

Accumulation and distribution of Zn in the shoots and reproductive structures of the halophyte plant species *Kosteletzkya virginica* as a function of salinity

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Received: 5 March 2013 / Accepted: 18 May 2013
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Abstract *Kosteletzkya virginica* is a wetland halophyte that is a good candidate for rehabilitation of degraded salt marshes and production of oil as biodiesel. Salt marshes are frequently contaminated by heavy metals. The distribution of Zn in vegetative and reproductive organs of adult plants, and the NaCl influence on this distribution remain unknown and were thus explored in the present study. Plants were cultivated in a nutrient film technique system, from seedling stage until seed maturation in a control, Zn (100 μ M), NaCl (50 mM) or Zn + NaCl medium. Photosynthesis, ion nutrition, malondialdehyde and non-protein thiol concentrations were quantified. Zinc distribution in reproductive organs was estimated by a laser ablation-inductively coupled plasma-mass spectrometry procedure (LA-ICP-MS). Adult plants accumulated up to 2 mg g⁻¹ DW Zn in the shoots. Zinc reduced plant growth, inhibited photosynthesis and reduced seed yield. Zinc accumulation in the seeds was only two times higher in Zn-treated plants

than in controls. Exogenous NaCl neutralized the damaging action of Zn and modified the Zn distribution through a preferential accumulation of toxic ions in older leaves. Zinc was present in seed testa, endosperm and, to a lower extent, in embryo. Additional NaCl induced a chalazal retention of Zn during seed maturation and reduced final Zn seed content. It is concluded that NaCl 50 mM had a positive impact on the response of *K. virginica* to Zn toxicity and acts through a modification in Zn distribution rather than a decrease in Zn absorption.

Keywords Heavy metals · Seashore mallow · Salinity · NaCl · Zinc · Phytoremediation

Abbreviations

A	Net carbon assimilation rate
BP	Basal part of the plant
E	Instantaneous transpiration
F ₀	Minimal fluorescence level
F _m	Maximal fluorescence level
FP	Mean number of flowers per plant
g _s	Stomatal conductance
LA-ICP-MS	Laser ablation-inductively coupled plasma-mass spectrometry
LN	Number of leaves
LR	Mean length of the ramifications
MDA	Malondialdehyde
NR	Number of ramifications
NFT	Nutrient film technique
NPQ	Non-photochemical quenching
PSII	Photosystem II
qP	Photochemical quenching coefficient
SL	Mean length of the main stem
UP	Upper part of the plant
ΦPSII	Actual PSII efficiency

Electronic supplementary material The online version of this article (doi:10.1007/s00425-013-1903-3) contains supplementary material, which is available to authorized users.

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Ψ_w	Leaf water potential
Ψ_s	Osmotic potential
WC	Water content

Introduction

Zinc is an essential element for all living organisms. Its metabolic functions are based on its strong tendency to form tetrahedral complexes with N-, O- and S-ligands and it therefore plays both a functional and a structural role in enzyme reactions. Zinc excess in the environment, however, frequently occurs as a result of anthropic emissions (Callender and Rice 2000; Reck et al. 2006). It may accumulate at high concentrations in estuarine sediments where it compromises the ecosystem stability and constitutes a serious threat for human health (Sáinz et al. 2002; Morillo et al. 2004). Among the major symptoms of Zn toxicity in plants, photosynthesis inhibition, modification in the plant water status and oxidative stress are frequently reported (Vaillant et al. 2005; Tewari et al. 2008; Lefèvre et al. 2010; Redondo-Gómez et al. 2011).

Several studies reported that salinity may directly interfere with heavy metal absorption by the roots and translocation to the shoots (López-Chucken and Young 2005; Lefèvre et al. 2009a). Since wetland species are of special interests for phytoremediation and restoration of heavy metal contaminated salt marshes, special attention should be paid to those ecosystems (Otte et al. 1991; Weis and Weis 2004; Rai 2008).

Comino et al. (2005) mentioned that interactions between the two types of constraint (salinity on the one hand and heavy metals on the other hand) may vary according to plant species. Hyperaccumulating plant species able to accumulate high concentrations of heavy metals display contrasting behaviour in response to salinity (Whiting et al. 2003). Salinity was reported to reduce Zn accumulation by the hyperaccumulating species *Noccaea caerulescens* (Comino et al. 2005). Heterogeneity was reported for halophyte plant species exposed to high external doses of zinc. Redondo-Gómez et al. (2011) recently reported that salinity has a stimulatory effect on zinc accumulation by the invader obligate halophyte *Spartina densiflora*. Mahon and Carman (2008) also reported a stimulating effect of salinity on heavy metal accumulation in several other salt marsh species.

Kosteletzkya virginica (L.) Presl (Malvaceae; syn. *K. pentacarpos*) is a perennial facultative halophyte species growing in brackish areas of tidal wetlands along the southern Atlantic coast of the United States. This species has been introduced in China, where it was integrated in

developmental schemes for alternative saline agriculture (He et al. 2003; Ruan et al. 2008). Of special interest is the seed oil content of some selected lines which may contain more than 20 % lipids with a high level of unsaturated fatty acids (Ruan et al. 2008, 2012). In contrast to the above-mentioned wetland halophyte species, salinity reduces Zn accumulation in stems but had no impact on leaf Zn concentration in young seedlings (Han et al. 2012a). To the best of our knowledge, no data are available for adult plants exposed to Zn and NaCl for long periods. Data are also crucially lacking concerning Zn distribution in reproductive organs, in fruits and mature seeds, which are of special interest in relation to the putative use of this promising plant for biofuel production.

Although plants employ efficient strategies to limit the translocation of non-essential heavy metals such as Cd or Pb to developing embryos in maturing seeds (Ernst and Nelissen 2000), the essential role of Zn during the germination process and early seedling growth may lead to Zn accumulation in embryonic tissues. The aims of the present study were therefore (1) to determine Zn impact on plant growth, development and physiological parameters (2) to analyse the influence of moderate doses of salt on plant response to Zn excess (3) to quantify the seed set and analyse mineral distribution in fruits and seeds through the use of a microanalytical laser ablation strategy.

Materials and methods

Plant material and culture conditions

Seeds of *K. virginica* were issued from a field plantation in Jinhai Agricultural Experimental Farm of Yancheng (Jiangsu Province) and kindly provided by Prof. P. Qin, University of Nanjing (PR China). Seeds were sown in trays filled with a perlite vermiculite mix (1:3 v/v) moistened regularly with a half-strength modified Hoagland nutrient solution and seedlings were grown in a phytotron under a 12-h photoperiod [mean light intensity (PAR) = 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by Osram Sylvania (Danvers, MA) fluorescent tubes (F36W/133-T8/CW) with 27 °C/23 °C day/night temperature and 70 %/50 % atmospheric humidity]. Fifteen days after sowing, seedlings were transferred into a greenhouse and fixed on polyvinyl chloride plates floating on aerated half-strength modified Hoagland nutrient solution. The nutrient solution contained the following chemicals (in mM): 2.0 KNO₃, 1.7 Ca(NO₃)₂, 1.0 KH₂PO₄, 0.5 NH₄NO₃, 0.5 MgSO₄ and (in μM) 17.8 Na₂SO₄, 11.3 H₃BO₃, 1.6 MnSO₄, 1 ZnSO₄, 0.3 CuSO₄, 0.03 (NH₄)₆Mo₇O₂₄ and 14.5 Fe-EDDHA. Minimum temperatures were 16–18 °C and daily maxima were 24–28 °C. Natural light was supplemented by Philips

lamps (Philips Lighting S.A., Brussels, Belgium) (HPLR 400 W) in order to maintain a light irradiance of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ (PAR) at the top of the canopy.

After 15 days of acclimation, seedlings were gently removed from the tank and introduced in a nutrient film technique (NFT) device consisting in an 8-m-long and 0.25-m-broad gully with a slope of 1.5 %. Seedlings were laterally adapted in specific orifices to the gully, with the root system inward (Fig. 1a). Gullies were then closed with a dark plastic sheet to avoid algae contamination and solution evaporation. Fifteen plants were placed at equal distances in the four gullies. A 50-L tank behind each gully was used to supply nutrient solution (see composition above) at a flow of 3 L min^{-1} generated by an Eheim 1260 circulating pump. Nutrient solution was renewed each week but was daily controlled for pH stability. Gullies were distributed in a greenhouse under natural light, and temperature conditions as stated above. Humidity ranged between 48 and 70 %. Environmental conditions were daily recorded by a Hobo® Onset Computer (Burne, MA, USA) and no significant difference was recorded between the gullies. Shoots were regularly fixed to a vertical cable thus avoiding lodging for shoots growing up to 4 m high.

After 2 weeks, four treatments were applied (one treatment per gully): (1) control, (2) $100 \mu\text{M ZnCl}_2$, (3) 50 mM NaCl and (4) $100 \mu\text{M ZnCl}_2 + 50 \text{ mM NaCl}$. Heavy metal and salt doses were chosen on the basis of previous results (Han 2013). The dose of 50 mM NaCl had no stimulating impact on the growth of the halophyte *K. virginica* and should therefore help us to determine the metabolic impact of salt on the plant response to Zn stress independently of dilution effects resulting from growth stimulation. In each case, pH was adjusted to 6.0. Treatments were maintained for a total period of 16 weeks.

Results obtained from Visual MINTEQ 3.0 geochemical model showed that NaCl reduced Zn activity in the nutrient solution by 4.6 % only.

Plant growth and water status

The number of leaves (LN), the number of ramifications (NR), the mean length of the main stem (SL) and the mean length of the ramifications (LR) were recorded every week for each plant. Plants were harvested after 16 weeks of treatment. Heavy metals have a drastic influence on the young seedling architecture in *K. virginica* (Han et al. 2012a, b, 2013), but also on architecture of adult plants from other species (Lefèvre et al. 2009b, 2010); we therefore considered separately the upper part of the plant (UP, containing 50 % of the leaves on the main stem) and the basal part (BP), corresponding to the older part. Ramifications produced by those parts were also considered separately.

Leaf water potential (Ψ_w) and osmotic potential (Ψ_s) were determined between 12:00 and 14:00 p.m. Leaf water potential (Ψ_w) was evaluated immediately after sampling by the pressure chamber method (PMS Instrument Co., Orlando, USA) using the main stem and three ramifications on each plant. For osmotic potential determination (Ψ_s), tissues were quickly collected, cut into small segments, placed in Eppendorf tubes perforated with four small holes and immediately frozen in liquid nitrogen. After being encased individually in a second intact Eppendorf tube, they were allowed to thaw for 30 min and centrifuged at $15,000g$ for 15 min at 4°C . The collected tissue sap was analysed for Ψ_s estimation. Osmolarity (c) was assessed with a vapour pressure osmometer (Wescor 5500; Wescor, Inc., Utah, USA) and converted from mosmol kg^{-1} to MPa using the following formula: $\Psi_s \text{ (MPa)} = -c$



Fig. 1 Experimental device used for NFT culture of *Kosteletzkya virginica* mature plants. Young seedlings were introduced laterally in a closed gully exhibiting a slope of 1.5 % while a circulating nutrient

solution was provided from a tank through an Eheim pump at a flow rate of 3 L min^{-1} (a). Mature shoots were fixed on vertical cables acting as a support to avoid lodging (b)

(mosmol kg⁻¹) $\times 2.58 \times 10^{-3}$ according to the Van't Hoff equation.

Leaf stomatal conductance (g_s) was measured between 1 and 2 p.m. with an automatic porometer (AP 4, Delta-T Devices, Cambridge, UK) after 4 and 12 weeks on 5 plants per treatment.

Photosynthetic parameters

Net photosynthesis was estimated for a leaf located at the middle portion of the main stem, so that the considered leaf always had more or less the same physiological age. The net carbon assimilation rate (A) under 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) and the instantaneous transpiration (E) were measured using a portable, open system infrared gas analyzer (IRGA, LCA4 8.7 ADC, Hertshire, UK) using a PLC Parkinson leaf cuvette on intact leaves for 1 min (20 records min⁻¹), with an air flow of 300 ml min⁻¹. Air taken in the greenhouses was sent to a chamber in which a leaf portion of 6.25 cm² was introduced. Measurements (5 per treatment) were made between 10:00 and 12:00 a.m. in six attached leaves for each treatment.

Chlorophyll fluorescence measurements were carried out using a portable pulse-modulated chlorophyll fluorimeter (FMS2; Hansatech, King's Lynn, UK). The recordings were made on a leaf located at the middle portion of the main stem. Leaves were acclimated to darkness for 20 min. The minimal fluorescence level (F_0) was estimated by measuring the modulated light (0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The maximal fluorescence level (F_m) with all photosystem II (PSII) reaction centers open was determined by a 0.8-s saturating pulse at 8,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in dark-adapted leaves. The leaves were then continuously illuminated with white actinic light (320 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 3 min; the steady-state value of fluorescence (F_s) was recorded. A second saturating pulse at 8,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was imposed to determine the maximal fluorescence level in the light-adapted state (F_m'). The actinic light was removed and the minimal fluorescence level in the light-adapted state (F_0') was determined by illuminating the leaf with a 3-s pulse of far-red light. Using both light and dark fluorescence properties, the following parameters were calculated according to Maxwell and Johnson (2000):

Maximal efficiency of PSII photochemistry in the dark-adapted state, $F_v/F_m = (F_m - F_0)/F_m$;

Photochemical quenching coefficient, $qP = (F_m' - F_s)/(F_m' - F_0')$;

Non-photochemical quenching, $NPQ = (F_m - F_m')/F_m'$; and

Actual PSII efficiency, $\Phi_{PSII} = (F_m' - F_s)/F_m'$.

Chlorophyll (Chl *a* and Chl *b*) and total carotenoid (xanthophylls + β -carotene) concentrations were quantified on the oldest leaf of the UP. Samples (ca. 150 mg FW) were ground in the dark in 8 mL acetone 80 % and centrifuged at 1,000g for 10 min at 4 °C. The absorbance of the sample was read at three different wavelengths (663.2, 646.8 and 470 nm) using a spectrophotometer (DU[®] 640, Beckman). Each measurement was repeated three times. The pigments concentrations were calculated according to Lichtenthaler (1987).

Non-protein thiol and malondialdehyde quantification

Leaves were harvested from shoots of four plants per treatment after 4 and 16 weeks of treatment and frozen in liquid nitrogen. The total non-protein thiol concentration was determined according to De Vos et al. (1992) by grinding 200 mg FW of tissue in 2 mL of 5 % (w/v) sulfosalicylic acid + 6.3 mM diethylenetriaminepentaacetic acid (pH < 1) at 0 °C with quartz sand in a mortar. The homogenate was centrifuged at 10,000g for 10 min at 4 °C. The clear supernatants were collected and used for the determination of thiols using Ellman's reagent. Three hundred microliters of supernatant were mixed with 630 μL of 0.5 M KH₂PO₄ and 25 μL of 10 mM 5,5-dithiobis 2-nitrobenzoic acid (final pH 7.0). The absorbance at 412 nm was recorded after 2 min, and the thiol concentration was estimated using an extinction coefficient of 13,600 M⁻¹ cm⁻¹.

Malondialdehyde (MDA), routinely used as an indicator of membrane lipid peroxidation, was determined by the thiobarbituric acid reaction as described by Heath and Packer (1968). For each sample, 250 mg fresh tissue was ground in a pre-chilled mortar, and homogenized in 5 mL of 5 % (w/v) trichloroacetic acid. The homogenates were centrifuged at 10,000g for 10 min and filtered through Whatman filter paper (11 μm). A 2-mL aliquot of thiobarbituric acid (0.67 % (w/v)) was added to 2 mL of supernatant: the mixture was heated at 100 °C for 30 min and then quickly cooled on ice. Samples were centrifuged at 5,000g for 1 min and the absorbance was measured at 532 nm. The non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

Mucilage extraction and quantification

Leaves, stems, and roots of *K. virginica* were harvested from ten plants per treatment at the end of the stress period. Tissues were oven-dried at 40 °C for 1 week and then ground into a fine powder using a ball mill and passed through a 0.315-mm mesh sieve. The swelling index, providing semi-quantitative information on the quantity of

mucopolysaccharides in the plant tissues, was determined as described previously (Ghanem et al. 2010), using 0.5 g of homogenized powdered material.

Mineral concentration

For each sample (leaf, stems, flowers, fruit, whole seeds and extracted mucilage), digestion of dry matter (50–100 mg) was accomplished at 80 °C in 67 % (v/v) HNO₃. After evaporation to dryness the residual minerals were dissolved in a drop of aqua regia and diluted with de-ionized water and then filtered. The element concentrations were determined using an atomic absorption spectrometer (Thermo Scientific ICE 3300; Waltham, US-MA) or by inductively coupled plasma atomic emission spectroscopy (ICP-AES, iCap6500, Thermo Scientific) depending on the element to be quantified. All measurements were performed in triplicate.

Reproductive parameters and laser ablation approach

The date of flowering corresponds to the macroscopic appearance of the first flower. The mean number of flowers per plant (FP) was recorded at 4-day intervals, starting on the flowering date. Three weeks after the start of flowering, five flowers were collected at the upper part (UP) of four plants per treatment. Flowers collected at 2 p.m. were used for pollen viability and stigma receptivity assessment. Pollen viability was determined with fluorescein diacetate (Dafni and Firmage 2000) on at least 500 grains issued from a minimal number of seven anthers per flower. Stigma receptivity was qualitatively assayed by the method of Dafni et al. (2005).

A quadrupole ICP-MS (Agilent 7500ce, Palo Alto, USA) interfaced with a 213 nm Nd:YAG laser ablation system (New Wave Research UP 213, Fremont, SA) was used for microanalysis of Zn and also Ca, Na and K in flowers, pollen and mature seeds. Organs were cut with a clean razorblade, put on an object glass and subjected to laser ablation in spot analysis (for pollen) or line scanning/mapping (for stamen, style, ovary and seeds) mode using the following operational conditions: laser energy density, 1 J cm⁻² (spot) or 2 J cm⁻² (line scanning/mapping); pulse rate 10 Hz; beam diameter, 30 µm (spot) or 55 µm (line scanning/mapping); dwell time, 6 s, line scan speed 10 µm s⁻¹. Helium (0.95 L min⁻¹) was used to transport the ablated material from the ablation chamber to the inductively coupled plasma (ICP); argon was added as a make-up gas before the torch of ICP. The ions formed in the ICP were extracted in the quadrupole mass spectrometer and separated according to their mass-to-charge ratios. The mass spectrometer was set up in time-resolved mode, measuring one point per mass and acquiring each of the

masses ²³Na, ³⁹K, ⁴³Ca and ⁶⁶Zn. Measurements of the background gases (He/Ar mixture) served to establish a blank signal for all masses.

Statistical treatment of the data

The experiment was repeated three times (spring 2009, spring 2010 and spring 2011) and gave similar results. Shapiro–Wilk and Bartlett/Levene tests were performed to check normality and variance equality of the data, respectively. Log transformation of the raw data was performed when required. Percentage values were Arcsin transformed before analysis. Data were subjected to statistical analysis using SAS software (SAS System for Windows, version 9.1). The statistical significance of the results was analysed by the Student–Newman–Keuls test (SNK) at the 5 or 1 % significance level.

Quantitative and qualitative colocalization analysis of elements intensities was determined on the maps of elemental distribution in maturing seeds through ImageJ software with the “intensity correlation analysis” plug-in, generating Pearson’s correlation coefficient (*r*), Mander’s overlap coefficient (*R*) and intensity correlation quotient (ICQ) (http://www.macbiophotonics.ca/imagej/colour_analysis.htm). Mander’s overlap coefficient ranges between 1 and 0, with 1 being high-colocalisation, 0 being low. Values for Pearson’s correlation coefficient range from 1 to −1, where a value of 1 represents perfect correlation, a value of −1 represents perfect exclusion and zero represents random localisation. The ICQ values are distributed between −0.5 and +0.5, with random staining represented by ICQ ~ 0, segregated staining by 0 > ICQ > −0.5 and dependent staining: 0 < ICQ < +0.5 (Li et al. 2004).

Results

Plant growth and development

All tested plants remained alive until the end of the treatment. The presence of 100 µM Zn reduced the shoot elongation (Fig. 2a). However, the presence of NaCl strongly mitigated the deleterious impact of Zn on the shoot elongation process. The number of leaves on the main stem (Fig. 2b) increased with time in controls, NaCl and NaCl + Zn treatments while no new leaves appeared after 12 weeks in the Zn-treated plants. The presence of 100 µM in the nutrient solution did not inhibit the ramification process itself: the number of lateral branches was even the highest in plants exposed to Zn treatment at week 4 and 16 (Fig. 2c). However, it is worth noticing that Zn drastically inhibited the elongation process of these lateral branches (Fig. 2d), which bore only a few leaves compared

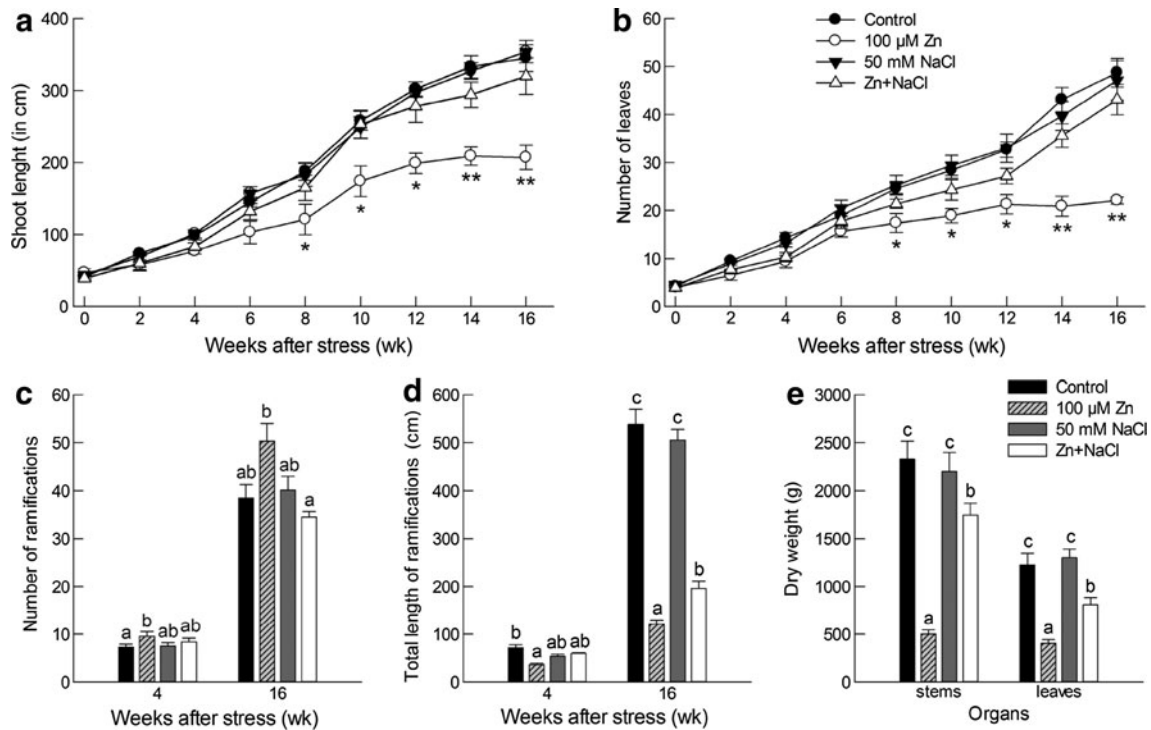


Fig. 2 Impact of zinc (100 μ M), NaCl (50 mM) and mixed-treatment (Zn + NaCl) on the growth parameters of *Kosteletzkya virginica* cultivated for a period of 16 weeks in the NFT device. The shoot height (a) and total number of leaves (b) were recorded each week. The number of ramifications (c) and the total length of the ramifications (d) were recorded after 4 and 16 weeks of treatment. The total dry weight was recorded for stems and leaves at the end of

the treatment (e). Each value is the mean of five replicates and vertical bars are SE. For a given duration of stress, according to Student–Newman–Keuls test, asterisks in a and b indicate a significant difference between treatments and the control at $P < 0.05$ (*) and $P < 0.01$ (**) while different letters in c, d and e indicate significant difference at $P < 0.05$

to control or NaCl-treated plants, while those exposed to the mixed treatment (Zn + NaCl) displayed an intermediate behaviour at week 16.

At the time of harvest, the stem dry weight decreased in response to Zn and Zn + NaCl treatment, although the mean value was higher for plants exposed to the latter treatment compared to the former (Fig. 2e). A similar trend was observed for the leaves (Fig. 2e). It thus appears that the presence of NaCl mitigates Zn toxic effects although its impact differs as a function of the parameter considered.

Plant water status

The mean leaf water content (WC) after 16 weeks of treatment was lower than after 4 weeks of treatment, independent of treatment or plant part (Table 1). Zinc decreased the WC already after 4 weeks of treatment in UP and BP. Deleterious impact of Zn on the leaf WC was even more marked after 16 weeks of treatment, especially for leaves from the BP, where the WC was lower than 70 %. In most cases NaCl mitigated such a deleterious impact of Zn on the leaf WC.

The presence of salt, either in the absence or presence of Zn, significantly decreased Ψ_w and Ψ_s . As far as the Zn treatment is concerned, a significant decrease in Ψ_w was recorded for the leaves located in BP and on ramification, but not for those located in UP. For all plant parts, the lowest Ψ_s value was recorded for the mixed treatment (Zn + NaCl). When turgor potential (Ψ_p) was approximated by the difference $\Psi_p = \Psi_w - \Psi_s$, the lowest turgor was always recorded for the Zn treatment, and was especially low and close to 0 (between 0.02 and 0.06 MPa) for leaves located on the ramifications (detailed data not shown). Stomatal conductance (g_s ; Table 1) only marginally decreased in response to 50 mM NaCl, while it significantly decreased in all plant parts of Zn-treated plants compared to control. Once again, the concomitant presence of NaCl and Zn reduced the effect of zinc and allowed the leaves to avoid strong stomatal closure, as suggested by g_s values recorded at the end of the stress period.

Photosynthesis-related parameters

Net photosynthesis is indicated in Fig. 3a. It remained constant for control plants throughout the duration of the

Table 1 Water content (WC, in %), water potential (Ψ_w , in MPa), osmotic potential (Ψ_s , in MPa) and stomatal conductance (g_s , in $\text{mmol m}^{-2} \text{s}^{-1}$) in leaves of *Kosteletzkya virginica* maintained in control conditions, or in the presence of 100 μM Zn, 50 mM NaCl or Zn + NaCl

Plant part	Treatment	WC (%)		Ψ_w (MPa)		Ψ_s (MPa)		g_s ($\text{mmol m}^{-2} \text{s}^{-1}$)	
		4	16	4	16	4	16	4	16
Basal part	Control	88.8a	78.3b	-0.88a	-1.23b	-1.47b	-1.65bc	218c	207bc
	Zn	80.4b	68.4c	-1.45c	-1.37c	-1.70b	-1.74c	85a	118a
	NaCl	87.4a	79.2ab	-1.68d	-1.74d	-2.03d	-2.26e	163b	177b
	Zn + NaCl	91.4a	77.6b	-1.72d	-1.78d	-2.45e	-2.78f	174b	163b
Upper part	Control	88.5a	81.3a	-0.94ab	-1.05a	-1.54b	-1.58b	422e	371d
	Zn	83.2b	76.2b	-0.88a	-0.98a	-1.25a	-1.32a	211c	123a
	NaCl	90.3a	82.4a	-1.12b	-1.17ab	-2.07d	-2.15d	401e	358d
	Zn + NaCl	87.5a	79.4ab	-1.35c	-1.88de	-2.47e	-2.87f	361de	299c
Ramifications	Control	91.3a	82.5a	-1.04b	-1.06a	-1.63bc	-1.58b	321d	333d
	Zn	89.5a	76.9b	-1.97e	-1.94e	-1.99c	-2.03d	81a	107a
	NaCl	88.2a	81.8a	-1.68d	-1.67d	-2.54e	-2.66f	348d	221bc
	Zn + NaCl	89.6a	83.0a	-1.87de	-1.97e	-2.87f	-3.09g	118a	153b

Leaves were harvested after 4 and 16 weeks of treatment, separately for leaves from the basal part, the upper part, or the ramifications from the upper part. Leaves were harvested from 4 plants per treatment and 3 leaves were collected from each part of the individual plants. For a given parameter and a given duration of treatment, means followed by different letters are statistically different at $P < 0.05$

treatment and was unaffected by 50 mM NaCl. Zinc excess had no effect on the A value before week 8, but abruptly decreased it afterwards. Such a decrease occurred later and was more progressive for plants exposed to the mixed Zn + NaCl treatment.

The F_v/F_m ratio was drastically affected by Zn excess, already at week 6 but remained constant thereafter, while a more progressive decline was observed for plants exposed to the mixed treatment. Non-photochemical quenching (NPQ; Fig. 3c) increased with time for all treatments ($P < 0.05$), except for controls. The highest NPQ value was recorded for the mixed treatment. In contrast, the qP value remained constant (Fig. 3d), except for plants exposed to the mixed treatment, where a small although significant decrease was observed at the end of the treatment. The quantum efficiency of PSII (ΦPSII) followed a similar pattern to that of F_v/F_m (Fig. 3e). The instantaneous water use efficiency (WUE_i ; defined as the A/E ratio) remained constant in controls and NaCl treated plants (Fig. 3f), and slightly decreased in Zn-treated plants but strongly increased for plants exposed to the mixed treatment.

The Chl a /Chl b ratio remained constant, whatever the treatment (mean value of 3.98 ± 0.07). Zinc treatment induced a decrease in total Chl (Chl a + Chl b ; Table 2) and in carotenoid concentration in both the basal and upper parts of the plant. Sodium chloride increased Chl concentrations in the upper parts of the plants, compared to controls. Mixed treatment reduced Chl concentration in the basal part of the plant, and had no detrimental effect on the carotenoid concentration.

It could thus be assumed that exogenous NaCl improves the photosynthesis of *K. virginica* exposed to Zn toxicity.

Malondialdehyde, non-protein thiol and mucilage quantification

The malondialdehyde concentration was higher in the BP than in the UP in control plants (Table 2). Salinity slightly increased the MDA concentration, especially in BP, while Zn drastically increased it in all plant parts. The presence of NaCl in the Zn-containing solution reduced MDA accumulation in the UP compared to Zn treatment alone (Table 2). The non-protein thiol concentration increased in response to NaCl and Zn treatments. The simultaneous presence of those mineral agents increased non-protein thiol content in the BP of the plant but decreased it in the UP. An opposite picture was registered for the mucilage content, indirectly quantified by the swelling index (Table 2). The swelling index increased as a response to NaCl treatment and was unaffected in response to Zn excess. The simultaneous presence of NaCl and Zn led to an obvious increase in the swelling index in the UP ($P < 0.01$) and ramifications ($P < 0.05$) but not in the BP.

Mineral concentration in the vegetative organs

The mineral concentrations in stems and in leaves from BP, UP and ramifications are given in Fig. 4 for plants harvested at the end of the treatment. Zinc excess induced a strong increase in Zn concentration in all analysed organs

Fig. 3 Impact of zinc (100 μM), NaCl (50 mM) and mixed-treatment (Zn + NaCl) on the photosynthesis-related parameters of *Kosteletzkya virginica* cultivated for a period of 16 weeks in the NFT device. The net CO_2 assimilation rate (a), maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m ; b), non-photochemical quenching (NPQ; c), photochemical quenching (qP; d), the quantum efficiency of PSII (ΦPSII ; e) and instantaneous water use efficiency (WUE; f) were recorded weekly for five plants per treatment. Vertical bars are associated with SE for a given duration of stress, asterisks indicate a significant difference between treatments and the control at $P < 0.05$ (*) and $P < 0.01$ (**) according to Student–Newman–Keuls test. Note that the vertical scale is not the same for different parameters

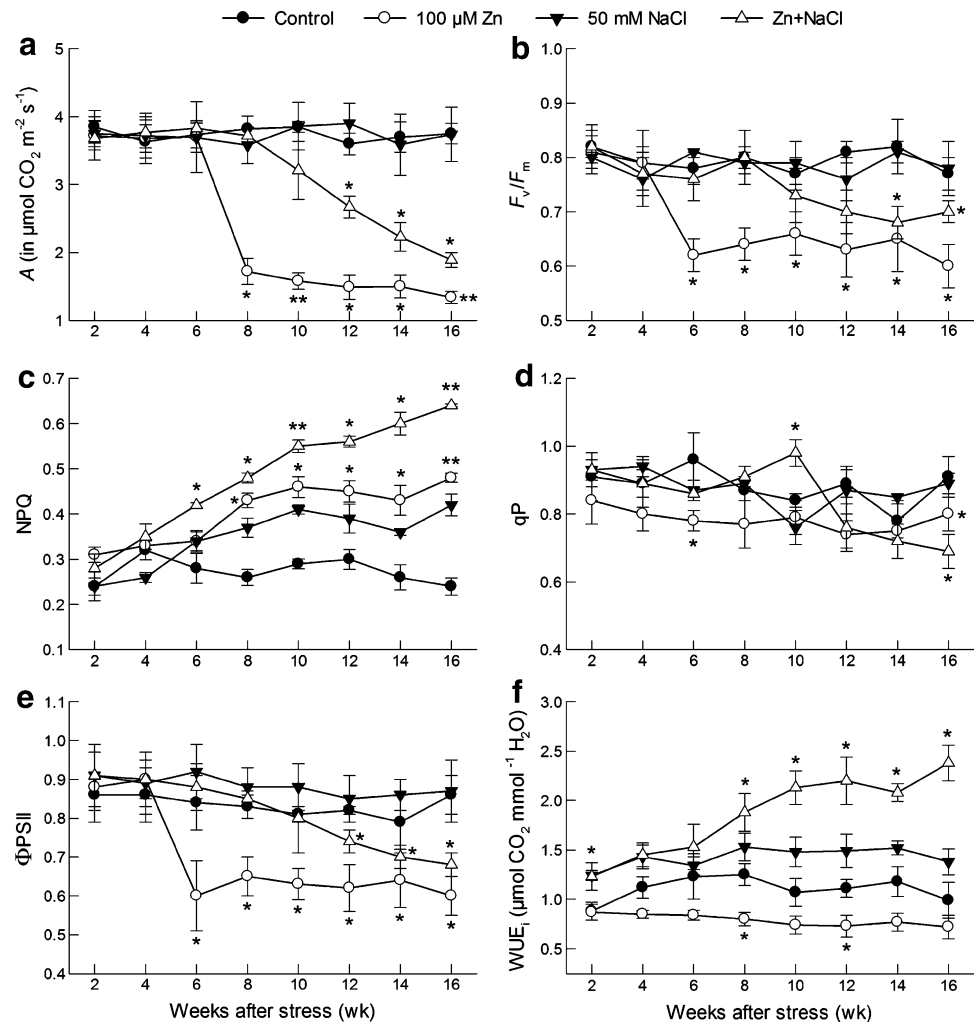


Table 2 Total chlorophyll concentration (Chl, in mg g^{-1} DW), carotenoids (in mg g^{-1} DW), malondialdehyde (MDA, nmol g^{-1} DW), non-protein thiols ($\mu\text{mol g}^{-1}$ DW) and mucilage swelling index in leaves and stems of *Kosteletzkya virginica* maintained in control conditions, or in the presence of 100 μM Zn, 50 mM NaCl or Zn + NaCl

Plant part	Treatment	Total Chl (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	MDA (nmol g^{-1} FW)	Total thiol ($\mu\text{mol g}^{-1}$ FW)	Swelling index
Basal part	Control	12.3 $\pm$ 0.9c	2.4 $\pm$ 0.1c	45.1 $\pm$ 3.8b	11.6 $\pm$ 1.2a	6.4 $\pm$ 0.5a
	Zn	7.8 $\pm$ 0.3a	0.5 $\pm$ 0.1a	97.2 $\pm$ 7.1e	31.6 $\pm$ 2.4c	6.3 $\pm$ 0.8a
	NaCl	11.6 $\pm$ 0.5bc	2.2 $\pm$ 0.2c	63.0 $\pm$ 5.4c	24.8 $\pm$ 0.9b	11.7 $\pm$ 1.6c
	Zn + NaCl	8.1 $\pm$ 0.4a	2.5 $\pm$ 0.3c	76.4 $\pm$ 5.8d	52.8 $\pm$ 3.6d	6.7 $\pm$ 0.7ab
Upper part	Control	13.3 $\pm$ 0.6c	3.2 $\pm$ 0.4d	22.4 $\pm$ 2.3a	12.3 $\pm$ 2.6a	7.4 $\pm$ 0.9b
	Zn	7.9 $\pm$ 0.2a	1.1 $\pm$ 0.1b	77.8 $\pm$ 4.9d	21.3 $\pm$ 1.1b	6.9 $\pm$ 0.5ab
	NaCl	17.1 $\pm$ 0.3d	2.9 $\pm$ 0.2cd	33.4 $\pm$ 4.5ab	19.7 $\pm$ 0.9b	13.6 $\pm$ 1.1c
	Zn + NaCl	10.1 $\pm$ 0.1b	2.4 $\pm$ 0.4c	36.3 $\pm$ 5.2ab	10.4 $\pm$ 0.7a	18.5 $\pm$ 2.1d
Ramifications	Control	12.8 $\pm$ 0.5c	2.8 $\pm$ 0.3cd	21.8 $\pm$ 0.8a	13.2 $\pm$ 0.9a	5.9 $\pm$ 0.2a
	Zn	8.3 $\pm$ 0.4ab	1.7 $\pm$ 0.2b	87.9 $\pm$ 7.9e	22.9 $\pm$ 1.9b	5.7 $\pm$ 0.4a
	NaCl	12.4 $\pm$ 0.9c	3.0 $\pm$ 0.3d	27.9 $\pm$ 1.2a	18.6 $\pm$ 1.2b	6.4 $\pm$ 0.6a
	Zn + NaCl	13.1 $\pm$ 0.8c	3.3 $\pm$ 0.1d	48.8 $\pm$ 5.6b	14.7 $\pm$ 0.9a	17.5 $\pm$ 1.0d

Leaves were harvested after 16 weeks of treatment from the basal part, the upper part, or the ramifications of the upper part. Leaves were harvested from 4 plants per treatment and 3 leaves were collected from each part of the individual plants. For a given parameter, means followed by different letters are statistically different at $P < 0.05$

compared to controls and this element was evenly distributed among the four different parts. Additional NaCl, however, strongly modified the pattern of Zn accumulation because it increased Zn accumulation in the BP but obviously decreased it in the UP and in the ramifications compared to Zn treatment alone. Control plants contained a low amount of Na (added as Na₂SO₄), and Zn alone had no strong influence on the accumulation of this element in the absence of salt (details not shown). Salinity induced an expected Na accumulation (Fig. 4b). In the concomitant presence of Zn, Na accumulation was 40 % higher than in the absence of Zn. Zinc, salinity or their combination had no clear impact on K and Ca nutrition. Zinc excess reduced the Mg concentration in all plant organs, especially in the ramifications. Salinity reduced this negative impact of Zn, more particularly in the younger parts of the shoot (ramification and UP).

When the extracted mucilage was analysed for mineral content, Zn appeared to increase in Zn + NaCl treatment compared to Zn treatment alone, especially in leaves harvested from UP (Table 3). Such a process appeared to be negatively correlated with Ca retention by the mucilage which decreased in response to NaCl + Zn treatment, while it remained rather high in the presence of Zn alone.

Salinity thus modifies not only mineral content, but also ion distribution in Zn-treated plants.

Reproductive parameters and laser ablation approach

The presence of Zn excess in the absence of salt delayed flowering by more than 10 days compared to controls, while NaCl and Zn + NaCl-treated plants flowered at the same time as the control (detailed data not shown). Zinc treatment reduced the total number of flowers per plant and the mean number of fruits, but had no impact on the mean number of seeds per fruit (Table 4). The pollen viability and stigma receptivity (detailed data not shown) were not affected by the Zn treatment. The mean seed weight was reduced by Zn treatment.

The bulk Zn concentration in flowers and fruits increased in response to the Zn treatment but remained lower than in the vegetative organs (Table 4). The Zn + NaCl treatment reduced Zn accumulation in the flowers but not in the fruit compared to Zn treatment alone. Sodium was not detected in control and Zn-treated flowers or fruits, while it accumulated to a similar extent in the presence of NaCl on the one hand and Zn + NaCl on the other hand, reaching a mean value of 1.27, 0.84 and 0.63 mg g⁻¹ DW for flowers, fruits and seeds, respectively.

The laser ablation approach allowed us to detect a Zn-induced decrease in K and increase in Zn content in pollen grains (Fig. 5). Once again, the effect was partly mitigated

by the concomitant presence of 50 mM NaCl. Zinc also surprisingly increased the pollen Ca content. Sodium accumulation was detected in pollen collected on plants exposed to Zn + NaCl (NaCl-treated plants were not analysed for pollen by laser ablation). It was also clear that most of the Zn in the female organs (scanned longitudinally, from ovary to stigma) was detected in the ovary itself and did not accumulate in style and in stigmas (Fig. 5e). The LA-ICP-MS signals produced by the ovary zone from Zn + NaCl-treated plants were extended to the whole length of the organ, while the signals produced by Zn-treated ones is mainly limited to the basal area of the ovary.

Maturing seeds still included in the fruits were longitudinally cross-sectioned and analysed with LA-ICP-MS for mineral distribution (Fig. 6; only fruits from control, Zn and Zn + NaCl treatments were considered). The seed coat (*T*) consisted of a testa derived from the outer integument of the ovule, and the tegment derived from the inner integument. Seeds were still in close contact with a spongy pericarp (*P*). Integuments are torn due to the shrinkage tension resulting from partial desiccation and an air space is present around the embryo (*V*). The endosperm (*E*) is visible and consists in an outer zone which is one to several cell layers thick and an inner zone penetrating between the folds of the cotyledons.

In control plants, most of the Zn signal originated from the seed coat (Fig. 6). In the Zn-treated plants, Zn accumulated to some extent in the fruits tissues, in the seed coat and in the chalazal cells (*c*), but mainly in the endosperm. In the Zn + NaCl-treated plants, Zn displayed a different distribution: it exhibited a strong retention in the chalazal area and accumulated in the seed coat while the Zn signals in the endosperm were lower than for Zn-treated seeds. A moderate Zn signal was observed in the embryo of Zn + NaCl-treated plants which appeared stronger than in the embryo of control and Zn-treated plants. The Na signals were found to be the highest in the pericarp and to some extent in the seed coat and the chalazal cells from control plants. In response to Zn + NaCl treatment, Na accumulated mainly in pericarp, endosperm and chalazal cells. Likewise Na, the K signals were found to be the highest in the pericarp and to some extent in the seed coat and the chalazal cells from control plants. The Zn + NaCl treatment strongly increased the K level in the endosperm compared to control and Zn-treated plants. A moderate Ca signal was found in control maturing seeds, while seeds harvested from Zn-treated plants exhibited a stronger Ca signal especially within the endosperm. Seeds from Zn + NaCl-treated plants had a basal Ca level similar to the controls, except for pericarp and endosperm where the signal intensity was higher. A trend of Zn-K colocalisation in seeds of control plants was suggested by high *r* and *R* coefficients (supplementary information). In response to

Fig. 4 Impact of zinc (100 μ M), NaCl (50 mM) and mixed-treatment (Zn + NaCl) on the mineral concentration in leaves from the basal/upper part and the stems/leaves from the ramifications of *Kosteletzkya virginica* cultivated for a period of 16 weeks in the NFT device (**a** Zn, **b** Na, **c** K, **d** Ca, **e** Mg, **f** Mn). Each histogram is the mean of five replicates and vertical bars are associated with SE. For a given part of organ investigated, different letters indicate significant difference at $P < 0.05$ according to Student–Newman–Keuls test

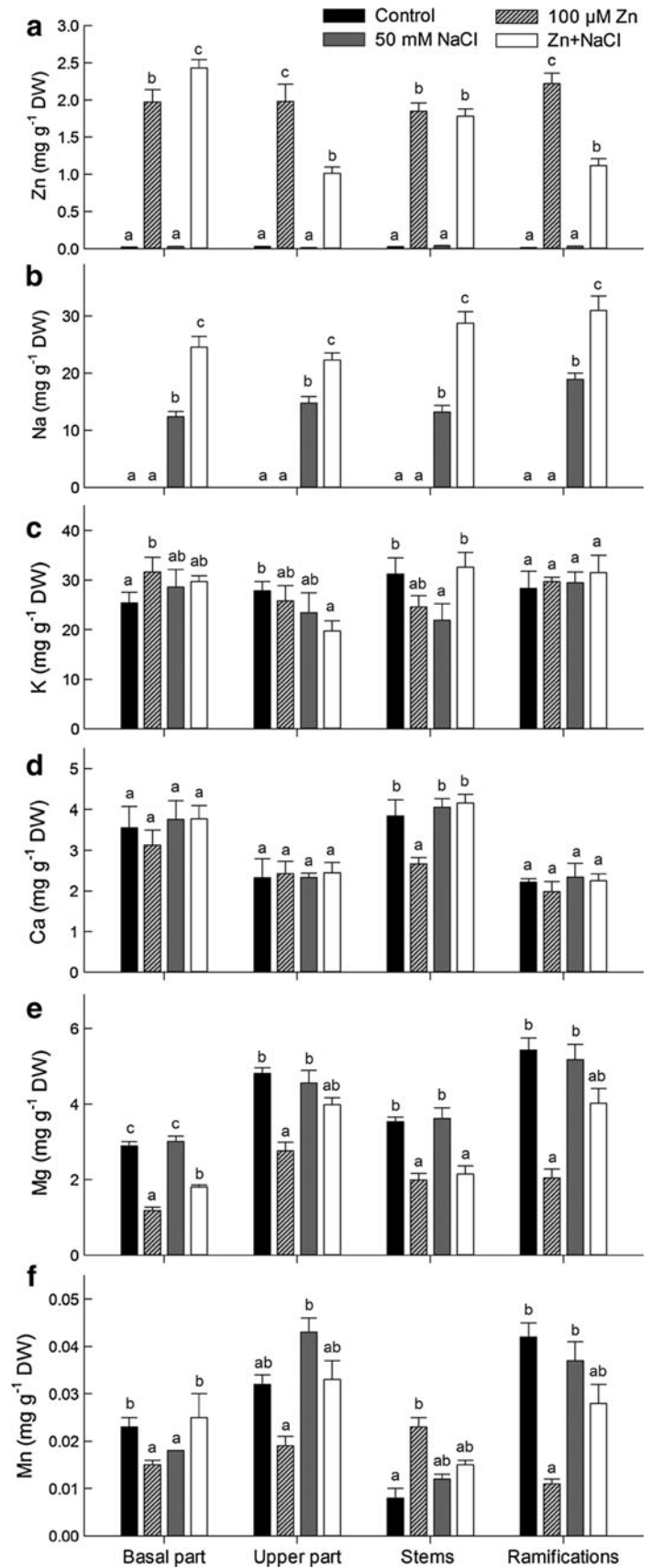


Table 3 Zinc and calcium concentration in mucilage of *Kosteletzkya virginica* maintained in control conditions, or in the presence of 100 μM Zn, 50 mM NaCl or Zn + NaCl

Element	Plant parts	Control	Zn	NaCl	Zn + NaCl
Zinc (mg g^{-1} DW)	Basal part	$0.08 \pm 0.01\text{a}$	$8.3 \pm 0.7\text{c}$	$0.12 \pm 0.02\text{a}$	$13.4 \pm 0.9\text{d}$
	Upper part	$0.06 \pm 0.01\text{a}$	$7.6 \pm 0.5\text{bc}$	$0.09 \pm 0.01\text{a}$	$14.3 \pm 0.2\text{d}$
	Stem	$0.11 \pm 0.02\text{a}$	$6.2 \pm 0.2\text{b}$	$0.10 \pm 0.01\text{a}$	$7.4 \pm 0.1\text{c}$
	Ramifications	$0.05 \pm 0.00\text{a}$	$5.9 \pm 0.1\text{b}$	$0.10 \pm 0.02\text{a}$	$13.2 \pm 1.0\text{d}$
Calcium (mg g^{-1} DW)	Basal part	$60.3 \pm 5.4\text{de}$	$64.2 \pm 3.7\text{e}$	$45.8 \pm 2.9\text{b}$	$36.4 \pm 2.3\text{ab}$
	Upper part	$54.2 \pm 3.7\text{cd}$	$56.7 \pm 3.8\text{d}$	$51.1 \pm 4.2\text{c}$	$35.3 \pm 3.0\text{a}$
	Stem	$47.6 \pm 5.1\text{bc}$	$50.4 \pm 4.6\text{c}$	$49.6 \pm 3.9\text{c}$	$28.1 \pm 2.4\text{a}$
	Ramifications	$48.9 \pm 2.8\text{bc}$	$59.2 \pm 3.7\text{d}$	$37.2 \pm 2.7\text{b}$	$31.6 \pm 2.5\text{a}$

For a given parameter, means followed by different letters are statistically different at $P < 0.05$. Mucilage was extracted after 16 weeks of treatment, separately from leaves from the basal part, the upper part, the stem and the ramifications of the upper part. Each value is the mean of 5 replicates $\pm$ SE. For a given element, means followed by different letters are statistically different at $P < 0.05$

Table 4 Reproductive parameters of *Kosteletzkya virginica* maintained in control conditions, or in the presence of 100 μM Zn, 50 mM NaCl or Zn + NaCl

Parameter	Control	Zn	NaCl	Zn + NaCl
No. of flowers per plant	$79.2 \pm 5.3\text{b}$	$52.6 \pm 2.4\text{a}$	$83.4 \pm 3.8\text{bc}$	$96.0 \pm 2.1\text{c}$
No. of fruits per plant	$39.5 \pm 2.8\text{b}$	$26.3 \pm 1.7\text{a}$	$42.6 \pm 3.9\text{b}$	$45.4 \pm 4.5\text{b}$
No. of seeds per fruit	$4.7 \pm 0.1\text{a}$	$4.7 \pm 0.1\text{a}$	$4.6 \pm 0.2\text{a}$	$4.8 \pm 0.1\text{a}$
No. of flowers open per day	$3.76 \pm 0.42\text{b}$	$1.21 \pm 0.25\text{a}$	$3.33 \pm 0.51\text{b}$	$3.91 \pm 0.36\text{b}$
Mean 100-seeds weight (g)	$1.72 \pm 0.11\text{b}$	$0.93 \pm 0.07\text{a}$	$1.68 \pm 0.09\text{b}$	$1.84 \pm 0.12\text{b}$
Zn concentration in flowers (mg g^{-1} DW)	$0.013 \pm 0.001\text{a}$	$0.98 \pm 0.07\text{d}$	$0.021 \pm 0.003\text{b}$	$0.41 \pm 0.04\text{c}$
Zn concentration in fruits (mg g^{-1} DW)	$0.017 \pm 0.002\text{a}$	$0.45 \pm 0.05\text{b}$	$0.018 \pm 0.001\text{a}$	$0.47 \pm 0.04\text{b}$
Zn concentration in seeds (mg g^{-1} DW)	$0.030 \pm 0.003\text{a}$	$0.69 \pm 0.10\text{c}$	$0.022 \pm 0.002\text{a}$	$0.31 \pm 0.03\text{b}$

Each value is the mean of 4 replicates $\pm$ SE. For a given parameter, means followed by different letters are statistically different at $P < 0.05$

Zn treatment, the Zn increase in seed endosperm and chalaza was associated with a K increase in endosperm and chalaza and Ca increase in endosperm, as represented by the white areas indicating an overlapping of the elements. However, all the correlations between these elements decreased when applying NaCl to the nutrient solution and Zn appeared therefore more concentrated in seed coat and chalaza than other elements (supplementary information).

Discussion

Zinc excess impairs plant growth and development in *Kosteletzkya virginica*

The present study demonstrates that the salt-marsh halophyte *K. virginica* is able to remain alive despite a high rate of Zn accumulation in its photosynthetic tissues. Han et al. (2012a) already demonstrated that *K. virginica* is able to cope with short-term exposure to Zn stress at the seedling stage but no data are available for mature adult plants. The present work confirms that such properties are also valid at

the adult stage. The external dose of 100 μM ZnCl_2 used in the present work is lower than external doses used in other hydroponic culture experiments (up to 1 mmol L^{-1} ; Redondo-Gómez et al. 2011). In recent work by Cambrollé et al. (2012) even a much higher dose (70 mmol L^{-1}) was used, but the exposure time was shorter than in our experiments. In fact *K. virginica* was exposed to Zn toxicity for almost 4 months, and thus faced Zn toxicity at almost all phenological stages (except germination). Since the solution was frequently renewed (see section “Materials and methods”), the shoot was able to accumulate up to 2 mg g^{-1} DW Zn; such an accumulation was similar to that recorded for other halophyte plant species such as *Atriplex halimus* (Lutts et al. 2004), *Avicennia marina* (McFarlane and Burchett 1999), *S. densiflora* (Redondo-Gómez et al. 2011) and *Halimione portulacoides* (Cambrollé et al. 2012), despite the fact that higher external doses of Zn were used in these studies. If we consider the mean water content of the shoot, and if we express the ion concentration on a water content basis rather than a dry weight basis, it appears that *K. virginica* exhibited a bioaccumulation factor of more than 30, illustrating an unusual ability

Fig. 5 Impact of zinc (100 μ M) and mixed-treatment (Zn + NaCl) on the mineral concentration in pollen grains (**b** Zn, **c** Na, **d** K, **e** Ca) and on the Zn distribution (**g**) in the female organs of *Kosteletzkya virginica* cultivated for a period of 16 weeks in the NFT device using a laser ablation spot analysis (**b–e**) or line scanning (**g**) approach as detailed in section “[Materials and methods](#)”. Detailed photos of pollen grain and female organs of *K. virginica* are presented in **a** and **f** figures

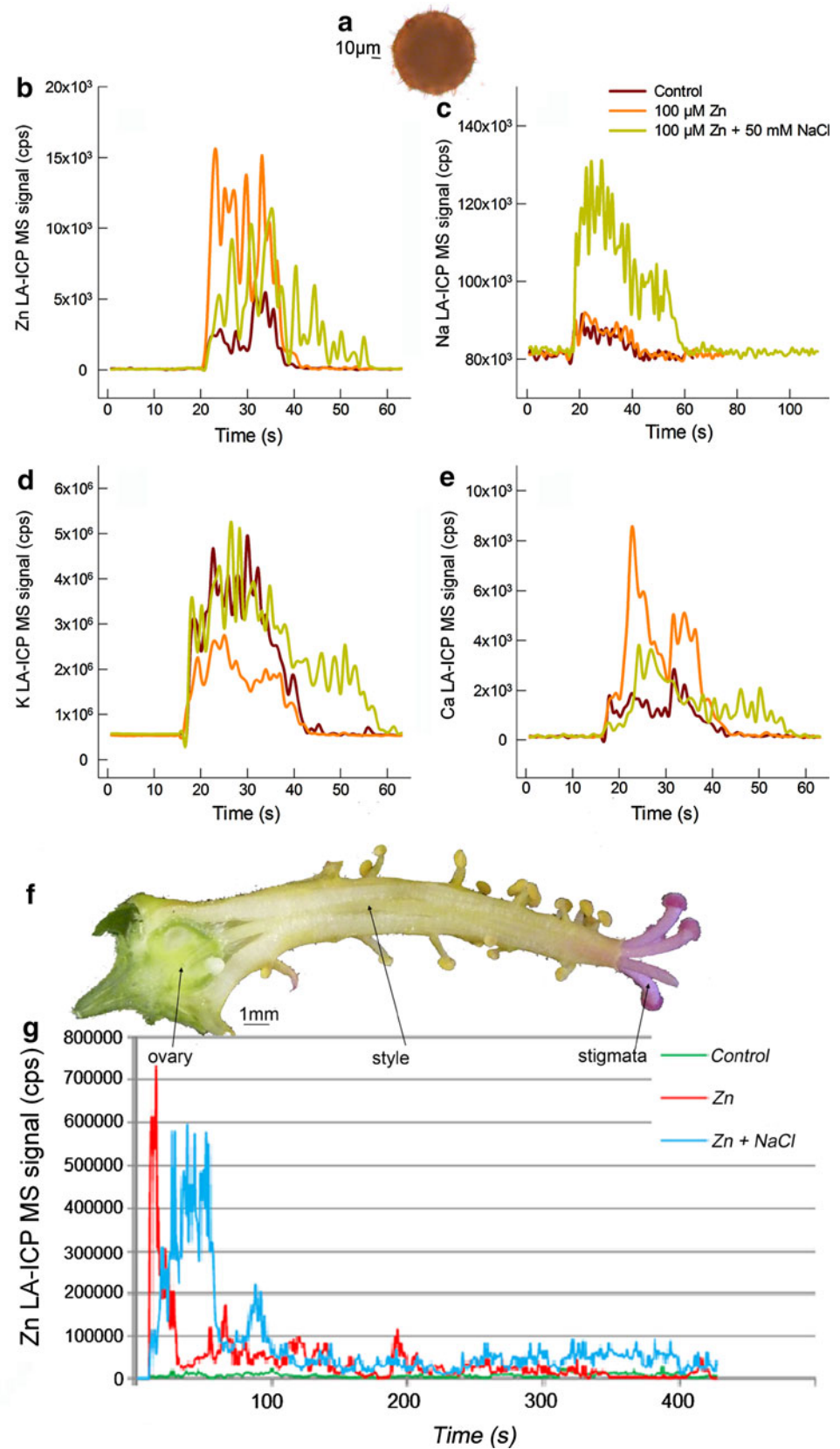
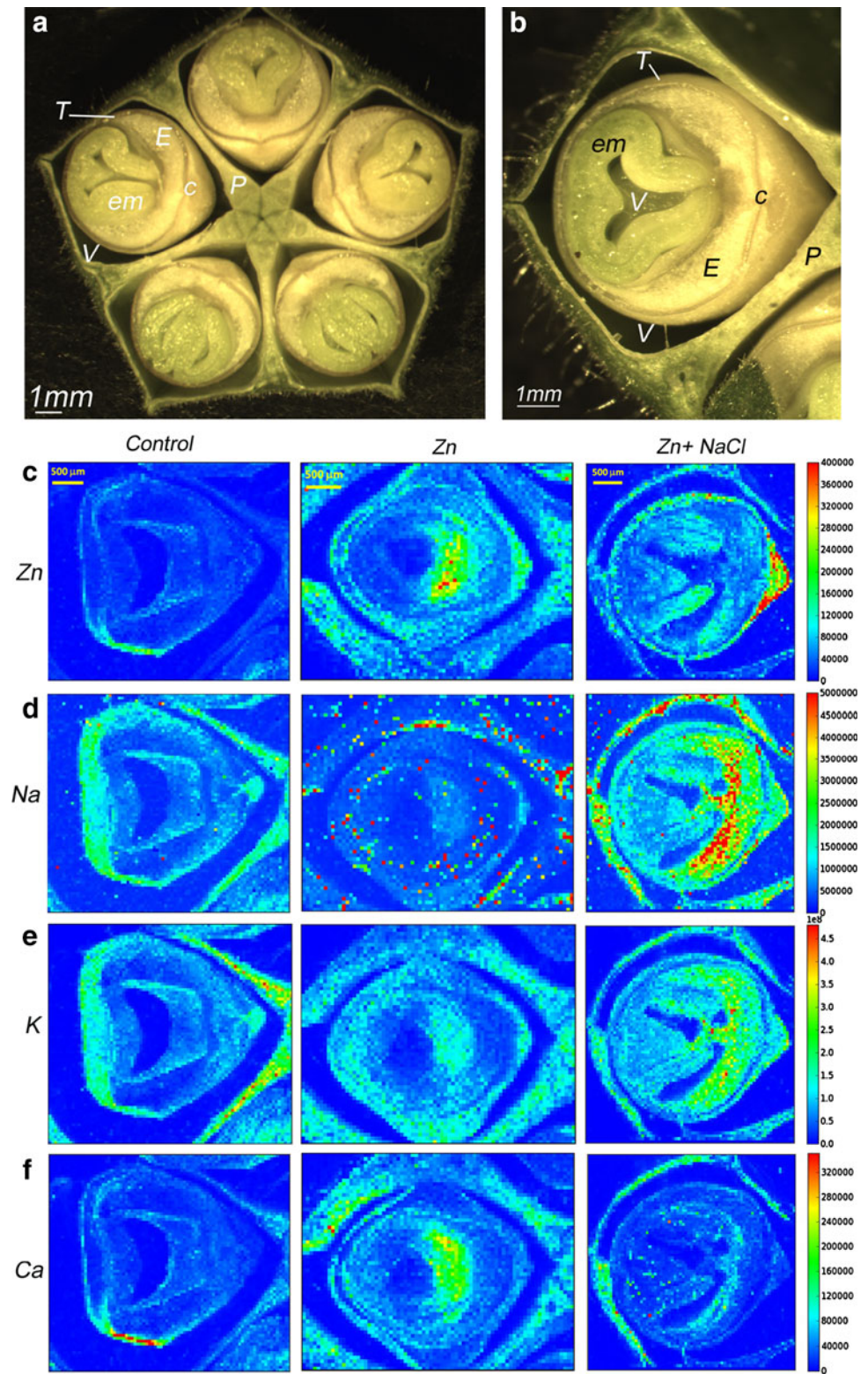


Fig. 6 Transversal section of a maturing fruit and seeds (a), section of a maturing seed used for laser ablation (b) and the elemental distribution in maturing seeds (c Zn, d Na, e K, f Ca) of *Kosteletzkya virginica* subjected to control, zinc (100 μ M), and mixed treatment [Zn (100 μ M) + NaCl (50 mM)] for a period of 16 weeks by cultivation in the NFT device. Analysis was performed using a laser ablation elemental mapping approach as detailed in section “Materials and methods”. Elemental densities (in counts per second) in c–f are visualized as jet colours with lower image densities mapped onto “cool” colours and higher densities onto “hot” colours; for a given element the scale is the same for all three treatments. The structural details of the seed section are demonstrated in B (*T* seed coat, *V* air space, *E* endosperm, *em* Embryo, *P* pericarp, *c* chalaza)



of this plant to take up and accumulate Zn. According to Lin et al. (2012), a Zn concentration of 2 mg g⁻¹ DW may be considered as close to a “hyperaccumulating” status. According to Han et al. (2012a), an important proportion of

the absorbed zinc is retained by the root system in *K. virginica*. Our NFT system did not allow us to have an access to this root compartment since the root system developed in the gullies as an intricate network. Whatever

the proportion of Zn retained by the roots, a bioaccumulation factor higher than 30 clearly suggests that *K. virginica* might be considered as a useful tool to remove zinc from contaminated substrates.

Such a conclusion should however be mitigated considering the deleterious impact of Zn on plant growth since the mean total shoot dry weight was estimated at 3.75 and 1.04 kg per plant for control and Zn-treated individuals, respectively. When considering the mean Zn concentration of each plant part in this latter case, it appears that one single plant of *K. virginica* would be able to remove 1.81 g of Zn. In the case of Zn + NaCl-treated plants, such a removal may be as high as 4.76 g of Zn per plant. Considering that average density of plants in the field plantation is 150,000 individuals per hectare (He et al. 2003), one may expect to remove 270 kg Zn ha⁻¹ from polluted areas in the absence of salt and up to 700 kg Zn ha⁻¹ in the presence of a moderate salinity.

Even if Zn did not induce plant mortality, it clearly impacts water status and photosynthesis. A decrease in cell elongation may be considered as a consequence of the turgor drop. In contrast to salt-treated plants, Zn-treated plants were apparently unable to perform osmotic adjustments to guarantee turgor maintenance when Ψ_w decreased. Other authors have also mentioned that Zn-induced perturbations in the V-ATPases may be responsible for growth inhibition since these enzymes play a key role in the trans-Golgi network involved in the synthesis and trafficking of cell wall components (Brüx et al. 2008; Fukao et al. 2011).

Photosynthesis appeared sensitive to Zn excess. This could be due both to stomatal causes [g_s being strongly decreased in Zn-treated plants, as reported in *S. densiflora* (Redondo-Gómez et al. 2011)] or to non-stomatal limitations in relation to the alteration of the electron transport, as recently evidenced by Cambrollé et al. (2012) in *H. portulacoides*. Zinc was reported to affect the PSII chemistry by an interaction with the donor side of PSII, thus inhibiting photosynthetic CO₂ fixation and the Hill reaction (Mateos-Naranjo et al. 2008). The recorded decrease in F_v/F_m suggests that a similar problem occurs in *K. virginica*. The reported increase in NPQ may therefore be considered as an attempt to dissipate excess energy in order to avoid ROS synthesis. The global consequence of F_v/F_m decrease and NPQ increase was a clear decrease in quantum efficiency of PSII (Φ_{PSII}).

Besides its effect on vegetative growth, zinc also has a detrimental impact on the reproductive phase of development. Flowering was delayed and most-quantitative parameters related to the flowering process were affected by Zn excess (Table 4). The underlying causes of perturbations are not necessarily related to a direct zinc accumulation. In the Zn-treated plants, the zinc concentration in

the whole flower was only two times higher than in the controls, suggesting that Zn translocation was tightly regulated. Pollen viability remained unaffected, although Zn was present in the pollen of Zn-treated plants. In contrast, there was no zinc accumulated in the style and stigma which may explain that the stigma receptivity remained unaffected; additionally, the seed set was similar in controls and Zn-treated plants (ca. 50.6 %).

Seeds were analysed for Zn distribution before full maturation. Ozturk et al. (2006) reported that this stage corresponds to the highest Zn content within seeds. Developing seeds depend mainly on uptake through the phloem from the vegetative parts of the plant for their supply of carbohydrates, amino acids, and minerals. To date, some data are available for Zn distribution in cereals (Ozturk et al. 2006; Tauris et al. 2009; Wang et al. 2011) or hyperaccumulators (Mesjasz-Przybyłowicz et al. 1999; Ernst and Nelissen 2000; Vogel-Mikuš et al. 2007), but no data are available for *K. virginica*. Even if Zn is present in the endosperm, the low level of accumulation in the bulk seeds appears compatible with the use of this plant species for biodiesel production in marginal areas, as recommended by Ruan et al. (2008, 2012).

Salinity reduces Zn toxicity in the halophyte *Kosteletzkya virginica*

Low to moderate NaCl doses, in the absence of heavy metals, are reported to increase plant growth in numerous halophyte species (Ruan et al. 2010 for review). According to Ghanem et al. (2010) 100 mM NaCl indeed slightly stimulated plant growth in *K. virginica*. The present study refers to the use of 50 mM NaCl, which is not a growth-stimulating dose, in order to decipher the metabolic impact of NaCl on heavy-metal stress resistance, independently of any diluting effect of growth stimulation. It is noteworthy that NaCl had no significant impact on the plant behaviour in the absence of Zn stress, but that it clearly improved the plant's behaviour in the presence of Zn excess. Indeed, NaCl increased the photosynthesis in Zn-treated plants, restored growth and improved the efficiency of the flowering process. Clearly, NaCl neutralized the damaging action of Zn, as reported by Kholodova et al. (2005) in the halophyte *Mesembryanthemum crystallinum*. In another halophyte species, *S. densiflora*, Redondo-Gómez et al. (2011) reported a different situation, where salinity and zinc stress have synergic rather than antagonistic effects. This suggests that different halophyte species may have a distinct response to heavy metal toxicity in saline conditions.

One of the major positive impacts of NaCl on Zn-treated plants is an obvious increase in WUE_i (Fig. 3f), which could be related to an increase in g_s , allowing

photosynthesis maintenance. Photosynthesis may then provide metabolic energy and biochemical building blocks for synthesis of osmolytes, thus explaining the efficient osmotic adjustment performed by *K. virginica* in the presence of NaCl. More precisely, *K. virginica* belongs to the Malvaceae family which produces high amounts of glycinebetaine, a quaternary ammonium compound known to protect chloroplast structures from the deleterious effect of ion toxicities (Ohnishi and Murata 2006). This compound might be involved in the Φ PSII increase in Zn + NaCl-treated plants compared to Zn-treated ones.

Positive impact of NaCl is associated with a modification in Zn distribution within the plant

Considering the whole shoot system, salinity did not reduce the mean Zn concentration in Zn + NaCl-treated plants in comparison to the Zn treatment. Taking into account that Zn-induced growth inhibition was mitigated by NaCl, the total amount of Zn removed from the solution was higher for Zn + NaCl treatment than for Zn treatment. Salinity clearly modified the Zn distribution in the shoot, increasing Zn accumulation in the old leaves of the basal part and reducing it in the young leaves from the upper part. Such a preferential accumulation in old leaves has already been reported for other ion toxicities (Lutts et al. 1999; Lefèvre et al. 2010) and may be considered as an attempt to preserve young, photosynthetically active tissues while old leaves, already engaged in a natural-occurring senescence programme, are not crucial for plant survival anymore.

It is interesting to note that the salt-induced uneven Zn distribution was associated with two distinct types of response in BP and UP. Indeed, the basal part presented an increase in the total thiol content, which suggests that peptides such as phytochelatins might be induced for subsequent vacuolar sequestration in these leaves (De Vos et al. 1992). Such an increase was not recorded in UP where the swelling index increased, suggesting a higher Zn sequestration by the mucilage. An increase in Zn concentration in the extracted mucilage (Table 3) further supports this hypothesis. Moreover, the fact that the Zn concentration in mucilage was higher than in the bulk tissue also suggests that it might act as a protecting agent against Zn toxicity.

Salinity reduced the Zn accumulation in dry seeds. LA-ICP-MS has been demonstrated as a sensitive and efficient technique to study the element distribution in seeds (Wirth et al. 2009; Wang et al. 2011) and other biological structures (Gholap et al. 2010). It allowed us to pinpoint that NaCl restricts Zn translocation to the maturing seed at the chalazal pole. In maturing seeds of angiosperms, assimilates are unloaded from the phloem symplastically through the chalaza and then into the apoplast via transfer cells

which constitute the interface between the maternal tissues and the apoplastic space of the endosperm cavity. Tauris et al. (2009) demonstrated that many genes coding for Zn transporters are expressed in the transfer cells and that Zn may accumulate in their vacuoles for temporary storage. Wang et al. (2011) postulated that those transporters may be affected under Zn excess and our data support the hypothesis that this process is even more affected by the presence of additional NaCl. This hypothesis could explain both chalazal retention (Fig. 5) and a decrease in the Zn content of the whole seeds (Table 4). As a consequence, the presence of salt, which decreases Zn seed content and improved yield parameters, should be considered as a positive factor for biodiesel production by *K. virginica* in Zn-polluted areas.

Acknowledgments This work was supported by the Belgian Science Policy (BELSPO): Chinese-Belgian Cooperation Project [200441505], by the Fonds National de la Recherche Scientifique (Crédit aux Chercheurs—Conventions no 1.5.111.10F, 1.5117.11), and by Wallonie-Bruxelles-International (Convention mixte: WBI—République de Slovénie). R.-M. Han is grateful to the Université catholique de Louvain (UCL) for the award of a research fellowship. The authors are grateful to Mr B. Capelle and Mrs B. Van Pee for their precious technical assistance.

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