Stomatal and mesophyll conductances to CO₂ are the main limitations to photosynthesis in sugar beet (*Beta vulgaris*) plants grown with excess zinc

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Received: 11 December 2009
Accepted: 21 February 2010

doi: 10.1111/j.1469-8137.2010.03241.x

**Summary**

- The effects of zinc (Zn) toxicity on photosynthesis and respiration were investigated in sugar beet (*Beta vulgaris*) plants grown hydroponically with 1.2, 100 and 300 μM Zn.
- A photosynthesis limitation analysis was used to assess the stomatal, mesophyll, photochemical and biochemical contributions to the reduced photosynthesis observed under Zn toxicity.
- The main limitation to photosynthesis was attributable to stomata, with stomatal conductances decreasing by 76% under Zn excess and stomata being unable to respond to physiological and chemical stimuli. The effects of excess Zn on photochemistry were minor. Scanning electron microscopy showed morphological changes in stomata and mesophyll tissue. Stomatal size and density were smaller, and stomatal slits were sealed in plants grown under high Zn. Moreover, the mesophyll conductance to CO₂ decreased by 48% under Zn excess, despite a marked increase in carbonic anhydrase activity. Respiration, including that through both cytochrome and alternative pathways, was doubled by high Zn.
- It can be concluded that, in sugar beet plants grown in the presence of excess Zn, photosynthesis is impaired due to a depletion of CO₂ at the Rubisco carboxylation site, as a consequence of major decreases in stomatal and mesophyll conductances to CO₂.

**Key words:** *Beta vulgaris* (sugar beet), CO₂ mesophyll conductance, CO₂ stomatal conductance, photosynthesis limitations, zinc (Zn) excess.

**Abbreviations:** $A_n$, net photosynthesis; AOX, alternative oxidase; APX, ascorbate peroxidase; BL, DL, MCL, NSL and SL, biochemical conductance, diffusional conductance, mesophyll conductance, and nonstomatal and stomatal limitations, respectively; $C_a$, $C_C$ and $C_i$, atmospheric, chloroplastic and substomatal CO₂ concentrations, respectively; CA, carbonic anhydrase; Chl, chlorophyll; ETR, electron transport rate; $F_{PSII}$, actual photosystem II efficiency; FAAS, flame atomic absorption spectroscopy; $F_o$, minimal Chl fluorescence in the dark; $Fv/Fm$, maximum potential photosystem II efficiency; $g_m$ and $g_s$, CO₂ mesophyll and stomatal conductances, respectively; $J_{max}$, *in vivo* maximum rate of electron transport driving regeneration of RuBP; Mn-SOD, manganese-superoxide dismutase; PPFD, photosynthetic photon flux density; PSII and PSI, photosystems II and I, respectively; $V_{c,max}$, *in vivo* maximum rate of Rubisco carboxylation; WC, water content.
Introduction

Zinc (Zn) availability is generally high in low-pH soils (Foy et al., 1978; Chaney, 1993), and the Zn concentration in plants can reach toxic levels (Broadley et al., 2007). Zn is a major industrial pollutant of terrestrial and aquatic environments (Barak & Helmke, 1993) in most regions of the world, including North and South America, Europe, Africa and Asia (Forstner, 1995). Zn toxicity occurs when leaf concentrations reach 400–500 μg g⁻¹ dry weight (Marschner, 1995). Zn toxicity includes symptoms similar to those of cadmium (Cd) or lead (Pb) toxicity (Foy et al., 1978; Larbi et al., 2002; Fodor et al., 2005). Excess Zn reduces growth, leading to stunted plants (Horler et al., 1980; Sagardoy et al., 2009) and produces changes in root growth and morphology (Vaillant et al., 2005; Broadley et al., 2007). Young leaf chlorosis in plants grown in high Zn has been suggested to be caused by deficiencies in iron (Fe) or magnesium (Mg) (Marschner, 1995; Sagardoy et al., 2009). The ratio chlorophyll (Chl) \( a : Chl \ b \) has been found to decrease with Zn toxicity (Monnet et al., 2001; Schuerger et al., 2003; Vaillant et al., 2005), suggesting that Chl \( a \) is more affected than Chl \( b \). However, in other species, leaves of Zn-treated plants may remain green when Zn concentrations are high, suggesting that inhibition of leaf expansion could have a greater effect than Chl breakdown or reduced biosynthesis (Horler et al., 1980; Sagardoy et al., 2009). Therefore, the effects of Zn excess in plants are highly variable, depending on the Zn concentration and the species in question.

Zn toxicity may inhibit photosynthesis at various steps and through different mechanisms. Generally, Zn toxicity decreases net photosynthesis (\( A_{v} \)) (Van Assche et al., 1980; Vaillant et al., 2005; Dhir et al., 2008; Mateos-Naranjo et al., 2008), but it is a subject of debate whether this decrease is limited by stomatal conductance (\( g_{s} \)), mesophyll conductance (\( g_{m} \)), increased respiration, decreased photochemistry, impaired biochemistry, or a combination of several of these factors (Sharma et al., 1995; Di Baccio et al., 2009; Shi & Cai, 2009). It has been observed that high Zn concentrations could cause blockage of xylem elements (Robb et al., 1980), large decreases in root biomass (Vaillant et al., 2005) and decreases in leaf water content (Bonnet et al., 2000), all of which may lead to impaired \( g_{s} \), in turn decreasing transpiration rates and photosynthesis (Schuerger et al., 2003; Vaillant et al., 2005; Sagardoy et al., 2009). Increased mesophyll resistance to CO\(_{2}\) diffusion has also been suggested to occur with Zn excess (Van Assche et al., 1980; Prasad & Strzalka, 1999). However, the effects of Zn toxicity on mesophyll diffusion conductance to CO\(_{2}\) (\( g_{m} \)) have only been assessed using a parameter that combines \( g_{m} \) and carboxylation activity. To date, no study has analyzed the effects of Zn toxicity on \( g_{m} \), in spite of the fact that Zn is a cofactor of carbonic anhydrases (CAs), which facilitate \( g_{m} \) by catalyzing the interconversion between CO\(_{2}\) and HCO\(_{3}^{-}\) in the weakly alkaline chloroplast stroma (Evans et al., 2009).

Respiratory metabolism also interacts with photosynthesis, and respiratory mutants (especially those with altered alternative oxidase (AOX) activity) show decreased photosynthesis, as well as decreased \( g_{s} \) and \( g_{m} \) (reviewed by Flexas et al., 2008). The effects of Zn excess on respiration are a matter of controversy. On the one hand, in vitro studies have suggested that Zn inhibits the activity of the mitochondrial cytochrome bc1 complex, probably by interfering with the enzyme’s reaction with ubiquinol at the UQH\(_{2}\) binding niche that oxidizes UQH\(_{2}\), the quinol oxidase (\( Q_{o} \) site (Link & von Jagow, 1995), as well as the activity of AOX (Affourtit & Moore, 2004). On the other hand, in vivo studies have shown that respiration is enhanced progressively with increasing Zn doses (Ismail & Azooz, 2005), and use of inhibitors of cytochrome bc1 and AOX has suggested that there is a specific increase in AOX activity (Webster & Gadd, 1999), although, to the best of our knowledge, no report on the in vivo activity of AOX under Zn excess has been published. Regardless of the mechanisms involved, Zn-induced alterations in respiration would result from metabolic effects that could potentially reduce net photosynthesis.

The effects of Zn excess on the photochemistry and biochemistry of photosynthesis are also a matter of controversy. In some studies, the quantum yield of photosystem II (PSII)-related electron transport, estimated at low photosynthetic photon flux density (PPFD), was not affected in Zn-treated plants (Van Assche & Clijsters, 1986a), in agreement with studies showing only small decreases (from 0.82 to 0.70) of the maximum potential PSII efficiency (\( F_{v}/F_{m} \)) under Zn excess (Schuerger et al., 2003; Dhir et al., 2008; Sagardoy et al., 2009). However, other studies have shown an inhibition of thylakoid electron transport rates (ETRs) by high Zn concentrations (Kim & Jung, 1993). Specific effects on PSII photochemistry, related to competitive substitution of manganese (Mn) by Zn at the site of water photolysis inhibiting photosynthetic electron transport and oxygen evolution, have also been reported (Van Assche & Clijsters, 1986a; Ralph & Burchett, 1998). Consistent with this, Zn excess was found to decrease PSII efficiency (\( \Phi_{PSII} \)) and noncyclic photophosphorylation (Van Assche & Clijsters, 1986a; Bonnet et al., 2000; Schuerger et al., 2003; Sagardoy et al., 2009), although, alternatively, \( \Phi_{PSII} \) could be limited by excess Zn-mediated reductions in dark photosynthetic processes. Other studies have shown no changes in the ADP : ATP and NADP\(^{+} \) : NADPH ratios with changes in Zn concentration (Dhir et al., 2008). In some studies, the decrease in \( F_{v}/F_{m} \) was associated with increased levels of minimal Chl fluorescence in the dark (\( F_{o} \)) (Vaillant et al., 2005), suggesting PSII photoinactivation or photooxidation, consistent with the observation that lipid peroxidation was found to be enhanced in plants treated with Zn excess.
The activity of antioxidant enzymes such as Mn-superoxide dismutase (Mn-SOD) and ascorbate peroxidase (APX) increased in plants exposed to high concentrations of Zn (del Río et al., 1985; Chaoui et al., 1997; Bonnet et al., 2000). Catalase (CAT) activity was decreased in high-Zn plants (Chaoui et al., 1997; and references therein), which may suggest decreased photorespiratory activity. However, photosynthetic ETR: $A_N$ ratios increased under Zn excess (from c. 9 in controls to 31–77; recalculated from Monnet et al., 2001), which is probably indicative of increased electron consumption diverted to photorespiration or to alternative processes.

Regarding other photoprotection mechanisms, leaf reflectance increases under Zn toxicity (Horler et al., 1980). Sagardoy et al. (2009) reported low xanthophyll cycle activity, with low de-epoxidation values, under Zn excess. While the latter may suggest that the formation of the thylakoid pH gradient may be impaired under excess Zn, Dhir et al. (2008) showed an increase in trans-thylakoid ΔpH under Zn toxicity as a consequence of increased activity of photosystem I (PSI). Finally, specific effects on Rubisco carboxylase capacity (Van Assche & Clijsters, 1986b; Monnet et al., 2001; Mateos-Naranjo et al., 2008) and the Calvin cycle (Chaney, 1993) have been reported, although other studies have reported only slight Zn effects on Rubisco (Van Assche et al., 1980; Dhir et al., 2008).

The aim of this work was to investigate the effects of Zn toxicity on photosynthesis and respiration rates in the model plant sugar beet (Beta vulgaris). A complete analysis of photosynthetic activity has been used to separate stomatal, mesophyll and biochemical contributions to the reductions in photosynthesis (Grassi & Magnani, 2005), similar to those used for water stress and recovery (Grassi & Magnani, 2005; Galmés et al., 2007a;b; Flexas et al., 2009; Gallé et al., 2009) and leaf ageing (Grassi & Magnani, 2005; Flexas et al., 2007, 2009).

Materials and Methods

Plant material and growth conditions

Sugar beet (Beta vulgaris L. ‘Orbis’) seeds were germinated and grown in vermiculite for 3 wk, in a growth chamber at a constant temperature (25°C) and a PPFD of 600 μmol m$^{-2}$ s$^{-1}$ with a 16 h : 8 h light/dark regime. Plants were then moved to a greenhouse, and transplanted into 20-l plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution (Terry, 1980) with 45 μM Fe(III)-EDTA. Once plants were established, experiments with different concentrations of Zn were initiated. Zn (ZnSO$_4$) concentrations were 1.20 μM (control), 100 μM and 300 μM. Treatments used were based on a previous study (Sagardoy et al., 2009). Half-strength Hoagland’s solution was added, when necessary, several times throughout the experiment. Plants were used for measurements 10–14 d after the treatments were imposed. Measurements were performed in young, fully expanded leaves, 3 to 4 h into the light period.

Growth parameters

At the end of the experiment, 14 d after the start of the treatments, plants were collected and separated into roots and shoots. Fresh weights (FWs) and dry weights (DWs) were determined for each fraction, and water content (WC) was calculated as (FW – DW)/FW. Leaf areas were measured with an AM-100 leaf area meter (ADC, Herts, UK). Eight plants per treatment were used.

Mineral analysis

For nutrient analysis, plant tissues were washed with pure water and dried in an oven at 60°C for 76 h to constant weight. Samples were then dry-ashed and dissolved in HNO$_3$ and HCl following the Association of Official Analytical Chemists (AOAC) procedure (Association of Official Analytical Chemists, Washington, DC, USA); Zn was determined by flame atomic absorption spectroscopy (FAAS) (Igartua et al., 2000).

Gas exchange and chlorophyll fluorescence measurements

Stomatal conductance ($g_s$) was estimated nondestructively with a portable leaf porometer (SC-1; Decagon Devices, Pullman, WA, USA). Porometry was used for rapid assessment of stomatal status throughout the experimental period, in order to ensure all experiments were performed under similar initial experimental conditions.

Leaf gas exchange and Chl fluorescence were measured simultaneously using an open gas exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) with an integrated Chl fluorescence chamber head (Li-6400-40 leaf chamber fluorometer; Li-Cor, Inc.) according to Flexas et al. (2007). These measurements were typically carried out on days 10–13 after the start of the treatments. All measurements were performed at 25°C and 1500 μmol photons m$^{-2}$ s$^{-1}$ (10% blue light). The reference CO$_2$ concentration ($C_i$) was set at 400 μmol CO$_2$ mol$^{-1}$ air, and vapor pressure deficit (VPD) was kept at 2.0 ± 0.2 kPa.

In addition to net photosynthesis ($A_N$) and $g_s$, the sub-stomatal CO$_2$ concentration ($C_i$) was calculated. The possible occurrence of $C_i$ overestimation was evaluated in preliminary experiments in all treatments as follows. Average $g_s$ values were found to be much higher (data not shown) than those typically causing cuticular-associated $C_i$ overestimations (Boyer et al., 1997; Flexas et al., 2009). Also, leaf Chl fluorescence images (obtained using a Fluor-
CAM; PSI Instruments, Brno, Czech Republic; data not shown) demonstrated that patchiness did not occur, indicating that heterogeneous stomata closure did not cause errors in the calculation of Ci (Terashima, 1992).

The incorporated fluorometer allowed determination of the actual PSII efficiency ($\Phi_{\text{PSII}} = (F'_{m} - F')/F'_{\text{ref}}$, where $F'$ and $F'_{m}$ are the steady-state and maximum Chl fluorescentes, respectively) (Genty et al., 1989). $F'_{m}$ was determined with a light-saturating pulse of c. 8000 µmol photons m$^{-2}$ s$^{-1}$.

The ETR was then calculated as $\Phi_{\text{PSII}} \times$ PPFD $\times$ $\alpha$. In this equation, $\alpha$ (a term that includes the product of leaf absorbance and the partitioning of absorbed quanta between PSI and PSII) was determined for each treatment as the slope of the relationship between PSI and PSII absorbance and the partitioning of absorbed quanta (after Bernacchi et al., 2004). $C$ measurements at saturating light, whereas $C$ was taken as in Florez-Sarasa et al. (2007). The electron partitioning through the alternative oxidase pathway ($\tau_\text{o}$) was calculated as $(\Delta n - \Delta c)/(\Delta a - \Delta c)$, where $\Delta n$, $\Delta c$, $\Delta a$ are the oxygen isotopic fractionation in the absence of inhibitors, in the presence of SHAM (hydroxy-salicylic acid) and in the presence of KCN, respectively. For inhibitor treatments, leaf discs were incubated in the presence of 10 mM KCN for 30 min and the $\Delta a$ value obtained was 32‰. As cytochrome oxidase pathway discrimination has been shown to be remarkably constant in several species (Ribas-Carbo et al., 2005), a $\Delta c$ value of 20.0‰ was used. The individual activities of the cytochrome oxidase ($v_{\text{cyt}}$) and alternative oxidase ($v_{\text{alt}}$) pathways were obtained from the total oxygen uptake rate ($V_o$) and $\tau_o$ as $V_o(1 - \tau_o)$ and $V_o\tau_o$, respectively.

Four discs per treatment were collected for respiratory measurements 10–12 d after Zn treatments were imposed.

**Respiratory measurements**

An isotope ratio mass spectrometer (Delta Plus XP; Thermo LCC, Bremen, Germany) with a dual-inlet system from the Serveis Científico Tècnicos of the Universitat de les Illes Balears (UIB) was used for respiratory measurements. Analysis of respiration and oxygen-isotope fractionation was performed in 10-cm$^2$ leaf discs at a controlled temperature of 25°C as in Florez-Sarasa et al. (2007). The electron partitioning through the alternative oxidase pathway ($\tau_\text{o}$) was calculated as $(\Delta n - \Delta c)/(\Delta a - \Delta c)$, where $\Delta n$, $\Delta c$, $\Delta a$ are the oxygen isotopic fractionation in the absence of inhibitors, in the presence of SHAM (hydroxy-salicylic acid) and in the presence of KCN, respectively. For inhibitor treatments, leaf discs were incubated in the presence of 10 mM KCN for 30 min and the $\Delta a$ value obtained was 32‰. As cytochrome oxidase pathway discrimination has been shown to be remarkably constant in several species (Ribas-Carbo et al., 2005), a $\Delta c$ value of 20.0‰ was used. The individual activities of the cytochrome oxidase ($v_{\text{cyt}}$) and alternative oxidase ($v_{\text{alt}}$) pathways were obtained from the total oxygen uptake rate ($V_o$) and $\tau_o$ as $V_o(1 - \tau_o)$ and $V_o\tau_o$, respectively.

Experiments on stomatal conductance responses and application of the Ball–Woodrow–Berry model

Stomatal conductance responses were studied with the Li-6400 gas exchange system using three different treatments: leaf desiccation (DES treatment); exogenous application of abscisic acid (ABA treatment); and changes in relative humidity (RH treatment). The DES treatment was carried out by cutting the leaf petiole in air and letting the leaf desiccate for ≥ 90 min under ambient conditions. Measurements were taken every 1 min, using four leaves per treatment. Exogenous application of ABA consisted of a single application of 100 µM ABA (ABA was previously dissolved in MeOH) to the plant nutrient solution. Measurements were taken in attached leaves for 4 d after the start of the treatment, using four replicates per treatment. In the RH treatment, 70%, 50% and 30% RH values were used. Measurements were carried out once $g_s$ had reached a steady-state
level (typically 20–40 min after changing the RH value), using four leaves per treatment. DES and RH experiments were carried out in plants not treated with ABA.

To assess the effects of Zn stress on the stomatal conductance response to environmental conditions and its coupling to photosynthesis, the model proposed by Ball et al. (1987) was used, plotting \( g_s \) vs \( \frac{(A_N H_S)}{C_i} \), where \( A_N \) is the net photosynthesis rate (in \( \mu mol CO_2 \cdot m^{-2} \cdot s^{-1} \)), \( C_i \) is the CO2 concentration at the leaf surface (\( \mu mol \cdot mol^{-1} \)) and \( H_S \) is the RH value (%). Data obtained from the \( A_N-C_i \) curves and RH and ABA measurements (9–25 data points depending on the treatment) were used.

**Xylem sap collection**

In order to analyze ABA concentration, sugar beet xylem sap was obtained using leaf petioles as described elsewhere (López-Millán et al., 2000). Malate dehydrogenase (MDH, EC 1.1.1.37) was used as a cytosolic contamination marker by checking the activity in xylem sap against the corresponding activities in petiole total homogenates.

Xylem from six plants per treatment was analyzed for ABA following the procedures described in Albacete et al. (2008) on a high-performance liquid chromatography (HPLC)/mass spectrometry (MS) system (CEBAS-CSIC, Murcia, Spain).

**Enzyme assays**

Extracts used for the measurement of carbonic anhydrase (CA, EC 4.2.1.11) activity were prepared by grinding fresh leaf discs (0.95 cm²) in 1 ml of extraction buffer (50 mM HEPES-NaOH, pH 8.3, 0.5 mM EDTA, 10 mM dithiothreitol (DTT), 10% (v/v) glycerol and 1% (v/v) Triton X-100) at 4°C. Extracts were centrifuged at 2400 \( g \) for 10 min at 4°C. Supernatants were put in Eppendorf tubes and frozen at −20°C until assayed. CA activity was measured using a method adapted from that of Gillon & Yakir (2000). Assays were carried out in 7-ml flat-bottom glass vials placed on ice with continuous stirring. A volume of 4.5 ml of reaction buffer (Na-Barbital 20 mM, pH 8.3) was supplemented with 75 μl of extract, and 1.5 ml of CO₂-saturated water (at 0°C) was added to start the reaction. CA activity was obtained from the reaction time of pH change from 8.3 to 7.3.

**Scanning electron microscopy**

Leaf samples were taken from sugar beet plants grown according to Sagardoy et al. (2009), and electron microscopy was used to study leaf surfaces (scanning electron microscopy (SEM); ICB-CSIC, Zaragoza, Spain) and cryo-fractured leaf pieces (low temperature-SEM (LT-SEM); Cryotrans CT-1500, Oxford, UK). A Hitachi S-3400 N microscope (Krefeld, Germany) was used to visualize hydrated leaf surfaces. Fresh pieces were trimmed to an adequate size, mounted on stubs and observed directly (uncoated) with an accelerating voltage of 1 kV. Also, fresh leaf sections were mounted on aluminum stubs with adhesive (Gurrs, O.C.T. B.D.H, UK; Gurr®, OCT compound, BDH, Poole, UK), cryofixed in slush nitrogen (−196°C), cryotransferred to a vacuum chamber at −180°C, and fractured using a stainless steel spike. Once inside the microscope, samples underwent superficial etching in a vacuum (at −90°C and 2 kV for 120 s), and were overlaid with gold (Au) for observation. Fractured samples were observed at low temperature using a Zeiss digital scanning microscope (DSM 960), employing secondary and back-scattered electrons (SEM-BSE).

Hydrated leaf surfaces were analyzed by measuring stomatal density and pore size on the adaxial and abaxial sides of the leaf. Stomatal density was calculated from SEM images by measuring the number of stomata in a given leaf surface. Stomatal density data are the mean ± SE of 10 (adaxial) and three (abaxial) images in each treatment. Pore size was measured manually using the Adobe Photoshop CS3 image analysis software. Pore size data are the mean ± SE of 50 (adaxial) and 25 (abaxial) stomata from the same images used in stomatal density measurements.

The spaces occupied by intercellular air spaces and sub-stomatal cavities were quantified using the SEM images (nine images from control leaves and eight from 300 μM Zn-grown plants) and the ImageJ Image Processing and Analysis software from the Wright Cell Imaging Facility (http://www.uhnresearch.ca/facilities/wcif). Chloroplast size was measured manually in each SEM image by counting the number of pixels occupied by the axis of the chloroplast relative to the scale, using the Adobe Photoshop CS3 image analysis software (for 18 chloroplasts each in the control and high-Zn treatments, using nine and eight images, respectively).

**Protoplast and chloroplast isolation and size measurements**

Intact protoplasts and chloroplasts were isolated as described in González-Vallejo et al. (2000) and using the chloroplast isolation kit (Sigma), respectively, and measured using the Adobe Photoshop CS3 image analysis software. Data shown are for 40 protoplasts and 30 chloroplasts in each treatment (obtained using eight different images in each case).

**Results**

**Growth parameters**

High Zn concentrations in the nutrient solution reduced whole-plant DW and leaf area (Table 1), and also shoot and root DW (data not shown) in sugar beet plants grown in a
growth chamber conditions (Sagardoy et al., 2009). However, although there was a tendency for some parameters to decrease with excess Zn, Duncan analysis showed no significant differences at P < 0.05 in \( F_{v}/F_{m} \) (\( P < 0.061 \)), ETR (\( P < 0.056 \)) and \( \Phi_{PSII} \) (\( P < 0.056 \)) (Table 2). There were significant differences in \( F_{v}/F_{m} \) only when Tukey’s analysis (\( P < 0.027 \)), a softer statistical test, was used. Stomatal conductance (\( g_{s} \)) was reduced by 70% in excess Zn, whereas mesophyll conductance was reduced by 44%, and in both cases differences between the 100 and 300 \( \mu M \) Zn treatments were not significant. Similar \( g_{s} \) changes were observed when using a leaf porometer (Fig. 1).

The slope of the relationship between \( \Phi_{PSII} \) and \( \Phi_{CO2} \) (\( \alpha \)) was found to be 0.44 in all Zn treatments (not shown). The intercept of the relationship, which indicates the amount of electrons channeled to alternative sinks (Laisk & Loreto, 1996; Long & Bernacchi, 2003), was very close to zero, being slightly negative in the controls (−0.024) and in plants grown at intermediate Zn concentrations (−0.031) and positive but of similar magnitude (0.025) in the 300 \( \mu M \) Zn-grown plants (not shown). As a negative \( \Phi_{PSII} \) is not possible, these differences are mere statistically non-significant deviations from the origin.

### Photosynthetic parameters and respiratory measurements

Leaves of plants grown in high Zn showed 50% decreases in photosynthetic rate (\( A_{N} \)), with no significant differences between the 100 and 300 \( \mu M \) Zn treatments (Table 2). However, different zinc (Zn) concentrations in sugar beet plants grown in hydroponics with different zinc (Zn) concentrations

Table 1 Growth parameters, water content (WC) and zinc (Zn) concentrations in sugar beet plants grown in a glasshouse in hydroponics with different Zn concentrations for 14 d

<table>
<thead>
<tr>
<th>Zn treatment</th>
<th>1.2 ( \mu M )</th>
<th>100 ( \mu M )</th>
<th>300 ( \mu M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW (g per plant)</td>
<td>2.8 ± 0.4 ( ^{a} )</td>
<td>1.3 ± 0.2 ( ^{b} )</td>
<td>0.8 ± 0.2 ( ^{b} )</td>
</tr>
<tr>
<td>Area per leaf (cm(^{2} ))</td>
<td>136.7 ± 5.5 ( ^{a} )</td>
<td>48.3 ± 5.2 ( ^{b} )</td>
<td>29.8 ± 3.0 ( ^{b} )</td>
</tr>
<tr>
<td>WC (%)</td>
<td>96.8 ± 0.2 ( ^{a} )</td>
<td>91.1 ± 0.1 ( ^{b} )</td>
<td>87.6 ± 0.3 ( ^{c} )</td>
</tr>
<tr>
<td>Zn in roots (( \mu g ) g(^{-1} ) DW)</td>
<td>136.1 ± 15.0 ( ^{a} )</td>
<td>218.4 ± 2.9 ( ^{b} )</td>
<td>202.9 ± 0.1 ( ^{i} )</td>
</tr>
<tr>
<td>Zn in shoots (( \mu g ) g(^{-1} ) DW)</td>
<td>129.7 ± 11.9 ( ^{a} )</td>
<td>1223.7 ± 64.5 ( ^{b} )</td>
<td>1184.3 ± 102.7 ( ^{b} )</td>
</tr>
</tbody>
</table>

Data are the mean ± SE of eight replicates.

Different letters indicate significant differences (Duncan’s test) at \( P < 0.05 \).
At the measuring CO₂ concentration (400 µmol CO₂ mol⁻¹ air), Ci and Cc were lower (30–38% and 35–48%, respectively) in plants grown in an excess of Zn, although no differences were found between the two high Zn concentrations (Table 2). The AN vs Ci and AN vs Cc curves also showed that, in excess Zn, Ci reached values of 700–800 µmol CO₂ mol⁻¹ air, whereas Cc never reached values of 400 µmol CO₂ mol⁻¹ air (Fig. 2). Analysis of the data shown in Fig. 2 indicated that there were no significant differences between treatments in slope (Vc,max); P < 0.198) and (Jmax), although the latter parameter could not be calculated in the 300 µM Zn treatment (Table 2).

The AN vs Ci curve for Zn-treated plants saturated at values below the saturation values for control plants, which would appear to reflect a biochemical limitation that is not associated with CO₂ availability. However, when AN was expressed on a Cc basis, CO₂ availability was found to be clearly responsible for the decreased photosynthesis. Data from the limitation analysis also showed that biochemical limitations were negligible in excess Zn-grown plants (Table 3). In other words, the ‘apparent’ biochemical limitation observed in the AN vs Ci curves can be fully explained by the decreased mesophyll conductance in excess Zn-treated plants.

Similar to Grassi & Magnani, (2005) the control treatment can be defined as the actual reference where all three parameters (gs, gm and Vc,max) were at their maximum: gs and gm declined in response to Zn treatment, whereas Vc,max did not change significantly (although it tended to increase; Table 2). Therefore, the same Vc,max value was used for all treatments, so that all biochemical limitations (BL) were 0 (Table 3). Using the AN, gs, gm and Vc,max values found, stomatal conductance limitation (SL) was estimated to account for 79%–86% of the total limitation (TL), whereas nonstomatal limitations (NSL = MCL + BL) were only 14% and 21% in the 100 and 300 µM Zn-treated plants, respectively (Table 3). The implications of the fact that Vc,max values tended to increase with excess Zn concentration (400 µmol CO₂ mol⁻¹ air), Ci and Cc were lower (30–38% and 35–48%, respectively) in plants grown in an excess of Zn, although no differences were found between the two high Zn concentrations (Table 2). The AN vs Ci and AN vs Cc curves also showed that, in excess Zn, Ci reached values of 700–800 µmol CO₂ mol⁻¹ air, whereas Cc never reached values of 400 µmol CO₂ mol⁻¹ air (Fig. 2). Analysis of the data shown in Fig. 2 indicated that there were no significant differences between treatments in slope (Vc,max); P < 0.198) and (Jmax), although the latter parameter could not be calculated in the 300 µM Zn treatment (Table 2).

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Zn merit some consideration. Although mathematically one could calculate a negative limitation, such a value would imply the alleviation of a pre-existing limitation in the control plants. But, if control plants had some limitation, then the net photosynthesis displayed by them would not be really maximal and therefore control plants could not be used as a reference. Establishing a hypothetical optimal state may be uncertain, as maximum values in all three parameters (\(g_s\), \(g_m\) and \(V_{c,max}\)) would result in net photosynthesis values higher than any of those measured but still unknown; that is, the total limitation could not be properly stated and hence a limitation analysis would not be possible. Because of this, even when \(V_{c,max}\) values were (non-significantly) higher in treated than control plants, BL was considered null and not negative, as explained by Flexas et al. (2009). Nevertheless, we also performed the analysis allowing negative limitations to occur. The results were identical in 100 \(\mu M\) plants, whereas in 300 \(\mu M\) plants SL, MCL and BL become 49, 6 and -7, respectively, as compared with 42, 5 and 0 when BL was forced to 0. In other words, the main conclusion remains the same, that is, that SL was by far the most important limitation and BL did not occur in response to high Zn.

Stomatal conductance experiments

The response of stomatal conductance was studied by inducing stomatal closure, in a short-term (DES) and a long-term (ABA) treatment, and also by using different vapor pressure deficits (RH treatment). After detachment, \(g_s\) increased transiently for a few minutes in control plants. This transient \(g_s\) increase was smaller at 100 \(\mu M\) Zn and absent at 300 \(\mu M\) Zn (Fig. 3a). These data show the progressive inability of stomata in Zn-treated plants to respond to hydraulic stimuli. In the long-term experiments with ABA, \(g_s\) decreased markedly in the controls whereas in plants grown in 300 \(\mu M\) Zn it was not affected (Fig. 3b). Also, when relative humidity was reduced, \(g_s\) decreased in the controls but not in the two high-Zn treatments (Fig. 3c).

The Ball–Berry model (\(g_s\) vs \((A_{N,H})/C_o\)) distinguished clearly between control and 300 \(\mu M\) Zn-treated plants (Fig. 4a). However, when ABA was used, stomata closed in the controls and the data for controls and 300 \(\mu M\) Zn-treated plants had similar slopes (Fig. 4b).

Biochemical parameters and respiratory measurements

CA activity in leaves was similar in control and 100 \(\mu M\) Zn-treated plants, whereas in plants grown at 300 \(\mu M\) Zn it increased markedly (by 80%; Table 4). Xylem ABA concentrations were reduced by 70% at 100 \(\mu M\) Zn compared with the controls, whereas at 300 \(\mu M\) Zn changes were not statistically significant (Table 4).

Leaf respiration increased 2-fold in both high-Zn treatments when compared with the control. The increase was more marked for the cytochrome oxidase pathway (COP;
plants grown in hydroponics with different zinc (Zn) concentrations in xylem sap from sugar beet (Ismail & Azooz, 2005; Vaillant et al., 2008). In a previous study, we described changes in gas exchange properties and photosynthetic pigments in sugar beet grown under high Zn at lower PPFDs than those used in the present study (Sagardoy et al., 2009).

Discussion

The aim of the present study was to investigate the causes of the decrease in photosynthesis that occurs under Zn stress. Both 100 and 300 μM ZnSO₄ resulted in high Zn concentrations in roots and shoots, far above the leaf critical toxicity concentrations (400–500 μg g⁻¹ DW; Marschner, 1995), with shoot Zn concentrations being similar in the 100 and 300 μM Zn treatments. Both biomass and photosynthetic rates decreased markedly in Zn-treated plants compared with the controls. Decreased growth and photosynthesis under excess Zn have already been described for other species (Ismail & Azooz, 2005; Vaillant et al., 2005; Mateos-Naranjo et al., 2008). In a previous study, we described changes in gas exchange properties and photosynthetic pigments in sugar beet grown under high Zn at lower PPFDs than those used in the present study (Sagardoy et al., 2009).

Table 4 Carbonic anhydrase (CA) activity in leaf extracts and abscisic acid (ABA) concentration in xylem sap from sugar beet plants grown in hydroponics with different zinc (Zn) concentrations

<table>
<thead>
<tr>
<th>Zn treatment</th>
<th>1.2 μM</th>
<th>100 μM</th>
<th>300 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA (μmol CO₂ m⁻² s⁻¹)</td>
<td>305.5 ± 12.3 a</td>
<td>328.8 ± 29.6 a</td>
<td>550.5 ± 44.7 b</td>
</tr>
<tr>
<td>ABA (ng ml⁻¹)</td>
<td>111.4 ± 22.7 a</td>
<td>33.5 ± 5.0 b</td>
<td>63.6 ± 7.1 ab</td>
</tr>
</tbody>
</table>

Data are the mean ± SE of five and six replicates, respectively. Different letters indicate significant differences (Duncan’s test) at P < 0.05.

2.3-fold) than for the alternative oxidase pathway (AOP; 1.8-fold) respiration pathway (Table 5).

Scanning electron microscopy images

Leaf surface samples were scanned at ×300 and ×3000. Both the adaxial (Fig. 5c) and abaxial (Fig. 5d) epidermis of high-Zn plants had a smoother appearance than those of control plants (Fig. 5a,b). Moreover, excess Zn induced a decrease in both stomatal density and size (Table 6, Fig. 5). A closer look at stomata showed structural differences, and plants grown at 300 μM Zn (Fig. 6c,d; adaxial and abaxial sides, respectively) had rounder stomata and smaller stomatal slits than the control plants (Fig. 6a,b; adaxial and abaxial sides, respectively). In some cases, the stomatal slit of high-Zn leaves appeared to be completely sealed.

Mesophyll anatomical features and the arrangement of chloroplasts around the mesophyll cell plasma membrane were different in plants grown under high Zn concentrations than in the controls. LT-SEM showed a more compact mesophyll tissue in Zn-treated plants (Fig. 7c) than in the controls (Fig. 7a), with smaller cells. Isolated protoplasts were smaller in 300 μM high-Zn plants than in controls (33 ± 1 vs 46 ± 3 μm, respectively; n = 40). Also, intercellular spaces and abaxial and adaxial substomatal cavities were smaller in the high-Zn leaves. The space occupied by intercellular air spaces decreased by 31% in the highest Zn treatment, from 9.8% (controls) to 6.8% (300 μM Zn-grown plants) of the whole mesophyll, whereas that occupied by the substomatal cavities decreased by 27%, from 5.2% (controls) to 3.8% (300 μM Zn-grown plants) of the mesophyll.

Chloroplast length was reduced in high-Zn leaves (Fig. 7d) compared with controls (Fig. 7b); data obtained from the SEM images were 2.8 ± 0.2 vs 3.9 ± 0.1 μm (n = 18) in 300 μM high-Zn and control plants, respectively. Isolated chloroplasts were also smaller in high-Zn plants (3.0 ± 0.2 μm) than in the controls (4.5 ± 0.3 μm) (images not shown; n = 30). High Zn concentrations reduced the apparent adherence of chloroplasts to the mesophyll cell plasma membrane (Fig. 7d) compared with control plants (Fig. 7b).

Discussion

The aim of the present study was to investigate the causes of the decrease in photosynthesis that occurs under Zn stress. Both 100 and 300 μM ZnSO₄ resulted in high Zn concentrations in roots and shoots, far above the leaf critical toxicity concentrations (400–500 μg g⁻¹ DW; Marschner, 1995), with shoot Zn concentrations being similar in the 100 and 300 μM Zn treatments. Both biomass and photosynthetic rates decreased markedly in Zn-treated plants compared with the controls. Decreased growth and photosynthesis under excess Zn have already been described for other species (Ismail & Azooz, 2005; Vaillant et al., 2005; Mateos-Naranjo et al., 2008). In a previous study, we described changes in gas exchange properties and photosynthetic pigments in sugar beet grown under high Zn at lower PPFDs than those used in the present study (Sagardoy et al., 2009).
The most important effect of high Zn on photosynthetic parameters was a 70% decrease in stomatal conductance, with mesophyll conductance decreasing by 44%, whereas other possible causes for photosynthetic rate decreases such as PSII photochemistry were not significantly affected. A photosynthesis limitation analysis (Grassi & Magnani, 2005) revealed that, of a total photosynthesis limitation of 42–48% under excess Zn, up to 38–42% could be accounted for by SLs and only 4–5% could be accounted for by MCLs, whereas significant BLs did not occur. Furthermore, the decrease in stomatal conductance was caused by physical and/or structural stomatal changes, whereas hydraulic and chemical signaling, which usually control stomatal closure (Christmann et al., 2005, 2007), were not involved. Stomata of high Zn-treated plants did not respond at all to either chemical or hydraulical signals, and the concentration of ABA in the xylem was decreased rather than increased under excess Zn, indicating that stomatal closure was not mediated by ABA signals. Also, \( g_s \) in Zn-treated plants was unaffected by exogenous ABA and changes in VPD. In summary, Zn-treated plants showed a stomatal closure similar to that of control plants supplied

<table>
<thead>
<tr>
<th>Zn treatment</th>
<th>1.2 ( \mu \text{M} ) adaxial</th>
<th>1.2 ( \mu \text{M} ) abaxial</th>
<th>300 ( \mu \text{M} ) adaxial</th>
<th>300 ( \mu \text{M} ) abaxial</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_t ) (( \mu \text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1} ))</td>
<td>0.37 ± 0.04 (^a)</td>
<td>0.81 ± 0.02 (^b)</td>
<td>0.80 ± 0.05 (^b)</td>
<td>0.72 ± 0.02 (^b)</td>
</tr>
<tr>
<td>( v_{\text{cyt}} ) (( \mu \text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1} ))</td>
<td>0.25 ± 0.03 (^a)</td>
<td>0.59 ± 0.02 (^b)</td>
<td>0.58 ± 0.04 (^b)</td>
<td>0.57 ± 0.02 (^b)</td>
</tr>
<tr>
<td>( v_{\text{alt}} ) (( \mu \text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1} ))</td>
<td>0.12 ± 0.02 (^a)</td>
<td>0.22 ± 0.01 (^b)</td>
<td>0.23 ± 0.01 (^b)</td>
<td>0.21 ± 0.01 (^b)</td>
</tr>
</tbody>
</table>

Data are the mean ± SE of four replicates. Different letters indicate significant differences (Duncan’s test) at \( P < 0.05 \).

Table 6 Stomatal density and pore size in the abaxial and adaxial epidermis of hydroponically grown control and 300 \( \mu \text{M} \) zinc (Zn)-grown sugar beet plants

<table>
<thead>
<tr>
<th>Zn treatment</th>
<th>1.2 ( \mu \text{M} ) adaxial</th>
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<th>300 ( \mu \text{M} ) adaxial</th>
<th>300 ( \mu \text{M} ) abaxial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (stomata mm(^{-2}))</td>
<td>218 ± 7 (^a)</td>
<td>223 ± 3 (^a)</td>
<td>172 ± 5 (^b)</td>
<td>156 ± 12 (^b)</td>
</tr>
<tr>
<td>Pore size (range in ( \mu \text{m} ))</td>
<td>9–22</td>
<td>10–18</td>
<td>7–14</td>
<td>6–15</td>
</tr>
<tr>
<td>Pore size (mean in ( \mu \text{m} ))</td>
<td>14.5 ± 0.3 (^a)</td>
<td>14.0 ± 0.5 (^a)</td>
<td>10.8 ± 0.2 (^b)</td>
<td>10.1 ± 0.4 (^b)</td>
</tr>
</tbody>
</table>

Stomatal density data are the mean ± SE of 10 (adaxial) and three (abaxial) images, and pore size data are the mean ± SE of 50 (adaxial) and 25 (abaxial) stomata from the same images used in stomatal density measurements (Fig. 5). Different letters indicate significant differences (Duncan’s test) at \( P < 0.05 \).

Fig. 5 Images of the leaf surface (magnification ×300) of sugar beet plants grown in hydroponics from control (a, adaxial; b, abaxial) and 300 \( \mu \text{M} \) zinc (Zn) (c, adaxial; d, abaxial) treatments, taken with scanning electron microscopy (SEM), showing alterations in guard cell development caused by excess Zn (lower stomatal density on the leaf surface; numbers are presented in Table 6). Stomatal density data were obtained from 10 (adaxial) and three (abaxial) images for each treatment.
with exogenous ABA, according to a Ball–Woodrow–Berry analysis.

Zn-stressed plants had a lower stomatal frequency and a smaller stomatal size than control plants, and similar characteristics were found in *Phaseolus vulgaris* grown in high Zn (Van Assche et al., 1980). Scanning electron microscopy showed that stomata of 300 µM Zn-grown plants were round in shape and had a shorter slit than the stomata of control plants, and in many cases the slit was apparently sealed with unidentified, wax-like substances. Preliminary experiments (using a 10-s wash with chloroform:MeOH 2 : 1, a 10-s wash with hexane or a sequential combination of the two procedures) confirmed the waxy nature of these substances. Further studies are needed to elucidate the

Fig. 6 Images of stomata (magnification ×3000) of sugar beet plants grown in hydroponics from control (a, adaxial; b, abaxial) and 300 µM zinc (Zn) (c, adaxial; d, abaxial) treatments, taken with scanning electron microscopy (SEM), showing reductions in pore size (numbers are presented in Table 6) and the sealing of some stomata in response to Zn toxicity. Pore size data were obtained from 50 (adaxial) and 25 (abaxial) stomata from the same images used in stomatal density measurements.

Fig. 7 Images of leaf cross-sections (a, c) and mesophyll cell details (b, d) of sugar beet plants grown in hydroponics from control (a, b) and 300 µM zinc (Zn) (c, d) treatments, taken with low-temperature scanning electron microscopy (LT-SEM), showing the excess Zn-mediated reduction in the intercellular spaces in the mesophyll and in the substomatal cavities. We used nine images from control leaves and eight from 300 µM Zn-grown plants, and the images shown are representative of each treatment. Note the different scales in (b) and (d). Light-colored objects on the right in (d) are chloroplasts.
nature of this stomatal seal, and the mechanism for its accumulation under excess Zn.

The causes of the decreased CO₂ mesophyll conductance were also investigated using LT-SEM. High Zn-grown plants had a lower leaf porosity than control plants, with the surface of leaf mesophyll cells being less exposed to intercellular air spaces than those of control leaves. Also, chloroplasts were smaller and the interaction of chloroplasts with cell membranes was hampered as a result of the changes in shape, and both factors would increase the length of the CO₂ diffusion pathway in the cytosol. All these factors have been shown to be determinants of gₛₘ under very different experimental conditions (Sharkey et al., 1991; Flexas et al., 2008; Evans et al., 2009; Li et al., 2009).

The photochemistry-related parameters Fᵥ/Fᵥₒ, ETR and Φₚₛₛᵢ were not decreased significantly under high Zn, consistent with previous observations in sugar beet grown in relatively low PPFDs (Sagardoy et al., 2009) as well as in other species (Van Assche & Clijsters, 1986a; Schuerger et al., 2003; Dhir et al., 2008). This indicates that decreased photosynthesis was not caused by impaired leaf photochemistry. Zn treatments did not induce the operation of alternative sinks for electrons. On the one hand, this evidence comes from the close to 0 intercept of the relationship between gₛₘ and the ETR, which suggests that there were no changes either in leaf absorbance or in energy partitioning between PSI and PSII. Photosynthetic biochemistry was also unaffected by Zn, based on the in vivo estimates of Vₑ,ₘₐₓ and Jₑ,ₘₐₓ. Nevertheless, Zn excess increased the activity of CA, a metalloprotein with Zn in its active center, although only at 300 μM Zn. Zn was previously reported to inhibit CA in vitro at high concentrations (Ivanov et al., 2007). Although it has been suggested that CA is involved in the regulation of mesophyll conductance to CO₂ (Gillon & Yakir, 2000), gₛₘ did not increase under excess Zn, but instead decreased by 44% compared with the control. This decrease, moderate when compared with the much greater decrease in stomatal conductance, may be related to the increase in CA activity.

Dark respiration increased markedly in Zn-treated plants, as observed previously in other species (Ismail & Azooz, 2005). The increase in total respiration was associated with significant increases in the activity of both cytochrome (φᵥ) and alternative (φₐ) pathways. Data obtained in this study are not consistent with previous data suggesting a Zn-induced preferential increase in AOX (Webster & Gadd, 1999), or an inhibitory effect of Zn on AOX (Affourtit & Moore, 2004). The cytochrome pathway is associated with the growth component of respiration and results in high ATP production, whereas AOX is associated with the maintenance component of respiration and results in lower ATP production (Florez-Sarasa et al., 2007). In the case of the sugar beet plants in our study, where growth of high-Zn plants was severely reduced, increased ATP synthesis through increased cytochrome respiration would probably be used to increase ion uptake, exchange and compartmentalization (Lambers et al., 2005), to minimize the impact of Zn toxicity. The direct effect of increased respiration on the decrease in net photosynthesis would have been very small (approx. 3%); assuming that the measured rates of dark respiration also applied during the light period, the increased respiration would have decreased the total reduction in photosynthesis induced by excess Zn from 42% to 39% and from 48% to 45% under 100 μM and 300 μM Zn, respectively. The actual effect was probably even smaller, as the rates of respiration in the light are often lower than in the dark (Priault et al., 2006; Juszczuk et al., 2007).

In conclusion, 100–300 μM Zn resulted in large reductions in sugar beet biomass (> 50%) and photosynthetic rates (40–50%), whereas leaf respiration rates doubled through increased activity of both the cytochrome and alternative pathways, probably resulting in increases in capacities for ion compartmentalization and Zn exclusion. Under excess Zn, stomatal conductance was reduced by 70%, and stomata became insensitive to environmental variables such as leaf water status, exogenously applied ABA and VPD. In high Zn-treated plants, stomata were round in shape and smaller than in control plants and, in many cases, were covered by a wax-like seal of unknown nature. Excess Zn, therefore, affected primarily stomatal conductance, apparently through alterations of guard cell development (lower stomatal density on the leaf surface) and guard cell function. Leaf photochemistry and photosynthetic biochemistry were not significantly affected by high Zn. Mesophyll conductance to CO₂ also showed 44% decreases, despite concomitant 2-fold increases in CA, possibly as a result of changes in mesophyll ultrastructure and chloroplast size and arrangement with respect to the mesophyll cell plasma membrane.

Acknowledgements

This work was supported by Spanish Ministry of Science and Innovation grants AGL2007-61948 (to J.A.) and BFU2008-01072/BFI (to M.R-C.). R.S. was supported by
an I3P-CSIC predoctoral fellowship. We thank P. Pons, M. Truyols and G. Cabot for assistance with growing plants. J. Bota for help with CA measurements, I. Tacchini and F. Pinto for help with SEM and LT-SEM, respectively, and J. M. Andrés for the use of the SEM apparatus. Finally, we would like to thank Dr. Biel Martorell for his technical help with the IRMS and all the staff at the Serveis Científico-Tècnics of the Universitat de les Illes Balears for their help during the running of these experiments.

References


