BIOLOGIA PLANTARUM 54 (3): 502-508, 2010

Leaf structural modifications in *Populus* × *euramericana* subjected to Zn excess

D. DI BACCIO*, A. MINNOCCI and L. SEBASTIANI

BioLabs, Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 33, I-56127 Pisa, Italy

Abstract

In previous experiments elevated but sub-symptomatic applications of Zn (0.1 mM and 1 mM) caused impairments in growth parameters and photosynthetic performance of *Populus* × *euramericana* (Dode) Guinier clone I-214. The aim of this work was to evaluate leaf morphological and anatomical traits in this clone in response to the same Zn concentrations. The results showed that Zn treatments induced variations in leaf dry mass, area, mesophyll thickness, intercellular spaces, stomatal density and size. Stronger modifications, especially concerning stomata characteristics induced by 1 mM Zn, were consistent with physiological impairments while those induced by 0.1 mM Zn suggested a compensatory strategy for maintaining functional integrity.

Additional key words: cryo-scanning electron microscopy, intercellular spaces, mesophyll thickness, poplar, stomatal density.

Introduction

Pollution with heavy metals, including micronutrients such as copper and zinc (Hermle *et al*. 2007, Gatti 2008, Mourato *et al*. 2009, Wang *et al*. 2009), is one of the major environmental problems worldwide. Zinc is an essential metal in trace amounts for plants and organisms, but becomes toxic when present in bioavailable forms at excessive levels, producing dangerous effects on leaf performance and plant growth similar to those induced by cadmium (Lunáčková *et al*. 2003/4, Wójcik and Tukiendorf 2005, Scebba *et al*. 2006, Markovska *et al*. 2009). Although information about the quantitative Zn requirement of forest trees is sparse, the typical Zn concentration required for adequate growth of most crops is 15 - 20 mg kg^{-1}(DM) (Marschner 1995), and in contaminated soils it is easy to reach this threshold (Broadley *et al*. 2007). Zinc inputs to the environment are largely due to anthropogenic sources, such as fossil fuel combustion, mine waste, agrochemicals, particles from galvanized surfaces and rubber mulches (Broadley *et al*. 2007 and references within).

Poplar and willow plantations hold particular promise as renewable energy crops, being fast-growing trees with the ability to tolerate and accumulate high levels of heavy metals such as Zn and Cd, although a significant clonal variation results due to their uptake (Lunáčková *et al*. 2003/4, Laureysens *et al*. 2004, 2005, Wójcik and Tukiendorf 2005, Giachetti and Sebastiani 2006, 2007, Hermle *et al*. 2007). Furthermore, *Populus nigra* has shown to be useful as a biomonitor in heavy metal contaminated regions (Djingova *et al*. 1995). Although these tree species have favourable characteristics as ideal plants for phytoremediation (Sims *et al*. 2006, Shah and Nongkynrih 2007), studies on the physiological and molecular tolerance/defence mechanisms and heavy metal accumulation remain relatively unknown. This situation will soon be improved thanks to the recent genome sequence project of *Populus trichocarpa* (Tuskan *et al*. 2006), and by the fact that *Populus* has now been internationally accepted as a model system for physiological and molecular tree studies.

Poplar clone I-214 is commonly used in Italian plantations and studied for its response/tolerance to heavy metals and air pollution stresses (Sebastiani *et al*. 2004, Tognetti *et al*. 2004, Di Baccio *et al*. 2008). Some

Received 13 September 2008, *accepted* 12 June 2009.

Abbreviations: cryo-SEM - cryo-scanning electron microscopy; DM - dry mass; FH - frozen-hydrated; f_{ias} - fraction of mesophyll volume occupied by intercellular air spaces; FM - fresh mass; GCL - guard cell length; LA - leaf area; LAR - leaf area ratio; LMR - leaf mass ratio; LPI - leaf plastochron index; LTD - leaf tissue density; S_d - stomatal density; SLA - specific leaf area; S_{mes} - area of mesophyll surfaces directly exposed to intercellular air spaces on a leaf area basis; SN - stomata number; V - volume.

Acknowledgements: We thank Dr. Lorenzo Vietto (Agricultural Research Council, Unit for Intensive Wood Production, Casale Monferrato, Alessandria, Italy) for having provided cuttings of I-214 clone. This research was supported by "Ministero degli Affari Esteri, Direzione Generale per la Promozione e la Cooperazione Culturale", Roma, Italy. We are really grateful to Dr. R. Sahai for language revision.

* Corresponding author; fax: (+39) 050 883495, e-mail: dibaccio@sssup.it

physiological and molecular aspects of I-214 response to the excess of Zn and other heavy metals in hydroponic, pot and lysimeter experiments have been published recently (Di Baccio *et al*. 2003, 2005, 2009, Giachetti and Sebastiani 2006). Alterations of photosynthetic performance and growth parameters were found in clone I-214 subjected to the sub-symptomatic applications of 0.1 and 1 mM Zn, and leaves were found to be the organ that retained the higher amounts of Zn (Di Baccio *et al*. 2003).

In this article, we point out if these physiological impairments due to Zn were caused by leaf morphological and anatomical modifications. Stomatal density, guard cell length, mesophyll thickness and intercellular air spaces of frozen-hydrated (FH) leaves were determined by cryo-scanning electron microscopy (cryo-SEM).

Materials and methods

Plants and treatments: Woody cuttings of the hybrid poplar [*Populus* × *euramericana* (Dode) Guinier] clone I-214 were provided by the Agricultural Research Council, Unit for Intensive Wood Production (Casale Monferrato, Italy). They were rooted in mould in a nursery and in spring (late March) 30 homogenously rooted cuttings, uniform in size, were selected for the experiments (bud break started in mid-March). After the roots had been washed carefully with deionized water, the rooted cuttings were transplanted into pots (12 dm^3) filled with a sand-*Vermiculite* (1:1, v/v) with Zn content (total: 41 ± 3 μg g^{-1}; exchangeable: 3.2 ± 0.4 μg g^{-1}). All plants were supplied with macro- and micronutrients by a modified Hoagland's solution at pH 6.7, and kept in a greenhouse at Pisa, Italy. The nutrient solution was applied weekly and Zn (1 μM, control) was added as $ZnSO_4 \cdot 7\,H_2O$. Within the greenhouse, the maximum radiation on the top leaves was *ca*. 1500 μmol(photon) m^{-2} s^{-1}, while the maximum air temperature (30 ± 2 °C) and relative humidity (70 - 75 %) resulted in a leaf-to-air vapour difference of 2 kPa.

Considering 1 μM (control) as the adequate physiological requirement of Zn for plant mineral nutrition (Taiz and Zeiger 1998), we tested Zn concentrations 100- and 1000-fold higher than that in control solution. In addition to control, Zn treatments (0.1 and 1 mM; 1 dm^3 solutions to each pot) were applied twice, once in June and once in August.

Leaf morphology: At the end of the experiments (end of September), after 3.5 months of Zn treatments, four plants of each treatment were harvested, washed quickly with distilled water and separated into leaves, stem, roots and woody cutting. Total leaves (25 - 36) were separated using the leaf plastochron index (LPI) into two groups: younger (LPI = 1 - 7) and older (LPI > 7) leaves. LPI is is a plant leaf classification system based on the first fully open, but not yet completely expanded, apical leaf (Dickmann 1971); leaves of LPI = 1 - 4 were still expanding, while leaves with a higher LPI were fully expanded. Visual toxicity symptoms (such as leaf chlorosis) were not observed on any group of leaves of the treated plants. The younger leaves undergo most relevant physiological, biochemical and molecular modifications (Di Baccio *et al*. 2003, 2005, 2008) and, for this reason, the analyses were focused on the younger leaves.

Leaf fresh mass (FM) was immediately recorded, then the leaves were oven dried at 60 °C to a constant mass for dry mass (DM) determinations. Specific leaf area (SLA) was determined as the projected leaf area (m^2) per leaf DM (kg). The leaf area ratio (LAR) was calculated using the equation LAR = SLA × LMR, where leaf mass ratio (LMR) is the fraction of the total biomass allocated to the leaves [mass of leaf (kg)/mass of plant (kg)]. Leaf tissue density (LTD) was obtained from the ratio of FM (g) and leaf volume (V; cm^3), calculated by the product of leaf area and leaf thickness determined on frozen-rehydrated (FH) samples by cryo-SEM. Mesophyll volume ($V_{mesophyll}$) was calculated by the product of leaf area and total mesophyll thickness. Leaf area (LA), excluding petiole, was measured using a scanner and an image analysis software (*Scion Image*, release *4.0.2*, *Scion Corporation*, Frederick, MD, USA). The two main orthogonal leaf diameters (D_1 and D_2) were recorded for each leaf of the plants from each Zn treatment. At the end of the experiment the product ($D_1 \times D_2$) of the two main orthogonal leaf diameters was calculated for each leaf of each plant and correlated to the corresponding LA using three linear regressions (one for each Zn treatment, r^2 values ranging from 0.97 to 0.98). In order to estimate if the three linear regressions were different from each other, a homogeneity test for the regression coefficients (slope and intercept) was applied at 1 % level of significance.

Leaf anatomy: Observations of stomatal density (S_d; adaxial and abaxial surfaces) and mesophyll thicknesses were made on six portions (10 mm diameter discs) of 4 younger leaves collected at the end of the treatment from 4 plants for each of the Zn doses. The leaf portions were selected between second-order veins, the central part of the leaf blade, immediately plunged in liquid N_2, and stored there until observation. The FH samples were mounted on aluminium stubs with *Tissue-Tek* (*Miles*, Elkhart, IN, USA). Specimens were moved to a cryo-preparation chamber (*SCU 020*, *Bal-Tech*, Liechtenstein), freeze-fractured by a motor-driven fracturing microtome at -120 ºC, surface etched for 5 min at -80 ºC under high vacuum (< 2×10^{-4} Pa), and sputter-coated with 10 nm of gold in an argon atmosphere (< 2.2×10^{-2} Pa) to produce an electrically conductive surface. FH specimens were then transferred into a cryo-stage (-180 °C) inside the scanning electron

microscope (SEM, model *515*, *Philips*, Eindhoven, The Netherlands). Slow-scan images were digitised at 768 × 576 pixels (256 grey levels) and analysed with *AnalySIS 2.1* (*Soft-Imaging Software*, Münster, Germany). Number of stomata of five fields (574.4 × 430.8 μm) per sample was determined. Of these stomata the guard cell length (GCL) or stoma size (μm) was measured according to Gratani *et al*. (2006) (Fig. 1*A*).

The thickness of leaf layers (total leaf thickness, adaxial and abaxial epidermis thickness, palisade and spongy mesophyll thickness, number of palisade cell layers) was examined on transversally freeze-fractured planes (274.8 × 206.1 μm). The intercellular leaf spaces on a leaf area basis (S_{mes}) and the fraction of mesophyll volume occupied by intercellular air spaces (f_{ias}) were determined as described in Marchi *et al*. (2008), using, respectively, the following equations: $S_{mes} = 1.36 (L_m/W)$ and $f_{ias} = 1 - (A_m/tW)$, where, A_m is the total cross-sectional area of mesophyll cells, t is the mesophyll thickness between the two epidermises, W is the width of the leaf section and L_m represents the cross-sectional length of mesophyll tissue exposed to intercellular air spaces. For S_{mes}, the factor 1.36 accounts for cell surfaces that were not uniformly perpendicular to the plane of the section, and is the average value for prolate and oblate spherid-shaped cells with an average width/length dimension ratio of 0.67 (Evans *et al*. 1994).

Zinc determinations: After morphological and anatomical trait measurements, the younger leaves from each Zn treatment were dried and ground into a powder using a laboratory mill (*IKA-Werke*, Staufen, Germany). Aliquots were used for residual water determination at 105°C. The total content of Zn was determined after digestion in concentrated HNO_3 by atomic absorption spectrophotometry (model *373*, *Perkin Elmer*, Norwalk, CT, USA), as previously described (Di Baccio *et al*. 2003). Chemical analyses were validated by blanks.

Experimental design and statistical analysis: The experiment was set up in a completely randomized design with four replicate plants ($n = 4$) for each Zn treatment, and analyses were done on all younger leaves. For anatomical traits, the analyses were performed on discs randomly sampled from 4 younger leaves. One-way analysis of variance (*ANOVA*) was applied in order to evaluate the effect of Zn concentrations. Statistical analysis was conducted by using *CoStat* version *6.203* (*CoHort Software*, Monterey, CA, USA). Separation of means was performed using LSD-test at $P \leq 0.05$.

Results

The dry mass of young fully expanded leaves (LPI ≤ 7) decreased by 35.6 % in 1 mM Zn treated plants, while remained stable in 0.1 mM Zn treated plants when compared to the control (Table 1). No significant changes were observed in DM (%) and FM to DM ratio. The total leaf area per plant (LA_{tot}) was 2-fold lower in both Zn treatments compared to the control. The area of a single leaf (MLA) and SLA also remarkably decreased by 55 and 57 % and 36 and 38 % at 0.1 and 1 mM Zn, respectively. LAR, LMR, V and $V_{mesophyll}$ and LTD did not show modifications with Zn treatments (Table 1). Zinc content in leaves was 1.3-fold higher in plants subjected to 1 mM Zn treatment than in the control, while no differences were observed in 0.1 mM Zn treated plants (Table 1).

Table 1. Morphological traits and Zn content of leaves (LPI ≤ 1-7) in *P.* × *euramericana* clone I-214 subjected to 1 μM (control), 0.1 mM and 1 mM Zn treatments. Measurements were taken out at the end of one (first) growing season. Data are means of four plants ($n = 4$) per Zn treatment ± SE. Different letters indicate significant differences at $P \leq 0.05$ in the same line. Abbreviations: DM - dry mass, FM - fresh mass, LA_{total} - total leaf area per plant, MLA - mean leaf area (LA_{total}/leaf number), SLA - specific leaf area, LAR - leaf area ratio, LMR - leaf mass ratio, V - leaf volume, $V_{mesophyll}$ - mesophyll leaf volume, LTD - leaf tissue density.

Parameter	Control	0.1 mM Zn	1 mM Zn
DM [g plant^{-1}]	6.66 ± 0.02a	5.08 ± 0.37ab	4.29 ± 0.70b
DM [% of FM]	31.17 ± 0.64	27.77 ± 0.77	30.30 ± 2.61
FM/DM	3.21 ± 0.06	3.61 ± 0.10	3.35 ± 0.30
LA_{tot} [m^2 plant^{-1}]	1.51 ± 0.06a	0.73 ± 0.001b	0.72 ± 0.01b
MLA [dm^2 leaf^{-1}]	2.96 ± 0.01a	1.34 ± 0.001b	1.26 ± 0.002b
SLA [m^2 kg^{-1}]	22.71 ± 3.45a	14.50 ± 1.21b	14.07 ± 1.14b
LAR [m^2 kg^{-1}]	0.90 ± 0.23	0.63 ± 0.02	0.78 ± 0.04
LMR [kg kg^{-1}]	0.04 ± 0.006	0.04 ± 0.001	0.06 ± 0.002
V [cm^3 leaf^{-1}]	2.19 ± 0.31	1.97 ± 0.16	1.83 ± 0.27
$V_{mesophyll}$ [cm^3 leaf^{-1}]	1.90 ± 0.27	1.74 ± 0.14	1.57 ± 0.23
LTD [g cm^{-3}]	1.91 ± 0.22	1.70 ± 0.06	1.64 ± 0.08
Leaf Zn content [μg g^{-1}(DM)]	103.90 ± 10.22b	105.05 ± 23.00b	144.77 ± 23.10a

Table 2. Anatomical traits measured on freeze-fractured planes of frozen-hydrated leaves in *P.* × *euramericana* clone I-214 subjected to Zn 1 μM (control), 0.1 mM and 1 mM treatments. Measurements were taken out at the end of the first growing season. Means ± SE, n = 4. SN - stomata number per leaf, GCL - guard cell length, S_d - stomatal density, S_{mes} - intercellular leaf spaces on a leaf area basis, f_{ias} - fraction of mesophyll volume occupied by intercellular air spaces.

Parameter	Control	0.1 mM	1 mM
Adaxial SN × 10^6	2.20 ± 0.3	2.50 ± 0.2	1.40 ± 0.2
Abaxial SN × 10^6	3.00 ± 0.4a	3.40 ± 0.3a	1.50 ± 0.4b
Total SN × 10^6	5.20 ± 0.7a	5.80 ± 0.5a	2.90 ± 0.4b
adaxial/abaxial SN ratio	0.71 ± 0.01b	0.74 ± 0.02b	0.97 ± 0.04a
Adaxial GCL [μm]	30.52 ± 0.54a	29.56 ± 0.64a	26.54 ± 0.37b
Abaxial GCL [μm]	31.57 ± 0.11	30.50 ± 0.73	29.89 ± 0.75
Adaxial S_d [mm^{-2}]	128.50 ± 3.74b	184.67 ± 6.67a	114.00 ± 3.18c
Abaxial S_d [mm^{-2}]	181.50 ± 2.32b	250.33 ± 4.91a	117.00 ± 5.38c
Total thickness [μm]	130.70 ± 1.4b	147.20 ± 1.6a	145.20 ± 1.9a
Adaxial epidermis [μm]	9.80 ± 0.5	9.10 ± 0.5	10.20 ± 0.7
Palisade mesophyll [μm]	55.70 ± 1.2b	62.20 ± 1.6a	60.80 ± 1.0a
Spongy mesophyll [μm]	57.70 ± 1.1b	67.40 ± 1.8a	63.60 ± 1.4a
Mesophyll thickness [μm]	113.33 ± 1.85c	129.51 ± 2.10a	124.39 ± 1.01b
Palisade/spongy mesophyll	0.97 ± 0.03	0.93 ± 0.04	0.96 ± 0.03
Abaxial epidermis [μm]	7.50 ± 0.6	8.40 ± 0.5	9.20 ± 0.5
Adaxial/abaxial epidermis	1.38 ± 0.12	1.10 ± 0.06	1.18 ± 0.15
S_{mes}	23.34 ± 1.54b	31.82 ± 1.58a	32.16 ± 1.09a
f_{ias}	0.51 ± 0.03a	0.33 ± 0.02b	0.29 ± 0.03b

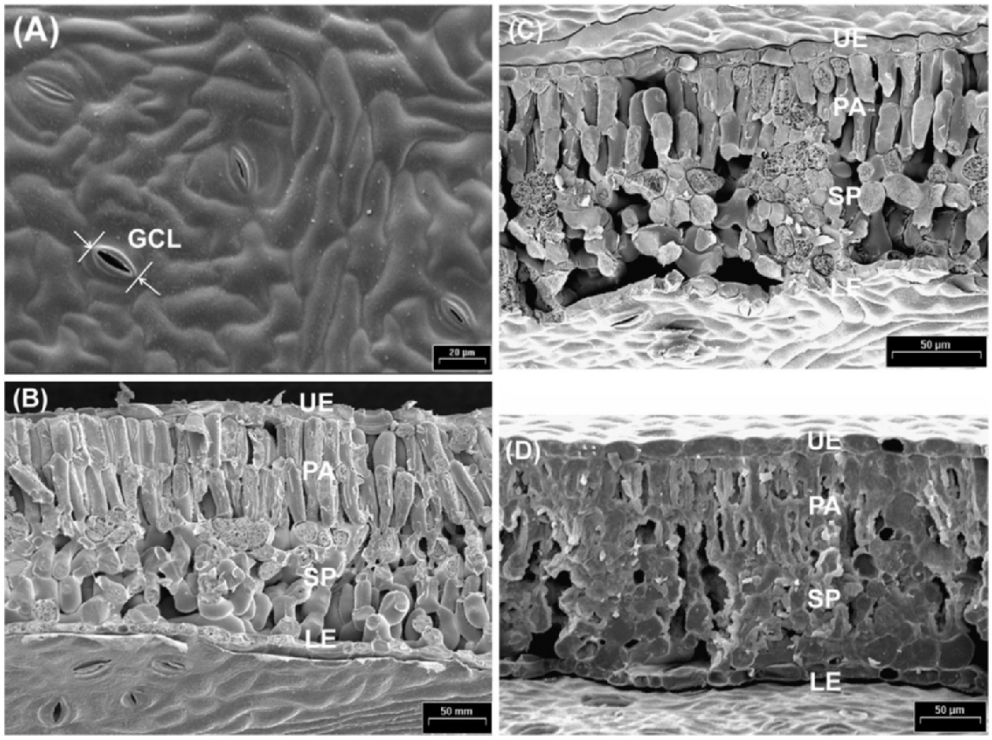

Fig. 1. Cryo-SEM images of frozen-hydrated leaves in *P.* × *euramericana* clone I-214. Type of frame utilized for stomata guard cell length (GCL) measurements (*A*); leaf transversal freeze-fractures of plants grown in suitable Zn amount (control: *B*), Zn 0.1 mM (*C*) and Zn 1 mM (*D*). UE - upper epidermis, PA - palisade mesophyll, SP - spongy mesophyll, LE - lower epidermis.

Stomata numbers on the leaf epidermes were reduced considerably by 1 mM Zn (Table 2). The adaxial to abaxial stomata ratio became 1.4-fold higher at 1 mM Zn. The GCL (Fig. 1*A*) on the adaxial surface was 13 %

lower at 1 mM Zn, while no changes were observed on the abaxial surface. The S_d showed the same trend on both adaxial and abaxial surfaces (Table 2), increasing at 0.1 mM Zn and decreasing at 1 mM Zn. In particular, on the adaxial surface the S_d showed a 44 % increase in the presence of 0.1 mM Zn and a 11 % decrease at 1 mM Zn. On the abaxial surface the S_d was enhanced by 38 % by 0.1 mM Zn and reduced by 36 % by 1 mM Zn (Table 2).

Total leaf thickness showed a 12.6 and 11.1 % increase in the plants treated with 0.1 and 1 mM Zn, respectively, compared to the control (Table 2). In both cases this was due to the enhancement of the palisade and spongy mesophyll thickness, since the mesophyll thickness was 14.3 and 10 % higher in 0.1 mM and 1 mM Zn treated leaves, respectively. The palisade to spongy mesophyll ratio was not changed in any Zn treatment, as well as the number of palisade cell layers (Table 2 and Fig. 1*B*,*C*,*D*). The S_{mes} and the f_{ias} showed an opposite trend in Zn 0.1 mM and 1 mM treated plants compared to the control. The S_{mes} increased by 36 % at 0.1 mM Zn and 38 % at 1 mM Zn, while f_{ias} decreased by 35 % and 43 % in presence of 0.1 mM and 1 mM Zn, respectively (Table 2).

Discussion

Information on tree performance under heavy metal contaminations is scarce, particularly on leaf function (Hermle *et al*. 2007). Visual symptoms of leaf injury caused by excess of Zn or Cd consist of chlorosis, stipples, desiccation, necrosis and red-brown coloration of leaf margins or veins; in severe cases, necrotic lesions lead to the death of the entire leaf (Wójcik and Tukiendorf 2005, Hermle *et al*. 2007, Wang *et al*. 2009). No visible symptoms of Zn toxicity were observed in the leaves of the *P.* × *euramericana* clone I-214 treated with 0.1 and 1 mM Zn, confirming previous observations (Di Baccio *et al*. 2003). Although it depends on the plant species and age, this fact demonstrates that these types of treatments were sub-symptomatic. In fact plant interaction with heavy metals, such as Zn, which are also micronutrients, is complex, depending not only on the ion type and concentrations, but also on the plant growth stage and nutrient conditions (Broadley *et al*. 2007 and references within).

The DM of fully expanded leaves from the top shoot at the end of the treatment period was reduced by 1 mM Zn treatment (Table 1), as just observed previously in older leaves (Di Baccio *et al*. 2003). Nevertheless, the DM (% of FM) and FM/DM ratio were not affected by Zn treatments (Table 1), suggesting no alterations of water status and biomass allocation (Di Baccio *et al*. 2004). The LA_{total} of this rank of leaves was remarkably decreased by 52 % at both Zn treatments (Table 1). The steady-state (0.1 mM Zn) and reduced (1 mM Zn) leaf biomass and LA did not correspond to a reduction in leaf number (data not shown). This means that increasing the Zn concentration affected the area expansion of leaves before their biomass allocation. The leaf area displayed per unit of leaf mass (SLA) decreased at both Zn concentrations. Although dependent on plant species, genotype, age and growth conditions, a strict correlation between SLA and plant responses to stress has been demonstrated (Wieser *et al*. 2003, Gratani *et al*. 2006, Monclus *et al*. 2006). The smaller but thicker leaves (lower SLA) may be a defence mechanism or compensatory strategy for reducing transpiration rate (Table 1). The efficiency of this response can be confirmed by stable LAR, leaf density and volume. Also the leaf biomass allocation (LMR) remained stable, showing that the reduction of whole plant biomass at 1 mM Zn treatment (data not shown) was not sufficient to modify the relative leaf mass. Differences found in LA expansion did not affect changes in leaf shape. This was demonstrated by the coefficient homogeneity among the three regression curves built between the product of the two main orthogonal leaf diameters and the corresponding LA for each Zn treatment (data not shown).

As expected, the leaf Zn content increased more in 1 mM Zn treated plants in comparison with those subjected to 0.1 mM Zn and in controls (Table 1). These variations were previously correlated with impairments in carbon assimilation and chlorophyll concentrations (Di Baccio *et al*. 2003). The leaf Zn concentration followed the same trend as the Zn content, showing that the Zn uptake was directly proportional to biomass production. To understand if the differences in Zn accumulation could be explained by leaf structural variations, anatomical traits were further analysed by cryo-SEM, which enables the real highlighting of leaf structural features. In fact, this experimental tool permits the observation of the leaf samples in their natural hydrated state, avoiding preparation artefacts and maintaining the original gas fraction of mesophyll (Minnocci *et al*. 1999, Tognetti *et al*. 2004, Marchi *et al*. 2008).

Leaf thickness is characteristic of plant species (Gratani *et al*. 2003), but it changes depending on treatment and environmental conditions (Minnocci *et al*. 1999, Tognetti *et al*. 2004, Gratani *et al*. 2006, Di Baccio *et al*. 2009) or developmental stages (Marchi *et al*. 2008). Total leaf thickness increased at 0.1 mM and 1 mM Zn, as was just observed in I-214 grown in hydroponic systems with higher Zn concentrations (Di Baccio *et al*. 2009) and in soil amended with organic materials from tanneries containing heavy metals (Tognetti *et al*. 2004). This increment in mesophyll thickness was due to the contemporary enhancements of palisade and spongy mesophyll layer thickness (Table 2), nevertheless the number of cell layers remained the same (Fig. 1*B*,*C*,*D*). This increase, however, was not sufficient to enhance the leaf volume, as discussed before, or to modify the

palisade to spongy mesophyll ratio. As suggested for SLA, the higher mesophyll thickness found may be explained as an attempt to increase the leaf assimilatory area in response to the stress (Lin *et al*. 2001). This probably also explains the increase in S_{mes} calculated in the same conditions (Table 2). Indeed, the S_{mes} is strongly and positively related to the photosynthetic capacity (Terashima *et al*. 2001, Marchi *et al*. 2008). However, treatments with 0.1 and 1 mM Zn reduced the f_{ias}, suggesting modifications of mesophyll structure and organization, which appeared less densely packed. This may cause variations of mesophyll conductance and, consequently, photosynthetic function, as previously shown with the same Zn treatments (Di Baccio *et al*. 2003).

Apart from the biochemical and structural modifications of the mesophyll, impairments in leaf photosynthesis capacity can be due to stomata malfunctioning. Although poplar shows large clonal variability in stomatal characteristics (Al Afas *et al*. 2006, Tognetti *et al*. 2004), variations in stomatal density directly affect biomass production (Al Afas *et al*. 2006). In I-214 both adaxial and abaxial S_d increased at 0.1 mM Zn and decreased at 1 mM Zn treatment (Table 2). These modifications corresponded to the strong reduction in MLA at both Zn doses (Table 1). In the case of 0.1 mM Zn the S_d increase was not dependent on an effective enhancement of total SN (Table 2). On the other hand, at 1 mM Zn the S_d decrease correlated with strong decrease of total SN, principally due to the decrease of abaxial SN. In this case impairments modified also the adaxial/abaxial stomata ratio (Table 2). This index is characteristic in amphistomatous leaves (Matyssek *et al*. 1998, Soares *et al*. 2007). However, at 1 mM Zn the stomata on the abaxial surface were lower in number but unmodified in size (GCL) in comparison with the control. In the same condition, the adaxial stomata were unmodified in number but smaller in size. As with LA and mesophyll modifications, differences in stomata number, density and size may be explained as adaptive mechanisms to the stress caused by Zn excess. We believe that these morphological and anatomical modifications in response to Zn excess may explain physiological affections previously observed in I-214 (Di Baccio *et al*. 2003, 2009, Tognetti *et al*. 2004).

As a consequence of both 0.1 and 1 mM Zn treatments, the leaves of I-214 poplar clone did not display visual symptoms of injury (chlorosis, necrosis and leaf senescence), but variations in dry mass, area, SLA, mesophyll thickness, intercellular spaces, stomata number, density and size. Lower Zn concentration induced mostly leaf modifications leading to maintaining or restoring functional integrity under stress. However, the higher Zn concentration altered some structural traits, especially stomata characteristics, affecting their functionality.

In summary we can conclude that morphological and anatomical modifications of leaves are involved in the I-214 poplar response to Zn excess, highlighting the key role of this organ, but knowledge on the leaf structure and cell distribution under heavy metal stress is still insufficient.

References

Al Afas, N., Marron, N., Ceulemans, R.: Clonal variation in stomatal characteristics related to biomass production of 12 poplar (*Populus*) clones in a short rotation coppice culture. - Environ. exp. Bot. **58**: 279-286, 2006.

Broadley, M.R., White, P.J., Hammond, J.P., Zelko, I., Lux, A.: Zinc in plants. - New Phytol. **173**: 677–702, 2007.

Di Baccio, D., Castagna, A., Paoletti, E., Sebastiani, L., Ranieri, A.: Could the differences in O_3 sensitivity between Eridano and I-214 poplar clones be related to different antioxidant defence and secondary metabolism induction in response to O_3 influx? - Tree Physiol. **28**: 1761-1772, 2008.

Di Baccio, D., Kopriva, S., Sebastiani, L., Rennenberg, H.: Does glutathione metabolism have a role in the defence of poplar against zinc excess? - New Phytol. **167**: 73-80, 2005.

Di Baccio, D., Navari-Izzo, F., Izzo, R.: Seawater irrigation: antioxidant defence responses in leaves and roots of a sunflower (*Helianthus annuus* L.) ecotype. - J. Plant Physiol. **161**: 1359-1366, 2004.

Di Baccio, D., Tognetti, R., Minnocci, A., Sebastiani, L.: Responses of *Populus* × *euramericana* clone I-214 to zinc excess: carbon assimilation, structural modifications, metal distribution and cellular localization. - Environ. exp. Bot. **67**: 153-163, 2009.

Di Baccio, D., Tognetti, R., Sebastiani, L., Vitagliano C.: Responses of *Populus deltoides* × *Populus nigra* (*Populus* × *euramericana*) clone I-214 to high zinc concentrations. - New Phytol. **159**: 443-452, 2003.

Dickmann, D.I.: Photosynthesis and respiration by developing leaves of cottonwood (*Populus deltoides* Bartr.). - Bot. Gaz. **132**: 253-259, 1971.

Djingova, R., Wagner, G., Peshev, D.: Heavy metal distribution in Bulgaria using *Populus nigra* **'**Italica' as a biomonitor. - Sci. Total Environ. **172**: 151-158, 1995.

Evans, J.R, Von Caemmerer, S., Setchell, B.A., Hudson, G.S.: The relationship between CO_2 transfer conductance and leaf anatomy in transgenic tobacco with a reduce content of Rubisco. - Aust. J. Plant Physiol. **21**: 475-495, 1994.

Gatti, E.: Micropropagation of *Ailanthus altissima* and *in vitro* heavy metal tolerance. - Biol. Plant. **52**: 146-148, 2008.

Giachetti, G., Sebastiani, L.: Metal accumulation in poplar plant grown with industrial wastes. - Chemosphere **64**: 446-454, 2006.

Giachetti, G., Sebastiani, L.: Effects of tannery waste on growth dynamics and metal uptake in *Salix alba* L. - Plant Biosystems **141**: 22-30, 2007.

Gratani, L., Covone, F., Larcher, W.: Leaf plasticity in response to light of three evergreen species of the Mediterranean maquis. - Trees **20**: 549-558, 2006.

Gratani, L., Meneghini, M., Pesoli, P., Crescente, M.F.: Structural and functional plasticity of *Quercus ilex* seedlings of different provenances in Italy. - Trees **17**: 515-521, 2003.

Hermle, S., Vollenweider, P., Günthardt-Goerg, M.S.,

McQuattie, C.J., Matyssek, R.: Leaf responsiveness of *Populus tremula* and *Salix viminalis* to soil contaminated with heavy metals and acidic rainwater. - Tree Physiol. **27**: 1517-1531, 2007.

Laureysens, I., Blust, R., De Temmerman, L., Lemmens, C., Ceulemans, R.: Clonal variation in heavy metal accumulation and biomass production in a poplar coppice culture: I. Seasonal variation in leaf, wood and bark concentrations. - Environ. Pollut. **131**: 485-494, 2004.

Laureysens, I., De Temmerman, L., Hastir, T., Van Gysel, M., Ceulemans, R.: Clonal variation in heavy metal accumulation and biomass production in a poplar coppice culture: II. Vertical distribution and phytoextraction potential. - Environ. Pollut. **133**: 541-551, 2005.

Lin, J., Jach, M.E., Ceulemans, R.: Stomatal density and needle anatomy of Scots pine (*Pinus sylvestris*) are affected by elevated CO_2. - New Phytol. **150**: 665-674, 2001.

Lunáčková, L., Šottníková, A., Masarovičová, E., Lux, A., Streško, V.: Comparison of cadmium effect on willow and poplar in response to different cultivation conditions. - Biol. Plant. **47**: 403-411, 2003/4.

Marchi, S., Tognetti, R., Minnocci, A., Borghi, M., Sebastiani, L.: Variation in mesophyll anatomy and photosynthetic capacity during leaf development in a deciduous mesophyte fruit tree (*Prunus persica*) and an evergreen sclerophyllous Mediterranean shrub (*Olea europaea*). - Trees **22**: 559-571, 2008.

Markovska, Y.K., Gorinova, N.I., Nedkovska, M.P., Miteva, K.M.: Cadmium-induced oxidative damage and antioxidant responses in *Brassica juncea* plants. - Biol. Plant. **53**: 151-154, 2009.

Marschner, H. (ed.): Mineral Nutrition of Higher Plants. 2nd Ed. - Academic Press, London 1995.

Matyssek, R., Günthardt-Goerg, M.S., Schmutz, P., Saurer, M., Landolt, W., Bucher, J.B.: Response mechanisms of birch and poplar to air pollutants. - J. Sustain. Forest. **6**: 3-22, 1998.

Minnocci, A., Panicucci, A., Sebastiani, L., Lorenzini, G., Vitagliano, C.: Physiological and morphological responses of olive plants to ozone exposure during a growing season. - Tree Physiol. **19**: 391-397, 1999.

Monclus, R., Dreyer, E., Villar, M., Delmotte, F.M., Delay, D., Petit, J.-M., Barbaroux, C., Le Thiec, D., Bréchet, C., Brignols F.: Impact of drought on productivity and water use efficiency in 29 genotypes of *Populus deltoides* × *Populus nigra*. - New Phytol. **169**: 765-777, 2006.

Mourato, M.P., Martins, L.L., Campos-Andrada, M.P.: Physiological responses of *Lupinus luteus* to different copper concentrations. - Biol. Plant. **53**: 105-111, 2009.

Scebba, F., Arduini, L., Ercoli, L., Sebastiani, L.: Cadmium effects on growth and antioxidant enzymes activities in *Miscanthus sinensis*. - Biol. Plant. **50**: 688-692, 2006.

Sebastiani, L., Scebba, F., Tognetti, R.: Heavy metal accumulation and growth responses in poplar Eridano (*Populus deltoides* x *maximowiczii*) and I-214 (*P.* × *euramericana*) exposed to industrial waste. - Environ. exp. Bot. **52**: 79-88, 2004.

Shah, K., Nongkynrih, J.M.: Metal hyperaccumulation and bioremediation. - Biol. Plant. **51**: 618-634, 2007.

Sims, R.E.H., Hastings, A., Schlamadinger, B., Taylor, G., Smith, P.: Energy crops: current status and future prospects. - Global Change Biol. **12**: 2054-2076, 2006.

Soares, A.S., Driscoll, S.P., Olmos, E., Harbinson, J., Arrabaça, M.C., Foyer, C.H.: Adaxial/abaxial specification in the regulation of photosynthesis and stomatal opening with respect to light orientation and growth with CO_2 enrichment in the C4 species *Paspalum dilatatum*. - New Phytol. **177**: 186-198, 2007.

Taiz, L., Zeiger, E.: Mineral nutrition. - In: Taiz, L., Zeiger, E. (ed.): Plant Physiology. 2nd Ed. Pp. 103-124. Sinauer Associates, Sunderland 1998.

Terashima, I., Miyazawa, S.I., Hanba, Y.T.: Why are sun leaves thicker than shade leaves? Consideration based on analyses of CO_2 diffusion in the leaf. - J. Plant Res. **114**: 93-105, 2001.

Tognetti, R., Sebastiani, L., Minnocci, A.: Gas exchange and foliage characteristics of two poplar clones grown in soil amended with industrial waste. - Tree Physiol. **24**: 75-82, 2004.

Tuskan, G.A., Di Fazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., Putnam, N., Ralph, S., Rombauts, S., Salamov, A., Schein, J., Sterck, L., Aerts, A., Bhalerao, R.R., Bhalerao, R.P., Blaudez, D., Boerjan, W., Brun, A., Brunner, A., Busov, V., Campbell, M., Carlson, J., Chalot, M., Chapman, J., Chen, G.-L., Cooper, D., Coutinho, P.M., Couturier, J., Covert, S., Cronk, Q., Cunningham, R., Davis, J., Degroeve, S., Déjardin, A., De Pamphilis, C., Detter, J., Dirks, B., Dubchak, I., Duplessis, S., Ehlting, J., Ellis, B., Gendler, K., Goodstein, D., Gribskov, M., Grimwood, J., Groover A., Gunter, L., Hamberger, B., Heinze, B., Helariutta Y., Henrissat B., Holligan D., Holt R., Huang W., Islam-Faridi, N., Jones, S., Jones-Rhoades, M., Jorgensen, R., Joshi, C., Kangasjärvi, J., Karlsson, J., Kelleher, C., Kirkpatrick R., Kirst M., Kohler A., Kalluri U., Larimer F., Leebens-Mack, J., Leplé, J.-C., Locascio, P., Lou, Y., Lucas, S., Martin, F., Montanini, B., Napoli, C., Nelson, D.R., Nelson, C., Nieminen, K., Nilsson, O., Pereda, V., Peter, G., Philippe, R., Pilate, G., Poliakov, A., Razumovskaya J., Richardson P., Rinaldi C., Ritland K., Rouzé, P., Ryaboy, D., Schmutz, J., Schrader, J., Segerman, B., Shin, H., Siddiqui, A., Sterky, F., Terry A., Tsai C.-J., Uberbacher E., Unneberg P., Vahala J., Wall, K., Wessler, S., Yang, G., Yin, T., Douglas, C., Marra, M., Sandberg, G., Van de Peer, Y., Rokhsar, D.: The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). - Science **313**: 1596-1604, 2006.

Wang, H., Liu, R.L., Jin, J.Y.: Effects of zinc and soil moisture on photosynthetic rate and chlorophyll fluorescence parameters of maize. - Biol. Plant. **53**: 191-194, 2009.

Wieser, G., Hecke, K., Tausz, M., Häberle, K.H., Grams, T.E.E., Matyssek, R.: The influence of microclimate and tree age on the defense capacity of European beech (*Fagus sylvatica* L.) against oxidative stress. - Ann. Forest Sci. **60**: 131-135, 2003.

Wójcik, M., Tukiendorf, A.: Cadmium uptake, localization and detoxification in *Zea mays*. - Biol. Plant. **49**: 237-245, 2005.