TOLERANCE OF TWO HYDROPONICALLY GROWN *SALIX* GENOTYPES TO EXCESS OF ZINC

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ABSTRACT

Woody cuttings from two *Salix* genotypes (I genotype - clone LUC-31, *Salix alba* and II genotype - clone STOTT, *Salix viminalis*) were grown hydroponically for 14 days at increasing concentrations of Zn: (control - Hoagland nutrient solution), + 50, + 100 and +150 μM Zn. Genotype tolerance to excess of Zn, based on root elongation test, was evaluated. The changes in growth, Zn, Fe, Cu and Mn concentrations as well as photosynthetic performance were used as additional evaluation criteria. Photosynthetic pigments concentrations in Zn-exposed cuttings of II genotype decreased more as compared with I genotype, which corresponded well with the higher leaf Zn accumulation, lowered Fe concentrations as well as lowered photosynthetic rate. Based on the indicators used I genotype (*Salix alba*) was classified as more tolerant to excess Zn than II genotype (*Salix viminalis*).

Key Words: Salix, zinc, tolerance, photosynthesis, mineral nutrients

INTRODUCTION

Phytostabilization of metal-contaminated soils is an environmentally friendly approach using both soil amendments for metal immobilisation and tolerant plants for site revegetation (Vangronsveld and Cunningham, 1998). The use of willow (*Salix*) for soil stabilization in former mining sites due to its ability to tolerate high soil metal concentrations has been recently proposed (Dickinson, 2000; Hammer et al., 2003). Actually, a large variation in the tolerance to excess heavy metals exists within *Salix* clones (Greger and Landberg, 1999; Punshon and Dickinson, 1999; Rulford et al., 2002; Vyslouzilova et al. 2003) indicating that the potential of *Salix* to tolerate elevated metal levels is still far from being fully unravelled.

Generally, easily and quickly measurable growth parameters, such as root length, root biomass as well as roots numbers are preferable in the screening studies for metal tolerance (Watson et al., 2003), but for more accuracy some physiological and biochemical indicators should complement the former ones (Vangronsveld and Clijsters, 1992). For example, some photosynthetic parameters, e.g. gas exchange, photosynthetic pigments content, etc. may be helpful in the screening studies as many metal-induced plant disorders may be finally result in effects on photosynthetic performance (Krupa and Baszynski, 1995).

Zinc is an essential micronutrient for plants, but at supra optimal concentrations, which are occurring in some industrially polluted soils, it can become phytotoxic (Cuypers et al., 1999). This effect may be due to Zn-induced (1) decrease in the content of essential nutrients as Fe, Cu and Mn (Siedleska, 1995; Ebbs and Kochian, 1997), (2) oxidative damage to membranes (Weckx and Clijsters, 1997; Cuypers et al., 2001), despite of its known effect to protect membrane integrity when present at normal concentrations (Ernst et al., 1992; Cakmak, 2000) and (3) disturbances in photosynthetic performance (Vangronsveld and Clijsters, 1994). Excess Zn may affect photosynthesis at different sites, including photosynthetic pigments, photosynthetic electron transport, RubisCo activity, etc.; a substitution of Zn for Mg and Mn has been suggested as a possible mechanism.

Plant performance of *Salix* species at elevated Zn concentrations is only occasionally investigated in more detail (Landberg and Greger, 2002), which contrasts to the significant attention for these species in phytoremediation programmes. Therefore, we decided to study the zinc tolerance of two hydroponically grown *Salix* genotypes to excess Zn using, additionally to the more common morphological effects, Zn accumulation and some photosynthetic parameters.

MATERIAL AND METHODS

Plant Material and Growth Conditions

Woody cuttings (25 cm long) from 1-year-old shoots from two *Salix* genotypes (*Salix alba*; clone LUC 31 and *Salix viminalis*; clone STOTT, named in the text as I genotype and II genotype, respectively), grown in the collection garden of the Hasselt University (Belgium), were rooted for one month in tap water. After that they were transferred for 14 days to ½ strength modified Hoagland nutrient solution (HNS) combined by increasing concentrations of Zn [(HNS-control), + 50, + 100, and + 150 μM]. The experiments were conducted in a greenhouse at 14/10 hours (day/night) photoperiod, 65 ± 5% / 75 ± 5% air humidity, 22 ± 2 / 18± 2 °C temperature (day/night) and about 150 μmol m^{-2} s^{-1} photosynthetic photon flux density at leaf level. The solutions were continuously aerated and refreshed every 3 days. All treatments were arranged in 3 replicates (3 liter-pots), each consisting of 3 cuttings.

Growth Test

Before the treatment period the root tips were stained black in a stirred suspension of finely powdered active carbon (according to the procedure described by Schat and Ten Bookum, 1992), followed by rinsing in deionised water as shown by Gupta et al. (1999). The root elongation at the end of the treatment period was measured as a difference between the black zone and the tip. Tolerance indexes, representing percentage of the root growth in presence of Zn to that without Zn, were calculated as described by Schat and Ten Bookum (1992). The so-called “effect”

concentrations - EC_{50} were calculated using the regression equations, describing the tolerance indexes dependence on the external Zn concentrations. At the end of the treatment period, cuttings were harvested and divided into roots, woody cuttings, and new growing shoots (named in the text shoots). Fresh weight of both roots and shoots were determined as well as the percentage of dry weight after drying of samples for 48 hours at 70 °C.

Mineral Analysis

Samples of both roots and leaves were thoroughly washed with deionised water and used for mineral analysis. Samples were processed through dry mineralisation at 600 °C for 7 hours. The ash was dissolved in 20% HCl and the concentrations of Zn, Fe, Cu and Mn were determined by inductively coupled plasma - atomic emission spectrometry (ICP-AES; PERKIN ELMER, Optima 3000 DV).

Photosynthetic Parameters

Leaf gas exchange (net photosynthetic rate - A, transpiration rate – E and stomatal conductance - gs) was measured on the youngest fully expanded leaf (from the top) at the end of the treatment period. The analyses were performed between 10.00 and 12.00 a.m. using an LCA-4 (ADC, England) under the conditions in the greenhouse desribed above. Chlorophylls *a* and *b* and total carotenoids were extracted in 100% acetone, measured spectrophotometrically and calculated according to the formulae of Lichtenthaler (1987). Soluble protein content in the leaves was determined using the method of Bradford (1976).

Statistical Analysis

Statistical analysis was performed using one-way ANOVA (for $P<0.05$). A regression analysis was applied to establish trends.

RESULTS

Growth Responses

Dry mass of the both *Salix* genotypes as well as their tolerance indices to excess Zn are shown in Fig. 1. The dry mass of II genotype was significantly higher then I genotype (Fig. 1 A, B), which corresponds to its much higher stem productivity under field conditions. After 14 days growth in a half strength Hoagland solution without additional Zn (controls) the total weight of both roots and shoots reached 1.95 ± 0.25 and 2.90 ± 0.32 g DW in the cuttings of I genotype and II, respectively. It should be mentioned that although the cuttings were carefully selected for uniformity they showed high variation in both shoot and root growth.

Growth retardation (Fig. 1 A, B) and some chlorosis of the upper leaves, mainly in II genotype, were detected at higher Zn treatments. The applied range of excess Zn caused almost linear suppression of dry mass of both roots and shoots, being similar for both clones. For example, the exposure to 100 μM Zn produced 28% and 25% inhibition of the total weight of I genotype and II, respectively. The roots of II genotype showed a stronger biomass decrease, except at 50 μM Zn treatment.

Additional dry weight, excess Zn also retarded root growth. The length of the longest roots in both clones was gradually reduced (data not shown). The Zn tolerance indices based on root elongation test, showed a similar pattern for both *Salix* clones (Fig. 1C). The calculated EC_{50} values (obtained by linear regression equations) were 114 and 121 μM Zn for clones 1 and 2, respectively.

Nutrients Content

In control cuttings, root Zn concentrations of both *Salix* clones were within the normal range (Punshon and Dickinson, 1997). However, the root Zn concentrations in the Zn-exposed cuttings (Table 1 and 2), rose gradually being higher in II genotype. For example, the maximum root Zn concentration achieved at 150 μM Zn treatment in II genotype was 560 ± 28, whereas the respective

value in I genotype was 486 ± 34 mg kg^{-1} DW. In the leaves, similar trends of Zn accumulation have been observed in both clones, resulting in higher maximum concentrations in II genotype (656 ± 37 mg Zn kg^{-1}) as compared with I genotype.

The concentrations of Cu, Fe and Mn in plant organs were different in clones 1 and 2 but in normal concentration range (Punshon and Dickinson, 1999; Rulford et al., 2002). In the roots, Mn concentrations tended to decrease in both clones when the external Zn level increased. Excess of Zn did not induce significant changes in Cu concentrations of both clones, but decreased Fe concentrations in both leaves and roots of II genotype.

Photosynthetic Performance

The net photosynthetic rate (A) in both *Salix* genotypes under control conditions was relatively similar (4.02 – 4.27 μmol CO_2 m^{-2} s^{-1}), but the transpiration rate (E) and stomata conductance (g_s) were much higher in II genotype (Fig. 2). In general, excess of Zn retarded A in both clones as well as E in II genotype at levels of 100 μM Zn and higher. Both E and gs did not show significant due to excess of Zn, but some lowered values were found in II genotype at higher Zn treatments.

The most pronounced difference between the responses of both clones to excess of Zn was the change in their photosynthetic pigments concentrations (Table 3). While I genotype showed a significant decrease only in chlorophyll *a* content at the highest Zn treatment, significant decreasing trends in chl. *a*, chl. *b* and total carotenoids were observed in II genotype. These results corresponded well with the observed chlorotic symptoms in II genotype, especially at 150 μM Zn treatment.

The soluble protein content in the leaves of both clones was different, being higher in II genotype (Table 3). The mean values at control treatments were 3.63 ± 0.13 (I genotype) and 5.75 ± 0.36 (II genotype). Zn treatments tended to decrease leaf protein content in both clones.

DISCUSSION

The so-called “effect” toxicity is often used to establish the external metal concentration causing a certain decrease in plant growth or yield. The usefulness of root growth as a parameter in metal tolerance tests relies on the fact that metal-imposed root growth reduction is due to direct effects on both cell division and elongation (Ernst et al., 1992). The EC_{50} value based on root elongation tests, suggested that both *Salix* genotypes had similar tolerances for increased external Zn concentrations (I genotype - 114 μM; II genotype – 121 μM) (Fig. 1C). Landberg and Greger (2002) considered *Salix* clones as resistant when they showed not more than a 20% decrease of root dry weight when growing at 70 μM Zn for 20 days on $Ca(NO_3)_2$ solution. Following this suggestion and using the regression equations shown in Fig. 1 (A, B) both genotypes may be classified as resistant to excess of Zn.

In general, zinc concentrations in plants are between 15-100 mg kg^{-1} DM (Hagemeyer, 1999) and a leaf Zn concentration of 400 mg kg^{-1} dry matter can be considered as an average critical toxicity level (Kiekens, 1990). However, it may vary depending on the species and the method of cultivation. In our study, II genotype assimilated and allocated to the leaves more Zn than I genotype. While Zn concentrations in the roots of I genotype were higher then in the leaves, the opposite situation was observed in II genotype. As a result of this pattern, the achieved leaf Zn concentrations in II genotype reached the toxic Zn threshold at the higher treatments.

Excess Zn diminished Fe concentrations in both roots and leaves of II genotype. A similar trend was observed in many studies and mostly explained by the similar radii of their hydrated ions (Marschner, 1986). The lowered Mn concentrations in roots may be due to both, decreased root growth and probably unsuberized areas, where ions are mostly taken up.

The observed decline in net photosynthetic rate (A) of Zn-exposed *Salix* genotypes may be due to both stomata and mesophyll-related factors. Obviously, in our study, the decrease of A was more related to non-stomatal constrains (Fig. 2) since E and g_s showed insignificant changes. Some influence of stomatal opening may be supposed in II genotype at higher Zn concentrations but

obviously, the negative effect of Zn was more related to the decreased pigments content. In fact, the observed drop in photosynthetic pigments was higher than a decrease in A, especially in II genotype. This is not surprising since light absorption by the leaves and overall photosynthetic activity are not linearly related to chlorophyll content (Terry, 1980). Since the Fe deficiency level for *Salix* is not clearly established, we may only suggest that, at least partially, the decrease of chlorophyll content in II genotype could be due to Zn-induced Fe deficiency.

The results obtained in this study suggest II genotype (*Salix viminalis*) being less tolerant to excess Zn as compared with I genotype (*Salix alba*). The difference between the genotypes was not really clear when using morphological parameters. At least partially, this may be due to a contribution of stem reserves of the cuttings to root and shoot growth as well as significant variation observed, even in well-selected and visibly uniformed cuttings. Among the used physiological indicators, the photosynthetic pigment concentrations showed the most pronounced response of excess of Zn. Photosynthetic pigments in Zn-exposed cuttings declined stronger in II genotype than in I genotype, which corresponded well with its higher leaf Zn accumulation as well as decreased Fe levels. Our data illustrate the importance of involving physiological and eventually also biochemical parameters for the evaluation of metal tolerance of *Salix* clones.

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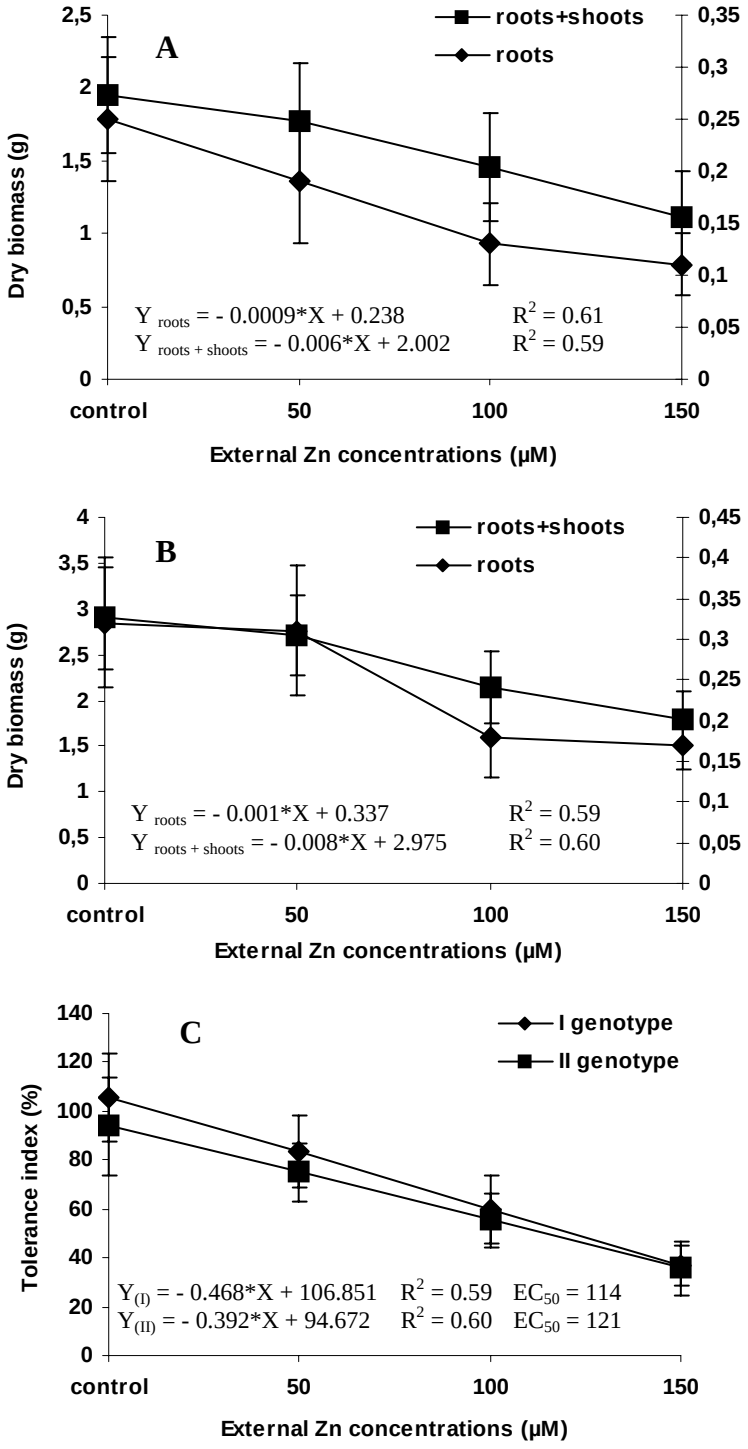

Fig. 1. Dry mass of two *Salix* clones (A – I genotype; B – II genotype) exposed to excess of Zn as well as their tolerance indexes (C), based on root elongation test. On the right axis are presented the roots data.

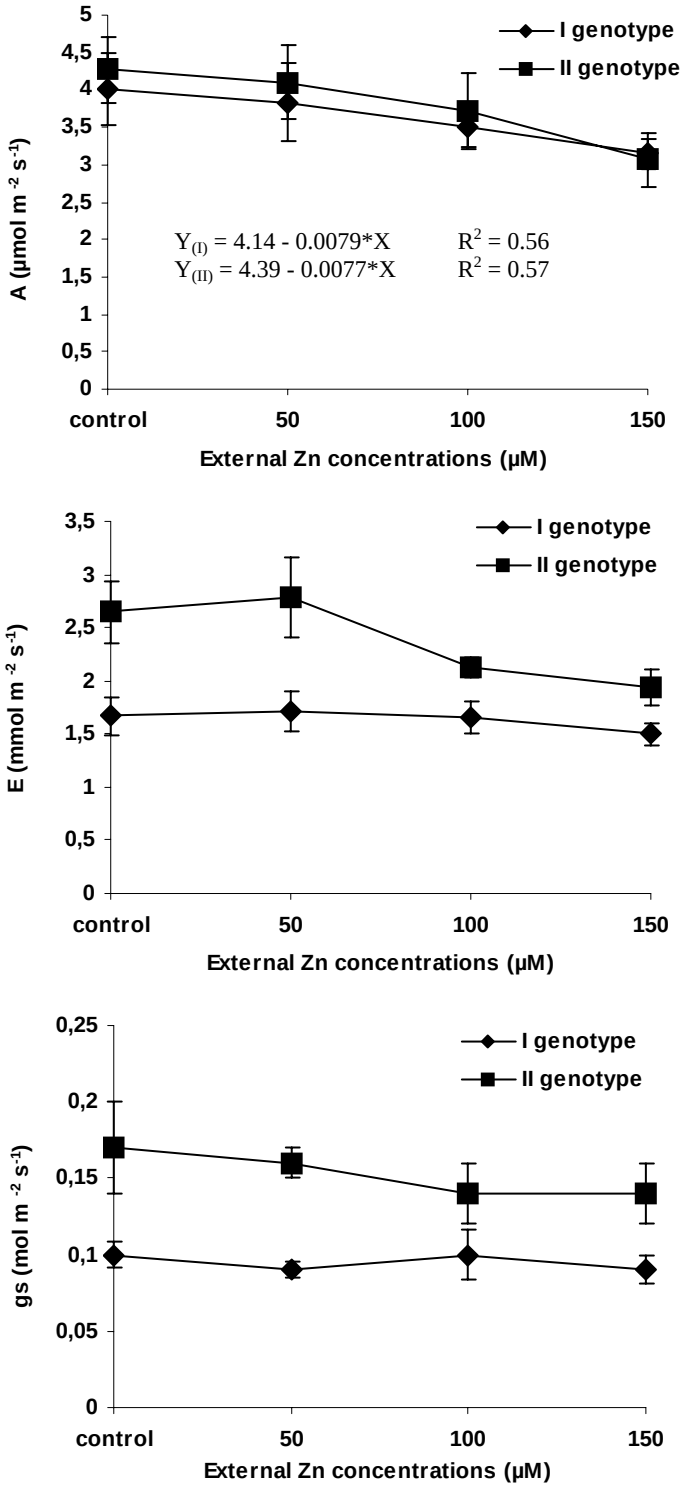

Fig. 2. Leaf gas exchange (A – photosynthetic rate; E – transpiration rate; gs – stomatal conductance) of two *Salix* clones exposed to excess of Zn

Table 1. Concentrations of several nutrients in both roots and leaves of *Salix* cuttings (I genotype), exposed to excess of Zn

Treatments	Content of some nutrients in roots (mg kg^{-1} DW)			
(μM Zn)	Zn	Fe	Cu	Mn
Control	126 ± 16	149 ± 5	55 ± 6	29 ± 5
+ 50	311 ± 26	165 ± 25	48 ± 5	19 ± 1
+ 100	442 ± 17	159 ± 38	45 ± 8	18 ± 2
+ 150	486 ± 34	164 ± 32	51 ± 3	14 ± 1
Nutrient	Regression equations			R^2
Zn	Y = 3.03*X + 136.7			0.84
Mn	Y = - 0.08*X + 26.4			0.80
Treatments	Content of some nutrients in leaves (mg kg^{-1} DW)			
(μM Zn)	Zn	Fe	Cu	Mn
Control	91 ± 4	61 ± 9	34 ± 4	25 ± 1
+ 50	286 ± 21	40 ± 2	29 ± 8	18 ± 1
+ 100	297 ± 35	39 ± 3	28 ± 1	19 ± 3
+ 150	375 ± 18	40 ± 5	28 ± 3	14 ± 1
Nutrient	Regression equations			R^2
Zn	Y = 1.71*X + 134.1			0.82
Mn	Y = -0.06*X + 23.7			0.70

Table 2. Concentrations of several nutrients in both roots and leaves of *Salix* cuttings (II genotype), exposed to excess of Zn

Treatments	Content of some nutrients in roots (mg kg^{-1} DW)			
(μM Zn)	Zn	Fe	Cu	Mn
Control	72 ± 10	234 ± 23	32 ± 3	36 ± 5
+ 50	302 ± 34	250 ± 27	35 ± 7	19 ± 3
+ 100	564 ± 43	196 ± 17	33 ± 1	15 ± 1
+ 150	560 ± 28	176 ± 24	33 ± 5	10 ± 2
Nutrient	Regression equations			R^2
Zn	Y = 3.47*X + 113.4			0.88
Fe	Y = -0.48*X + 251.8			0.60
Mn	Y = -0.16*X + 32.4			0.82
Treatments	Content of some nutrients in leaves (mg kg^{-1} DW)			
(μM Zn)	Zn	Fe	Cu	Mn
Control	118 ± 13	58 ± 3	34 ± 1	62 ± 2
+ 50	305 ± 23	57 ± 4	27 ± 1	60 ± 6
+ 100	506 ± 33	43 ± 1	34 ± 3	63 ± 3
+ 150	656 ± 37	40 ± 2	30 ± 2	61 ± 1
Nutrient	Regression equations			R^2
Zn	Y = 3.65*X + 121.3			0.98
Fe	Y = -0.14*X + 59.8			0.82

Table 3. Photosynthetic pigments and soluble protein concentrations in two *Salix* clones, exposed to excess of Zn

Treatments (μM Zn)	Photosynthetic pigments and soluble protein content (mg kg^{-1} FW)			
	Chl. a	Chl. b	Total Car.	Protein
I genotype				
Control	1.94 ± 0.27	0.66 ± 0.13	0.44 ± 0.09	3.63 ± 0.13
+ 50	1.85 ± 0.11	0.61 ± 0.03	0.42 ± 0.04	3.78 ± 0.16
+ 100	1.76 ± 0.12	0.62 ± 0.05	0.39 ± 0.01	2.95 ± 0.42
+ 150	1.44 ± 0.10	0.53 ± 0.09	0.35 ± 0.04	2.57 ± 0.37
Parameter	Regression equations			R^2
Protein	Y = -0.007*X + 3.87			0.64
II genotype				
Treatments (μM Zn)	Photosynthetic pigments and soluble protein content (mg kg^{-1} FW)			
	Chl. a	Chl. b	Total Car.	Protein
Control	1.73 ± 0.08	0.60 ± 0.05	0.34 ± 0.03	5.75 ± 0.36
+ 50	1.67 ± 0.10	0.57 ± 0.05	0.34 ± 0.01	5.21 ± 0.45
+ 100	1.32 ± 0.08	0.48 ± 0.08	0.28 ± 0.02	4.63 ± 0.54
+ 150	0.84 ± 0.09	0.32 ± 0.04	0.17 ± 0.04	4.51 ± 0.51
Parameter	Regression equations			R^2
Chl. a	Y = -0.006*X + 1.87			0.89
Chl. b	Y = -0.002*X +0.64			0.77
Total Car.	Y = -0.001*X + 0.36			0.76
Protein	Y = -0.009*X +5.69			0.60