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# The cytokinin *trans*-zeatine riboside increased resistance to heavy metals in the halophyte plant species *Kosteletzkya pentacarpos* in the absence but not in the presence of NaCl

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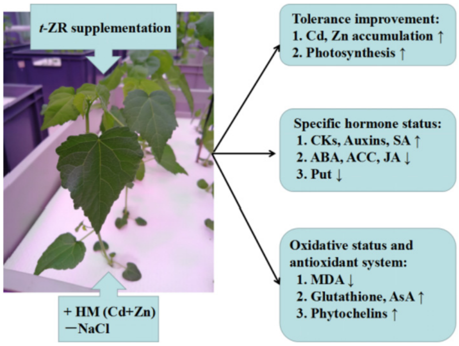
## HIGHLIGHTS

- Cd and Zn have different impacts on the plant behavior and their combination induces a specific plant hormonal status.
- Salinity helps the plant to cope with heavy metal toxicities.
- Cytokinin depletion is involved in Zn-induced senescence.
- Cytokinin assume key function in heavy-metal resistance but its efficiency is lower in the presence of NaCl.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Heavy metals such as cadmium and zinc constitute major pollutants in coastal areas and frequently accumulate in salt marshes. The wetland halophyte plant species *Kosteletzkya pentacarpos* is a promising species for phytostabilization of contaminated areas. In order to assess the role of the antisenescing phytohormone cytokinin in heavy metal resistance in this species, seedlings were exposed for two weeks to Cd (10 μM), Zn (100 μM) or Cd + Zn (10 μM + 100 μM) in the presence or absence of 50 mM NaCl and half of the plants were sprayed every two days with the cytokinin *trans*-zeatine riboside (10 μM). Zinc reduced the endogenous cytokinin concentration. Exogenous cytokinin increased plant growth, stomatal conductance, net photosynthesis and total ascorbate and reduced oxidative stress estimated by malondialdehyde in Zn-treated plants maintained in the absence of NaCl. Heavy metal induced an increase in the senescing hormone ethylene which was reduced by cytokinin treatment. Plants exposed to the mixed treatment (Cd + Zn) exhibited a specific hormonal status in relation to accumulation of abscisic acid and depletion of salicylic acid. Non-protein thiols (glutathione and phytochelatins) accumulated in response to Cd and Cd + Zn. It is concluded that toxic doses of Cd and Zn have different impacts on the plant behavior and that the simultaneous presence of the two elements induces a specific physiological constraint at the plant level. Salinity helps the plant to cope with heavy metal toxicities and

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the plant hormone cytokinin assumes key function in Zn resistance but its efficiency is lower in the presence of NaCl.

## 1. Introduction

Heavy metals in soil may have three origins: i) the alteration of geological materials, ii) atmospheric deposition and iii) agricultural and industrial inputs. Human activities could be a significant and major cause of heavy metal dissemination: combustion, specific industrial processes (mining, chemical, steel, metallurgy, …) as well as agricultural managements contribute to heavy metal pollution (Kumar et al., 2019). Metal ions such as zinc and copper are essential to plants but may also have toxic effects if their concentration in solution exceeds a certain threshold value. Non-essential metals such as Cd, Hg and Pb are not necessary for life and can interfere with metabolic processes, even in trace amounts (Shi et al., 2018).

Cadmium and zinc are often occurring concomitantly in polluted areas and both elements are harmful for human health (Meena et al., 2018; Kicińska et al., 2019; Xia et al., 2019). Coastal areas are prone to heavy metal contamination in relation to rapid urbanization and industrialization processes (Wang et al., 2013). Wetland plant species may consequently accumulate high amounts of heavy metals (Bonanno et al., 2017; Meena et al., 2018) and some of them have been recommended as promising plant materials for phytoextraction and bioremoval of trace metals from contaminated water and sediments (Chandra et al., 2017; Chowdhury et al., 2017). In coastal areas, plants are also exposed to high salinity levels. Salt is an important factor influencing toxicity, mobility and transfer of metals in estuarine wetlands (Bai et al., 2019). Salinity also directly acts on the plant behavior and only halophyte plant species are adapted to cope with heavy metals in salt-affected areas (Lutts and Lefèvre, 2015). According to Wen et al. (2019), coastal groundwater quality in numerous areas suffers from both a saline water intrusion and heavy metal pollution. Polluted areas are almost always characterized by the presence of several heavy metals (Wang et al., 2013; Meena et al., 2018; Bai et al., 2019; Kicińska et al., 2019; Wen et al., 2019). Despite this frequent polymetallic contamination, most studies dealing with the plant response to heavy metal usually consider plant exposure to one single toxic element, although some recent data clearly demonstrated that elements such as Cd and Zn may interact in a specific manner in terms of plant absorption, translocation, distribution and speciation (Cheng et al., 2018; Zhou et al., 2018a; Wu et al., 2019).

*Kosteletzkya pentacarpos* (syn. *K. virginica*) is a perennial facultative halophyte plant species from the Malvacea family originating from the Atlantic coast of the US and which has been introduced in China some decades ago. Beside its economic interest as a potential cash crop for alternative saline agriculture (He et al., 2003; Qin et al., 2015), *K. pentacarpos* has also been recommended as an interesting material for phytostabilization purposes. This species is indeed able to cope with Cu (Han et al., 2012a), Cd and Zn (Han et al., 2012b, 2013a; 2013b) pollution. In this halophyte species, salinity reduces Cd uptake and translocation (Han et al., 2012b) and modifies Zn distribution within plant tissues (Han et al., 2013b). It also influences the root biosorption capacities in relation to a modification in mucilage content and composition (Lutts et al., 2016).

Heavy metals accumulation lead to leaf senescence which could lead to premature death of plants. According to Zhou et al. (2018a) leaf senescence in *K. pentacarpos* is related to ethylene oversynthesis in response to Cd but not in response to Zn and the toxicity of the Cd + Zn treatment is mainly due to a senescing process occurring as a result of a modification in the polyamines status rather than to simple additive effects of Cd and Zn. Beside ethylene and polyamines, the plant hormones cytokinins also assume key functions in the regulation of leaf sensescence, acting mainly as *anti*-senescing compounds (Albacete et al., 2009; Wang et al., 2019). Exogenous cytokinins were accordingly shown to improve plant resistance to heavy metals in *Lupinus termis* (Gadallah and El-Enany, 1999), maize (Lukatkin et al., 2007), *Alyssum murale* (Cassina et al., 2011), *Helianthus annuus* (Cassina et al., 2012), *Solanum melongena* (Singh and Prasad, 2014) and tomato (Singh et al., 2018). All these plant species are typically terrestrial plants and, to the best of our knowledge, no data are available regarding cytokinin treatment on the wetland plant species *K. pentacarpos* exposed to a polymetallic treatment. Moreover, salinity itself may have a strong impact on senescing hormones independently of heavy metal pollution (Albacete et al., 2009; Rivero et al., 2009; Lutts and Lefèvre, 2015; Singh et al., 2018) but there is a crucial lack of information regarding salt and heavy metal interaction in relation to plant phytohormonal status. This information is however of paramount importance for managing phytoremediation of coastal saline polluted areas. Although salinity may be due to different types of salts, NaCl is commonly used to mimic salinity for coastal areas since seawater contains mainly NaCl (more than 98%). The tested hypotheses are i) that exogenous CKs application may delay senescence in response to Cd, Zn and Cd + Zn in *K. pentacarpos* and ii) these effect might be influenced by the presence of NaCl in the external medium and the nature of considered heavy metal treatment.

## 2. Material and methods

### 2.1. Plant material and growth conditions

Seeds of *Kosteletzkya pentacarpos* were harvested on mature plants growing in our greenhouses (Earth and Life Institute, Université catholique de Louvain, Louvain-la-Neuve, Belgium). These plants were issued from seeds kindly provided by Prof. Qin (University of Nanking, PR of China). Seeds were briefly surface-sterilized using 0.2% sodium hypochlorite solution and then sown in trays containing a mixture of peat and loam (1:1 v/v) regularly moistened with distilled water and placed in a phytotron at 28 °C/22 °C under a 16 h day/8 h night period. Light intensity was 245 $\mu$mol $m^{-2}$ $s^{-1}$ provided by fluorescent lamps (Master TL-D reflex Super 80 58W/840 from Philips) and relative humidity was maintained at 75 ± 5%.

Young seedlings of uniform size bearing two intact cotyledons and a first developing leaf were selected for transfer to hydroponic system. A total number of 480 seedlings were distributed among 32 tanks (15 plants per tank) each containing 25 L of aerated Hoagland nutrient solution containing (in mM): 5 $KNO_3$, 5.5 $Ca(NO_3)_2$, 1 $NH_4H_2PO_4$, 0.5 $MgSO_4$, and (in $\mu$M) 25 KCl, 10$H_3BO_4$, 1 $MnSO_4$, 0.25 $CuSO_4$, 1 $ZnSO_4$, 10 $(NH_4)_6Mo_7O$ and 1.87 g $L^{-1}$ Fe-EDTA. Solution was renewed every week and pH was adjusted daily to 5.7 using 5 M KOH.

Each tank contained 20 seedlings fixed on a polyvinylchloride plate floating at the top of the solution. Environmental conditions for plant growth remained the same as previously described for germination. Solutions was renewed each week and tanks randomly rearranged in the phytotron. After 3 weeks, the plants had 6 leaves and were then exposed to heavy metal stress in the presence or absence of 50 mM NaCl. Heavy metal treatment consisted in i) control (no additional heavy metal) ii) Cd 10 μM, iii) Zn 100 μM and iv) Cd 10 μM + Zn 100 μM. Heavy metals doses were chosen on the basis of previous work (Han et al., 2012b, 2013a, 2013b; Zhou et al., 2018a, 2018b) and corresponds to moderate levels of pollution recorded in wetland coastal ecosystems (Wang et al., 2013; Meena et al., 2018; Bai et al., 2019; Kumar et al., 2019). Heavy metals were added as chloride salts purchased from Sigma Chemical (Belgium). Considering the additional presence or absence of salt (NaCl 50 mM), a total number of 8 treatments was thus considered, each treatment involving four tanks. Half of the plants within each treatment were then exposed to exogenous cytokinin: *trans*-zeatine riboside (*t*-ZR) was purchased from Sigma Chemica and the required amounts was dissolved in 2 mL acetone; the volume allowing a final concentration of 10 μM was obtained by adding sterilized double distilled water with Tween-20 (0.1%, v/v) as a leaf surfactant. The treated plants were sprayed with *t*-ZR solution every 2 day at 10 a.m. until run-off while non-treated plants were sprayed with a similar volume of sterile deionized water containing Tween-20. After 7 applications (two weeks of treatment), plants were harvested for further analysis pooling leaves n°3, 4, 5 and 6 for each individual plant. Leaves were quickly rinsed in deionized water for 10 s to remove adhering *t*-ZR from the leaf surface.

### 2.2. Mineral concentration

Plant samples were dried at 70 °C for 72 h; 50–100 mg of leaves were then digested in 68% $HNO_3$ and acid evaporated to dryness on a sand bath at 80 °C. Minerals were incubated with a mix of HCl 37%-$HNO_3$ 68% (3:1) slightly evaporated and dissolved in distilled water. Cations, phosphorus and sulfur were quantified by Inductively Coupled Plasma-Optical Emission Spectroscopy (Varian, type MPX).

### 2.3. Phytohormone content

The ethylene production was measured by an ethylene detector ETD-300 (Sensor Sense, Nijmegen, The Netherlands). Samples were treated with the method detailed by Cristescu et al. (2002) with slight modifications as recommended by Chmielowska-Bak et al. (2013). Free polyamines (PAs) were extracted and dansylated according to Quinet et al. (2014). Samples were injected onto a Nucleodur $C_{18}$ Pyramid column (125 × 4.6 mm internal diameter, 5 μm particle size; Macherey-Nagel) maintained at 40 °C. The mobile phase consisted of a water/ACN gradient from 40 to 100% ACN and the flow was 1.0 mL $min^{-1}$. Analyses were performed by a Shimadzu HPLC system coupled to a RF-20A fluorescence detector (Shimadzu, 's-Hertogenbosch, The Netherlands) with an excitation wavelength of 340 nm and an emission wavelength of 510 nm.

Other phytohormones (abscicic acid (ABA), auxins, salicylic acid (SA), jasmonic acid (JA), cytokinins (CK) and their metabolites and the precursor of ethylene aminocyclopropane carboxylic acid (ACC)) were extracted and purified from fresh leaves according to Dobrev and Kaminek (2002) and Dobrev and Vankova (2012). Hormonal quantification was performed by HPLC (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP; Applied Biosystems) as described previously (Djilianov et al., 2013) using isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data processing was carried out with Analyst 1.5 software (Applied Biosystems).

### 2.4. Stomatal conductance, net photosynthesis, chlorophyll content and carbon isotope discrimination

Before leaf harvest, the instantaneous $CO_2$ assimilation under ambient conditions (400 ppm $CO_2$) ($A$) was measured using an infrared gas analyser (LCA4 8.7 ADC, Bioscience, Hertfordshire, UK). Leaf stomatal conductance ($g_s$) was measured on 5 plants per treatment using an AP4 diffusion porometer (Delta-TDevices Ltd., Cambridge, UK). All measurements were performed on leaf n°5 between 2 p.m. and 4 p.m.. Total chlorophyll (a+b) concentrations were measured according to Lichtenthaler (1987).

Leaves dried in an oven and used for mineral content were also used for carbon isotope discrimination. Isotopic and elemental measurements were performed using Optima mass spectrometer (Micromass, UK) coupled to a C-N-S elemental analyser (Carlo Erba, Italy). The C concentration is expressed in percent relative to the total dry weight. The reference material used was IAEA CH-6 ($\delta^{13}C = -10.4 \pm 0.2‰$). Carbon isotope composition ($\delta^{13}C$) values were obtained in part per thousand (‰) relative to Vienna Pee Dee Belemnite (vPDB) according to the following formula: $\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 10^3$ where $R = {}^{13}C/{}^{12}C$. Carbon isotope discrimination ($\Delta^{13}C$) was calculated according to the formula of Farquhar and Richards (1984): $\Delta^{13}C = [(\delta_a - \delta_p)/(1 + \delta_p)] \times 10^3$ where $\delta_p$ is the $\delta^{13}C$ of the leaf sample and $\delta_a$ is the $\delta^{13}C$ of the atmospheric $CO_2$ (−8‰).

### 2.5. Oxidative-stress related compounds and non protein thiols

The level of lipid peroxidation was measured as 2-thiobarbituric acid-reactive substances, mainly malondialdehyde (MDA) according to Heath and Packer (1968).

The ascorbate (AsA and DHA) were determined according to Wang et al. (1991) on the basis on $Fe^{3+}$-$Fe^{2+}$ reduction by ascorbate in acid solution. Reduced (GSH) and total (GSHt) glutathione

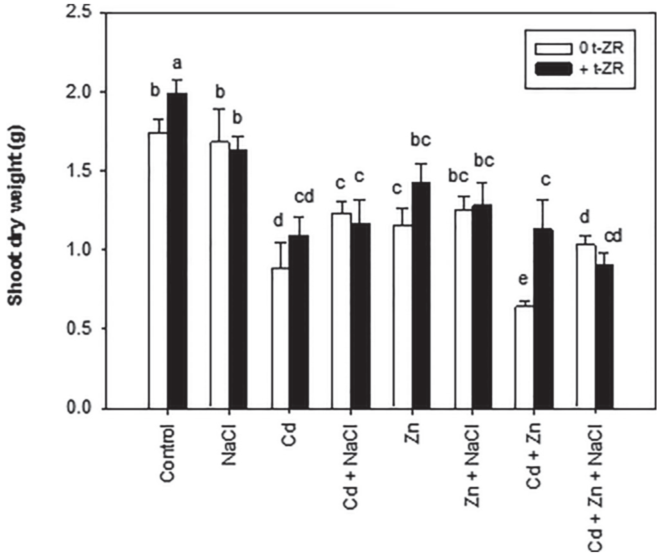

**Fig. 1.** Shoot dry weight (g) of *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 μM), zinc (100 μM), Cd + Zn (10 μM + 100 μM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 μM t-zeatin riboside (*t*-ZR; 10 μM). Each value is the mean of 5 replicates and vertical bars are standard errors. Values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

quantification were determined by Shimadzu HPLC system (Shimadzu, ‘s-Hertogenbosch, The Netherlands) equipped with a Nucleodur C18 Pyramid column (125 × 4.6 mm internal diameter; 5 μm particle size) (Macherey-Nagel, Düren, Germany) according to Cereser et al. (2001). The total non-protein thiols (NPT) concentration was determined according to De Vos et al. (1992) using Ellman's reagent. Phytochelatins content was evaluated as the difference between NPT and GSH levels (Schäfer et al., 1997).

### 2.6. Statistical treatment

All analysis were performed on 5 biological replicates, except carbon isotope discrimination and hormonal profiling performed on three replicates. For biochemical analysis, technical triplicates were performed for each sample to check the accuracy of the technical procedures. Normality of the data was verified using Shapiro-Wilk tests and the data were transformed when required. ANNOVA 3 were performed at a significant level of $P < 0.05$ using SAS Entreprise Guide (SAS 9.4 system for windows) considering the type of heavy metal treatment, the salinity, and the application of exogenous cytokinin as main factors. Post-hoc analyses were performed using Student-Newman-Keuls test at 5% level.

## 3. Results

### 3.1. Shoot dry weight

Salinity had no impact on the shoot dry weight (DW) (Fig. 1) comparatively to non-salinized control. Cadmium and zinc applied separately significantly decreased the shoot weight by 39% and 22%, respectively ($P < 0.05$). In both cases, NaCl at least partly mitigated the deleterious impact of heavy metals on shoot growth. The mixed treatment (Cd + Zn) was the most detrimental in terms of shoot DW and NaCl also partly restored the shoot dry weight. Cytokinin application in the form of 10 μM zeatine-riboside (*t*-ZR) had a slight, although significant impact on the shoot DW of non-salinized controls ($P < 0.05$) while it had no impact on this parameter in plants exposed to NaCl, Cd or Cd + NaCl. In contrast, exogenous CK obviously improved the shoot DW in plants exposed to Cd + Zn in the absence of NaCl, but surprisingly, NaCl reduced the beneficial impact of *t*-ZR in these plants.

### 3.2. Mineral content

Cadmium (Table 1) was detected in Cd- and Cd + Zn-treated plants only (detection limit: 0.013 mg/L). Salinity reduced leaf Cd accumulation in both cases in the absence of *t*-ZR, the lowest concentration being recorded in salt-treated Cd + Zn-exposed plants. Exogenous *t*-ZR application increased the leaf Cd concentration in the absence of salt but did not modify it in the presence of NaCl. Leaf Zn concentration (Table 1) was similar in control and in Cd-treated plants and neither NaCl nor *t*-ZR had any significant impact on this parameter for these plants. Zinc excess strongly increased leaf Zn concentration. The leaf Zn concentration (Table 1) was lower in plants exposed to Cd + Zn than to Zn in the absence of salt and salinity decreased it to a higher extent in plants exposed to the mixed heavy metal treatment. It is noteworthy that exogenous *t*-ZR application significantly increased Zn content in all plants exposed to Zn excess ($P < 0.05$).

**Table 1**
Cadmium and zinc concentration (in mg kg$^{-1}$ DW) in leaves of seedlings of *Kosteletzkya pentacarpos* cultivated in the presence of Cd (10 μM), Zn (100 μM) or Cd + Zn (10 μM + 100 μM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 μM t-zeatin riboside (*t*-ZR; 10 μM). Each value is the mean of 5 replicates ± standard errors. For a given element, values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.(-: not detected).

| | Cadmium (mg kg$^{-1}$ DW) | | Zinc (mg kg$^{-1}$ DW) | |
|---|---|---|---|---|
| | No *t*-ZR | + *t*-ZR | No *t*-ZR | + *t*-ZR |
| Control | – | – | 179 ± 10 a | 158 ± 7 a |
| NaCl | – | – | 201 ± 16 a | 187 ± 18 a |
| Cd | 489 ± 34 e | 541 ± 23 f | 212 ± 15 a | 191 ± 20 a |
| Cd + NaCl | 278 ± 19 b | 263 ± 25 b | 184 ± 21 a | 203 ± 11 a |
| Zn | – | – | 1071 ± 77 e | 1322 ± 103 f |
| Zn + NaCl | – | – | 843 ± 65 c | 1019 ± 89 e |
| Cd + Zn | 322 ± 41 c | 408 ± 32 d | 988 ± 49 d | 1284 ± 113 f |
| Cd + Zn + NaCl | 107 ± 11 a | 122 ± 17 a | 532 ± 19 b | 918 ± 64 cd |

Sodium concentration always remained low in plants maintained in the absence of NaCl (Fig. 2A). Exposure to NaCl increased the leaf Na concentration in the absence and in the presence of heavy metals but to a lower extent in plants exposed to Cd or Cd + Zn treatments than in plants exposed to Zn alone. In salt-treated plants, exogenous *t*-ZR reduced Na concentration in the absence of heavy metal and in Zn-treated plants but not in Cd-exposed ones. It is noteworthy that Cd but not Zn significantly decreased the Fe (Fig. 2B) and Mn (Fig. 2C) leaf concentration ($P < 0.05$) and that exogenous *t*-ZR allowed the plant to limit the deleterious impact of heavy metals on Fe and Mn content, mainly in the absence of NaCl. Sulfur concentration (Fig. 2D) increased within the leaf in response to Cd but not in response to Zn. Salinity had no impact on the sulfur content while *t*-ZR increased S content in plants exposed to Cd alone or to Cd + Zn. Treatments had no significant impact on the phosphorous and potassium concentration (data not shown).

### 3.3. Hormonal profiling

The global hormonal status is provided in Fig. 3 for total cytokinins (Fig. 3A), total auxin (Fig. 3B), total gibberellins (Fig. 3C) and in Fig. 4 for abscisic acid (4A), salicylic acid (Fig. 4B), jamonates (Fig. 4C) and aminocyclopropane carboxylic acid (Fig. 4D).

In plants that did not receive exogenous *t*-ZR treatment and that were not exposed to NaCl, Cd alone did not impact the total endogenous CK concentration while Zn and Cd + Zn treatment significantly decreased it ($P < 0.05$). The presence of NaCl increased endogenous CKs in all plants exposed to heavy metal treatments. Similarly, exogenous application of *t*-ZR increased endogenous CKs in all plants. In salt-treated plants, the impact of *t*-ZR on endogenous CKs remained low but was significant in all cases ($P < 0.05$). Salt and exogenous *t*-ZR not only had an impact on the total CKs but also modified the proportion of various compounds as detailed in Table 2. Beside active CKs, CK deactivation forms (*N*7- and *N*9-glucosides), the CK storage forms (*O*-glucose) and CK-phosphates (immediate precursors) were detected. The active forms only constitute a minor proportion of the total CKs but it increased in response to NaCl in Cd-treated plants and in Zn-treated ones. Exogenous *t*-ZR application significantly increased the percentage of bioactive cytokinins in all treatment ($P < 0.05$), except in plants exposed to Cd + NaCl treatment. In the absence of exogenous *t*-ZR, exposure to Zn significantly decreased the CK-*O*-glucoside proportion ($P < 0.05$), especially in the absence of salt. Spraying with *t*-ZR significantly increased the storage CK-*O*-glucoside forms ($P < 0.05$) except, once again, in Cd + NaCl treatment. The treatments had only a limited impact on the CK-phosphate fraction. The CK-*N*-fraction was by far the most important one and always represented more than 85% of the detected cytokinins. The percentage of CK-*N*-glucoside was always lower in plants exposed to exogenous *t*-ZR than in non-treated plants sprayed with water, the difference being significant for Zn- and Cd + Zn-treated plants

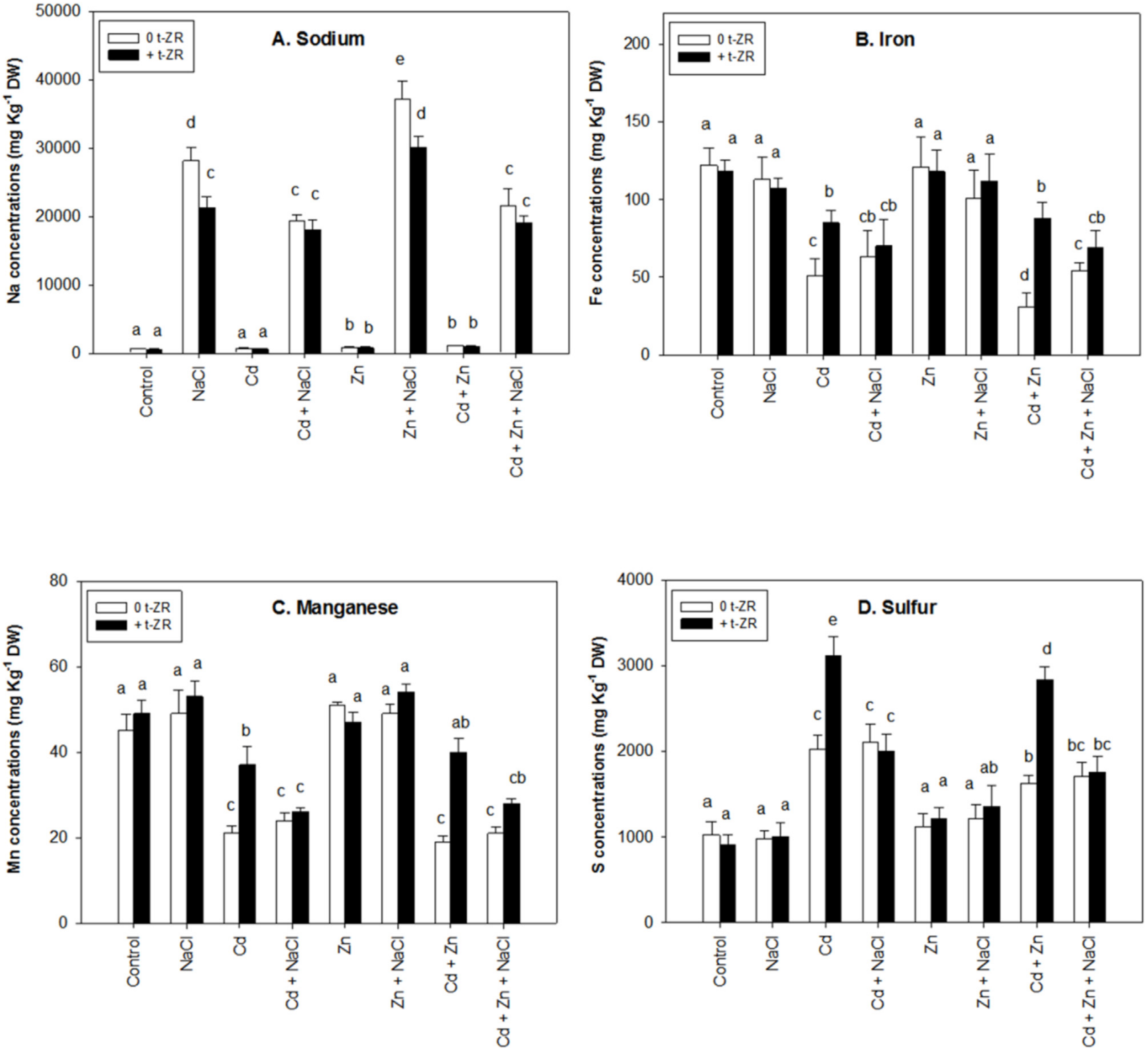

**Fig. 2.** Leaf Na (A), Fe (B), Mn (C) and S (D) concentrations (mg $kg^{-1}$ DW) of *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 μM), zinc (100 μM), Cd + Zn (10 μM + 100 μM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 μM t-zeatin riboside (*t*-ZR; 10 μM). Each value is the mean of 5 replicates and vertical bars are standard errors. Values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

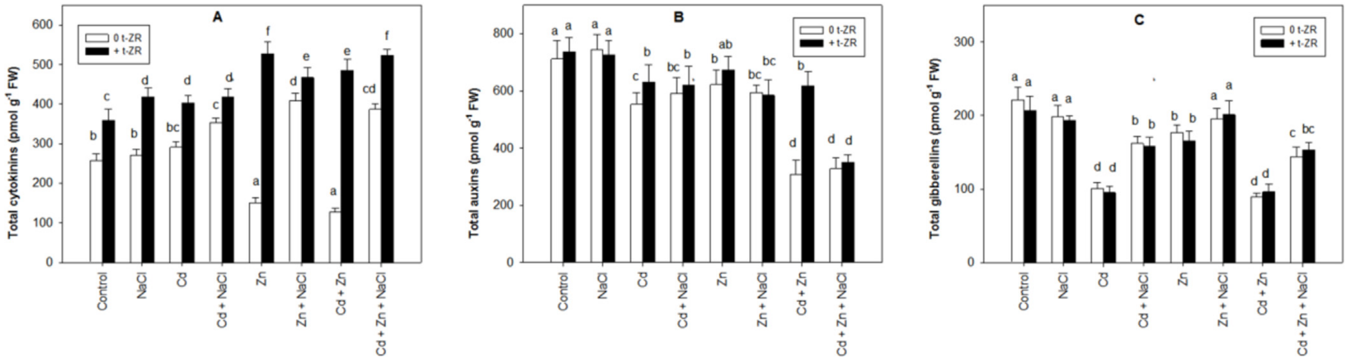

**Fig. 3.** Leaf total cytokinins (A), auxins (B) and gibberellins (C) concentrations (pmol $g^{-1}$ FW) of *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 μM), zinc (100 μM), Cd + Zn (10 μM + 100 μM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 μM t-zeatin riboside (*t*-ZR; 10 μM). Each value is the mean of 3 replicates and vertical bars are standard errors. Values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

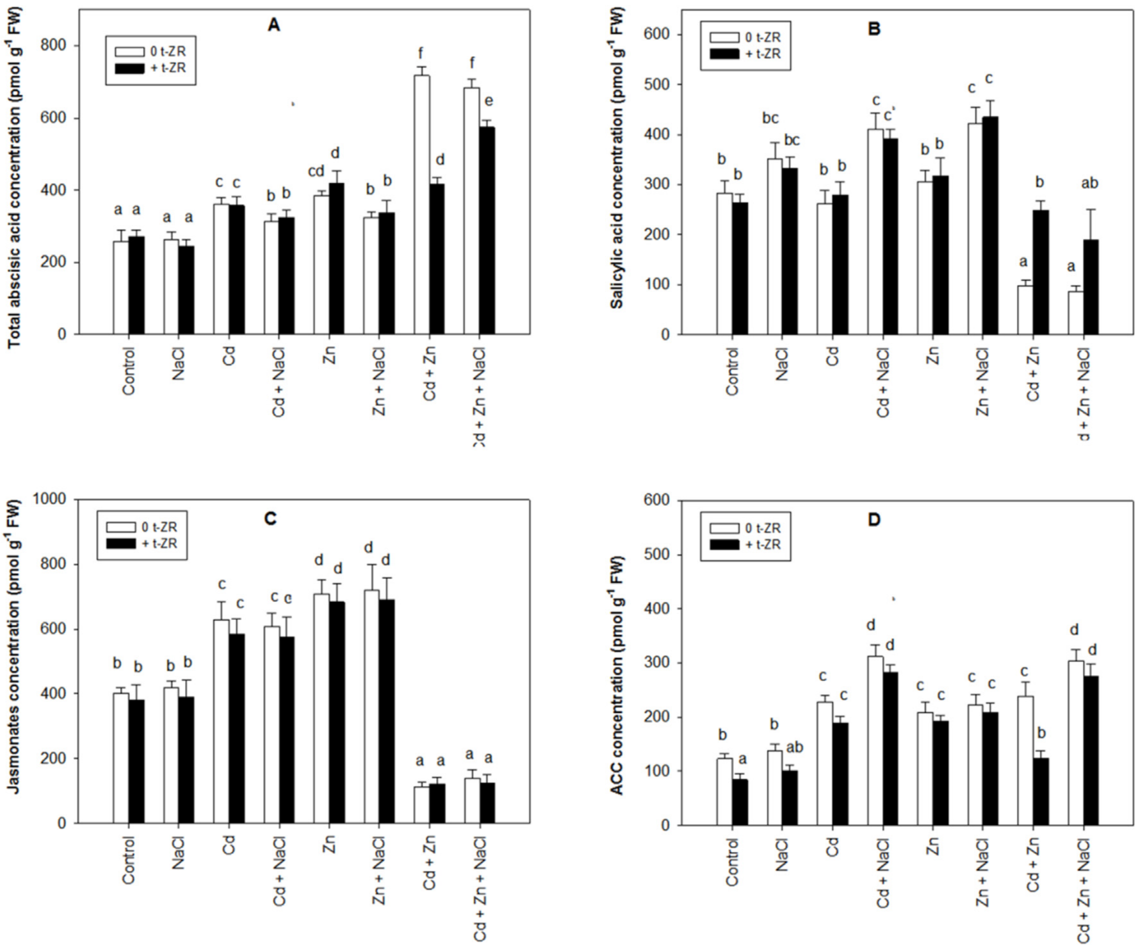

**Fig. 4.** Leaf total abscisic acid (A), salicylic acid (B), jasmonates (C) and aminocyclopropane carboxylic acid (D) concentrations (pmol $g^{-1}$ DW) of *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 μM), zinc (100 μM), Cd + Zn (10 μM + 100 μM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 μM t-zeatin riboside (*t*-ZR; 10 μM). Each value is the mean of 3 replicates and vertical bars are standard errors. Values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

**Table 2**
Relative percentage of bioactive cytokinins (CK; free bases and ribosides), CK-*N*-glucoside (deactivation form), CK-*0*-glucoside (storage forms) and CK-phopsohate (immediate biosynthetic precursors) in leaves of seedlings of *Kosteletzkya pentacarpos* cultivated in the presence of Cd (10 μM), Zn (100 μM) or Cd + Zn (10 μM + 100 μM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 μM t-zeatin riboside (*t*-ZR; 10 μM). For a given class of compounds, values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.(-: not detected).

| | Bioactive CK | | CK-*0*-glucoside | | CK-phosphate | | CK-*N*-glucoside | |
|---|---|---|---|---|---|---|---|---|
| | No *t*-ZR | + *t*-ZR | No *t*-ZR | + *t*-ZR | No *t*-ZR | + *t*-ZR | No *t*-ZR | + *t*-ZR |
| Control | 2.50 b | 5.41 e | 3.18 c | 4.50 de | 2.06 a | 2.25 a | 92.2 b | 87.8 ab |
| NaCl | 3.25 c | 4.84 de | 4.24 d | 5.81 f | 2.98 b | 2.48 ab | 89.5 ab | 86.8 a |
| Cd | 2.34 b | 4.12 d | 4.03 d | 5.21 e | 2.14 a | 1.71 a | 91.5 b | 88.9 ab |
| Cd + NaCl | 5.91 fg | 5.18 e | 5.11 e | 5.36 e | 1.79 a | 3.11 bc | 87.1 a | 86.3 a |
| Zn | 2.84 bc | 6.17 g | 1.81 b | 6.03 g | 1.88 a | 2.94 b | 93.5 b | 84.8 a |
| Zn + NaCl | 5.81 f | 6.23 g | 2.22 b | 4.18 d | 2.12 a | 2.53 ab | 89.8 ab | 87.1 a |
| Cd + Zn | 1.79 a | 5.27 e | 0.64 a | 5.47 ef | 2.33 a | 2.13 a | 95.2 b | 87.1 a |
| Cd + Zn + NaCl | 4.84 de | 5.93 fg | 3.25 c | 5.01 e | 3.01b | 3.28 c | 88.9 ab | 85.8 a |

maintained in the absence of salt ($P < 0.05$).

Total auxin (Fig. 3B) comprises IAA, but also other compounds such as IAA-Asp, IAA-GLU and oxIAA. Cadmium and Zn applied separately had only a small effect on the total auxin content which slightly decreased. In contrast, the simultaneous presence of Cd and Zn had a strong deleterious impact on the total auxin concentration: in these plants, IAA decreased by more than 60% while IAA-Asp exhibited 80% decrease (detailed data not shown). Salinity did not afford protection in terms of auxin content but exogenous *t*-ZR application partly restored the endogenous concentration of auxin in the absence but not in the presence of NaCl. Cadmium and mixed treatment reduced the total GA concentration (Fig. 3C) in plants cultivated in the absence of salt. Adding NaCl mitigated the deleterious impact of heavy metals on GA content while exogenous application of *t*-ZR had no effect on endogenous GA concentration.

A specific hormonal status for plants exposed to the mixed treatment Cd + Zn was also recorded for ABA, SA and jasmonates concentrations as indicated in Fig. 4. In all cases, the impact of

Cd + Zn constraint was by far higher than the recorded impact of Cd or Zn applied separately. Total ABA increased in response to Cd or Zn, even if NaCl attenuated the impact of heavy metals. The recorded increase in ABA for plants exposed to Cd + Zn was higher than the sum of increase recorded for Cd and Zn. While exogenous *t*-ZR had no impact on ABA content in plants exposed to one single heavy metal, it clearly reduced the ABA concentration in plants exposed to Cd + Zn, and to a higher extent in the absence than in the presence of NaCl.

In contrast to ABA, endogenous salicylic acid (SA; Fig. 4B) was depleted in plants exposed to the mixed Cd + Zn treatment while plants exposed to heavy metals separately remained only marginally and non-significantly affected. Benzoic acid, a precursor of SA, was also obviously decreased in plants exposed to mixed toxicity and dropped from 740 pmol $g^{-1}$ FW to less than 200 pmol $g^{-1}$ FW in Cd + Zn-treated plants (detailed data not shown). Once again, the plants exposed separately to Cd or Zn were not significantly modified for their benzoic acid content. Salinity increased SA concentration in Zn- or Cd-treated plants but had no impact on SA content in plants exposed to Cd + Zn. In contrast, *t*-ZR application had no impact on SA content in plants exposed to one single heavy metal, but it increased it in plants exposed to mixed heavy metal toxicity. Jasmonates concentration (Fig. 4C) increased in plants exposed to Cd or Zn but it decreased in plants exposed to mixed toxicity Cd + Zn. Salinity and *t*-ZR had no significant impact on JA concentration. Aminocyclopropane carboxylic acid (ACC; Fig. 4D) increased in response to all heavy metal treatments. Salinity increased ACC concentration in plants exposed to Cd or to Cd + Zn toxicities and had no significant impact on ACC concentration in Zn-treated plants. Exogenous treatment with *t*-ZR decreased ACC content in plants cultivated in the absence of heavy metals (control and NaCl-treated plants) and in plants exposed to the mixed treatment in the absence but not in the presence of salt.

Cadmium and Cd + Zn treatment increased the endogenous content of the diamine putrescine (Put) but reduced the triamine spermidine (Spd) and the tetramine spermine (Spm). As a consequence, the Put/Spd + Spm ratio (Table 3) strongly increased in response to these treatments. Application of *t*-ZR had no effect on the Put/(Spd + Spm) ratio in Cd-treated plants but it almost alleviated the increase for plants exposed to Cd + Zn treatment in the absence of NaCl: this effect was due both to a decrease in stress-induced Put and to an increase in Spd while Spm remained unaffected by *t*-ZR. The effect exogenous application of *t*-ZR on Put/Spd + Spm ratio was lower in Cd + Zn + NaCl-treated plants. Ethylene is acting as a major senescing agent in plant tissues and is overproduced by *K. pentacarpos* in response to heavy metals (Table 3): Cd and Zn applied separately had a similar impact on ethylene synthesis from a quantitative point of view but ethylene synthesis was clearly the highest for the Cd + Zn-treated plants. NaCl reduced ethylene synthesis in plants exposed to Cd alone or in combination (Cd + Zn) while exogenous *t*-ZR had exactly an opposite effect since it reduced ethylene synthesis in Zn-treated plants only.

**Table 3**
Putrescine/(spermidine + spermine) ratio (Put/(Spd/Spm)) and ethylene synthesis (nL $g^{-1}$ FW $h^{-1}$) in leaves of seedlings of *Kosteletzkya pentacarpos* cultivated in the presence of Cd (10 μM), Zn (100 μM) or Cd + Zn (10 μM + 100 μM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 μM t-zeatin riboside (*t*-ZR; 10 μM). Each value is the mean of 3 replicates ± standard errors. For a given parameter, values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

| | Put/(Spd + Spm) | | Ethylene (nL $g^{-1}$ FW $h^{-1}$) | |
|---|---|---|---|---|
| | No *t*-ZR | + *t*-ZR | No *t*-ZR | + *t*-ZR |
| Control | 2.3 ± 0.44 a | 2.1 ± 0.23 a | 0.32 ± 0.05 a | 0.36 ± 0.1 a |
| NaCl | 2.2 ± 0.36 a | 2.5 ± 0.11 a | 0.28 ± 0.04 a | 0.34 ± 0.09 a |
| Cd | 11 ± 1.2 d | 9.8 ± 0.78 d | 1.7 ± 0.18 d | 1.6 ± 0.12 d |
| Cd + NaCl | 4.2 ± 0.35 c | 4.0 ± 0.21 bc | 1.1 ± 0.15 c | 1.3 ± 0.05 cd |
| Zn | 2.9 ± 0.15 ab | 2.6 ± 0.09 a | 1.6 ± 0.08 d | 0.45 ± 0.01 b |
| Zn + NaCl | 3.0 ± 0.29 b | 2.9 ± 0.22 c | 1.6 ± 0.11 d | 1.1 ± 0.08 c |
| Cd + Zn | 17 ± 1.8 f | 4.5 ± 0.35 c | 2.8 ± 0.17 f | 2.2 ± 0.19 e |
| Cd + Zn + NaCl | 13 ± 2.2 e | 8.0 ± 0.12 d | 1.3 ± 0.14 cd | 1.2 ± 0.13 c |

### 3.4. *Stomatal conductance, net photosynthesis, chlorophyll content and carbon isotope discrimination*

Salinity had no significant impact on the stomatal conductance ($g_s$) in plants cultivated in the absence of heavy metals (Fig. 5A). Exposure to Cd decreased stomatal conductance. In control and Cd-treated, exogenous application of *t*-ZR induced a significant increase in $g_s$ values ($P < 0.05$). A very low stomatal conductance was recorded for Zn-treated plants and for plants exposed to Cd + Zn. In Zn-treated plants, stomatal closure was partly reduced by NaCl while exogenous *t*-ZR had a clear significant positive impact on stomatal conductance in plants exposed to Zn in the absence of salt ($P < 0.05$). Similarly, exogenous *t*-ZR prevented stomatal closure in plants exposed to Cd + Zn in the absence of salt but this effect was less pronounced in the presence of NaCl.

Salinity and *t*-ZR had no impact on net photosynthesis (*A*; Fig. 5B) in plants cultivated in the absence of heavy metals (Fig. 5B). Cadmium and Zn reduced *A* values and NaCl partly mitigated the deleterious effect of heavy metals on net photosynthesis. Exogenous application of *t*-ZR significantly reduced heavy metals impact on net photosynthesis in plants maintained in the absence of NaCl ($P < 0.05$) but it had no significant effect on plants cultivated in the presence of salt. The total chlorophyll content (Table 4) was also reduced in response to Cd and Cd + Zn but the recorded decrease in Zn-treated plants was lower and it mainly concerned *Chl* b and not *Chl* a (detailed data not shown). Cytokinin application increased the total chlorophyll concentration, including in control plants. Carbon isotope discrimination ($\Delta^{13}C$) was reduced by Cd and Zn exposure; NaCl had no impact on $\Delta^{13}C$ values in Cd-treated plants but it limited the recorded $\Delta^{13}C$ decrease in plants exposed to Zn and to Cd + Zn. Exogenous *t*-ZR had no impact on $\Delta^{13}C$ values, except for plants exposed to these treatments (see Table 4).

### 3.5. *Oxidative-stress related compounds and non-protein thiols*

The leaf malondialdehyde concentration (Fig. 6A) increased to similar values in Cd- and in Cd + Zn-treated plants and in both cases, NaCl decreased MDA concentration ($P < 0.05$). In Zn-treated plants, MDA also significantly increased but NaCl did not reduce MDA content. In contrast, *t*-ZR decreased MDA concentration in these plants in the absence of NaCl and a similar effect was noticed for Cd + Zn-exposed plants. Exogenous *t*-ZR had no impact on MDA content of Cd-treated plants.

The total ascorbate pool containing AsA and DHA is represented in Fig. 6B. Total ascorbate pool slightly increased in response to NaCl. Cadmium had a moderate stimulating impact on ascorbate concentration but Zn-treated plants presented a decrease in ascorbate pool that was prevented by the concomitant application of NaCl. *t*-ZR application increased the total ascorbate pool mainly in Zn and in Zn + Cd-treated plants. The proportion of dehydroascorbate remained constant whatever the treatment (mean value of 12.3 ± 1.7%, data not shown).

Total glutathione (GSH + GSSG; Fig. 6C) increased in response to Cd exposure in plants exposed to Cd alone or to Cd + Zn and glutathione accumulation was reinforced by NaCl. In contrast, total glutathione was only marginally increased in plants exposed to Zn in the absence of Cd, although NaCl increased glutathione

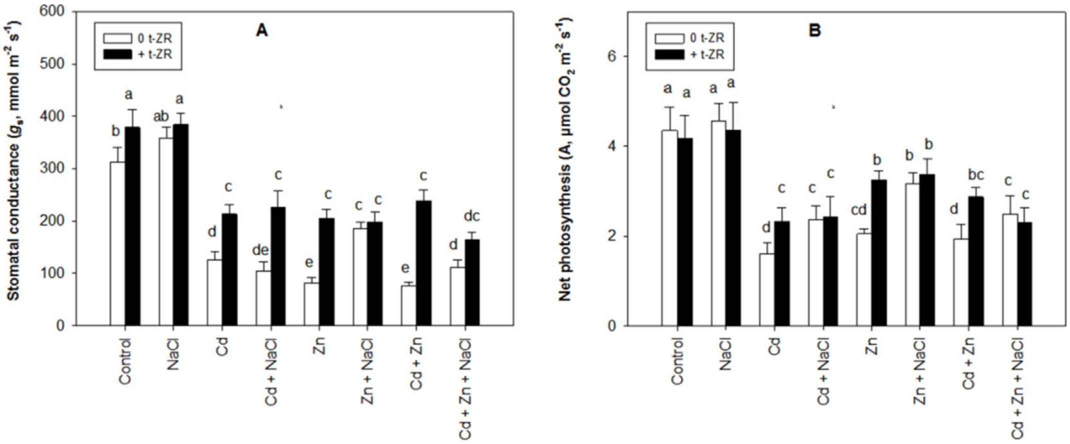

**Fig. 5.** Leaf stomatal conductance ($g_s$, mmol m$^{-2}$ s$^{-1}$; A) and net photosynthesis ($A$, µmol CO$_2$ m$^{-2}$ s$^{-1}$; B) in *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 µM), zinc (100 µM), Cd + Zn (10 µM + 100 µM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 µM t-zeatin riboside (*t*-ZR; 10 µM). Each value is the mean of 5 replicates and vertical bars are standard errors. Values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

**Table 4**

Total chlorophyll (in mg g$^{-1}$ FW) and carbon isotope discrimination ($\Delta^{13}$C, ‰) in leaves of seedlings of *Kosteletzkya pentacarpos* cultivated in the presence of Cd (10 µM), Zn (100 µM) or Cd + Zn (10 µM + 100 µM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 µM t-zeatin riboside (*t*-ZR; 10 µM). Total chlorophyll value is the mean of 3 replicates ± standard errors and carbon isotope discrimination value is the mean of 5 replicates ± standard errors. For a given parameter, values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

| | Total chlorophyll (mg g$^{-1}$ FW) | | Carbon isotope discrimination ($\Delta$C$^{13}$, ‰) | |
|---|---|---|---|---|
| | No *t*-ZR | + *t*-ZR | No *t*-ZR | + *t*-ZR |
| Control | 13 ± 0.2 d | 15 ± 0.2 e | 25.32 ± 0.12 a | 25.25 ± 0.15 a |
| NaCl | 14 ± 0.3 d | 15 ± 0.2 e | 25.01 ± 0.08 a | 24.98 ± 0.08 a |
| Cd | 7.9 ± 0.2 a | 9.0 ± 0.2 b | 23.17 ± 0.21 b | 23.22 ± 0.10 b |
| Cd + NaCl | 9.5 ± 0.1 b | 11 ± 0.4 c | 23.14 ± 0.11 b | 23.16 ± 0.42 b |
| Zn | 11 ± 0.2 c | 12 ± 0.1 d | 21.08 ± 0.23 d | 23.47 ± 0.09 b |
| Zn + NaCl | 12 ± 0.3 d | 14 ± 0.2 d | 22.14 ± 0.47 c | 22.27 ± 0.12 c |
| Cd + Zn | 7.6 ± 0.3 a | 10 ± 0.3 bc | 21.07 ± 0.31 d | 23.18 ± 0.16 b |
| Cd + Zn + NaCl | 8.9 ± 0.1 b | 9.8 ± 0.3 bc | 22.01 ± 0.14 c | 22.24 ± 0.30 c |

accumulation in this material. Exposure to *t*-ZR increased total glutathione in Cd- and Cd + Zn-treated plants in the absence of NaCl. The GSSG/GSH ratio increased from 0.173 in controls to 0.292, 0.345 and 0.421 in Cd-, Zn- and Cd + Zn-treated plants and neither NaCl nor *t*-ZR had significant impact on this ratio (data not shown). The concentration of non-protein thiols exhibited a similar trend (Fig. 6D) and it mainly occurred in Cd- and Cd + Zn-treated plants. In these plants, additional NaCl did not increase NPT concentration. Non-protein thiols concentration was similar in controls and in Zn-treated plants. Exposure to *t*-ZR decreased NPT concentration in Cd-treated plants. The phytochelatins (PC; Table 5) concentration was estimated as the difference between NPT and total glutathione. It increased in plants exposed to Cd and was slightly reduced by salinity. The maximal value was recorded in plants exposed to the mixed treatment (Cd + Zn) in the absence of NaCl. The *t*-ZR treatment reduced PC concentration in all plants exposed to Cd (both in Cd- and Cd + Zn-treated plants) but had no impact on plants exposed to Zn alone.

## 4. Discussion

Although *K. pentacarpos* is mainly encountered in salt marshes (He et al., 2003; Qin et al., 2015), it should not be regarded as an obligate halophyte plant species: the plant indeed grows well in the absence of NaCl and a moderate NaCl dose of 50 mM did not significantly improve plant growth (Fig. 1). Nevertheless, NaCl was able to improve plant behavior in heavy-metal treated plants. Such a positive effect may be, at least partly, explained by a decrease in heavy metal accumulation. Han et al. (2012b) hypothesized that Cd may be fixed by root mucilage while Lutts et al. (2016) provided evidences that salinity increased the root mucilage pectic compounds and hemicellulose involved in metal binding. In mixed treatment (Cd + Zn), toxic elements may also compete for root absorption and both elements accumulated to lower concentrations in the mixed than in the non-mixed treatment. Cheng et al. (2018) demonstrated that Cd reduced Zn uptake in *Carpobrotus rossii* while Wu et al. (2019) recently showed that Zn inhibited Cd uptake in *Brassica chinensis* mainly by acting on the expression of gene coding for the iron-regulated transporters BcIRT1 putatively involved in Cd uptake. The fact that Fe concentration in *K. pentacarpos* (Fig. 2B) decreased in Cd-treated plants suggests that Cd may also impact Fe nutrition in this species. However, in contrast to *B. chinensis*, Zn did not improve Fe nutrition in the presence of Cd. Even if Zn itself was reported to interact with IRT (Caldelas and Weiss, 2017; Gupta et al., 2016), it has to be noticed to 100 µM Zn did not reduce Fe content in the leaves while 10 µM Cd did.

In contrast to these “competition process”, Li et al. (2009) mentioned that Cd uptake and translocation could be enhanced by Zn excess but these authors considered a hyperaccumulating plant species (*Sedum alfredii*), although Tkalec et al. (2014) reported a similar observation for tobacco which is not a hyperaccumulating species. As indicated by Cherif et al. (2012), one should distinguish the impact of Zn provided at a physiological dose from the additional deleterious impact resulting from a Zn excess. Moreover, the present work reported ion concentration in the leaves only and root compartment was not considered, while numerous authors mentioned that root absorption and root-to-shoot translocation may be differently regulated (Han et al., 2010; Cheng et al., 2018).

The specific physiological status of plants exposed to the mixed heavy metal toxicity was also evident from a phytohormonal point of view: auxin, ABA, SA and JA were more markedly affected in response to mixed treatment than in the case of exposure to one single heavy metal. Jasmonates (Fig. 4C) strongly decreased in response to mixed toxicity while they increased when plants were exposed to only one heavy metal, thus clearly showing that the

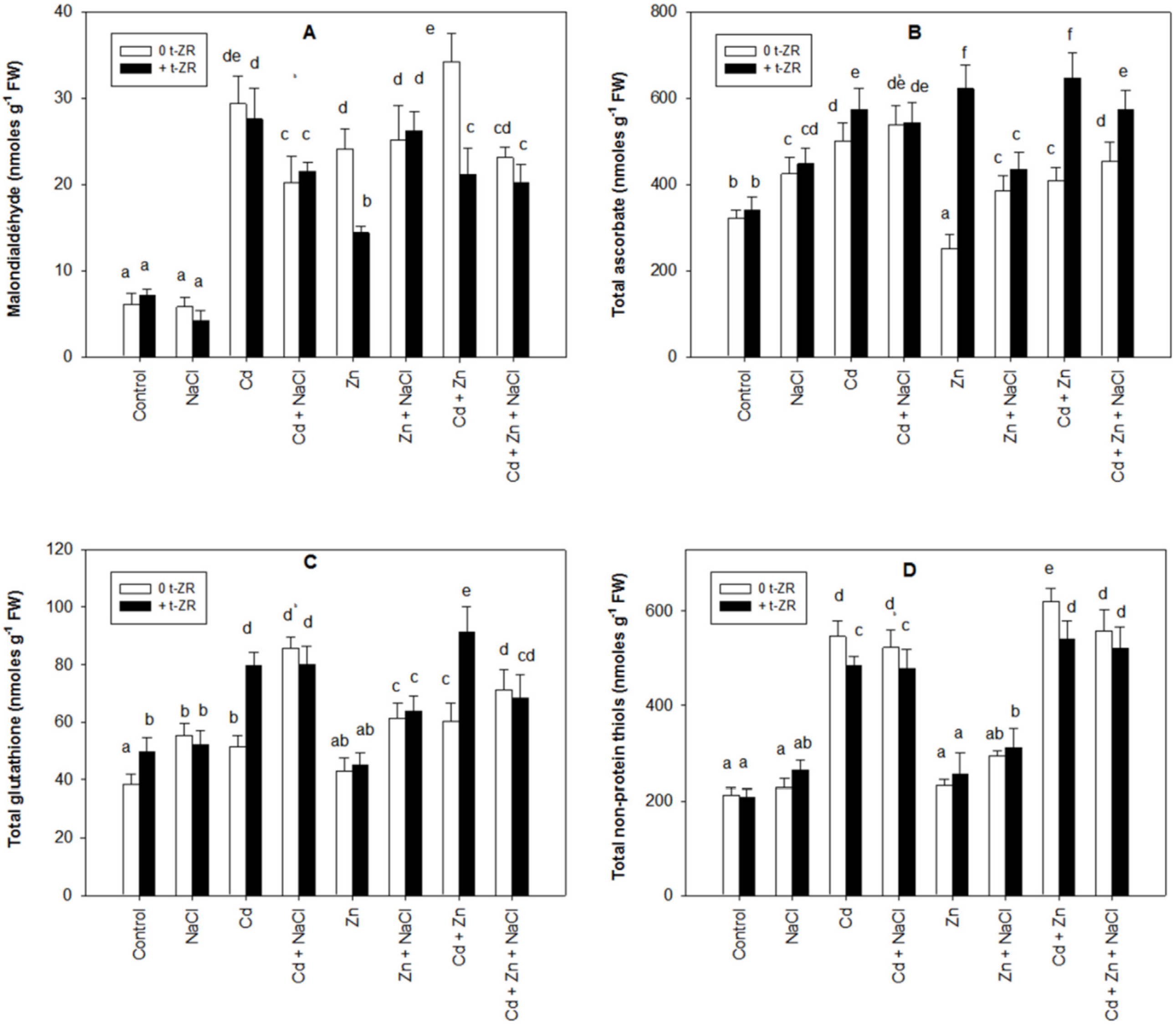

**Fig. 6.** Leaf malondialdehyde (MDA, nmol $g^{-1}$ FW; A), total ascorbate (nmol $g^{-1}$ FW; B), total glutathione (nmol $g^{-1}$ FW; C) and total non-protein thiols (nmol $g^{-1}$ FW; D) in *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 μM), zinc (100 μM), Cd + Zn (10 μM + 100 μM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 μM t-zeatin riboside (*t*-ZR; 10 μM). Each value is the mean of 5 replicates and vertical bars are standard errors. Values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

**Table 5**
Phytochelatin concentration (in nmol $g^{-1}$ FW) in leaves of seedlings of *Kosteletzkya pentacarpos* cultivated in the presence of Cd (10 μM), Zn (100 μM) or Cd + Zn (10 μM + 100 μM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 μM t-zeatin riboside (*t*-ZR; 10 μM). Each value is the mean of 5 replicates ± standard errors. Values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

| | Phytochelatin (mg $g^{-1}$ FW) | |
|---|---|---|
| | No *t*-ZR | + *t*-ZR |
| Control | 173 ± 25 a | 157 ± 32 a |
| NaCl | 171 ± 17 a | 211 ± 10 a |
| Cd | 494 ± 19 d | 405 ± 14 b |
| Cd + NaCl | 437 ± 11 c | 398 ± 13 b |
| Zn | 189 ± 22 a | 209 ± 7 a |
| Zn + NaCl | 216 ± 18 a | 196 ± 15 a |
| Cd + Zn | 561 ± 32 e | 450 ± 18 cd |
| Cd + Zn + NaCl | 487 ± 26 d | 413 ± 21 c |

response to Cd + Zn was not simply an additive effect of separate toxicities.

We provide evidences that CK may assume positive functions in the response of *K. pentacarpos* to heavy metal in the absence of NaCl. Cadmium was reported to decrease endogenous CK in *Deschampsia cespitosa* (Hayward et al., 2013) or soybean (Hashem, 2014). In *K. pentacarpos*, Cd had no impact on CK concentration but Zn drastically reduced it. In Zn-treated plants, exogenous *t*-ZR had a positive impact on plant behavior which is corresponding to our hypothesis that stress-induced decrease in CKs may be, at least partly, responsible for Zn toxicity. In Zn-treated pants, *t*-ZR obviously increased the bioactive forms and reduced deactivation forms of *t*-ZR, thus supporting our hypothesis. Acing as an antisenescing hormone, CK may help the plant to cope with stress symptoms. Increase of endogenous CK concentration through the over-expression of *ipt* gene was reported to enhance Zn tolerance in tobacco (Pavlíková et al., 2014) while exogenous kinetin application attenuated the toxic effects of Zn in maize (Lukatkin et al., 2007). It is noteworthy that the positive impact of *t*-ZR was not related to a decrease in endogenous Cd or Zn, as clearly stated in Table 1. This might be related to the fact that CK increased stomatal conductance, hence transpiration stream, leading to a higher accumulation of toxic elements. It also implies that the beneficial effect of *t*-ZR should be related to an improvement of tolerance mechanisms to accumulated elements rather than to the avoidance resulting from a decrease in Cd or Zn accumulation. According to Rivero et al. (2009), the putative function of CK on stressed plants is not necessarily associated only with stomatal regulation. In our work, the impact of *t*-ZR on ion accumulation was not similar for all elements and *t*-ZR even decreased Na accumulation in salt-treated plants (Fig. 2A) despite an increase in the $g_s$ value (Fig. 5A). This suggests that foliar application of *t*-ZR may influence root properties in terms of mineral nutrition. In the present work, we applied exogenous CK in the form of *t*-ZR. Veselov et al. (2018) recently demonstrated that zeatin-riboside is a major form for shoot-to-root transport of zeatin-type cytokinins. It may thus not be excluded that *t*-ZR translocated to the root may act on ion transporters and thus influence mineral nutrition independently of the passive translocation by transpirational flux. Cytokinins and abscisic acid are commonly considered to have antagonist effects, especially in stress conditions (Zhou et al., 2016). ABA concentration moderately increased in plants exposed to Cd or Zn and *t*-ZR did not reduce it. A different picture arose from mixed treatment where a higher increase in ABA was recorded while *t*-ZR reduced ABA concentration. *Trans*-zeatin riboside increased chlorophyll concentration in all plants and is also clearly related to its well-known *anti*-senescing properties. According to Merewitz et al. (2011) and Singh and Prasad (2014), this may help the plant to maintain optimal net photosynthesis and Δ values independently of a direct impact on stomatal closure.

The fact that the positive effect of CK was, from a relative point of view, lower in salt-treated plants remains puzzling. A hypothesis may be that heavy metal reduced the endogenous CK concentration while NaCl increased it. If this is the case, then exogenous *t*-ZR may improve plant behavior in plants exposed to heavy metal in the absence of salt (where endogenous CK was low) but did not afford clear advantage in the presence of NaCl (where endogenous CK remained high enough to allow the plant to efficiently cope with ion toxicity). This might be the case in plants exposed to Zn alone or in combination with Cd (Cd + Zn) where endogenous CK was low in the absence of salt but remained high in the presence of NaCl. In contrast, this is probably not valid for plants exposed to Cd alone because this treatment did not decrease CK levels. Moreover, the fact that *t*-ZR increased Zn concentration in plants exposed to Zn excess while NaCl decreased it is an indirect proof that NaCl impact on plant response is not limited to its stimulation in CK endogenous content.

Heavy metals are known to induce premature senescence in plant tissues but we propose that in *K. pentacrapos*, senescence implies distinct targets in response to Cd on the one hand and to Zn on the other hand. Our previous study demonstrated that Cd-induced senescence in *K. pentacarpos* was mainly due to ethylene oversynthesis in relation to an increase in Put concentration and to a decrease in Spd and Spm (Zhou et al., 2018b): exogenous treatment with the inhibitor of ethylene synthesis aminovinylglycine (AVG) reduced Cd-induced senescence but had only a limited impact on plant response to Zn, suggesting that other key factors are involved in Zn-treated plants. The present works suggests a central role for CK depletion in Zn-induced senescence in *K. pentacarpos*.

Non-protein thiols, and especially phytochelatins, are involved in plant tolerance to heavy metal toxicity. In our study, PC mainly increased in response to Cd but not in response to Zn, confirming a higher affinity for binding and subsequent sequestration for the former element comparatively to the latter. As a consequence, Cd-induced increase in S content (Fig. 2D) may be regarded as an attempt to provide enough sulfur for thiol group synthesis. Although exogenous *t*-ZR had a positive effect on S content, it decreased PC concentration in the Cd-treated plants. Mohan et al. (2016) recently demonstrated that CK depletion is required to trigger the accumulation of phytochelatins and glutathione in the model plant species *Arabidopsis thaliana* exposed to As. In *K. pentacarpos*, CK content was not reduced by Cd, leading to a different situation but the fact that exogenous *t*-ZR may contribute to heavy metal tolerance despite a decrease in endogenous PC is in line with the view of Cassina et al. (2011) who suggested that CK does not trigger non-protein thiol accumulation. According to Hayward et al. (2013), ABA is acting as a stress signal inducing PC synthesis in *Deschampsia cespitosa* exposed to Cd while CK had an opposite effect. In *K. pentacarpos*, ABA accumulated to similar extend in Cd and in Zn-treated plants while PC accumulation occurred in the former case but not in the latter, suggesting the involvement of other parameters in NPT regulation in this species.

Glutathione has a dual function in response to heavy metals: it directly acts as an important antioxidant in plant tissues but also serves as a precursor of PC. In Cd-treated plants, glutathione accumulated in response to *t*-ZR or in response to NaCl but it apparently did not allow to reduce oxidative stress since *t*-ZR did not reduce MDA content in those plants. This contrasts with previous data obtained by Singh et al. (2018) on tomato and Singh and Prasad (2014) on *Solanum melongena*. It has been demonstrated that in glycophyte species, CK reduces oxidative stress in plants exposed to salinity in the absence of heavy metals (Albacete et al., 2009; Wu et al., 2012) but this is not the case in the halophyte *K. pentacarpos* since plants exposed to 50 mM NaCl did not exhibit any oxidative stress symptoms. Beside glutathione, ascorbate also plays a key role in the ascorbate-glutathione cycle and *t*-ZR increased the total pool of ascorbate in plants exposed to Zn alone or in combination with Cd in the absence of NaCl. This increase in the endogenous ascorbate pool occurred concomitantly with a decrease in the MDA content.

## 5. Conclusions

Taken together, the present work shows that toxic doses of Cd and Zn have different impacts on the plant behavior and that the simultaneous presence of the two elements induces a specific physiological constraint at the plant level, which is illustrated by a specific hormonal profiling characterized by high amounts of ABA and low levels of JA and SA. Cytokinin depletion appeared to be involved in Zn-induced premature senescence. Salinity helps the plant to cope with heavy metal toxicities through a decrease in toxic ion absorption. The plant hormone cytokinin assume key function in heavy-metal resistance tolerance, especially in Zn-treated plants where exogenous *t*-ZR increased both plant growth and heavy metal concentration, offering a promising perspective for phytoextraction processes. Efficiency of exogenous CK is however reduced by the presence of NaCl and this is only partly explained by a NaCl-induced increase in endogenous CK content.

Plant species suitable for phytostabilization must remain alive and able to grow in the presence of high concentrations of heavy metals. Most studies dealing with heavy metal pollution consider one single pollutant while we show here that mixed toxicity induces a specific physiological constraint on the plant, and this needs to be considered since most polluted sites in coastal areas are contaminated by several heavy metals present simultaneously (Bai et al., 2019). Identification of precise parameters linked to the ability of the plant to cope with heavy metals may be useful for selecting the most efficient plant material. We suggest here that CK endogenous concentration might be considered as a valuable criteria of tolerance to heavy metal stress and that exogenous application of CK-type compounds might be considered in the future as a strategy to improve plant tolerance to Zn contamination.

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