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Salinity influences the interactive effects of cadmium and zinc on ethylene and polyamine synthesis in the halophyte plant species *Kosteletzkya pentacarpos*

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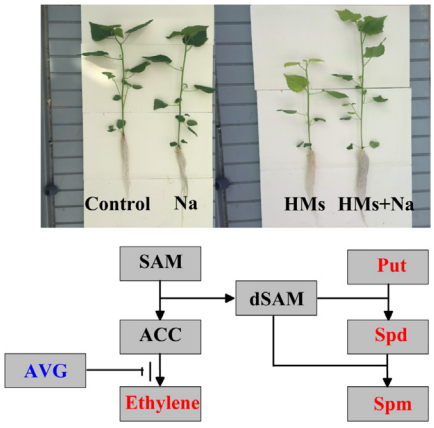
HIGHLIGHTS

- Mixed treatment (Cd + Zn) was more toxic than exposure to single heavy metals (Cd or Zn).
- Leaf senescence was induced by ethylene in response to Cd but not in response to Zn.
- Salinity reduced heavy metal accumulation and improved plant tolerance to Cd and Zn.
- Salinity reduced ethylene synthesis and increased polyamine concentrations.
- Toxicity of Cd + Zn was due to a specific physiological status and not to additive effects.

GRAPHICAL ABSTRACT



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ABSTRACT

Salt marshes are major sinks for heavy metals where plants are often exposed to polymetallic contamination and high salinity. Seedlings from the wetland halophyte plant species *Kosteletzkya pentacarpos* were exposed during three weeks to nutrient solution containing 10 μM $CdCl_2$, 100 μM $ZnCl_2$ or a combination of the two metals (Cd + Zn) in the presence or absence of 50 mM NaCl. Synthesis of the senescing hormone ethylene was quantified together with the concentration of protecting polyamines (spermidine and spermine) and their precursor putrescine and analyzed in relation to senescence markers (soluble protein, malondialdehyde, chlorophyll content and assessment of cell membrane stability). Salinity reduced the deleterious impact of heavy metals on plant growth and decreased accumulation of the pollutants in the plants. Heavy metals increased ethylene synthesis but NaCl decreased it in plants exposed to Cd or to the combined treatment (Cd + Zn) but not in plants exposed to Zn alone. Putrescine increased while spermine and spermidine decreased in Cd-treated plants. Zinc had only a marginal impact on polyamine concentration. The highest putrescine and spermine concentrations were observed in plants exposed to the combined treatment. The inhibitor of ethylene synthesis (AVG; aminovynilglycine) partially restored plant growth, reduced putrescine content and increased spermidine

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and spermine concentration, leading to an attenuation of senescence, mainly in Cd-treated plants. Combined treatment induced a specific physiological status in *K. pentacarpos* which could not be fully explained by an additive effect of Cd and Zn. Results are discussed in relation to specificities of heavy metals impacts on plant response.

1. Introduction

Heavy metals contamination is an important pollution compromising ecosystem stability in several areas in the world (Luo et al., 2017; Meena et al., 2018). Elements such as Cd, Pb, Ni, Cu, Zn, Hg and As induce major risks for human health through contamination of food chain and drinking water (Peralta-Videa et al., 2009; Chowdhury et al., 2016). The situation is especially serious in coastal areas with a high population density. Salt marshes constitute important sinks for the accumulation of heavy metals released by industrial activities (Bai et al., 2012; Mesa et al., 2016). Halophyte plant species have been recommended as an interesting material for phytoremediation purposes (Ruan et al., 2010; Lutts and Lefèvre, 2015). These plants indeed exhibit a fascinating capacity to cope with toxic ions in their environment and salinity has been demonstrated to afford specific advantages in terms of resistance to heavy metals in those species (Ghnaya et al., 2007; Manousaki et al., 2009; Wali et al., 2015, 2016).

Kosteletzkya pentacarpos (formely known as *Kosteletzkya virginica*) is a perennial wetland halophyte plant species from the Malvacea family. This species is a promising plant material for saline agriculture (He et al., 2003). Since several years, however, *K. pentacarpos* is also considered as an interesting plant for phytoremediation purposes. Although the plant is sensitive to copper in the absence of salt, NaCl improves the plant growth in the presence of this toxic element (Han et al., 2012a). A similar observation was reported for Cd and Zn (Han et al., 2012b). In these studies, the recorded salt-induced improvement of heavy metal resistance was related to both a decrease in heavy metal absorption and to an improvement of tolerance mechanisms allowing the plant to cope with the accumulated pollutants. Beside its interest for phytoextraction, *K. pentacarpos* is also a promising species for rhizofiltration approaches and salinity may improve the root properties in relation to Cd and Zn biosorption (Lutts et al., 2016).

The putative interest of a given plant species for phytoextraction of toxic ions depends on the maintenance of the growing processes allowing biomass production and on tolerance mechanisms allowing heavy metal accumulation in the shoot harvestable parts (Buscaroli, 2017). Both processes may be influenced by the hormonal status of the stressed plants. Indeed, plant survival and growth may be hampered by stress-induced senescence frequently occurring as a result of ethylene oversynthesis (Koyama, 2014). Han et al. (2013) demonstrated that Cd exposure increased the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC; the immediate precursor of ethylene) in *K. pentacarpos*, but ethylene synthesis itself was not measured. According to these authors, salinity contributed to delaying Cd-induced senescence in relation to a decrease in ACC synthesis and an increase in endogenous antioxidant.

Beside ethylene, polyamines (PAs) also influence senescing processes in plants. Polyamines are small aliphatic amines behaving as polycations at cellular pH. They assume a wide range of crucial functions in plant growth and development, from seed germination to flowering processes (Tiburcio et al., 2014; Vera-Sirera et al., 2010). They are also directly involved in plant responses to abiotic and biotic stresses (Fariduddin et al., 2013; Pál et al., 2015; Liu et al., 2015). Polyamines contribute to protein and DNA protection, stabilization of biological membranes and other cellular structures, as well as scavenging of reactive oxygen species (ROS) (Gupta et al., 2013, 2016; Tiburcio et al., 2014). Polyamines interact with cell wall components and lignification processes (Vera-Sirera et al., 2010; Bala et al., 2016). They also assume key functions in the regulation of mineral nutrition and ion homeostasis (Pottosin and Shabala, 2014; Schachtman, 2015). The diamine putrescine (Put) is produced either from ornithine or arginine. Subsequent addition of aminopropyl groups provided by decarboxylated S-adenosylmethionine (dSAM) induces the synthesis of the triamine spermidine (Spd) and the tetramine spermine (Spm). S-adenosylmethionine (SAM) is also the precursor of ACC and the polyamine and ethylene pathways may thus appear to be competitive. Polyamines (especially Spd and Spm) are thought to exhibit antisenescing properties (Pandey et al., 2000) but this specific point is still controversial (Sobiezczuk-Nowicka, 2017). To the best of our knowledge, these important compounds were never studied in *K. pentacarpos*.

Most studies dealing with the physiological impact of heavy metals in plants, including those which consider the influence of salinity, usually consider one single heavy metal. This may appear as an unrealistic oversimplification of field conditions since polluted sites are almost always contaminated by several heavy metals (Ren et al., 2018). This is especially the case in coastal areas of China which are now facing serious environmental problems in relation to polymetallic contaminations (Chen et al., 2018). Distinct heavy metals may have additive, synergistic or antagonist effects depending on the considered plant species and studied physiological properties (Hesami et al., 2018; Dotaniya et al., 2018). Cadmium and zinc are frequently simultaneously present in contaminated areas and share numerous chemical properties. However, they exhibit distinct levels of toxicity in plants. The plant responses to Cd and Zn are triggered by series of signal transduction pathways and involve a large set of physiological and molecular cues. Some of them are common for Cd and Zn response while others are specific to each pollutant (Lin and Aarts, 2012). The impacts of a mixed toxicity induced by the simultaneous presence of Cd and Zn on ethylene and polyamine synthesis in halophyte species, as well as the influence of salinity on these responses, are poorly documented.

The aim of the present work was to analyze the heavy-metal induced leaf senescence process in *Kozteltskya pentacarpos* exposed to individual heavy metals (Cd or Zn) or to combined treatment (Cd + Zn) in the presence or absence of NaCl. Ethylene synthesis and polyamines profile were quantified for plants exposed to treatment during three weeks in nutrient solution. An additional set of experiments was performed in the presence of an inhibitor of ethylene synthesis (aminovinylglycine (AVG), a potent inhibitor of ACC synthase) to obtain additional information on the role of ethylene on stress-induced senescence processes in *K. pentacarpos*.

2. Material and methods

2.1. Plant material and growing conditions

Seeds of *Kosteletzkya pentacarpos* were harvested from Jinhai Agricultural Experimental Farm of Yancheng (Jiangsu Province) and kindly provided by Prof. P. Qin, University of Nanjing (PR China). Germination was performed in trays filled with a perlite and vermiculite mix (1:3 *v/v*) and moistened regularly with a half − strength modified Hoagland nutrient solution. Seedlings were grown in a phytotron under a 12 h photoperiod [mean light intensity (PAR) = 150 μmol m^{-2} s^{-1} provided by Osram Sylvania (Danvers, MA) fluorescent tubes (F36W/133-T8/CW) with 25 °C/23 °C day/night temperature and 70%/50% atmospheric humidity]. Fifteen days after sowing, seedlings were fixed on polyvinylchloride plates floating on aerated half-strength modified Hoagland nutrient solution and transferred in 50L tanks into a greenhouse. The nutrient solution contained the following chemicals (in mM): 2.0 KNO_3, 1.7 $Ca(NO_3)_2$, 1.0 KH_2PO_4, 0.5 NH_4NO_3, 0.5 $MgSO_4$ and (in μM) 17.8 Na_2SO_4, 11.3 H_3BO_3, 1.6 $MnSO_4$, 1 $ZnSO_4$, 0.3 $CuSO_4$, 0.03 $(NH_4)_6Mo_7O_{24}$ 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 minimal light irradiance of 120 μmol m^{-2} s^{-1}(PAR) at the top of the canopy. After two weeks of growth in control conditions, plants were distributed in four groups 1) control 2) Cd 10 μM 3) Zn 100 μm and 4) Cd 10 μM + Zn 100 μM. Zinc and cadmium were added in the form of anhydrous chloride salts ($ZnCl_2$ and $CdCl_2$; purchased from Sigma Aldrich; Belgium). Within each group, half of the plants were exposed to 50 mM NaCl while the remaining half were maintained in the absence of stress. For all solutions, pH was set to 5.7 and readjusted every 4 d. Solubility of added heavy metals was confirmed by the Visual MINTEQ09 software. Treatments were maintained during three weeks on 24 plants per treatment (heavy metal x salt). Leaves were then harvested: the three leaves located in the middle part of the stem, and which were already present and still elongating at the time of stress exposure, were considered for subsequent analysis.

A second set of experiments was repeated in the same environmental condition, but some plants were at the same time treated with 10 μM aminovinylglycine (AVG; a potent inhibitor of ACC-synthase) as an inhibitor of ethylene synthesis which was added to the nutrient solution. This experiment has been independently repeated three times.

2.2. Ion quantification

Dried samples were ground to a fine powder using a porcelain mortar and a pestle, digested with a mix of 37% HCl and 68% HNO_3 (3:1) and the mixture was slightly evaporated to dryness on a sand bath at 80 °C. Minerals were dissolved in HCl 0.1 N. Ion concentrations were determined by SOLAAR S4 atomic absorption spectrometry (Thermo Scientific, Cambridge, UK). For each treatment, six plants per treatment were considered and each analysis was performed on technical triplicates.

2.3. Ethylene detection

The ethylene production was estimated through an online photo-acoustic ethylene detector ETD-300 (Sensor Sense) (Cristescu et al., 2002). Leaves harvested on six plants per treatment were placed in glasses box of 400 mL on filter paper Whatman n°1 moistened with nutrient solution. The box was covered with a Plexiglas plate with an inlet and outlet for gas flow, and tightly closed. The measurements were conducted in stop-and-flow mode with each cuvette being alternatively flushed during 20 min with a flow of 3 l h^{-1}. The flow from each cuvette was directed into a photoacoustic cell where ethylene was quantified. The obtained results were analyzed with the use of Valve Controller software and expressed as nanoliters per hour per g of leaf FW. Measurements were performed with three experimental repetitions.

2.4. Polyamine quantification

For polyamine (PAs) extraction, 200 mg of leaves were ground in liquid nitrogen in a mortar and were then homogenized with 500 μL of cold $HClO_4$ 4% (v/v) containing 5 mg.L^{-1} 1,7-diaminoheptane (internal standard) by vortexing vigorously. Samples were left to stand for 1 h at 4 °C. The homogenate was centrifuged at 13,000 g at 4 °C during 20 min. The pellet was re-extracted with 500 μL $HClO_4$ 4% (v/v) and re-centrifuged. The two supernatants were used for free polyamines determination. For fluorescence detection, PAs were derivatized by dansylation according to Lefèvre et al. (2001). After that, the dried extract was dissolved in 1 mL of methanol, filtered through 0.45 μm microfilters (Chromafil PES-45/15, Macherey-Nagel) and 5 μL of sample were injected on a Nucleodur C18 Pyramid column (125 × 4.6 mm internal diameter; 5 μm particle size) (Macherey-Nagel, Düren, Germany) maintained at 40 °C. HPLC-FLD (High performance liquid chromatography coupled with a fluorescence detector) analyses were performed on a Shimadzu HPLC system, equipped with a solvent delivery unit LC-20AT, a SIL-HTc autosampler and a RF-20A Fluorescence Detector (Shimadzu, 's-Hertogenbosch, The Netherlands) with the excitation wavelength at 340 nm and the emission wavelength at 510 nm. The flow of the mobile phase was 1.0 mL min^{-1}. The mobile phase consisted of water (eluent A) and acetonitrile (eluent B). The gradient program was as follows: 40% B to 91% B (20 min), 91% B to 100% B (2 min), 100% B (8 min), 100% B to 40% B (1 min) and column equilibration at 40% B during 4 min. Free PAs were quantified using six-points calibration curves with custom-made external standard solutions and internal standard (1,7-diaminoheptane), ranging from 3.125 to 100 μM and every ten injections, a check standard solution was used to confirm the calibration of the system. Internal standard gave information about the recovery of the extraction and derivatization during the evaluation of PAs content.

2.5. Senescence related parameters

Senescence-related parameters involve estimation of total soluble protein, chlorophyll and malondialdehyde (MDA) concentration and analysis of membrane permeability as detailed in Lutts et al. (1996).

Briefly, soluble proteins were extracted from 2.0 g of fresh mass in 10 mL of 0.1 M Tris-HCl (pH 7.5) after centrifugation at 40,000 g for 30 min at 4 °C. Proteins in the supernatant were determined according to Lowry et al. (1951). Chlorophyll was extracted from a median segment of the lamina which was weighed before grinding and cold extracted in acetone 80%. The absorbance of the extract was estimated at 645 and 663 nm and chlorophyll and carotenoid concentrations were calculated according to Lichtenthaler (1987). Malondialdehyde was estimated on the basis of thiobarbituric reaction according to Dhindsa and Matowe (1981).

Membrane permeability was estimated 1) using the electrolyte leakage (EL) method (Blum and Ebercon, 1981) and 2) using the leakage of UVA-absorbing substances and calculation of the relative leakage ratio (RLR) according to Redmann et al. (1986).

2.6. Statistical treatment of the data

All analysis were performed on at least 6 biological replicates. 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 data were transformed when required. ANOVA 2 were performed at a significance level of $P < 0.05$; $P < 0.01$ or $P < 0.001$ using SAS Enterprise Guide 6.1 (SAS 9.4 system for windows) considering the type of heavy metal exposure (Cd, Zn or Cd + Zn) and the salinity level as main factors. Post-hoc analyses were performed using Student-Newman-Keuls test at a 5% level. Results are presented as means ± standard errors.

3. Results

3.1. Plants cultivated in the absence of the inhibitor AVG

Salinity had no deleterious impact on plant height (Table 1) in the absence of heavy metals. Cadmium (10 μM) and Zn (100 μM) applied separately reduced plant height. Salinity partly reduced the deleterious impact of Cd on the stem elongation. The combined treatment (Cd + Zn) strongly reduced plant height and was more detrimental than heavy metals applied separately. Salinity also significantly reduced the deleterious impact of the combined treatment on the main stem length.

Cadmium accumulated in the leaves in response to Cd applied alone and, to a lower extent, in response to the combined treatment (Cd + Zn) (Fig. 1A). Salinity significantly reduced Cd accumulation in both cases. Zinc accumulated to similar values in Zn-treated plants and in plants exposed to combined treatments (Fig. 1B). Salinity reduced Zn accumulation but the recorded decrease was higher for plants exposed to a combined stress (Cd + Zn). Sodium accumulation was important in plants exposed to NaCl, while in the absence of salt, the recorded leaf Na content remained rather low. Cadmium drastically reduced Na accumulation in the leaves while Zn increased it (Fig. 1C). Under combined treatment (Cd + Zn), Na accumulated in salt-treated plants to a similar extent than in plants exposed to NaCl in the presence of Cd alone.

Salinity slightly (but significantly) increased ethylene synthesis in the absence of heavy metals (Fig. 2). Cadmium induced a burst in ethylene synthesis which was partly reduced by a simultaneous exposure to salinity. Zinc treatment also increased ethylene synthesis but to a lower extent than Cd and salinity had no impact on ethylene synthesis by Zn-treated plants. The highest ethylene synthesis was recorded in plants exposed to the combined heavy metal stress (Cd + Zn). In this case, salinity strongly reduced the ethylene synthesis which was even lower than in the Cd-treated plants.

Table 1
Height (in cm) of plants of *Kosteletzkya pentacarpos* cultivated during three weeks on a nutrient solution containing 10 μM Cd, 100 μM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the mean of 6 replicates ± S.E. Values followed by the same letter are not significantly different according to the SNK test ($P < 0.05$).

Treatment	Plant height (cm)
Control	52.37 ± 2.31 a
NaCl	55.18 ± 1.15 a
Cd	33.04 ± 2.54 c
Cd + NaCl	41.66 ± 3.8 b
Zn	38.07 ± 1.26 b
Zn + NaCl	44.69 ± 2.38 b
Cd + Zn	23.18 ± 1.75 d
Cd + Zn + NaCl	37.08 ± 2.05 bc

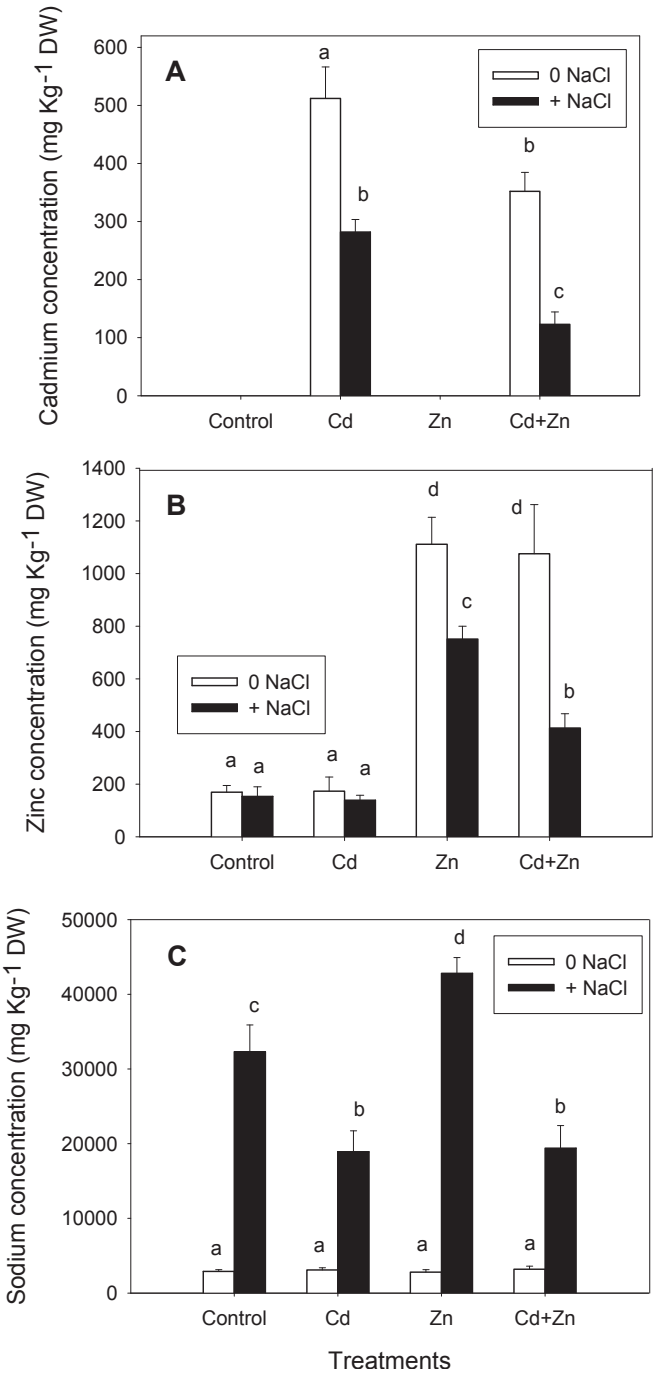

Fig. 1. Total concentration (mg kg^{-1} DW) of Cd (A), Zn (B) and Na (C) in the leaves of *Kosteletzkya pentacarpos* exposed during three weeks in nutrient solution containing 10 μM Cd, 100 μM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the mean of 6 replicates ± S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

In the absence of heavy metals, salinity slightly decreased Put concentration in the leaves (Fig. 3A). Cadmium increased Put concentration in the leaves while NaCl limited such increase. Zinc did not increase Put concentration in the leaves but addition of NaCl to Zn-treated plants slightly increased it. The maximal Put concentration in the leaves was recorded for plants exposed to the combined treatment (Cd + Zn) but in this case, NaCl was unable to reduce Put accumulation. Spermidine remained similar in control and in NaCl-treated plants (Fig. 3B). Spermidine concentration

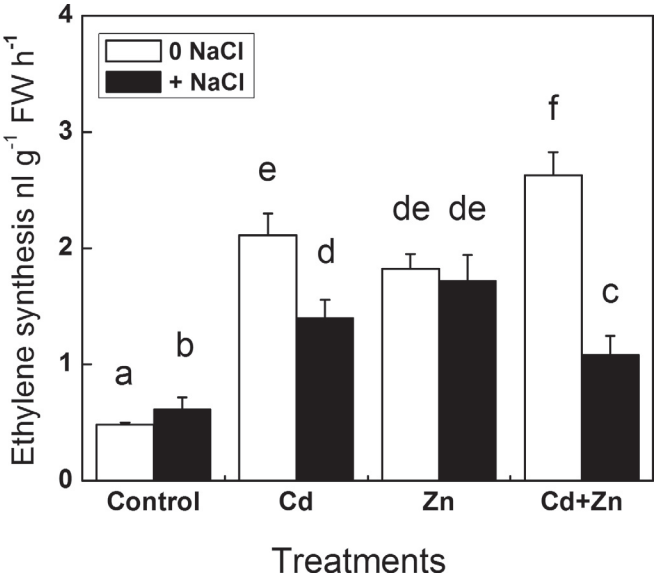

Fig. 2. Ethylene synthesis (nl g^{-1} FW h^{-1}) in detached leaf of *Kosteletzkya pentacarpos* exposed during three weeks in nutrient solution containing 10 μM Cd, 100 μM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the mean of 6 replicates ± S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

decreased in Cd-treated plants and additional NaCl restored level of Spd similar to the control. Zinc did not significantly increase Spd concentration and NaCl had no impact on Spd accumulation in Zn-treated plants. Very low amounts of Spd was recorded for plants exposed to the combined treatment but NaCl mitigated the Cd + Zn-induced decrease in leaf Spd. In the absence of heavy metals, spermine accumulation was increased by NaCl (Fig. 3C). Cadmium strongly decreased Spm and NaCl counteracted such a decrease by more than 50%. Zinc had no impact on Spm concentration but salinity increased it on Zn-treated plants. In the absence of salt, the highest Spm concentration was found in plants exposed to the combined treatment. For these plants, salinity reduced the Spm concentration.

In the absence of NaCl, Cd and combined treatment reduced soluble protein and chlorophyll content and increased the MDA concentration (Table 2). Zinc had a lower deleterious impact than Cd on these senescence-related parameters: it had no effect on the total soluble protein content, and it only slightly increased leaf MDA. Zinc had no impact on Chl a concentration but decreased Chl b. Salinity only slightly reduced the impact of Cd and Cd + Zn stress on protein concentration but significantly reduced MDA in heavy-metal treatments.

The cell membrane stability estimated through electrolyte leakage (EL) and relative leakage ratio procedure is presented in Fig. 4. The two procedures provided contrasting data. Salinity increased electrolyte leakage in all treatments except the combined one (Fig. 4A). As far as plants cultivated in the absence of salt are concerned, the electrolyte leakage was increased by Cd but not by Zn. The highest electrolyte leakage value was observed for plants exposed to combined treatment (Cd + Zn). Values recorded for RLR estimation are provided in Fig. 4B. RLR values, in contrast to EL, were poorly affected by the salt treatment in plants that were not exposed to heavy metals. In the absence of salt, Cd and Zn applied separately increased the EL values, and to a higher extent in the former than in the latter. Salinity slightly reduced the RLR values in Cd-treated plants. Combined treatment also induced an increase in RLR values but in this case, NaCl was not significantly able to reduce it.

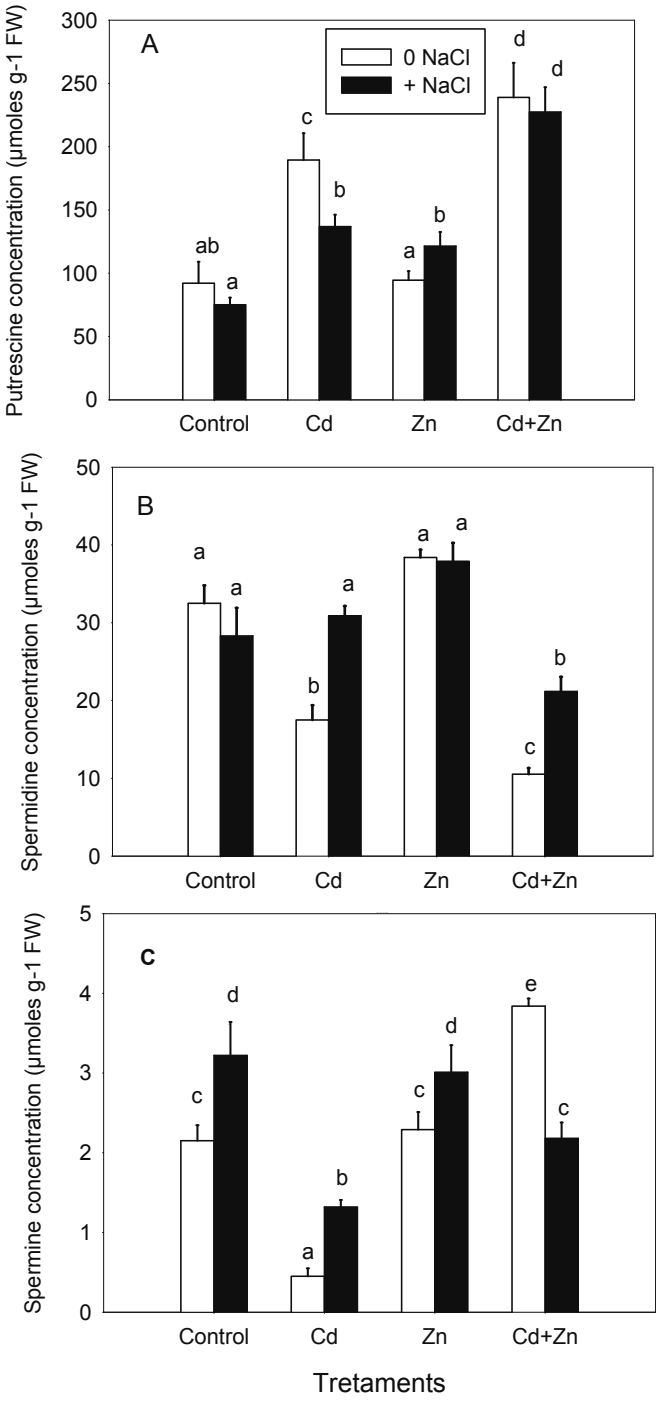

Fig. 3. Putrescine (A), spermidine (B) and spermine (C) concentration (μmol g^{-1} FW) in the leaves of *Kosteletzkya pentacarpos* exposed during three weeks in nutrient solution containing 10 μM Cd, 100 μM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the mean of 6 replicates ± S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

3.2. Behavior of treated plants concomitantly exposed to inhibitor of ethylene synthesis

Addition of AVG to the nutrient solutions strongly reduced ethylene synthesis in all treatments (Fig. 5 versus Fig. 2). For each treatment, the impacts of the inhibitor on physiological and biochemical parameters involved in the plant response to salt and

Table 2
Total soluble proteins, malondialdehyde (MDA) and chlorophyll concentration on median leaves harvested on plants of *Kozteletskya pentacarpos* cultivated during three weeks on a nutrient solution containing 10 μM Cd, 100 μM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the mean of 6 replicates ± S.E. Values followed by the same letter are not significantly different according to the SNK test ($P < 0.05$).

Treatments	Protein	MDA	Chl a	Chl b
	mg g^{-1} FW	nmol g^{-1} FW	mg g^{-1} FW	mg g^{-1} FW
Control	33.8 ± 1.2a	5.02 ± 0.31a	10.91 ± 1.07a	2.33 ± 0.11a
NaCl	31.9 ± 1.7a	6.13 ± 1.19a	10.87 ± 0.85a	2.40 ± 0.08a
Cd	13.9 ± 0.7c	31.7 ± 1.90d	7.61 ± 0.75d	1.48 ± 0.21c
Cd + NaCl	17.3 ± 3.0bc	23.3 ± 1.54c	8.32 ± 0.54c	1.79 ± 0.12b
Zn	34.1 ± 2.1a	12.9 ± 0.89b	10.82 ± 0.95a	1.54 ± 0.15c
Zn + NaCl	32.3 ± 1.7a	19.1 ± 1.13c	10.51 ± 0.66 ab	1.84 ± 0.08b
Cd + Zn	23.5 ± 1.1b	28.5 ± 1.71d	8.07 ± 0.33cd	1.94 ± 0.09b
Cd + Zn + NaCl	26.5 ± 0.5b	10.5 ± 0.90b	9.11 ± 0.47b	2.12 ± 0.21 ab

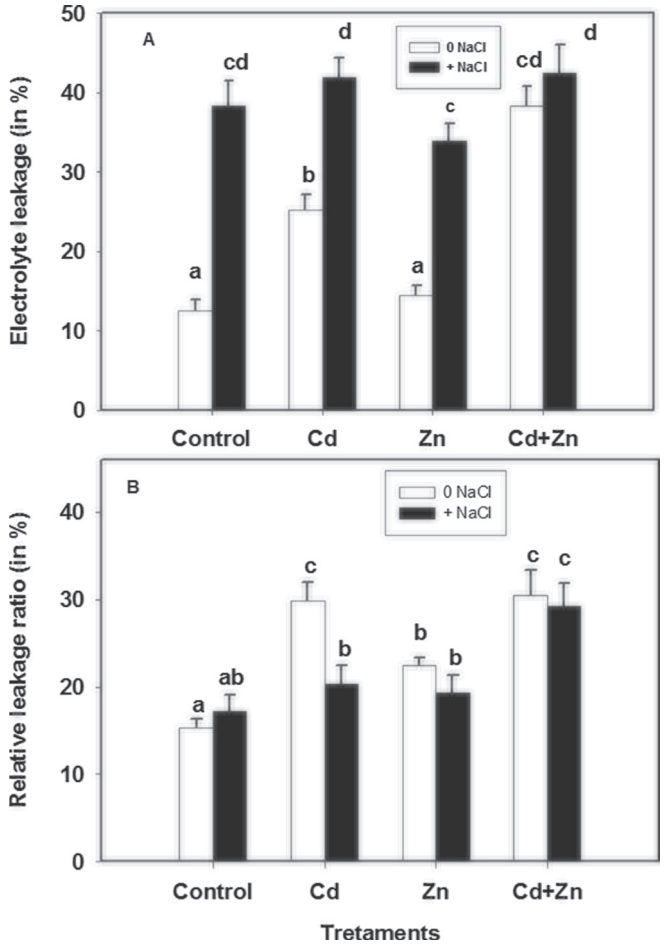

Fig. 4. Electrolyte leakage (in %, A) and relative leakage ratio (in %, B) in leaf segment of *Kosteletzkya pentacarpos* exposed during three weeks to nutrient solution containing 10 μM Cd, 100 μM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the mean of 6 replicates ± S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

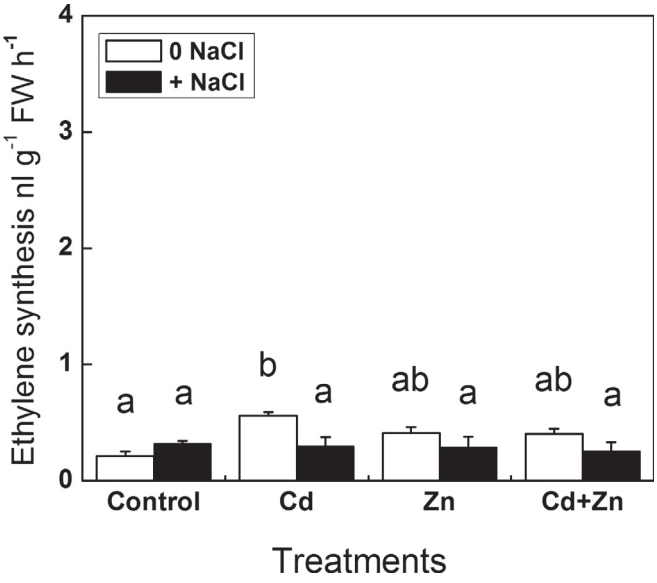

Fig. 5. Ethylene synthesis (nL g^{-1} FW h^{-1}) in detached leaves of AVG-treated plants of *Kosteletzkya pentacarpos* exposed during three weeks in nutrient solution containing 10 μM Cd, 100 μM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. All plants were cultivated in the presence of 10 μM AVG. Each value is the mean of 6 replicates ± S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

heavy metals are listed in Table 3 and presented as relative percentage of the same parameter recorded on plants maintained under similar conditions but cultivated in the absence of AVG. Partial inhibition of ethylene synthesis had no impact on control plants or plant exposed to NaCl alone. It only decreased the MDA produced by salt-treated plants comparatively to plants that were cultivated in the absence of the inhibitor. In contrast, AVG treatment had an obvious positive effect on plants exposed to Cd since it improved plant elongation and all-senescence related parameters, as indicated by significant increases in protein and chlorophyll concentrations and decreases in MDA, EL and RLR values. A similar trend was observed for plants simultaneously exposed to Cd + NaCl. It has however to be mentioned that inhibition of ethylene had no impact on Cd accumulation by these plants but clearly improved membrane stability and reduced MDA concentration in leaves. Ethylene inhibition decreased Put concentration and increased Spd and Spm concentrations in Cd-treated plants. From a relative point of view, the positive impact of AVG was more marked for plants exposed to Cd than for plants exposed to Cd + NaCl.

The impact of AVG on Zn-treated plants was clearly different. Even if ethylene inhibition decreased the zinc concentration in the leaves, it did not improve the senescence-related parameters, and even increased the MDA concentration in the leaves of Zn-treated plants. AVG applied on Zn-treated plants did not modify their polyamines profile.

AVG was especially efficient for plants exposed to the combined treatment Cd + Zn: indeed it significantly improved elongation and reduced Cd accumulation. It also increased the protein content and reduced MDA concentration and RLR values. The recorded changes, once again, were more marked for plants exposed to Cd + Zn in the absence of NaCl than in the presence of salt. The recorded modifications were associated with an AVG-induced modification of polyamines profiling characterized by a decrease in Put concentration and an important increase in both Spd and Spm content.

4. Discussion

The present work confirms that Cd and Zn had a negative impact on *K. pentacarpos* and that the combined treatment of the two heavy metals was the most toxic treatment in terms of plant growth. In all cases, NaCl afforded partial protection which could be related to a NaCl-induced decrease in heavy metal absorption. The

Table 3
Impact of 10 μM AVG (aminovinylglycine) on physiological and biochemical parameters involved in the response of *Kozteltskya pantacarpos* exposed to 10 μM Cd, 100 μM Zn or combined treatment (Cd + Zn) in the absence or presence of 50 mM NaCl. Each parameter is expressed as a relative value (in percentage) of the same parameters recorded for plants exposed to similar treatments but in the absence of AVG. * or ** indicate that significant differences were recorded for a given parameter between plants treated or not with AVG at $P < 0.05$ or $P < 0.01$, respectively considering three independent experiments (PH: Plant height; ND: not detected).

Treatments	PH	Cd	Zn	Na	Put	Spd	Spm	Prot	MDA	Chl a	Chl b	EL	RLR
Control	81.7	ND	104.1	90.3	80.6	105.7	110.3	111.5	97.6	114.7	87.9	105.7	89.1
NaCl	85.9	ND	99.6	101.5	87.9	98.3	103.7	102.3	90.4*	98.7	89.6	97.6	91.9
Cd	133.2**	106.6	90.5	124.6*	67.8**	144.6**	165.2**	125.9**	78.5*	121.7**	134.2**	81.2*	77.4**
Cd + NaCl	119.4*	93.4	108.5	103.2	74.5**	118.7*	154.8**	116.7*	80.6*	132.1**	125.6**	99.8	79.8**
Zn	119.3*	ND	78.9*	119.7*	87.4	108.8	99.9	97.5	120.1*	102.3	97.3	101.0	90.0
Zn + NaCl	108.5	ND	84.2*	111.4	83.3*	104.4	106.1	94.2	107.6	97.8	89.7	104.9	91.8
Cd + Zn	145.6**	65.7**	87.8	95.4	54.6**	134.5**	156.7**	132.9**	76.4*	100.5	97.6	85.4*	68.8**
Cd + Zn + NaCl	123.2*	70.3**	93.2	103.1	61.8**	143.8**	132.6**	111.4*	84.5*	109.7*	88.9	97.6	71.3**

positive impact of NaCl on heavy metal resistance in halophyte plant species was previously reported (Ghnaya et al., 2007; Manousaki et al., 2009; Lutts and Lefèvre, 2015; Ghabriche et al., 2017). Wali et al. (2015) even demonstrated that in *Sesuvium portulacastrum*, NaCl changes the chemical form of accumulated Cd by increasing the proportion of Cd bound to pectates and chlorides. In some cases, the positive impact of NaCl on halophyte species may be due to a salt-induced growth stimulation leading to a diluting effect of accumulated heavy metals (Lutts and Lefèvre, 2015). Zhou et al. (2018) recently demonstrated that *K. pentacarpos* exposed to a mixed Cd + Zn toxicity increased its endogenous concentration of phytochelatins and that NaCl increased the efficiency of Cd sequestration by these protecting peptides.

Conversely, heavy metals also had an impact on Na accumulation since Cd decreased leaf Na concentration while Zn increased it, thus confirming the study of Han et al. (2012b). Under combined treatment, Na accumulation by salt-treated plants was similar to the values observed in the presence of Cd, suggesting that the Cd effect was prevailing. Wali et al. (2017) recently reported that for some halophyte plants species, Cd may reduce salinity tolerance but this was not observed in our case, mainly because the NaCl dose used in the present work was very low (50 mM).

In the present study, and for the considered dose of heavy metals, Cd was more toxic than Zn. Cadmium impact may have occurred through a senescence process triggered by ethylene oversynthesis. Indeed, ethylene synthesis clearly increased in response to Cd exposure (Fig. 2). The presence of NaCl, which improved plant growth under Cd stress, decreased ethylene synthesis. The addition of AVG inhibited ethylene synthesis and concomitantly improved plant growth in Cd-treated plants. It is noteworthy that the beneficial impact of AVG on Cd-treated plants was not associated to significant reduction of Cd accumulation, suggesting that AVG improved tissue tolerance to accumulated toxic compounds. Ethylene is well documented as a stress-induced hormone acting as a senescing compound (Pandey et al., 2000; Koyama, 2014). Cadmium had a drastic effect on senescence-related parameters and induced a decrease in protein and chlorophyll content together with an increase in MDA and in membrane permeability. For this last parameter, data provided by the classical electrolyte leakage or by relative leakage ratio of UV-absorbing substances differed, especially for NaCl-treated plants. Salinity indeed always strongly increased EL values and it might be suggested that Na partly accumulated in the apoplasm and leaked during leaf segments incubation, thus contributing to the recorded electrical conductivity, although it did not cross any biological membrane. This hypothesis is supported by the fact that EL values increased in Cd + NaCl treated plants comparatively to Cd alone while MDA issued from membrane lipid peroxidation decreased, which at first sight appears somewhat contradictory. Mucilage, which is present in all organs of *K. pentacarpos*, may also adsorb cations. It has been reported that mucilage present around the stomata and in intercellular spaces at the leaf level increased in reponse to NaCl (Ghanem et al., 2010). Mucilage may weakly retain Na (Lutts et al., 2016) and a desorption process occurring during the incubation of the leaf segment prior to measurement of electrical conductivity could thus not be ruled out. Once again, the inhibition of ethylene synthesis by AVG in Cd-treated plants attenuated the senescence-related parameters, confirming that ethylene is a major factor responsible for Cd injuries in *K. pentacarpos*. Since NaCl reduced both Cd accumulation and ethylene synthesis in Cd-treated plants, it might be argued that Cd itself may trigger ethylene synthesis in this wetland species.

Although Zn also increased ethylene synthesis, the situation appeared quite different than for Cd. Salinity indeed only marginally improved the growth of Zn-treated plants (Table 1) and it did not reduce ethylene synthesis by these plants. Nevertheless, salinity did clearly reduced Zn accumulation and this observation suggests that, in contrast to Cd, Zn was not able to directly trigger ethylene synthesis. Moreover, inhibiting ethylene synthesis by AVG did not mitigate the senescence-related processes and this also suggests that ethylene was not the major responsible of senescence in Zn-treated plants.

Differences between Cd and Zn impacts on *K. pentacarpos* were also obvious in relation to the modification of PAs concentration. Although some studies reported that Zn induce modification in polyamine accumulation in several species (Franchin et al., 2007; Castiglione et al., 2009; Rouphael et al., 2016), our study provided experimental evidences that this was not the case for *K. pentacarpos* exposed to 100 μM Zn. Indeed, in the absence of NaCl, PAs titers were similar in control and in Zn-treated plants. In contrast, Cd clearly impacted PAs concentrations: it increased Put content but clearly decreased Spd and Spm. A Cd-induced increase in Put concentration was already reported in *Malus hupehensis* (Jiang et al., 2012), *Potamogeton crispus* (Yang et al., 2010) and *Atriplex halimus* (Lefèvre et al., 2009). Franchin et al. (2007) reported that, for a given plant species, PAs modification is heavy metal-dependent. It has often been suggested that Put may have negative effects on plants since it causes membrane depolarization, increases solute leake and eventually lead to apoptotic cell death (Yang et al., 2010; Jiang et al., 2012; Tiburcio et al., 2014; Pál et al., 2015). In our study, Spd and Spm decreased concomitantly with Put accumulation. In contrast to Put, Spd and Spm assume protective functions in relation to reactive oxygen species scavenging and protection of numerous cellular structures and compounds (Fariduddin et al., 2013; Minocha et al., 2014; Tiburcio et al., 2014; Liu et al., 2015). We hypothesize that Put accumulation occurring together with Spd and Spm decrease resulted from ethylene oversynthesis in Cd-treated plants since S-adenosylmethionine is a

precursor for both ethylene on the one hand and for Spd and Spm synthesis produced from Put on the other hands. The fact that the inhibitor of ethylene synthesis AVG applied to Cd-treated plants markedly increased Spm and Spd concentration while decreasing Put content supports such hypothesis. NaCl, which also decreased ethylene synthesis, had similar effect on PAs concentration in Cd-treated plants.

Endogenous PAs concentrations result from a balance beween synthesis, conjugation and catabolism (Gupta et al., 2013, 2016; Sobiezczuk-Nowicka, 2017). Diamine oxidase catalyses the oxidation of Put at the primary aminogroup and was shown to be strongly increased by Cd in *Potamogeton crispus* (Yang et al., 2010). Similarly, Jiang et al. (2012) reported that H_2O_2 produced by polyamine oxidase is partly responsible for cell death in roots of *Malus hupehensis* exposed to Cd stress. Only the free soluble fraction was considered in the present work. Polyamine conjugation is however an important process for cell tolerance to ionic toxicity and heavy metals were reported to have an impact on polyamine conjugation to phenolic acid or binding to macromolecules. In the halophyte *Inula crithmoides*, Cd was reported not only to increase the free PAs, but also the conjugated and bound fractions as well (Ghabriche et al., 2017).

Several observations suggest that the combined treatment induced a specific physiological status in *K. pentacarpos* which could not be fully explained neither by an additive effect of Cd and Zn, nor by the sole effect of the most detrimental Cd. First, Cd accumulation was reduced in Cd + Zn-treated plants comparatively to Cd-treated ones and this could be partly explained by the fact that Cd enters in the plant through non-selective transporters devoted to the absorption of Zn which is an essential element for all living organisms (Lin and Aarts, 2012; Hesami et al., 2018). Hence, competition occurring in the presence of Zn excess might explain the recorded decrease in Cd accumulation. The second puzzling observation was that NaCl, which strongly reduced ethylene synthesis in those plants, did not reduce Put concentration which is not in agreement with our hypothesis that ethylene decrease should favor Put conversion to Spd and Spm as a result of an increased availability of the common precursor SAM. An alternative hypothesis could be that the combined treatment acted upstream of Spd synthesis and increased Put synthesis from ornithine and/or arginine. In poplar, Franchin et al. (2007) indeed reported that transcripts corresponding to ornithine decarboxylase and arginine decarboxylase accumulated in a heavy-metal specific manner and a similar effect might have occurred in *K. pentacarpos* exposed to the combined treatment. A third observation was that the combined treatment (Cd + Zn) increased Spm concentration in the absence of salt and that salinity decreased Spm while NaCl had an opposite effect on plants exposed to Cd alone or to Zn alone. Finally, the last unexpected observation was that AVG significantly reduced Cd accumulation when plants were exposed to the combined treatment while it had absolutely no impact when plants were exposed to Cd in the absence of Zn excess. This observation suggests that ethylene may somewhat influence Cd absorption in the presence of Zn excess, which, to the best of our knowledge, was never previously reported. In another halophyte plant species (*Solanum chilense*) ethylene increased stomatal conductance and AVG decreased transpiration as a result of partial stomatal closure (Gharbi et al., 2017). Stomatal conductance was not measured in the present study but i) there is no reason to claim that ethylene interacts with stomata only in the case of combined treatment and ii) if Cd decrease was due to reduction of transpiration stream, a similar reduction should have been observed for Zn and Na accumulation, which was not the case (Table 3). At this stage, we do not have explanation for this effect, although it has been consistently observed during 3 independent experiments.

5. Conclusions

Cadmium and zinc have contrasting impacts on the halophyte plant species *Kozteletskya pentacarpos*. Cadmium hastened senescence processes in relation to ethylene oversynthesis. It increased membrane permeability and oxidative stress while it decreased protein, chlorophyll, Spd and Spm concentrations. Salinity decreased ethylene synthesis and attenuated senescence symptoms. In contrast, salinity did not reduce ethylene synthesis in Zn-treated plants and ethylene was not the major factor inducing senescence in these plants. Plants exposed to combined treatments (Cd + Zn) exhibited a specific physiological status and their response to pollutants could not be explained by additive effects of cadmium and zinc. Inhibition of ethylene synthesis increased Spd and Spm concentration and reduced Cd accumulation in those plants, suggesting that this plant hormone may influence Cd absorption in the presence of Zn excess.

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