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High Zn concentration does not impair biomass, cutting radial growth, and photosynthetic activity traits in *Populus alba* L.

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Abstract

Purpose Evaluate the efficiency of *Populus alba* clone Villafranca in the uptake and translocation of Zn from contaminated soils.

Materials and methods The effects of 48 days of zinc treatment (Zn_t) on the growth and the photosynthetic activities of *P. alba* L. clone Villafranca were studied using $ZnSO_4$ (375 ppm per unit of soil dry weight) added in sand and peat moss substrate at the beginning of the treatment (T_0) and again after 30 days (T_1) in order to reach a target Zn concentration of 375 ppm at T_0 and 750 ppm at T_1 per unit of soil dry weight.

Results and discussion Zn uptake in the different organs was analyzed after 30 (T_1) and 48 days (T_2) from the beginning of treatment, showing the following order: root ≫ leaves ≥ woody cutting = stem. The leaf area increased by 12 % in comparison to control plants at the end of second treatment (48 days). Cutting radial growth showed a high synchronicity in the growth rate fluctuation among control and Zn_t plants, but a higher increase in radial diameter of Zn_t cutting was observed starting from day 38 (after 8 days of second Zn_t) reaching after 48 days 38 % higher than control plants.

Conclusions Although our data of leaf Zn concentration were in the range usually reported as toxic for plants, Villafranca clone in Zn_t substrate were unaffected in terms of net CO_2 assimilation and stomatal conductance to water vapor.

Keywords Bio-concentration factor · CO_2 assimilation · Poplar · Radial growth · Translocation factor

1 Introduction

Recently, there has been an upsurge of interest on plant responses to heavy metals (HMs) because metal pollution is still increasing worldwide modifying the ecosystems' stability (Manara 2012). Fast-growing tree species, such as those belonging to Salicaceae, have been evaluated for biomass production in heavy metal-contaminated lands (Unterbrunner et al. 2007; Guerra et al. 2011). Among Salicaceae, poplars have wide species distributions that frequently cover markedly different soil pollution ranges, making this group of species an ideal system for the study of tree adaptation to HMs. Crossing these two factors (*HMs × poplars*) for many years, researchers have focused on the study of the responses of poplar under high concentrations of Cu, Cd, Cr, Zn, etc., using in vitro approaches (Franchin et al. 2007), hydroponic systems, or experiments in pot/lysimeters containing soil contaminated with HMs (Di Baccio et al. 2003; Sebastiani et al. 2004, 2014; Giachetti and Sebastiani 2006; Borghi et al. 2008; Durand et al. 2010; Romeo et al. 2014a).

The range of total Zn concentrations in soils reported in the literature change around the world; in England, soils

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contaminated from various sources showed a mean Zn content of 97 mg kg^{-1} (full range 5–3548 mg kg^{-1}) (Alloway 2009). In France, the Zn in soil starts to 17 mg kg^{-1} in sandy soils up to 132 mg kg^{-1} in very clayey soils (>50 % clay) (Baize 1997); in Australia, the range is 2–180 mg kg^{-1} with a mean of 34 mg kg^{-1} (Alloway 2009). The high concentrations of Zn found in contaminated soils may cause symptoms of toxicity like yield reduction, chlorosis, and interference with ion uptake (Sagardoy et al. 2009; Hassan and Aarts 2011). The optimum Zn concentration inside plant tissues for adequate growth is estimated in 15–150 mg Zn kg^{-1} dry weight (DW) (Markert 1992). The guideline for safe limits of Zn in agricultural soils is 300 ppm (as reported in Table 5 of Nagajyoti et al. 2010), but anthropogenic inputs can enrich soil. In fact, the Zn concentration in sewage sludge ranges from 91 to 49,000 ppm, in phosphatic fertilizers from 50 to 1450 ppm, and in farmyard manure from 15 to 556 ppm (Nagajyoti et al. 2010).

Populus species include several cultivars: One of them was the *Populus alba* L. clone 'Villafranca' (Romeo et al. 2014a), obtained at the Poplar Research Institute, Casale Monferrato (Italy) crossing a female *P. alba* from Villafranca Piemonte (Piedmont, Italy) and a male specimen of the same species from Lucca (Tuscany, Italy). This genotype showed a good biomass production and a remarkable re-sprouting ability after coppicing (Confalonieri et al. 2000). Moreover, Villafranca has been previously studied for its high Zn uptake traits in hydroponic studies aimed to compare several poplar species (Romeo et al. 2014a) and successfully used as a model plant in transformation experiments to get new varieties with traits related to herbicide tolerance and insect pest tolerance (Confalonieri et al. 2000).

This study was planned under controlled environmental conditions with the objectives to evaluate the efficiency of *P. alba* clone Villafranca in the uptake and translocation of Zn from artificially polluted soil in order to simulate a monometallic pollution. With this proposal, the radial growth profile was monitored during the experiment in order to study the secondary growth in terms of biomass production in relation to Zn treatment.

2 Material and methods

2.1 Plant materials and Zn treatments

Woody stem cuttings obtained from 1-year-old cutback stems of *P. alba* L. clone Villafranca were planted into sand. After rooting (1 month), plants were placed in 1-dm^3 pots containing sand and peat moss (25:75, *v/v*, pH 6.9) to allow stem bud sprouting and shoot growth. Plants were cultivated in a growth chamber at 21±2 °C, 70±5 % of relative humidity, and an irradiance of 1000 μmol m^{-2} s^{-1} provided during 16-h photoperiod. After 6 weeks, plants were transferred into pots containing 1.6 kg of soil DW and concomitantly pruned to second axillary bud in order to make sure that the new leaves were entirely formed under Zn exposure (day 0).

A solution of $ZnSO_4 \times 7H_2O$ was added to soils of treated plants (Zn_t) at the beginning of the experiment (T_0) and then again after 30 days (T_1) in order to reach a target Zn concentration of 375 ppm at T_0 and 750 ppm at T_1 per unit of soil dry weight; sulfate was balanced with K_2SO_4 in the case of control plants. The whole experimental period was 48 days (T_2). Plants were manually irrigated every 2 days with tap water avoiding leaching.

2.2 Growth parameters

Leaf area, leaf number, and stem length were measured every 3 days. After 30 (T_1) and 48 (T_2) days from the beginning of the experiment, six plants from each groups of treatments were collected and divided into root, woody cutting, stem (newly formed stem during the treatment), and leaves.

Leaf mass ratio (LMR), stem mass ratio (SMR), and root mass ratio (RMR) were calculated as the fraction of the total biomass allocated to the leaves, stem, and root [organ mass (g)/mass of plant (g)] to whole plant (Hunt 1978). The translocation factor (Tf) was calculated as ratio of Zn concentration in aerial part (leaves, stem, and woody cutting) to root. The Zn bio-concentration factor (BCF) of root system and aerial part (woody cutting, stem, and leaves) was calculated as the ratio of Zn concentration in specific organ to Zn soil concentration.

According to Morabito et al. (2006), individual leaf width (Lw, cm) was manually measured periodically in order to estimate not destructively the leaf area (La, cm^2) using the following formula: La $= (0.71 \times Lw^2) + (1.114 \times Lw)$, $R^2 = 0.98$. Leaf area ratio (LAR, cm^2 g^{-1}) was calculated as the fraction of the total leaf area and total biomass allocated to the leaves.

2.3 Zn determination

Each plant was separated into roots, stems, woody cuttings, and leaves. Then, the plant organs were oven dried at 70 °C for 7 days. Dried plant organs were grounded with a laboratory grinder, and 200 mg (±0.5 mg) of samples were treated by an acid mixture (3 ml of 37 % hydrochloric acid and 6 ml of nitric acid 65 %) and digested with a pressurized closed-vessel microwave system (Multiwave 3000, Anton Paar GmbH, Germany). The digested samples were adjusted to 50 ml with deionized water and used for the determination of Zn by atomic absorption spectrophotometer (model 373; Perkin-Elmer, Norwalk, CT, USA).

Table 1 Growth traits of *Populus alba* clone Villafranca in control and Zn_t conditions, after 30 (T_1) and 48 (T_2) days from the beginning of the experiment that corresponds to 375 and 750 ppm Zn treatment, respectively

	T_1			T_2		
	Control	Zn_t	*t* test	Control	Zn_t	*t* test
LMR	0.17±0.010	0.20±0.010	ns	0.38±0.020	0.34±0.030	ns
SMR	0.07±0.008	0.11±0.006	ns	0.22±0.009	0.24±0.009	ns
RMR	0.03±0.006	0.04±0.005	*	0.04±0.001	0.05±0.005	ns
LAR	168±12	172±6	ns	176±5	267±49	ns

At each time of sampling, leaf mass ratio (*LMR*), stem mass ratio (*SMR*), root mass ratio (*RMR*) and leaf area ratio (*LAR*, $cm^2 \ g^{-1}$) were analyzed with *t* test analysis at 0.05 probability level. Values are means ± standard error (*n*= 5 for data at T_1; *n*=6 for data at T_2). *ns* not significant, *$P \le 0.05$

2.4 Cutting radial growth

Cutting radial growth during the 48 days of treatment was measured every 15 min with an automatic point dendrometer using six plants per treatment. A linear motion sensor (AB Electronics Ltd., Romford, Essex, UK) was mounted and adjusted on woody cutting so that its diameter changes mechanically impinged on it, forcing the electric tension out of the variable resistance of the sensor connected as a tension divider. The variable tension was digitized by an analog-to-digital converter (Adlink Technology Inc., Taiwan) plugged into a PC-based recording data system. The software piloting this device and storing the collected data was developed in Visual Basic for Applications and integrated in Excel's spreadsheet (Microsoft, Redmond, Washington, USA) (Morabito et al. 2006). The daily ΔR was calculated by dividing the stem radius diurnal cycle into three distinct phases: (i) contraction phase (the period between morning maximum and afternoon minimum), (ii) expansion phase (the period from the minimum to the following morning maximum), and (iii) stem radius increment phase (part of the expansion phase from the time when the stem radius exceeds the morning maximum until the subsequent maximum). The difference between maximum expansion and the beginning of the third phase represents the positive ΔR. When the maximum value of the previous cycle was not reached, negative ΔR (cm) was calculated (Giovannelli et al. 2007).

2.5 Photosynthetic activities

Net CO_2 assimilation (A) and leaf stomatal conductance to water vapor (g_s) were measured on the last fully expanded leaf for six plants for each treatment and were conducted with a portable photosynthetic system (*Ciras-2*, *PP Systems*, Hitchin, Herts, UK) on days 14, 20, 23, 27, 30, 35, 40, and 43 from the beginning of experiment. From these data, the water use efficiency was estimated (WUE=A/g_s).

2.6 Statistical analysis

The experiment was set up in a completely randomized design (*n*=5 for data at T_1; *n*=6 for data at T_2). Data were subjected to statistical analyses. Two-way ANOVA for statistical evaluation of the effects produced by Zn, organ, and interaction of these two factors was applied to data regarding Zn concentration and Zn content. Means were subjected to Fisher's LSD test at 0.05 probability level. Unpaired *t* test analysis at 0.05 probability level was performed on data about leaf mass ratio, stem mass ratio, root mass ratio, leaf area ratio, leaf area, stem length, leaf number, CO_2 assimilation, stomatal conductance, and WUE. Statistical analysis was performed using NCSS 2000 Statistical Analysis System Software.
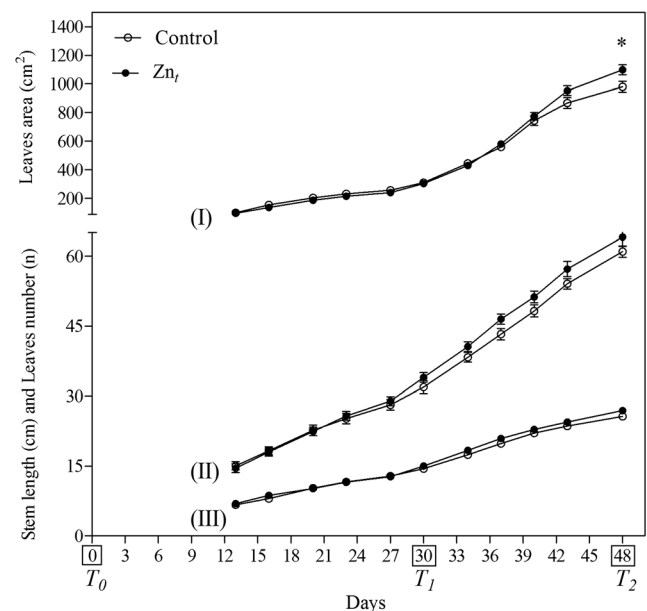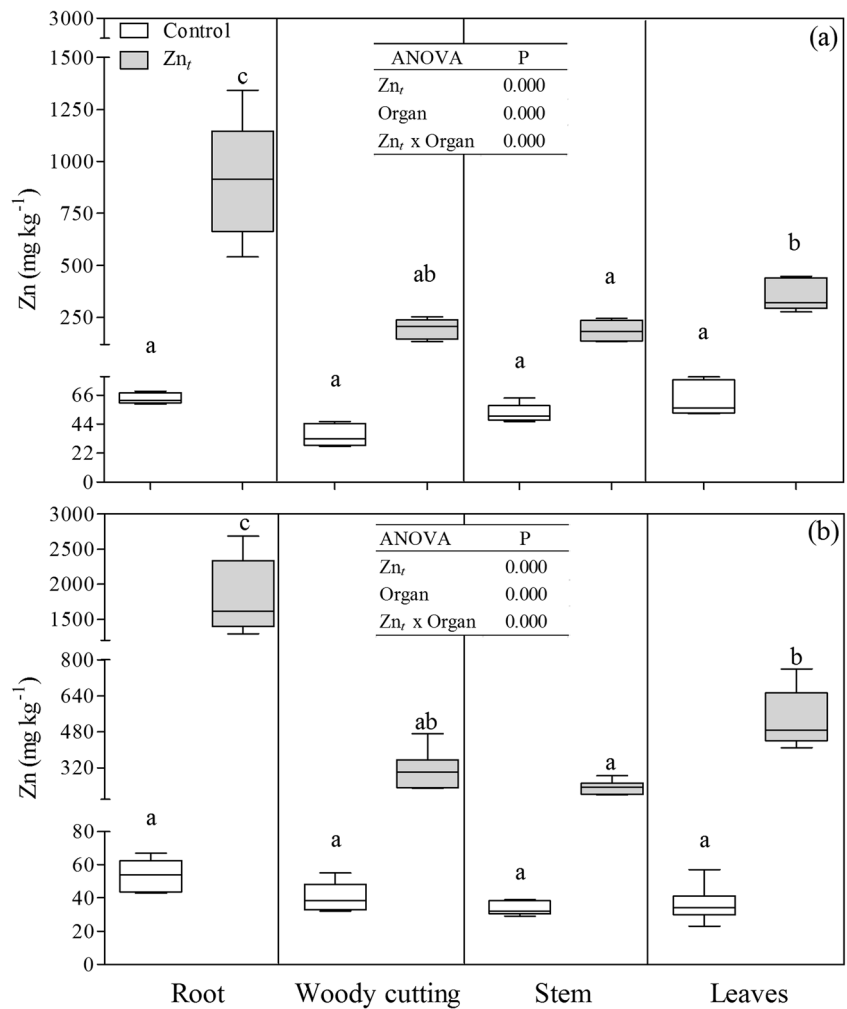

Fig. 1 Leaf area (*I*), stem length (cm) (*II*), and leaf number (*n*) (*III*) of Villafranca clone. T_0 corresponds to the first Zn treatment, T_1 to the second, and T_2 is the end of experiment. Values are means ± standard error (*n*=5 for data at T_1; *n*=6 for data at T_2). Means were subjected to *t* test analysis at 0.05 probability level. *$P \le 0.05$

3 Results and discussion

In this experiment, the addition of Zn to substrate was deliberately designed to reach a concentration value two times higher than the one imposed in agricultural soil by the European Union guideline (Nagajyoti et al. 2010). As a consequence of Zn treatments, the leaves of Villafranca poplar clone did not display visual symptoms of injury (chlorosis, necrosis, or leaf senescence). Moreover, dry weight did not differ in

response to Zn although an increment of RMR was observed after 30 days of Zn exposure compared to control (Table 1). As previously demonstrated by Romeo et al. (2014b), Zn excess condition can induce an increase of root branching, considered as an early strategy to facilitate water and nutrition absorption in the root system under Zn excess conditions.

In this study, it has been shown that the growth responses of Villafranca clone to Zn application differ between organs and do not correspond to Zn accumulation. The total leaf area was
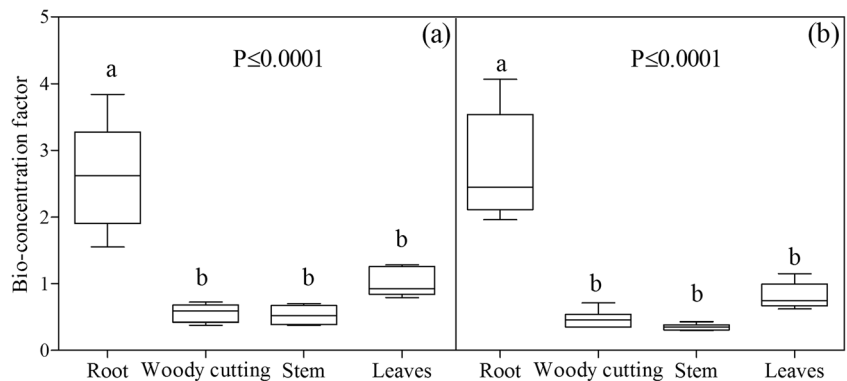
not affected in Zn_t plants up to day 48 (T_2), when a 12 % increase occurred in comparison to control plants (Fig. 1). As also observed by Di Baccio et al. (2010), this means that increasing Zn concentration can affect the area expansion of leaves before their biomass allocation.

No effect on stem length and leaf number were found comparing the two sets of plants, that reached at the end of the experimental period an average stem length of 64±1.99 cm and a leaf number equal to 27±0.51 (Fig. 1).

Although plant biomass was unaffected under Zn exposure, the heavy metal was uptaken in the different plant organs in the following order: root ≫ leaves ≥ woody cutting = stem.

At the end of exposure (T_2), root Zn concentration of Zn_t plants was 1808±218 mg kg^{-1}, the double compared to T_1 (907±130 mg kg^{-1}) (Fig. 2a, b). Furthermore, at each sampling time (T_1 and T_2), root Zn concentration was, on average, three times of magnitude higher than in leaves. Stems and woody cuttings of Zn_t plants exhibited the lowest Zn concentrations relatively to other organs (Fig. 2a, b). Similar trend of Zn accumulation was previously found by Romeo et al. (2014a) in hydroponic experimental condition.

The capability of Villafranca clone to extract and accumulate Zn in the plant was evaluated calculating the plant/soil concentration quotients (bio-concentration factor) as reported in Fig. 3a, b. The BCF ranged between 0.38 and 3.83 at T_1 and between 0.30 and 4.06 at T_2. The BCF indicated that Villafranca poplar clone had the same capability to accumulate Zn in different organs independently of timing of exposure. Moreover, in Villafranca clone, the root BCF represented approximately 55 and 62 % of the whole plant Zn uptaken from the soil at time T_1 and T_2, respectively.

The root system plays a key role in the interaction between heavy metal and plants. In fact, the Tf, that is indicative of the plant capability to accumulate Zn in the aerial part, was lower than 1 (Fig. 4a, b) at both time of sampling. In particular, a reduction of 30 % of Tf was observed at the end of experiment (T_2) as a consequence of an increase in root concentrations. Similar behavior was observed in *Populus nigra* and *P. alba* under Zn treatment in a hydroponic system (Dos Santos Utmazian et al. 2007).

Fig. 5 Zn content in root, woody cutting, stem, and leaves of *Populus alba* clone Villafranca after 30 (**a**) and 48 (**b**) days from the beginning of the experiment. Data, $n=5$ for (**a**) and $n=6$ for (**b**) were analyzed by two-way ANOVA, and values having different letters are significantly different according to Fisher's LSD test at 0.05 probability level

Villafranca clone did not show a metal indicator phenotype under Zn supply, and the highest Zn concentration was found at root level. Zn distribution in plant is also in agreement with data found in our previous experiment (Romeo et al. 2014a), confirming that this clone regulates Zn uptake independently to the substrate and experimental conditions applied.

As expected, the Zn content increased more in treated plants in comparison with the control ones, following the order: leaves > woody cutting = root ≥ stem (Fig. 5). Similar

Fig. 4 Translocation factor calculated on *Populus alba* clone Villafranca at time T_1 (**a**) and T_2 (**b**), respectively ($n=5$ for data at T_1; $n=6$ for data at T_2). Results were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test ($P<0.05$). *Different letters* represent significant differences among organs
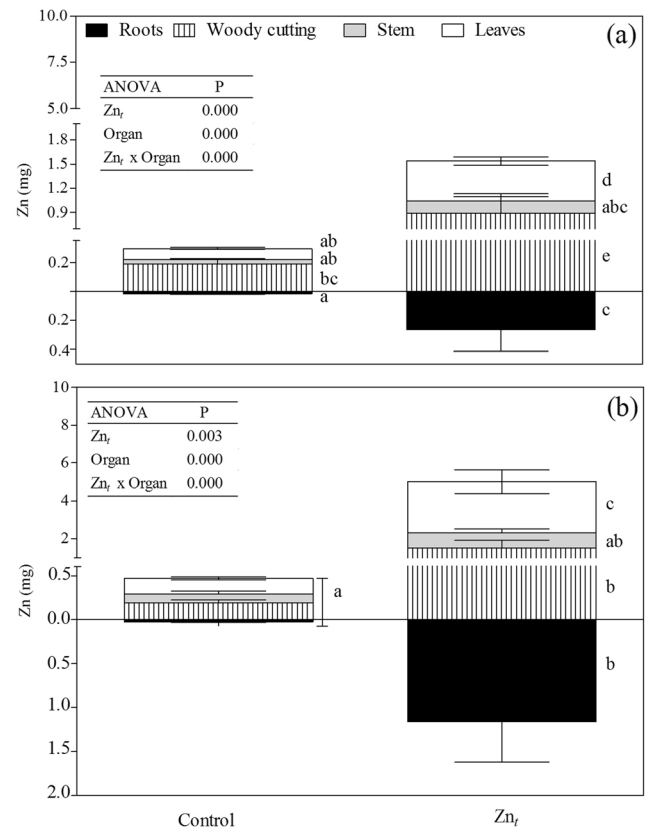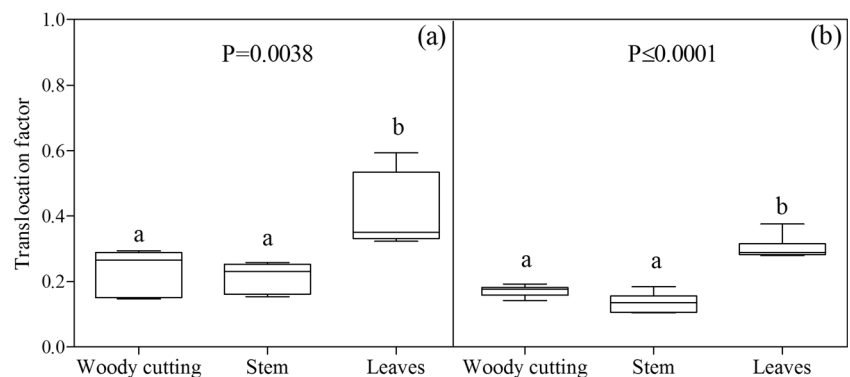
results were obtained by Langer et al. (2009) on *Populus x canescens* grown under different Zn doses and by Hermle et al. (2006) in *Populus tremula*.

In leaves, Zn increased from T_1 to T_2 reaching values in the range 0.28 to 2.71 mg (Fig. 5a, b). Considering the theoretical harvestable parts (stem + woody cutting + leaves), the Zn content was about five times higher than the root content at each sampling time: 1.5 vs. 0.3 and 5.0 vs. 1.1 mg (T_1 and T_2, respectively).

The Zn concentration did not follow the same trend of Zn content, showing that the Zn accumulation was majorly influenced by biomass production. Considering Zn concentration of the harvestable portion (stem) of poplar plants and hypothesizing a plantation density of 10,000 plants ha^{-1} (short rotation forestry), producing 119 t of dry biomass ha^{-1} in an 11-year cycle (Bonari 2001), our experimental values correspond to 27.61 kg ha^{-1} of Zn exported.

Combining different measurements of Villafranca clone performance provided a more accurate view about the net response of this poplar under Zn conditions. As reported by Körner (2003), secondary growth shows higher sensitivity to environmental conditions than photosynthesis, and so, the radial growth profile was monitored during the experiment in order to give information about secondary growth in terms of biomass production in relation to Zn_t. Cutting radius changes show a high synchronicity in the growth rate fluctuation among control and Zn_t plants (Fig. 6a), although after the 38th day, Zn_t plants show an increase in radial diameter compared to control plants. This increase reached a value 38 % higher than in control plants after 48 days of treatment, as well as showed comparing the diurnal cycle of stem radius variation from 38 to 48 days (Fig. 6b).

Data about cutting radial growth were usually used for long-term experiments under different stress conditions such as heavy metals (Fedorkov 2007) or climatic change (Oberhuber et al. 2014), but high-resolution measurements of cutting radial growth variation could also provide a means to study short-term dynamics of tree growth. Radial growth is usually proportionally attributed to leaf area (Yang and Midmore 2005) and stem length, as also demonstrated under our experimental condition (Fig. 7a, b). In particular, both treatments showed similar linear correlation between these parameters indicating that under Zn_t, plants follow the trend of control plants.

Moreover, the analyses of the positive ($\Delta R+$) and negative ($\Delta R-$) frequency distribution (ΔR), between treatments were performed (data not showed), and the occurrence of $\Delta R+$ and $\Delta R-$ did not change significantly between Zn_t and control

Fig. 6 **a** Time course (days) of cutting radial growth (cm) of *Populus alba* clone Villafranca grown in control (*empty circles*) and Zn_t (*filled circles*). Data are means ± standard error (*n*=6). **b** Cutting radial growth over Zn_t and control plants from 38 to 48 days and regression analysis
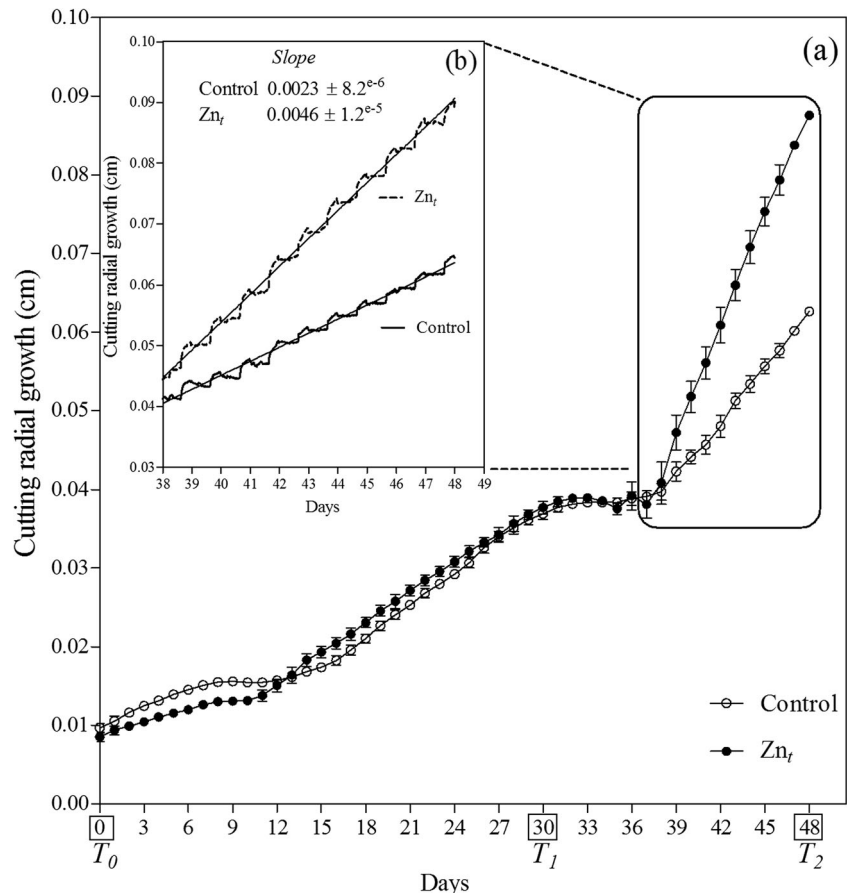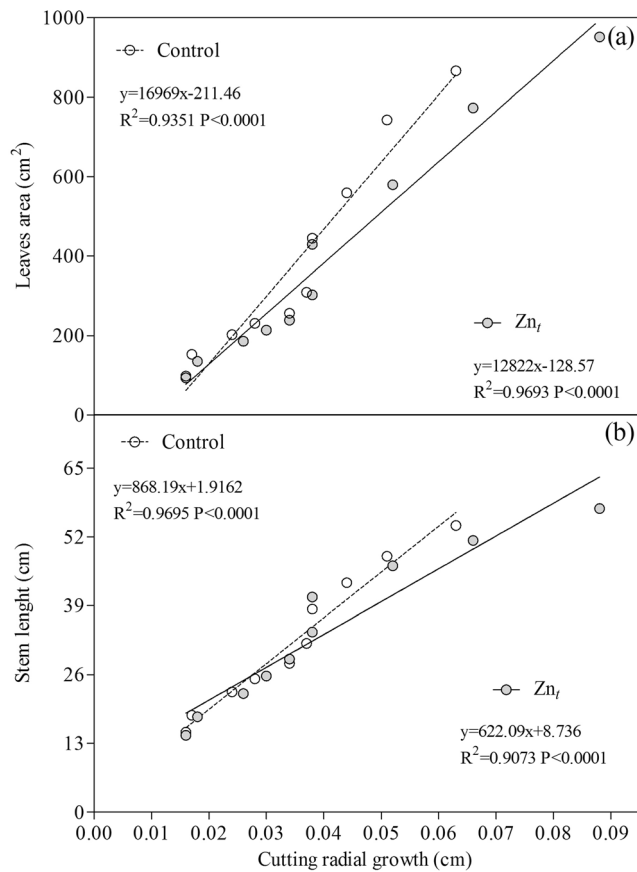
Fig. 7 Best fit first-order linear equations ($y=ax+b$) between average of cutting radial growth (cm) and leaf area (cm^2) (**a**) and cutting radial growth (cm) and stem length (cm) (**b**)

exchange parameters indicating an efficient plant homeostasis aimed to maintain the metal content within phytotoxic limits (Bernardini et al. 2015). The inhibitory effects of Zn on photosynthetic processes were observed by the authors in all clones analyzed only when Zn concentrations reached values of 750 and 1500 mg kg^{-1} in the soil.

Comparing the results obtained in this experiment to hybrid *Populus deltoids* × *P. nigra* (Di Baccio et al. 2003), *Salix viminalis*, and *P. tremula* (Hermle et al. 2006), Villafranca clone could be considered able to tolerate Zn stress. When data about gas exchange were correlated to Zn concentration at leaf levels, we found that g_s could be used as an integrative
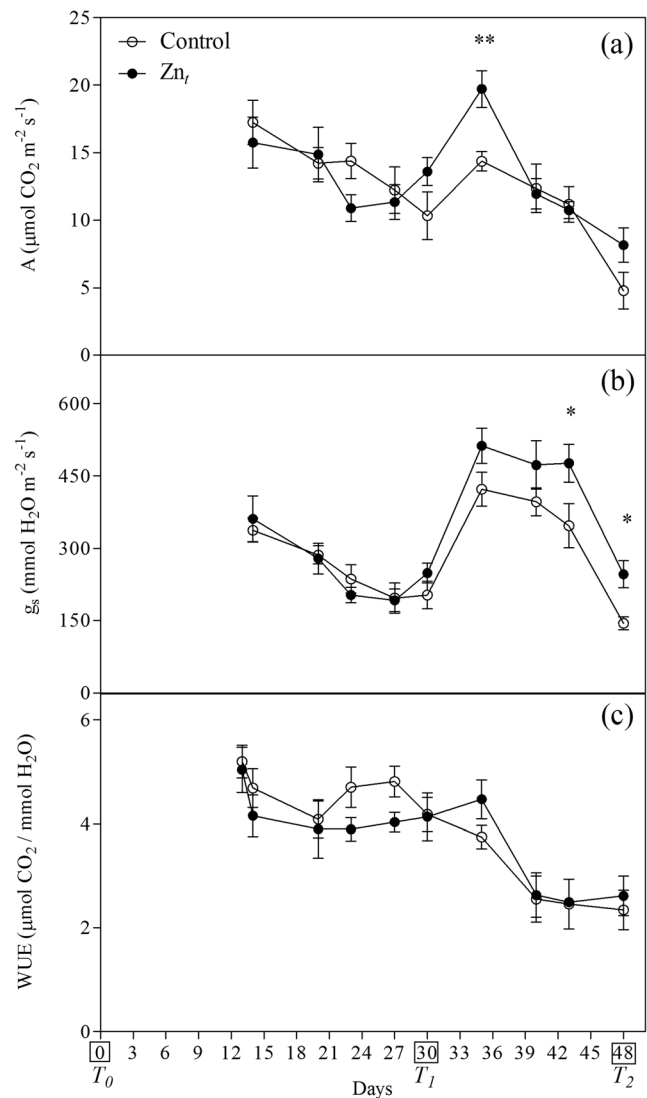

Fig. 8 Time course of CO_2 assimilation (**a**) stomatal conductance g_s (**b**), and water use efficiency (WUE) (**c**) of *Populus alba* clone Villafranca submitted to control conditions (*empty circles*) and Zn$_t$ (*filled circles*). Means ± standard error for control and Zn$_t$ ($n=6$) were compared. For each time, results were subjected to t test analysis at 0.05 probability level. *Asterisks* indicate significant difference between control and Zn$_t$. *$P \leq$ 0.05, **$P \leq 0.01$

plants (X^2 test, $P<0.05$). ΔR+ and ΔR− were 92 and 88 % and 7 and 11 % for control and Zn$_t$, respectively.

The performances of the whole plant evaluated by cutting radial growth are indicative of a plant morphological adjustment in response to applied Zn and could be used as an important parameter able to determine the outcomes of plant–environment interactions.

Several authors reported that photosynthesis is usually affected by high heavy metal concentrations differing among heavy metal applied, plant species, and cultivar (Borghi et al. 2008; Azzarello et al. 2012; Fernàndez et al. 2012; Mukhtar et al. 2013). As reported by Marschner (1995), Zn toxicity occurs when leaf concentration range is between 400 and 500 mg kg^{-1} dry weight. Although our data of leaf Zn concentration were in the range 358–535 mg kg^{-1} (T_1 and T_2, respectively), leaves of plants grown in Zn$_t$ substrate were unaffected for CO_2 assimilation (Fig. 8a–c). These results are in agreement with previous data reported by Durand et al. (2010) for *P. tremula* × *P. alba* genotype under Zn excess (265 mg kg^{-1} in soil) that also did not exhibit a dry mass reduction like Villafranca clone in our experimental condition. Under 300 ppm of ZnCl$_2$, the ecophysiological response of five Salix clones showed the absence of reductions in gas
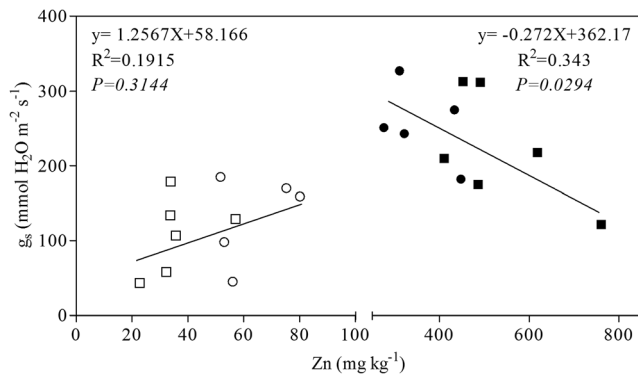
Fig. 9 Linear correlation between Zn concentration and g_s (stomatal conductance) in leaves of *Populus alba* clone Villafranca at T_1 and T_2 sampling data. *Open symbols* represent control data at T_1 (*empty circles*) and T_2 (*empty squares*). *Close symbols* represent Zn_t data at T_1 (*filled circles*) and T_2 (*filled squares*)

indicator able to give information on Zn uptake. In fact, although the net CO_2 assimilation was not affected by Zn exposure, we observed significant negative correlation between g_s and Zn concentration only when optimum plants' Zn uptake was exceeded (Fig. 9).

4 Conclusions

Taking into account all data accumulated during this experiment, but also previous results obtained on different experimental condition (Romeo et al. 2014a), *P. alba* Villafranca could be considered as a Zn-resistant plant. In fact, although the foliar Zn concentration was above the normal ranges for plants, Villafranca clone did not show any toxicity symptoms like necrosis or chlorosis. More sensitive analyses like cutting radial growth measurements revealed that this clone continues to improve its growth although under Zn supply. Applying data of Zn uptake observed in this experimental conditions to a hypothetical model of short rotation forestry application, the clone Villafranca will be able to accumulate up to 2.5 kg ha^{-1} year^{-1} Zn in the harvestable portion. These results suggest that this clone could be a suitable tree for in-field application of phytoremediation approach on contaminated land polluted by high Zn concentration.

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