess in shoots, main and fine roots, whereas Mg concentrations decreased significantly only in shoots and main roots. Phosphorus concentrations increased in shoots and increased slightly in main roots, but did not change significantly in fine roots. Calcium concentrations were unaffected in roots and increased only in shoots of plants grown with 50 and 100 μM Zn. All of the macronutrient concentrations found, except for N, were in the normal ranges used for sugar beet (Jones *et al.* 1991).

Micronutrient concentrations were also affected by high Zn in the nutrient solution (Fig. 3). Iron concentrations decreased by 40% in shoots (from approximately 110 to 70 $\mu g{\cdot}g^{-1}$ DW) and increased by 25% in main roots in response to Zn excess. Manganese concentrations decreased progressively with Zn excess in shoots and fine roots, but shoot Mn concentrations always remained

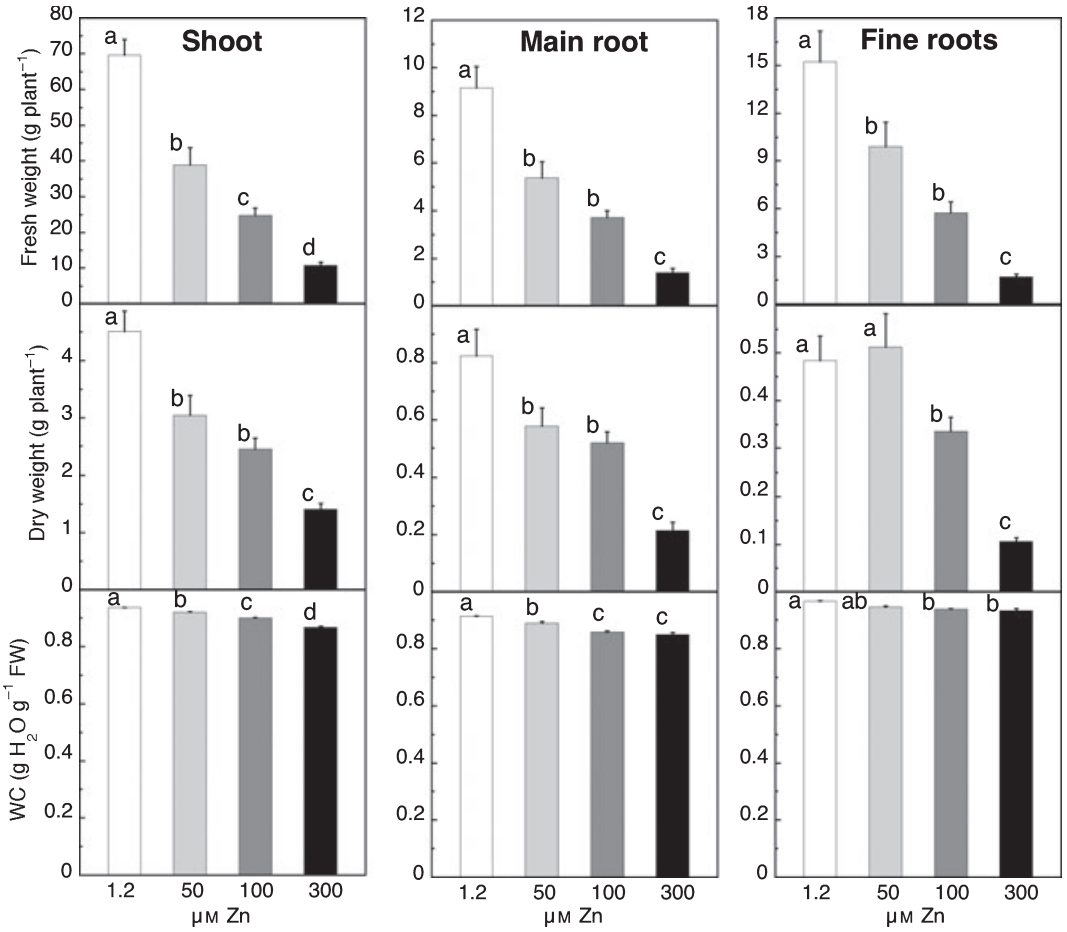

Fig. 1. Fresh and dry mass (in g per plant) and water content (in g·g^{-1} FW) of shoots, main roots and fine roots of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 10 days. Data are means ± SE (four different batches of plants, four to eight replicates in each batch). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the $P \leq 0.05$ probability level.

above 50 μg·g^{-1} DW. Copper concentrations did not change significantly with Zn excess in shoots and main roots, although Cu concentrations in fine roots increased progressively, reaching approximately 120 μg·g^{-1} DW in plants grown with 300 μM Zn, a value threefold higher than that found in control plants.

Zinc concentrations increased significantly in all plant parts with high Zn in the nutrient solution. Increases were approximately fourfold in shoots, five- to sevenfold in main roots and two- to threefold in fine roots. Shoot Zn concentrations in plants grown with high Zn were 233–259 μg·g^{-1} DW. Total Zn amounts extracted per plant were 367 μg in the 1.2 μM Zn treatment (263, 32 and 72 μg in shoot, main root and fine root, respectively), 968 μg in the 50 μM Zn treatment (709, 118 and 141 μg in shoot, main root and fine root, respectively), 844 μg in the 100 μM Zn treatment (618, 125 and 101 μg in shoot, main root and fine root, respectively), and 468 μg in the 300 μM Zn treatment (367, 57 and 44 μg in shoot, main root and fine root, respectively). Therefore, Zn allocation (as a percentage of total Zn) for shoot/-main root/fine root was 72/8/20 in control plants, 73/12–15/12–15 in the 50 and 100 μM Zn treatments, and 78/12/9 in the 300 μM Zn treatment.

Effects of excess Zn on photosynthetic pigment composition

Leaf chlorosis was already seen in the 50 μM Zn treatment and, accordingly, concentrations of all major photosynthetic pigments on a leaf area basis were decreased when compared to those found in control plants (Fig. 4). In leaves of plants grown with 100 and 300 μM Zn, decreases in lutein, β-carotene, Chl *a* and Chl *b* were more marked than at 50 μM Zn, with reductions of approximately 40–50% when compared to the controls. However, total concentrations of violaxanthin cycle pigments (V + A + Z) were very similar, at 50 and 100 μM Zn, and decreased further only in the 300 μM Zn treatment (Fig. 4). In the case of neoxanthin, no further significant decreases were found at Zn concentrations above 50 μM in the nutrient solution.

In the 50 μM Zn treatment, the (V + A + Z)/Chl ratio did not change with respect to the value found in control

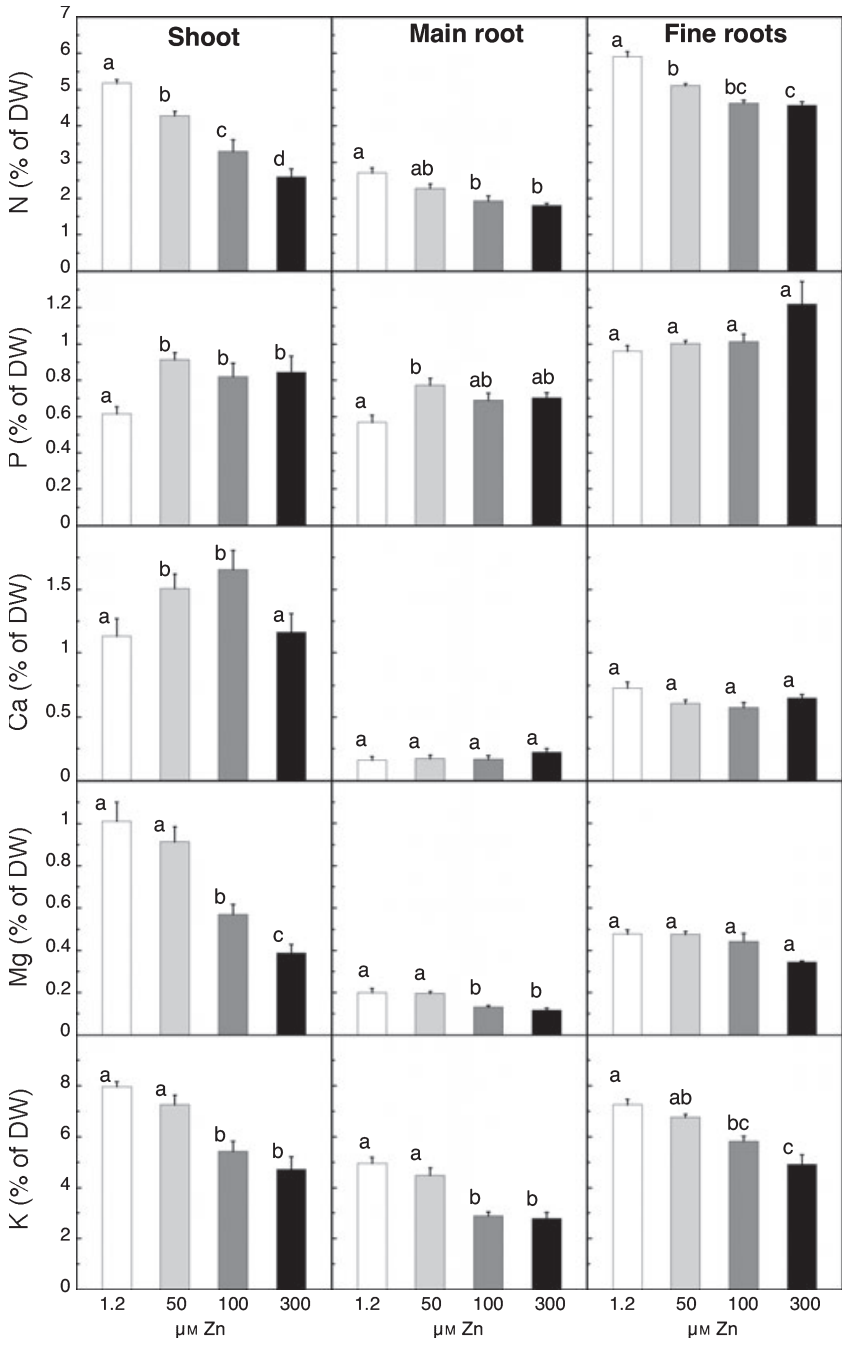

Fig. 2. Macronutrient concentrations (in % DW) in shoots, main roots and fine roots of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 10 days. Data are means ± SE (12–20, 8–16 and 4–8 samples for shoot, main and fine roots, respectively). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the $P \leq 0.05$ probability level.

plants, but the (A + Z)/(V + A + Z) ratio increased, indicating extensive de-epoxidation of violaxanthin cycle pigments (Fig. 5). In the 100 μM Zn treatment, however, both the (V + A + Z)/Chl ratio and the (A + Z)/(V + A + Z) ratio were markedly increased, while in the highest Zn treatment, 300 μM, neither the (V + A + Z)/Chl nor the (A + Z)/(V + A + Z) ratios changed when compared to control ratios. Chl *a/b* ratios in leaves of sugar beet plants treated with 50 and 100 μM $ZnSO_4$ were higher than in leaves of plants grown with 300 μM $ZnSO_4$, with control plants having an intermediate value (Fig. 5).

Effects of excess Zn on gas exchange

No significant differences were measured in gas exchange parameters (P_N, E, g_S, or C_i) in plants grown with 50 and 100 μM Zn in the nutrient solution when compared to controls (Fig. 6). However, in the 300 μM Zn treatment E, g_S, and C_i decreased by 73, 82 and 24%, respectively,

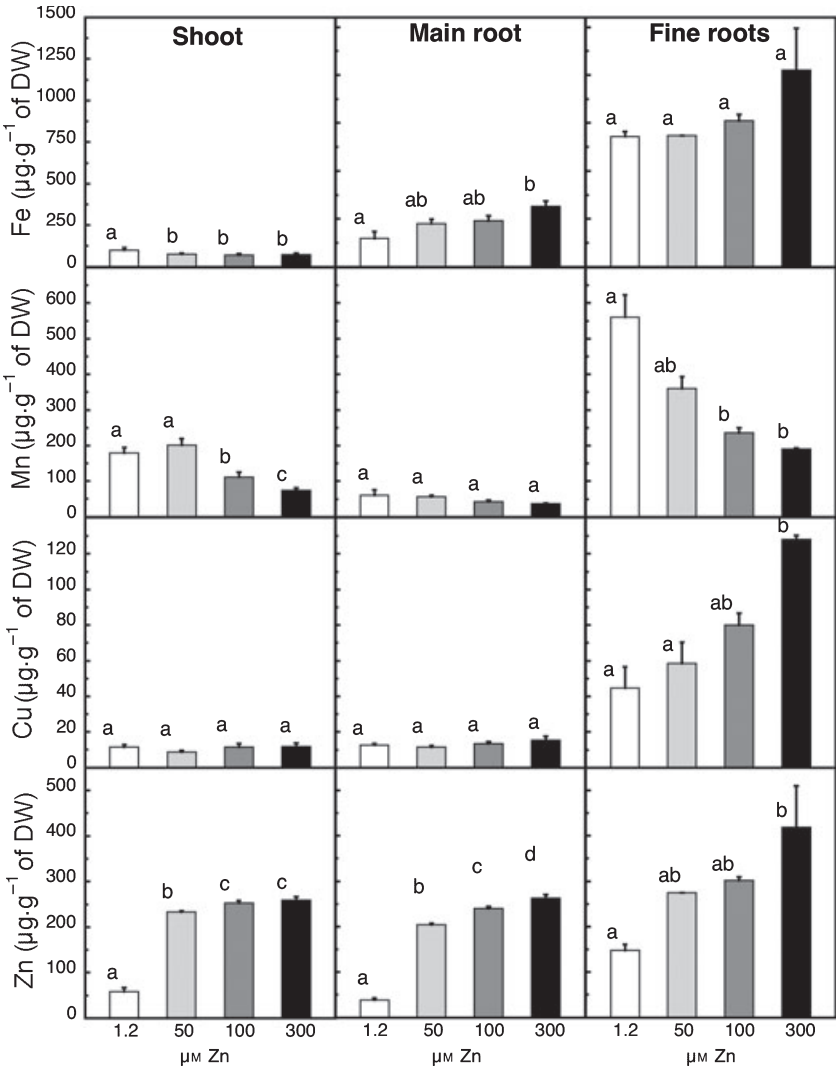

Fig. 3. Micronutrient concentrations (in $\mu g \cdot g^{-1}$ DW) in shoots, main roots and fine roots of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 µM Zn) for 10 days. Data are means ± SE (12–20, 8–16 and 4–8 samples for shoot, main and fine roots, respectively). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the $P \leq 0.05$ probability level.

when compared to control plants, indicating marked stomatal closure. In these plants, P_N rates did not decrease significantly when compared to values found in control plants.

Effects of excess Zn on chlorophyll fluorescence parameters

Leaves of plants grown with the highest Zn concentration (300 µM) showed slight but significant decreases in F_v/F_m ratios (Fig. 7). Upon illumination, these leaves showed decreases in actual PSII efficiency (Φ_{PSII}) associated with decreases in intrinsic PSII efficiency (Φ_{exc}), since photochemical quenching (qP) did not change significantly when compared to control values (Fig. 7). No changes in chlorophyll fluorescence parameters were found in plants grown with 50 µM Zn, and only small, not significant, changes were found in plants grown with 100 µM Zn. Non-photochemical quenching, however, was markedly increased (twofold) both in the 100 and 300 µM Zn treatments (Fig. 7).

Effects of excess Zn on root Fe(III)-chelate reductase activity

Roots of sugar beet became brownish when grown with excess Zn, and in the 50 and 100 µM Zn treatments, some yellow root tips were observed 7 day after treatment onset. Whole root FC-R activity increased in plants grown with 50 µM Zn for 7 and 10 days when compared to control values (Table 2). However, in the time period studied, root FC-R activity of plants grown with 100 and 300 µM Zn did not show major changes when compared to controls.

DISCUSSION

Zn excess had different effects in sugar beet plants, and the type and extent of the effects were dependent on the Zn concentration in the nutrient solution. In general, excess Zn reduced plant growth, and leaves showed symptoms of chlorosis and signs of damage. Effects were also

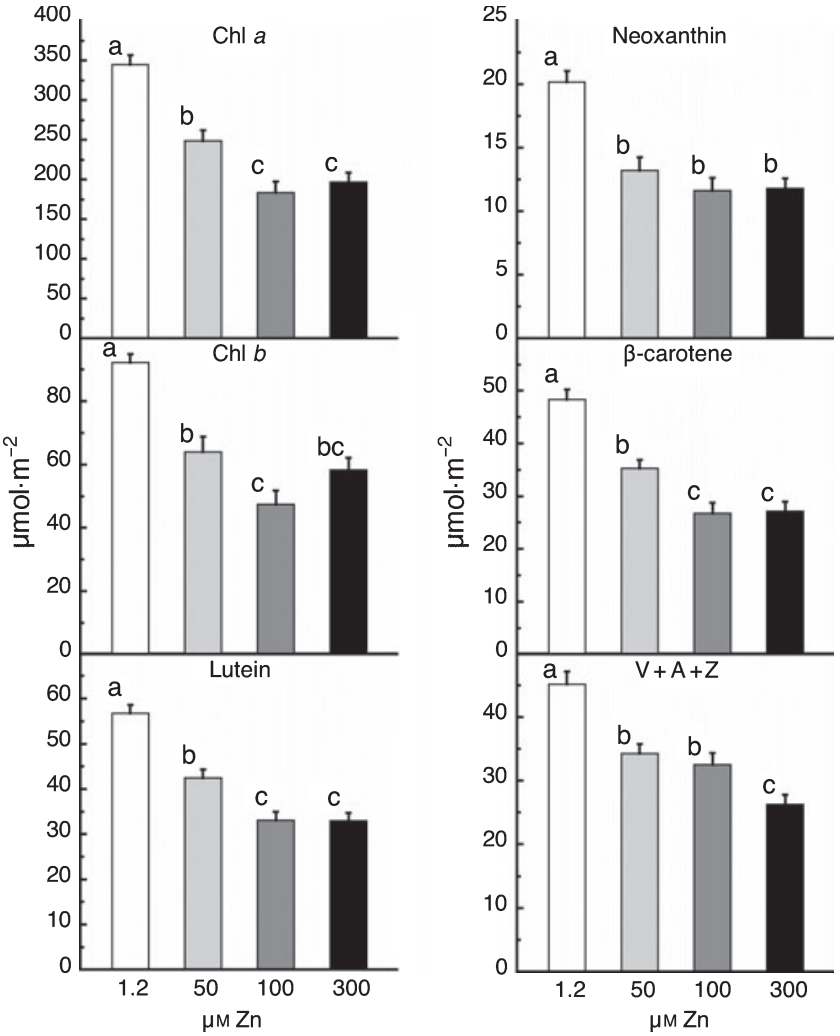

Fig. 4. Leaf concentrations of photosynthetic pigments (carotenoids and chlorophylls, in $\mu mol \cdot m^{-2}$) in sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 10 days. Data are means ± SE (20 or more replications per treatment). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the $P \leq 0.05$ probability level.

apparent in roots, with depressed growth and browning. Effects on photosynthetic rates, photosynthetic pigments and chlorophyll fluorescence were markedly different, depending on the Zn concentration in the nutrient solution.

A moderate Zn treatment (50 μM) led to Zn(II) concentrations in the nutrient solution of approximately 45 μM and to shoot concentrations of approximately 230 μg Zn g^{-1} DW. At this Zn concentration, shoot and root growth were already markedly affected when compared to controls. Shoots had less Fe and N, and more P and Ca than control plants. Leaves had mild chlorosis and less Fe than control plants, with decreases in the concentrations of all photosynthetic pigments. Although no significant changes were seen in gas exchange or chlorophyll fluorescence parameters, de-epoxidation of the xanthophyll cycle (V + A + Z) pigments did occur, indicating slight thylakoid stress. In roots, 50 μM Zn led to some yellow tips, and to a slight increase in whole root FC-R activity. All these data indicate that, besides the marked decrease in growth, 50 μM Zn in the nutrient solution caused moderate Fe deficiency. In Fe-deficient sugar beet, moderate decreases in photosynthetic pigments do not affect photosynthetic rates (Larbi *et al.* 2006). This behaviour is similar to that observed in 50 μM Zn-treated sugar beet plants.

The intermediate Zn treatment (100 μM Zn) led to nutrient solution Zn(II) concentrations of approximately 90 μM, but shoots had only marginally higher concentrations of Zn (approximately 250 μg Zn g^{-1} DW) than those of plants grown with 50 μM Zn. In these plants, shoot and root growth were further decreased. Shoot concentrations of N, Mg, K and Mn decreased when compared with values found at 50 μM Zn. Concentrations of P and Ca were still higher, and Fe was lower, than in the controls. Leaves had stronger chlorosis and a similar Fe concentration than that at 50 μM Zn, with the concentrations of photosynthetic pigments decreasing further, except for neoxanthin and V + A + Z pigments, thus leading to increases in the ratio (V + A + Z)/Chl. No significant changes were seen in gas exchange and in most chlorophyll fluorescence parameters, but both

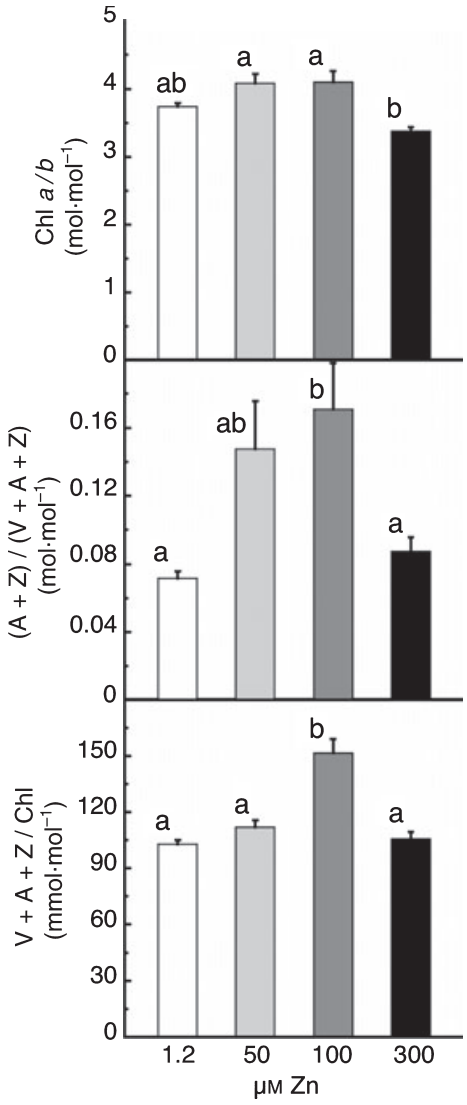

Fig. 5. Ratios (A + Z)/(V + A + Z) (in mol/mol), (V + A + Z)/Chl (in mmol/mol) and Chl *a*/*b* (in mol/mol) in leaves of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 10 days. Data are means ± SE (20 or more replications per treatment). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the P ≤ 0.05 probability level.

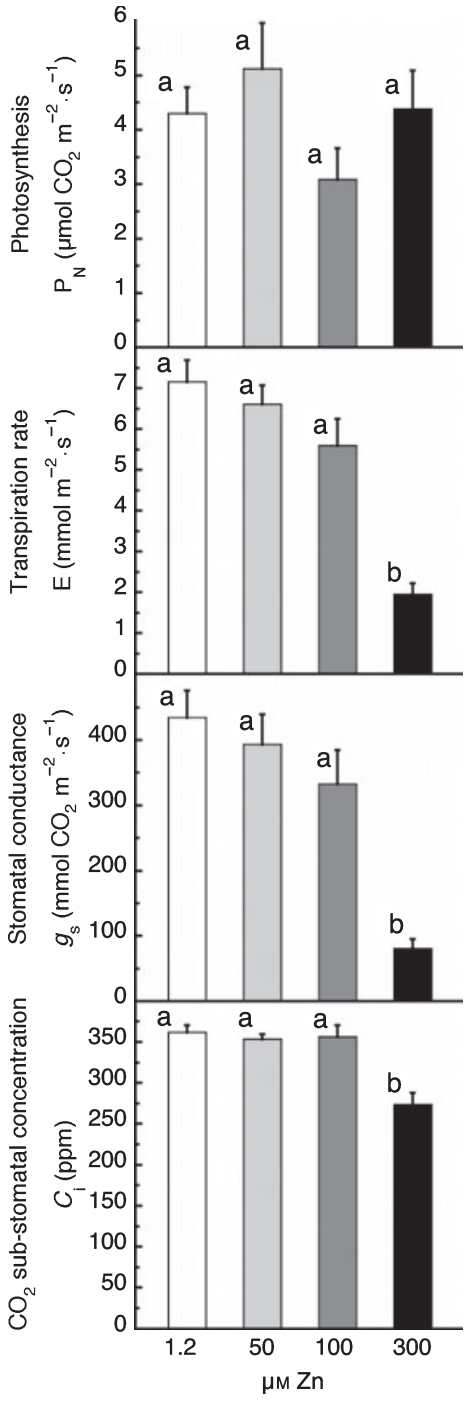

Fig. 6. Gas exchange parameters in leaves of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 9 days. The incident PPFD was between 130 and 170 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Data are means ± SE (two to three sets of measurements, 6–10 replications each). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the P ≤ 0.05 probability level.

de-epoxidation of V + A + Z pigments and an increase in NPQ occurred, indicating marked thylakoid stress. In roots, the 100 μM Zn treatment led to some yellow tips, but no change in whole root FC-R activity was observed. This may be associated to a deleterious effect of high Zn on enzyme activity, as shown to occur with other heavy metals such as Cd and Pb (Larbi *et al.* 2002; Chang *et al.* 2003). All these data indicate that 100 μM Zn in the nutrient solution caused strong decreases in growth and significant photosynthetic stress that was not necessarily related to Fe deficiency.

Using an even higher Zn treatment (300 μM), Zn(II) concentrations in the nutrient solution were estimated to be approximately 270 μM, but leaves still had approximately 250 μg Zn g^{-1} DW. This treatment led to further decreases in shoot and root growth. Leaves had interme-

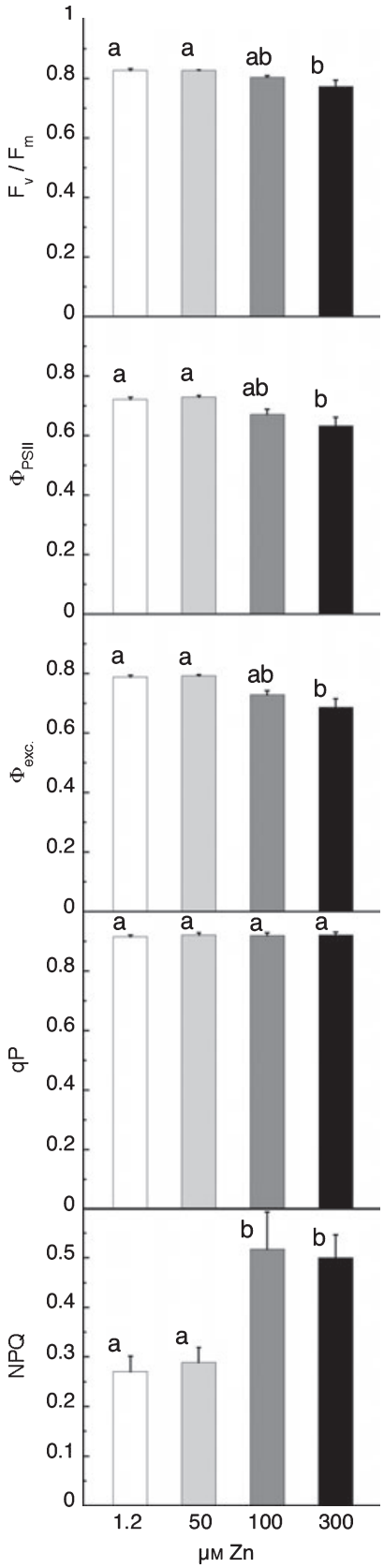

Fig. 7. Modulated Chl fluorescence parameters in leaves of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 9 days. The incident PPFD was between 200 and 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Data are means ± SE (three sets of measurements, 13–17 replications each). Bars marked with the same letter were not significantly different (LSD Bonferroni test) at the $P \leq 0.05$ probability level.

Table 2. Root Fe-reductase activity (in nmol Fe reduced g^{-1} FW min^{-1}) measured in intact plants grown in nutrient solution containing different concentrations of Zn.

total Zn concentration (μM)	day 4	day 7	day 10
1.2 (control)	3.05 ± 0.31	1.81 ± 0.12	2.03 ± 0.39
50	2.68 ± 0.24	3.62 ± 0.42**	3.84 ± 0.22**
100	2.25 ± 0.13	1.52 ± 0.24	1.93 ± 0.19
300	1.89 ± 0.20*	1.67 ± 0.38	2.23 ± 0.26

Data are means ± SE of two batches of plants (four replications each). Symbols * and **indicate significant differences from the control values (LSD Bonferroni test) at the $P \leq 0.05$ and $P \leq 0.01$ probability levels, respectively.

diate chlorosis, between that found at 50 and 100 μM Zn, and the Fe concentration was similar to that found at 50 μM Zn, but the ratio (V + A + Z)/Chl, the de-epoxidation state of the V + A + Z pigments and photosynthetic rates were not changed when compared to control values. Stomatal conductance, transpiration, C_i, F_v/F_m, Φ_{PSII}, and Φ_{exc} decreased, whereas the NPQ was still quite high. Similar results were reported with four different *Datura* species grown in vermiculite with much higher Zn concentrations (5 mM) (Vaillant *et al.* 2005). In roots, the 300 μM Zn treatment led to the appearance of some yellow tips, but no change in whole root FC-R activity was observed. All these data indicate that 300 μM Zn in the nutrient solution causes a strong decrease in growth, stomatal closure and signs of photosynthetic stress, again not necessarily related to Fe deficiency. The lack of effect on violaxanthin cycle pigments suggests that formation of the thylakoid pH gradients may be strongly impaired by high Zn. The fact that Zn excess causes marked stomatal closure with little effect on C fixation rates points to the possibility that a futile cycle may exist in plants grown in high Zn, where the C respired may be re-fixed by photosynthesis. Also, carbonic anhydrase, a Zn-containing enzyme whose activity correlates with leaf Zn concentration (Hacisalihoglu & Kochian 2003; López-Millán *et al.* 2005), could contribute to the low C_i values found. This possibility should be explored in further studies.

Our results indicate that Zn homeostasis is tightly controlled in sugar beet, since when the Zn concentration in the nutrient solution increased (from 50 to 300 μM Zn), Zn shoot concentrations only increased marginally, from

236 to 259 $\mu g \cdot g^{-1}$ DW, and Zn allocation to the shoot was little changed. Therefore, sugar beet can be used as a good model plant to study Zn homeostasis in non-hyperaccumulator plant species. Treatment with two doses of sewage sludge containing Zn and other metals led to sugar beet leaf Zn concentrations of approximately 75 $\mu g \cdot g^{-1}$ DW (Singh & Agrawal 2007). In four *Datura* species grown in vermiculite with high concentrations of Zn in the nutrient solution, leaf Zn concentrations were higher than 300–500 $\mu g \cdot g^{-1}$ DW (Vaillant *et al.* 2005). Different mechanisms have been implicated in the regulation of Zn homeostasis, including downregulation of Zn root uptake systems, Zn chelation by low-molecular weight compounds, and/or subcellular compartmentalisation of excess Zn in the apoplast or vacuoles (Hall 2002). Most of the knowledge to date comes from the study of Zn hyperaccumulator plants, such as *Arabidopsis halleri* and *Thlapsi caerulescens*, in which the major strategy for Zn detoxification consists of metal sequestration in vacuoles from mesophyll cells (Lasat *et al.* 1998; Kobae *et al.* 2004; Kupper *et al.* 2004). However, in non-hyperaccumulators other mechanisms might make a higher contribution to cope with excess Zn. Further studies should be directed to analyse, in sugar beet plants treated with excess Zn, the underlying mechanisms that contribute in this species to control Zn homeostasis, with special emphasis on chemical speciation in xylem sap, subcellular Zn distribution and the storage forms in vacuoles and/or apoplastic compartments.

Data presented here indicate that sugar beet is not a Zn accumulator and is unlikely to have potential for Zn bioremediation. This contrasts with data obtained for Cd and Pb with the same plant species (Larbi *et al.* 2002). This results from the fact that high Zn causes a very strong growth decrease, whereas the concentration of Zn in tissues does not increase greatly (*i.e.*, 260 μg Zn g^{-1} DW in plants grown with 300 μM Zn in the nutrient solution). In consequence, the amount of Zn removed per plant was larger with 50 than with 100 or 300 μM Zn in the nutrient solution. This is in contrast to Cd and Pb, since sugar beet shoots may contain up to 500 μg Cd or Pb g^{-1} DW in plants grown with 50 μM Cd or 2 mM Pb in the nutrient solution (Larbi *et al.* 2002). Sugar beet plants took up approximately 4, 2 and 1% of the total nutrient solution Zn in the treatments containing 50, 100 and 300 μM Zn, respectively. Considering a possible shoot dry mass production of 1.5 $ton \cdot ha^{-1}$ (usual values in a sugar beet commercial crop are approximately 3 $ton \cdot ha^{-1}$), and a possible leaf Zn concentration of 200 $\mu g \cdot g^{-1}$ DW, metal removal would be approximately 300–600 g Zn ha^{-1}, an amount clearly insufficient for Zn phytoremediation.

In summary, Zn toxicity in sugar beet caused a range of effects, depending on the Zn concentration in the nutrient solution. These included growth decreases, changes in the concentrations of different elements and signs of increased photosynthetic energy dissipation through the violaxanthin cycle pigments. At the highest Zn concentrations tested, plants exhibited a different adaptation strategy, closing stomata and further reducing growth.

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