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Interaction between Heavy Metal Pollutants (Cd and Zn) is Influenced by the Presence of Salinity in the Halophyte Plant Species *Kosteletzkya pentacarpos*

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Nothing is difficult to a willing heart.

世上无难事，只怕有心人。

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Abbreviations

A	Net photosynthesis
ABA	Abscisic acid
ABTS	2,2 -azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
ACC	1-aminocyclopropane-1-carboxylic acid
ADF	Acid detergent fiber residue
ADL	Acid detergent lignin
AOAD	antioxidant activity measured in dichloromethane extract
AOAM	Antioxidant activity measured in methanol extract
Ara	Arabinose
AsA	Ascorbate
AVG	Aminovinylglycine
BADH	Betaine aldehyde dehydrogenase
BF	Bioaccumulation factor
BSA	Bovine serum albumin
CAT	Catalase
CAX	Cation exchange
CDF	Cation diffusion facilitator
Chl	Chlorophyll
CK	Cytokinin
CMO	Choline monooxygenase
CTR	Copper transporter
d	Berger-Parker dominance index
D	Simpson diversity index
DHA	Dehydroascorbate
DNPH	Dinitrophenylhydrazine
dSAM	Decarboxylated S-adenosylmethionine
DTT	Dithiothreitol
DW	Dry weight
E	Instantaneous transpiration rate
EDDS	Ethylenediamine-N, N'-disuccinic acid
EL	Electrolyte leakage
F'_m	Maximum fluorescence in the light

F'_v	Variable fluorescence in the light
F_0	Steady-state yield of fluorescence in the absence of a photosynthetic light
F_m	Maximum fluorescence in the absence of a photosynthetic light
FRAP	Ferric reducing ability of plasma
F_s	Steady-state value of fluorescence
Fuc	Fucose
F_v	Variable fluorescence in the absence of a photosynthetic light
F_v/F_m	Maximum quantum yield of PSII
FW	Fresh weight
Gal	Galactose
GB	Glycinebetaine
GDP	Gross domestic production
Glc	Glucose
g_s	Stomatal conductance
GSA	Glutamic γ-semialdehyde
GSH	Reduced glutathione
GSHt	Total glutathione
GSSG	Oxidized glutathione
H	Shannon's diversity index
HACC	Hyperpolarization activated Ca channels
HMs	Heavy metals
HMW	High molecular weight
HPLC	High liquid pressure chromatography
IAA	Indole acetic acid
J	Pielou's evenness index
JA	Jasmonic acid
LBs	Number of lateral branches
LLB	Leaves on the later branches
LMS	Leaves on the main stem
LMW	Low molecular weight
LN	Number of leaves
Ma	Margalef richness index
Man	Mannose

MDA	Malondialdehydes
MRG	Metal-rich granule
MS	Main stem
MTs	Metallothioneins
NDF	Neutral detergent fiber residue
NF	Number of flowers
NFR	Number of fruits
NPQ	Non-photochemical quenching
NPT	Non-protein thiol
NRAMP	Natural resistance associated macrophage protein
NSCC	Non selective cation channel
OAT	Ornithine-δ-aminotransferase
OM	Organic matter
OPA	Ortho-phthaladelyde
P5CR	Pyrroline-5-carboxylate reductase
P5CS	Δ^1-pyrroline-5-carboxylate synthetase
PAs	Polyamines
PCs	phytochelatins
PDH	Proline dehydrogenase
ProT	Proline transporter
Put	Putrescine
PVP	Polyvinylpyrrolidone
QAC	Quaternary ammonium compounds
qP	Photochemical quenching coefficient
Rha	Rhamnose
RLR	Relative leakage radio
ROS	Reactive oxygen species
SA	Salicylic acid
SAM	S-adenosylmethionine
SNPK	No-polluted soil + plant
SNPKA	No-polluted soil + plant + NaCl
SNPKE	No-polluted soil + plant + EDDS
SNPKEA	No-polluted soil + plant + NaCl + EDDS
SOD	Superoxide dismutase
Spd	Spermidine
SPK	Polluted soil + plant

SPKA	Polluted soil + plant + NaCl
SPKE	Polluted soil + plant + EDDS
SPKEA	Polluted soil + plant + NaCl + EDDS
Spm	Spermine
TBA	Thiobarbituric acid
TEA	Tetraethylammonium
TF	Translocation factor
t-ZR	*tran*-zeatin riboside
VST	Van Soest method
WC	Water content
Xyl	Xylose
ZIP	ZRT, IRT related Protein
$\Delta^{13}C$	Carbon isotope discrimination
Φ_{PSII}	Quantum yield of PSII

General Introduction and Scientific Question

Numerous areas in the world are contaminated with high amounts of heavy metals resulting from industrial and mining activities. These pollutants represent a risk for human health and ecosystem stability. Agricultural soils may also be contaminated as a result of atmospheric fall out of contaminated dusts produced by surrounding industrial places, or through the use of low-quality fertilizers and low-quality water for irrigation purposes.

Coastal areas are frequently acting as sink for numerous pollutants: anthropogenic sources of heavy metals derived from mining, smelting, petrochemical, electronic industry and municipal waste are ultimately discharge into the marine environments where they can be bioaccumulated by marine organisms and biomagnified through the food chain. The situation is especially challenging for China where numerous coastal areas are seriously polluted as a consequence of rapid industrialization and urbanization. In almost all cases, these sites are characterized by the simultaneous presence of several heavy metals and, even organic pollutants, leading to a very complex pollution constraint on living organisms.

Since two decades, a large number of studies are reporting the impact of heavy metal toxicity on plants, not only because plant are essential components of ecosystems, but also because they can be used to stabilize polluted environments or even reduce the level of contamination. Indeed, besides a wide range of sophisticated technics well adapted to remove heavy metals from contaminated substrates, phytoremediation consisting in the use of plants to stabilize or extract pollutants, receive a considerable interest from the scientific community. Phytoremediation is by far cheaper than other *in situ*- or *ex-situ* approaches and it is thus applicable to large contaminated areas while other strategies are limited to very small surfaces. In October 2019, more than 13234 available articles referenced in the Scopus

databank comprises « phytoremediation » in the title, the abstract and/or keywords.

However, there is still a « gap » between fundamental research performed in the laboratory, and real field situations. In order to improve our knowledge on plant behavior, scientists are frequently working under fully controlled environmental conditions, on artificially-contaminated substrate or nutrient solution, and consider one single pollutant. This last point is undoubtedly the most limiting factor in terms of knowledge transfer from the lab to the field because, as stated above, more than 95% of polluted areas present an excess of more than one pollutant. The presence of several heavy metals in the soil is adding an additional level of complexity on the plant behavior since different heavy metals may interact at both the soil level and the plant level. At the soil level, bioavailability of a given element may be directly influenced by the presence of other elements. At the plant level, heavy metal absorption is also influenced by the presence of other elements by non-specific transporters. The physiological consequences of accumulated may be categorized in the five following classes:

1) Mineral nutrition, implying the absorption, translocation and accumulation/distribution of elements. In the case of heavy metals, element speciation is an important aspect of mineral nutrition
2) Plant water status: impact of heavy metals on hydric, osmotic and turgor potentials, transpiration, water use efficiency, osmotic adjustment, stomatal regulation
3) Photosynthesis, implying to consider gas exchange (net photosynthesis), stability of the photosynthetic machinery which could be determined by chlorophyll fluorescence analysis, pigment concentrations, Calvin cycle activity….
4) Plant hormonal status: phytohormone are controlling all aspects of plant growth and development. In response to abiotic constraints, and especially in response to mineral toxicities, plants are encountering a precocious senescing process hampering plant growth and survival: it is clear that the

phytohormonal status is one of the main determinants of this process

5) Oxidative stress is also produced in response to abiotic stress and management of reactive oxygen compounds is an important determinant of plant resistance.

It is clear that these classes of injuries should not be regarded as independent, but they are influencing each other in a precise dynamic scheme depending on the nature and the intensity of stress. The ultimate consequence of this complex interaction is the modification of plant growth and development and, in the most extreme situation, plant death. It may also be hypothesized that the consequence of interactions is even not necessarily the same at all developmental stage.

From a conceptual point of view, two heavy metals simultaneously present in the environment may act according to a synergistic, antagonist or additive mode. An additive mode implies that two separate pollutants are acting on the same physiological parameters independently. A synergistic mode implies that the alteration of a given parameter is, from a quantitative point of view, higher than the sum of modification individually induced by each heavy metal. An antagonist impact might be explained if the considered heavy metals inhibit the absorption of each other. Moreover, considering the mode of interaction may vary depending on the fact that we quantify physiological modification by referring to external pollutant or to the internal accumulated pollutant. Because heavy metals are acting on almost all aspects of plant physiology, one may hypothesize that the mode of interaction may be of one type for a given set of parameters, and of another type for another set of parameters. Hence, a comprehensive approach of interaction implies to consider several parameters belonging to each of the 5 classes of above-mentioned injuries.

Specificity of coastal areas phytomanagement also results from the presence of high soluble salts concentrations, mainly in the form of NaCl. It implies that the chosen plant species should not only be able to cope with heavy metals, but with salinity as well. Independently of the presence of heavy metals, high electrical conductivities are damaging for most plant species. This implies that halophyte (plants naturally resistant to salinity) are more suitable than glycophyte plant species (salt-sensitive) for phytoremediation of heavy metals contaminated coastal areas. Nevertheless, salinity and heavy metals toxicity should not be considered independently because i) salinity influences heavy metals bioavailability and speciation in the soil solution, ii) salinity also acts directly on the 5 classes of physiological injuries listed above. Moreover, moderate doses of salt may stimulate plant growth in halophyte plant species, leading to a « dilution » of accumulated heavy metals expressed on a fresh basis.

Kosteletzkya pentacarpos (formerly referred as *K. virginica*) is a promising wetland halophyte plant species for phytomanagement of heavy metal-contaminated salt areas. Ruiming Han (PhD Thesis, 2013) conducted numerous experiments demonstrating that the plant is, to some extent, able to cope with heavy metals such as cadmium and zinc, and that low doses of NaCl which did not stimulate plant growth although it clearly improved the plant resistance to heavy metals, suggesting that salinity induces a beneficial modification of the plant physiological status allowing it to improve its resistance to accumulated Cd or Zn and that this modification is not due to any diluting effect.
In the work of R. Han, however, *K. pentacarpos* was exposed to one single heavy metal (Cd or Zn) and was cultivated in nutrient solution only.

The global aim of the present study is to assess the response of the halophyte wetland plant *Kosteletzkya pentacarpos* species to a complex heavy metal pollution implying the simultaneous

presence of cadmium and zinc excess, to determine and understand the putative influence of NaCl on the plant physiological and biochemical behavior, and to compare the plant behavior in nutrient solution and contaminated soil.

The tested hypothesis is that a « mixed » toxicity is considered by the plant as a specific environmental constraint inducing a specific physiological status which should not be considered as the simple additive impacts of each pollutant. Salinity may improve the capacity of the plant to cope with a mixture of Cd and Zn and this improvement is not only due to a decrease in heavy metals absorption or growth stimulation but also to a modification of the plant physiological properties sustaining heavy metal resistance.

Outline of Study

To test this hypothesis, the performed experimental work was divided in two distinct parts (see Figure 1 below): the first part is dealing with plant exposure to the complex pollution and/or salinity in nutrient solution, allowing to precisely control the stress intensity. The second part characterized *K. pentacarpos* growing on an artificially multi-metals and As contaminated soil in semi-controlled greenhouses.

In part I, five distinct chapters will be considered with the aim to provide an answer to the following questions:

- Is *K. pentacarpos* able to survive and to grow in the simultaneous presence of Cd and Zn, and what are: the rate of heavy metal accumulation and their subcellular distribution ii) the impact of mixed pollution on photosynthetic activities and oxidative status and iii) the influence of NaCl on the above-mentioned parameters? (Chapter 1).
- What are the influences of heavy metal pollution on senescence process and does complex pollution (Cd+Zn) have a different impact than individual pollution (Cd or Zn) in the absence and in the presence of NaCl? Senescence processes (photosynthesis, cell membrane stability, oxidative stress) are analyzed in relation to the plant phytohormonal status of the stressed plants, more especially considering ethylene and polyamine content (Chapter 2).
- What is the impact of the simultaneous presence of Cd and Zn on mineral concentration, the capacity of carbon fixation and oxidative status in the absence and in the presence of NaCl as well as exogenous cytokinin in *K. pentacarpos*? Does the complex pollution (Cd or Zn) induce a special plant hormone status in the absence and in the presence of NaCl as well as exogenous cytokinin in *K. pentacarpos*? The main nutrient elements as well as heavy metal concentrations and the

considered parameters (stomatal conductance, net chlorophyll, chlorophyll concentration and carbon isotope discrimination) related to carbon fixation capacity are determined. The main phytohormone including total auxin, cytokinin, gibberellins, ethylene (and their metabolites) and ABA, jasmonate acid, salicylic acid, polyamines are analyzed to give a global view of plant hormone status (Chapter 3).

- Is *K. pentacarpos* triggering an adapted response in terms of osmotic adjustment through osmocompatible solutes accumulation and modification on their transfer when exposed to NaCl and mixed pollution? Two major organic solutes (proline and glycinebetaine) are quantified and enzyme activities involved in their synthesis and catalysis are analyzed in relation to the expression of the corresponding gene expression (Chapter 4).
- Is mucilage produced by *K. pentacarpos* involved in heavy metal resistance? Do Zn and salinity make a modification on the mucilage amount and the polysaccharide structure in *K. pentacarpos*? The total amount as well as the component of mucilage and the structure of polysaccharide will be quantified for plants exposed to Zn in the absence and in the presence of NaCl (Chapter 5).

Those 5 chapters will allow us to gain an overview of the plant capacity to cope with mixed pollution when heavy metals are fully available (as could be the case in nutrient solutions). In field conditions, however, plants are growing on soil substrate and bioavailability of the pollutant is an important factor to consider, especially due to the fact that salinity may strongly influence pollutant availability. Hence, **in the second part** of the present thesis, *K. pentacarpos* will be cultivated in an artificially (spiked) polluted soil containing Cd and Zn, but also As and Pb, two pollutant frequently reported in contaminated salt marshes in the eastern cote of China. Both short-term (few weeks; Chapter 6) and long-term (several months; Chapter 7) experiments will be performed in an adapted column device in order to give an answer to the two following

questions:

- What are: i) the rate of multi-pollutant accumulation in *K. pentacarpos* growing in a polycontaminated substrate, ii) changes of oxidative status induced by combination of heavy metals and As and iii) the influence of NaCl on plant uptake, antioxidant system, heavy metal bioavailability and pollutant leaching? The research objects are included, which are consisted of soil (pollutants bioavailability), plant tissue (mineral content and global antioxidant activity) and leachate (volume and pollutants concentration) (Chapter 6).
- What is the influence of mixed pollution and the additional NaCl as well as EDDS on the plant morphology (stem and leaf development) and reproduction (flower and seed number) during its whole growing cycle? What is the effect of NaCl and EDDS addition on i) pollutants bioavailability in soil and ii) pollutants accumulation in different types of stem (main stem and lateral branch) and leaf (on main stem and on lateral branch) on long-term time basis? What is the modification of polysaccharide structure induced by multi-pollutant, NaCl and EDDS? (Chapter 7)

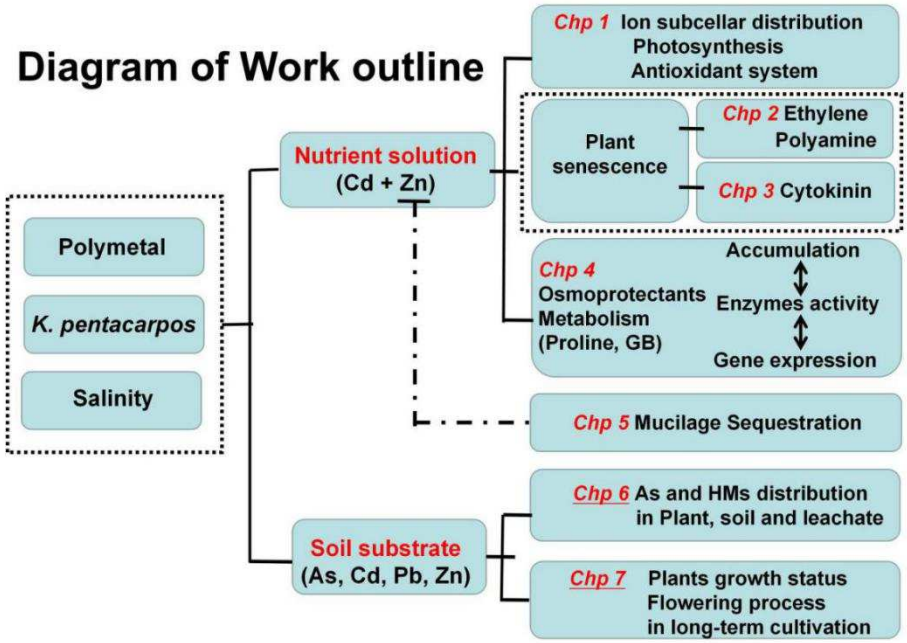

Figure 1. The diagram of the work outline

Literature Review

1. Heavy Metal Stress

1.1. Heavy metal

The enormous variety of matter in the world is made from different combinations of substances called elements. A chemical element is a species of atoms having the same number of protons in their atomic nuclei, which is not able to be detached by chemical method. In accordance with elements properties, it is classified into metallic and non - metallic. The term “heavy metal” is being widely used to refer metals and metalloids, whose densities are higher than 5 g/cm^3 such as chromium (Cr), zinc (Zn), lead (Pb), cadmium (Cd), arsenic (As) as well as mercury (Hg) (Yang et al. 2018).

In atmosphere, hydrosphere and lithosphere, heavy metals enter the biosphere through natural processes or anthropogenic activities. In case of a natural process, heavy metals reach to biosphere as a consequence of natural calamities such as volcanoes and floods. In soil and water, modifications in pH and redox potential often results in the conversion of heavy metals from insoluble to soluble form, leading to a progressive increase in concentration. However, anthropogenic activities, such as smelting, sewage irrigation, chemical production, agricultural development as well as fuel combustion, significantly accelerate this process, causing an obvious increase in heavy metal concentration up to toxic levels (Fig 1) (Calatayud et al. 2018, Marrugo-Negrete et al. 2017, Nadgorska-Socha et al. 2017).

The industrial revolution strongly improved the efficiency of production compared to previous technologies in ancient time. But it is also associated with large amounts of heavy metal waste released

into environment. In the global world, more than 30,000 tons Cr and 800,000 tons of Pb emission from industrial process have caused greatly serious heavy metal pollution (Yang et al. 2018). It is reported that from 2003 to 2008, the annual discharge of municipal sewage sharply increased from 45 billion tons to 57 billion tons. Due to the over-exploitation and utilization of land, the heavy metal pollution in soil has been greatly serious. In China, the total pollutant over-standard rate of soil (the Grade II[1] environmental quality standard for soils in China (GB15618-1995)) was 16.1% in 2014, including Cd (7%), As (2.7%) and Pb (1.5%) (Yang et al. 2018).

Throughout the whole world, cities located in the coastal region take the lead of development like New York, Los Angeles, London, Hong Kong and Shanghai. However, the rapid development relying on the industry and the agriculture is followed by an extremely high environmental price, although the gross domestic production (GDP) from coastal area accounts for a large part of total (Gao et al. 2013). For instance, in 2015, 21 billion tons of pollutant were released to coastal environments in China. The concentration of Cd, Pb and As in sediments near Chinese Bohai Sea are up to 248, 753, and 397 mg/kg dry weight respectively, which are strongly worse than Grade III (Manzoor et al. 2018). Now, we are aware of the seriousness of this issue and deeply realize the threaten and harm to all livings, resulting from heavy metal pollution.

[1] Guideline values (mg/kg dry weight soil): Grade I: Cd (0.5), Pb (60), As (20); Grade II: Cd (1.5), Pb (130), As (65); Grade III: Cd (5), Pb (250), As (93)

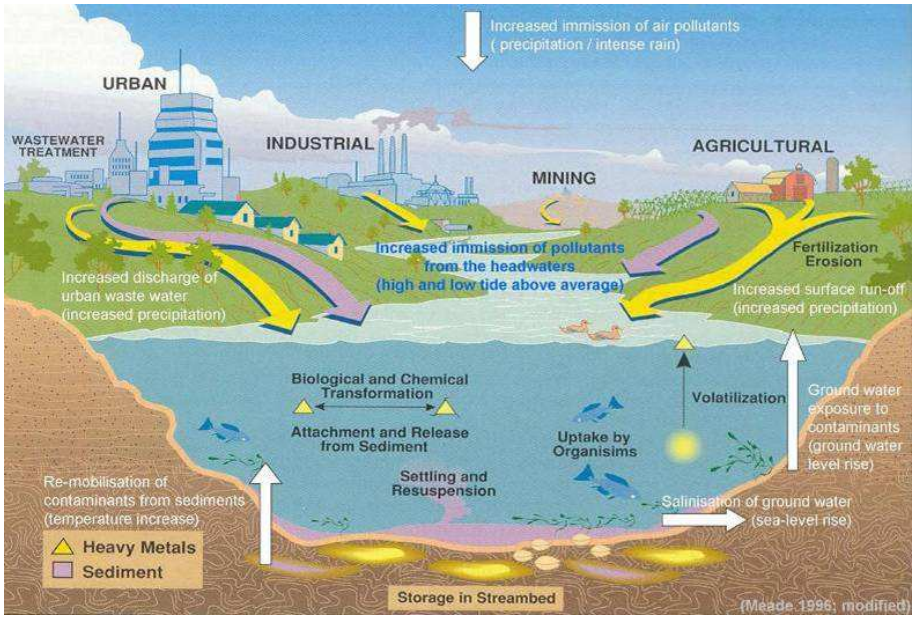

Figure 1 Sources of sinks of heavy metals (Hennes, 2007)

1.2. Heavy Metal Bioavailability and Hyperaccumulating Species

Although the measured concentration of heavy metals is very high in polluted areas, it is not enough to assess any actual occurring adverse effects on the soil ecosystem. In soil, heavy metals are present under various chemical species. Until now, the term bioavailability has not been consistently defined. It has been reported (Lanno et al. 2004) that bioavailability should be considered as a dynamic process, which results from three steps: environmental availability (a physiochemically controlled desorption process), environmental bioavailability (a physiologically controlled uptake process) as well as toxicological bioavailability (Peijnenburg et al. 1997). On the other hand, bioavailability is not only difficult to be defined, but also it is affected by a wide range of soil properties. One of the most important factors is pH. In general, the bioavailability of heavy metals from soil decreases with increasing pH, resulting from higher adsorption and precipitation of heavy metals in alkaline and neutral environments

(Peijnenburg et al. 1997).

When plants can absorb high doses of heavy metals and do not exhibit toxic symptoms of injury, they are commonly called 'hyperaccumulating' plant species. One of the main criteria to consider a plant species as hyperaccumulator is that a given metal/metalloid concentration must be reached in the aerial part of the plant. Baker and Brooks (1989) suggested that threshold concentrations for hyperaccumulation in plants are 100 μg g^{-1} dry weight for Cd, 1,000 μg g^{-1} dry weight for Ni, Cu, Co, Pb, and 10,000 μg g^{-1} dry weight for Zn and Mn. It has been found hundreds of hyperaccumulators, which are included in 45 different families. A second criteria is that heavy metal concentration in the shoots should be higher than in the roots and this property explain why hyperaccumulator are often used as model plant species to identify transporters involved in xylem loading.

1.3. Cadmium

Occurrence in environment

In the periodic table of elements, the 48th element in group 12 is cadmium, whose density is 8.65 g/cm^3. It is naturally present in surface rocks and relatively stable in its insoluble solid state. However, over exploitation by human beings transfers Cd to soluble compounds, which made soil and water polluted although its concentration is lower than other heavy metals. What is notable is even if under low concentration, Cd still has high toxicity to most organisms, because it is not essential (Das et al. 1997).

Absorption and translocation in plants

Cadmium can enter root through different families of transporter like ZIP (Wu et al. 2019) or cation channels such as hyperpolarization

activated calcium channels (HACC) (Mei et al. 2014). These transporter and cation channels are not specific, leading to deficiency of other nutrients such as Ca, Mg, Zn, K (Khaliq et al. 2019). Data from lots of studies shows that most of Cd is accumulated in root, only small parts are transported to shoots, suggesting that it is not readily translocated in the phloem (Rizwan et al. 2019).

Impact on plant physiology

Cadmium makes toxicity in all plant life span, even under 1 μM/g dry weight of plant tissue. Most of research shows that the Cd is in competitive relation with other elements such as K, Zn, Mg, Ca in the same transmembrane carrier (Rivette et al, 1997). It could penetrate the root through the cortical tissue. When Cd enters the roots, it can complex with organic acids, phytochelatins, small peptides and go to the xylem through apoplastic and symplastic pathway (Salt et al, 1995). Generally, most of Cd ion is retained in root (Han et al, 2012). However, small part still can be transferred to the shoot and has deleterious effect on plant, although the concentration is low. The noticeable symptoms of Cd toxicity in plants comprise (i) leaf necrosis and chlorosis, (ii) growth retardation, (iii) modification in plant morphology, especially ramification number, (iv) inhibition of lateral root growth, due to a series of changes such as inhibition of cell division and cell elongation v) impairment of plant photosynthesis and vi) alteration in the plant hormonal status (Han et al. 2012, Han et al. 2013). Cd can cause inhibition of photosynthesis when it accumulate in chloroplast, resulting from decreasing pigment (chlorophyll and carotene) concentration, disturbing the electronic transmission chain, alteration of chloroplast structure and biosynthesis as well as reducing stomatal conductance (Lu et al. 2018, Ma et al. 2018). Furthermore, the biomass production sharply decreases due to negative effect on photosynthesis under Cd stress. On the other hand, Cd toxicity is also related to the production of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide

(H_2O_2), although it does not participate in Fenton-type ROS-producing reactions (Hu et al. 2019). The ROS can lead to macromolecules disruption, such as protein and DNA degradation, and membrane damage (Newkirk et al. 2018).

Mechanisms of tolerance

Facing to rapidly deteriorating environment, plants have already evolved to tolerate heavy metal stress before it reaches the concentration limits (Fig 1.2 B). On one hand, when Cd is absorbed by plant, it would be fixed in cell wall. Previous study showed that galacturonic acid residue plays a vital role in metallic cation binding (Kim et al, 1996). On the other hand, the production of chelating molecules helps plants to improve their ability to tolerate Cd. Many studies suggest that Cd is prone to bound to S - containing ligands (Calatayud et al. 2018, Jia et al. 2016). The non - protein thiol group, which is rich in S is roughly defined as the sum of glutathione and phytochelatins (PCs). PCs are a family of peptides bases on glutathione, with the general structure (γ - Glu - Cys) n - Gly (n=2-12) (Lue-Kim &Rauser 1986). To protect the cytosol from free Cd ions, PCs are induced to chelate Cd, forming complexes which are thought to be translocated in the vacuole (Vatamaniuk et al. 2000, Xu et al. 2018). In addition to the synthesis of PCs, glutathione also has its own function, mainly as redox-homeostatic buffer, alleviating the oxidative damages from ROS (Ling et al. 2018, Semida et al. 2018). Hence, to deal with Cd toxicity, plants have to find the best compromise between the use of glutathione in the management of oxidative stress and its integration in PCs to reduce the toxicity by binding metal. On the other hand, metallothioneins (MTs) is another group of compounds rich in cysteine residues (Le Beau et al. 1985). Several studies indicate that cadmium is able to induce MTs - genes expression, leading to the synthesis of MT so that it can be chelated by MTs (Cobbett 2000, Karenlampi et al. 2000).

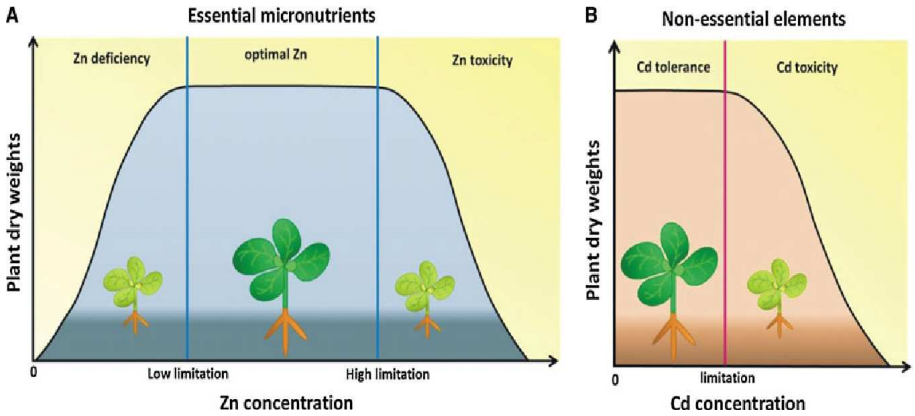

Figure 2 Response curves of plants to Zn (A) and Cd (B) (Alloway, 1995)

1.4. Zinc

Zinc is the 30th element in the periodic table of elements with a density of 7.14 g/cm^3. It is the 24th most abundant element of the earth's crust and the second most abundant transition metal in organisms after iron (Fe). Chemical and physical weathering of parent rocks is the primary input of Zn. It is present in rocks of diverse types, including Zn sulfide, sulfate, oxide, carbonate, phosphate and silicate minerals. In non - polluted agricultural soils, the concentration of Zn ranges from 10 µg to 300 µg (Ayoubi et al. 2019). However, human activities such as waste combustion, mining, steel industry, oil exploitation and sewage disposal strongly increase the amount of Zn in the soil and water, which may be 20 times higher than its natural input (Lin et al. 2019, Nahar et al. 2018).

Function in organism

Zinc assumes key biological functions and contributes to maintain normal growth and development in most organisms including human beings. More than 300 proteins contain Zn, which are classified as zinc finger proteins (Olechnowicz et al. 2018, Weinthal et al. 2010). Meanwhile, it is the only metal present in all six enzyme classes

(oxidoreductase, transferase, hydrolases, lyases, isomerases and ligases), which plays the functions of activities regulation as co-factors and conformational stabilization and enzymes folding (Olechnowicz et al. 2018). Apart from proteins, membrane lipids and DNA/RNA molecules are common structures presenting Zn binding sites (Rom et al. 2006). Zn is also involved in various plant physiological processes, including hormone regulation, maintenance of photosynthesis and signal transduction through kinases. In the world, zinc is the most common plant micronutrient deficiency. The symptom of Zn deficiency in plant is including root apex necrosis, stem elongation inhibition, leaf chlorosis and necrosis. Zinc deficiencies have a deleterious impact on human health (Khatun et al. 2018).

Impact on plant physiology

To deal with essential nutrients, plants experience three stage from deficiency, enough to excess (Fig. 2 A). Despite the deficiency of zinc for plants, especially for cereals, is ubiquitous all over the world, the soil contaminated by excessive zinc is still severe, due to unreasonable anthropogenic activities. When plants are stressed by high concentration of Zn, the first symptom is inhibition of root growth and stem elongation (Han et al. 2012). Due to interference of nutrients absorption and inhibition of electron transport, dysfunction in photosynthesis process occurs. Besides, large amount of zinc is able to induce more ROS production, leading to redox status unbalance and oxidative damage (Morina et al. 2010).

Mechanisms of tolerance

Even though most of plant species are sensitive to high concentration of zinc, plants have selected strategies to cope with zinc excess in the surrounding environment. Unlike cadmium, the production of organic acid such as malate, citrate as well as oxalate play an important role

on detoxification of excessive zinc (Sarret et al. 2002, Wang et al. 1992). It has been reported that Zn might stimulate citrate production in the hyperaccumulating *Thlaspi caerulescens* root. In the shoots, zinc, malate as well as oxalate are correlated. The Zn-organic complex can be transferred to vacuoles and be compartmentalized. Beside organic acid, glutathione is also able to bind to Zn, due to its S - containing ligands (Kupper et al. 2004). Furthermore, glutathione has two types, reduced (GSH) and oxidized (GSSG) which the mutual conversion alleviates the damage produced by ROS under Zn stress.

1.5. Combination of Cd and Zn Pollution

Around Zn mines and smelters, cadmium is very often associated with zinc. Both elements belong to group II transition elements, with similar physical and chemical properties (Yun et al. 2018). As previously stated, Zn is an essential micro-nutrient while Cd is non - essential even toxic under low concentration. Since both are often present in nature simultaneously, it is quite interesting to analyze how they can interact and how living organisms are able to discriminate them.

Different types of relation between Cd and Zn may exist: synergistic, antagonistic and independent effects have been detected in recent researches. In a previous research (Nan et al. 2002), cadmium and zinc interactions as well as their transfer in soil-crop systems under actual field conditions were studied. They concluded that the effects of the two metals were synergistic under field conditions, in which increasing Cd and Zn contents in soils (Cd: 0.14 - 19.3 mg/kg soil Zn: 43.5 - 565 mg/kg soil) could increase the accumulations of Zn or Cd in the spring wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.)

In contrast, it has been reported that low concentration of Zn addition to soil (30 mg/kg soil) could effectively improve physiological performance and mineral absorption of wheat in cadmium stress

condition (Sarwar et al. 2015). A decrease in Cd accumulation may be accompanied with the increase of Zn accumulation in plant tissue. Furthermore, the foliar application of Zn (3 g/L) at booting stage in rice is more effective to ameliorate the adverse of Cd and decease grain - Cd content than adding Zn in soil directly. Same results are presented by other studies (Saifullah et al. 2013).

In Cherif's opinion (2011), Cd and Zn are clearly antagonistic for ion absorption in *Solanum lycopersicum*, while different concentration of Zn has different interactions with Cd in other aspects of plant responses. When Zn was supplied from 10 to 150 μmol/L, it clearly reduced Cd accumulation in *Solanum lycopersicum*. At low level (10 μmol/L), the additional Zn restored and enhanced functional activity of enzymatic antioxidant (SOD, CAT, APX as well as GR), compared to Cd alone. However, at high level (100 - 150 μmol/L), the excessive Zn combined with Cd strongly induced oxidative stress, which is even more severe than that for Cd alone or excess Zn alone. In another study of Cherif, it was revealed that Zn supplementation at 10 and 50 μmol in combination with Cd protected the photochemical function, while high Zn concentration (100 and 150 μmol) exacerbated the negative impacts of Cd and aggravated the R_{Fd} (690) and R_{Fd} (730), which could be explained by irreversible damages to the photosynthetic apparatus (Cherif et al. 2012).

1.6. Remediation of Polluted Area

To face with serious heavy metals pollution in soil, various cleanup techniques are available. They are classified as physical, chemical and biological remediation. For the physical and chemical remediation, although they can remove heavy metals quickly, the cost is extremely high. In USA, $6 – 8 billion are spent annually in remediation efforts, with global costs in the range of $25 – 50 billion (Glass, 1999; Tsao, 2003). Moreover, physical and chemical treatments affect the soil properties and structure, cause soil disturbance, affect biodiversity,

create secondary contamination problems and interfere with the normal function of soil ecosystem (Bhargava, et al., 2012).

Unlike physical and chemical remediation, phytoremediation is cheap and eco-friendly. It consists in the use of plants to remove or neutralize contaminants, which holds great promise for unobtrusively and cost effectively treating soil contaminated with pollutants (van der Lelie et al., 2001). It consists in numerous approaches, including phytoextraction, phytostabilization, phytovolatilization, phytofiltration and phytodegradation (Fulekar et al, 2009; Marques et al, 2009). The selected plants grow in the heavy metal contaminated soil and uptake heavy metals through roots. Part of the heavy metals is transferred to the aboveground shoot system. The metal-rich aboveground biomass is then harvested at maturity. The ultimate consequence is that a fraction of the soil contaminant is removed. The main advantages of phytoremediation are low cost of the operation (compared to classical remediation techniques) and aesthetic aspects, making it suitable for remediating large contaminated sites in populated areas (van der Lelie et al., 2001). Besides, there is no secondary contamination. However, there are still some limitations for phytoremediation. On one hand, the main obstacles to large-scale applications of phytoremediation technologies are the time required for remediation, the pollutant levels tolerated by the plants used, and the fact that only the bio-available fraction of the contaminant will be treated (van der Lelie et al., 2001). On the other hand, how to deal with the harvested biomass that is rich in pollutants is a realistic problem, although some studies were focused on it in recent years (Moser et al. 2013; Tang et al. 2013). In the future, a more reasonable and feasible way to handle the plant material for phytoremediation should be developed to avoid the secondary pollution.

2. Salinity

2.1. Salinity and Sodicity

Salinity corresponds to the amount of salt dissolved in water or soil, which is estimated by electrical conductivity (EC) measuring the ability to conduct an electric current. In general, when the EC value is higher than 4 dS/m (around 40 mM NaCl) in the water - saturated soil paste, substrate is regarded as salt-affected and may exhibit three characteristics: (i) high salt concentration, (ii) high sodium cation (Na^+) concentration, and (c) high pH related to high CO_3^{2-} concentration (Daliakopoulos et al. 2016).

Soil salinisation is a widespread phenomenon affecting 932.2 Mha in the world (Rengasamy, 2006). In relation to salinity, sodicity is mainly leading by alkaline hydrolysis in high concentration of CO_3^{2-}, HCO_3^- and SiO_3^{2-}. Distinguishing chemical features of saline and sodic soil is mainly related to pH and EC (saline soil < 8.5, sodic soil > 8.5; saline soil > 4 dS/M, sodic soil < 4 dS/M, respectively). In coastal area, resulting from seawater intrusion and erosion as well as human activities, the soil is always saline and sodic simultaneously, leading to the loss of the emerging resources, goods and services of soil, reducing agricultural production, thus making a threat to human health (Daliakopoulos et al. 2016, Szabo et al. 2016).

Soil salinisation implies accumulation of soluble salts in soil layer; although it may occur naturally, including weathering, releasing and transport from parent rock (Feitz and Lundie, 2002). It is dramatically increased as a result of human activities. From the last century, salt accumulation is sharply enhanced in soil layer, which is the direct consequence of unreasonable usage of non-adapted irrigation system. After flood irrigation, amount of extra water with abundant salt remains in the surface, leading to water logging. As a result, soil with

salt deposit becomes salinized after evaporation. In addition, to improve the yield of crops, excessive fertilizers are frequently applied to soil, creating eutrophication which is also related to soil salinization (Siebe, 1998). Last but not least, salinisation may also be associated to soil pollution. Residues from mining as well as chemical industry contain high amounts of salt which destroys soil structure and texture, changes the microbial community and reduces soil productivity. In accordance to Van-Camp's report, approximately 4 Mha of European soil were evaluated to have a moderate to high level of degradation due to secondary salinisation, which including Italy, Span, Hungry, Greece, Portugal, Poland, the Dalmatian coast of the Balkans Slovakia and Romania (Van-Camp L, 2004).

2.2. Physiological Impact of Salinity on Plants

In general, when plants are stressed by high concentration of salt, two main responses occur. Within minutes to several days, plants suffer mainly from osmotic potential decrease, resulting in stomatal closure, CO_2 availability decrease for carbon fixation and cell expansion inhibition, leading to ion-independent growth reduction (Jha and Subramanian 2014). After several weeks of stress, the second phase takes place, in which the representative characteristic is cytotoxicity. The metabolic processes slow down with generation of ROS, causing premature senescence, and ultimately cell death (Jha and Subramanian 2014).

2.3. Salt Stress Resistance and Halophyte Plant Species

To cope with salt stress, plants have evolved various ways to reduce the damage. Regulation of ion homeostasis and compartmentalization is one of the most crucial process for plant normal growth (Sharma et al, 2018). It also plays a vital role on salinity tolerance in plants. The excessive Na^+ is transferred to plant vacuole in older tissue. Meanwhile, the amount of compatible solutes, such as proline,

trehalose and carbohydrates are produced to act as osmotic protection (Bates et al, 1973). On the other hand, salt accumulation in plant tissue leads to oxidative stress with the increasing generation of ROS. In plants, the antioxidant system could be classified to two groups: enzymatic antioxidants like SOD, CAT, POX, and non-enzymatic antioxidants like reduced glutathione (GSH) and ascorbate (AsA). Both of them are able to neutralize excessive electron to maintain homeostasis in plant. Last but not least, plant hormone regulation also plays a function to enhance resistance ability to salt stress (Ryu and Cho, 2015).

Depending on the plant response to salinity, species are classified as halophyte (salt-resistant) or glycophyte (salt-sensitive) (Flowers et al, 1986). The prefi “halo-” and root “-phytes” are translated as salt and plant, respectively. In general, a halophyte is a plant that can grow in the environment of 80 mmol/L salt (Menzel and Lieth, 2013). All over the world, more than 1560 halophyte species have been found, which are distributed in sea, salt marsh, land as well as salty desert. It can be classified in many ways. Stocker (1928) distinguishes 3 kinds of halophytes which are aqua-halines, terrestro-halines as well as aero-halines. On the basis of tolerance mechanisms, they are classified as euhalophyte, recretohalophyte and pseudohalophyte (Flowers et al, 1986). In euhalophyte, the leaves and stems become succulent to restore more water, regulating the osmotic potential. Recretohalophyte has evolved a special organ, salt glands, trichomes and vesicle, which secrete inorganic ion usually at the leaf surface. The pseudohalophyte, also called salt - dilution halophyte, can selectively absorb salt ion and transfer and restore it to the upper part, avoiding damage by high salinity.

3. Impact of Salinity on Plant Response to Heavy Metals

3.1. Impact of Salinity on HMs properties

As we all know, high concentration of heavy metal has a detrimental impact on plant growth and development (Han et al, 2012). What we need to clarify is the definition of "high concentration". Sometimes, despite under high total concentration of heavy metal condition, plants are still able to grow, because of low bioavailability of heavy metal. Heavy metal bioavailability is the fraction of total heavy metal in soil or water which is available to the organism (Petruzzelli, 1989). It is affected by abiotic factors such as soil structure, soil composition, soil acidity as well as redox potential, and biotic factors such as root activities as well as rhizosphere microorganisms (Zhang et al, 2014).

In addition to factors mentioned in the last paragraph, the influence of salinity on heavy metal bioavailability and speciation is an important, although still neglected aspect. In Acosta's research (2011), the impact of salinity ($CaCl_2$, $MgCl_2$, NaCl, and Na_2SO_4) on mobility of each heavy metal (Cu, Pb, Zn, Cd) in soil was studied. An obvious influence on the mobilization of metals was shown with the increasing ionic strength of four salt: (1) $CaCl_2$ has the strongest effect on the mobilization of heavy metal (except Cu). It may indicate that heavy metals are more efficiently replaced by Ca^{2+} on exchange sites as well as more readily made a complex with chloride (2) Among four kinds of heavy metal, an increase in NaCl promotes the highest release of Cd. Cd is easy to form complex with chloride to $CdCl^{+}$ rather than SO_4^{2-} (Acosta et al. 2011).

Studies focused on water and sediment from estuaries are important in understanding the cycling of heavy metals in response to salinity, which has an impact on moving the absorption edge of heavy metal to higher pH. It is indicated that in desorption experiments, Zn or Cd

presented intermediate reversibility and desorption was higher in seawater than in freshwater, implying the importance of chlorocomplexation controlling their behavior (Hatje et al. 2003). Meanwhile, Chu (2014) also presented that the increase in salinity may enhance the mobility of Cd, Zn as well as Pb, indicating that these metals may be remobilized during saline tidal flooding. Hence, heavy metal speciation and bioavailability are affected not only by exchange of divalent or monovalent cation, but also by forming complex with chloride or other anions (Chu et al. 2014).

3.2. HMs Absorption and Translocation in Plant under salinity condition

The absorption of heavy metal by plant is affected by diverse factors including plant species, the age and growth stage of the plant, soil structure and texture, heavy metal speciation and bioavailability, as well as seasonal changes.

No doubt that the root plays a critical role in nutrient absorption which acts as a bridge between soil and plant. Hence, the first step of heavy metal absorption occurs in rhizosphere. Soluble organic substances which consist of low - molecular - weight (LMW) organic acids, high - molecular - weight (HMW) polysaccharides and other organic acids, is able to be released from roots (Graham and Stangoulis 2003). Data from Mucha's study shows that the halophyte species *J. maritimus*, is able to release LMW organic compounds (malonate and oxalate) that can act as complexing agents of Pb, Cr, Cu, Zn, Ni and Cd (Mucha et al. 2005). Similarly, it has been presented that in the root of the most salt-tolerant cereal crop, barley (*Hordeum vulgare L.*), 76 known metabolites are identified, including 29 amino acids and amines, 20 organic acids and fatty acids, and 19 sugars and sugar phosphates by GC – MS, which promote the uptake of lanthanum (Han et al. 2005, Shelden et al. 2016).

After heavy metals enter plants, they are conveyed to the aerial parts by transporters. Numerous families regarding to metal transporters are found in glycophytic plant species including copper transporter (CTR) (Senovilla et al. 2018), cation diffusion facilitator (CDF) (Migocka et al. 2018), zinc-iron permease (ZIP) (Guerinot 2000), cation exchange (CAX) (Pittman & Hirschi, 2016), natural resistance – associated macrophage protein (NRAMP) (Gupta et al. 2017), non – selective cation channel (NSCC) (Demidchik & Maathuis, 2007) and heavy metal ATPases (HM-ATPase) (Takahashi et al. 2012). However, for halophyte species, there is no available data on this aspect although the hypothesis is raised that halophyte and glycophyte have similar metal transport characteristics. This should be tested in future.

In halophyte, the available data shows that a moderate concentration of salt could enhance plant growth, which aims to dilute the heavy metal concentration, thus decreasing toxicity. On the other hand, salinity not only reduces heavy metal accumulation but also improves plant tolerance to accumulated pollutants. It has been demonstrated that 50 mM NaCl is able to delay senescence in Cd – treated *Kosteletzkya pentacarpos* through reducing the concentration of aminocyclopropane carboxylic acid and increasing the concentration of cytokinin (zeatin and zeatin riboside) (Han et al. 2013).

There are two factors to estimate heavy metal accumulation and translocation in plant which are bioaccumulation factor (BF) as well as transfer factor (TF). The BF is an indicator of the plant's ability to accumulate the heavy metal in harvestable organs comparatively to its mean concentration in the environment. It is affected by many factors such as plant type, plant growth stage, biomass and plant physiology (especially in terms of water flow and translocation). The TF is representative of the plant's ability to translocate pollutant from the root to the shoot system, which is classified on the basis of concentration (TF_c) and on the basis of the total amount (TF_a) of translocated pollutants. Several studies (Pellegrini et al. 2017,

Petranich et al. 2017, Feng et al. 2018) indicate that in halophyte, the BF is always high while TF is relatively low. It just confirms with high accumulation of heavy metal in root and low translocation in shoot.

Until now, there is no available data regarding to the major chemical form of heavy metal translocation in halophyte. Mucilage is a polysaccharide mixture commonly found in various organs of halophyte plant. The high water-binding capacity of hydroxyl groups allows it to make a contribution to long – distance transport. In *Kosteletzkya pentacarpos*, it is observed that a very high proportion of rhamnose and uronic acid of mucilage from stem tissue (Ghanem et al. 2010). The rhamnose is regarded as one of the most important components in pectin, which can be used as cationic exchangers for fixing metal cation in aqueous solution. The hypothesis that mucilage may play a vital role in heavy metal translocation in halophyte should be verified in future.

3.3. The use of Halophyte plant Species for phytoremediation

With the development of economy and society, some areas which have high concentrations of soluble salt like coastal line suffer from serious pollution especially heavy metal contamination (Rodriguez-Estival et al. 2019, Zhang et al. 2016). Halophyte species now are receiving more attention, due to their salt tolerance as well as potential to phytoremediation of heavy metal polluted soil. It has been demonstrated that halophytes like *Atriplex halimus*, *Atriplex nummularia*, *Mesembryanthemum crystallinum*, *Sesuvium portulacastrum*, *Tamarix smyrnensis*, *Salicornia sp*. may be useful tools phytoremediation. A lot of works are focused on the heavy metal toxicity in plants, and preliminary mechanisms of resistance set up by halophytes are revealed (Fig 3) (Lutts and Lefevre, 2015). The first response of plant to excessive heavy metal is nutrient imbalance due to ionic absorption and toxicity, which is referred to primary stress.

Meanwhile, the secondary stress including water stress and oxidative stress is induced indirectly. It may be considered that secondary stresses, such as impairment of plant water status or oxidative stress are induced by various type of ion toxicities, implying that the salt-tolerance mechanisms selected by halophytes might, to some extent, be an advantage for plants exposed to heavy metal.

In coastal area, despite the bioavailable concentration of heavy metal is much lower than it of salt, it still can result in water stress indirectly. Halophyte species are adapted to heavy metal stress allowing them to cope with heavy metal toxicity. In the schematic representation (Fig 3), four kinds of substance to handle excessive heavy metal are summarized, which is including metal chelators, osmo-protecting compounds, antioxidant compounds and antioxidant enzymes. A lot of studies have verified that when halophyte accumulates significant amounts of heavy metal, the content of total protein thiol increases (Han et al. 2013, Liu et al. 2016). Besides, other compounds like MTs and organic acid also play a vital role on metal sequestration. Furthermore, in relation to plant osmotic potential, proline, glycinebetaine and other osmo-compounds are induced when plant under heavy metal stress. High concentration of osmo-compounds is able to enhance the resistance ability to excessive heavy metal.

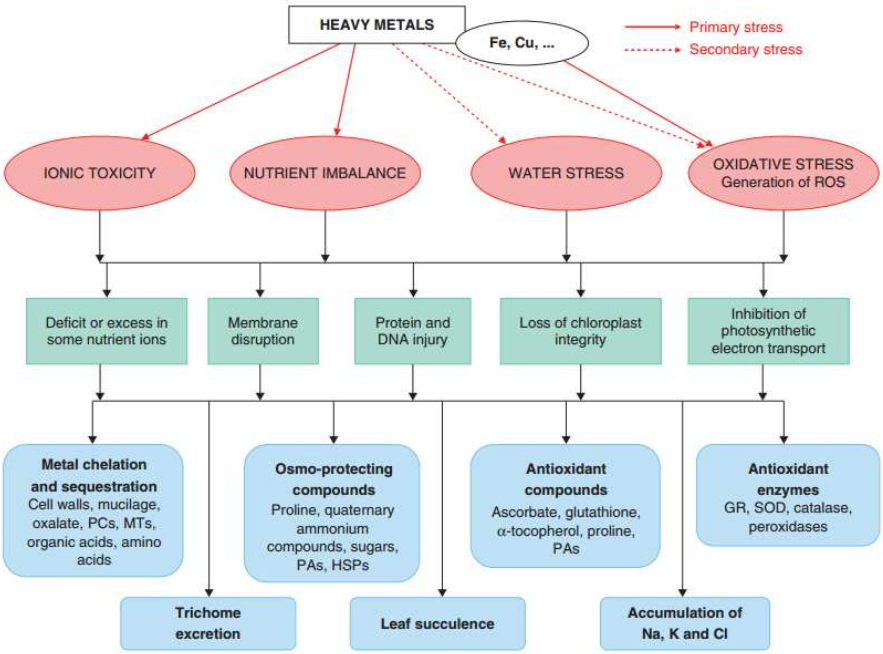

Figure 3 Schematic representation of heavy metal toxicity in plants and the mechanisms of resistance set up by halophytes (Lutts and Lefevre 2015).

4. *Kosteletzkya pentacarpos*

4.1. Botanical Classification and Ecological Distribution

Kosteletzkya pentacarpos (L.) Ledebour (syn. = *Kosteletzkya virginica* (L.) Presl. ex Gray) is one species of salt-dilution halophytes, generally known as "seashore mallow" and "saltmarsh mallow", which naturally distribute from Louisiana to Florida and north along the Atlantic coast to Delaware and the state of New York (Blits and Gallagher, 1990). For the botanical naming of the seashore mallow, it is indicated that this species should be named *Kosteletzkya pentacarpos* (L.) Ledebour and *Kosteletzkya virginica* (L.) Presl. ex Gray has to be relegated to synonymy according to the international Code of Botanical Nomenclature's rule that the correct name should be the one chosen by the person who first unites them (Blanchard, 2013). Hence, in my study, *Kosteletzkya pentacarpos* is being used. In taxonomy, *Kosteletzkya pentacarpos* (L.) Ledebour has been classified as the following table 1:

Table 1 Classification of *Kosteletzkya pentacarpos*

Classification	
Kingdom	*Plantae* - Plants
Subkingdom	*Tracheobionta* - Vascular plants
Phylum	*Spermatophyta* - Seed plants
Subphylum	*Magnoliopsida* - flowering plants
Class	*Magnoliopsida* - Dicotyledons
Subclass	*Dilleniidae*
Order	*Malvales*
Family	*Malvaceae*
Genus	*Kosteletzkya* - Kosteletzkya
Species	*Kosteletzkya pentacarpos* (L.) Ledebour - saltmarsh mallow

4.2. Phenological and Morphological Properties

As a perennial plant species, *K. pentacarpos* grows and blooms over the spring and summer, dies back every autumn and winter, and then returns in the spring from their rootstock.



Figure 4 The morphology of *K. pentacarpos*. (A) The roots are from 6 months plants, cultivating in the greenhouse in Belgium. (B) Plants that are growing for 3 months in the greenhouse in Belgium. The flower (C) and seeds (D) of *K. pentacarpos.*

It has a well-developed root system (Fig. 4 A). The roots form quite a strong taproot, surrounded by a dense network of lateral roots. The developed root system is in favor of increasing surface area, improving absorption nutrients from soil. Also, secretion of a gelatinous substance which is mucilage (see experimental part of the present thesis) is a common phenomenon, especially in Malvaceae. In *K. pentacarpos*, mucilage content increased in the root in response to

salt stress (Ghanem et al. 2010). It consists of polysaccharide, acts a vital role in energy reserve and cation exchange.

The stem height of *K. pentacarpos* can be up to 1 - 1.5 meters for mature plants and it has plenty of lateral branches (Fig. 4 B). The bark skin is green and the stem is highly fibrotic, which is also rich in mucilage. Besides the increase of mucilage quantity in salt stress condition, changes also occur in neutral monosaccharide components in stem, mainly for rhamnose as well as uronic acid. It may play an important function on the tolerance to salinity (Ghanem et al. 2010).

In an adult plant, the average area of *K. pentacarpos* mature leaves is 600 cm^2 with various shapes such as the linear, shield - shape, heart - shape, as well as halberd - shape (Fig. 4 B). The blades are covered by tiny fluff. Some of them have short petioles and others could be sessile.

During the flowering process, *K. pentacarpos* shows the characteristics of infinite inflorescence (Fig. 4 C). The flowering stage can last to 60 days. There are more than 100 flowers per plant. Each flower has 5 petals with the calyx composed of five sepals. The style extends outwards, with the pistil located in the center of this column. A staminal column is formed by 20 - 30 yellow stamens.

As we observed, the seed of *K. pentacarpos* is brown in kidney - shape (Fig. 4 D). Five seeds in five chambers make up a capsule. The diameter of the seed is 0.25 - 0.29 cm. The weight of one thousand is 16 grams and after selected without shriveled is 17.6 grams. the seeds of *K. pentacarpos* have high nutritional value with protein content around 27.4 - 29.6 % and oil content over 20% (Qin et al. 2015). In accordance with the data from gas chromatography, the proportion of unsaturated fatty acid is 70% much higher than saturated fatty acid which is 30%. Meanwhile, the content of sodium, potassium and calcium in 100 seeds is 15 mg, 1248 mg and 205 mg, respectively,

which is suitable for human's nutrition demand.

4.3. Interest for Saline Agriculture

Globally, there is 130 million hm^2 land, which approximately half of it located in the coastal area. In the past decades, about 13 million km^2 soil has been submerged under seawater or high salinization (Daliakopoulos et al. 2016). With time going on, more and more soil will become saline, where 99% of plant species are killed under high concentration of salt conditions. Due to the lack of resources, saline agriculture is now considered as a promising alternative (Ladeiro, 2012). In general, saline agriculture implies the cultivation of salt land and breeding of wild domesticated and transgenic salt - tolerant plants to provide various products. Transgenesis is not a suitable option for ecological and social considerations (Fitt et al, 2008). The use of halophyte, which already display high level of tolerance is thus an interesting option. All domesticated cultivated species are glycophyte and only coton or sugar beet have halophyte ancestors. Some halophyte species might however be used as food, forages, medicine, fiber, chemical material as well as greening.

Kosteletzkya pentacarpos has great ecological and economic value. Since 1993, it has been introduced to China. In recent 25 years, it is proven that *Kosteletzkya pentacarpos* grows very well in coastal area (salt concentration: 5‰ - 10‰) of Jiangsu province as well as several other coastal provinces, including Liaoning, Tianjin, Shandong, Zhejiang, and Fujian, from north (23°30' N) to south (38°45'N) of China (Qin et al. 2015, Ruan et al. 2008, Ruan et al. 2009). Now, it has already been regarded as the main tool halophyte species for coastal saline ecological engineering, due to its high biomass, potential of carbon fixation, and production of abundant bioactivator such as polysaccharide, flavonoid and saponins. The process of *Kosteletzkya pentacarpos* ecological engineering is proposed (Qin Pei, 2017). At first, the cultivation of *Kosteletzkya pentacarpos* obviously

improved the soil physical and chemical quality. From the data of table 2, the organic matter, total nitrogen, hydrolyzed nitrogen available phosphorus and exchanged capacity are significantly increasing (Zhang et al. 2014), while the EC is significantly decreasing compared to bare land after 6 years cultivation. The soil structure is obviously improved, in relation to soil aggregation (data not shown). In addition, plant diversity index in those ecosystems is significantly enhanced after 5 years cultivation (table 3), which may result from improvement of soil nutrients and reduction of salt so that more and more plant species easily settle down.

Table 2 Soil physical and chemical properties after 5 years cultivation of *Kosteletzkya pentacarpos* in Jinhai farm. Each value is the mean of 3 replicates ±S.E. For a given parameter, values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test (Qin Pei, 2017).

	Bare land	Plantation area
Organic matter (g/kg soil)	6.5 ± 0.07 a	12 ± 0.35 b
Total nitrogen (g/kg soil)	0.29 ± 0.08 a	0.73 ± 0.05 b
Total phosphorus (g/kg soil)	0.65 ± 0.04 a	0.72 ± 0.02 a
Total potassium (g/kg soil)	20.5 ± 1.5 a	20 ± 0.71 a
Available phosphorus (g/kg soil)	11 ± 0.33 a	16 ± 0.07 b
Hydrolyzed nitrogen (g/kg soil)	32 ± 2.6 a	37 ± 0.31 b
Available potassium (g/kg soil)	440 ± 41 b	232 ± 12 a
Exchange capacity (mol/kg soil)	7.1 ± 0.11 a	16 ± 0.45 b
Electrical conductivity (mS/cm)	9.4 ± 0.59 b	1.4 ± 0.07 a

Table 3 Comparison of plant diversity index Berger - Parker dominance index (d), Margalef richness index (Ma), Simpson diversity index (D), Shannon's diversity index (H), Pielou's evenness index (J) between control place and *Kosteletzkya pentacarpos* plantation area in Jinhai farm after 5 years. Each value is the mean of 3 replicates ± S.E. For a given parameter, values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test

Index	Control	Plantation area
d	1.05 ± 0.17 a	2.3 ± 0.54 b
Ma	3.04 ± 10.5 a	7.4 ± 1.6 a
D	0.04 ± 0.14 a	0.67 ± 0.06 b
H	0.55 ± 0.19 a	1.2 ± 0.18 b
J	0.80 ± 0.26 a	0.91 ± 0.63 a

After harvesting *Kosteletzkya pentacarpos*, its seeds, roots and leaves can be achieved to hierarchically multi-level development (Fig. 4). Previous data shows that seeds are regarded as material for oil production containing high quantity and quality of amino acid (especially glutamate and aspartic acid) and oil (> 20%). In terms of roots, the fine powder contains high amount of polysaccharide (690 mg/g), saponins (69.4 mg/g) and flavonoids (11.9 mg/g), which has already produced wine (Chinese name: 海滨锦葵露酒), feed additives as well as health care products. At last, the flower of *Kosteletzkya pentacarpos* is considered as the auxiliary material for cooking (Qin et al. 2015).

4.4. Interest for Phytoremediation

Apart from high salinity tolerance ability in *Kosteletzkya pentacarpos*, this halophyte plant species is also proven to have great interest for phytoremediation in heavy metals polluted area. When plants were treated in 10 μM Cu, 100 μM Zn and 10 μM Cd respectively, heavy metals were mostly accumulated in plant root up to 1500 mg/kg dry weight (DW) Cu, 5000 mg/kg DW Zn as well as 1500 mg/kg DW Cd.

However, the retention of Cu, Zn and Cd are 30, 5 and 3 times less in leaf (heavy metal concentration in stem is similar to it in leaf) (Han et al, 2011, Han et al, 2012).

Although all plants exposed to Cu, Zn and Cd stress were alive, the accumulation of heavy metals indeed had a negative impact on plant growth and development (Han et al, 2012; Han et al, 2013). Results showed a strong inhibition effect of Cd on leaf emergence, lateral branch development and leaf expansion. Heavy metals induced a significant decrease in plant dry weight, water content, osmotic potential and leaf water potential. The metal toxicity inhibited the photosynthesis efficiency with reduction in chlorophyll concentration. Cd induced oxidative stress in relation to an increase in $O_2^{\bullet -}$ and H_2O_2 concentration and lead to a decrease in endogenous glutathione (GSH) and α-tocopherol in the leaves. Cd not only increased leaf zeatin and zeatin riboside concentration but also increased the senescing compounds 1-aminocyclopropane-1-carboxylic acid (ACC) and abscisic acid (ABA) (Han et al. 2013).

In the presence of heavy metal excess, salinity decreased Cu, Cd and Zn accumulation in plant organs (Han et al, 2012). Salinity also delayed the Cd-induced leaf senescence: NaCl reduced the deleterious impact of Cd on photosynthesis apparatus through an improvement of F_v/F_m, Y(II) and ETR (Han et al, 2012). Salt reduced oxidative stress in Cd-treated plants through an increase in GSH, α-tocopherol and ascorbic acid synthesis and an increase in glutathione reductase activity. Additional salt reduced ACC and ABA accumulation in Cd+NaCl-treated leaves comparing to Cd alone. Exogenous NaCl neutralized the damaging action of Zn and modified the Zn distribution through a preferential accumulation of toxic ions in older leaves (Han et al. 2013). Dry powder of the roots from the wetland halophyte species *Kosteletzkya pentacarpos* grown in the presence or in the absence of 50 mM NaCl was used for biosorption of Cd and Zn. The use of roots collected from plants grown for 16 weeks in the

presence of NaCl improved sorption process, especially for Zn (Lutts et al. 2016).

In summary, the positive impact of NaCl on *K. pentacarpos* response to polymetallic pollution made this species a promising candidate for revegetation of heavy metal contaminated salt areas. Although salinity reduces heavy metal accumulation in general, it does improve plant growth, alleviate toxicity and decrease oxidative damage induced by heavy metals so that it extends the plant life to make it better for phytoremediation (Han et al, 2013). Indeed, it still has a problem that how to enhance the efficiency of phytoremediation because salinity decreases HMs translocation. In addition, soil is a quite complex environment, which comprises organisms as well as numerous organic substances, including organic pollutants (multi-pollutant). In future, the strategy to improve phytoremediation potent of seashore mallow in complex multi-polluted environment appears as an interesting challenge.

Reference

Acosta JA, Jansen B, Kalbitz K, Faz A, Martinez-Martinez S (2011): Salinity increases mobility of heavy metals in soils. Chemosphere 85, 1318-24

Alloway B (1995): Heavy metals in soils. Springer

Arora S, Rao GG (2017): Bio-amelioration of Salt-Affected Soils Through Halophyte Plant Species. 71-85

Ayoubi S, Adman V, Yousefifard M (2019): Use of magnetic susceptibility to assess metals concentration in soils developed on a range of parent materials. Ecotoxicol Environ Saf 168, 138-145

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. plant and soil 39 (1): 205-207

Bhargava A, Carmona FF, Bhaegava M, Srivastava S. 2012. Approaches for enhanced phytoextraction of heavy metals. Journal of Environmental Management 105: 103-120.

Blanchard OJ, Jr. (2013): Experimental hybridization and chromosome pairing in *Kosteletzkya* (Malvaceae, Malvoideae, Hibisceae), and possible implications for phylogeny and phytogeography in the genus. Comp Cytogenet 7, 73-101

Blits KC, Gallagher JL (1990): Effect of NaCl on lipid content of plasma membranes isolated from roots and cell suspension cultures of the dicot halophyte *Kosteletzkya virginica* (L.) Presl. Plant Cell Rep 9, 156-9

Calatayud S, Garcia-Risco M, Rojas NS, Espinosa-Sanchez L, Artime S, Palacios O, Canestro C, Albalat R (2018): Metallothioneins of the urochordate *Oikopleura dioica* have Cys-rich tandem repeats, large size and cadmium-binding preference. Metallomics 10, 1585-1594

Cherif J, Mediouni C, Ben Ammar W, Jemal F (2011): Interactions of zinc and cadmium toxicity in their effects on growth and in antioxidative systems in tomato plants (*Solanum lycopersicum*). J Environ Sci (China) 23, 837-44

Cherif J, Derbel N, Nakkach M, von Bergmann H, Jemal F, Lakhdar ZB (2012): Spectroscopic studies of photosynthetic responses of tomato plants to the interaction of zinc and cadmium toxicity. J Photochem Photobiol B 111, 9-16

Chu B, Chen X, Li Q, Yang Y, Mei X, He B, Li H, Tan L (2014): Effects of salinity on the transformation of heavy metals in tropical estuary wetland soil. Chemistry and Ecology 31, 186-198

Cobbett CS (2000): Phytochelatins and their roles in heavy metal detoxification. Plant Physiol 123, 825-32

Das P, Samantaray S, Tout GR (1997): Studies on cadmium toxicity in plants: A review. Environmental Pollution 98(1): 29-36

Demidchik V, Maathuis FJ (2007): Physiological roles of nonselective cation channels in plants: from salt stress to signalling and development. New Phytol 175, 387-404

Feng JX, Lin YY, Yang Y, Shen QQ, Huang JR, Wang SG, Zhu XS, Li ZF (2018): Tolerance and bioaccumulation of Cd and Cu in *Sesuvium portulacastrum*. Ecotoxicology and Environmental Safety 147: 06-312

Fitt GP, Mares CL, Llewellyn DJ (2008). Field evaluation and potential ecological impact of transgenic cottons (*Gossypium hirsutum*) in Australia. Biocontrol Science and Technology: 535-548

Flowers TJ, Hajibagheri MA, Clipson NJW (1986): Halophyte. The Quarterly review of biology, 61: 313-337.

Gao X, Li P, Chen CT (2013): Assessment of sediment quality in two important areas of mariculture in the Bohai Sea and the northern Yellow Sea based on acid-volatile sulfide and simultaneously extracted metal results. Mar Pollut Bull 72, 281-8

Ghanem ME, Han RM, Classen B, Quetin-Leclerq J, Mahy G, Ruan CJ, Qin P, Perez-Alfocea F, Lutts S (2010): Mucilage and polysaccharides in the halophyte plant species *Kosteletzkya virginica*: localization and composition in relation to salt stress. J Plant Physiol 167, 382-92

Glass DJ. 1999. U. S. and International Markets for Phytoremediation, 1999-2000; D. Glass Associates, Inc.: Needham, MA.

Graham RD, Stangoulis JC (2003): Trace element uptake and distribution in plants. J Nutr 133, 1502S-5S

Guerinot ML (2000): The ZIP family of metal transporters. Biochim Biophys Acta 1465, 190-8

Guo X, Wang W, Yuan X, Yang Y, Tian Q, Xiang Y, Sun Y, Bai Z (2019): Heavy

metal redistribution mechanism assisted magnetic separation for highly-efficient removal of lead and cadmium from human blood. J Colloid Interface Sci 536, 563-574

Gupta DK, Chatterjee S, Datta S, Voronina AV, Walther C (2017): Radionuclides: Accumulation and Transport in Plants. Rev Environ Contam Toxicol 241, 139-160

Han F, Shan XQ, Zhang J, Xie YN, Pei ZG, Zhang SZ, Zhu YG, Wen B (2005): Organic acids promote the uptake of lanthanum by barley roots. New Phytol 165, 481-92

Han R-M, Lefèvre I, Ruan C-J, Beukelaers N, Qin P, Lutts S (2011): Effects of Salinity on the Response of the Wetland Halophyte *Kosteletzkya virginica* (L.) Presl. to Copper Toxicity. Water, Air, & Soil Pollution 223, 1137-1150

Han R-M, Lefèvre I, Ruan C-J, Qin P, Lutts S (2012): NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regulation 68, 97-109

Han RM, Lefevre I, Albacete A, Perez-Alfocea F, Barba-Espin G, Diaz-Vivancos P, Quinet M, Ruan CJ, Hernandez JA, Cantero-Navarro E, Lutts S (2013): Antioxidant enzyme activities and hormonal status in response to Cd stress in the wetland halophyte *Kosteletzkya virginica* under saline conditions. Physiol Plant 147, 352-68

Han RM, Muriel Q, André E, Johannes Teun van Elteren, Destrebecq F, Vogel-Mikuš K, Cui GL, Debeljak M, Lefèvre I, Lutts S (2013): Accumulation and distribution of Zn in the shoots and reproductive structures of the halophyte plant species *Kosteletzkya virginica* as a function of salinity. Planta 238 (3): 441-457

Hatje V, Payne TE, Hill DM, McOrist G, Birch GF, Szymczak R (2003): Kinetics of trace element uptake and release by particles in estuarine waters: effects of pH, salinity, and particle loading. Environ Int 29, 619-29

Hennes S (2007): Guidance for the use and application of sediment quality targets for the protection of sediment-dwelling organisms in Minnesota. Book

Hu Y, Lu L, Tian S, Li S, Liu X, Gao X, Zhou W, Lin X (2019): Cadmium-induced nitric oxide burst enhances Cd tolerance at early stage in

roots of a hyperaccumulator *Sedum alfredii* partially by altering glutathione metabolism. Sci Total Environ 650, 2761-2770

Jha Y and Subramanian RB (2014): PGPR regulate caspase-like activity, programmed cell death, and antioxidant enzyme activity in paddy under salinity. Physiology and Molecular Biology of Plants 20 (2): 201-207

Jia H, Wang X, Dou Y, Liu D, Si W, Fang H, Zhao C, Chen S, Xi J, Li J (2016): Hydrogen sulfide - cysteine cycle system enhances cadmium tolerance through alleviating cadmium-induced oxidative stress and ion toxicity in *Arabidopsis* roots. Sci Rep 6, 39702

Karenlampi S, Schat H, Vangronsveld J, Verkleij JA, van der Lelie D, Mergeay M, Tervahauta AI (2000): Genetic engineering in the improvement of plants for phytoremediation of metal polluted soils. Environ Pollut 107, 225-31

Khaliq MA, James B, Chen YH, Ahmed Saqib HS, Li HH, Jayasuriya P, Guo W (2019): Uptake, translocation, and accumulation of Cd and its interaction with mineral nutrients (Fe, Zn, Ni, Ca, Mg) in upland rice. Chemosphere 215, 916-924

Khatun MA, Hossain MM, Bari MA, Abdullahil KM, Parvez MS, Alam MF, Kabir AH (2018): Zinc deficiency tolerance in maize is associated with the up-regulation of Zn transporter genes and antioxidant activities. Plant Biol (Stuttg) 20, 765-770

Kim RY, Yoon JK, Kim TS, Yang JE, Owens G, Kim KR (2015): Bioavailability of heavy metals in soils: definitions and practical implementation--a critical review. Environ Geochem Health 37, 1041-61

Kupper H, Mijovilovich A, Meyer-Klaucke W, Kroneck PM (2004): Tissue- and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by x-ray absorption spectroscopy. Plant Physiol 134, 748-57

Ladeiro B (2012): Saline Agriculture in the 21st Century: Using Salt Contaminated Resources to Cope Food Requirements. Journal of Botany (2012).

Lanno R, Wells J, Conder J, Bradham K, Basta N (2004): The bioavailability of chemicals in soil for earthworms. Ecotoxicology and Environmental Safety 57(1): 39-47

Le Beau MM, Diaz MO, Karin M, Rowley JD (1985): Metallothionein gene cluster is split by chromosome 16 rearrangements in myelomonocytic leukaemia. Nature 313, 709-11

Li LZ, Tu C, Peijnenburg W, Luo YM (2017): Characteristics of cadmium uptake and membrane transport in roots of intact wheat (*Triticum aestivum* L.) seedlings. Environ Pollut 221, 351-358

Lin W, Wu K, Lao Z, Hu W, Lin B, Li Y, Fan H, Hu J (2019): Assessment of trace metal contamination and ecological risk in the forest ecosystem of dexing mining area in northeast Jiangxi Province, China. Ecotoxicol Environ Saf 167, 76-82

Ling S, Cui X, Zhang X, Liu B, He C, Wang J, Qin W, Zhang Y, Gao Y, Bai G (2018): Glutathione-protected gold nanocluster decorated cadmium sulfide with enhanced photostability and photocatalytic activity. J Colloid Interface Sci 530, 120-126

Liu X, Shen X, Lai Y, Ji K, Sun H, Wang Y, Hou C, Zou N, Wan J, Yu J (2016): Toxicological proteomic responses of halophyte *Suaeda salsa* to lead and zinc. Ecotoxicol Environ Saf 134P1, 163-171

Lu Q, Zhang T, Zhang W, Su C, Yang Y, Hu D, Xu Q (2018): Alleviation of cadmium toxicity in *Lemna minor* by exogenous salicylic acid. Ecotoxicol Environ Saf 147, 500-508

Lue-Kim H, Rauser WE (1986): Partial characterization of cadmium-binding protein from roots of tomato. Plant Physiol 81, 896-900

Lutts S, Lefevre I (2015): How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? Ann Bot 115, 509-28

Lutts S, Qin P, Han RM (2016): Salinity influences biosorption of heavy metals by the roots of the halophyte plant species *Kosteletzkya pentacarpos*. Ecological engineering 95: 682-689

Ma YL, Wang HF, Wang P, Yu CG, Luo SQ, Zhang YF, Xie YF (2018): Effects of cadmium stress on the antioxidant system and chlorophyll fluorescence characteristics of two Taxodium clones. Plant Cell Rep 37, 1547-1555

Manzoor R, Zhang T, Zhang X, Wang M, Pan JF, Wang Z, Zhang B (2018): Single and combined metal contamination in coastal environments in China: current status and potential ecological risk evaluation. Environ Sci Pollut

Res Int 25, 1044-1054

Marrugo-Negrete J, Pinedo-Hernandez J, Diez S (2017): Assessment of heavy metal pollution, spatial distribution and origin in agricultural soils along the Sinu River Basin, Colombia. Environ Res 154, 380-388

Mei X, Li S, Li Q, Yang Y, Luo X, He B, Li H, Xu Z (2014): Sodium chloride salinity reduces Cd uptake by edible amaranth (*Amaranthus mangostanus* L.) via competition for Ca channels. Ecotoxicol Environ Saf 105, 59-64

Menzel U, Lieth H (2003): HALOPHYTE Database V. 2.0 update. In Lieth H, Mochechenko M. Cash Crop Halophyte. Dordrecht, the Netherlands: Kluwer

Migocka M, Malas K, Maciaszczyk-Dziubinska E, Papierniak A, Posyniak E, Garbiec A (2018): Cucumber metal tolerance protein 7 (CsMTP7) is involved in the accumulation of Fe in mitochondria under Fe excess. Plant J 95, 988-1003

Morina F, Jovanovic L, Mojovic M, Vidovic M, Pankovic D, Veljovic Jovanovic S (2010): Zinc-induced oxidative stress in *Verbascum thapsus* is caused by an accumulation of reactive oxygen species and quinhydrone in the cell wall. Physiol Plant 140, 209-24

Moser BR, Dien BS, Seliskar DM, Gallagher JL (2013): Seashore mallow (*Kosteletzkya pentacarpos*) as a salt-tolerant feedstock for production of biodiesel and ethanol. Renewable Energy 50: 833-839

Mucha AP, Almeida CMR, Bordalo AA, Vasconcelos MTSD (2005): Exudation of organic acids by a marsh plant and implications on trace metal availability in the rhizosphere of estuarine sediments. Estuarine, Coastal and Shelf Science 65, 191-198

Nadgorska-Socha A, Kandziora-Ciupa M, Trzesicki M, Barczyk G (2017): Air pollution tolerance index and heavy metal bioaccumulation in selected plant species from urban biotopes. Chemosphere 183, 471-482

Nahar K, Chowdhury MAK, Chowdhury MAH, Rahman A, Mohiuddin KM (2018): Heavy metals in handloom-dyeing effluents and their biosorption by agricultural byproducts. Environ Sci Pollut Res Int 25, 7954-7967

Nan Z, Li J, Zhang J, Cheng G (2002): Cadmium and zinc interactions and their transfer in soil-crop system under actual field conditions. Sci Total Environ

285, 187-95

Newkirk GM, Wu H, Santana I, Giraldo JP (2018): Catalytic Scavenging of Plant Reactive Oxygen Species In Vivo by Anionic Cerium Oxide Nanoparticles. J Vis Exp

Nikalje GC, Suprasanna P (2018): Coping with Metal Toxicity - Cues From Halophytes. Front Plant Sci 9, 777

Oh K, Li T, Cheng HY, Hu XF, Lin Q, Xie YH (2013): A Primary Study on Assessment of Phytoremediation Potential of Biofuel Crops in Heavy Metal Contaminated Soil. Applied Mechanics and Materials 295-298: 1135-1138

Olechnowicz J, Tinkov A, Skalny A, Suliburska J (2018): Zinc status is associated with inflammation, oxidative stress, lipid, and glucose metabolism. J Physiol Sci 68, 19-31

Pittman JK, Hirschi KD (2016): CAX-ing a wide net: Cation/H(+) transporters in metal remediation and abiotic stress signalling. Plant Biol (Stuttg) 18, 741-9

Pellegrini E, Petranich E, Acquavita A, Canário J, Emili A, Covelli S (2017): Mercury uptake by halophytes in response to a long-term contamination in coastal wetland salt marshes (northern Adriatic Sea). Environmental Geochemistry and Health 39 (6): 1273-1289

Peijnenburg W, Posthuma L, Eijsackers H, Allen H (1997): A conceptual framework for implementation of bioavailability of metals for environmental managementpurposes. Ecotoxicology and Environmental Safety 37,163-172

Petranich E, Acquavita A, Covelli S (2017): Potential bioaccumulation of trace metals in halophytes from salt marshes of a northern Adriatic coastal lagoon. Journal of Soils and Sediments 17 (7): 1986-1998

Petruzzelli G (1989): Recycling wastes in agriculture: heavy metal bioavailability. Agriculture, Ecosystems & Environment, 27 (1-4), 493-503

Qin P, Han R-m, Zhou M-x, Zhang H-s, Fan L-s, Seliskar DM, Gallagher JL (2015): Ecological engineering through the biosecure introduction of *Kosteletzkya virginica* (seashore mallow) to saline lands in China: A review of 20 years of activity. Ecological Engineering 74, 174-186

Rengasamy P (2006): World salinization with emphasis on Australia. J Exp Bot 57, 1017-23

Rizwan M, Ali S, Ali B, Adrees M, Arshad M, Hussain A, Zia Ur Rehman M, Waris AA (2019): Zinc and iron oxide nanoparticles improved the plant growth and reduced the oxidative stress and cadmium concentration in wheat. Chemosphere 214, 269-277

Rodriguez-Estival J, Sanchez MI, Ramo C, Varo N, Amat JA, Garrido-Fernandez J, Hornero-Mendez D, Ortiz-Santaliestra ME, Taggart MA, Martinez-Haro M, Green AJ, Mateo R (2019): Exposure of black-necked grebes (*Podiceps nigricollis*) to metal pollution during the moulting period in the Odiel Marshes, Southwest Spain. Chemosphere 216, 774-784

Rom S, Gilad A, Kalifa Y, Konrad Z, Karpasas MM, Goldgur Y, Bar-Zvi D (2006): Mapping the DNA- and zinc-binding domains of ASR1 (abscisic acid stress ripening), an abiotic-stress regulated plant specific protein. Biochimie 88, 621-8

Ruan C-J, Li H, Mopper S (2008): *Kosteletzkya virginica* displays mixed mating in response to the pollinator environment despite strong inbreeding depression. Plant Ecology 203, 183-193

Ruan CJ, Mopper S, da Silva JAT, Qin P, Zhang QX, Shan Y (2009): Context-dependent style curvature in *Kosteletzkya virginica* (Malvaceae) offers reproductive assurance under unpredictable pollinator environments. Plant Systematics and Evolution 277, 207-215

Ryu H and Cho YG (2015): Plant hormones in salt stress tolerance. Journal of Plant Biology 58 (3): 147-155

Saifullah, Sarwar N, Bibi S, Ahmad M, Ok YS (2013): Effectiveness of zinc application to minimize cadmium toxicity and accumulation in wheat (*Triticum aestivum* L.). Environmental Earth Sciences 71, 1663-1672

Salzman MB, Smith EM, Koo C (2002): Excessive oral zinc supplementation. J Pediatr Hematol Oncol 24, 582-4

Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse A, Marcus MA, Manceau A (2002): Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. Plant Physiol 130, 1815-26

Sarwar N, Ishaq W, Farid G, Shaheen MR, Imran M, Geng M, Hussain S (2015): Zinc-cadmium interactions: Impact on wheat physiology and mineral acquisition. Ecotoxicol Environ Saf 122, 528-36

Semida WM, Hemida KA, Rady MM (2018): Sequenced ascorbate-proline-glutathione seed treatment elevates cadmium tolerance in cucumber transplants. Ecotoxicol Environ Saf 154, 171-179

Senovilla M, Castro-Rodriguez R, Abreu I, Escudero V, Kryvoruchko I, Udvardi MK, Imperial J, Gonzalez-Guerrero M (2018): Medicago truncatula copper transporter 1 (MtCOPT1) delivers copper for symbiotic nitrogen fixation. New Phytol 218, 696-709

Sharma SS, Dietz K-J, Mimura T (2018): Vacuolar compartmentalization as indispensable component of heavy metal detoxification in plants. Plant cell and environment 39(5): 1112-1126

Shelden MC, Dias DA, Jayasinghe NS, Bacic A, Roessner U (2016): Root spatial metabolite profiling of two genotypes of barley (*Hordeum vulgare* L.) reveals differences in response to short-term salt stress. J Exp Bot 67, 3731-45

Siebe (1998): Nutrient inputs to soils and their uptake by alfalfa through long-term irrigation with untreated sewage effluent in Mexico. Soil use and management 14: 119-122

Stocker O (1928): Das Halophytenproblem. Ergeb. Biol 3: 265-353.

Szabo S, Hossain MS, Adger WN, Matthews Z, Ahmed S, Lazar AN, Ahmad S (2016): Soil salinity, household wealth and food insecurity in tropical deltas: evidence from south-west coast of Bangladesh. Sustain Sci 11, 411-421

Takahashi R, Bashir K, Ishimaru Y, Nishizawa NK, Nakanishi H (2012): The role of heavy-metal ATPases, HMAs, in zinc and cadmium transport in rice. Plant Signal Behav 7, 1605-1607

Tsao D. 1999. The industrialist's perspective-Can phytoremediation really deliver what industry needs? In IBC's 4th Annual International Conference on Phytoremediation, Toronto, Ontario, Canada; June 23-25, 1999; International Business Communications: Southborough, MA.

Van-Camp L, Bujarrabal B, Gentile AR, Montanarella L, Olazabal C, Selvaradjou SK (2004): REPORTS OF THE TECHNICAL WORKING

GROUPS: ESTABLISHED UNDER THE THEMATIC STRATEGY FOR SOIL PROTECTION.

Van Der Lelie D, Schwitzguebel JP, Glass DJ, Vangronsveld J, Baker A (2001): Assessing phytoremediation's progress in United States and Europe. Environmental Science and Technology 1: 447-452.

Vatamaniuk OK, Mari S, Lu YP, Rea PA (2000): Mechanism of heavy metal ion activation of phytochelatin (PC) synthase: blocked thiols are sufficient for PC synthase-catalyzed transpeptidation of glutathione and related thiol peptides. J Biol Chem 275, 31451-9

Wang J, Evangelou BP, Nielsen MT, Wagner GJ (1992): Computer, Simulated Evaluation of Possible Mechanisms for Sequestering Metal Ion Activity in Plant Vacuoles: II. Zinc. Plant Physiol 99, 621-6

Weinthal D, Tovkach A, Zeevi V, Tzfira T (2010): Genome editing in plant cells by zinc finger nucleases. Trends Plant Sci 15, 308-21

Wu X, Zhu ZB, Chen JH, Huang YF, Liu ZL, Zou JW, Chen YH, Su NN, Cui J (2019): Transcriptome analysis revealed pivotal transporters involved in the reduction of cadmium accumulation in pak choi (*Brassica chinensis* L.) by exogenous hydrogen-rich water. Chemosphere 216, 684-697

Xu X, Zhang S, Xian J, Yang Z, Cheng Z, Li T, Jia Y, Pu Y, Li Y (2018): Subcellular distribution, chemical forms and thiol synthesis involved in cadmium tolerance and detoxification in *Siegesbeckia orientalis* L. Int J Phytoremediation 20, 973-980

Yang Q, Li Z, Lu X, Duan Q, Huang L, Bi J (2018): A review of soil heavy metal pollution from industrial and agricultural regions in China: Pollution and risk assessment. Sci Total Environ 642, 690-700

Yun SW, Baveye PC, Kim DH, Kang DH, Lee SY, Kong MJ, Park CG, Kim HD, Son J, Yu C (2018): Analysis of metal(loid)s contamination and their continuous input in soils around a zinc smelter: Development of methodology and a case study in South Korea. Environ Pollut 238, 140-149

Zhang C, Yu ZG, Zeng GM, Jiang M, Yang ZZ, Cui F, Zhu MY, Shen LQ, Hu L (2014): Effects of sediment geochemical properties on heavy metal bioavailability. Environment International 73: 270-281

Zhang HS, Qin FF, Qin P, Pan SM (2014): Evidence that arbuscular mycorrhizal

and phosphate-solubilizing fungi alleviate NaCl stress in the halophyte *Kosteletzkya virginica*: nutrient uptake and ion distribution within root tissues. Mycorrhiza 24, 383-95

Zhang Z, Wang JJ, Ali A, DeLaune RD (2016): Heavy metal distribution and water quality characterization of water bodies in Louisiana's Lake Pontchartrain Basin, USA. Environ Monit Assess 188, 628

CHAPTER 1 NaCl impact on *Kosteletzkya pentacarpos* seedlings simultaneously exposed to cadmium and zinc toxicities

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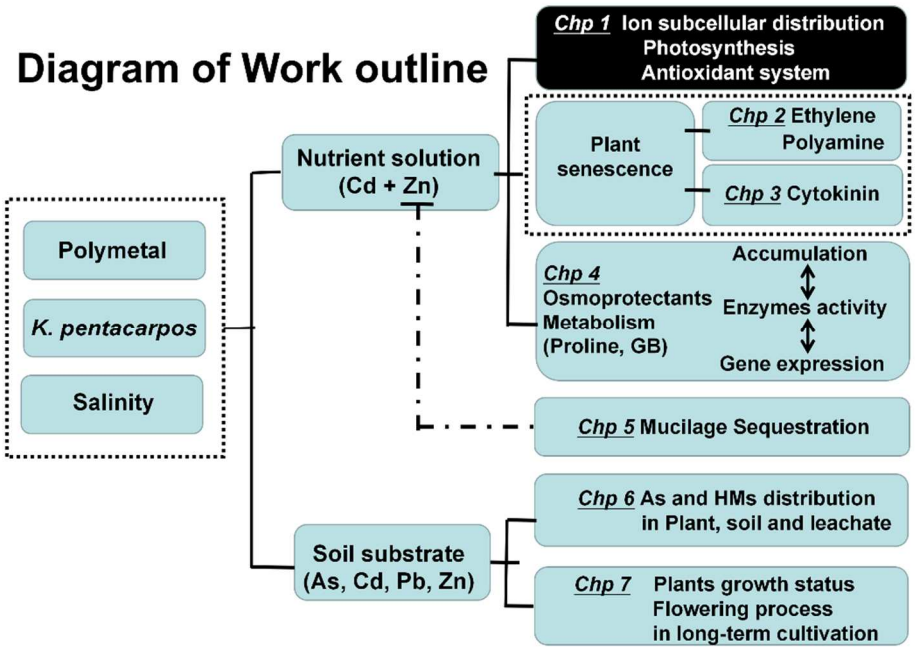
Diagram of Work outline
Chp 1 Ion subcellular distribution
Photosynthesis
Antioxidant system
Plant
senescence
Chp 2 Ethylene
Polyamine
Chp 3 Cytokinin
Nutrient solution
(Cd + Zn)
Polymetal
K. pentacarpos
Salinity
Chp 4
Osmoprotectants
Metabolism
(Proline, GB)
Accumulation
Enzymes activity
Gene expression
Chp 5 Mucilage Sequestration
Chp 6 As and HMs distribution
in Plant, soil and leachate
Soil substrate
(As, Cd, Pb, Zn)
Chp 7 Plants growth status
Flowering process
in long-term cultivation

Chapter 1

Abstract

Data regarding NaCl impact on halophyte plant species exposed to a polymetallic contamination remain scarce. Seedlings of the salt-marsh species *Kosteletzkya pentacarpos* were simultaneously exposed to cadmium (10 µM) and zinc (100 µM) in the absence or presence of 50 mM NaCl. Heavy metal exposure reduced plant growth and increased Cd and Zn concentrations in all organs. Cd and Zn accumulation reduced net photosynthesis in relation to stomatal closure, decreased in chlorophyll concentration and alteration in chlorophyll fluorescence-related parameters. Salinity reduced Cd and Zn bioaccumulation and translocation, with a higher impact on Cd than Zn. It mitigated the deleterious impact of heavy metals on photosynthetic parameters. NaCl reduced the heavy metals-induced oxidative stress assessed by malondialdehyde, carbonyl and H_2O_2 concentration. Subcellular distribution revealed that Cd mainly accumulated in the cell walls but NaCl increased it in the cytosol fraction in the leaf and in the metal-rich granule fraction in the roots. It had no impact on Zn subcellular distribution. The additional NaCl contributed to a higher sequestration of Cd on phytochelatins and stimulated glutathione synthesis. The positive impact of NaCl on *K. pentacarpos* response to polymetallic pollution made this species a promising candidate for revegetation of heavy-metal contaminated salt areas.

Keywords Halophyte, Heavy metal, Cadmium, Zinc, Phytoremediation, Salinity

1. Introduction

The increasing anthropogenic activities, such as mining, agriculture, metallurgy, combustion of fossil fuels and military operations, have led to widespread contamination of the environment (Ogundele et al. 2017; Różański et al. 2017). In addition to organic contamination, heavy metals such as cadmium, zinc, lead, copper, mercury and nickel constitute the most hazardous soil pollutants. Although some of them (Cu, Zn) are essential elements for living organisms, their presence in the soil at high concentrations are deleterious to plant growth and survival. Heavy metals impair plant photosynthesis, induce oxidative stress, affect plant mineral nutrition, compromise the plant water and hormonal status (Tattibayeva et al. 2016; Wang et al. 2015; Shahid et al. 2014).

The majority of studies devoted to heavy metal impact on plant behavior consider one single heavy metal. However, in real metal-polluted environmental settings, several heavy metals often coexist which interact with each other in a complex way. This is especially the case for Cd and Zn, which are frequently simultaneously present on polluted soils (Mani et al. 2015). Although these elements share several physico-chemical properties, they have quite different impact on plant physiology since Zn is an essential element while Cd has no recognized biological functions. Cadmium and zinc may have different distributions in heavy-metal treated plants (Lefèvre et al. 2014) and may bind to different complexing compounds (Lefèvre et al. 2016). However, their putative interaction in the case of polymetallic contamination remain poorly documented. It might be argued that cadmium and zinc interact, to some extent, not only in terms of absorption but also in terms of plant response to accumulated ions (Qiu et al. 2011). Moreover, the overall plant response is directly influenced by ion distribution and the presence of NaCl may somewhat affect both processes in a complex way (Lutts and Lefèvre 2015).

Heavy metal pollution is frequently reported in coastal areas leading to imbalance of soil ecological system and serious threat to food safety (El Nemr and El-Said 2017). Hence, it is of crucial importance to study the behavior of plants simultaneously exposed to salinity and heavy metal conditions. Halophyte species have been considered as promising material for phytoremadiation of heavy-metal contaminated sites and moderate doses of salt is thought to play a role in the protection against heavy metal toxicity in these species (Lutts and Lefèvre 2015). Once again, such a protective effect has often been tested on plants exposed to one single heavy metal stress (Nawaz et al. 2017; Zhang et al. 2016). For instance, salinity improved zinc tolerance by the halophyte *Spartina densiflora* in relation to maintenance of photosynthetic apparatus and mineral nutrition (Redondo-Gomez et al. 2011). Salinity also improved Cd tolerance in *Sesuvium portulacastrum*, which could be explained by an increase in glutathione (GSH), proline concentration, maintenance of the redox balance and photosynthesis (Wali et al. 2015, 2016) although it has been recently demonstrated by Wali et al. (2017) that Cd hampered salt tolerance in this species.

Kosteletzkya pentacarpos (L.) Presl. (formerly designed as *Kosteletzkya virginica*) is a perennial dicot halophyte species of Malvaceae family, and it is recommended as a potential resource for food, feed, biodiesel as well as health care (Halchak et al. 2011; Qin et al. 2015; Vaughn et al. 2013). *Kosteletzkya pentacarpos* is able to cope with a high level of salinity in its natural environment (up to 420 mM NaCl), exhibiting a high selectivity for K over Na (Blits and Gallagher 1990). Ghanem et al. (2010) demonstrated that mucilage produced by the plant may be involved in Na fixation in stem, preventing accumulation of this toxic element in photosynthetic leaves.

Kosteletzkya pentacarpos is also, to some extent, able to cope with heavy metal pollution in salt marsh conditions and it could therefore be recommended as an interesting tool for phytomanagement of polluted coastal areas (Han et al. 2012). Han et al. (2013a) demonstrated that NaCl differently interfered with Cd and Zn

toxicities in this wetland species. Cadmium increased the leaf K concentration while Zn had an opposite effect. Salinity reduced Cd accumulation to a higher extent than Zn accumulation. Distribution of heavy metals among plant organs also appeared differently affected by salinity since Cd was reduced mainly in the leaves while Zn was reduced in the roots. Management of heavy metal oxidative stress by *K. pentacarpos* appeared as a crucial component of resistance to Cd (Han et al. 2013b) or Zn (Han et al. 2013a). These data, however, were obtained for plants exposed to one single pollutant (Cd or Zn) but this species never tested in the simultaneous presence of the two heavy metals.

The present work was undertaken in order to answer to the following questions: (1) How does the addition of NaCl influence the growth response of *K. pentacarpos* simultaneously exposed to cadmium and zinc stresses? (2) What is the effect of NaCl on Cd and Zn absorption and distribution in the case of polymetallic contamination? (3) What is the impact of a mixed pollution on the management of antioxidative status by the plant and NaCl influence on this property?

2. Materials and Methods

2.1. Plant material and culture condition

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 12h photoperiod [mean light intensity (PAR) = 150 μmoles 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 light irradiance of 300 µmol m^{-2} s^{-1}(PAR) at the top of the canopy.

After 10 days of acclimation in the absence of stress (25 days after sowing), NaCl, $CdCl_2$ and $ZnCl_2$ were added to containers in order to create four treatments: (1) Control; (2) 50 mM NaCl; (3) 10 µM $CdCl_2$+100 µM $ZnCl_2$ (HMs); (4) 10 µM $CdCl_2$+ 100 µM $ZnCl_2$ + 50 mM NaCl (HMs+Na). Solutions were readjusted every 2 days and renewed every week. The pH of solutions was set to 5.7 ± 0.02 with KOH. Three replications with 12 plants per replication and per treatment were used for the measurement of different parameters.

2.2. Growth assessment

After two weeks of treatment stem height, number of lateral branches (LBs), leaf number on the main stem (LN), number of leaves on LBs and total length of LBs were recorded. Plants were then harvested. Roots were quickly rinsed in sterile deionized water for 30 s to remove ions from the free space and gently blotted dry with a paper towel. Roots, stems and leaves of each plant were separated and weighed for fresh weight determination. Before harvest, total leaf area of each plant was measured with a leaf area meter (AM300 Leaf area meter; ADC BioScientific Ltd., Hoddesdon, UK). Material was then incubated in an oven for 72h at 70 °C for dry weight determination.

2.3. Evaluation of ion concentration

Dried samples were ground to a fine powder using a porcelain mortar

and a pestle, digested in 35% HNO_3 and evaporated to dryness on a sand bath at 80 °C. The minerals were incubated with a mix of 37% HCl and 68% HNO_3 (3:1) and the mixture was slightly evaporated. Minerals were dissolved in HCl 0.1N. Ion concentrations were determined by SOLAAR S4 atomic absorption spectrometry (Thermo Scientific, Cambridge, UK). For each treatment, three separated plants were considered and each analysis was performed on technical triplicates.

Translocation factor (TF) is representative of the plant's ability to translocate pollutant from the root to the shoot system. In the present study, it was estimated for Cd and Zn on the basis of concentration (TF_c) and on the basis of the total amount (TF_a) of translocated pollutants according to:

TF_c Concentration in the shoot (mg g^{-1} DW) / Concentration in the roots (mg g^{-1} DW)

TF_a Total amount in the shoot (mg) / Total amount in the roots (mg)

The bioaccumulation factor (BF) is an indicator of the plant's ability to accumulate the heavy metal in harvestable organs comparatively to its mean concentration in the environment. Since a nutrient solution was used in the present experiment, we expressed shoot Cd and Zn concentration on a tissue water content basis for BF calculation according to:

BF Shoot concentration of heavy metal (µM) / concentration in the nutrient solution (µM)

2.4. Subcellular distribution of Cd and Zn

The subcellular distribution of Cd and Zn in *K. pentacarpos* roots and shoots was performed at 4 °C according to Weigel and Jager (1980) with some modification (Li et al. 2011): 0.2 g fresh material was ground with liquid nitrogen and homogenized in the 5.0 mL buffer

solution (0.25 M sucrose, 1.0 mM dithioerythritol and 50 mM Tris-HCl (pH 7.5)). The homogenate was centrifuged at 2500 *g* for 20 min. The supernatant of this first step was regarded as cytosol fraction. After adding 2.0 mL ultra-pure water, the pellet was heated at 100 °C for 2 min, followed by adding 2.0 mL NaOH (1M) and heated again at 70 °C for 1h. Samples were then centrifuged at 10,000 *g* for 15 min. The supernatant and pellet were designated as cell debris fraction (containing mainly cell walls) and metal-rich granule fraction (granules containing heavy metals bound to sulfur, iron, calcium and carbonates) respectively. The three factions were separately analyzed for ion content by spectrometry as above described.

2.5. Photosynthesis-related parameters

Photosynthetic-related parameters were determined on leaves located at the middle portion of the main stem. A portable pulse - modulated chlorophyll fluorimeter (FMS2; Hansatech, King's Lynn, UK) was used to determine the chlorophyll fluorescence. All measurements were performed in the middle part of the abaxial side of the leaves. Leaf portions were acclimated to darkness for 30 min. The minimal fluorescence level (F_0) was measured by estimating the modulated light (0.1µmol m^{-2} s^{-1}). The maximal fluorescence level (F_m) with all photosystem II (PSII) reaction centers closed was determined by a 0.8 s saturating pulse at 18,000 µmol m^{-2} s^{-1} in dark-adapted leaves. Leaf was then continuously illuminated with white actinic light (600 mmol m^{-2} s^{-1}) for 3 min. The steady-state value of fluorescence (F_s) was recorded and a second saturating pulse at 18,000 µmol m^{-2} s^{-1} was imposed to determine maximal fluorescence level in the light-adapted state (F'_m). The actinic light was removed and the minimal fluorescence level in the light-adapted state (F'_0) was determined by illuminating the leaf with a 3 s pulse of far-red. Using both light and dark fluorescence parameters, the maximal efficiency of PSII photochemistry in the dark-adapted state (*Fv/Fm*), the photochemical quenching coefficient (qP), the non-photochemical quenching (NPQ) and the actual PSII efficiency (ΦPSII) were calculated according to Maxwell and Johnson (2000).

Chlorophyll (Chl *a* and Chl *b*) and total carotenoid (xanthophylls and β-carotene) concentrations were quantified on the whole leaves from seven plants per treatment after acetone extraction as previously described (Han et al. 2012). Net photosynthesis (*A*) was recorded with an infrared gas analyser (LCA4 8.7; ADC Bioscientific, Hoddesdon, Hertfordshire, UK) using a PLC Parkinson leaf cuvette on intact leaves for 1 min (20 records min^{-1}) with an air flow of 300 $mL.min^{-1}$. Air taken in the greenhouses was sent to a chamber into which a leaf portion of 3.5 cm^2 was introduced. The net CO_2 assimilation rate and instantaneous transpiration rate (*E*) were estimated on leaves located at the middle part of the main stem. Leaf stomatal conductance (g_s) was measured using a diffusion porometer (AP4; Delta-TDevices Ltd., Cambridge, UK). Five plants were measured for each treatment, and all measurements were performed around at midday (between 12:30 a.m. and 2:30 p.m.).

2.6. Oxidative stress parameters and non-enzymatic antioxidants

The level of lipid peroxidation was measured as 2-thiobarbituric acid-reactive substances, mainly malondialdehyde (MDA) (Heath and Packer 1968). The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. Carbonyl assay was performed using the Reznick and Packer (1994) spectrophotometric method detecting the product of the reaction of dinitrophenylhydrazine (DNPH) with protein carbonyls to form protein hydrazones.

For hydrogen peroxide quantification, samples (0.5 g FW) were ground to powder in the presence of 5 mL 5% TCA. The mixture was centrifuged at 10,000 *g* for 20 min at 4 °C. The supernatant was adjusted to pH 8.4 with 17 M ammonia solution and then filtered. The filtrate was divided into aliquots of 1 mL. To one of these (the blank) 8 µg of CAT (10,000 U mg^{-1}) were added and samples were then kept at room temperatures for 10 min. To both aliquots (with and without CAT), 1 mL of colorimetric reagent was added. The reaction solution was incubated for 10 min at 30 °C. Absorbance at 505 nm was

determined. The colorimetric reagent contained 10 mg of 4-aminoantipyrine, 10 mg of phenol and 5 mg of peroxidase (150 U mg^{-1}) dissolved in 50 ml of 100 mM acetic buffer (pH 5.6) (Zhou et al. 2006).

For ascorbate extraction, frozen tissues were homogenised in ice cold 5% metaphosphoric acid solution (1:5, *w/v*) and then centrifuged at 20,000 *g* and 4 °C for 10 min. Total ascorbate (AsA + DHA) contents were determined according to Wang et al. (1991) on the basis on Fe^{3+}-Fe^{2+} reduction by ascorbate in acid solution. Fe^{2+} forms a red chelate with bathophenanthroline absorbing at 534 nm. The ascorbate (reduced form) assay mixture contained 0.1 mL of the extract, 0.5 mL of absolute ethanol, 0.6 M trichloroacetic acid, 3 mM bathophenantroline, 8 mM H_3PO_4 and 0.17 mM $FeCl_3$. The final total volume was 1.5 mL and the mixture were allowed to stand at 30 °C for 90 min. The absorbance of the coloured solution was read at 534 nm. The total ascorbate assay mixture contained 0.1 mL of the sample, 0.15 mL of 3.89 mM dithiothreitol and 0.35 mL of absolute ethanol in a total volume of 0.6 mL. Then, the reaction mixture was left standing at room temperature for 10 min. After reduction of dehydroascorbate to ascorbate, 0.15 mL of 20% trichloroacetic acid was added and the colour was developed by adding 0.15 mL of 0.4% (*v/v*) H_3PO_4-ethanol, 0.3 mL of 0.5% (*w/v*) bathophenantroline-ethanol and 0.15 mL of 0.03% (*w/v*) $FeCl_3$-ethanol. Dehydroascorbate (DHA) concentrations were estimated from the difference of "total ascorbate" and ascorbate concentration. Standard curve in the range 0-10 µmol ascorbate was used.

For reduced (GSH) and total (GSHt) glutathione quantification, 200 mg of frozen samples were extracted and derivatized by orthophthalaldehyde according to Cereser et al. (2001). GSHt was quantified after a reduction step of oxidized glutathione (GSSG) by dithiotreitol. Extracts were filtered through 0.45 µm microfilters (Chromafil PES-45/15, Macherey-Nagel) prior to injection and OPA derivatives were separated on a reversed-phase HPLC column with an acetonitrile-sodium acetate gradient system and detected

fluorimetrically. 5 µL of sample were injected into a Shimadzu HPLC system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a Nucleodur C18 Pyramid column (125 x 4.6 mm internal diameter; 5 µm particle size) (Macherey-Nagel, Düren, Germany). Derivatives were eluted in acetonitrile gradient in a 50 mM sodium acetate buffer pH 6.2 at 30 °C at a flow rate of 0.7 mL min^{-1}. Fluorimetric detection was performed with a spectra system Shimadzu RF-20A fluorescence detector at 420 nm after excitation at 340 nm. GSH was quantified using nine-point calibration curves with custom-made external standard solutions ranging from 0.0625 to 50 µM and every ten injections, a check standard solution was used to confirm the calibration of the system. The recovery was determined using GSH as an internal standard.

To estimate the total global antioxidant activity, ferric reducing ability of plasma (FRAP) was assayed according to Benzie and Strain (1996) considering the ability of plant extract to reduce ferric to ferrous ion at low pH and to produce a colored ferrous-tripyridyltriazine complex which was spectrophotometrically detected at 593 nm. A second assay was performed using the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) decolorization procedure according to Pellegrini et al. (1999) assays were performed. Results are expressed in µM Trolox equivalents (TE) / g fresh mass.

2.7. Evaluation of total non-protein thiol (NPT) and phytochelatin (PC) content

The total NPT concentration was determined according to De Vos et al. (1992): 200 mg fresh weight of tissue was ground in 2 mL of 5 % (w/v) sulfosalicylic acid plus 6.3 mM diethylenetriaminepentaacetic acid (pH < 1) at 0 °C with quartz sand in a mortar. The homogenate was centrifuged at 10,000 g for 10 min at 4 °C. The supernatants were collected and used for the determination of thiols using Ellman's reagent. Three hundred microliters of supernatant were mixed with 630 µL of 0.5 M KH_2PO_4 and 25 µL of 10 mM 5,5-dithiobis

2-nitrobenzoic acid (final pH 7.0). The absorbance at 412 nm was recorded after 2 min, and the NPT concentration was estimated using an extinction coefficient of 13,600 M^{-1} cm^{-1}. Phytochelatins content was evaluated as difference between NPT and GSH levels (Schäfer et al. 1997).

2.8. Statistical analysis

For each treatment, three 50 L tanks containing 12 plants each were used in a randomized complete block design. Tissue material from five individual plants were dried in 70 °C oven, which were used to analyze for growth, water status and ionic determination, and seven remaining individual plants were frozen in liquid nitrogen immediately and analyzed for pigment content, HMs subcellular distribution, MDA, carbonyl, H_2O_2, antioxidant (GSH, GSSH, AsA and DHA), NPT, PCs as well as FRAP and ABTS. Each analysis was performed on technical triplicates. All of parameters data were subjected to an analysis of variance, one-way ANOVA, using SPSS software, with the treatment considered as the main factor. The statistical significance of the results was analyzed by Turkey test at 5% level ($P < 0.05$).

3. Results

3.1. Plant growth and water status

All plants remained alive until the end of the experiment. However, after 5 - 7 days of treatment, chlorosis and necrosis were observed on HM-treated plants only. Salinity had no significant impact on morphological properties (Table 1). In contrast, HMs in the absence of NaCl reduced the number of leaves on main stem (LN), number of lateral branches (LB) and number of leaves on lateral branches. The main stem length, the total branch length and the total leaf area were also clearly affected by HM treatment. The addition of NaCl partly alleviated the toxicity of HMs on all morphological parameters, except

on LN.

Table 1 Morphological parameters in seedlings of *Kosteletzkya pentacarpos* exposed during two weeks to heavy metals (HMs; $CdCl_2$ 10 µM + $ZnCl_2$ 100 µM) in the presence or in the absence of 50 mM NaCl. Each value is the mean of 5 ± S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

Parameters	Control	50 mM Na	HMs	HMs+Na
LN on main stems	15 ± 0.60 b	15 ± 0.80 b	6.4 ± 0.8 a	7.2 ± 0.8 a
LN on LBs	16 ± 0.70 c	16 ± 0.40 c	5.2 ± 0.4 a	8.8 ± 0.4 b
No. of LBs	7.6 ± 0.50 c	7.4 ± 0.50 c	3.2 ± 0.4 a	4.0 ± 0.7 b
Main stem length (cm)	45 ± 0.63 c	44 ± 1.4 c	27 ± 0.96 a	36 ± 0.67 b
Total branch length (cm)	33 ± 1.6 c	33 ± 1.2 c	7.2 ± 0.77 a	12 ± 1.4 b
Leaf area (cm^2)	862 ± 8.9 c	863 ± 9.6 c	186 ± 3.6 a	372 ± 6.4 b

LN: number of leaves; LB: lateral branches

Heavy metals had a detrimental impact on root and leaf dry weight (Fig. 1) which were indeed reduced by 63% and 44%, respectively. Addition of NaCl significantly improved the root dry weight by 51%, when compared to HMs alone but it had no impact on the leaf dry weight. As shown in Fig. 1 (c and d) salinity did not affect water content in roots and leaves. In contrast, HMs led to significant water loss. The presence of NaCl was however unable to improve this parameter.

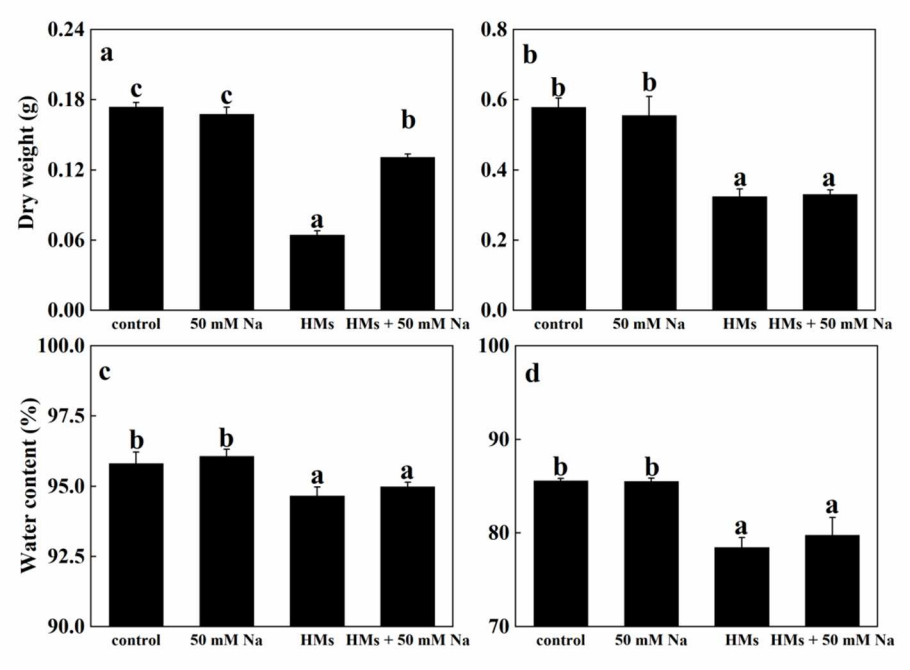

Figure 1 Root (a) and leaf (b) dry weight, root (c) and leaf (d) water content in seedlings of *Kosteletzkya pentacarpos* exposed during two weeks to heavy metals (HM; $CdCl_2$ 10 µM + $ZnCl_2$ 100 µM) in the presence or in the absence of 50 mM NaCl. Each value is the mean of 5 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

3.2. Accumulation and subcellular distribution of Cd and Zn

As expected, Cd was not detected in control or NaCl-treated plants. After two weeks of HMs treatment, the total concentration of Cd was higher in the roots than in the leaves (Table 2). The presence of 50 mM NaCl however reduced Cd concentration in all organs but to a higher extent in the leaves (74%) than in the roots (37%). In roots and leaves the majority of accumulated Cd was detected in the cell debris fraction while less than 25% was present in the cytoplasm. NaCl had a contrasting impact on Cd accumulation in cytoplasm depending on the considered organ since it decreased it in the roots while it increased it

in the leaves. The presence of 50 mM NaCl increased Cd recorded in the root metal-rich granule (MRG) fraction and in the leaf cell debris fraction.

Table 2 Total concentration (µg g^{-1} DW) and subcellular distribution of Cd and Zn in seedlings of *Kosteletzkya pentacarpos* exposed during two weeks to heavy metals (HM; $CdCl_2$ 10 µM + $ZnCl_2$ 100 µM) in the presence or in the absence of 50 mM NaCl. Each value is the mean of 3 replicates ±S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

	Root							
	Cadmium (µg g^{-1} DW)				Zinc (µg g^{-1} DW)			
	Total	Cytoplasm	Cell debris	MRG[2]	Total	Cytoplasm	Cell debris	MRG
Control	-	-	-	-	451 ± 5 a	45%	49%	6%
Na	-	-	-	-	434 ± 16 a	40%	54%	6%
HMs	423 ± 12 b	21%	74%	5%	2514 ± 65 c	45%	50%	5%
HMs + Na	266 ± 8 a	8%	76%	16%	2213 ± 28 b	39%	54%	7%
	Leaf							
	Cadmium (µg g^{-1} DW)				Zinc (µg g^{-1} DW)			
	Total	Cytoplasm	Cell debris	MRG	Total	Cytoplasm	Cell debris	MRG
Control	-	-	-	-	149 ± 11 a	25%	53%	22%
Na	-	-	-	-	150 ± 12 a	28%	51%	21%
HMs	314 ± 10 b	16%	76%	8%	1014 ± 9 c	25%	49%	26%
HMs + Na	82 ± 7 a	27%	66%	7%	566 ± 10 b	27%	54%	19%

[2] MRG: metal – rich granules

The additional NaCl did not affect Zn concentration in plants exposed to non-contaminated solution. In the presence of heavy metals, zinc accumulated to a higher extent in the roots than in the leaves. NaCl only slightly reduced Zn concentration in the roots (12%) while it reduced Zn accumulation in the shoots by more than 44%. In roots of control plants, Zn was present in similar proportion in cytoplasm and cell debris fraction and only a minor proportion was recorded in root MRG fractions. In contrast, MRG fraction contained more or less 20% of leaf Zn and the presence of this element in cytoplasm was clearly lower than in roots. It is noteworthy that NaCl had no obvious impact on Zn distribution in roots and leaves.

Table 3 Bioaccumulation factor (BF) and translocation factor (TF_C[3]and TF_a[4]) in seedlings of *Kosteletzkya pentacarpos* exposed during two weeks to heavy metals (HM; $CdCl_2$ 10 µM + $ZnCl_2$ 100 µM) in the presence or in the absence of 50 mM NaCl. Each value is the mean of 3 replicates ±S.E. For a given parameter, values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

	Cadmium			Zinc		
	BF	TF_C	TF_a	BF	TF_C	TF_a
Control	-	-	-	4.3±0.2 c	0.42±0.04 b	1.2±0.1 b
Na	-	-	-	4.3±0.3 c	0.37±0.05 ab	1.1±0.2 b
HMs	2.5±0.1 a	0.56±0.03 a	2.1±0.1 a	1.4±0.2 b	0.35±0.02 ab	1.4±0.1 c
HMs+Na	1.1±0.1 b	0.30±0.03 b	0.65±0.06 b	0.95±0.27 a	0.31±0.02 a	0.7±0.06 a

[3] TF_c: HM Concentration in the shoot (mg g^{-1} DW) / HM Concentration in the roots (mg g^{-1} DW)

[4] TF_a: HM Total amount in the shoot (mg) / HM Total amount in the roots (mg)

NaCl also reduced Cd and Zn accumulation in the stems (from 318 to 109 µg g^{-1} DW for Cd in HM and HM+NaCl-treated plants, and from 841 to 388 µg g^{-1} DW for Zn; detailed data not shown). The bioaccumulation factor recorded in HM-treated plants (Table 3) was higher for Cd than for Zn; in both cases, the presence of NaCl reduced BF values, but its impact was higher for Cd than for Zn. For both elements, translocation factor estimated on a total amount basis (TF_a) was higher than translocation factor estimated on a concentration basis (TF_c). As far as Zn is concerned, TF_a was slightly higher in HM-treated plants than in controls. NaCl reduced Cd TF_c by 47% and TF_a by more than 60%. In contrast, NaCl had no significant impact on the Zn TF_c values, although it reduced TF_a by 50%.

Sodium concentration increased from 0.52 ± 0.03 to 12.9 ± 2.0 mg g^{-1} DW in the roots of control and NaCl-treated plants, respectively. The presence of heavy metals in the solution significantly decreased the root Na concentration to 5.6 ± 0.04 in HM + Na-treated plants. Heavy metals in the solution however, did not reduce the leaf Na which was 13.2 ± 2.9 mg g^{-1} DW in NaCl-treated plants and 14.0 ± 0.2 mg g^{-1} DW in HM+NaCl-treated ones (detailed data not shown).

3.3. **Photosynthesis - related parameters**

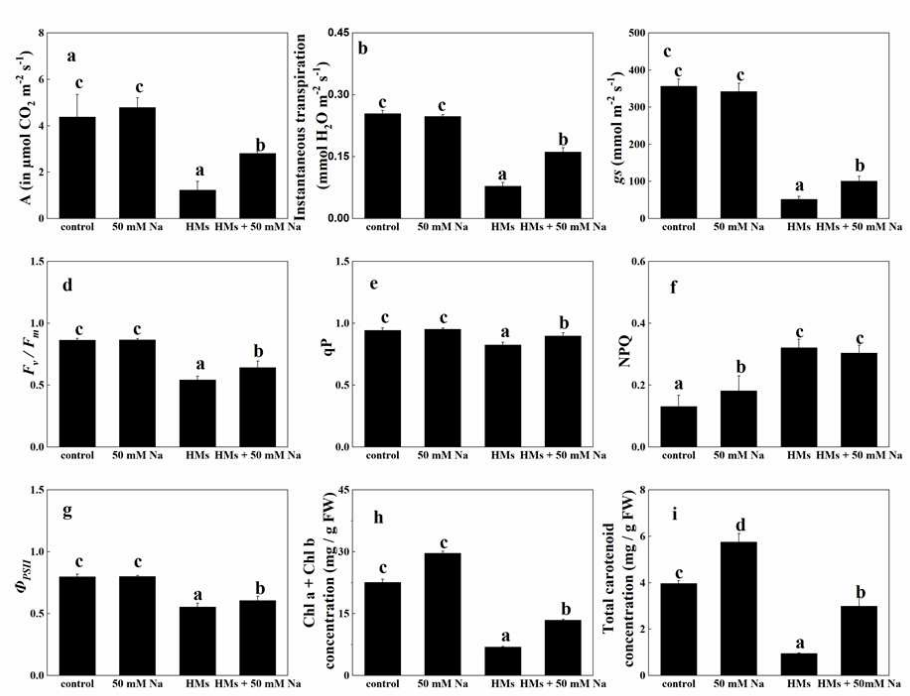

Fig. 2 Net photosynthesis (*A*; a), instantaneous transpiration (*E*; b), stomatal conductance (g_s; c) maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m: d), photochemical quenching (q_P; e), non-photochemical quenching (NPQ; f), actual PSII efficiency (ΦPSII; g) , total chlorophyll (Chl a+Chl b; h) total carotenoid (i) in seedlings of *Kosteletzkya pentacarpos* exposed during two weeks to heavy metals (HM; $CdCl_2$ 10 µM + $ZnCl_2$ 100 µM) in the presence or in the absence of 50 mM NaCl. Value of pigment content is the mean of 3 replicates and others is the mean of 5 replicates. Vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

In the absence of HM, addition of NaCl had no impact on net CO_2 assimilation rate, instantaneous transpiration, and stomatal conductance (Fig. 2. a, b and c). However, after two weeks of exposure to heavy metals stress, *A, E* and g_s values decreased sharply.

Addition of NaCl to HMs treatment significantly mitigated the deleterious effects of pollutant on these parameters.

Heavy metals also had a direct impact on fluorescence-related parameters. The maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m) was significantly reduced when *K. pentacarpos* was exposed to HMs (Fig. 2d) and a similar observation was made for qP (Fig. 2e) and ΦPSII (Fig. 2g). The presence of NaCl in HM-contaminated solutions slightly increased F_v/F_m, qP and ΦPSII comparatively to values recorded in absence of salt. The presence of NaCl in the absence of HM slightly increased NPQ values (Fig. 2f). The recorded increase in NPQ was however higher in plants exposed to HM and the addition of NaCl to polluted solution had no impact on this parameter.

Plants exposed to 50 mM NaCl had higher concentration of total chlorophyll and total carotenoid comparatively to controls (Fig. 3 h and i). In contrast, HMs induced a sharp decrease in total chlorophyll and carotenoid concentration. Addition of NaCl to HM-containing solution reduced the negative impact of Cd and Zn on these photosynthetic pigments' concentration which, however, remained lower than in controls.

3.4. Oxidative stress parameters and non-enzymatic antioxidants

As indicated in Fig.3, the additional NaCl alone did not induce MDA or carbonyl increase neither in the roots (Fig. 3a and 3b) nor in the leaves (Fig 3d and 3e) suggesting that no oxidative stress occurred in response to NaCl. In contrast, both parameters increased in HM-treated plants and such an increase was associated to an increase in H_2O_2 in roots (Fig. 3c) and leaves (Fig. 3f). The addition of NaCl to contaminated solution reduced MDA, carbonyl and H_2O_2 in roots as well as carbonyl and H_2O_2 in leaves. The additional NaCl alone had no impact on the total anti-oxidant activity estimated by FRAP (Fig.4a and 4b) or ABTS (Fig 4c and 4d) while HM strongly increased this total antioxidant activity. In all cases, the total antioxidant activity of

HM-treated plants was reduced by NaCl addition.

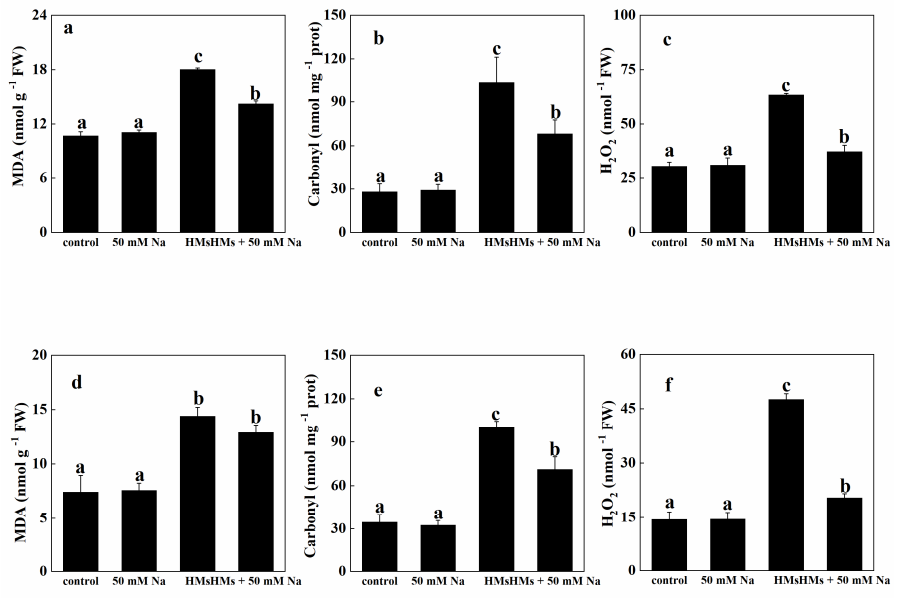

Fig. 3 Concentration of malondialdehyde (root: a; leaf: d) carbonyl (root: b; leaf: e) and hydrogen peroxide (root: c; leaf: f) in seedlings of *Kosteletzkya pentacarpos* exposed during two weeks to heavy metals (HM; $CdCl_2$ 10 µM + $ZnCl_2$ 100 µM) in the presence or in the absence of 50 mM NaCl. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

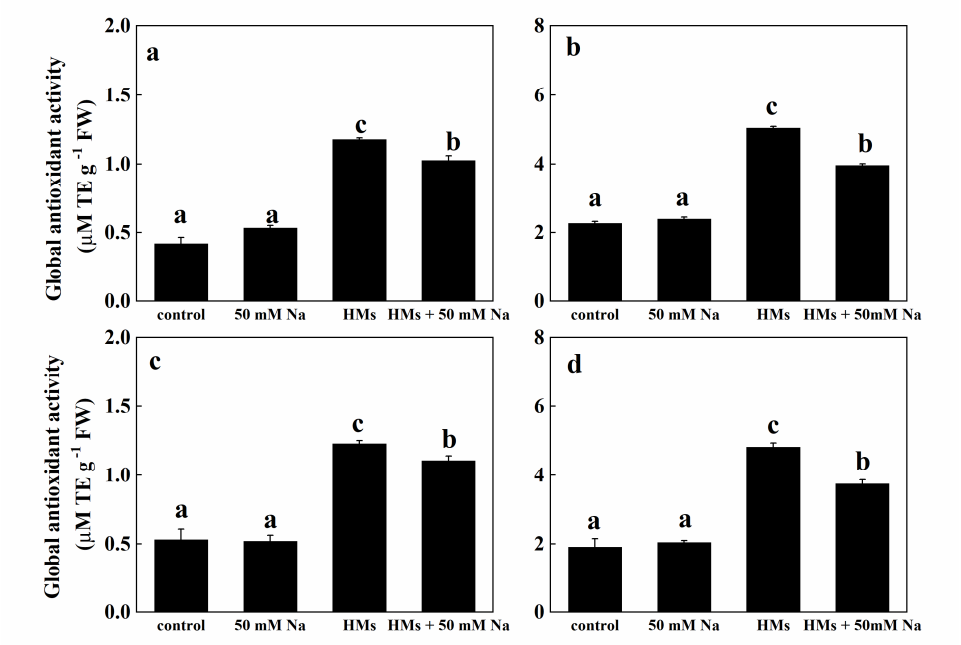

Fig. 4 Total antioxidant (estimated in terms of trolox equivalent per g fresh weight) quantified by FRAP (5a and 5b) and ABTS (5c and 5d) methods in seedlings of *Kosteletzkya pentacarpos* exposed during two weeks to heavy metals (HM; $CdCl_2$ 10 μM + $ZnCl_2$ 100 μM) in the presence or in the absence of 50 mM NaCl. Values are given separately for roots (a and c) and leaves (b and d). Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

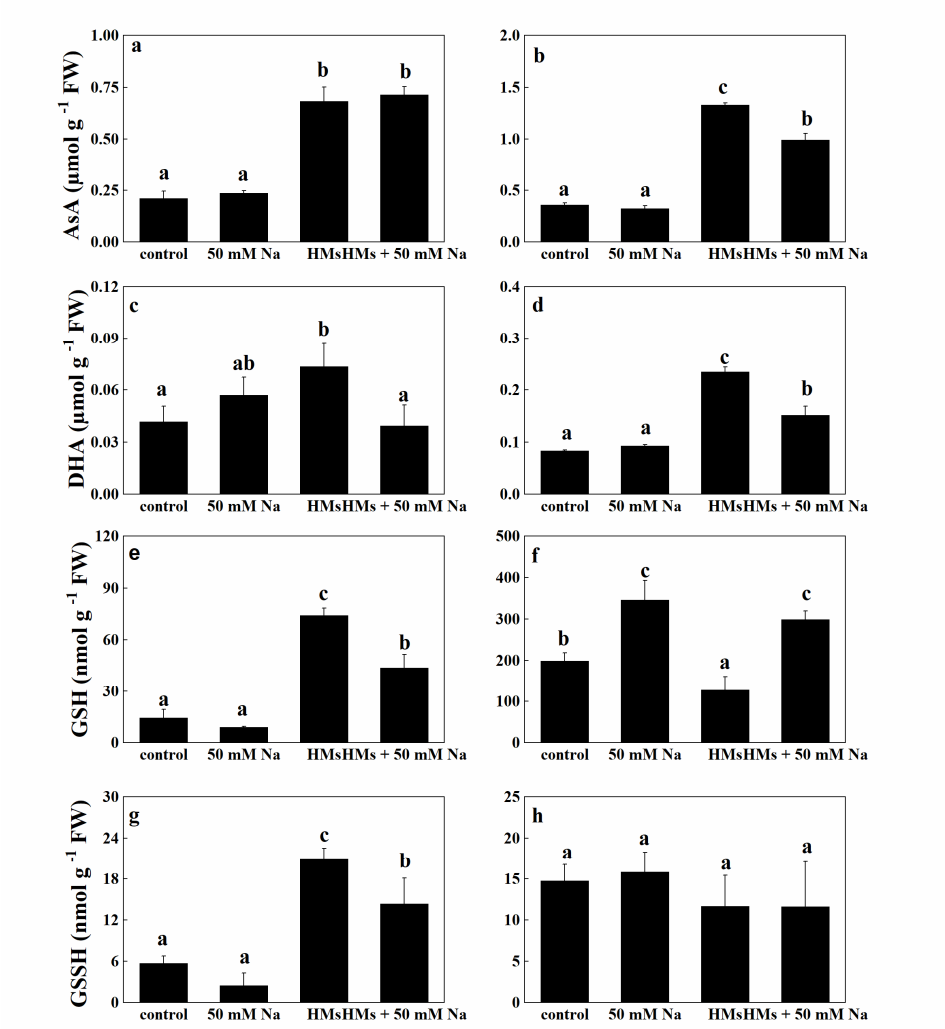

Fig. 5 Concentration of ascorbic acid (AsA; root: a; leaf: d) dehydroascorbate (DHA; root: c; leaf: d), reduced glutathione (GSH; root: e; leaf: f), oxidized glutathione (GSSG; root: g; leaf: f) in seedlings of *Kosteletzkya pentacarpos* exposed during two weeks to heavy metals (HM; $CdCl_2$ 10 µM + $ZnCl_2$ 100 µM) in the presence or in the absence of 50 mM NaCl. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

In roots, AsA and DHA on the one hand (Fig. 5a and 5c), GSH and GSSG on the other hand (Fig. 5e and 5g) were strongly increased by

HM treatment. NaCl added to HM-containing solution did not affect root AsA concentration but reduced root DHA to values similar to control plants. It also reduced GSH and GSSG but in this case, values remained higher than in control or NaCl-treated plants. As far as leaves were concerned, HM also increased AsA and DHA values (Fig. 5b and 5d) and the addition of NaCl mitigated such a HM-induced increase. NaCl significantly increased the leaf GSH concentration (Fig. 5f) while HM treatment reduced it. The mixed treatment (NaCl+HM) increased leaf GSH concentration to values similar to those recorded for NaCl-treated plants. The leaf GSSG concentration remained unaffected by the treatments (Fig. 5h).

3.5. Evaluation of total non – protein thiol (NPT) and phytochelatin (PC) content

Non-protein thiols were lower in the roots than in the leaves (Table 4), whatever the considered treatment. NaCl in the absence of heavy metals had no impact on the NPT concentrations. Exposure to HMs induced an increase in NPT values; Phytochelatins (PC) constituted 90% and 92% of NPT in roots and leaves, respectively. It is noteworthy that in *K. pentacarpos* exposed to Cd+Zn solutions, PC concentration was higher in the leaves than in the roots. Addition of NaCl to HM-containing solution reduced both NPT and PC values.

Table 4 Concentration of non - protein thiol (NPT) and phytochelatin (PC) (μmol g^{-1} FW) in seedlings of *Kosteletzkya pentacarpos* exposed during two weeks to heavy metals (HM; $CdCl_2$ 10 μM + $ZnCl_2$ 100 μM) in the presence or in the absence of 50 mM NaCl. Each value is the mean of 3 replicates ±S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

	Root		Leaf	
	NPT	PC	NPT	PC
Control	0.24 ± 0.01 a	0.22 ± 0.01 a	0.34 ± 0.04 a	0.14 ± 0.03 a
Na	0.26 ± 0.04 a	0.25 ± 0.04 a	0.47 ± 0.11 a	0.12 ± 0.07 a
HMs	0.71 ± 0.02 c	0.63 ± 0.02 c	1.2 ± 0.1 c	1.1 ± 0.1 c
HMs + Na	0.55 ± 0.02 b	0.51 ± 0.01 b	0.81 ± 0.10 b	0.51 ± 0.08 b

4. Discussion

In coastal areas, heavy metal excess is often associated with high levels of salinity and contaminated areas are frequently characterized by multiple pollution. Zinc are commonly containing 0.5-5% of Cd and both elements are simultaneously present in polluted places. Although salinity impact on heavy metal absorption by plants has been studied in the case of monometallic exposure (Han et al. 2013a; 2013b; Wali et al. 2015; Ghabriche et al. 2017) information regarding salt impacts on plants facing a polymetallic situation remain scanty.

The present study demonstrates that 50 mM NaCl clearly mitigated the deleterious impact of Cd+Zn on the halophyte *Kosteletzkya pentacarpos*. Heavy metals indeed affected all morphological parameters. Han et al. (2012) reported that Cd clearly impacted plant ramification in this species while Zn did not. In the present study, a drastic effect on lateral branches growth was observed, suggesting that the presence of Zn in excess did not reduce Cd toxicity. Hormone synthesis and distribution within organs may be modified by heavy metals and have a strong impact on plant architecture (Li et al. 2009;

Yan et al. 2016; Sofo et al. 2017). Han et al. (2013b) reported that in *K. virginica*, salinity reduced the synthesis of 1-aminocyclopropane-1-carboxylic acid (precursor of ethylene) and abscisic acid, which are both acting as senescing hormones. We may thus hypothesize that such a salt-induced modification may also help to improve growth of plants exposed to a mixed toxicity and delayed senescence may explain a higher concentration in photosynthetic pigments.

NaCl directly interferes with heavy metal absorption. Cadmium concentration was clearly lower in roots and leaves of plants in the presence of NaCl than in plants exposed to heavy metals in the absence of NaCl. From a relative point of view, the recorded decrease was higher for Cd than for Zn. Such a decrease could not be attributed to a decrease in transpiration rate since E and g_s both increased in salt-treated plants exposed to heavy metals. Competition may occur between Cd and Zn and the interactive pattern could be antagonist in some cases, additive or synergistic in other cases depending on the tested species, the external doses and environmental conditions (Sun et al. 2005; Qiu et al. 2011; Cherif et al. 2012; Tkalec et al. 2014). All these studies, however, focused on Cd and Zn interaction in the absence of salt. In our study performed under salt conditions, it is noteworthy that the NaCl-induced recorded decrease in Cd accumulation was higher than the salt-induced decrease in Cd accumulation when plants were, under similar experimental conditions, exposed to Cd alone as we previously reported (Han et al. 2012; 2013b): NaCl reduced leaf Cd concentration by 74% when Zn excess was simultaneously present, while in the case of exposure to Cd alone, NaCl-induced decrease in leaf Cd was not higher than 44% (Han et al. 2012).

The overall consequence of these processes was that the bioaccumulation factor was reduced for both Cd and Zn in the presence of NaCl. Hence, if plants are used for phytoextraction purposes aiming at removing Cd and Zn from contaminated soils, salinity does not appear as a positive environmental factor. Some new

strategies to recover and recycle Cd and Zn from harvested biomass start to be available but are more efficient when a small amount of highly contaminated biomass has to be manipulated (Hazotte et al. 2017). In our study, NaCl reduced the total amount of Cd and Zn in the shoot by 69.3 and 43.1%, respectively, confirming that NaCl-induced growth stimulation did not compensate the salt-induced decrease in heavy metal accumulation for pollutant removal.

Heavy metals translocation from roots to shoots was also strongly inhibited by NaCl. As a consequence, the total amount of heavy metals retained by the roots increased in NaCl-treated plants and to a higher extent for Zn (78%) than for Cd (25%). It has been recently demonstrated that NaCl increased the heavy metal (especially Zn) biosorption efficiency of roots from *K. pentacarpos* in relation to a salt-induced increase in mucilage pectic compounds and hemicellulose involved in heavy metal binding (Lutts et al. 2016). Although the mucilage in *K. pentacarpos* is mainly present in intercellular spaces (Ghanem et al. 2010) and hemicellulose is a major component of the cell wall, our data suggested that NaCl did not strongly increase the proportion of root Cd sequestered in cell walls which are recovered in the cell debris fraction. At the leaf level, Cd proportion in the cell wall was even lower in NaCl-treated plants than in those facing pollution in the absence of salt.

Beside cell wall sequestration, heavy metal complexation to cysteine-rich phytochelatin contributes to heavy metal resistance, especially for Cd (Sun et al. 2005; Lefèvre et al. 2016). In our study, heavy metal exposure increased PC concentration in leaves and roots, confirming that *K. pentacarpos* is able to trigger this protective mechanism. Addition of NaCl to polluted medium reduced PC concentration but the recorded decrease (20 and 50% in roots and leaves, respectively) was lower than the recorded decrease in Cd (38 and 73% in roots and leaves, respectively) which leads us to hypothesize that the proportion of Cd sequestered in PC increased in NaCl-treated plants. The Cd-PC complex is considered to accumulate in the vacuole where low-molecular weight-PC polymerize to form

high-molecular weight insoluble complexes which are thought to be detected in the MRG fraction (Adams et al. 1997). This might explain that Cd proportion in MRG increased by 300% in our analyzed root sample

Protection against oxidative stress is also an important component of heavy metal resistance in plants (Tkalec et al. 2014; Shadid et al. 2014; Yan et al. 2016). In the absence of heavy metals, no oxidative stress was recorded in salt-treated *K. pentacarpos*, confirming the halophytic nature of this species. This is also supported by the fact that Na was quite efficiently translocated to the shoots. NaCl was able to reduce MDA, carbonyl and H_2O_2 in HM-treated plants. Chloroplasts are important sites for reactive oxygen species (ROS) synthesis and Cherif et al. (2012) demonstrated that high Zn concentration exacerbate the negative effect of Cd on chloroplast structure. In our work, NaCl improved all fluorescence-related parameters (except NPQ), suggesting that salt contribute to protect the chloroplast structure. This protecting effect, together with the NaCl-induced increase in stomatal conductance and pigment concentration in HM-treated plants probably contributed to the salt-induced improvement of net photosynthesis in plants challenging with Cd+Zn.

The total antioxidant activity slightly decreased in plants exposed to Cd+Zn in the presence of NaCl, suggesting that ROS synthesis rather than ROS detoxification was improved in the presence of salt. Glutathione, which is an important antioxidant was however increased by NaCl but was depleted in HM-treated plants in the absence of salt. GSH assumes a dual function in the presence of heavy metals since it acts both as an antioxidant and as a precursor of PC (Schäfer et al. 1997). Hence, the recorded GSH depletion recorded in HM-treated plants could be attributed to the stimulation of these protective compounds, as previously reported in other species (De Vos et al. 1992; Sun et al. 2005). Our data however suggest that NaCl was able to restore GSH content which could be due both to a direct stimulation of its synthesis and to a lower requirement of this precursor for PC synthesis considering the NaCl-induced decrease in HM

accumulation.

5. Conclusion

These results demonstrate that 50 mM NaCl improved the ability of the halophyte plant species *Kosteletzkya pentacarpos* to cope with the simultaneous presence of Cd (10 µM) and Zn (100 µM). It increased plant growth in relation to a decrease of Cd and Zn absorption. The recorded decrease was higher for Cd than for Zn. NaCl also reduced heavy metal translocation to the shoot, which in turn, allows to maintain photosynthetic activities. Exogenous NaCl increased GSH content and improved the antioxidative status of plants exposed to heavy metals. It compromises the use of *K. pentacarpos* for phytoextraction and heavy metal removal from contaminated soils but its positive impact on plant growth could favor the use of this plant for revegetation of HM-polluted sites in salt marsh and coastal areas.

Reference

Adams SM, Shorey CD, Byrne M (1997) An ultrastructural and microanalytical study of metal-ionn content in granular concretions of the freshwater mussel *Hydriella depressa*. Micron 28:1-11

Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay Analytical biochemistry 239:70-76

Blits KC, Gallagher JL (1990) Salinity tolerance of *Kosteletzkya virginica* 1. Shoot growth, ion and water relations. Plant Cell Environ 13:409-418

Cereser C, Guichard J, Drai J, Bannier E, Garcia I, Boget S, Parvaz P, Revol A (2001) Quantitation of reduced and total glutathione at the femtomole level by high-performance liquid chromatography with fluorescence detection: application to red blood cells and cultured fibroblasts J Chrom B: Biomed Sci Appl 752:123-132

Cherif J, Derbel N, Nakkach M, vopn Bergmann H, Jemal F, Ben Lakhdar Z (2012) Spectroscopic studes of photosynthetic responses of tomato plants to the interaction of zinc and cadmium toxicity. J Photochem Photobiol B Biol 111:9-16

De Vos CR, Vonk MJ, Vooijs R, Schat H (1992) Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus* Plant Physiol 98:853-858

El Nemr A, El-Said GF (2017) Assessment and ecological risk of heavy metals in sediment and molluscs from the Mediterranean coast. Water Environ Res 89:195-210

Feitz AJ and Lundie S (2002) Soil salinisation: a local life cycle assessment impact category. The International Journal of Life Cycle Assessment 4 (7): 244-249

Ghabriche R, Ghanya T, Mnasri M, Zaier H, Baioui R, Vromman D, Abdelly C, Lutts S (2017) Polyamine and tyramine involvement in NaCl-induced improvement of Cd resistance in the halophyte *Inula chritmoides* L. J Plant Physiol 6:136-144

Ghanem ME, Han RM, Classen B, Leclerq J, Mahy G, Ruan CJ, Pérez-Alfcoea F, Lutts S. (2010) Mucilages and polysaccharides in the halophyte plant species *Kosteletzkya virginica* (L.) Presl.: localization and composition in relation to salt-induced physiological modifications. J Plant Physiol

167:382-392

Halchak JL, Seliskar DM, Gallagher JL (2011) Root system architecture of *Kosteletzkya pentacarpos* (Malvaceae) and belowground environmental influences on root and aerial growth dynamics Am J Bot 98:163-174

Han RM, Lefèvre I, Ruan CJ, Qin P, Lutts S (2012) NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regul 68:97-109

Han RM, Quinet M, André E, Van Elteren J, Destrebecq F, Vogel-Mikus K, Cui G, Debeljak, Lefèvre I, Lutts S (2013a) Accumulation and distribution of Zn in the shoots and reproductive structures of the halophyte plant species *Kosteletzkya virginica* as a function of salinity Planta 238:441-457

Han RM, Lefèvre I, Albacete A, Pérez-Alfocéa F, Baeba-Espín G, Díaz-Vivancos P, Quinet M, Ruan CJ, Hernández JA, Cantero-Navarro E, Lutts S (2013b) Antioxidant enzyme activities and hormonal status in response to Cd stress in the wetland halophyte *Kosteletzkya virginica* under saline conditions. Physiol Plant 147:352-368

Hazotte C, L ubie B, Rees F, Morel JL, Simonnot MO (2017) A novel process to recover cadmium and zinc from the hyperaccumulator plant *Noccaea caerulescens.* Hydrometallurgy 174: 56-65.

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. II. Role of electron transfer Arch Biochem Biophys 125:850-857

Lefèvre I, Vogel-Mikuš K, Jeromel J, Vavpetič P, Planchon s, Arčon I, Van Elteren J, Lepoint G, Gobert S, Renaut J, Pelicon P, Lutts S (2014) Differential cadmium and zinc distribution in relation to their physiological impact in the leaves of the accumulating *Zygophyllum fabago* L. Plant Cell Environ 37:1299-1320

Lefèvre I, Vogel-Mikuš K, Arčon I, Lutts S (2016) How do roots of the metal resistant perenial bush *Zygophyllum fabago* cope with cadmium and zinc toxicities? Plant Soil 404:193-207

Li D, Zhou D, Wang P, Li L (2011) Temperature affects cadmium-induced phytotoxicity involved in subcellular cadmium distribution and oxidative stress in wheat roots Ecotoxicol Environ Saf 74:2029-2035

Li T, Yang X, Lu L, Islma E, He Z (2009) Effects of zinc and cadmium interactions on root morphology abd metal translocation in a hyperaccumulating species under hydroponic conditions. J Hazard Mat 169:734-741

Lutts S, Lefevre I (2015) How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? Ann Bot 115:509-28

Lutts S, Qin P, Han RM (2016) Salinity influences biosorption of heavy metals by the root of the halophyte species *Kosteletzkya pentacarpos*. Ecol Eng 95:682-689

Mani D, Kumar C, Patel NK (2015) Integrated micro-biochemical approach for phytoremediation of cadmium and zinc contaminated soils. Ecotoxicol Environ Saf 111:86-95

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence--a practical guide J Exp Bot 51:659-668

Nawaz I, Iqbal M, Bliek M, Schat H (2017) Salt and heavy metal tolerance and expression levels of candidate tolerance genes among four extremophile Cochlearia species with contrasting habitat preferences. Sci Total Environ 584-585:731-741

Ogundele LT, Owoade OK, Hopke PK, Olise FS (2017) Heavy metals in industrially emitted particulate matter in Ile-Ife, Nigeria. Environ Res 156:320-325

Pellegrini N, Re R, Yang M, Rice-Evans CA (1999) Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying the 2,2'-azobis(3-ethylenebenzothiazoline-6-sulfonic) acid radical cation decolorization assay. Meth Enzymol 299:379-389

Qin P, Han RM, Zhou MX, Zhang HS, Fan LS, Seliskar DM, Gallagher JL (2015) Ecological engineering through the biosecure introduction of *Kosteletzkya virginica* (seashore mallow) to saline lands in China: A review of 20 years of activity. Ecol Eng 74:174-186

Qiu RL, Thangavel P, Hu PJ, Senthilkumar P, Ying RR, Tang YT (2011) Interaction of cadmium and zinc on accumulation and sub-cellular distribution in leaves of hyperaccumulator *Potentilla griffithii*. J Hazard Mat 186:1425-1430

Redondo-Gomez S, Andrades-Moreno L, Mateos-Naranjo E, Parra R, Valera-Burgos J, Aroca R (2011) Synergic effect of salinity and zinc stress on growth and photosynthetic responses of the cordgrass, *Spartina densiflora.* J Exp Bot 62:5521-5530

Reznick AZ, Packer L (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Meth Enzymol 233:357-363

Różański S, Jaworska H, Matuszczak K, Nowak J, Hardy A (2017) Impact of

highway traffic and the acoustic screen on the content and spatial distribution of heavy metals in soils. Environ Sci Pollut Res 24:12778-12786

Schäfer HJ, Greiner S, Rausch T, Haag-Kerwer A (1997) In seedlings of the heavy metal accumulator *Brassica juncea* Cu^{2+} differentially affects transcript amounts for γ-glutamylcysteine synthetase (γ-ECS) and metallothionein (MT2) FEBS lett 404:216-220

Shahid M, Pourrut B, Dumat C, Nadeem M, Aslam M, Pinelli E (2014) Heavy-metal-induced reactive oxygen species: phytotoxicity and physicochemical changes in plants. Rev Environ Contam Toxicol 232:1-44

Sofo A, Bochicchio R, Amato M, Rendina N, Vitti A, Nuzzaci M, Altamura MM, Falasca G, Della Rovere F, Scopa A (2017) Plant architecture, auxin homeostasis and phenol content in *Arabidopsis thaliana* grown in cadmium- and zinc-enriched media. J Plant Physiol 216:174-180

Sun Q, Wang XR, Ding SM, Yuan XF (2005) Effects of interactions between cadmium and zinc on phytochelatin and glutathione production in wheat (*Triticum aesticum* L.). Environ Toxicol 20:195-201

Tattibayeva D, Nebot C, Miranda JM, Abuova AB, Baibatyrov TA, Kizatova MZ, Cepeda A, Franco CM (2016) A study on toxic and essential elements in wheat grain from the Republic of Kazakhstan. Environ Sci Pollut Res 23:5527-5537

Tkalec M, Štefanić PP, Cvjetko P, Šikić S, Pavlica M, Balen B (2014) The effect of cadmium-zinc interactions on biochemical responses in tobacco seedlings and adult plants. PLoS ONE 9: e87582

Vaughn SF, Moser BR, Dien BS, Iten LB, Thompson AR, Seliskar DM, Gallagher JL (2013) Seashore mallow (*Kosteletzkya pentacarpos*) stems as a feedstock for biodegradable absorbents. Biomass Bioenergy 59:300-305

Wali M, Fourati E, Hmaeid N, Ghabriche R, Poschenrieder C, Abdelly C, Ghnaya T (2015) NaCl alleviates Cd toxicity by changing its chemical forms of accumulation in the halophyte *Sesuvium portulacastrum*. Environ Sci Pollut Res Int 22:10769-10777

Wali M, Gunsé N, Llugany M, Corrales I, Abdelly C, Poschenrieder C, Ghnaya T (2016) High salinity helps the halophyte *Sesuvium portulacastrum* in defense against Cd toxicity by mainatining redox balance and photosynthesis. Planta 244:333-346

Wali M, Martos S, Pérez-Martín L, Abdelly C, Ghnaya T, Poschenrieder C,

Gunsé B (2017) Cadmium hampers salt tolerance of *Sesuvium portulacastrum*. Plant Physiol Biochem 115:390-399

Wang SY, Jiao HJ, Faust M (1991) Changes in ascorbate, glutathione, and related enzyme activities during thidiazuron-induced bud break of apple. Physiol. Plant 82:231-236

Wang Y, Wang S, Nan Z, Ma J, Zang F, Chen Y, Li Y, Zhang Q (2015) Effects of Ni stress on the uptake and translocation of Ni and other mineral nutrition elements in mature wheat grown in sierozems from northwest of China. Environ Sci Pollut Res 22:19756-19763

Weigel HJ, Jager HJ (1980) Subcellular distribution and chemical form of cadmium in bean plants Plant Physiol 65:480-482

Yan H, Filardo F, Hu X, Zhao X, Fu DH (2016) Cadmium stress alters the redox reaction and hormone balance in oilseed rape (*Brassica napus* L.) leaves. Environ Sci Pollut Res 23:3758-3769

Zhang C, Sale PW, Tang C (2016) Cadmium uptake by *Carpobrotus rossii* (Haw.) Schwantes under different saline conditions. Environ Sci Pollut Res 23:13480-13488

Zhou B, Wang J, Guo Z, Tan H, Zhu X (2006) A simple colorimetric method for determination of hydrogen peroxide in plant tissues. Plant Growth Regul 49:113-118

Interchapter 1-2

What do we know?

- All plants survive and grow in the simultaneous presence of Cd and Zn.
- Heavy metal exposure increases Cd and Zn concentrations in all organs and both heavy metals accumulate to a higher extent in roots than in shoots. In terms of subcellular distribution, Cd mainly accumulates in the cell walls in roots and leaves, while Zn is evenly distributed in cytoplasm and cell walls.
- Cd and Zn accumulation reduces net photosynthesis in relation to stomatal closure, decreases in chlorophyll concentration and alteration in chlorophyll fluorescence-related parameters. Some of these modifications could be related to a heavy metal-induced oxidative stress.
- Salinity reduces Cd and Zn bioaccumulation and translocation, with a higher impact on Cd than Zn. It increases Cd in the cytosol fraction in the leaf and in the metal-rich granule fraction in the roots, while it has no impact on Zn subcellular distribution. NaCl mitigates the deleterious impact of heavy metals on photosynthetic parameters as well as reduces the oxidative damage.

What are the questions?

It is well established from data available in the literature that heavy metals induce precocious senescence in plants. Symptoms of senescence in leaves involve a decrease in photosynthetic pigments and protein concentrations, as well as an increase in cell membrane permeability leading to a decrease in cell viability. Ethylene is

considered as the main phytohormone triggering leaf senescence in plants. Previous work (Han et al., Physiol. Plant., 147: 352-368) confirmed that Cd exposure induced an increase in the precursor of ethylene in *K. pentacarpos* but no information is available on the impact of mixed toxicity on ethylene synthesis itself. We intend to precise the role of ethylene in senescence of *K. pentacarpos* exposed to mixed toxicity and to check if the NaCl-induced improvement recorded in the Chapter 1 could be due to a decrease in ethylene production. Ethylene biosynthetic pathway is also related to synthesis of polyamines frequently considered to strongly influence senescing process in plants but we still do not know if polyamines titers are or not modified in response to mixed toxicity.

What is our strategy?

Senescence processes (assessed by quantifying photosynthesis, cell membrane stability, oxidative stress) will be analysed in relation to the ethylene biosynthesis and polyamines profilein plants exposed to mixed toxicity (Cd+Zn) and compared with plants exposed to individual heavy metals (Cd or Zn). An inhibitor of ethylene synthesis (aminovinylglycine (AVG)) will be used to obtain additional information on the role of ethylene on stress-induced senescence processes in *K. pentacarpos*.

CHAPTER 2 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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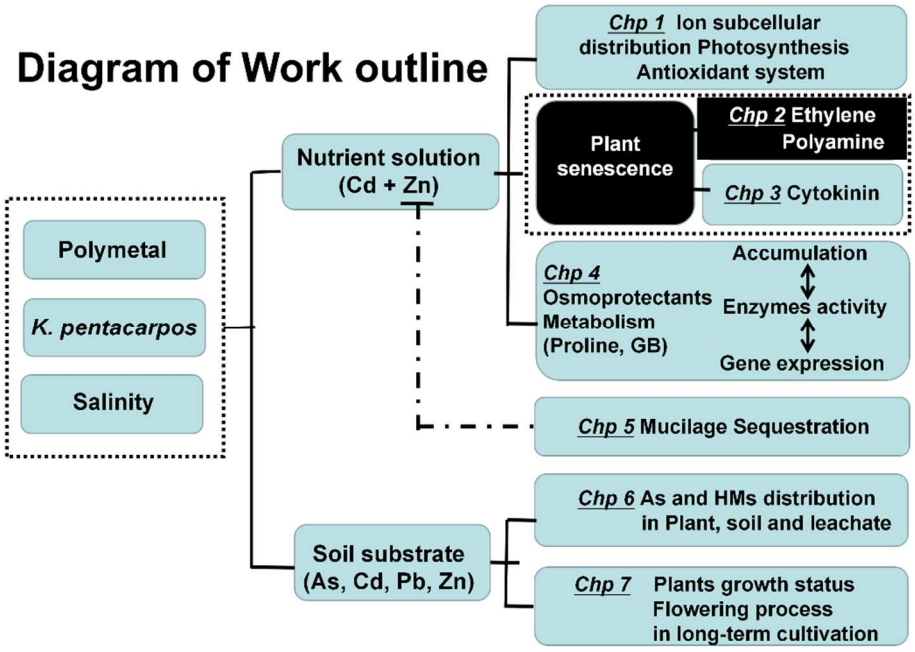
Diagram of Work outline
Chp 1 Ion subcellular distribution Photosynthesis Antioxidant system
Plant senescence
Chp 2 Ethylene Polyamine
Chp 3 Cytokinin
Nutrient solution (Cd + Zn)
Polymetal
K. pentacarpos
Salinity
Chp 4 Osmoprotectants Metabolism (Proline, GB)
Accumulation
Enzymes activity
Gene expression
Chp 5 Mucilage Sequestration
Chp 6 As and HMs distribution in Plant, soil and leachate
Soil substrate (As, Cd, Pb, Zn)
Chp 7 Plants growth status Flowering process in long-term cultivation

Chapter 2

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 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.

Keywords: cadmium, halophyte, phytoremediation, polyamines, salinity, zinc

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 and 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. Besides 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 delay Cd-induced senescence in relation to a decrease in ACC synthesis and an increase in endogenous antioxidant.

Besides 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 and 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 *Kosteletzkya 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 12h 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 0.1N. 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 x 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 Lichenthaler (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 was 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.

Table 1. Height (cm) of plants of *Kosteletzkya pentacarpos* cultivated 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 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 50 mM	55.18 ± 1.15 a
Cd 10 µM	33.04 ± 2.54 c
Cd 10 µM + NaCl 50 mM	41.66 ± 3.8 b
Zn 100 µM	38.07 ± 1.26 b
Zn 100 µM + NaCl 50 mM	44.69 ± 2.38 b
Cd 10 µM + Zn 100 µM	23.18 ± 1.75 d
Cd 10 µM + Zn 100 µM + NaCl 50 mM	37.08 ± 2.05 bc

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.

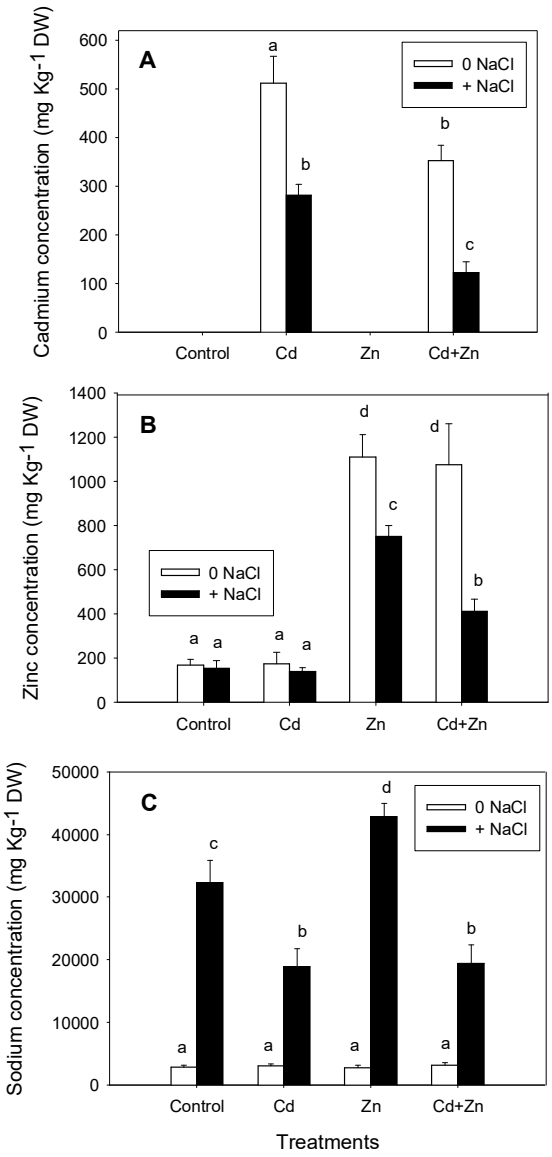

Fig. 1 Total concentration (mg kg^{-1} DW) of Cd (A), Zn (B) and Na (C) in the leaves of *Kosteletzkya pentacarpos* exposed during three weeks in nutrient solution containing 10 µM Cd, 100 µM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the mean of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

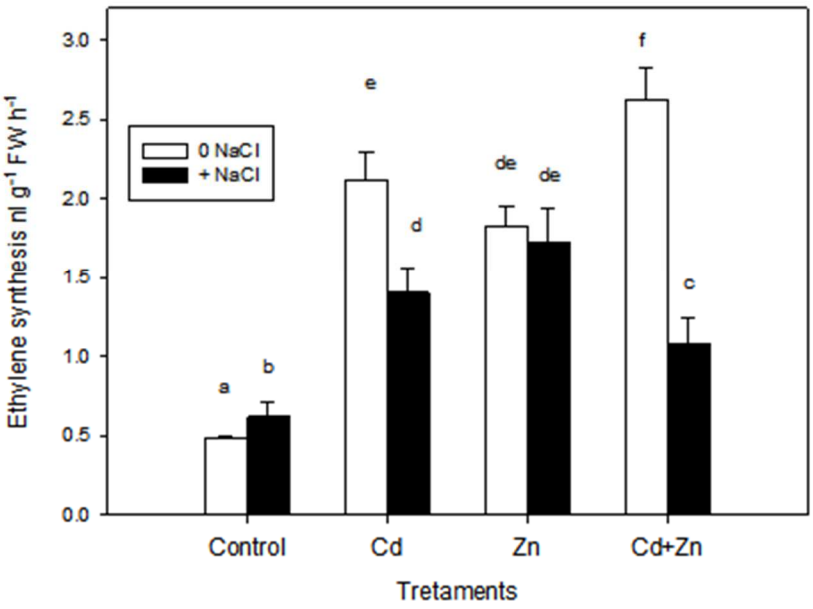

Fig. 2 Ethylene synthesis (nL g^{-1} FW h^{-1}) in detached leaf of *Kosteletzkya pentacarpos* exposed during three weeks in nutrient solution containing 10 µM Cd, 100 µM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the mean of 6 replicates ± S.E.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

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 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.

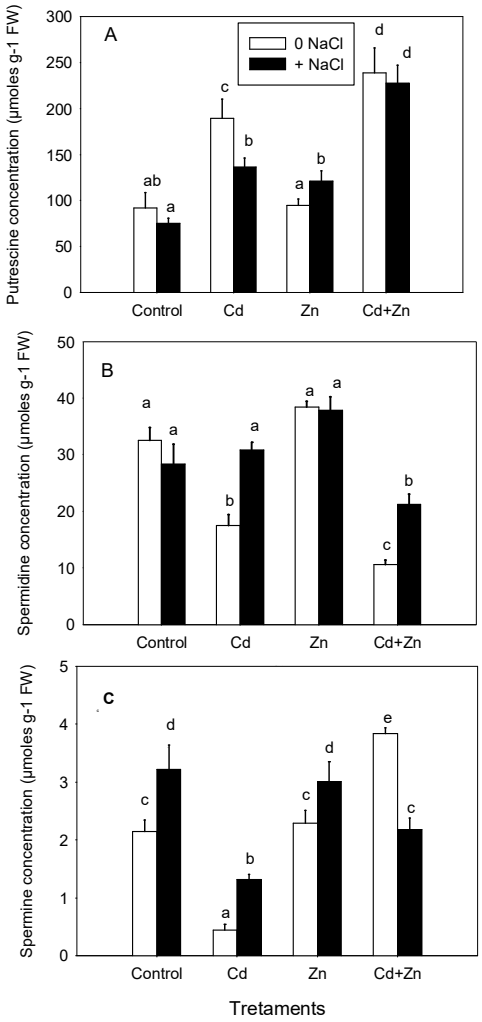

Fig. 3 Putrescine (A), spermidine (B) and spermine (C) concentration (µmol g^{-1} FW) in leaf of *Kosteletzkya pentacarpos* exposed during three weeks in nutrient solution containing 10 µM Cd, 100 µM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the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

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.

Table 2. Total soluble proteins (mg g^{-1} FW), malondialdehyde (MDA, mg g^{-1} FW) and chlorophyll concentration (mg g^{-1} FW) on median leaves harvested on 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 Tukey test ($P > 0.05$).

Treatment	Protein (mg g^{-1} FW)	MDA (mg g^{-1} FW)	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)
Control	33.8 ± 1.2 a	5.0 ± 0.31 a	10.9 ± 1.07 a	2.3 ± 0.11 a
Na	31.9 ± 1.7 a	6.1 ± 1.19 a	10.9 ± 0.85 a	2.4 ± 0.08 a
Cd	13.9 ± 0.7 c	32 ± 1.90 d	7.6 ± 0.75 d	1.5 ± 0.21 c
Cd+Na	17.3 ± 3.0 bc	23 ± 1.54 c	8.3 ± 0.54 c	1.8 ± 0.12 b
Zn	34.1 ± 2.1 a	13 ± 0.89 b	10.8 ± 0.95 a	1.5 ± 0.15 c
Zn+Na	32.3 ± 1.7 a	19 ± 1.13 c	10.6 ± 0.66 ab	1.8 ± 0.08 b
Cd+Zn	23.5 ± 1.1 b	29 ± 1.71 d	8.1 ± 0.33 cd	1.9 ± 0.09 b
Cd+Zn+Na	26.5 ± 0.5 b	11 ± 0.90 b	9.1 ± 0.47 b	2.1 ± 0.21 ab

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.

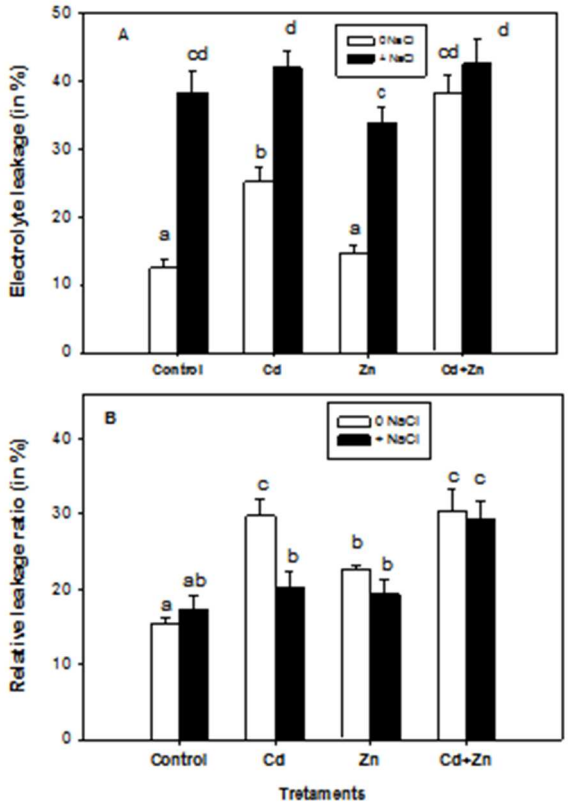

Fig. 4 Electrolyte leakage (in %, A) and relative leakage ratio (in %, B) in leaf segment of *Kosteletzkya pentacarpos* exposed during three weeks in nutrient solution containing 10 µM Cd, 100 µM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. Each value is the mean 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 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.

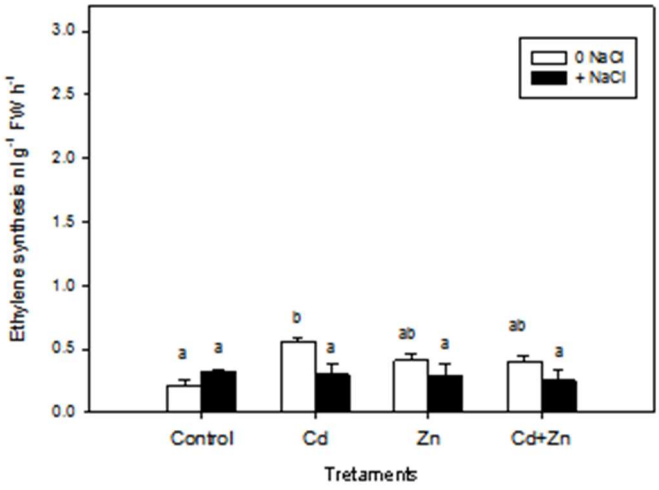

Fig. 5 Ethylene synthesis (nL g^{-1} FW h^{-1}) in detached leaves of AVG-treated plants of *Kosteletzkya pentacarpos* exposed during three weeks in nutrient solution containing 10 µM Cd, 100 µM Zn or combined treatment (Cd + Zn) in the absence or in the presence of NaCl 50 mM. All plants were cultivated 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.

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.

Table 3. Impact of 10 µM AVG (aminoethoxyvinyl glycine) on physiological and biochemical parameters involved in the response of *Kosteletzkya 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 maintained under 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. (ND: not detected)

Treatment	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
Na	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 + Na	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 + Na	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	97.8	85.4*	68.8**
Cd+Zn+Na	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**

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 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). In chapter 1, it 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 response 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; Gupta et al., 2016; Sobiezcuk-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 *Kosteletzkya 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.

Reference

Bai, J., Xiao, R., Zhang, K., Gao, H., 2012. Arsenic and heavy metal pollution in wetland soils from tidal freshwater and salt marshes before and after flow sediment regulation regime in the Yellow River delta China. J. Hydrol. 450/451, 244-253.

Bala, S., Asthir, B., Bains, N.S., 2016. Syringaldazine peroxidase stimulates lignification by enhancing polyamine catabolism in wheat during heat and drought stress. Cer. Res. Comm. 44, 561-571.

Blum, A., Ebercon A., 1981. Cell membrane stability as a measure of drought and heat tolerance in wheat. Crop Sci. 21, 43-47.

Buscaroli, A., 2017. An overview of indexes to evaluate terrestrial plants for phytoremediation purposes. Ecol. Ind. 82, 367-380.

Castiglione, S., Todeschini, V., Franchin, C., Torrigiani, P., Gastaldi, D., Cicatelli, A., Rinaudo, C., Berta, G., Biondi, S., Lingua, G., 2009. Clonal differences in survival capacity, copper and zinc accumulation and correlation with leaf polyamine levels in poplar: A large-scale field trial on heavily polluted soil. Environ. Poll. 157, 2108-2117.

Chen, Q., Pan, X.D., Huang, B.F., Han, J.L., 2018. Distribution of metals and metalloids in dried seaweeds and health risk to population in southestern China. Scient. Rep. 8, 3578.

Chowdhury, S., Mazumder, M.A.J., Al-Attas, O., Husain, T., 2016. Heavy metals in drinking water: occurrences, implications, and future needs in developing countries. Sci. Total Environ. 569/570, 476-488.

Cristescu, S.M., De Martinis, D., Te Lintel Hekkert, S., Parker, D.H., Harren, F.J.M., 2002. Ethylene production by *Botrytis cinerea* in vitro and in tomato. Appl. Environm. Microbiol. 68, 5342-5350.

Dhindsa, R.S., Matowe, W., 1981. Drought tolerance in two mosses: correlated with enzymatic defence against lipid peroxidation. J. Exp. Bot., 32, 79-91.

Dotaniya, M.L., Rajendiran, S., Vassanda Coumar, M., Meena, V.D., Saha, J.K., Kundu, S., Kumar, A., Patra A.K., 2018. Interactive effect of cadmium and zinc on chromium uptake in spincah grown in Vertisol of Central India. Int. J. Environ. Sci. Technol. 15, 441-448.

Fariduddin, Q., Varshney, P., Yusuf, M., Ahmad, A., 2013. Polyamines: potent modulators of plant responses to stress. J. Plant Let. 8, 1-16.

Franchin, C., Fossati, T., Pasquini, E., Lingua, G., Castiglione, S., Torrigiani, P.,

Biondi, S., 2007. High concentrations of zinc and copper induce differential polyamine responses in micropropagated white poplar (*Populus alba*). Physiol. Plant., 130, 77-90.

Ghabriche, R., Ghnaya, T., Mnasri, M., Zaier, H., Baioui, R., Vromman, D., Abdelly, C., Lutts, S., 2017. Polyamine and tyramine involvement in NaCl-induced improvement of Cd resistance in the halophyte *Inula chrithmoides* L. J. Plant Physiol. 216, 136-144.

Ghanem, M.E., Han, R.M., Classen, B., Leclerq, J., Mahy, G., Ruan, C.J., Pérez-Alfcoea F., Lutts, S., 2010. Mucilages and polysaccharides in the halophyte plant species *Kosteletzkya virginica* (L.) Presl.: localization and composition in relation to salt-induced physiological modifications. J. Plant Physiol. 167, 382-392.

Gharbi, E., Martínez, J.P., Benahmed, H., Lepoint, G., Vanpee, B., Quinet, M., Lutts, S., 2017. Inhibition of ethylene synthesis reduces salt-tolerance in tomato wild relative species *Solanum chilense*. J. Plant Physiol. 210, 24-37.

Ghnaya, T., Slama, I., Messedi, D., Grignon, C., Ghorbel, M.H., Abdelly, C., 2007. Cd-induced growth reduction in the halophyte *Sesuvium portulacastrum* is significantly improved by NaCl. J. Plant Res. 1210, 309-316.

Gupta, K., Dey, A., Gupta, B., 2013. Plant polyamines in abiotic stress responses. Acta Physiol. Plant. 35, 2015-2036.

Gupta, K., Sengupta, A., Chakraborty, M., Gupta, B., 2016. Hydrogen peroxide and polyamines act as double edged swords in plant abiotic stress responses. Front. Plant Sci. 7, 1343.

Han, R., Lefèvre, I., Ruan, C.J., Beukelaers, N., Qin, P., Lutts, S., 2012a. Effects of salinity on the response of the wetland halophyte *Kosteletzkya virginica* (L.) Presl. to copper toxicity. Water, Air Soil Poll. 223, 1137-1150.

Han, R, Lefèvre, I., Ruan, C.J., Qin, P., Lutts, S. 2012b. NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regul. 68, 97-109.

Han, R., Lefèvre, I., Albacete, A., Pérez-Alfocea, F., Barba-Espín, G., Díaz-Vivancos, P., Quinet, M., Ryan, C.J., Hernández, J.A., Cantero-Navarro, E., Lutts, S. 2013. Antioxidant enzyme activities and hormonal status in response to Cd in the wetland halophyte *Kosteletzkya virginica* (L.) Presl. under saline conditions. Physiol. Plant. 147, 352-368.

He, Z.X., Ruan, C.J., Qin, P., Seliskar, D., Gallagher, J.L., 2003. *Kosteletzkya*

virginica (L.) Presl., a halophytic species with potential for agroecotechnology in Jiangsu Province, China. Ecol. Eng., 21, 271-276.

Hesami, R., Salimi, A., Ghaderian, S.M., 2018. Lead, zinc, and cadmium uptake, accumulation and phytoremediation by plants growing around Tang-e Douzan lead-zinc mine, Iran. Environ. Sci. Poll. Res., 25, 8701-8714.

Jiang, Q.Q., Yang, H.Q., Sun, X.L., Li, Q., Ran, K., Zhang, X.R., 2012. Relationship between polyamines metabolism and cell death in roots of *Malus hupehensis* Rehd. under cadmium stress. J. Integr. Agric. 11, 1129-1136.

Koyama, T., 2014. The roles of ethylene and transcription factors in the regulation of onset of leaf senescence. Front. Plant Sci. 5, 650.

Lefèvre, I., Gratia, E., Lutts, S., 2001. Discrimination between the ionic and the osmotic components of salt stress in relation to free polyamines accumulation in rice (*Oryza sativa* L.). Plant Sci. 161, 943-952.

Lefèvre, I., Marchal, G., Meerts, P., Corréal, E., Lutts, S., 2009. Chloride salinity reduces cadmium accumulation by the Mediterranean halophyte species *Atriplex halimus* L. Environ. Exp. Bot. 65, 142-152

Lichtenthaler, H.K.,1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Meth. Enzymol. 148, 350-382

Lin, Y.F., Aarts, M.G.M., 2012. The molecular mechanisms of zinc and cadmium stress response in plants. Cell. Mol. Life Sci. 69, 3187-3206.

Liu, J.H., Wang, W., Wu, H., Gong, X., Moriguchi, T., 2015. Polyamines function in stress tolerance: from synthesis to regulation. Front Plant Sci. 6, 827.

Lowry, O.H., Rosebrought, L.N., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193, 265-275.

Luo, R., Li, J., Zhao, Y., Fan, X., Zhao, P., Chai, L. 2017. A critical review on the research topic system of soil heavy metal pollution bioremediation based on dynamic co-words network measures. Geoderma 305, 281-292.

Lutts, S., Lefèvre, I. 2015. How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? Ann. Bot. 115, 509-528.

Lutts, S., Kinet, J.M., Bouharmont, J., 1996. NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Ann. Bot., 78, 389-398.

Lutts, S., Qin, P., Han, R. 2016. Salinity influences biosorption of heavy metals

by the roots of the halophyte plant species *Kosteltzkya pentacarpos*. Ecol. Eng. 95, 682-689.

Manousaki, E., Kokkali, F., Kalogerakis, N. 2009. Influence of salinity on lead and cadmium accumulation by the saltr cedar (*Tamarix smyrnensis* Bunge). J. Chem. Technol. Biotechnol. 84, 877-883.

Meena, R.A.A., Satishkumar, P., Ameen, F., Yusoff, M.R.M., Gu, F.L., 2018. Heavy metal pollution in immobile and mobile components of lentic ecosystem – a review. Environ. Sci. Poll. Res., 25, 4134-4148.

Mesa, J., Mateos-Naranjo, E., Pajuelo, E., Caviedes, M.A., Rodriguez-Llorente, I.D., 2016. Heavy mertal pollution structrures soil bacteria community dynamics in SW Spain polluted salt marshes. Water Air Soil Poll. 227, 446.

Minocha, R., Majumdar, R., Minocha, S.C., 2014. Polyamines and abiotic stress in plants: a complex relationship. Front. Plant Sci. 5, 175.

Pál, M., Szalai, G., Janda, T., 2015. Speculation: polyamines are important in abiotic stress signaling. Plant Sci. 237, 16-23.

Pandey, S., Ranade, S.A., Nagar, P.K., Kumar, N., 2000. Role of polyamines and ethylene as modulators of plant senescence. J. Biosci. 25, 291-299.

Peralta-Videa, J.R., Lopez, M.L., Narayan, M., Saupe, G., Gardea-Rorresdey, J. 2009. The biochemistry of environmental heavy metal uptake by plants: implications for the food chain. Int. J. Biochem. Cell Biol. 41, 1665-1677.

Pottosin, I., Shabala, S., 2014. Polyamines control of cation transport across plant membranes: implications for ion homeostasis and abiotic stress signaling. Front Plant Sci. 5, 154.

Redmann, R.E., Haraldson, J., Gusta, L.V., 1986. Leakage of UV-absorbing substances as a measure of salt injury in leaf tissue of woody species. Physiol. Plant 67, 87-91.

Ren, J., Chen, J., Han, L., Wang, M., Yang, B., Du, P., Li, F., 2018. Spatial distribution of heavy metals, salinity and alkalinity in soils around bauxite residue disposal area. Sci. Tot. Environ., 628/629, 1200-1208.

Rouphael, Y., Colla, G., Bernardo, L., Kane, D., Trevisan, M., Lucini, L., 2016. Zinc excess triggered polyamines accumulation in lettuce root metabolome, as compared to osmotic stress under high salinity. Front. Plant Sci. 7, 842.

Ruan, C.J., Teixeira da Silva, J., Mopper, S., Qin, P., Lutts, S., 2010. Halophyte improvement for a salinized world. Crit. Rev. Plant Sci. 29: 329-359.

Schachtman, D.S., 2015. The role of ethylene in plant responses to K^+ deficiency. Front Plant Sci., 6,1153.

Sobiezczuk-Nowicka, E., 2017. Polyamine catabolism adds fuel to leaf senescence. Amino Acids 49, 49-56.

Tiburcio, A.F., Altabemlla, T., Bitrián, M., Alcázar, R., 2014. The roles of polyamines during lifespan of plants: from development to stress. Planta 240,1-18.

Vera-Sirera, F., Minguet, E.G., Singh, S.K., Ljung, K., Tuominen, H., Blázquez, M.A., Carbonell, J., 2010. Roles of polyamines in plant vascular development. Plant Physiol. Biochem. 48, 534-539.

Wali, M., Fourrati, E., Hmaeid, N., Ghabriche, R., Poschenrieder, C., Abdelly, C., Ghnaya, T., 2015. NaCl alleviates Cd toxicity by changing its chemicaml forms of accumulation in the halophyte *Sesuvium portulacastrum*. Environ. Sci. Pollut. Res. 22, 10769-10777.

Wali, M., Gunsè, B., Llugany, M., Corrales, I., Abdelly, C., Poschenrieder, C., Ghnaya, T., 2016. High salinity helps the halophyte *Sesuvium portulacastrum* in defense against Cd toxicity by maintaining redox balance and photosynthesis. Planta 244, 333-346.

Wali, M., Martos, S., Pérez-Martin, L., Abdelly, C., Ghnaya, T., Poschenrieder, C., Gunsé, B., 2017. Cadmium hampers salt tolerance of *Sesuvium portulacastrum*. Plant Physiol. Biochem. 115, 390-399.

Yang, H., Shi, G., Wang, H., Xu, Q., 2010. Involvement of polyamines in adaptation of *Potamogeton crispus* L. to cadmium stress. Aquatic Toxicol. 100, 282-288.

Interchapter 2-3

What do we know?

- Heavy metals increase ethylene synthesis in relation to increase in senescence parameters, but NaCl decreases it in plants exposed to Cd or to the combined treatment (Cd+Zn) but not in plants exposed to Zn alone.
- Putrescine increases while spermine and spermidine decreases in Cd-treated plants. Zinc has only a marginal impact on polyamine concentration. The highest putrescine and spermine concentrations are in plants exposed to the combined treatment.
- The inhibitor of ethylene synthesis, AVG, can partially restore plant growth, reduce putrescine content and increase spermidine and spermine concentration, leading to an attenuation of senescence, mainly in Cd-treated plants.

Combined treatment induces a specific plant hormonal status (in ethylene and polyamine) in *K. pentacarpos* which could not be fully explained by an additive effect of Cd and Zn. It appears that ethylene is mainly involved in Cd-induced senescence processes, but its implication in Zn-induced senescence is not clearly demonstrated at this stage under our experimental conditions.

What are the questions?

- Beside polyamines, cytokinins are also considered as important antisenescing phytohormones. What could be the involvement of cytokinins in the heavy-metal induced senescence in *K. pentacarpos*?

- Salinity itself has a strong impact on senescing hormones independently of heavy metal pollution but there is a crucial lack of information regarding salt and heavy metal interaction in relation to plant phytohormonal status in *K. pentacarpos*.

What is our strategy?

In chapter 3, the impact of an exogenous application cytokinin (in the form of trans-zeatin riboside, one of the most active form of cytokinin) will be studied in *K. pentacarpos* exposed in nutrient solution to Cd+Zn or individual elements in the presence and absence of NaCl. To reduce the cost, this compound will be applied by leaf spraying rather than addition to nutrient solution. The considered parameters (stomatal conductance, net chlorophyll, chlorophyll concentration and carbon isotope discrimination) related to carbon fixation capacity will be determined.

In order to provide a global overview of the plant hormonal status, the main phytohormone including total auxin, cytokinin, gibberellins, ethylene (and their metabolites) and ABA, jasmonic acid, salicylic acid, polyamines will analysed in *K. pentacarpos* exposed to combination of Cd and Zn in the presence and absence of NaCl or/and exogenous cytokinin.

CHAPTER 3 The cytokinin trans-zeatin 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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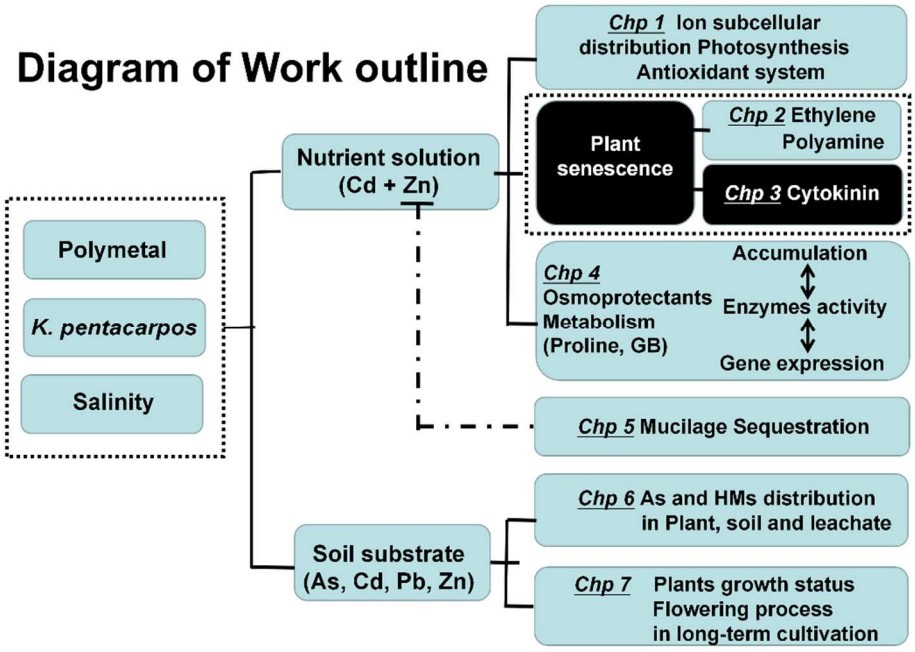
Diagram of Work outline
Chp 1 Ion subcellular distribution Photosynthesis Antioxidant system
Plant senescence
Chp 2 Ethylene Polyamine
Chp 3 Cytokinin
Nutrient solution (Cd + Zn)
Polymetal
K. pentacarpos
Salinity
Chp 4 Osmoprotectants Metabolism (Proline, GB)
Accumulation
Enzymes activity
Gene expression
Chp 5 Mucilage Sequestration
Chp 6 As and HMs distribution in Plant, soil and leachate
Soil substrate (As, Cd, Pb, Zn)
Chp 7 Plants growth status Flowering process in long-term cultivation

Chapter 3

ABSTRACT

Heavy metals such as cadmium and zinc constitute major pollutants in coastal areas and frequently accumulate in salt marshes. The wetland halophyte plant *Kosteletzkya pentacarpos* is a promising species for phytomanagement 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-zeatin 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 (MDA) 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 the plant hormone cytokinin assumes key function in Zn resistance but its efficiency is lower in the presence of NaCl.

Keywords: *Kosteletzkya pentacarpos*, cytokinin, salinity, zinc, cadmium

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; Kicinska 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; 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. Besides 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 chapter 2, 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 senescence, 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 effects 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 16h day/8h night period. Light intensity was 245 µmol 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 µM) 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 5M 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; Han et al., 2013a, 2013b) 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*-zeatin 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 two days 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 72h; 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 sulphur 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 Kamínek (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 ± 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 δ_p is the $\delta^{13}C$ of the leaf sample and δ_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 quantification were determined by Shimadzu HPLC system (Shimadzu,

's-Hertogenbosch, The Netherlands) equipped with a Nucleodur C18 Pyramid column (125 x 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 was 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 Enterprise 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 zeatin-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.

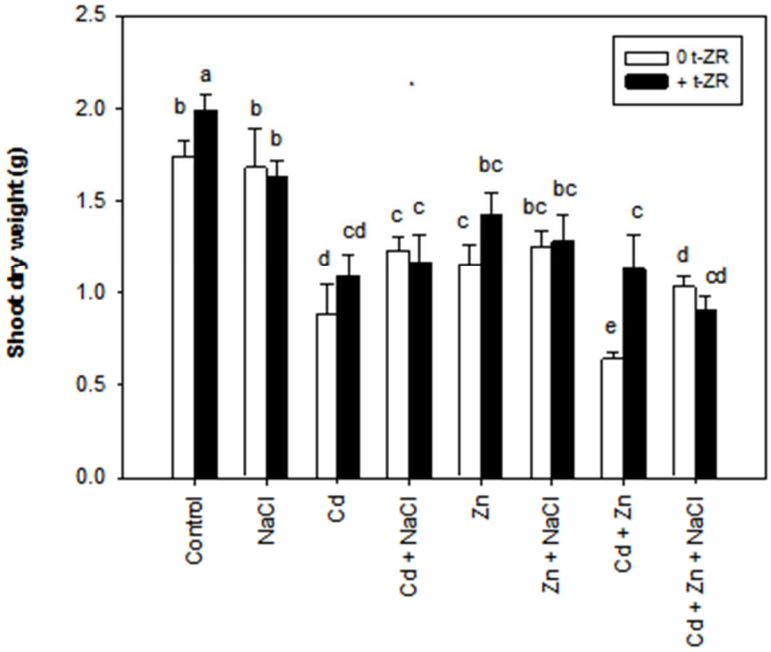

Figure 1. Shoot dry weight (g) of *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 µM), zinc (100 µM), Cd + Zn (10 µM + 100 µM) in the presence or in the absence of 50 mM NaCl. Plants were sprayed or not with 10 µM *t*-zeatin riboside (*t*-ZR;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

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).

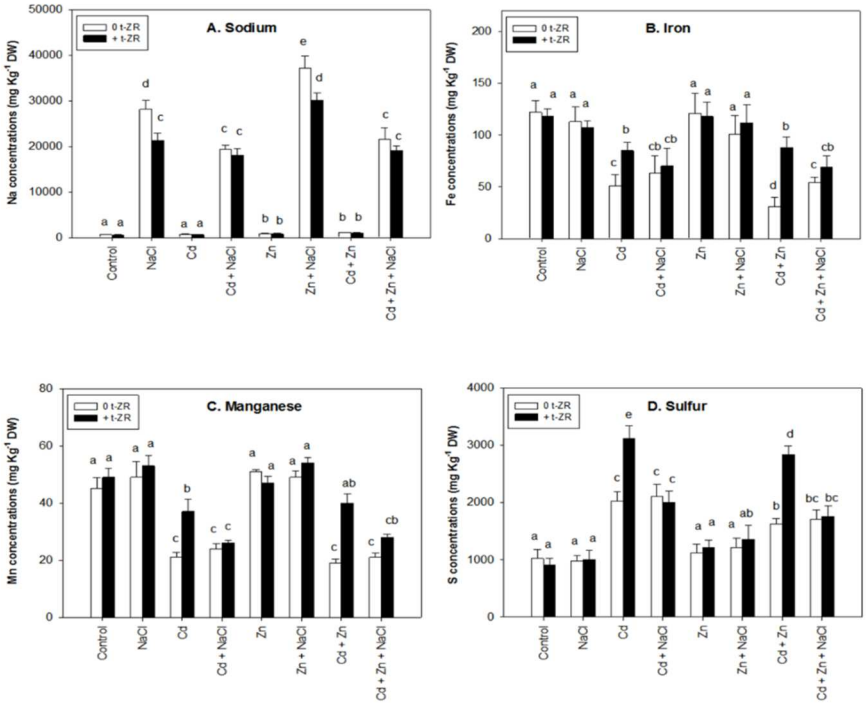

Figure 2. Leaf Na (A), Fe (B), Mn (C) and S (D) concentrations (mg kg^{-1} DW)

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.

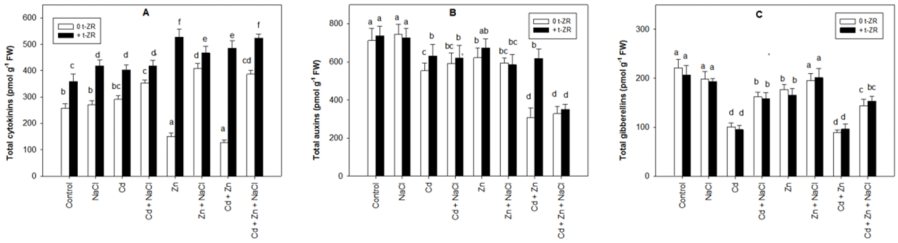

Figure 3. Leaf total cytokinins (A), auxins (B) and gibberellins (C) concentrations (pmol g^{-1} FW) of *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 µM), zinc (100 µM), Cd + Zn (10 µM + 100 µM) in the presence or in the absence of 50 mM 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.

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).

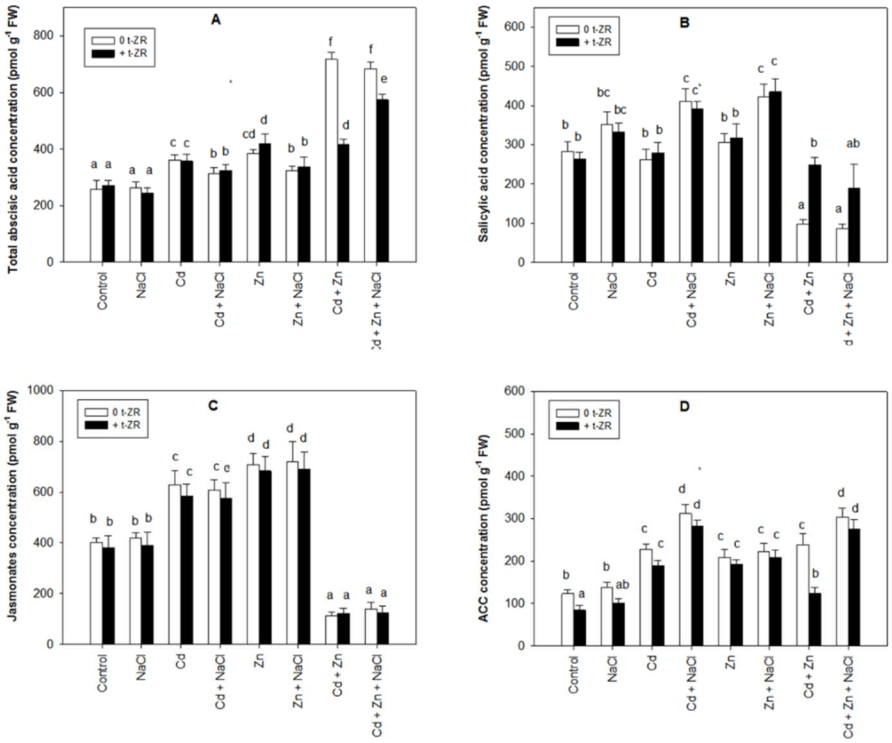

Figure 4. Leaf total abscisic acid (A), salicylic acid (B), jasmonates (C) and aminocyclopropane carboxylic acid (D) concentrations (pmol g^{-1} DW) of *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 µM), zinc (100 µM), Cd + Zn (10 µM + 100 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.

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

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

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.

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 element, 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

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.

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

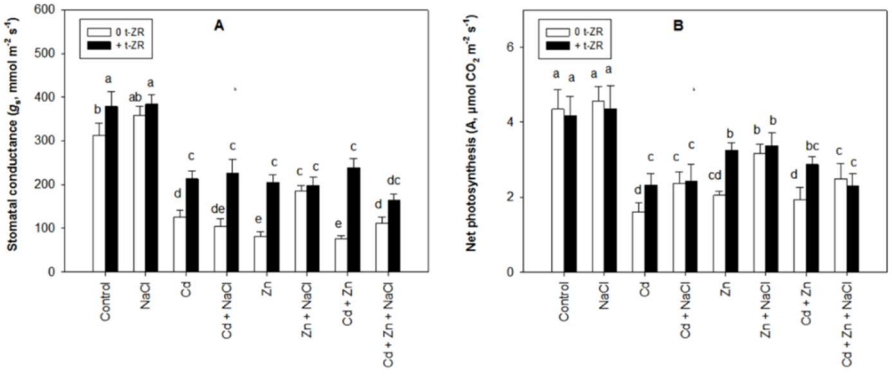

Figure 5. Leaf stomatal conductance (g_s, mmol m^{-2} s^{-1}; A) and net photosynthesis (A, µmol CO_2 m^{-2} s^{-1}; B) in *Kosteletzkya pentacarpos* seedlings

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.

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.

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 element, 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 (ΔC^{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 a	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

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

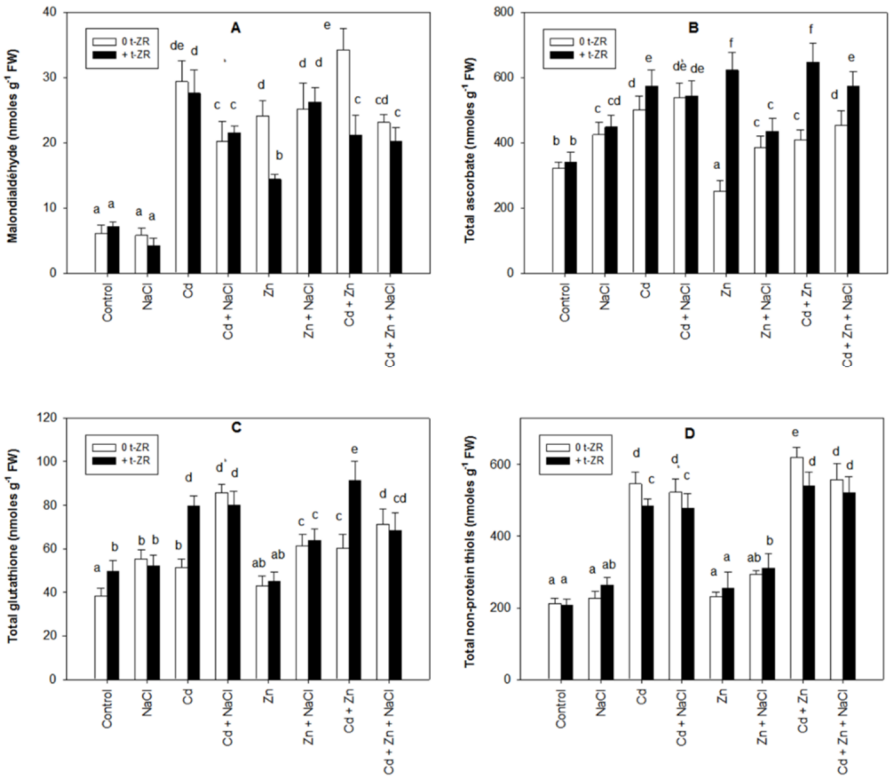

Figure 6. Leaf malondialdehyde (MDA, nmol g^{-1} FW; A), total ascorbate (nmol g^{-1} FW; B), total glutathione (nmol g^{-1} FW; C) and total non-protein thiols (nmol g^{-1} FW; D) in *Kosteletzkya pentacarpos* seedlings cultivated in nutrient solution and exposed for two weeks to cadmium (10 µM), zinc (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.

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).

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. For a given element, values exhibiting different letters are significantly different at $P < 0.05$ according to the Student-Newman-Keuls test.

	Phytochelatin concentration (in nmol 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

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 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 an 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 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 plants, *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 overexpression of *ipt* gene was reported to enhance Zn tolerance in tobacco (Pavlikova 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 (Chapter 2): 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.

Reference

Albacete, A., Martinez-Andujar, C., Ghanem, M., Acosta, M., Sanchez-Bravo, J., Asins, M.J., Cuartero, J., Lutts, S., Dodd, I.C., Pérez-Alfocea, F., 2009. Rootstock-mediated changes in xylem ionic and hormonal status are correlated with delayed leaf senescence and increased leaf area and crop productivity in salinized tomato. Plant Cell Environ. 32, 928-9238

Bai, J., Zhao, Q., Wang, W., Wang, X., Jia, J., Cui, B., Liu, X., 2019. Arsenic and heavy metals pollution along a salinity gradient in drained coastal wetland soils: depth distributions, sources and toxic risks. Ecol. Ind. 96, 91-98

Bonanno, G., Borg, J.A., Di Martino, V., 2017. Levels of heavy metals in wetland and marine vascular plants and their biomonitoring potential: a comparative assessment. Sci. Total Environ. 576, 796-806

Caldelas, C., Weiss, D.J., 2017. Zinc homeostasis and isotopic fractionation in plants: a review. Plant Soil 411, 17-46

Cassina, L., Tassi, E., Morelli, E., Giorgetti, L., Remorini D., Chaney, R.L., Barbafieri, M., 2011. Exogenous cytokinin treatments of an Ni hyper-accumulator *Alyssum murale* grown in a serpentine soils: implications for phytoextraction. Int. J. Phytorem. 13, 90-101

Cassina, L., Tassi, E., Pedron, F., Petruzzelli, G., Ambrosini, P., Barebafieri, M., 2012. Using a plant hormone and a thioligand to improve phytoremediation of Hg-contaminated soil from a petrochemical plant. J. Hazard. Mat. 231/232, 36-42

Cereser, C., Guichard, J., Drai, J., Bannier, E., Garcia, I., Boget, S., Parvaz, P., Revol, A., 2001. Quantitation of reduced and total glutathione at the femtomole level by high-performance liquid chromatography with fluorescence detection: application to red blood cells and cultured fibroblasts J Chrom B: Biomed Sci Appl 752, 123-132

Chandra, R., Yadav, S., Yadav, S., 2017. Phytoextraction potential of heavy metal by native plants growing on chlorolignin containing sludge of pulp and paper industry. Ecol. Eng. 98: 134-145

Cheng, M., Kopittke, P.M., Wang, A., Sale, P.W., Tang, C., 2018. Cadmium reduces zinc uptake but enhances its translocation in the cadmium-accumulator *Carpobrotus rossii* without affecting speciation. Plant Soil 430, 219-231

Cherif, J., Derble, A., Nakkach, M., Bergmann, H., Jemal, F., Lakhdar, Z.B., 2012. Spectroscopic studies of photosynthetic responses of tomato plants to the interaction of zinc and cadmium toxicity. J. Photochem. Photobiol. B: Biology 111, 9-16

Chmielowska-Bak, J., Lefèvre, I., Lutts, S., Deckert, J., 2013. Short term signaling responses in roots of young soybean seedlings exposed to cadmium stress. J. Plant Physiol. 170, 1585-1594

Chowdhury, R.N., Favas, P.J.C., Johnatan, M.P., Venkatachalam, P., Raja, P., Sarkar, S.L., 2017. Bioremoval of trace metals from rhizosediments by mangrove plants in Indian Sundarban Weltands. Marine Poll. Bull. 124, 1078-1088

Cristescu, S.M., De Martinis, D., Te Lintel Hekkert, S., Parker, D.H., Harren, F.J.M., 2002. Ethylene production by *Botrytis cinerea* in vitro and in tomatoes. Appl. Environ. Microbiol. 68, 5342-5350

De Vos, C.R., Vonk, M.J., Vooijs, R., Schat, H., 1992. Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus* Plant Physiol. 98, 853-858

Djilianov, D.L, Dobrev, P.I., Moyankova, D.P., Vaňková, R., Georgieva D.T., S. Gajdošová, S., Motyka, V., 2013. Dynamics of endogenous phytohormones during desiccation and recovery of the resurrection plant species *Haberlea rhodopensis*. J. Plant Growth Regul. 32, 564-574

Dobrev, P., Kaminek, M., 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid phase extraction. J Chromatogr A 950, 21-29

Dobrev, P.I., Vankova, R., 2012. Quantification of abscisic acid, cytokinin, and auxin content in salt-stressed plant tissues. Meth. Mol. Biol. 913, 251-261

Farquhar, G.D., Richards, R.A., 1984. Isotopic composition of plant carbon correlates with water-use efficiency in wheat genotypes. Austr. J. Plant Physiol. 11, 539-552

Gadallah, M.A.A., El-Enany, A.E., 1999. Role of kinetin in alleviation of copper and zinc toxicity in *Lupinus termis* plants. Plant Growth Regul. 29, 151-160.

Gupta, N., Ram, H., Kumar, B., 2016. Mechanisms of zinc absorption in plants: uptake, transport, translocation and accumulation. Review Environ. Sci. Biotechnol. 15, 89-109

Han, R.M., Lefèvre, I., Albacete, A., Pérez-Alfocea, F., Barba-Espín, G.,

Díaz-Vivancos, P., Quinet, M., Ryan, C.J., Hernández, J.A., Cantero-Navarro, E., Lutts, S., 2013 a. Antioxidant enzyme activities and hormonal status in response to Cd in the wetland halophyte *Kosteletzkya virginica* (L.) Presl. under saline conditions. Physiol. Plant. 147, 352-368

Han, R.M., Lefevre, I., Ruan, C.J., Beukelaers, N., Qin, P., Lutts, S., 2012 a. Effects of salinity on the response of the wetland halophyte *Kosteletzkya virginica* (L.) Presl. to copper toxicity. Water, Air, Soil Pollut. 223, 1137-1150

Han, R.M., Lefèvre, I., Ruan, C.J., Qin, P., Lutts, S., 2012 b. NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regul 68, 97-109

Han, R.M., Quinet, M., André, E., Teun van Elteren, J., Destrebecq, F., Vogel-Mikuš, K., Cui, G., Debeljak, M., Lefèvre, I., Lutts, S., 2013 b. Accumulation and distribution of Zn in the shoots and reproductive structures of the halophyte plant species *Kosteletzkya virginica* as a function of salinity. Planta 238, 441-457

Han, S.H., Kim, D-H., Lee, J.C., 2010. Cadmium and zinc interaction and phytoremediation potential of seven *Salix caprea* clones. J. Ecol. Field Biol. 33, 245-251

Hashem, H.A., 2014. Cadmium toxicity induces lipid peroxidation and alters cytokinin content and antioxidant enzyme activities in soybean. Botany 92, 1-7

Hayward, A.R., Coates, K.E., Galer, A.L., Hutchinson, T.C., Emery, R.J.N., 2013. Chelator profiling in *Deschampsia cespitosa* (L.) Beauv. reveals a Ni reaction, which is distinct from the ABA and cytokinin associated response to Cd. Plant Physiol. Biochem. 64, 84-91

He, Z.X., Ruan, C.J., Qin, P., Seliskar, D.M., Gallagher, J.L., 2003. *Kosteletzkya virginica*, a halophytic species with potential for agroecotechnology in Jiangsu provine, China. Ecol. Engin. 210, 271-276

Heath, RL, Packer, L., 1968. Photoperoxidation in isolated chloroplasts. II. Role of electron transfer. Arch. Biochem. Biophys. 125, 850-857

Kicińska, A., Smerczak, B., Jadczyszyn, J., 2019. Soil bioavailability of cadmium lead and zinc in the areas of Zn-Pb ore mining and processing (Bukowno, Olkisz). J. Ecol. Eng. 20, 84-92

Kumar, V., Sharma, A., Kaur, P., Sidhu, G.P.S., Bali, A.S., Bhardwaj, R., Thukral, A.K., Cerda, A., 2019. Pollution assessment of heavy metals in soils of

India and ecological risk assessment: A state of the art. Chemosphere 216, 449-462

Li, T., Yang, X., Lu, L., Islam, E., He, Z., 2009. Effects of zinc and cadmium interactions on root morphology and metal translocation in a hyperaccumulating species under hydroponic conditions. J. Hazard. Mat. 169, 734-41

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Meth. Enzymol. 148, 350-382.

Lukatkin, A.S., Gracheva, N.V., Grishenkova, N.N., Duikhovski, P.V., Brazaitite, A.A., 2007. Cytokinin-like growth regulators mitigate toxic action of zinc and nickel ions on maize seedlings. Russ. J. Plant Physiol. 54, 381-387

Lutts, S., Lefèvre, I., 2015. How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? Ann. Bot. 115, 509-528

Lutts, S., Qin, P., Han, R., 2016. Salinity influences biosorption of heavy metals by the roots of the halophyte plant species *Kosteltzkya pentacarpos*. Ecol. Eng. 95, 682-689.

Meena, R.A.A., Satishkumar, P., Ameen, F., Yusoff, A.R.M., Gu, F.L., 2018. Heavy metal pollution in immobile and mobile components of lentic ecosystems – a review. Environ Sci Poll. Res. 25, 4134-4148

Merewitz, E.B., Gianfagna, T., Huang, B., 2011. Photosynthesis, water use and root viability under water stress as affected by expression of SAG12-ipt controlling cytokinin synthesis in *Agrostis stolonifera*. J. Exp. Bot. 62, 383-395

Mohan, T.C., Castrillo, G., Navarro, C., Zarco-Fernandez, S., Ramireddy, E., Mateo, C., Zamarreño, A.M., Paz-Ares, J., Muñoz, R., Garcia-Mina, J.M., Hernández, L.E., Schmülling, T., Leyva, A., 2016. Cytokinin determines thiol-mediated arsenic tolerance and accumulation. Plant Physiol. 171, 1418-1426

Pavlíková, D., Pavlík, M., Procházková, D., Zemanová, V., Hnilička, F., Wilhelmová, N., 2014. Nitrogen metabolism and gas exchange parameters associated with zinc stress in tobacco expressing an ipt gene for cytokinin synthesis. J. Plant Physiol. 171, 559-564

Qin, P., Han, R.M., Zhou, M.X., Zhang, H.S., Fan, L.S., Seliskar, D.M., Gallagher, J.L., 2015. Ecological engineering through the biosecure introduction of *Kosteletzkya virginica* (seashore mallow) to saline lands in

China: a review of 20 years of activity. Ecol. Eng. 74, 174-186

Quinet, M., Bataille, G., Dobrev, P.I., Capel, C., Gómez, P., Capel, J., Lutts, S., Motyka, V., Angosto, T., Lozano, R., 2014. Transcriptional and hormonal regulation of petal and stamen development by STAMENLESS, the tomato (*Solanum lycopersicum* L.) orthologue to the B-class APETALA3 gene. J. Exp. Bot. 65, 2243-2256

Rivero, R.M., Shualev, V., Blumwald E., 2009. Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. Plant Pysiol. 150, 1530-1540

Schäfer, H.J., Greiner, S., Rausch, T., Haag-Kerwer,A., 1997. In seedlings of the heavy metal accumulator *Brassica juncea* Cu2+ differentially affects transcript amounts for γ-glutamylcysteine synthetase (γ-ECS) and metallothionein (MT2) FEBS lett 404:216-220

Shi, T., Ma, J., Wu, X., Ju, T., Lin, X., Zhang, Y., Li, X., Gong, Y., Hou, H., Zhao, L., Wu, F., 2018. Inventories of heavy metal inputs and outputs to and from agricultural soils: A review. Ecotox. Environ. Safety 164, 118-124

Singh, S., Prasad, S.M., 2014. Growth, photosynthesis and oxidative responses of *Solanum melongena* L. seedlings to cadmium stress: mechanism of toxicity amelioration by kinetin. Scien. Hortic. 176, 1-10

Singh, S., Singh, A., Srivastava, P.K., Prasad, S.M., 2018. Cadmium toxicity and its amelioration by kinetin in tomato seedlings vis-à-vis ascorbate glutathione cycle. J. Photochem. Photobiol. B: Biology 178, 76-84

Tkalec, M., Stefanic, P.P., Cvjetko, P., Sikic, S., Pavlica, M., Balen, B., 2014. The effect of cadmium-zinc interactions on the biochemical responses in tobacco seedlings and adult plants. PLoS ONE 9:e87582 doi: 10.1371/journal.pone.0087582.

Veselov, S.Y., Timergalina, L.N., Akhiyarova, G.R., Kudoyarova, G.R., Korobova, A.V., Ivanov, I., Arhipova, T.N., Prinsen, E., 2018. Study of cytokinin transport from shoots to roots of wheat plants is informed by a novel metyhod of differential localization of free cytokinin bases or their ribosylated forms by means of their specific fixation. Protoplasma 255, 1581-1594

Wang, S.Y., Jiao, H.J., Faust, M., 1991. Changes in ascorbate, glutathione, and related enzyme activities during thidiazuron-induced bud break of apple. Physiol. Plant. 82, 231-236

Wang, S.L., Xu, X.R., Sun, Y.X., Liu, J.L., Li, H.B., 2013. Heavy metal

pollution in coastal areas of South China: A review. Marine Poll. Bull. 76, 7-15

Wang, W., Hao, Q., Wang, W., Li, Q., Chen, F., Ni, F., Wang, Y., Fu, D., Wu, J., Wang, W., 2019. The involvement of cytokinin and nitrogen metabolism in delayed flag leaf senescence in a wheat stay-green mutant, tasg1. Plant Sci. 278, 70-79

Wen, X., Lu, J., Wu, J., Lin, Y., Luo, Y., 2019. Influence of coastal groundwater salinization on the distribution and risks of heavy metals. Sci. Total Environ. 652, 267-277

Wu, S., Shi, K., Hu, C., Guo, J., Tan, Q., Sun, X., 2019. Non-invasive microelectrode cadmium flux measurements reveals the decrease of cadmium uptake by zinc supply in pachkoi (*Brassica chinensis* L.). Ecotox. Environ. Saf. 168, 363-368

Wu, X., Zhu, Z., Li, X., Zha, D., 2012. Effects of cytokinin on photosynthetric gas exchange, chlorophyll fluorescence parameters and antioxidative system in seedlings of eggplant (*Solaum melongena* L.) under salinity. Acta Physiol. Plant. 34, 2105-2114

Xia, Z., Zhang, S., Cao, Y., Zhong, Q., Wang, G., Li, T., Xu, X., 2019. Remediation of cadmium, lead and zinc in contaminated soil with CETSA and MA/AA. J. Haz. Mat. 366, 177-183

Zhou, Y., Wang, D., Wang, N., Yu, J., Wang, Y., Wu, Q., Xu, W., Huang, R., 2016. Involvement of endogenous abscisic and cytokinin in photosynthetic performance of different stay green inbred lines of maize under drought. Int. J. Agric. Biol. 18, 1067-1074.

Interchapter 3-4

What do we know?

- Cytokinin depletion is involved in Zn-induced senescence (Chapter 3), while ethylene is mainly involved in Cd-induced senescence. Exogenous trans-zeatin riboside increases plant growth, stomatal conductance, net photosynthesis and total ascorbate and reduces oxidative stress in Zn-treated plants maintained in the absence of NaCl.
- Plants exposed to the mixed treatment (Cd+Zn) exhibit a specific hormonal status in relation to accumulation of ABA and depletion of SA.
- Salinity helps the plant to cope with heavy metal toxicities through a decrease in toxic ion absorption. However, cytokinin efficiency for reducing heavy metal injuries is reduced in the presence of NaCl.

In summary, cytokinin assume key function in heavy-metal resistance but its efficiency is lower in the presence of NaCl in *K. pentacarpos*.

What are the questions?

In chapter 1-3, the combination of Cd and Zn not only induces a modification of plant hormonal status, but also a decrease the leaf water content. This observation suggest that plants are encountering water stress, and this is confirmed by the impact of the treatment on stomatal conductance. Surprisingly, salinity treatment which is itself compromising the plant water status through its decreased in external osmotic potential seems to attenuate the negative impact of Cd and Zn on the plant water status.

To explain this puzzling information, we hypothesize that NaCl may induce an increase in osmocompatible solute which are also known to

assume protective functions in stressed plants in relation to protection of numerous cellular structures. Among these compounds, Proline is a ubiquitous osmolyte playing a vital role in resistance to water stress but its involvement in *K. pentacarpos* adaptation to short-term mixed Cd and Zn toxicity remains unclear.

In contrast to proline, glycinebetaine (GB) accumulates in a limited number of plant species. It has been proven that in some halophyte plant species (mainly ion Amaranthaceae and Malvaceae families), the accumulation of GB protects cellular (and especially chloroplast) structures from the deleterious impact of toxic ions. Hence, it is of great interest to discover the effect of salinity on the metabolism of GB in *K. pentacarpos* exposed to a complex of Cd and Zn.

What is our strategy?

Young plants of the halophyte *K. pentacarpos* will be grown in nutrient solution in the presence of Cd or Zn, or a combination of both heavy metals and in the presence or absence of NaCl 50 mM. To more precisely analyse the significance of osmolyte compounds accumulation in stressed tissues, we decide not only to quantify proline and glycinebaine concentration in relation to osmotic adjustment of stressed plants but also to quantify enzyme activities involved in their metabolism and the expression of the corresponding coding gene. Since osmolyte accumulation has been reported in the literature as a very fast response occurring few hours

CHAPTER 4 Effect of NaCl on proline and glycinebetaine metabolism in *Kosteletzkya pentacarpos* exposed to Cd and Zn toxicities

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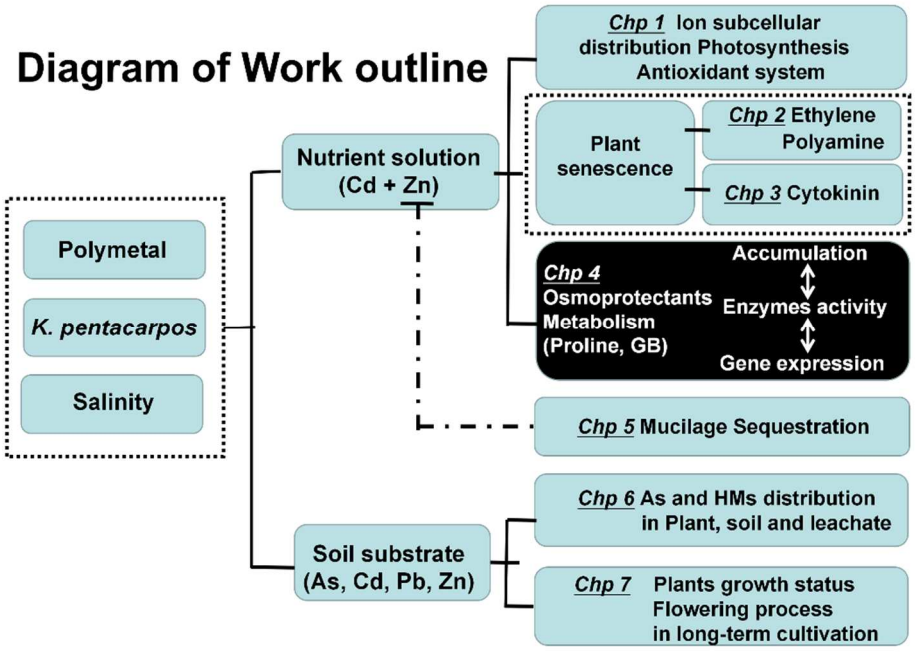


Diagram of Work outline
Chp 1 Ion subcellular distribution Photosynthesis Antioxidant system
Plant senescence
Chp 2 Ethylene Polyamine
Chp 3 Cytokinin
Nutrient solution (Cd + Zn)
Polymetal
K. pentacarpos
Salinity
Chp 4 Osmoprotectants Metabolism (Proline, GB)
Accumulation
Enzymes activity
Gene expression
Chp 5 Mucilage Sequestration
Chp 6 As and HMs distribution in Plant, soil and leachate
Soil substrate (As, Cd, Pb, Zn)
Chp 7 Plants growth status Flowering process in long-term cultivation

Chapter 4

ABSTRACT

Proline and glycinebetaine are osmolytes playing a role in resistance to salt and water stress but their involvement in plant adaptation to heavy metals remain unclear. Young plants of the halophyte *Kosteletzkya pentacarpos* were grown in nutrient solution in the presence of Cd (20 or 40 µM) or Zn (200 or 400 µM), or a combination of both heavy metals and in the presence or absence of NaCl 50 mM for 48h. Osmolytes concentrations, enzyme activities involved in their metabolism and expression of corresponding genes were determined in roots and leaves. Cadmium but not zinc increased proline and glycinebetaine in the leaves. Salinity reduced proline content in Cd-treated plants but increased it in plants exposed to Cd+Zn. Proline was produced through both glutamate and ornithine pathways while proline dehydrogenase was inhibited in response to heavy metals. Correlation between enzyme activities and corresponding gene expression was significant in the leaves but not in the roots. Gene coding for proline transport (*KvProT*) was upregulated in response to heavy metals. Low NaCl dose (50 mM) afford protection to heavy metal stress in *K. pentacarpos* and its effect on osmolyte synthesis depends on considered metal and plant organ

Keywords: halophyte, heavy metals, phytoremediation, salinity, seashore mallow, wetland

1. Introduction

Heavy metal pollution in the environment is a major problem resulting from human activities such as mining, industrial activities, use of pesticides or poor-quality fertilizers and sewage emission (Yang et al. 2018; Lin et al. 2019). Cadmium is a widespread and highly toxic pollutant. Its presence in contaminated soil is frequently associated with high amounts of Zn, and both elements share numerous chemical properties. When present in excess, those heavy metals affect plant growth and development, compromise plant survival, constitute a major risk for human health as a consequence of food chain contamination, and may affect the ecosystem stability (Patar et al. 2016; Kumar et al. 2019).

In coastal areas, plants are often concomitantly exposed to high levels of salinity and heavy metals. Halophyte plant species are consequently recommended as interesting tools for the phytomanagement of these specific contaminated zones (Lutts and Lefèvre 2015). These environmental constraints may interact to some extent and the presence of NaCl has been reported to influence heavy metal absorption in plants (Lefèvre et al. 2009; Lutts and Lefèvre 2015). Plants exposed to ion toxicities commonly suffer from oxidative stress resulting from the production of reactive oxygen species (ROS) and from alteration of the plant water status (Zhang et al. 2019; Sdouga et al. 2019). Organic compatible solutes such as proline or glycinebetaine are valuable osmoprotectants leading to a decrease in the internal osmotic potential allowing the plant to maintain water uptake and are acting as putative antioxidant compounds (Mansour and Ali 2017).

The amino acid proline accumulates under a wide range of environmental constraints (Kaur and Asthir 2015; Zhang and Becker 2015). It is synthesized from L-glutamate, which is reduced to glutamic γ-semialdehyde (GSA) by Δ^1-pyrroline-5-carboxylate synthetase (P5CS; EC 1.2.1.41); GSA spontaneously forms

pyrroline-5-carboxylate which is then converted to proline by pyrroline-5-carboxylate reductase (P5CR; EC 1.5.1.2). Proline may also be produced by an alternative pathway through the action of ornithine-δ-aminotransferase (OAT; EC 2.6.1.13) which converts ornithine to the proline precursor GSA (Skopelitis et al. 2006). Proline synthesis is a reversible process and this compound may be recycled to glutamate after the stress relief through the action of proline dehydrogenase (PDH; EC 1.5.5.2). P5CS and PDH activities are commonly considered as the rate-limiting steps for proline synthesis and catabolism, respectively (Ben Rejeb et al. 2014; Kavi Kishor and Sreenivasulu 2014). Proline oversynthesis was reported to occur as a result of transcriptional regulation leading to the overexpression of proline metabolism-related genes (Silva-Ortega et al. 2008; Kubala et al. 2015; Singh et al. 2016; Zegaoui et al. 2017; Guan et al. 2018; Sdouga et al. 2019). Proline distribution among organs and cell compartments is also an important component of plant response to environmental constraints: ProT is a proline transporter playing a key role in proline distribution between plant organs and the corresponding gene has been reported to be up-regulated by environmental constraints such as salt and drought (Chen et al. 2016).

While proline accumulation is a ubiquitous response in the plant kingdom, glycinebetaine (GB) accumulates in a limited number of plant species. Glycinebetaine is a fully N-methyl substituted derivative of glycine. As a quaternary ammonium compound, GB has a fascinating capacity to interact with biological membrane and to protect cellular structures from the deleterious impact of toxic ions and desiccation (Lutts 2000; Chen and Murata 2008; Figuerora-Soto and Valenzuela-Soto 2018). Glycinebetaine synthesis occurs in chloroplast and this compound stabilizes the oxygen-evolving PSII complex against high concentration of toxic ions and high temperature (Chen and Murata 2008). In higher plants, synthesis of GB includes two steps: dehydrogenation of choline by choline monooxygenase (CMO; EC 1.14.15.7) encoded by *CMO* gene and oxygenation of betaine aldehyde by betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8) encoded by *BADH* gene. The expression of *BADH* and

activity of BADH in plant exposed to abiotic stress conditions have been extensively studied since they are considered as the rate limiting step in GB synthesis (Mansour and Ali 2017; Figuerora-Soto and Valenzuela-Soto 2018).

Kosteletzkya pentacarpos (syn. *K. virginica*) is a perennial facultative halophyte species from the Malvaceae family and is native to the brackish marshes of mid-Atlantic and southeastern United States. The combination of Cd and Zn was reported to alter photosynthesis through an impact on the light phase (Chapter 1). It also increased oxidative stress and hasten senescence although additional NaCl was shown to reduce heavy metal impact on these parameters (Chapter 2 and 3). Proline is known to display complex interactions with plant senescence (Zhang and Becker 2015). Wang et al. (2015) demonstrated that proline accumulation in *K. pentacarpos* was more than six folds in 300 mM NaCl-treated plants than control plants already after 24h of exposure. Nevertheless, no data are available regarding the impact of heavy metals on proline metabolism in this species. Moreover, as a member of the Malvacea family, *K. pentacarpos* is able to produce and accumulate GB (Han et al. 2012a). To the best of our knowledge, no data are available on this protecting quaternary ammonium compounds in *K. pentacarpos* exposed to Cd and Zn and the influence of NaCl on GB content is not documented during short time.

The present work was therefore undertaken in order i) to determine the impact of Cd and Zn alone or in combination to proline and glycinebetaine content in *K. pentacarpos* and ii) to quantify the impact of salinity on the plant response in relation to enzyme activities involved in the osmoprotectant metabolism and the corresponding gene expression.

2. Material and methods

2.1. Plant material and growth conditions

Kosteletzkya pentacarpos seeds were kindly provided by Prof. P. Qin, University of Nanjing (PR China) and issued from Jinhai Agricultural Experimental Farm of Yancheng, Jiangsu province. 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 12h photoperiod [mean light intensity (PAR) = 150 µmoles 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, with 12 plants per tank. 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 light irradiance of 280 µmol m^{-2} s^{-1}(PAR) at the top of the canopy.

After 10 days of acclimation in the absence of stress (25 days after sowing), plants were distributed among seven groups: (1) Control (2) 20 µM $CdCl_2$ (3) 40 µM $CdCl_2$ (4) 200 µM $ZnCl_2$ (5) 400 µM $ZnCl_2$ (6) 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ and (7) 40 µM $CdCl_2$ + 400 µM $ZnCl_2$. Heavy metal doses were chosen on the basis of our previous results (Han 2013; Han et al. 2012b; Han et al. 2013a, 2013b) and may be considered as moderate (Cd 20 µM and Zn 200 µM) to high (Cd 40

μM and Zn 400 μM) pollution comparatively to data recorded in field conditions in coastal polluted wetlands (Bai et al. 2019; Yang et al. 2018; Kumar et al. 2019). For each group, half of the tanks received NaCl to reach a final dose of 50 mM and half of tanks remained unsalinized. The pH of solutions was set to 5.7 ± 0.02 with KOH. Twelve plants (three groups of 4 plants) per treatment were used for subsequent measurement of parameters after 48h heavy metal stress.

2.2. Plant growth and osmotic potential assessment

Roots were quickly rinsed in deionized water for 30s just before harvest to remove ions from the free space. Roots were then separated from shoots and leaves were separated from the stem. Roots and leaves were quickly frozen in liquid nitrogen then stored at -80°C until analysis, except subsamples of four plants per treatment incubated in an oven at 70°C for 72h to estimate dry weight and water content and to determine ion content.

For osmotic potential determination (Ψs), leaves quickly collected from three plants were cut into small segments, then placed in Eppendorf tubes perforated with small holes and immediately frozen in liquid nitrogen. Samples were then thawed at ambient temperature to rupture the membranes. After three freeze-thawing cycles, each tube was then encased in a second intact Eppendorf tube and centrifuged at 9,000 *g* for 10 min at 4 °C (Lutts et al. 1999a). The osmolarity of the collected sap was analyzed with a vapor pressure osmometer (Model 5500, WESCOR, Logan, Utah, USA).

2.3. Ion concentration

Dried samples were ground to a fine powder using a porcelain mortar and a pestle, digested in 35% HNO_3 and evaporated to dryness on a sand bath at 80 °C. The minerals were incubated with a mix of 37% HCl and 68% HNO_3 (3:1) and the mixture was slightly evaporated. Minerals were dissolved in HCl 0.1N and after full dissolution, the liquid was filtered on Whatmann n°2 filter paper. Ion concentrations

were determined by SOLAAR S4 atomic absorption spectrometry (Thermo Scientific, Cambridge, UK). For each treatment, four separated plants were considered and each analysis was performed on technical triplicates.

2.4. Determination of proline and quaternary ammonium compounds (QAC) content

Proline was extracted and quantified according to Bates et al. (1973) with slight modification as indicated in Kubala et al. (2015): 1 g FW of tissue was extracted with 5mL of 5% salicylic acid. After centrifugation at 5,000 *g,* free proline was specifically quantified using the ninhydrin method according to Bates et al. (1973): samples were incubated with 1 mL of 1% (w/v) solution of ninhydrin in 60% (v/v) acetic acid and heated at 95 °C for 20 min. Absorbance was read after chilling at 520 nm, standard curve being established using commercially available proline (Sigma-Aldrich).

Assays of glycinebetaine content in plant samples were performed according to Grieve and Grattan (1983) based on the ability of quaternary ammonium compounds to react with iodine. Dried samples were ground and mechanically shaken with 20 mL of dionized water at 20 °C. Extracts were diluted with 2N H_2SO_4 at 1:1 v/v and cooled in ice water for 1h. Cold KI-I_2 reagent (obtained from dissolving 15.7 g of iodine and 20 g of KI in 100 mL water) was added and samples stored at 0-4 °C for 16h and centrifuged at 15,000 *g* for 15 min at 0 °C. The pellet was then dissolved in 1,2-dichloroethane, incubated for 4.5 h and absorbance was read at 365 nm. Standard curve was established with commercial glycinebetaine (Sigma-Aldrich). Choline was specifically assessed by adding 40 mM sodium/potassium phosphate buffer (pH 7.4) instead of adding 2 N H_2SO_4.

2.5. Enzyme extraction and assays

Fresh samples (500 mg) were ground to a powder in liquid nitrogen and homogenized in the appropriate extraction buffer. For

proline-metabolizing enzymes (P5CS, PDH and OAT) extractions were carried out according to Lutts et al. (1999b). For P5CS and PDH extraction buffer consists in 50 mM Tris – HCl buffer (pH 7.4) containing: 0.6 M KCl, 7 mM $MgCl_2$, 3 mM EDTA (ethylene diamine tetraacetic acid), 1 mM DTT (dithiothreitol) and 5% (w/v) insoluble polyvinylpyrrolidone (PVP). Homogenate was filtered through 2 layers of Miracloth and centrifuged at 39,000 *g* for 20 min at 4 °C. After centrifugation, the supernatant was collected and desalted on a Sephadex G-25 column (GE Healthcare PD-10 column) equilibrated with 50 mM Tris – HCl (pH 7.4) supplied with 10% glycerol. The extraction buffer used for OAT consisted in 100 mM K-Pi buffer (pH 7.9) supplied with 1 mM EDTA, 15% glycerol, 10 mM β-mercaptoethanol. The homogenate was centrifuged at 15,000 *g*, 4 °C for 15 min. After centrifugation, the supernatant was treated with $(NH_4)_2SO_4$ at 60% saturation for 45 min. After ammonium sulfate treatment, sample was re-centrifuged at 15,000 *g* for 15 min and supernatant was desalted on Sephadex G-25 column (GE Healthcare PD-10 column) equilibrated with extraction buffer. Protein concentration in the extract was estimated according to Bradford (1976) procedure.

The P5CS activity was measured by monitoring the decrease in absorbance of NADH at 340 nm in 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM DTT, 1 mM Δ^1-pyrroline-5-carboxylate and 0.25 mM NADH. The PDH and OAT activity assays were conducted as previously detailed (Lutts et al. 1999b): PDH activity was quantified by monitoring the $NADP^+$ reduction at 340 nm in 0.15 M Na_2CO_3 buffer (pH 10.3) containing 15 mM proline and 1.5 mM $NADP^+$. The OAT activity was measured by monitoring the decrease in absorbance of NADH at 340 nm in 0.2 M Tris-KOH buffer (pH 8.0) containing 5 mM ornithine, 10 mM α-ketoglutarate and 0.25 mM NADH.

BADH activity was assayed independently by the betaine aldehyde-specific reduction of NAD^+ at 22 °C according to Weretilnyk and Hanson (1989). Extraction was performed in 0.1M Tricine-KOH, pH 8.5, 1 mM EDTA, 2 mM DTT and 0.6 M sucrose.

The reactions were carried out in a final volume of 1 mL containing 50 mM HEPES-KOH (pH 8.0), 10 mM EDTA, 1 mM NAD^+, 1 mM betaine aldehyde and 1 mg protein extract. One unit of BADH equals 1 nmol NAD^+ reduced min^{-1} mg^{-1} protein.

2.6. Gene expression analysis

Total RNA was extracted from 500 mg fresh roots and leaves of *K. pentacarpos* ground in liquid nitrogen. The powder was added to 7 mL of pre-heated (65 °C) extraction buffer (300 mM Tris HCl, 25 mM EDTA, 2 M NaCl, 2% (w/v) CTAB, 0.05 % (w/v) spermidine, 2% (w/v) PVP, 2 % (w/v) β-mercaptoethanol, pH 8 and incubated for 10 min at 65 °C. After centrifugation for 15 min at 4,000 *g* and 4 °C, the supernatant was collected and an equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added. Chloroform-isoamyl alcohol extraction was repeated twice. Then, 0.1 volume of 3M sodium acetate (pH 5.2) and 0.6 volume of isopropanol were added to the supernatant. After 30 min at -80 °C and centrifugation (30 min, 8400 *g*, 4 °C), the pellet was dissolved in 1 mL of TE buffer. Then 300 µl LiCl (10 M) were added and the samples were placed at 4 °C during 12 h. After centrifugation at 4 °C for 30 min, the pellet was washed with ethanol 70 %, dried and resuspended in 25 µl of DEPC-water. DNase treatment was performed using RQ1 RNAse-free DNase (Promega, Leiden, The Netherlands) according to manufacturer's instructions. RNA quality and concentration were verified by the NanoDrop ND-1000 (Isogen Life Science, De Meern, the Netherlands).

The cDNA synthesis was performed using 1 µg of RNA and the RevertAid H Minus First Strand Synthesis kit (Fermentas, St. Leon-Rot, Germany) following manufacturer's instructions. Genes involved in proline (*KvP5CS1*, *KvOAT*, *KvProT*, *KvPDH,* Wang et al (2015)) and glycine-betaine (*BADH)* metabolism were amplified by PCR using Dream Taq Green Polymerase (Fermentas, St Leon-Rot, Germany). Four independent PCR amplifications were conducted for each gene using the primer pairs, annealing temperatures, and number of cycles presented in table 1. Primers for *EF-1α*, *18SrRNA, KvP5CS1*,

KvOAT, *KvProT*, *KvPDH* were designed according to Wang et al (2015) and primers for *KvBADH* were designed based on *BADH* gene alignment (Figure S1). The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. Expression differences were analyzed by gel densitometry using Gelix One software and expressed as relative values compared to two reference genes (*EF-1α*, *18SrRNA)*.

Table 1 List of primers and amplification conditions used for semi-quantitative RT-PCR expression analysis in *Kosteletzkya pentacarpos* (syn. *K. virginica*)

Gene name	NCBI/SGN accession	Primer name	Sequence (5'-3')	*T*m (°C)	*No.* of cycles
EF-1α	NM_001327239	*EF*-F	GGTCATTCAAGTATGCCTGG	54	32
		EF-R	GAACCCAACATTGTCACCAG		
18SrRNA	AY739080	*18S*-F	GAGTATGGTCGCAAGGCTGAA	55	32
		18S-R	CCTCTAAATGATAAGGTTCAGTGG		
KvP5CS1	KR029088.1	*P5CS*-F	TTCAAGGGAAGCGTGTTGGT	57	35
		P5CS-R	AACAAGAGCGTCTGGTCGAG		
KvOAT	KR029089.1	*OAT*-F	TTGGGTGGTGGCGTAATACC	57	31
		OAT-R	ATAGTGTCGTGCGTGGGTTT		
KvProT	KR029091.1	*ProT*-F	TGCTTGCAGCTAAAGACGGA	57	31
		ProT-R	ACGCGCTTCCTCTAATACCG		
KvPDH	KR029090.1	*PDH*-F	GCTCGTTTATGCCGTCGAAC	57	35
		PDH-R	CCGATTCTTCTTGTGGGGCT		
KvBADH[5]		*BADH*-F	GTTGTGCTGCAATACTGAAG	55	32
		BADH-R	TAAGACGGGATGTTGCACTG		

[5] The primers design was present in figure S1

2.7. Statistical treatment

The data normality was verified by Shapiro-Wilk tests and homoscedasticity by Levene's tests. Differences among treatments were analyzed for statistical significance (P<0.05) using two-way ANOVAs with the type of heavy metal exposure (Cd, Zn, or Cd+Zn) and the NaCl concentration as main factors. Post-hoc analyses were performed using Tukey's comparison tests to investigate the differences among treatments. ANOVA and Tukey's tests were conducted using the SPSS 19 software. Correlations between relative gene expression, enzyme activities and final compound concentrations were performed using the OriginLab 8.0. software.

3. Results

3.1. Plant growth and water status

Short term (48h) treatment did not induce plant mortality but necrosis was observed in petiole and stem after 48h of acute heavy metal toxicity and was more obvious under Cd than Zn stress (Fig 1). The root dry weight (DW) was reduced by 33% and 42% in plants exposed to 20 µM Cd and 40 µM Cd alone, respectively (P < 0.05) (Fig 2A). Salinity partially alleviated Cd toxicity in terms of DW. Root DW was reduced by 15% in response to 400 µM Zn, while 200 µM had no significant impact. A low root DW weight was noticed in 20 µM Cd + 200 µM Zn treatment (24% decrease (P < 0.05)) but salinity significantly reduced the deleterious impact of heavy metals. The lowest leaf DW was observed in plants exposed to 40 µM Cd alone (35% decrease comparatively to controls) (Fig 5.2B). In contrast, Zn alone had no significant impact on the leaf DW while for mixed treatments only 40 µM Cd + 400 µM Zn treatment reduced the leaf DW. Such an impact was alleviated by concomitant exposure to NaCl.

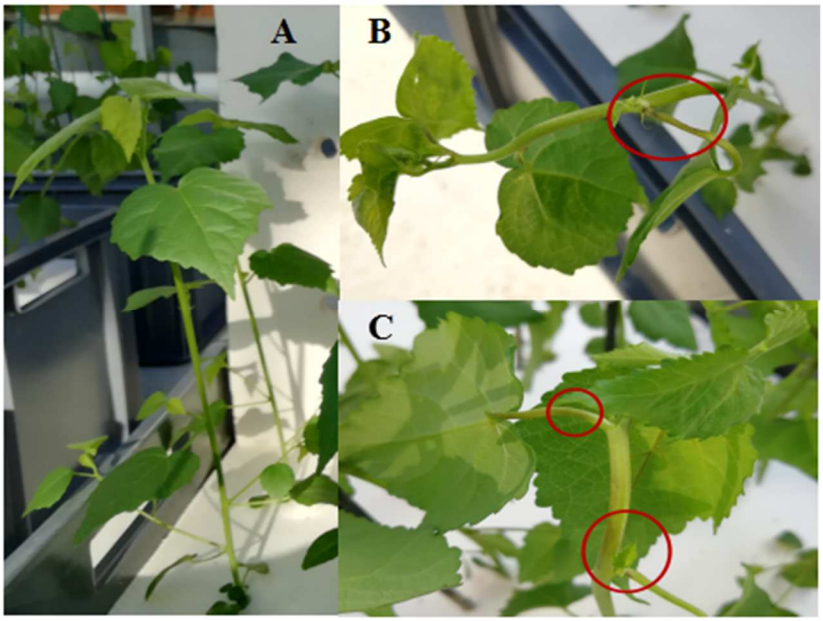


Figure 1. The morphology of *Kosteletzkya pentacarpos* maintained in control conditions (A) or exposed to 20 µM $CdCl_2$ (B) and 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ (C). After 48 h cadmium stress, shoot became necrotic, especially at the petiole and stem levels (red circle in photo B and C).

The 48h acute Cd and Zn toxicity did not affect the root water content (WC) except when 40 µM Cd was applied in the presence of NaCl (Fig. 2C). Leaf WC (Fig 2D) was significantly reduced in plants exposed to 20 µM Cd and 40 µM Cd alone ($P < 0.05$). NaCl however significantly mitigated deleterious impact of Cd on the leaf WC. Zinc alone had no effect on leaf WC but salinity slightly decreased it in the presence of 400 µM Zn. For combination of Cd and Zn treatment, there was no obvious change in leaf WC. As indicated in Fig. 3, NaCl had no impact on the leaf Ψs values in plants that were not exposed to heavy metals. Conversely, all heavy metal treatments except 200 µM Zn significantly decreased the leaf Ψs in the absence of NaCl, the lowest value being observed for plants exposed to 40 µM Cd. Salinity slightly increased Ψs in these plants but it led to an additional decrease in plants exposed to 200 µM Zn and to both mixed treatments.

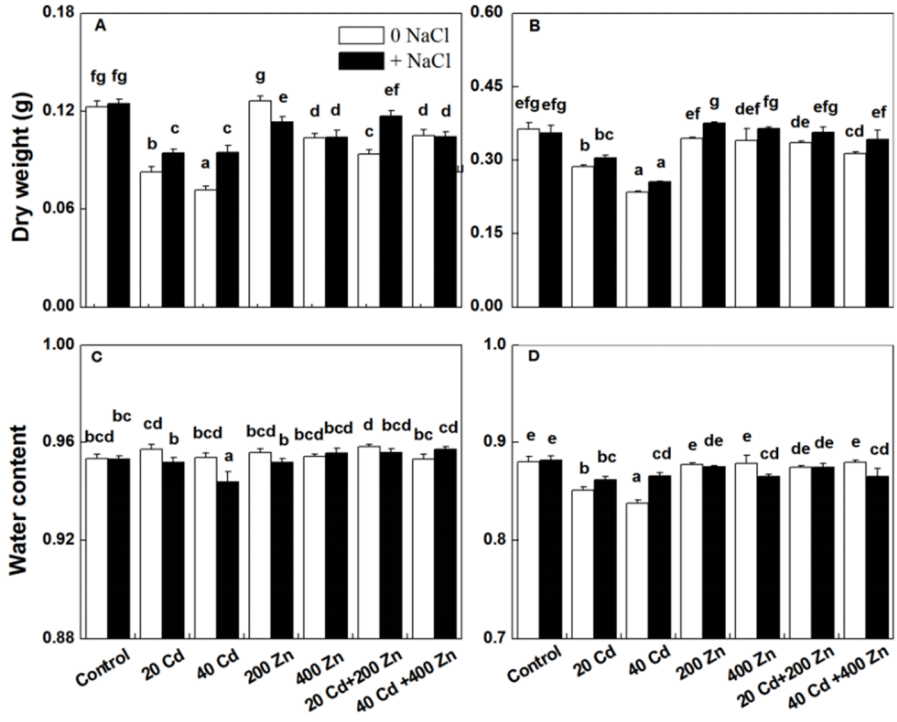


Figure 2. Root (A) and leaf (B) dry weight and root (C) and leaf (D) water content in seedlings of *Kosteletzkya pentacarpos* exposed to 20 µM $CdCl_2$, 40 µM $CdCl_2$, 200 µM $ZnCl_2$, 400 µM $ZnCl_2$, 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ and 40 µM $CdCl_2$ + 400 µM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl during 48h. Each value is the mean of four replicates, and vertical bars are SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

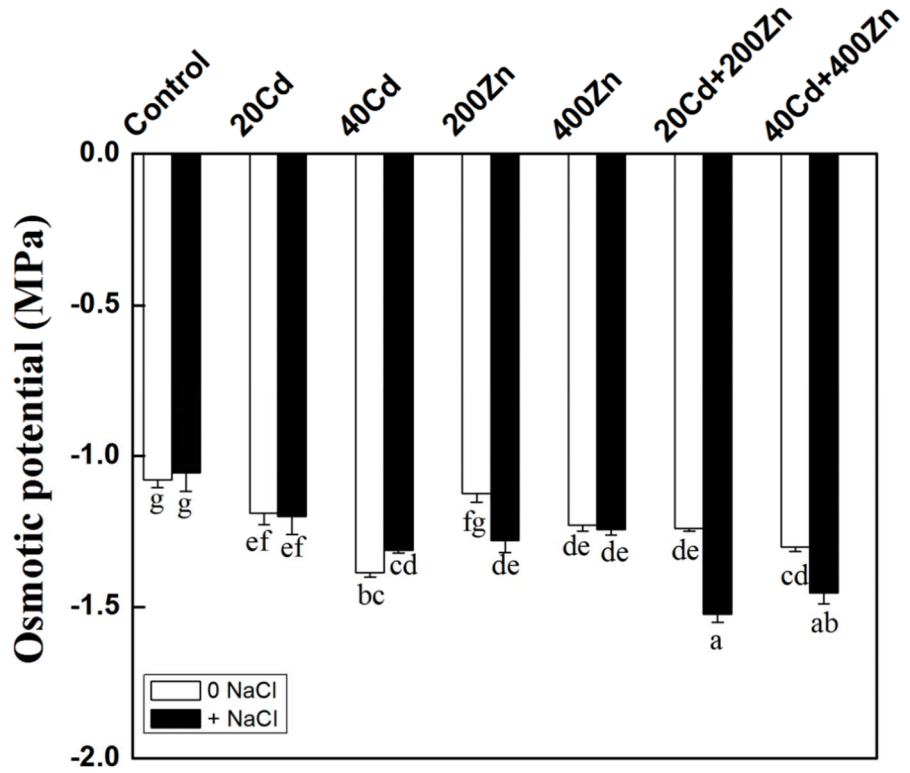


Figure 3. Leaf osmotic potential (Ψs) of *Kosteletzkya pentacarpos* exposed to 20 µM $CdCl_2$, 40 µM $CdCl_2$, 200 µM $ZnCl_2$, 400 µM $ZnCl_2$, 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ and 40 µM $CdCl_2$ + 400 µM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl during 48h. Each value is the mean of three replicates, and vertical bars are SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

3.2. Ion concentration in roots and leaves

Cadmium accumulated in roots and leaves of Cd-exposed plants (Table 2) but remained undetectable in the other treatments. The additional salinity strongly reduced Cd accumulation in all organs and in all Cd treatments in the presence and absence of Zn ($P < 0.05$). In root, in the combination of Cd and Zn, Zn had no effect on Cd accumulation, compared to treatment with Cd alone. In leaves, however, plants accumulated lower amounts of Cd when

concomitantly exposed to Zn in the mixed treatment ($P < 0.05$).

Table 2 Cadmium concentration in roots and leaves of *Kosteletzkya pentacarpos* exposed to 20 µM $CdCl_2$, 40 µM $CdCl_2$, 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ and 40 µM $CdCl_2$ + 400 µM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl during 48h. Each value is the mean of three replicates ± SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

Treatment	Cadmium (µg g^{-1} DW)	
	Root	Leaf
20 µM Cd	685 ± 40 c	128 ± 6.8 e
20 µM Cd + 50 mM NaCl	337 ± 41 ab	32 ± 5.5 b
40 µM Cd	1365 ± 293 d	173 ± 7.8 f
40 µM Cd + 50 mM Nacl	380 ± 55 abc	27 ± 5.2 b
20 µM Cd + 200 µM Zn	505 ± 26 bc	66 ± 1.7 c
20 µM Cd + 200 µM Zn + 50 mM NaCl	293 ± 17 ab	9.2 ± 1.7 a
40 µM Cd + 400 µM Zn	1063 ± 134 d	112 ± 4.7 d
40 µM Cd + 400 µM Zn + 50 mM NaCl	158 ± 9 a	21 ± 2.0 ab

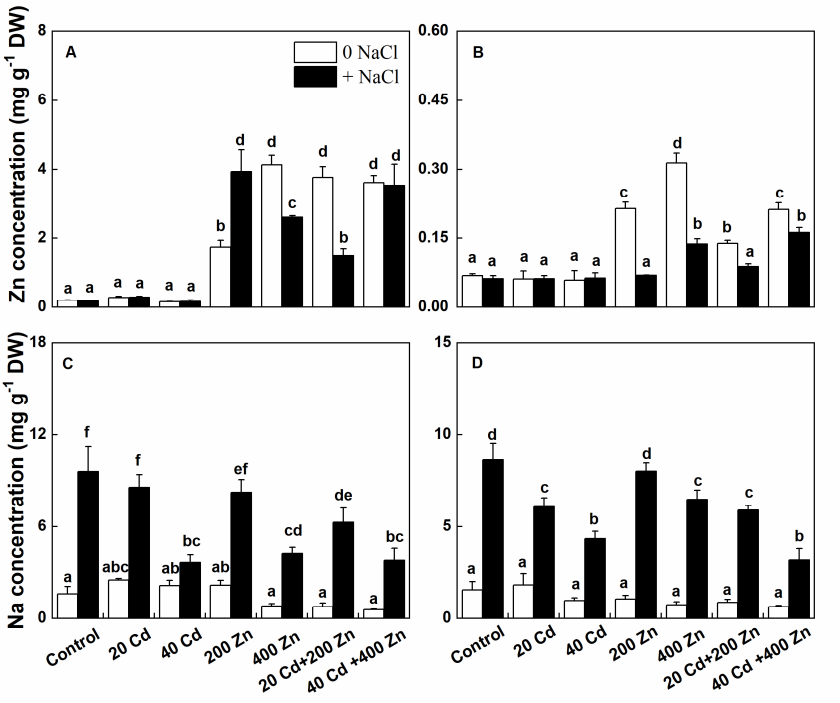


Figure 4. Zinc accumulation and Na concentration in root (A, C) and leaves (B, D) of *Kosteletzkya pentacarpos* exposed to 20 µM $CdCl_2$, 40 µM $CdCl_2$, 200 µM $ZnCl_2$, 400 µM $ZnCl_2$, 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ and 40 µM $CdCl_2$ + 400 µM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl during 48h. Each value is the mean of three replicates, and vertical bars are SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

Cadmium had no impact on Zn accumulation in roots and leaves (Fig. 4 A and B). Zinc accumulated in response to Zn 200 µM and salinity increased Zn accumulation in roots but decreased it in leaves. Zinc even accumulated to a higher extent in response to 400 µM Zn but in this case, salinity reduced Zn accumulation in both organs ($P < 0.05$). In plants exposed to mixed treatment (Cd+Zn), zinc accumulated to similar extent in roots of plants exposed to the two doses in the absence of NaCl while it accumulated to lower concentration than in plants exposed to Zn alone in the leaves. Salinity reduced Zn accumulation in the leaves of plants exposed to the mixed treatment (*P*

< 0.05) and in the roots of plants exposed to the lowest dose.

Heavy metals had no impact on the Na concentration in roots and leaves of plants maintained in the absence of NaCl (Fig. 4 C and D). As expected, salinity increased Na concentration in both organs. As far as roots are concerned, 40 µM Cd and 400 µM Zn decreased Na content, and a similar impact was observed for both mixed treatments. Cadmium (20 and 40 µM) and Zn (400 µM) also decreased Na accumulation in the leaves and an important decrease in Na accumulation in the leaves was recorded for plants exposed to 40 µM Cd + 400 µM Zn.

3.3. Proline and glycinebetaine accumulation in roots and leaves

After 48h of stress, proline did not accumulate in the roots, except for plants exposed to Cd 40 µM or Zn 200 µM in the presence of NaCl (Fig 5A). Salinity in the absence of heavy metal did not increase root and leaf proline content. In contrast, all heavy metal treatments significantly increased the leaf proline content, the highest accumulation being recorded in plants exposed to Cd. Salinity reduced leaf proline concentration in plants exposed to Cd but not in plants exposed to Zn. It is noteworthy that salinity increased leaf proline concentration in plants exposed to the mixed treatment.

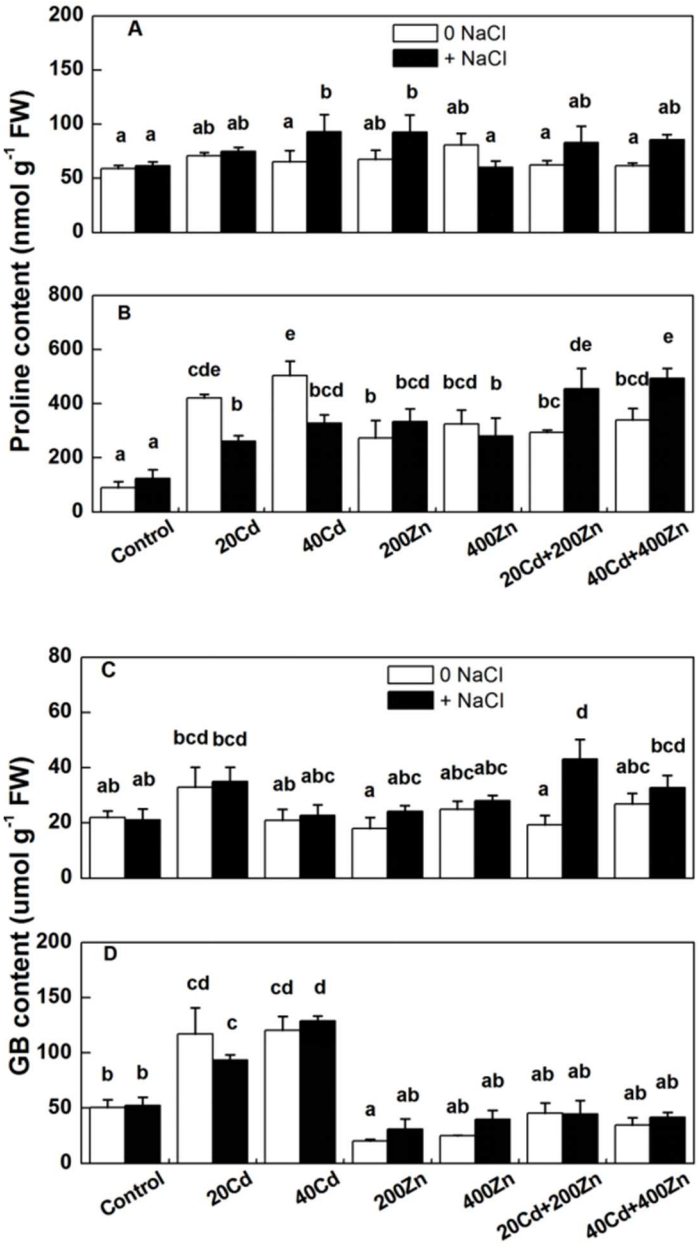


Figure 5. Proline and glycinebetaine concentrations in roots (A and C) and leaves (B and D) of *Kosteletzkya pentacarpos* exposed to 20 μM $CdCl_2$, 40 μM $CdCl_2$, 200 μM $ZnCl_2$, 400 μM $ZnCl_2$, 20 μM $CdCl_2$ + 200 μM $ZnCl_2$ and 40 μM $CdCl_2$ + 400 μM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl during 48h. Each value is the mean of three replicates, and vertical bars are SE.

Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

Glycinebetaine concentration was higher in the leaves than in the roots (Fig. 5C and D). Salinity had no significant impact on GB concentration in plants cultivated in the absence of heavy metals. Root GB concentration was not affected by heavy metals in plants cultivated in the absence of NaCl; salinity significantly increased root GB concentration in plants exposed to Cd 20 µM + Zn 200 µM, only ($P < 0.05$) (Fig. 5C). Cadmium induced GB accumulation in the leaves while Zn reduced it. Leaf GB concentration was not affected by the mixed treatment, whatever the heavy metal concentration. Salinity had no significant impact on leaf GB concentration.

3.4. Enzyme activities

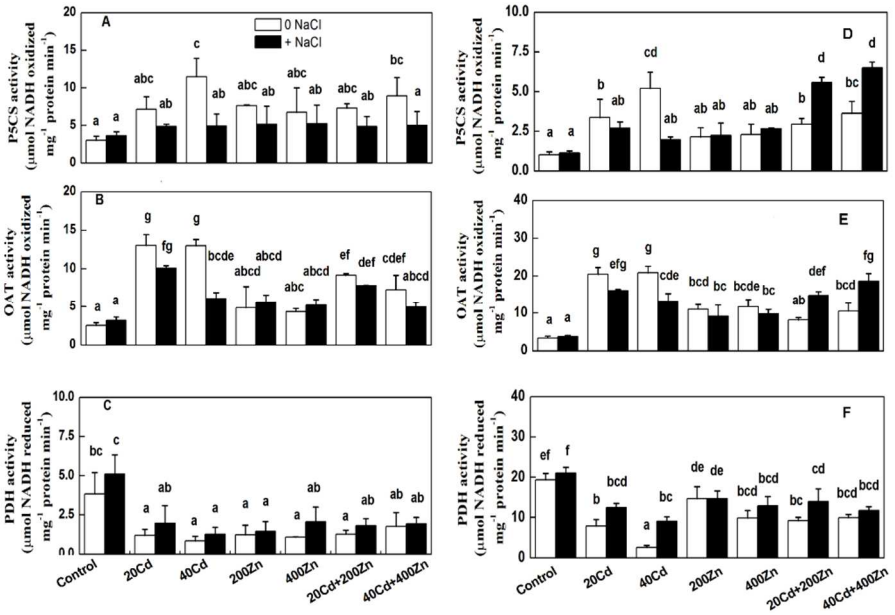


Figure 6. Activities of Δ^1-pyrroline-5-carboxylate synthetase (P5CS), ornithine-amino-transferase (OAT) and proline dehydrogenase (PDH) in roots (A-C) and leave (E-F) of *Kosteletzkya pentacarpos* exposed to 20 µM $CdCl_2$, 40 µM $CdCl_2$, 200 µM $ZnCl_2$, 400 µM $ZnCl_2$, 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ and

40 µM $CdCl_2$ + 400 µM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl during 48h. Each value is the mean of three replicates, and vertical bars are SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

Activities of proline-metabolizing enzymes are provided in Fig. 6. In the absence of NaCl, root P5CS activity (Fig. 6A) was significantly increased in response to Cd 40 µM and in the mixed treatment Cd 40 + Zn 400 and additional salinity significantly decreased the root P5CS activity in those plants. Although Zn alone slightly stimulated root P5CS activities, the recorded increase was not significant. Cadmium alone strongly increased the root OAT activities (Fig. 6B) and to a similar extent for both doses. Increase in root OAT was also observed in response to the mixed treatment at both doses, but value recorded remained lower than in plants exposed to Cd in the absence of Zn. Zinc alone had no impact on root OAT activities. Salinity significantly reduced OAT activities in plants exposed to Cd alone ($P < 0.05$). It is noteworthy that all heavy metal treatments decreased the root PDH activity (Fig. 6C) in plants cultivated in the absence of NaCl and additional presence of NaCl had no significant impact on the root PDH activities.

In the leaves of plants cultivated in the absence of NaCl, Cd increased P5CS activities proportionally to the Cd external concentration while Zn had no impact on this enzyme activity (Fig. 6D). The leaf P5CS activity also increased in response to the mixed treatment. Salinity decreased the leaf P5CS activity in plants exposed to Cd alone, but it increased it in plants exposed to the mixed treatment. All heavy metal treatments increased the leaf OAT activity (Fig. 6E) in plants cultivated in the absence of NaCl, the recorded increase being the highest in plants exposed to Cd 20 µM and Cd 40 µM, and the lowest in plants exposed to mixed treatment. Salinity had no impact on leaf OAT activity of the control plants; it slightly decreased OAT activities in plants exposed to Cd but increased it in plants exposed to mixed treatment. Cadmium at both doses, and Zn at the highest one (400 µM) decreased leaf PDH activities (Fig. 6F) and a significant decrease was

also recorded in the leaves of plants exposed to the mixed treatment. Salinity increased the leaf PDH activity in plants exposed to Cd 40 μM but it had no significant impact on other plants.

Betaine aldehyde dehydrogenase activity (BADH, Table 3) increased in the roots of plants exposed to Cd and to the mixed treatment but not in those of plants exposed to Zn. Salinity had no impact on the root BADH, except a significant increase in plants exposed to Cd 20 + Zn 200. As far as the leaves are concerned, all heavy metal treatments increased BADH activity with maximum values recorded in Cd-treated plants. Salinity decreased BADH activity in Cd-treated plants but had no impact on plants exposed to other treatments.

Table 3. Betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8) activities (µmol NAD^+ mg^{-1} prot min^{-1}) in roots and leaves of *Kosteletzkya pentacarpos* exposed 20 µM $CdCl_2$, 40 µM $CdCl_2$, 200 µM $ZnCl_2$, 400 µM $ZnCl_2$, 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ and 40 µM $CdCl_2$ + 400 µM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl during 48h. Each value was the mean of three replicates, and vertical bars are SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

	Roots		Leaves	
	0 mM NaCl	50 mM NaCl	0 mM NaCl	50 mM NaCl
Control	0.40 ± 0.06 a	0.27 ± 0.02 a	0.38 ± 0.02 a	0.30 ± 0.05 a
Cd 20 µM	1.7 ± 0.08 def	1.5 ± 0.30 de	1.1 ± 0.02 d	0.88 ± 0.14 c
Cd 40 µM	1.7 ± 0.32 ef	2.2 ± 0.07 f	1.7 ± 0.01 f	1.4 ± 0.01 e
Zn 200 µM	0.82 ± 0.20 abc	0.5 ± 0.12 a	0.71 ± 0.07 b	0.70 ± 0.07 b
Zn 400 µM	0.63 ± 0.20 ab	0.68 ± 0.16 ab	0.79 ± 0.01 bc	0.92 ± 0.02 c
Cd 20 µM + Zn 200 µM	1.1 ± 0.19 bcd	1.9 ± 0.24 f	0.88 ± 0.01 c	0.91 ± 0.01 c
Cd 40 µM + Zn 400 µM	1.5 ± 0.34 de	1.3 ± 0.11 cde	0.80 ± 0.01c	0.67 ± 0.02 b

3.5. Gene expression

Gene expression after 48h of treatment was analyzed in roots and leaves for three proline metabolizing enzymes (P5CS, OAT and PDH), for the proline transporter ProT, and for BADH (Fig. 7).

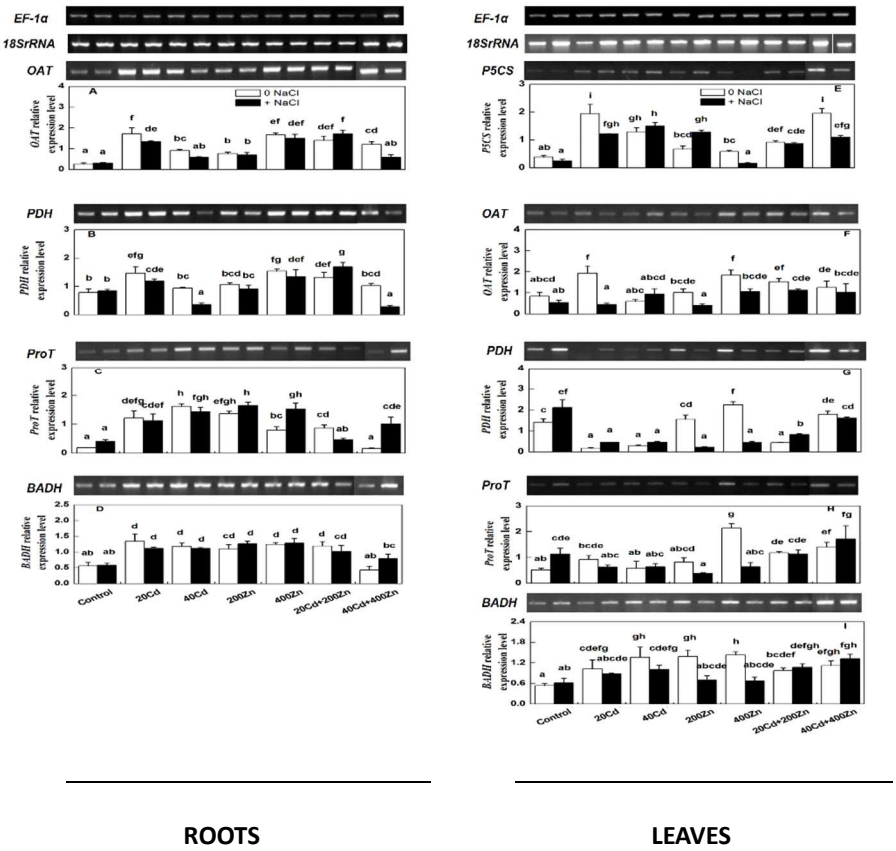


Figure 7. Semi-relative quantitative RT-PCR analysis of *KvP5CS*, *KvOAT*, *KvPDH*, *KvProT* and *KvBADH* in root (left column) and leaves (right column) of *Kosteletzkya pentacarpos* exposed to 20 µM $CdCl_2$, 40 µM $CdCl_2$, 200 µM $ZnCl_2$, 400 µM $ZnCl_2$, 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ and 40 µM $CdCl_2$ + 400 µM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl during 48h. Expression of *KvP5CS1* was not detected in the roots. Each value is the mean of four replicates, and vertical bars are SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

In the present work, expression of *KvP5CS1* was not detected in the roots whatever the considered treatment. The expression of all other genes remained unaffected by NaCl in the roots of plants that were not exposed to heavy metals. The expression of *KvOAT* was significantly increased by Cd and Zn and it peaked in roots of plants exposed to Cd

20 µM alone where the expression was 5.6 folds higher than in controls ($P < 0.05$). A high level of expression of this gene was also detected in roots of plants exposed to Zn 400 µM and to Cd 20 + Zn 200. Additional NaCl decreased *KvOAT* expression by 23 and 52% in roots exposed to Cd 20 and to Cd 40 + Zn 400. The expression of *KvPDH* also increased in roots in response to 20 µM Cd, 400 µM Zn and Cd 20 + Zn 200 and additional NaCl reduced *KvPDH* expression by 58 and 67% in Cd 40 µM and Cd 40 + Zn 400 ($P < 0.05$). The expression of gene coding for proline transporter (*KvProT*) was significantly increased by all heavy metal treatments and NaCl significantly increased *KvProT* expression in response to 400 µM Zn and to 40 Cd + 400 Zn. All heavy metal treatments (except Cd 40 + Zn 400) also increased *KvBADH* expression in the roots and additional NaCl had no impact on this gene expression.

In the leaves, *KvP5CS1* was upregulated by Cd and mixed treatment (Cd+Zn) and it increased by more than 4 folds in response to Cd 20 µM and Cd 40 + Zn 400: in both cases, additional NaCl decreased *KvP5CS1* expression ($P < 0.05$). Expression of *KvOAT* was significantly increased in plants exposed to Cd 20 µM, Zn 400 µM and mixed treatments; additional NaCl however decreased *KvOAT* expression in plants exposed to Cd 20 µM or Zn alone. Cadmium drastically down-regulated *KvPDH* in the leaves in the absence and presence of NaCl while a similar effect was noticed for Zn in the presence of NaCl only. In contrast, the expression of *KvProT* remained unaffected in Cd-treated plants while it significantly increased in the leaves of plants exposed to 400 µM Zn and to mixed treatment. *KvBADH* was also significantly up-regulated in the leaves of all plants exposed to heavy metals, although additional NaCl reduced *KvBADH* expression in plants exposed to Zn alone.

4. Discussion

Proline and glycinebetaine are zwitterionic compounds which possess a high solubility and are able to assume protective functions for

cellular structures and enzymatic proteins in plant tissues facing salt and water stress (Chen and Murata 2008; Szabados and Savouré 2010; Ben Rejeb et al. 2014; Kavi Kishor and Sreenivasulu 2014; Kubala et al. 2015; Mansour and Ali 2017; Zhang et al. 2019). Salinity induces a complex constraint at the plant level characterized by an osmotic component due to a marked decrease in external osmotic potential, and an ionic component related to the accumulation of Na^+ and Cl^-. In the present study, low level of salinity (50 mM) did not increase the proline and GB content in plants that were not exposed to heavy metals. This contrasts with the data provided by Wang et al. (2015) who demonstrated that NaCl may induce proline accumulation in *K. virginica* (syn. *K. pentacarpos*); these authors, however, used a short term exposure to a very high dose of NaCl (300 mM) and it may be hypothesized that under these circumstances, the osmotic component of salt stress prevailed over the ionic component. In our work, a lower NaCl (50 mM) was used and is relevant from the NaCl level encountered by the plant in its natural habitat (Han 2013): this dose did not compromise the plant water status but lead to an obvious Na^+ accumulation after 48h. Our data showed that Na^+ by itself was unable to directly trigger proline accumulation in this halophyte species. Ben Rejeb et al. (2014) explained that the transduction pathways leading to P5CS activation may differ for mild and severe salt stress.

In contrast, heavy metal exposure, and especially Cd treatment induced proline accumulation even if Cd exogenous concentration is rather low from an osmotical point of view and quite lower than 50 mM used for NaCl. It has however to be mentioned that Cd accumulation in leaf tissue induced a decrease in the leaf water content and that such limited desiccation might contribute to induce proline accumulation. This explanation, however, is not valid anymore for plants exposed to the mixed (Cd+Zn) treatment which also accumulated proline in the leaves but did not suffer from a water content decrease. It is also noteworthy that salt treatment of Cd-exposed plants reduced simultaneously both Cd and proline accumulation in leaves and that both Cd and proline accumulation were lower in the mixed treatment than in plants exposed to Cd alone,

supporting the hypothesis of a direct impact of toxic Cd on proline accumulation.

In numerous studies devoted to abiotic stresses, proline accumulation is attributed to increased P5CS activities (Szabados and Savouré 2010; Zhang and Becker 2015) and to inhibition of PDH (Lutts et al. 1999b; Szabados and Savouré 2010). In most cases, these modifications are, at least partly, attributed to the transcriptional regulation of the corresponding gene (Silva-Cortega et al. 2008; Kubala et al. 2015; Singh et al. 2016; Zegaoui et al. 2017; Guan et al. 2018; Sdouga et al. 2019). The expression of numerous genes is directly influenced by salinity in *K. virginica* and Tang et al. (2015) identified among them 66 genes involved in arginine and proline metabolism. Figure 8 represents the correlation analysis between gene expression, enzyme activities and corresponding metabolites separately in the roots (Fig. 8A) and in the leaves (Fig. 8B). From a global point of view, correlation was more obvious for leaves than for roots.

The absence of any detectable transcript for *KvP5CS1* in the roots was a puzzling result since root P5CS activity was obvious and even higher than in the leaves. Wang et al. (2015) reported *KvPSCS1* gene expression in the leaves but these authors did not analyze the root system. In most plant species, P5CS is encoded by two distinct genes which are differently regulated depending on the stress occurrence and developmental stage (Ben Rejeb et al. 2014; Kavi Kishor and Sreenivasulu 2014; Zhang and Becker 2015). Only one single gene was identified in *K. pentacarpos* until now, but it could not be excluded that a second gene exists and may account for the recorded root P5CS activity. In the leaves, in contrast to the root, P5CS activity clearly correlated with *KvPCS1* expression, and the proline content was correlated to P5CS activity (Fig. 8). It has been frequently reported that *P5CS* gene may be up-regulated by ABA in several plant species (Szabados and Savouré 2010; Kavi Kishor and Sreenivasulu 2014; Zhang and Becker 2015; Kaur and Asthir 2015). In a previous work, we demonstrated that ABA increased in the leaves of 20 µM Cd-treated plants but was then decreased in response to the additional

presence of NaCl. According to this study, the highest ABA accumulation occurred in response to the mixed treatment (Cd+Zn), and these data corroborate the whole pattern of *KvP5CS1* expression recorded in the leaves.

A highly significant correlation ($P < 0.01$) was also found between both leaf P5CS1 and OAT activities on the one hand and leaf proline content on the other hand. It has been considered that the ornithine pathway predominates in the mitochondria under high nitrogen supply whereas the P5CS pathway acts during abiotic stress (Kavi Kishor et al. 2015; Kaur and Asthir 2015). The present data however demonstrate that heavy metals may trigger OAT activation and that *KvOAT* expression and OAT activities both increased in response to Cd toxicity. The OAT gene expression was also increased in Zn-treated plants while *KvP5CS1* expression remained unaffected in these plants. Paradisone et al. (2015) also reported that Zn excess increased OAT activities in *Lactuaca sativa* even if proline is not clearly involved in Zn resistance in this species.

Leaf proline content was negatively correlated with Ψs ($r = -0.84$; $P < 0.001$) which support the involvement of proline in osmotic adjustment. In Cd-treated plants, however, NaCl decreased proline content but did not affect Ψs suggesting that other compounds (including sodium) might be involved in this process. Beside osmotic adjustment, proline may also assume other crucial functions in stressed tissues. It is considered to act as a free radical scavenger either directly or through the stimulation of endogenous antioxidant synthesis and antioxidative enzyme activities (Ben Rejeb et al. 2014). The chapter 1 reported that heavy metal induced oxidative stress in *K. pentacarpos* as indicated by an increase in malondialdehyde and H_2O_2 content while salinity reduced H_2O_2 production in these heavy metal-treated plants. Since H_2O_2 is involved in signal transduction leading to *P5CS1* gene activation (Szabados and Savouré 2010), the impact of NaCl on H_2O_2 may explain the recorded decrease in *KvP5CS1* gene expression and corresponding enzyme activities occurring in 20 µM Cd-treated plants as well as the recorded decrease

in leaf proline content. Proline also plays an important role in the cell wall architecture as a precursor of hydroxyproline present in high amounts in cell wall protein (Kavi Kishor et al. 2015). Hybrid-proline-rich protein (HyPRPs) are not only crucial players in cell elongation but also exhibit a high level of cysteine residues putatively involved in Cd-binding. In chapter 1, it demonstrated that a high proportion of Cd is bound to the cell wall in *K. pentacarpos*. Only free proline was quantified in the present study but data were reported after 48h of treatment while in chapter 1, it studied Cd distribution after two weeks of treatment. Hence, the hypothesis that Cd-induced proline may partly serve as a precursor for subsequent HyPRPs synthesis could not be ruled out, especially considering that proline was decreased by NaCl in Cd-treated plants which coincides with the observation of chapter 1 that NaCl also decreased the proportion of cell wall-bound Cd and increased the cytosolic fraction in *K. pentacarpos*.

Proline dehydrogenase is a mitochondrial enzyme involved in proline degradation. Expression of *AtProDH1* and *AtProDH2* is inhibited by water stress in the model plant species *Arabidopsis thaliana* (Ben Rejeb et al., 2014) and by salinity in a wide range of plant species (Szabados and Savouré 2010). Stress-induced decrease in the root PDH activity was observed in response to all heavy metal treatments (Fig. 6C) although transcript accumulated under these circumstances (Fig. 7). Such discrepancy may suggest that post-transcriptional inhibition of translation might occur, leading to an accumulation of transcript and an inhibition in enzyme activities. In the leaves, in contrast, a decrease in PDH activity may be related to an inhibition of the corresponding gene expression contributing to proline accumulation in Cd-treated plants but not in plants exposed to mixed toxicity. Proline dehydrogenase may also be involved in proline cycling: several lines of evidence suggest that the proline/P5C cycle is coupled to the maintenance of $NADP^+$/NADPH ratio and contribute to provide electron to the mitochondrial transport chain (Kavi Kishor and Sreenivasulu 2014; Kaur and Asthir 2015). Proline oxidation may provide up to 30 ATP through subsequent integration of resulting

glutamate in TCA cycle (Szabados and Savouré 2010) and it may thus be considered that stress-induced inhibition of *KvPDH* lead to a lack of energy which could at least partly explain stress-induced growth inhibition. From this point of view, proline accumulation should then be regarded as a symptom of injury or as an anticipating strategy allowing growth resumption after the stress relief.

Beside proline synthesis and proline degradation, proline transport is also an important component of stress tolerance and both the inter- and intracellular transport of this amino acid are critical for cellular homeostasis (Kavi Kishor et al. 2014). Although intracellular transport remains poorly understood, our knowledge of proline transport at the plant level benefits from the identification of several transporters localized at the plasma membrane and the expression of genes coding for those different transporters was found to be highly tissue specific (Szabados and Savouré 2010; Kavi Kishor et al. 2014). Some of them are clearly activated by external stress and assume important functions, especially in the growth zone of the root system (Kavi-Kishor et al. 2014; Chen et al. 2016). It is interesting to mention that in the present study *KvProT* gene was up-regulated by almost all heavy metal treatments at the root level, except in plants exposed to a high mixed toxicity while this mixed toxicity precisely stimulated its expression at the leaf level. Assessment of proline distribution between apoplasm and symplasm should help us to unravel the specific importance of proline transporter in resistance to heavy metal stress.

It is of special interest to mention that most proline transporters are also able to transport glycinebetaine (Fujiwara et al. 2010; Mansour and Ali 2017) suggesting that plant evolution selected similar transporters for translocation of distinct osmolytes. In the present study, however, correlation between proline content and GB content was not significant which suggest that synthesis of these compounds is regulated by different cues, as previously mentioned for other halophyte (Ben Hassine et al. 2008) even if their transport may use the same transporters.

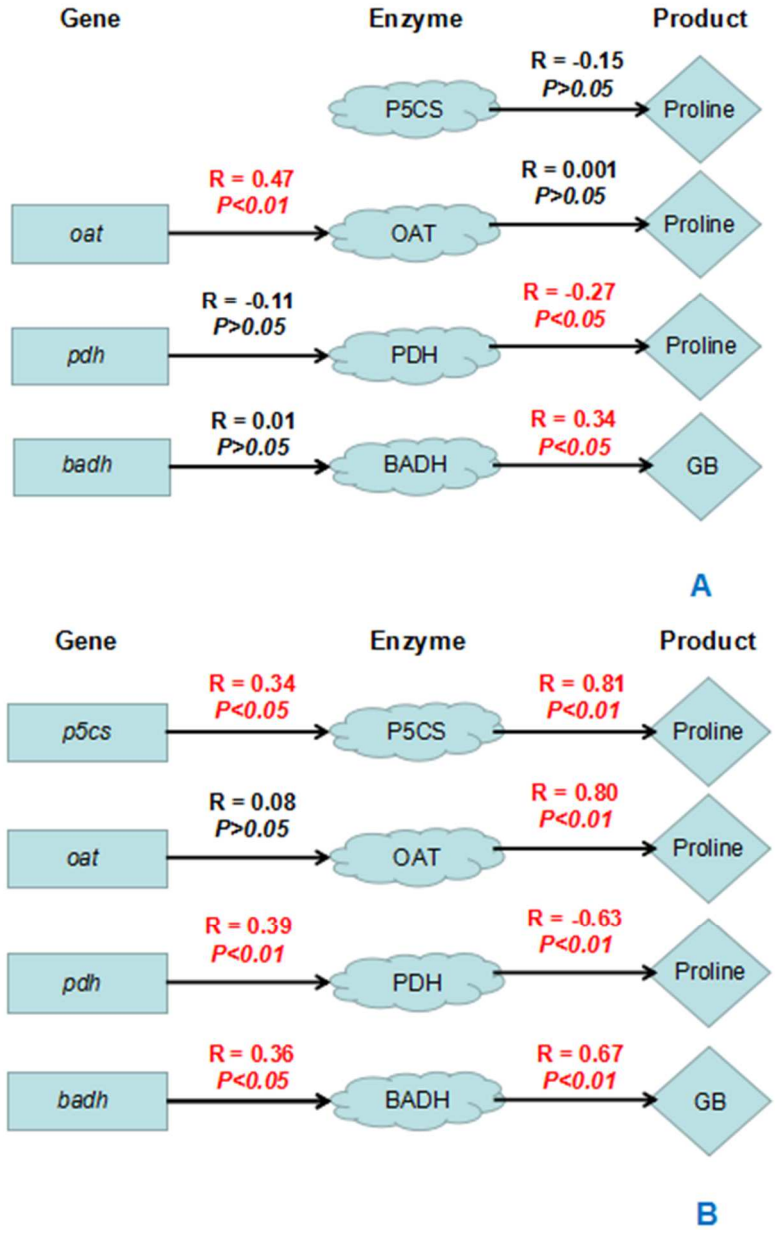


Figure 8. Correlation analysis between gene expression, enzyme activities and final product in *Kosteletzkya pentacarpos:* data are pooled for all treatments (Control, 20 µM $CdCl_2$, 40 µM $CdCl_2$, 200 µM $ZnCl_2$, 400 µM $ZnCl_2$, 20 µM $CdCl_2$ + 200 µM $ZnCl_2$ and 40 µM $CdCl_2$ + 400 µM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl) and are presented separately for roots (A) and

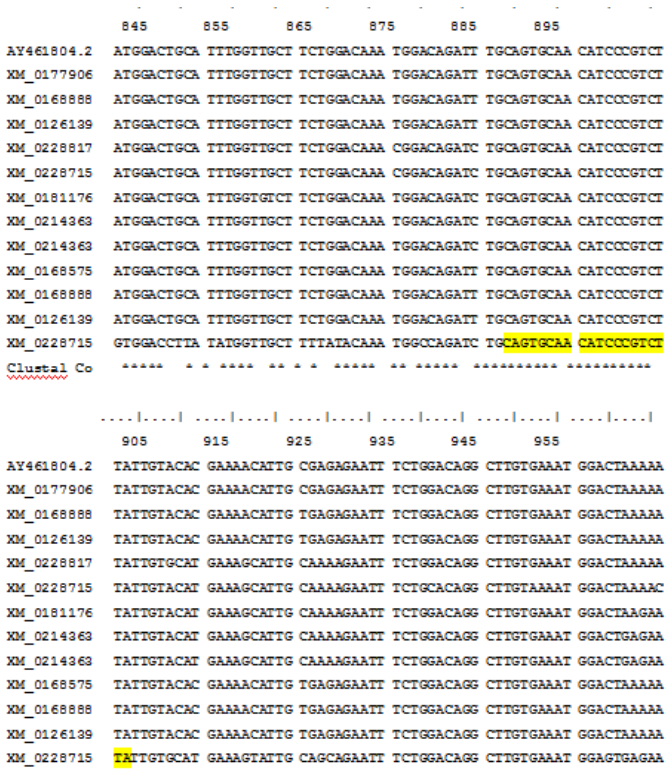
leaves (B). Significant correlation is presented in red and non-significant correlation in black letters.

Glycinebetaine synthesis is thought to occur in chloroplasts (Chen and Murata 2008) but GB was detected in the roots of *K. pentacarpos* (Fig. 5C). Except for the 20 Cd+200 Zn+NaCl treatment, heavy metals did not increase the root GB concentration. In contrast, all heavy metal treatments (except the mixed treatment at high doses) up-regulated the BADH gene expression in the root and all the treatments involving Cd increased the root BADH activity. This suggests i) that GB synthesis does not require fully mature chloroplast since *KvBADH* was expressed and BADH was active in the non-photosynhetic root tissue and ii) that neither gene expression nor BADH activities should be regarded as a limiting factor for root GB synthesis which could thus be limited *in vivo* by a poor availability of the choline precursor (Chen and Murata 2008; Figuerora-Soto and Valenzuela-Soto 2018; Mansour and Ali 21017). Exogenous Cd clearly increased GB concentration in the leaves while all treatments containing Zn decreased it. There was a clear correlation between *KvBADH* gene expression and BADH activities in the leaves (Fig. 8B). Glycinebetaine remained especially low in the leaves of plants exposed to the mixed treatment although those leaves exhibited a strong decrease in Ψs. When data are pooled for all treatments, there was no significant correlation between Ψs values and glycinebetaine concentration. As previously reported for proline, GB may assume numerous protective functions independently of its osmotic properties (Hossain et al. 2010; Figuerora-Soto and Valenzuela-Soto 2018). Mansour and Ali (2017) calculated that even in GB-accumulating plants, GB content is not sufficient to explain more than 15% of the recorded osmotic adjustment. Most studies dealing with GB involvement in heavy metal resistance are based on an exogenous GB application (Hossain et al. 2010; Farooq et al. 2016; Aamer et al. 2018; Yao et al. 2018).

Taken together, these data suggest that 50 mM NaCl did not represent a physiological constraint for the halophyte plant species *K. pentacarpos* and that it had no impact on physiological properties in

the absence of heavy metals. Rather, it helps the plant to cope with heavy metal toxicity but its impact on osmolyte synthesis depends on the nature and the doses of applied heavy metals. Cadmium indeed had a more pronounced effect that Zn on proline and GB content. In chapter 2, it is reported that Cd-induced senescence in *K. pentacarpos* was mainly due to ethylene and to putrescine (Put) accumulation. Since both compounds are produced from S-adenosylmethionine (SAM), these authors suggest that SAM is overproduced in response to Cd but not in response to Zn. As detailed by Kurepin et al. (2015), SAM is also acting as a precursor of cytosolic choline, which is then translocated to the chloroplast and used for BADH synthesis. Hence, a Cd-induced SAM overproduction may also be related to Cd-induced GB accumulation. According to the results in chapter 2, salinity reduces ethylene and Put synthesis in Cd-treated plants in *K. pentacarpos*, but we demonstrate here that it did not impact the GB content and we suggest that this synthesis remains a priority despite the salt-induced decrease in Cd absorption and translocation. Putrescine may be produced from ornithine or arginine. The present work demonstrates that Cd stimulated the proline synthesis by the ornithine pathway and it is therefore likely that Put was mainly produced from arginine. In a recent transcriptomic approach, Tang et al. (2015) indeed reported that several genes involved in arginine metabolism may be transcriptionally regulated in *K. pentacarpos* by environmental stress conditions, including high NaCl concentrations. In contrast, a moderate dose of NaCl which decreases proline synthesis (Fig. 5B) and OTA activities (Fig. 6) in Cd-treated plants also paradoxically decreased the Put concentration in *K. pentacarpos* but this could mainly be explained by an improved conversion of Put to higher polyamines (spermidine and spermine) which assume protective functions in stressed tissues.

5. Supplemental data



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XM_0228715  GTGGACCTTA TATGGTTGCT TTTATACAAA TGGCCAGATC TGCAGTGCAA CATCCCGTCT
Clustal Co  *****  * * ****  ** * *  *****  ** *****  ********** **********

            905       915       925       935       945       955
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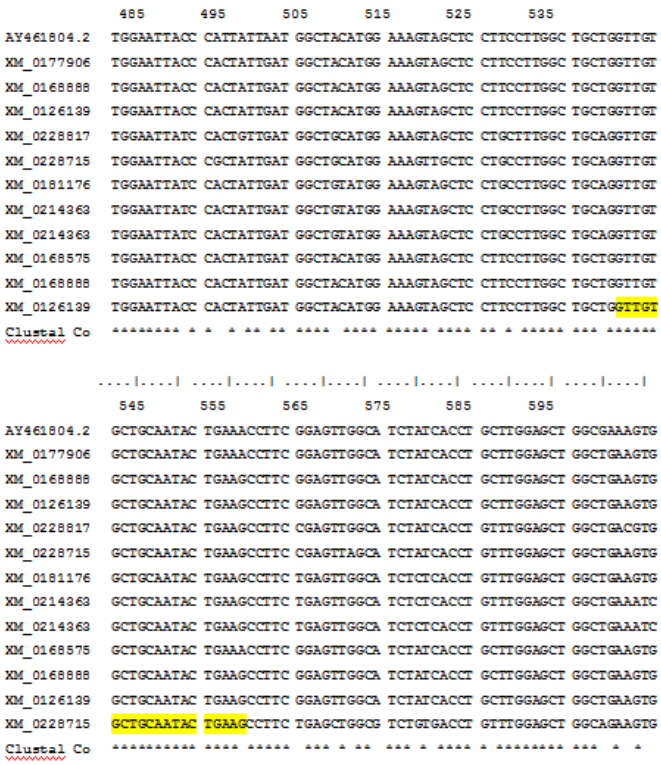

Figure S1. Aligment of *BADH (Betaine-aldehyde dehydrogenase)* cDNA sequences performed using BioEdit software and used to design the *KvBADH* primers. Primers were designed in conserved regions (in yellow). Genebank accession numbers: AY461804.2 (*Gossypium hirsutum*), XM_0177906 (*Gossypium arboreum*), XM_0168888 (*Gossypium hirsutum*), XM_0126139 (*Gossypium raimondii*), XM_0228817 (*Durio zibethinus*), XM_0228715 (*Durio zibethinus*), XM_0181176 (*Theobroma cacao*), XM_0214363 (*Herrania umbratica*), XM_0214363 (*Herrania umbratica*), XM_0168575 (*Gossypium hirsutum*), XM_0168888 (*Gossypium hirsutum*), XM_0126139 (*Gossypium raimondii*) and XM_0228715 (*Durio zibethinus*).

Reference

Aamer M, Muhammad UH, Abid A, Su Q, Liu Y, Adnan R, Muhammad AUK, Tahir AK, Huang G (2018) Foliar application of glycinebetaine (GB) alleviates the cadmium (Cd) toxicity in spinach through reducing Cd uptake and improving the activity of antioxidant systems. Appl Ecol Environ Res 16:7575-7583

Bai J, Zhao Q, Wang W, Wang W, Jia J, Cui B, Liu W (2019) Arsenic and heavy metals pollution along a salinity gradient in drained coastal wetland soils: depth distribution, sources and toxic risks. Ecol Ind 96:91-98

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205-207

Ben Hassine A, Ghanem M, Bouzid S, Lutts S (2008) An inland and a coastal population of the Mediterranean xero-halophyte species *Atriplex halimus* differ in their ability to accumulate proline and glycinebetaine in response to salinity and water stress. J Exp Bot 59:1315-1326

Ben Rejeb K, Abdelly C, Savouré A (2014) How reactive oxygen species and proline face stress together. Plant Physiol Biochem 80:278-284

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Chen THH, Murata N (2008) Glycinebetaine: an effective protectant against abiotic stress in plants. Trends Plant Sci 23:9

Chen J, Wu J, Lu Y, Cao Y, Zeng Y, Zhang Z, Wang L, Wang S (2016) Molecular cloning and characterization of a gene encoding the proline transporter protein in common bean (*Phaseolus vulgaris* L.) Crop J 4:384-390

Farooq MA, Ali S, Hameed A, Bharwana SA, Rizwan M, Ishaque W, Farid M, Mahmood K, Iqbal Z (2016) Cadmium stress in cotton seedlings: physiological, photosynthesis and oxidative damages alleviated by glycinebetaine. South Afr J Bot 104:61-68

Figuerora-Soto CG, Valenzuela-Soto EM (2018) Glycine betaine rather than acting only as osmolyte also plays a role as regulator in cellular metabolism. Biochimie 147:89-97

Fugiwara T, Mitsuya S, Miyake H, Hattori T, Takabe T (2010) Characterization of a novel glycinebetain/proline transporter gene expressed in the mestome

sheath and latteral root cap cells in barley. Planta 232:133-143

Grieve CM, Grattan SR (1983) Rapid assay for determination of water soluble quaternary ammonium componds. Plant Soil 70:303-307

Guan C, Huang YH, Cui X, Liu SJ, Zhou YZ, Zhang YW (2018) Overexpression of gene encoding the key enzyme involved in proline-biosynthesis (PuP5CS) to improve salt tolerance in switchgrass (*Panicum virgatum* L.). Plant Cell Rep 37:1187-1199

Han RM (2013) Sodium chloride improves heavy metal tolerance in the halophyte species *Kosteleyzkya virginica* independently of growth stimulation. PhD Thesis, Université catholique de Louvain, 309 p.

Han RM, Lefèvre I, Ruan C-J, Beukelaers N, Qin P, Lutts S (2012a) Effects of salinity on the response of the wetland halophyte *Kosteletzkya virginica* (L.) Presl. to copper toxicity. Water Air Soil Poll 223:1137-1150

Han RM, Lefèvre I, Ruan CJ, Qin P, Lutts S (2012b). NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regul 68:97-109

Han RM, Lefevre I, Albacete A, Pérez-Alfocea F, Barba-Espín G, Díaz-Vivancos P, Quinet M, Ryan CJ, Hernández JA, Cantero-Navarro E, Lutts S (2013a) Antioxidant enzyme activities and hormonal status in response to Cd in the wetland halophyte *Kosteletzkya virginica* (L.) Presl. under saline conditions. Physiol Plant 147:352-368

Han RM, Quinet M, André E, van Elteren J, Destrebecq F, Vogel-Mikuš K, Cui G, Debeljak M, Lefèvre I, Lutts S (2013b) Accumulation and distribution of Zn in the shoots and reproductive structures of the halophyte plant species *Kosteletzkya virginica* as a function of salinity. Planta 238:441-457

Hossain MA, Hasanuzzaman M, Fujita M (2010) Up-regulation of antioxidant and glyoxylase systems by exogenous glycinebetaine and proline in mung bean confer tolerance to cadmium stress. Physiol Mol Biol Plants 16:259-272

Kaur G, Asthir B (2015) Proline: a key player in plant abiotic stress tolerance. Biol Plant 59:609-619

Kavi Kishor PB, Sreenivasulu N (2014) Is proline accumulation per se correlated with stress tolerance or is proline homeostasis a more critical issue? Plant Cell Environ 37:300-311

Kavi Kishor PB, Kumari PH, Sunita MSL, Sreenivasulu N (2015) Role of proline in cell wall synthesis and plant development and its implication in

plant ontogeny. Front Plant Sci 6:544

Kubala S, Wojtyla L, Quinet M, Lechowska K, Lutts S, Garnczarska M (2015) Enhanced expression of the proline synthesis gene P5CSA in relation to seed osmopriming improvement of *Brassica napus* germination under salinity stress. J Plant Physiol 183:1-12

Kumar V, Sharma A, Kaur P, Sidhu GPS, Bali AS, Bhardwaj R, Thukral AK, Cerda A (2019) Pollution assessment of heavy metals in soils of India and ecological risk assessment: A state of the art. Chemosphere 216:449-462

Kurepin LV, Ivanov AG, Zaman M, Pharis RP, Allakherdiev SI, Hurry V, Hüner NPA (2015) Stress-related hormones and glycinebetaine interplay in protection of photosynthesis under abiotic stress conditions. Photosynth Res 126:221-235

Lefèvre I, Marchal G, Meerts P, Corréal E, Lutts S (2009) Chloride salinity reduces cadmium accumulation by the Mediterranean halophyte species *Atriplex halimus* L. Environ Exp Bot 65: 142-152

Lin W, Wu K, Lao Z, Hu W, Lin B, Li Y, Fan H, Hu J (2019) Assessment of trace metal contamination and ecological risk in the forest ecosystem of dexing mining area in northeast Jiangxi Province, China. Ecotoxicol Environ Saf 167:76-82

Lutts S (2000) Exogenous glycinebetaine reduces sodium accumulation in salt-stressed rice plants. Int Rice Res Notes 25:39-40

Lutts S, Bouharmont J, Kinet JM (1999a) Physiological characterization of salt-resistant rice somaclones. Austr J Bot 47:835-849

Lutts S, Majerus V, Kinet JM (1999b) NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings; Physiol Plant 105:450-458

Lutts S, Lefèvre I (2015) How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? Ann Bot 115:509-528

Mansour MMF, Ali EF (2017) Gluycinebetaine in saline conditions: an assessment of the current state of knowledge. Acta Physiol Plant 39:56

Paradisone V, Barrameda-Medina Y, Monteisinos-Pereira D, Romero L, Esposito S, Ruiz JM (2015) Roles of some nitrogenous compounds protectors in the resistance to zinc toxicity in *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco. Acta Physiol Plant 37:137

Patar A, Giri A, Boro F, Bhuyan K, Singha U, Giri S (2016) Cadmium pollution and amphibians: studies in tadpoles of *Rana limnocharis*. Chemosphere 144:1043-1049

Sdouga D, Ben Amor F, Ghribi S, Kabtni S, Tebini M, Branca F, Trifi-Farah N, Marghali S (2019) An insight from tolerance to salinity stress in halophyte *Portulaca oleracea* L.: physio-morphological, biochemical and molecular responses. Ecotox Environ Saf 172:45-52

Silva-Ortega CO, Ochoa-Alfaro AE, Reyes-Aguero JA, Aguado-Santacruz GA, Jimenez-Bremont JF (2008): Salt stress increases the expression of P5CS gene and induces proline accumulation in cactus pear. Plant Physiol Biochem 46:82-92

Singh V, Tripathi BN, Sharma V (2016) Interaction of Mg with heavy metals (Cu, Cd) in *Triticum aestivum* with special reference to oxidative and proline metabolism. J Plant Res 129: 487-97

Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED, Delis ID, Yakoumakis DI, Kouvarakis A, Papadakis AK, Stephanou EG, Roubelakis-Angelakis KA (2006): Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. Plant Cell 18:2767-81

Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. Trends Plant Sci 15:89-97.

Tang X, Wang H, Shao C, Shao h (2015) Global gene expression of *Kosteletzkya virginica* seedling responding to salt stress. PLoS ONE 10: e0124421

Wang H, Tang X, Wang H, Shao HB (2015) Proline accumulation and metabolism-related genes expression profiles in *Kosteletzkya virginica* seedlings under salt stress. Front Plant Sci 6:792

Weretilnyk EA, Hanson AD (1986) Betaine aldehyde dehydrogenase from spinach leaves: purification, *in vitro* translation of the mRNA and regulation by salinity. Arch Biochem Biophys 271:56-63

Yang Q, Li Z, Lu X, Duan Q, Huang L, Bi J (2018): A review of soil heavy metal pollution from industrial and agricultural regions in China: Pollution and risk assessment. Sci Total Environ 642:690-700

Yao WQ, Lei YK, Yang P, Li QS, Wang LL, He BY, Xu ZM, Zhou C, Ye HJ (2018) Exogenous glycinebetaine promotes soil cadmium uptaker by edible amaranth grown during subtropical hot season. Int J Environ Res Public Health 15:1794

Zegaoui Z, Planchais S, Cabassa C, Djebbar R, Belbachir OA, Carol P (2017) Variation in relative water content, proline accumulation and stress gene expression in two cowpea landraces under drought. J Plant Physiol

218:26-34
Zhang L, Becker DF (2015) Connecting proline metabolism and signaling pathways in plant senescence. Front Plant Sci 6:552
Zhang C, Shi S, Liu Z, Yang F, Yin G (2019): Drought tolerance in alfalfa (*Medicago sativa* L.) varieties is associated with enhanced antioxidative protection and declined lipid peroxidation. J Plant Physiol 232 :226-240

Interchapter 4-5

What do we know?

- Cadmium but not zinc increases proline and GB in the leaves. Salinity reduces proline content in Cd-treated plants but increases it in plants exposed to Cd+Zn. It has limited effect on GB concentration in both roots and leaves.
- Proline is produced through both glutamate and ornithine pathways while proline dehydrogenase is inhibited in response to heavy metals. Correlation between enzyme activities and corresponding gene expression is significant in the leaves but not in the roots. Gene coding for proline transport (*KvProT*) is upregulated in response to heavy metals.
- BADH activity increases in the roots of plants exposed to Cd and to the mixed treatment but not in those of plants exposed to Zn. Salinity had limited impact on the root BADH. In leaves, all heavy metal treatments increase BADH activity with up-regulation of *KvBADH* gene. Salinity decreases BADH activity in Cd-treated plants but had no impact on plants exposed to other treatments.

Heavy metals differently influence the metabolism of proline and GB in considered plant organs. And from a global point of view, the correlation between gene expression, enzyme activities and corresponding metabolites is more obvious for leaves than for roots *K. pentacarpos*.

What are the questions?

- Under water stress induced by heavy metals, *K. pentacarpos* triggers an adapted response in terms of osmotic adjustment through modification of proline and GB metabolism. In addition, mucilage also plays a vital role in drought resistance

due to its high water-binding capacity in plant. We still do not know if mucilage is produced by *K. pentacarpos*.

- Ghanem et al. (2010, J. Plant Physiol., 167: 382-392) showed that salinity modifies the composition of mucilage but once again, no data are available for heavy-metal treated plants. Therefore, it could be justified to analysis the composition of this polysaccharide in plants exposed to heavy metal toxicity in the presence and in the absence of salinity.
- We also showed in the Chapter 1 that cell wall constitutes an important site of ion retention. Because cellulose, hemicellulose and lignin do not have the same binding capacities, it appears interesting to compare the proportion of these components in cell wall of plants exposed or not heavy meyals.

What is our strategy?

The next experimental work will be carried out in Germany in the framework of a collaboration with the University of Kiel. It intends to quantify mucilage in *K. pentacarpos* and to analyse its composition, together with the analysis of cell wall polymers. Due to time limitation and considering the high amounts of required dry material to perform the analysis, we choose to focus on plants exposed to Zn excess in the presence or in the absence of NaCl.

CHAPTER 5 Influence of salinity on mucilage and polysaccharides in *Kosteletzkya pentacarpos* under zinc stress condition

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International Journal of Environmental Research as:

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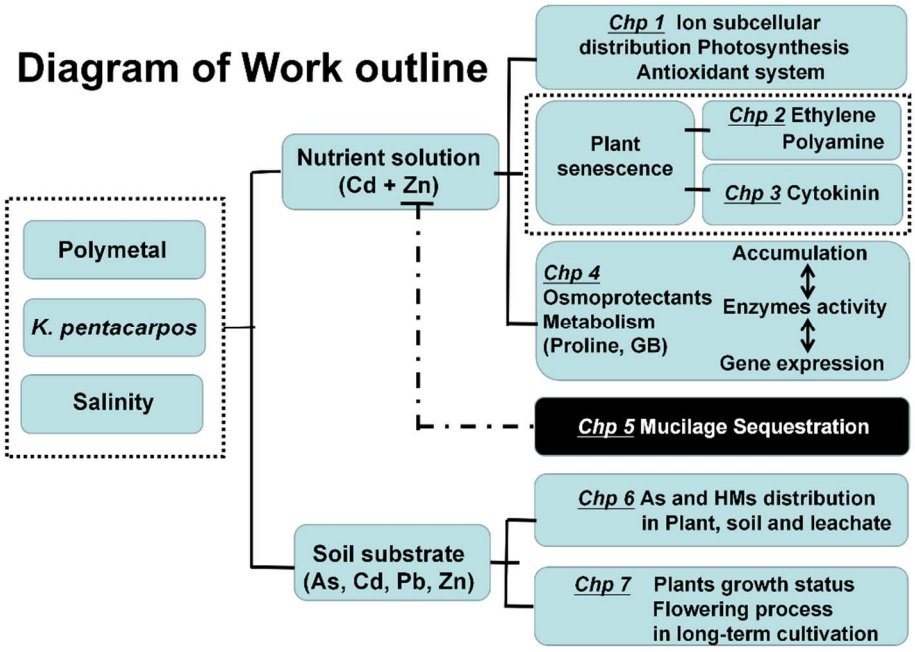
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Chp 1 Ion subcellular distribution Photosynthesis Antioxidant system
Plant senescence
Chp 2 Ethylene Polyamine
Chp 3 Cytokinin
Nutrient solution (Cd + Zn)
Polymetal
K. pentacarpos
Salinity
Chp 4 Osmoprotectants Metabolism (Proline, GB)
Accumulation
Enzymes activity
Gene expression
Chp 5 Mucilage Sequestration
Chp 6 As and HMs distribution in Plant, soil and leachate
Soil substrate (As, Cd, Pb, Zn)
Chp 7 Plants growth status Flowering process in long-term cultivation

Chapter 5

ABSTRACT

The halophyte species, *Kosteletzkya pentacarpos*, is known to produce high amount of mucilage, which may play a role in heavy metal tolerance. The quantity and composition of mucilage in *K. pentacarpos* exposed to zinc toxicity was therefore investigated. Exposure to 0.1 mM Zn had a detrimental impact on plant growth, leading to a significant decrease in dry weight and water content. Most of Zn was accumulated in the roots of plants exposed to Zn toxicity both in the absence or in the presence of NaCl. Salinity however partially alleviated the toxicity of zinc, increasing the plant biomass and decreasing zinc absorption by the root. Crude mucilage content increased in all plant organs in response to Zn stress. Furthermore, acid hydrolysis of crude mucilage extract in *K. pentacarpos* revealed that the main neutral monosaccharides constituents were rhamnose, arabinose, galactose and glucose. Zinc induced a rise in uronic acids in roots and stems but not in the leaves where a high constitutive proportion of uronic acids was observed in response to all treatment. Meanwhile, changes were also observed in neutral monosaccharides component in different plant organs. In accordance to the results from Van Soest method, Zn stress increased the content of hemicellulose as well as decreased the content of lignin and cellulose in the stem. The additional NaCl slightly increase the content of cellulose. We suggested that excessive zinc induced a modification in the composition and structure of polysaccharides. The mucilage may play a vital role in plants response exposed to Zn toxicity with moderate dose of salinity.

Keywords: Zinc, halophyte, mucilage, polysaccharides

1. Introduction

Following rapid social and economic development over the past several decades, heavy metal pollution in soil has been one of the hottest issues in the world. Although heavy metals are naturally in soil, local accumulation results from anthropogenic activities such as agriculture, urbanization, industrialization and mining (Gao et al. 2017; Men et al. 2018). Zinc is one of the most common elements in heavy metal polluted soil. Excessive Zn comes from various sources such as waste from ore extraction, coal and fuel combustion, the incineration of waste, sewage sludge and finally the use of fertilizer and pesticides containing zinc in agriculture (Rakotondrabe et al. 2018; Wang et al. 2017).

Zinc is an essential micro-element required for plant growth, development and reproduction (Javed et al. 2017, Park et al. 2017). It is the only metal present in all six enzyme classes (oxidoreductase, transferase, hydrolases, lyases, isomerases and ligases) (Baltaci et al. 2017). It acts as a catalytic cofactor (Barrameda-Medina et al. 2017) and is also involved in phytohormone regulation (Liu et al. 2017), signal transduction (Lin et al. 2005) and repairing process of PS Ⅱ complex during photoinhibition (Rizwan et al. 2017). However, high concentration of Zn has deleterious impact on plant, resulting in inhibition of photosynthesis, interference with nutrition absorption, synthesis of reactive oxygen species and damages in DNA and RNA (Leskova et al. 2017, Pramanick et al. 2017, Yu et al. 2015).

Numerous technics are available to remove heavy metals from a contaminated substrate or polluted water, but classical approaches are very expensive. Phytoremediation which implies the use of heavy-metal resistant plants appears as an attracting alternative (Ali et al. 2013). In areas simultaneously contaminated by salt and heavy metals, halophyte species have been recommended as a promising tool for phytomanagement (Lutts and Lefèvre 2015). One of the most efficient strategy to cope with high amounts of accumulated toxic ions

consists to store them in metabolically poorly active or inactive cell compartments and vacuolar sequestration of heavy metals associated or not with phytochelatins, as well as exclusion to apoplast and cell wall retention are well documented in the literature (see Sharma et al. (2016) and Berni et al. (2019) for review). Although less documented, heavy metal retention by mucilage is also an efficient option to limit toxic ion accumulation in cytosol.

Mucilage is composed of water soluble heteropolysaccharides of high molecular weight comprising a mixture of L-rhamnose, L-fucose, L-galactose, galacturonic acid, L-arabinose, D-xylose and D-galactose. Ii is frequently found at the root surface (Morel et al. 1986; 1987; Javed et al. 2013; Lapie et al. 2019), seeds (Elboutachfati et al. 2017; Kaur et al. 2018; Lodhi et al. 2019) and fruits (Ajala et al. 2016). The high water-binding capacity of hydroxyl and carboxyl groups in the polysaccharides allows mucilage to hydrate and thus store huge amounts of water which may offer plants the ability to overcome drought periods (Minjares-Fuentes et al. 2017; Schwartz et al. 2015). Mucilage issued from root exudation may also prevent toxic metal uptake through chelation of the cations by the carboxylic groups of the uronic acids, hydroxyls and carbonyl functions located on the polysaccharide chains (Morel et al. 1987; Javed et al. 2013; Lodhi et al. 2019; Lapie et al. 2019).

Kosteletzkya pentacarpos (L.) Presl. (syn. *Kosteletzkya virginica*) is a perennial dicot halophyte species of Malvaceae family, which is distributed from Louisiana to Florida and north along the Atlantic coast to Delaware and the state of New York (Islam et al. 1982; Zheng et al. 2017). It is recommended as a potential resource for food, feed, biodiesel as well as health care (Halchak et al. 2011; Qin et al. 2015; Vaughn et al. 2013). Beside a high level of resistance to salt stress, this species is also able to cope with heavy metals and the concomitant presence of NaCl was reported to improve its resistance to Cd and Zn (Han et al. 2013; chapter 1, 2, 3). This species produces high amounts of mucilage not only at the root level but also in stems and leaves where it was localized within the xylem vessels and in the leaf

epidermis (Ghanem et al. 2010). This study also demonstrated that salinity modified mucilage content and composition and that an important fraction of accumulated Na^+ was retained on mucilage. Lutts et al. (2016) also showed that dry root powder is an efficient material for heavy metals biosorption and that salinity improved the Zn biosorption process in relation to an increase in the amounts of mucilage pectic compounds and hemicellulose. A recent study demonstrated that for *K. pentacarpos* growing on a polycontaminated soil, salinity reduced the Zn leaching process and increased the proportion of Zn removed by the plant as a possible consequence of a Zn-induced modification of root mucilage content.

These data suggest that root mucilage contributes to heavy metal retention in the roots in order to limit toxic ions accumulation in the photosynthetic tissues. No data are available, however, regarding the influence of salinity and heavy metals on mucilage content and composition in the shoot part. The aims of the present work were i) to determine the impact of ion toxicities on mucilage and cell wall polymers (cellulose, hemicellulose and lignin) content in plants of *K. pentacarpos* exposed to deleterious concentration of Zn in the absence or in the presence of NaCl and ii) to compare this impact for roots, stems and leaves.

2. Materials and Methods

Seeds of *Kosteletzkya pentacarpos* (issued from Jinhai Agricultural Experimental Farm of Yancheng (Jiangsu Province, China)) were rinsed with sterile distilled water for three times and then placed in trays containing a perlite and vermiculite mix (1:3 v/v) regularly moistened with a half–strength modified Hoagland nutrient solution. Seedlings were grown in a phytotron under a 12h photoperiod [PAR= 150 μmoles 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 transferred and fixed on polyvinylchloride

plates floating on aerated half-strength modified Hoagland nutrient solution in 50L tanks. 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. The temperatures in the chamber were adjusted to 25 °C and 18 °C in the day and night, respectively. Light was supplemented by Philips lamps (Philips Lighting S.A., Brussels, Belgium) (HPLR 400 W) in order to maintain a light irradiance of 300 µmol m^{-2} s^{-1}at the top of the canopy.

Twenty-five days after sowing (acclimation without stress), NaCl, and $ZnCl_2$ were added to corresponding containers to induce four treatments: (1) Control; (2) 50 mM NaCl; (3) 0.1 mM $ZnCl_2$; (4) 0.1 mM $ZnCl_2$ + 50 mM NaCl (Zn + Na). The pH of solution was readjusted to 5.7 ± 0.02 with KOH every 2 days; the solution was renewed every week and tanks randomly rearranged in the phytotron at this occasion. For each treatment, three replications with 12 plants per replication were used for the measurement of physiological and biochemical parameters. After 4 weeks under stressed condition, roots, stems and leaves were separately harvested for analysis.

2.1. Growth parameters

Roots were quickly rinsed in sterile deionized water for 30 s to remove ions from the free space and gently blotted dry with a paper towel. Roots, stems and leaves of each plant were separated and weighed for fresh weight determination (n=5). Material was then incubated in an oven for 72h at 70 °C for dry weight determination. Water content was calculated as [(fresh weight - dry weight) / fresh weight] / 100.

2.2. Evaluation of ion concentration

Dried plant tissues were ground to a fine powder with porcelain mortar and pestle. 50-100 mg powder of each sample was digested in 3 ml 35% HNO_3 and evaporated to dryness at 80 °C on a sand bath

under the hood. Three ml of a mix of 37% HCl and 68% HNO_3 (3:1) was added and evaporated at 80 °C again. After full evaporation, the obtained minerals were dissolved in HCl 0.1N. The concentrations of Na as well as Zn were determined by SOLAAR S4 atomic absorption spectrometry (Thermo Scientific, Cambridge, UK). For each treatment, five separated plants were considered and each analysis was performed on technical triplicates.

The translocation factor (TF) was calculated in two ways: on the basis of concentration (TF_c) and on the basis of the total amount (TF_a) of translocated pollutants, respectively, using the following formula:

TF_c Concentration in the shoot ($mg.g^{-1}$ DW) / Concentration in the roots ($mg.g^{-1}$ DW)

TF_a Total amount in the shoot (mg) / Total amount in the roots (mg)

Besides, as an indicator of the plant's ability to accumulate the heavy metal in harvestable organs, the

Bioaccumulation factor (BF) was also calculated as an additional indicator of the plant's ability to accumulate the heavy metal in harvestable organs. Since a nutrient solution was used in the present experiment, we expressed shoot Zn concentration on a tissue water content basis for BF calculation according to:

BF Shoot concentration of heavy metal (µM) / concentration in nutrient solution (µM)

2.3. Mucilage analysis

The harvested Roots, stems and leaves of *K. pentacarpos* were oven-dried at 40 °C for one week. Dried material was then ground into a fine power and passed through a 0.315 mm mesh sieve. The crude mucilage in root, stem and leaf was determined as described in Classen and Blaschek (1998): 500 ml water was added to 5 g homogenized plant material; samples were stirred at room temperature for 40h, and then centrifuged at 20,000 *g* and 4 °C for 5 min. The supernatant was collected and the pellet was re-extracted once more

using the same procedure. All the aqueous supernatants were combined and concentrated to 150 ml by evaporation. 600 mL of a mixture of 96% ethanol and 1% acetic acid were then added and the obtained precipitate was washed with cold ethanol and lyophilized. The dry weight of the lyophilized precipitate as crude mucilage was determined.

Quantification of neutral monosaccharides in the mucilage of *K. pentacarpos* tissues was performed by acetylation analysis followed by gas liquid chromatography (GLC method) (Blakeney et al. 1983). 10 mg lyophilized crude mucilage were added to 1 ml 2M TFA as well as 50 µl Inositol-Standard in leak proof wheaton-vial. After heating for 1 h at 121 °C, the samples were transferred into small conical evaporator flask to evaporate until dryness. Dry samples were then reduced with 1 ml $NaBH_4$ in DMSO. The acetylation step was then performed with 1-methylimidazole and acetic anhydride. Afterward, the sample was extracted by adding dichloromethane. The lower phase was transferred into a GC-vial to analyze by gas liquid chromatography (HP 6890 Plus Series; Hewlett Packard) with a flame ionization detector.

In addition, quantification of uronic acids has been performed by silylation analyses and GLC following the protocol of Bleton et al. (1996).

2.4. Structural polysaccharides analysis

Concentration of lignin and structural polysaccharide (cellulose and hemicellulose) were determined according to Van Soest et al. (1991). Dry matter was crushed and exposed successively to a neutral detergent solution during 1h at 100 °C to obtain by filtration the NDF (neutral detergent fibers), then to an acid detergent solution during 1h to get the ADF fraction and then to sulfuric acid 72% during 3hto obtain the ADL fraction (acid detergent lignin fraction). The ADL fraction was incinerated at 550 °C during 3h, and the mass loss allowed us to calculate the lignin percentage. Cellulose and hemicellulose were obtained according to the following equations.

Hemicellulose content (g $100g^{-1}$ OM) = NDF - ADF

Cellulose content (g $100g^{-1}$ OM) = ADF - ADL

Lignin content (g $100g^{-1}$ OM) = ADL

2.5. Statistical analysis

For each treatment, three 50 L tanks containing 12 plants each were used in a randomized complete block design. Tissue materials of 5 *K. pentacarpos* were dried in 70 °C oven, which were used to analyze for growth parameters, ionic determination. The remaining plant materials were dried in 40 °C oven for mucilage analysis and structural polysaccharides analysis. All of parameters data were subjected to an analysis of variance, one-way ANOVA, using SPSS software, with the treatment considered as the main factor. The statistical significance of the results was analyzed by Turkey test at 5% level ($P < 0.05$).

3. Results and Discussion

3.1. Plant growth

All plants remained alive until the end of the treatment and salinity alone had no impact on plant dry weight and water content (Fig. 1). In halophyte plant species, moderate salinity may lead to plant growth stimulation leading to a dilution of the ion content within shoot (Lutts and Lefèvre 2015). Although such a growth stimulation has been reported for *K. pentacarpos* (syn. *K. virginica*) at 100 mM NaCl, our results corroborate the data of Han et al. (2012, 2013) who reported that 50 mM NaCl did not stimulate growth but nevertheless modified the physiological status of the plant in such a way that it more adequately respond to heavy metal toxicity.

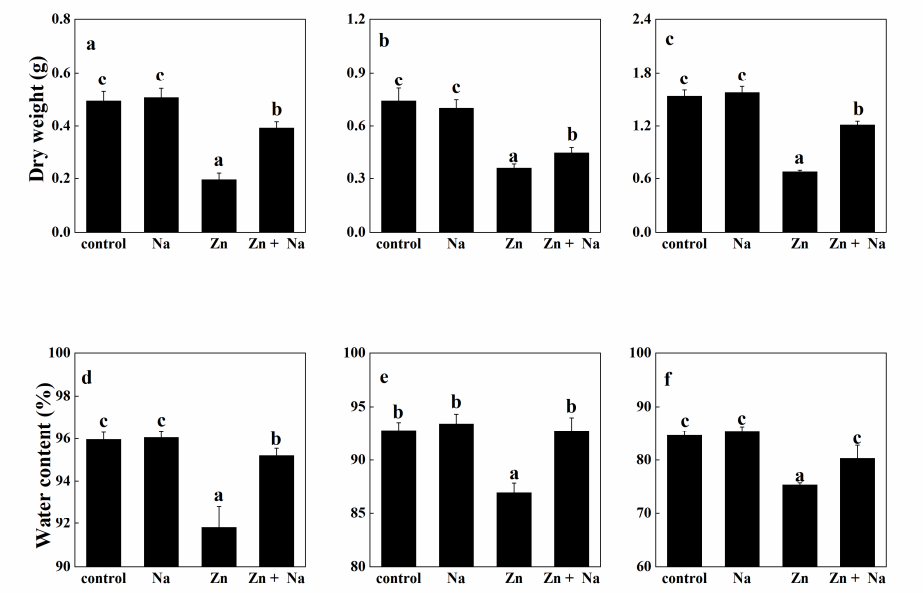

Fig. 1 Dry weight of root (a), stem (b), as well as leaf (c) and water content of root (d), stem (e) as well as leaf (f) in *Kosteletzkya pentacarpos* exposed during four weeks to 0.1 mM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

Plants exposed to 0.1 mM Zn already presented some symptoms of chlorosis and necrosis after one week of treatment. Zinc excess had a detrimental impact on plant growth with a decrease of 60%, 52% and 56%, for roots, stems, and leaves respectively after 4 weeks (Fig. 1 a-c). Addition of NaCl to the Zn-containing solution however significantly improved the growth of all organs when compared to Zn treated alone. As shown in Fig. 1 (d-f) salinity did not affect water content in plant. In contrast, Zn excess led to significant water loss in root, stem and leaf. However, the presence of NaCl was able to improve this parameter, especially in root and stem. Salt stress induces both an ionic component related to the presence of Na^+ and Cl^-, and an osmotic component related to the decrease in the external osmotic potential (Ψ_s) of the soil solution. Heavy metals such as Zn also

affects the plant water status but this impact should not be regarded as the consequence of a decrease in external Ψ_s since only low concentration are commonly used. In chapter 4, it was paradoxically demonstrated that 50 mM NaCl did not led to osmotic adjustment in *K. pentacarpos* while 200 µM $ZnCl_2$ induced a drop in Ψ_s which could be partly related to proline accumulation. According to this study, such an accumulation was reinforced by the concomitant presence of NaCl which could explain that, in the present study, NaCl partly alleviated the deleterious impact of Zn excess on organ water content.

Biomass production is directly related to the efficiency of the photosynthetic process which is sensitive to Zn excess in *K. pentacarpos* (Han et al. 2013). Zn was reported to have a negative effect on the PSII chemistry by an interaction with the donor side of PSII, leading to inhibition of the photosynthetic CO_2 fixation and the Hill reaction (Tang et al. 2013). If Zn-induced inhibition of photosynthesis is the underlying cause of dry weight decrease the fact that NaCl mitigated the deleterious impact of Zn suggest that Na^+ and/or Cl^- may positively act on photosynthesis, or that NaCl induces a decrease in Zn absorption and/or translocation. Although photosynthesis was not measured in the present study, it clearly appears from our data that NaCl itself did not improve plant growth in the absence of Zn, and that salt-induced improvement was also observed in non-photosynthetic tissues such as root and stems.

3.2. Ion content

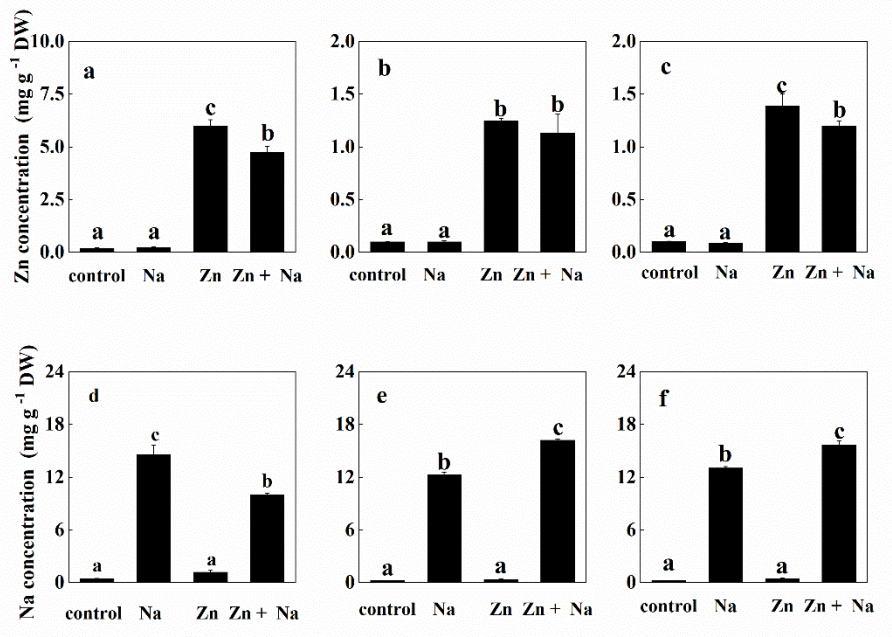

Fig. 2 Zn accumulation (mg kg^{-1} dry weight) in root (a), stem (b) as well as leaf (c) and Na concentration (mg kg^{-1} dry weight) in root (d), stem (e) as well as leaf (f) in seedlings of *Kosteletzkya pentacarpos* exposed during four weeks to 0.1 mM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

As expected, control plants absorbed and accumulate physiological concentration of Zn required for normal plant metabolism (Fig.2). Salinity did not affect Zn concentration in plants exposed to non-contaminated solution (Fig. 2 a-c). In our data, most of Zn accumulated in the roots of plants exposed to 0.1 mM Zn in the absence or presence of NaCl (69% and 67%, respectively). Salinity however significantly reduced Zn accumulation in root and leaves while it has no significant impact on Zn concentration in stems. These observations reinforced our hypothesis that NaCl-induced improvement of plant growth in Zn-treated plants may be due to a

decrease in Zn accumulation in the leaves. This is also supported by the fact that the presence of NaCl slightly reduced BF values. In contrast, there was no significant difference of TF_c and TF_a between Zn treatment and Zn+Na treatment (Table 1). This suggests that Zn concentrations were reduced to similar extent in shoots and roots and that NaCl-induced decrease in shoot Zn content was the consequence of a global decrease in Zn absorption, rather than an inhibition of Zn translocation. Zinc absorption and long-distance transport are under the control of a huge number of transporters, which differ in terms of precise location and affinities (Gupta et al. 2016). Some of them are involved in cellular absorption while other are involved in xylem loading in the roots and unloading in the leaves. It may be hypothesized that those transporters may be affected by the presence of 50 mM NaCl. It may also be hypothesized that the rise in the solution electrical conductivity modifies Zn speciation in the solution, and that the presence of chloro-complex ($ZnCl^+$) may reduce Zn absorption as previously suggested for Cd (Lefèvre et al. 2009).

Table 1 Bioaccumulation factor (BF) and translocation factor (TF_C and TF_a) in *Kosteletzkya pentacarpos* exposed during four weeks to 0.1 mM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

	BF	TF_C	TF_a
Control	3.9 ± 0.15 b	1.0 ± 0.04 c	2.3 ± 0.10 c
50 mM Na	3.9 ± 0.67 b	0.81 ± 0.08 b	1.8 ± 0.10 b
0.1 mM Zn	3.2 ± 0.45 ab	0.43 ± 0.01 a	1.2 ± 0.17 a
Zn + Na	2.5 ± 0.34 a	0.49 ± 0.04 a	1.1 ± 0.08 a

Sodium concentration increased from 0.45 ± 0.02 to 14.6 ± 1.0 mg g^{-1} DW in root of control and Na-treated plants, respectively (Fig. 2 d-f). The presence of Zn in the solution significantly decreased the root Na concentration to 10.0 ± 0.18 mg g^{-1} DW, while it significantly increased Na concentration in stem and leaf. This indicates that the excess of Zn increased Na translocation from the root to the shoot

even if NaCl conversely had no impact on Zn translocation.

3.3. Mucilage and polysaccharide analysis

The total amount of Zn accumulated within a given organ is not fully relevant from its real physiological impact since Zn may be accumulated in specific compartment where it has no or low physiological impact in terms of physiological disturbance (Lefèvre et al. 2014). This is especially the case for mucilage-producing plant species since divalent cations are known to bind more or less strongly to mucilage polysaccharide (Morel et al. 1986, 1987; Lapie et al. 2019; Lodhi et al. 2019). Mucilage deposit occurs in specific areas. Although mucilage at the root surface is reported in a wide range of species as a result of root exudation (Schwartz et al. 2015; Cai et al. 2013), wetland plant species (and especially halophytes) also produce high amounts of mucilage at the shoot part including leaves where mucilage may be involved in water absorption from the atmosphere (Jones et al. 2016).

The present work demonstrates that crude mucilage content was the highest for leaves (Fig. 3) and that in plants that were not exposed to Zn toxicity, mucilage content was similar in roots and stems. It is noteworthy that zinc toxicity obviously increased crude mucilage content in all organs and that in this case, crude mucilage content was slightly higher in stems than in roots. In shoots of *K. pentacarpos*, mucilage mainly accumulated within the xylem vessels and as deposit in leaf epidermis (Ghanem et al. 2010). It may thus be hypothesized that increase in stem mucilage content was an attempt to retain Zn excess during the translocation process in order to reduce translocation to photosynthetic leaves while Zn fixation on epidermal mucilage contribute to protect chloroplast in the mesophyll, as reported for other halophytes (Lefèvre et al. 2014). It has to be mentioned, however, that NaCl improved Zn resistance but did not modify the crude mucilage content, whatever the considered organ. This contrasts with the data provided by Ghanem et al. (2010) for *K. pentacarpos* exposed to higher salinity, and by Golkar et al. (2017)

who reported a salt-induced increase in mucilage content in *Plantago ovata*. It may nevertheless be argued that a smaller amount of Zn, as recorded in NaCl-treated plants, may be more efficiently retained by a similar amount of mucilage. Moreover, Zn binding is not only a matter of mucilage amounts but directly depends on mucilage composition, as demonstrated by mucilage-Zn^{2+}-pectinate controlled-release matrices used in pharmaceutical industry (Guru et al. 2018). A modification in the mucilage composition was also observed in response to non-ionic constraints such as water deficit (Minjares-Fuentes et al. 2017; Elboutachfaiti et al. 2017)

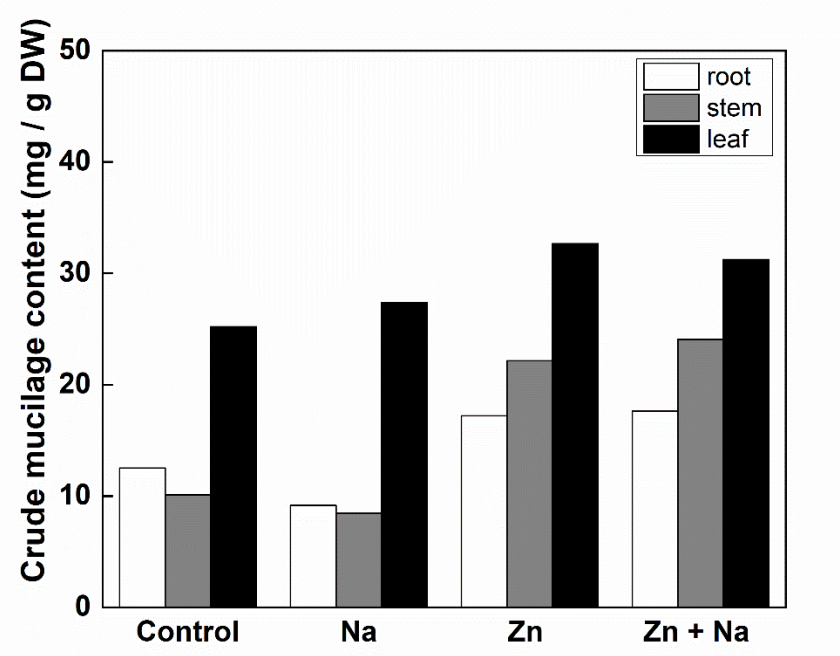

Fig. 3 Crude mucilage content (mg g^{-1} dry weight) in root, stem and leaf in *Kosteletzkya pentacarpos* exposed during four weeks to 0.1 mM $ZnCl_2$ in the presence or in the absence of 50 mM NaCl.

Acid hydrolysis of crude mucilage extract in *K. pentacarpos* revealed that the main neutral monosaccharides constituents were rhamnose, arabinose, galactose and glucose (Table 2). Zinc and salinity had different effects on the proportion of monosaccharides depending on

the considered organs. When plants were exposed to 0.1 mM Zn, the percentage of rhamnose in root decreased while the percentage of glucose as well as uronic acid increased, compared to control. NaCl increased the percentage of arabinose and galactose but decreased the percentage of glucose. In stem, the percentage of rhamnose, glucose and uronic acid increased while the percentage of arabinose decreased in Zn treatment compared to control. Salt increased the percentage of rhamnose and galactose in stem. Hence, in both root and stem of plants exposed to Zn toxicity, the percentage of uronic acids was much higher than control (Fig 4). Uronic acids are thought to play a key role in heavy metal sequestration through electrostatic interactions between carboxyl groups and positive charges of divalent cations and an increase in the proportion of uronic acids may contribute to an improved Zn retention independently of the total mucilage amount (Jones et al. 2016; Lodhi et al. 2019). In the root powder issued from *K. pentacarpos* and used for biosorption process, Lutts et al. (2016) demonstrated that root powder issued from plants cultivated from NaCl-treated was by far more efficient for Zn retention that powder issued from control plants, suggesting once again a modification in the mucilage composition. In the present case, however, NaCl added to Zn-treated plants induced a drop in uronic acids in roots and stems which were even lower than in control plants.

Table 2 Neutral monosaccharides composition (in mol % of total neutral monosaccharides) and uronic acid (Ua) content[6] (in % of dry weight of mucilage, Gal A + Glu A) of mucilage extracted from *K. pentacarpos* exposed to 0.1 mM $ZnCl_2$ in the absence or in the presence of 50 mM NaCl for four weeks. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

	Root				Stem				Leaf			
	Control	Na	Zn	Na + Zn	Control	Na	Zn	Na + Zn	Control	Na	Zn	Na + Zn
Rua	25±1.6b	27±3.1b	19±0.90a	15±0.32a	30±0.15a	31±0.25a	43±1.1b	29±5.1a	18±1.9ab	25±1.5c	14±0.66a	20±1.7b
Fuc	5.4±0.21 c	< 1.00	4.0±0.72b	2.6±0.15a	4.1±0.29b	< 1.00	3.1±0.23a	< 1.00	< 1.00	< 1.00	2.2±0.50a	1.9±0.30a
Ara	12±0.85ab	18±2.2bc	11±0.64a	19±3.3c	20±0.55bc	23±2.6c	14±0.50a	18±2.7ab	31±2.0a	27±1.4a	28±2.7a	30±0.59a
Xyl	5.5±0.92a	4.5±1.1a	6.1±0.45b	3.9±0.67a	9.2±0.91b	5.5±0.64a	3.3±0.68a	3.7±1.3a	2.0±0.36a	1.8±0.06a	2.8±0.12b	3.0±0.21b
Man	7.4±2.20a	6.6±1.3a	10±0.59a	7.5±1.4a	7.7±0.31c	7.4±0.25c	4.0±0.32a	6.5±0.20b	5.7±0.15b	5.5±0.29b	4.2±0.58a	4.0±0.60a
Gal	24±2.1a	30±2.9b	25±0.50a	33±0.8b	19±0.10ab	17±0.87a	20±0.21b	29±1.0c	34±2.4a	30±2.0a	36±4.3a	29±1.3a
Glc	20±0.45b	14±0.51a	24±1.3c	19±0.1b	10±0.87a	17±1.1c	13±1.5b	14±0.64bc	9.7±1.1a	10±1.1ab	13±0.56b	12±1.2 ab
Ua	4.5	4.2	12.3	1.4	8.7	3	15.9	4.8	19.72	21.64	20.03	19.82

[6] Rua: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal:galactose; Glc: glucose. Uronic acid content in mucilage is examined once without statistical analysis.

In leaves, there was no major change in uronic acid proportion in response to the various treatment but the content of acidic polysaccharides was very high in leaves already in the control plants and an additional increase in uronic acids would not necessarily afford an improvement in Zn sequestration. Interaction between uronic acids and toxic elements may be complex. It was also determined that uronic acids were significantly enhanced by 33% under arsenic stressed conditions (Deepika et al. 2016). Fox et al. (2012) confirmed that several functional groups present in mucilage could be involved in interaction with negatively charged ions such as arsenate.

In addition, in *K. pentacarpos*, the mucilage is mainly a mixture of pectic polysaccharides. A high amount of pectic rhamnogalacturonans may also have important function in metal binding capacity. It has been reported by Astier (2014) that an increase in pectin contents of Douglas fir trees was observed in response to increasing soil cadmium concentration in polluted soil. A concurrent reduction in methyl-esterification of pectin suggested that the structure of this major binding site could be modified as a reaction to cadmium contamination.

Van Soest sequential fiber solubilization method is widely used to assess the cellulose, hemicellulose and lignin contents of plants and to predict the nutritive value of these material. In our results (Table 3): 50 mM NaCl treatment in the absence of Zn had no impact on the contents of lignin, hemicellulose and cellulose. Zn 0.1 mM in the absence of NaCl significantly decreased the content of lignin as well as cellulose by 23% and 28% respectively ($P < 0.05$), while significantly increased hemicellulose content ($P < 0.05$).

Table 3 Contents of lignin, hemicellulose and cellulose in *K. pentacarpos* stem when plants were exposed to 0.1 mM $ZnCl_2$ in the absence or in the presence of 50 mM NaCl for four weeks, analyzed by the detergent fiber method (Van Soest method). Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test.

Treatment	Stem		
	Lignin (g $100g^{-1}$ OM)	Hemicellulose (g $100g^{-1}$ OM)	Cellulose (g $100g^{-1}$ OM)
Control	13 ± 1.2 c	7.3 ± 0.16 a	46 ± 0.53 c
Na	14 ± 0.86 c	7.4 ± 0.28 a	48 ± 2.7 c
Zn	10 ± 0.58 b	8.3 ± 0.52 b	33 ± 0.90 a
Zn + Na	7.4 ± 0.67 a	8.6 ± 0.71 b	41 ± 0.62 b

When plants were exposed to Zn stress, the additional NaCl significantly increased content of both hemicellulose and cellulose ($P < 0.05$), compared to Zn alone treatment. The cell wall is mainly composed of cellulose and matrix polysaccharides including pectins and hemicelluloses. In contrast to cellulose, which is generally regarded as chemically inactive, it has been widely reported that plant hemicellulose also has potential ability to bind with heavy metals (Wang et al., 2012; Hu et al., 2010). Yang (2011) indicated that 75% of cell wall aluminum accumulated in the hemicellulose 1 (HC 1) fraction after *Arabidopsis thaliana* was treated by Al stress for 24h. Meanwhile, a time-dependent kinetic study showed that only when the HC1 fraction was removed, the amount of Al adsorbed decreased sharply. In our results, when plants were exposed to Zn stress in the presence and absence of NaCl, the content of hemicellulose increased in the stem, which indicated that Zn induced more hemicellulose production to fix Zn in cell wall in stem. Lutts et al. (2016) also reported a same trend for roots where Zn, even in the presence of NaCl, significantly increased the hemicellulose content, leading us to hypothesize than Zn retention may occur both at the mucilage and within cell wall, although according to a recent study in chapter 1 cell

wall retention was more efficient for Cd than for Zn in the shoot of *K. pentacarpos*.

Reference

Ajala TO, Akin-Ajani OD, Ihuoma-Chidi C, Odeku OA (2016) *Chrysophyllum albidum* mucilage as a binding agent in paracetamol tablet formulations. J Phar Invest 46:565-573.

Ali H, Khan E, Sajad MA (2013) Phytoremediation of heavy metals: concepts and applications. Chemosphere 91:869-881.

Astier C, Gloaguen V, Faugeron C (2014) Phytoremediation of Cadmium-Contaminated Soils by Young Douglas Fir Trees: Effects of Cadmium Exposure on Cell Wall Composition. International Journal of Phytoremediation 16 (7-8): 790-803

Baltaci AK, Yuce K, Mogulkoc R (2017) Zinc metabolism and metallothioneins. Biol Trace Elem Res 183:22-31.

Barrameda-Medina Y, Blasco B, Lentini M, Esposito S, Baenas N, Moreno DA, Ruiz JM (2017) Zinc biofortification improves phytochemicals and amino-acidic profile in *Brassica oleracea* cv. Bronco. Plant Sci 258:45-51.

Berni R, Luyckx M, Xu X, Legay S, Sergeant K, Hausman JF, Lutts S, Cai G, Guerriero G (2019) Reactive oxygen species and heavy metal stress in plants: impact on the cell wall and secondary metabolism. Environ Exp Bot 61:98-106.

Blakeney AB, Harris PJ, Henry RJ, Stone BA (1983) A simple and rapid preparation of alditol acetates for monosaccharides analysis. Carbohydr Res 113: 291-299.

Bleton J, Mejanelle P, Sansoulet J, Goursaud S, Tchapla A (1996) Characterisation of neutral sugars and uronic acids after methanolysis and trimethylsilylation for recognition of plant gums. J Chrom A 720:27-49.

Cai M, Wang N, Xing C, wang F, Wu K, Du X (2013) Immobilization of aluminium with mucilage secreted by root cap and root border cells is related to aluminium resistance in *Glycine max* L. Environ Sci Pollut Res 20:8924-8933.

Classen B, Blaschek W (1998) High molecular weight acidic polysaccharides from *Malva sylvestris* and *Alcea rosea*. Planta Med 64:640-644.

Deepika KV, Raghuram M, Kariali E, Bramhachari PV (2016) Biological responses of symbiotic *Rhizobium radiobacter* strain VBCK1062 to the arsenic contaminated rhizosphere soils of mung bean. Ecotoxicol Environ Saf 134:1-10.

Elboutachfaiti R, Delattre C, Quéro A, Roulard R, Duchêne J, Mesnard F, Petit E (2017) Fractionation and structural characterization of six purified rhamnogalacturonans type I from flaxseed mucilage. Food Hydrocol 62:273-279.

Fox DI, Pichler T, Yeh DH, Alcantar NA (2012) Removing heay metals in water: the interaction of cactus mucilage and arsenate (As(V)).

Ghanem ME, Han RM, Classen B, Quetin-Leclerq J, Mahy G, Ruan CJ, Qin P, Pérez-Alfoceà F, Lutts S (2010) Mucilage and polysaccharides in the halophyte plant species *Kosteletzkya virginica*: localization and composition in relation to salt stress. J Plant Physiol 167:382-392.

Gao Y, Liu H, Liu G (2017) The spatial distribution and accumulation characteristics of heavy metals in steppe soils around three mining areas in Xilinhot in Inner Mongolia, China. Environ Sci Pollut Res 24:25416-25430.

Golkar P, Amooshahi F, Arzani A (2017) The effect of salt stress on physio-biochemical traits, total phenolic and mlucilage content of *Plantago ovata* Forsk under *in vitro* conditions. J Appl Bot Food Qual 90:224-231.

Gupta N, Ram H, Kumar B (2016) Mechanism of zinc absorption in plants: uptake, transport, translocation and accumulation. Rev Environ Sci Biotechnol 1589-109.

Guru P, Bera H, Das MP, Hasnain MS, Nayak AK (202018) Acelofenac-loaded *Plantago ovata* F. husk mucilage-Zn^{2+}-pectinate controlled release matrices. Starch 70:1700136.

Halchak JL, Seliskar DM, Gallagher JL (2011) Root system architecture of *Kosteletzkya pentacarpos* (Malvaceae) and belowground environmental influences on root and aerial growth dynamics. Am J Bot 98:163-174.

Han RM, Lefèvre I, Ruan C-J, Qin P, Lutts S (2012) NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regul 68:97-109.

Han R, Quinet M, Andre E, van Elteren JT, Destrebecq F, Vogel-Mikus K, Cui G, Debeljak M, Lefevre I, Lutts S (2013) Accumulation and distribution of Zn in the shoots and reproductive structures of the halophyte plant species *Kosteletzkya virginica* as a function of salinity. Planta 238:441-457.

Hu GH, Huang SH, Chen H, Wang F (2010) Binding of four heavy metals to hemicelluloses from rice bran. Food Research International 43(1): 203-206

Islam MN, Wilson CA, Watkins TR (1982) Nutritional evaluation of seashore

mallow seed, *Kosteletzkya virginica*. J Agric Food Chem 30:1195-1198.

Javed R, Usman M, Yucesan B, Zia M, Gurel E (2017) Effect of zinc oxide (ZnO) nanoparticles on physiology and steviol glycosides production in micropropagated shoots of *Stevia rebaudiana* Bertoni. Plant Physiol Biochem 110:94-99.

Jones BO, John OO, Luke C, Ochieng A, Bassey BJ (2016) Application of mucilage from Dicerocaryum eriocarpum plant as biosorption medium in the removal of selected heavy metal ions. J Environ Manag177:365-377.

Kaur M, Kaur R, Punia S (2018) Characterization of mucilages extracted from different flaxseed (*Linum usitatissiumum* L.) cultivars: A heteropolysaccharide with desirable functional and rheological properties. Intern J Biol Macromol 117:919-927.

Lefèvre I, Marchal G, Meerts P, Corréal E, Lutts S (2009) Chloride salinity reduces cadmium accumulation by the Mediterranean halophyte species *Atriplex halimus* L. Environ Exp Bot 65:142-152

Lefèvre I, Vogel-Mikus K, Jeromel J, Vavpetič P, Planchon S, Arčon I, T. van Elteren J, Lepoint G, Gobert S, Renaut J, Pelicon P, Lutts S (2014) Cadmium and zinc distribution in relation to their physiological impact in the leaves of the accumulating *Zygophyllum fabago* L. Plant Cell Environ 37:1299-1320.

Leskova A, Giehl RFH, Hartmann A, Fargasova A, von Wiren N (2017) Heavy metals induce iron deficiency responses at different hierarchic and regulatory levels. Plant Physiol 174:1648-1668.

Lin CW, Chang HB, Huang HJ (2005) Zinc induces mitogen-activated protein kinase activation mediated by reactive oxygen species in rice roots. Plant Physiol Biochem 43:963-968.

Liu Y, Liu D, Hu R, Hua C, Ali I, Zhang A, Liu B, Wu M, Huang L, Gan Y (2017) AtGIS, a C2H2 zinc-finger transcription factor from Arabidopsis regulates glandular trichome development through GA signaling in tobacco. Biochem Biophys Res Commun 483:209-215.

Lodhi BA, Abbas A, Jussain MA, Hussain SZ, Sher M, Hussain I (2019) Design, characterization and appraisal of chemically modified polysaccharide based mucilage from *Ocimum basilicum* (basil) seeds or the removal of Cd(II) from spiked high-hardness ground water. J Mol Liq 274:15-24.

Lutts S, Lefevre I (2015) How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? Ann Bot 115:509-528.

Lutts S, Qin P, Han RM (2016) Salinity influences biosorption of heavy metals by the oots of the halophyte plant species *Kosteletzkya pentacarpos*. Ecol Eng 95:682-689.

Men C, Liu R, Xu F, Wang Q, Guo L, Shen Z (2018) Pollution characteristics, risk assessment, and source apportionment of heavy metals in road dust in Beijing, China. Sci Total Environ 612:138-147.

Minjares-Fuentes R, Medina-Torres L, González-Laredo RF, Rodríguez-González VM, Eim V, Femenia A (2017) Influence of the water deficit on the main polysaccharides and the rheological properties of Aloe vera (*Aloe barbadensis* Miller) mucilage. Ind Crop Prod 109:644-653.

Morel JL, Andreux F, Habib L, Guckert A (1987) Comparison of the adsorption of maize root mucilage and polygalacturonic acid on montmorillonite homoionic to divalent lead and cadmium. Biol Fertil Soils 5:13-17.

Morel JL, Mench M, Guckert A (1986) Measurement of Pb^{2+}, Cu^{2+} and Cd^{2+} binding with mucilage exudates from maize (*Zea mays* L.) roots. Biol Fertil Soils 2:29-34

Park YR, Choi MJ, Park SJ, Kang H (2017) Three zinc-finger RNA-binding proteins in cabbage (*Brassica rapa*) play diverse roles in seed germination and plant growth under normal and abiotic stress conditions. Physiol Plant 159:93-106.

Pramanick P, Chakraborty A, Raychaudhuri SS (2017) Phenotypic and biochemical alterations in relation to MT2 gene expression in *Plantago ovata* Forsk under zinc stress. Biometals 30:171-184.

Qin P, Han RM, Zhou MX, Zhang HS, Fan LS, Seliskar DM, Gallagher JL (2015) Ecological engineering through the biosecure introduction of *Kosteletzkya virginica* (seashore mallow) to saline lands in China: A review of 20 years of activity. Ecol Eng 74:174-186.

Rakotondrabe F, Ndam Ngoupayou JR, Mfonka Z, Rasolomanana EH, Nyangono Abolo AJ, Ako Ako A (2018) Water quality assessment in the Betare-Oya gold mining area (East-Cameroon): multivariate statistical analysis approach. Sci Total Environ 610-611:831-844.

Rizwan M, Ali S, Hussain A, Ali Q, Shakoor MB, Zia-Ur-Rehman M, Farid M, Asma M (2017) Effect of zinc-lysine on growth, yield and cadmium uptake in wheat (*Triticum aestivum* L.) and health risk assessment. Chemosphere 187:35-42.

Schwartz N, Carminati A, Javaux M (2015) The impact of mucilage on root

water uptake - A numerical study. Water Resour Res 52:264-277

Sharma SS, Dietz KJ, Mimura T (2016) Vacuolar compartmentalization as indispensable component of heavy metal detoxification in plants. Plant Cell Environ 39:1112-1126.

Tang L, Ying RR, Jiang D, Zeng XW, Morel JL, Tang YT, Qiu RL (2013) Impaired leaf CO_2 diffusion mediates Cd-induced inhibition of photosynthesis in the Zn/Cd hyperaccumulator *Picris divaricata*. Plant Physiol Biochem 73:70-76.

Vaughn SF, Moser BR, Dien BS, Iten LB, Thompson AR, Seliskar DM, Gallagher JL (2013) Seashore mallow (*Kosteletzkya pentacarpos*) stems as a feedstock for biodegradable absorbents. Biomass Bioenergy 59:300-305.

Wang G, Zhang S, Xiao L, Zhong Q, Li L, Xu G, Deng O, Pu Y (2017) Heavy metals in soils from a typical industrial area in Sichuan, China: spatial distribution, source identification, and ecological risk assessment. Environ Sci Pollut Res Int 24:16618-16630

Yang JL, Zhu XF, Peng YX, Zheng C, Li GX, Liu Y, Shi YZ, Zheng SJ (2011) Cell Wall Hemicellulose Contributes Significantly to Aluminum Adsorption and Root Growth in Arabidopsis. Plant Physiol 155(4): 1885-1892.

Yu R, Fang X, Somasundaran P, Chandran K (2015) Short-term effects of TiO_2, CeO_2, and ZnO nanoparticles on metabolic activities and gene expression of *Nitrosomonas europaea*. Chemosphere 128:207-215.

Zheng H, Wang X, Chen L, Wang Z, Xia Y, Zhang Y, Wang H, Luo X, Xing B (2017) Enhanced growth of halophyte plants in biochar-amended coastal soil: roles of nutrient availability and rhizosphere microbial modulation. Plant Cell Environ 41:517-532.

Interchapter 5-6

What do we know?

- *Kosteletzkya pentacarpos* increased mucilage production in all plant organs in response to Zn excess. Such an increase may be considered as a strategy to fix accumulated Zn.
- Zinc induces a rise in uronic acids in roots and stems but not in the leaves where a high constitutive proportion of uronic acids is observed in response to all treatments. Meanwhile, changes are also observed in neutral monosaccharides components in different plant organs.
- Zn stress increases the content of hemicellulose but decreased the content of lignin and cellulose in the stem. The additional presence of NaCl slightly increases the content of cellulose.

An excess of zinc induces a modification in the composition and structure of polysaccharides. The mucilage may play a role in plants response exposed to Zn toxicity in the presence of moderate dose of salinity.

What are the questions?

- Until now, the part I (chapter 1 to chapter 5) is dealing with plant exposure to the mixed pollution and/or salinity in nutrient solution. All previous work published in the literature also consider nutrient solutions with fully available heavy metals. To the best of my knowledge, there is no available information regarding the physiological behaviour of this species grown on heavy metal polluted soils.
- In a soil substrate, bioavailability of the pollutant is an important factor influencing plant response and capacities of accumulation and must therefore be considered, especially

considering the fact that in salt-affected polluted substrate, NaCl influences the heavy metals bioavailability.

- Mobile heavy metals that are not absorbed by plants constitute a major ecological risk of leaching, leading to pollution of underground water. Hence, it is of crucial importance to more deeply understand the effect of plants on the concentration and amount of pollutants recovered in leakages.

What is our strategy?

As an attempt to mimic real field situation, soil collected from an experimental farm of Université catholique de Louvain will be sprayed by a mixture of Cd, Zn, Pb and As, considering that these four elements are frequently recorded in contaminated soil from salt marshes in eastern coast of China. *K. pentacarpos* will be grown on this spiked contaminated soil on short-term time basis to study its physiological response to heavy metals and As toxicity as well as capacity on phytoextraction. Furthermore, the heavy metal bioavailability will be determined in relation to quantification of leachate volume as well as pollutants in leachate. Plants will be irrigated by fresh tap water or by salt water in order to precisely determine the impact of salt on pollutant bioavailability and leaching.

CHAPTER 6 Salinity modifies heavy metals and arsenic absorption by the halophyte plant species *Kosteletzkya pentacarpos* and pollutant leaching from a polycontaminated substrate

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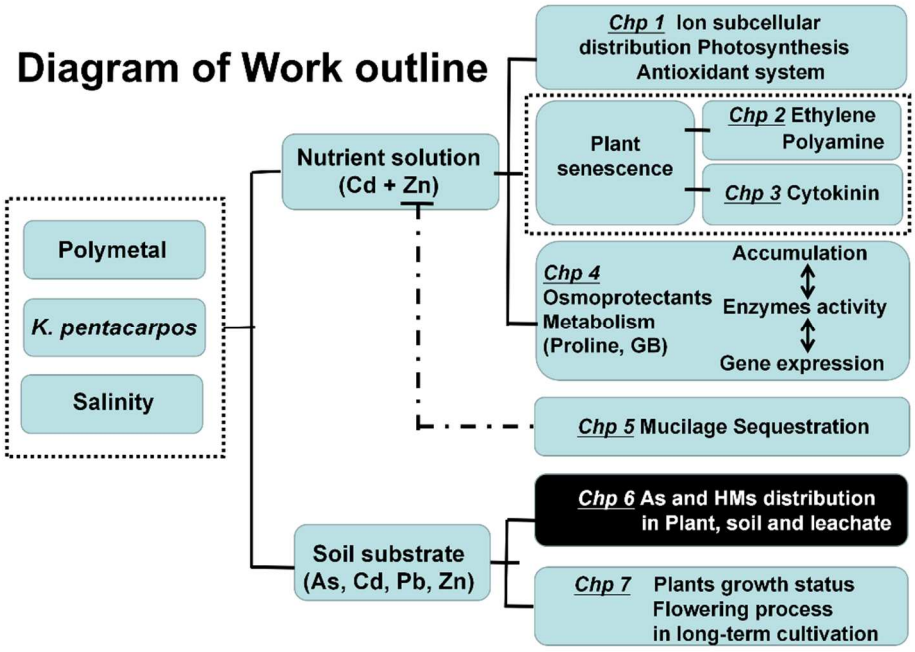
Diagram of Work outline
Chp 1 Ion subcellular distribution Photosynthesis Antioxidant system
Plant senescence
Chp 2 Ethylene Polyamine
Chp 3 Cytokinin
Nutrient solution (Cd + Zn)
Polymetal
K. pentacarpos
Salinity
Chp 4 Osmoprotectants Metabolism (Proline, GB)
Accumulation
Enzymes activity
Gene expression
Chp 5 Mucilage Sequestration
Chp 6 As and HMs distribution in Plant, soil and leachate
Soil substrate (As, Cd, Pb, Zn)
Chp 7 Plants growth status Flowering process in long-term cultivation

Chapter 6

ABSTRACT

Phytomanagement of polycontaminated soils is challenging, especially in areas simultaneously affected by salinity. The wetland halophyte plant species *Kosteletzkya pentacarpos* was cultivated in a column device allowing leachate harvest, on a polycontaminated spiked soil containing Cd (6.5 mg kg^{-1} DW), As (75 mg kg^{-1} DW), Zn (200 mg kg^{-1} DW) and Pb (300 mg kg^{-1} DW) and irrigated with salt water (final soil electrical conductivity 5.0 ms cm^{-1}). Salinity increased Cd bioavailability in the soil and Cd accumulation in the shoots while it had an opposite effect on As. Salinity did not modify Pb and Zn bioavailability and accumulation. Cultivating plants on the polluted soil drastically reduced the volume of leachate. In all cases, salinity reduced the total amounts of heavy metals removed by the leachate and significantly decreased the proportion of Cd and Zn removed by the plants. Heavy metal contamination induced a decrease in shoot dry weight and an increase in malondialdehyde (an indicator of oxidative stress); both symptoms were alleviated by the additional presence of NaCl but this positive impact was not related to increase in protecting phytochelatins synthesis. It is concluded i) that bioavailability estimated by the 0.01M $CaCl_2$ extraction procedure is not fully relevant from the heavy metal mobility, ii) that salinity decreased heavy metal percolation, especially in soils cultivated with *K. pentacarpos* and iii) that salinity improves plant tolerance to heavy metals in *K. pentacarpos* and that this species is a promising plant material for phytoremediation of polycontaminated soils.

Keywords: arsenic, cadmium, lead, phytoremediation, seashore mallow, zinc

1. Introduction

Mineral nutrition is of paramount importance for optimal plant growth and development. Although a reduced availability in nutrients induces numerous physiological disorders, an excess of elements may also compromise plant growth and survival. Ion toxicity may involve essential or non-essential elements and aluminum, ferrous iron, salinity (mainly NaCl) and heavy metals constitute important constraints for plant (Dufey et al., 2009; Lefèvre et al., 2014; Riaz et al., 2018; Singh et al., 2017). In numerous cases, several elements are simultaneously present in excess, leading to a complex stressing environment. This is especially the case for heavy metals and toxic metalloids. These persistent pollutants may occur as a consequence of geological processes but also result from human activities such as mining, smelting, fertilizers application, industrial activities and waste water discharge, wastes from incinerators (Clemente et al., 2012; Wang et al., 2013; Yang et al., 2018; Zhai et al., 2018; Liu et al., 2019; Wen et al., 2019). Coastal areas are especially prone to heavy metal pollution and wetlands soils act as a sink for numerous pollutants and face contamination with Cd, Zn, Pb and toxic metalloids like As (Bai et al., 2019; Liu et al., 2019; Wen et al., 2019).

As transitional region of land and ocean, coastal areas and sediments are typical zones suffering from the simultaneous presence of salinity and heavy metal in excess (Liu et al., 2019). In other areas, salt and heavy metal accumulation may also occur on a long term basis as a result of irrigation with brackish waters when high quality water, used in priority for human consumption, is not fully available (Wahla and Kirkham, 2008) and even in protected areas used in the past for excessive exploitation of natural resources (Bartowiak et al., 2017). It has been reported that salinity increases mobility of heavy metals in soils (Acosta et al., 2011; Du Laing et al., 2008; Zhao et al., 2013). Soil parameters controlling heavy metal chemistry and bioavailability are soil mineralogy, organic matter content, pH and electrical conductivity.

Salinity implies the competition of salt-derived cations with positively charged heavy metals for sorption sites on the solid phase and complexation capacity of salt-derived anions with heavy metals. Consequences of salinity on heavy metal mobility is well documented for Cd readily forming stable complexes with chloride ligands ($CdCl_n^{2-n}$) which are loosely bound to soil particles (McLaughlin et al., 1997; Filipović et al., 2018). Cd-chlorocomplex are less available than free Cd^{2+} for root absorption but Cd may nevertheless enter the roots directly or dissociating from the chlorocomplex after reaching binding sites at the root surface (Lefèvre et al. 2009; Filipović et al., 2018). Acosta et al. (2011) demonstrated that salinity impact on heavy metal mobility varies depending on the considered heavy metal but also depending on the precise nature of salinity and an increase in ionic strength by any salt has commonly lower impact on Zn and Pb mobilization than on Cd. In some cases, high salinity may even reduce heavy metal mobility as a consequence of Zn-containing (bechererite and hordaite) or Pb-containing (laurionite) precipitates (Tandon et al., 2018). Bai et al. (2019) demonstrated that salinity may significantly increase arsenic mobility in coastal wetland soils even if this element is commonly present in the soil in anionic forms (arsenate or arsenite, depending on the soil redox potential).

Phytoremediation is a biological tool frequently proposed as a promising alternative to expensive chemical and physiological strategies for management of heavy metal contaminated soil. Heavy metal phytoremediation of salt-affected soil is challenging since suitable plant species must be able to cope with both salt and pollutant constraints. Numerous halophyte species have been recommended for this purpose (Clemente et al., 2012; Cheng et al., 2012; 2018b; 2019; Lefèvre et al., 2009; Van Ootsen and Maggio, 2015; Lutts and Lefèvre, 2015; Vromman et al., 2016; Li et al., 2019). Indeed, tolerance to different toxic elements may at least partly rely on similar physiological properties in relation to ion sequestration, regulation of the plant water status, management of oxidative stress and protection of cellular structures.

Filipović et al., (2018) recently demonstrated that a salt-induced increase in heavy metal mobility does not necessarily implies an increase in phytoavailability. Salt may induce a particular speciation for pollutant which is less suitable for root absorption by unspecific transporter. Salinity also induces a water stress component leading to stomatal closure and thus a decrease in the transpiration stream which compromises root to shoot translocation (Lutts and Lefèvre, 2015). Cheng et al. (2012) demonstrated that in some mangrove species, salt-treated roots encountered anatomical changes and precocious lignification which strongly reduces Pb, Zn and Cu accumulation. Hence, despite an increase in heavy metal mobility, salinity often leads to a decrease in heavy metal accumulation in plants, even in halophyte species (Cheng et al., 2018b; 2019; Lefèvre et al., 2009; Lutts and Lefèvre, 2015; Van Oosten and Maggio, 2015). Mobilized heavy metals that are not absorbed by the roots constitute a major ecological risk of leaching leading to pollution of drainage water and contamination of aquifers (Wahla and Kirkham, 2008; Zhao et al., 2013).

Kosteletzkya pentacarpos (syn *Kosteletzkya virginica*) is a deep-rooted wetland halophyte plant species commonly found in salt marshes of coastal areas (He et al., 2003; Qin et al., 2015). Previous experiments demonstrated that moderate doses of salinity help the plant to cope with heavy metals such as Cu, Cd and Zn (Han et al., 2012a; 2012b; 2013a; 2013b). Additional works showed that mixed heavy metal treatment (Cd+Zn) induces a specific physiological constraint at the plant level comparatively to single treatment as assessed by a specific hormonal status, tissular distribution of pollutant or synthesis of osmoprotectants. In order to carefully and precisely control the stress intensities, those studies relied on plant culture in nutrient solution and did not pay attention to the interaction between roots and a solid substrate.

The aim of the present study was to quantify the impact of salinity on the relative proportion of Cd, Zn, Pb and As percolation and plant accumulation by the halophyte wetland plant species *Kosteletzkya pentacarpos* growing on a polycontaminated soil.

2. Material and Methods

2.1. Soil sampling and analysis

Soil was collected in the experimental farm of Université catholique de Louvain (Centre Alphonse de Marbaix, Corroy le Grand) on a field previously used for Miscanthus production. After removal of the organic layer, soil was collected in the first 20 cm, air-dried, sieved through a 4-mm mesh, homogenized and mixed with washed sand (ratio soil/sand: 4/1). Half of the harvested soil was maintained as a thin layer (3 cm) in a greenhouse and sprayed with heavy-metal containing solution in order to obtain a final concentration of 75, 6.5, 200 and 300 mg kg^{-1} for As, Cd, Zn and Pb, respectively (As was added as $Na_2HAsO_4.7H_2O$; Cd as $CdCl_2$, Zn as $ZnCl_2$ and Pb as $PbNO_3$). Doses were chosen in order to induce a medium toxicity according to the Belgian rules (http://environnement.wallonie.be/legis/solsoussol/sol003.htm). The remaining soil was used as unpolluted control. Spiked polluted soil was maintained for an additional period of one month to reach equilibrium. Soil electrical conductivity (EC) and pH-H_2O were determined using glass electrodes (WTW Multi 350i) in a 1:5 soil/water paste. Exchangeable base concentrations (Ca^{2+}, Mg^{2+}, Na^+ and K^+) were determined using percolation columns and ammonium acetate 1.0 M pH 7.0 (Page et al., 1982) and inductive-coupled plasma atomic emission spectrometry (ICP-AES; Thermo Jarrell Ash Iris Advantage). Cation exchangeable capacity (CEC) was assessed using the same percolation columns and KCl 1.0 M pH 3.0. Total N (NT) and organic C (Corg) were determined according to Kjeldahl and Walkley–Black methods (Page et al., 1982). Heavy metal concentrations were obtained by dissolving 0.5 g of soil in an open Teflon crucible with 3 mL HNO_3 (AnalaR NORMAPUR 65%) and 5 mL HF (AnalaR NORMAPUR 40%). The mixture was gently heated at 130 °C on a hot plate until complete dryness. The residue was redissolved with 2 mL $HClO_4$ (AnalaR NORMAPUR 70%), and the mixture was heated again until complete dryness. The residue was

redissolved with aqua regia (HCl AnalaR NORMAPUR 37% and HNO_3 65%) and filtered (Whatman-41). Finally, the solution was diluted to 50 mL with deionized water and analyzed with ICP-AES. Quality of the procedure was controlled by the use of certified samples (BCR CRM 141R and 142; Evisa). The proportions of clay, silt and sand in soil were 25.4±1.5, 51.1±3.4 and 23.5±2.0 %, respectively (table S1; NFX 31-107 sandard)). The values of pH_{water} (NFX31-130 standard) and CEC were 7.34±0.13 and 18.4±1.3 $cmol^{+}$ kg^{-1}, and EC was 0.83 $mS.cm^{-1}$. Other main characteristics of the used soil were also provided in table S1.

In order to assess soil heavy metal mobility, a 0.01 M $CaCl_2$ selective extraction was performed according to Houba et al. (2000) and Degryse et al. (2003): 2.5 g of soil and 25 mL of $CaCl_2$ were shaken during 24 h and then centrifuged at 3,000 *g* during 15 min (Sigma 3-30K, Germany). The supernatant was filtered (Whatman 2) and analyzed for heavy metal concentration with ICP-AES.

Soil was then introduced in plastic column (72 columns, 10.5 cm of diameter and 33 cms height; each column received 2.5 Kg of soil; 36 columns with polluted soil and 36 columns with control unpolluted soil). The bottom of the column was filled with 3 cms of gravel layer and a tap was adapted on the system to collect the leachates during the time course of experiment.

2.2. Plant material and growing conditions

Seeds of *Kosteletzkya pentacarpos* were surface-sterilized for 30 s in 75% ethanol (v/v) and germinated on a substrate of perlite/vermiculite (3:1) moistened with Hoagland solution in a phytotron under a 16h day/light photoperiod, a relative humidity of 51.3%, a mean temperature of 25 °C during the day and 23 °C during the night and a mean light intensity of 150 µmoles m^{-2} s^{-1} provided by Sylvania fluorescent tubes (F36W/133-T8/CW; Danvers, MA). Uniform young seedling at the three leaves stage and a mean height of 10.0 ± 1 cm was then transferred to the column (three seedlings per columns),

except on 12 columns which remained free of plants and were subsequently used as control.

Columns were randomly distributed in a greenhouse with semi-controlled environmental conditions (16h daylight photoperiod, natural light supplemented by Phillips lights 'Philips lighting SA, Bruxelles, HPLR 400W to provide a minimal light intensity of 280 $\mu moles.m^{-2}s^{-1}$ at the top of the canopy; mean temperature of 24.4 °C; mean relative humidity of 51%). Soil was preliminary watered before seedling transfer with deionized water to field capacity (volumetric soil water content of 27.8%). After 8 days of acclimatization, half of the columns were regularly irrigated at 2 days intervals with salt water providing a total amount of 6 gr NaCl in each column. Four treatments were thus considered: 1) controls (no pollution and no salt), 2) polluted, 3) NaCl 4) polluted + NaCl. Each treatment involved 15 columns with 3 plants per column, and for each treatment, 3 columns that did not contain any plant were used as additional control. Plants were cultivated during a total period of 84 days. Columns were irrigated to ensure mean soil humidity close to 80% of the field capacity controlled by a ProCheck Decagon Devices EC-5 soil moisture probe. Leachates at the bottom of the column were collected every 3 days, filtered and stored in the dark at 4 °C until analysis.

Three harvests were performed after 4, 8 and 12 weeks. At each harvest, 5 columns were considered and all the plants from a same column were collected. Stem length, number of leaves, stem and leaf fresh and dry weight (obtained after 72h of incubation in an oven at 68 °C) were considered. Roots, which cannot be accurately separated from the soil, were not considered. Soil was collected from columns at two different depths (10 and 20 cm) and pooled to constitute samples for subsequent assessments of soil heavy metal concentration and bioavailability.

2.3. Plant ion concentration

For mineral analysis, c.a. 50 mg dry material were ground to a fine powder using a porcelain mortar and a pestle, digested in 35% HNO_3 and evaporated to dryness on a sand bath at 80 °C. The remaining minerals were diluted in a mix of 37% HCl and 68% HNO_3 (3/1); the mixture was slightly evaporated and dissolved in HCl 0.1N. The samples used for Pb quantification were preliminary submitted to a progressive rise of temperature until 450 °C and maintained during 24h at this temperature to obtain ashes. Final metal concentrations in the sample were assessed by plasma emission spectrometry ICP-OES (Thermo Jarrell As Iris Advantage). Quality control was based on the use of certified samples (NIST: National Institute of Standard and Technology, Gaithersburg, Md; SRM 1573 (tomato leaves), SRM 1547 (peach leaves) and BCR 679 (white cabbage, trace elements). For each sample, all measurements were performed in technical triplicates. Mineral concentration was expressed on a dry weight basis.

2.4. Malondialdehyde (MDA) content and total antioxidant activities

The level of lipid peroxidation in the control and ash-treated plants was assessed from the concentration of malondialdehyde (MDA) as determined by the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Ground fresh shoots and roots (0.25 g) were homogenized in 5 mL of 5 % (w/v) trichloroacetic acid (TCA) containing 1.25 % glycerol. The homogenate was centrifuged (Sigma 3-30K, Germany) at 12000 *g* for 10 min at 4 °C and filtered on Wathman No. 1 filter paper. 2 mL of TBA (0.67%) was added to 2 mL of supernatant and the mixture was heated at 100 °C for 30 min. The reaction was stopped by placing the reaction tubes in an ice bath. The samples were centrifuged at 12,000 *g* for 1 min and their absorbance was measured at 532 nm (UV-1800 Shimadzu, Belgium). The results were corrected by subtracting the non-specific absorbance component as measured at 600 nm. The concentration of MDA (nmol g^{-1} DW) was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

To estimate the total global antioxidant activity, ferric reducing ability of plasma (FRAP) was assayed according to Benzie and Strain (1996) considering the ability of plant extract to reduce ferric to ferrous ion at low pH and to produce a colored ferrous-tripyridyltriazine complex which was spectrophotometrically detected at 593 nm: 1 g FW was frozen in liquid nitrogen, ground in the presence of 10 mL methanol, incubated during 12h at 4°C, and then centrifuged during 20 min at 10,000 *g* at 4°C (Sigma 3-30K, Germany). Supernatant contains the hydrophilic fraction (AOAM) and was stored at -20 °C until analysis. Pellets were dissolved in 10 mL dichloromethane, homogenized and incubated at 4 °C during 12h, and centrifuged again at 10,000 *g* during 20 min (Sigma 3-30K, Germany). The obtained supernatant corresponds to the hydrophobic fraction (AOAD) and was stored at -20°C. For final analysis, 150 µL of each fraction were separately added to 300 µL of freshly prepared FRAP reagent (25 mL acetate buffer pH 3.6, 2.5 mL of 2,4,6-tripyridyl-s-triazine and 2.5 mL $FeCl_3.6H_2O$ 20 mM). Standard curve was established with Trolox (50 to 800 µM).

2.5. Glutathione and total non-protein thiols

For reduced (GSH) and total (GSHt) glutathione quantification, 200 mg of frozen samples were extracted and derivatized by orthophthalaldehyde (OPA) according to Cereser et al. (2001). GSHt was quantified after a reduction step of oxidized glutathione (GSSG) by dithiotreitol. Extracts were filtered through 0.45 µm microfilters (Chromafil PES-45/15, Macherey-Nagel) prior to injection and OPA derivatives were separated on a reversed-phase HPLC column with an acetonitrile-sodium acetate gradient system and detected fluorimetrically. Five µL of sample were injected into a Shimadzu HPLC system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a Nucleodur C18 Pyramid column (125 x 4.6 mm internal diameter; 5 µm particle size) (Macherey-Nagel, Düren, Germany). Derivatives were eluted in acetonitrile gradient in a 50 mM sodium acetate buffer pH 6.2 at 30 °C at a flow rate of 0.7 mL min^{-1}. Fluorimetric detection was performed with a spectra system Shimadzu

RF-20A fluorescence detector at 420 nm after excitation at 340 nm. GSH was quantified using nine-point calibration curves with custom-made external standard solutions ranging from 0.0625 to 50 µM and every ten injections, a check standard solution was used to confirm the calibration of the system. The recovery was determined using GSH as an internal standard.

The total non-protein thiol (NPT) concentration was determined according to De Vos et al. (1992): 200 mg fresh weight of tissue was ground in 2 mL of 5 % (*w/v*) sulfosalicylic acid plus 6.3 mM diethylenetriaminepentaacetic acid (pH < 1) at 0 °C with quartz sand in a mortar. The homogenate was centrifuged at 10,000 *g* for 10 min at 4 °C (Sigma 3-30K, Germany). The supernatants were collected and used for the determination of thiols using Ellman's reagent. Three hundred microliters of supernatant were mixed with 630 µL of 0.5 M KH_2PO_4 and 25 µL of 10 mM 5,5-dithiobis 2-nitrobenzoic acid (final pH 7.0). The absorbance at 412 nm was recorded after 2 min, and the NPT concentration was estimated using an extinction coefficient of 13,600 M^{-1} cm^{-1}. Phytochelatins content was evaluated as difference between NPT and GSH levels (Schäfer et al. 1997*)*. Glutathione and NPT were quantified in the leaves of plants exposed to 8 and 12 weeks of treatment.

2.6. Statistical treatment of the data

Normality of the data was checked by the Shapiro-Wilk tests and homoscedasticity was verified using the Levene test. Data were transformed ($log10(x+1)$ or $asin(x/\Sigma x)$) when required to ensure normal distribution. Variance analysis (ANOVA I) was then performed at each harvest using the R software to determine the impact of the treatment on the recorded parameters. Post hoc analyses were performed using the Tukey test.

3. Results

3.1. Plant-related parameters

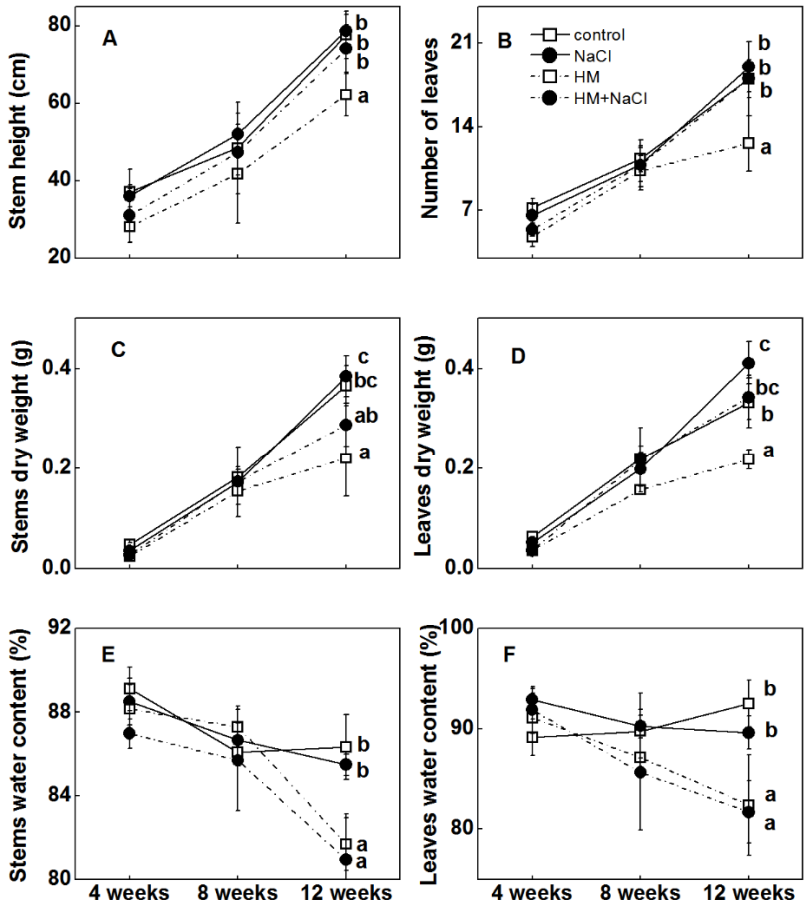

Fig. 1. Stem height (A), number of leaves (B), stem dry weight (C), leaf dry weight (D), stem water content (E) and leaf water content (F) in plants of *Kosteletzkya pentacarpos* cultivated during 4, 8 and 12 weeks on a non-polluted soil (control), the same soil added with NaCl (Na), with a mixture of heavy metals (HM) or with heavy metals and NaCl (HM+Na). Each value is the mean of 5 replicates and vertical bars are standard errors. Mean sharing a common letter are

not significantly different according to the Tukey test ($P > 0.05$).

Plant growth-related parameters are presented in Fig. 1. Stem height remained similar in control and in NaCl-treated plants (Fig. 1A) but decreased as a consequence of exposure to HM. However, the addition of NaCl to HM-treated plants allowed to significantly reduce the deleterious impact of pollution on stem elongation. A similar trend was observed for the number of leaves which drastically decreased in HM-treated plants after 8 weeks of treatment (Fig. 1B) but still increase in a similar way in plants exposed to the other treatments. The stem (Fig. 1C) and the leaves (Fig. 1D) dry weights displayed similar trends: they remained similar for all treatments up to 8 weeks but then started to differ and exhibited the lowest value for plants exposed to heavy metals and the highest value for controls (stem) or NaCl-treated plants (leaves). Once again, the presence of NaCl on HM-contaminated soil reduced the deleterious impact of those pollutants on plant growth. Heavy metals reduced water content in both organs (Fig. 1E and 1F) and to a similar extent in HM- and HM+NaCl-treated plants.

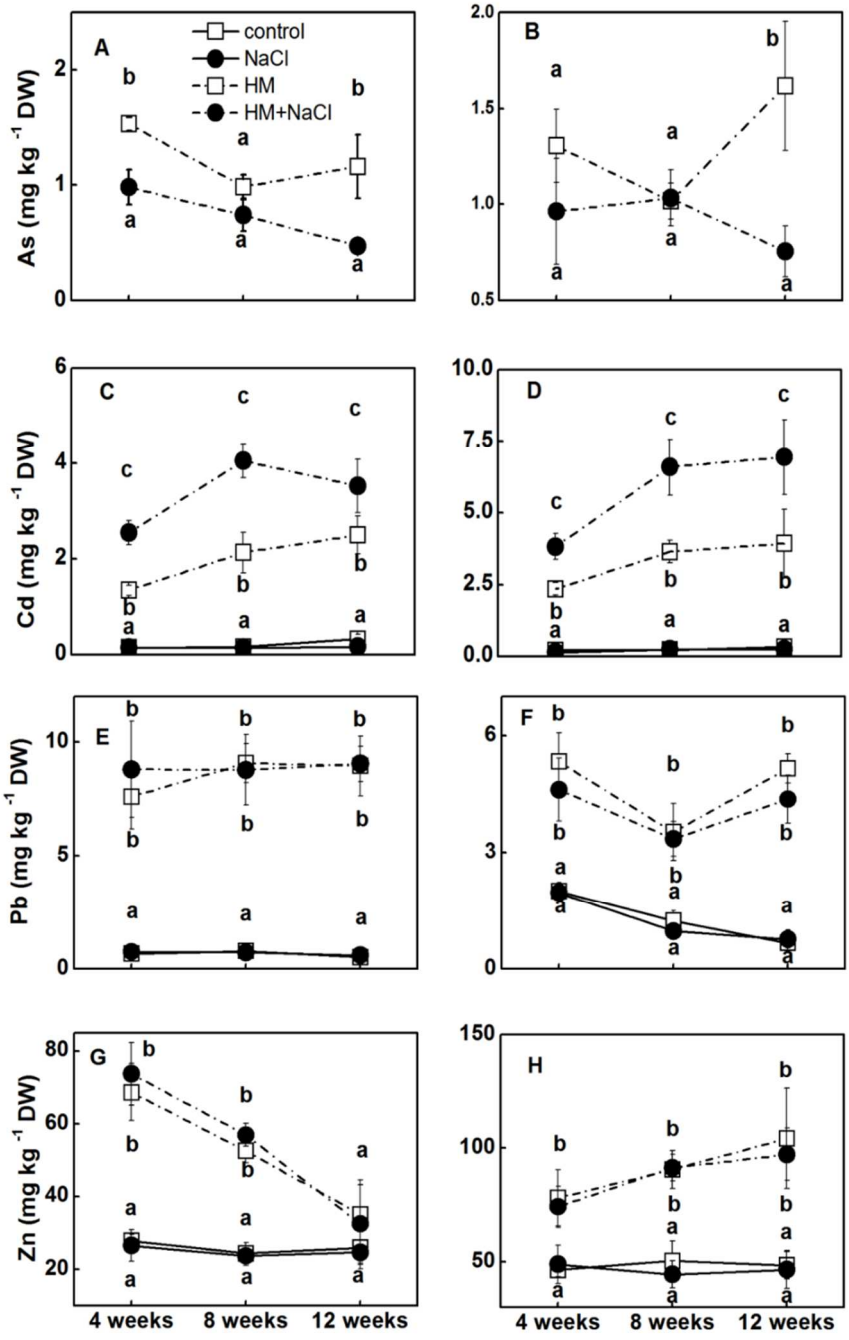

Fig. 2. Stem (A, C, E, G), and leaf (B, D, F, H) concentration in arsenic (A, B), cadmium (C, D), lead (E, F) and zinc (G, H) in plants of *Kosteletzkya pentacarpos*

cultivated during 4, 8 and 12 weeks on a non-polluted soil (control), the same soil added with NaCl (Na), with a mixture of heavy metals (HM) or with heavy metals and NaCl (HM+Na). Each value is the mean of 5 replicates and vertical bars are standard errors. Mean sharing a common letter are not significantly different according to the Tukey test ($P > 0.05$).

During the time course of the experiment, As was not detected in control and in NaCl-treated plants. Salinity reduced As accumulation in the stems after 4 and 12 weeks (Fig. 2A) and in the leaves after 12 weeks (Fig. 2B). Salinity had an opposite impact on Cd content: it increased it in both stems (Fig. 2C) and leaves (Fig. 2D) of plants maintained on contaminated soil. Lead was detected in low amounts in control and in NaCl-treated plants but it obviously increased in plants exposed to the contaminated substrate. It remained constant during the experiment in the stem and NaCl had no impact on Pb accumulation in plants (Fig. 2E and F). Zinc accumulation it the stem (Fig. 2G) significantly decreased with the duration of the treatment in plants exposed to the contaminated substrate while it remained constant in plants growing on uncontaminated substrate: at the end of the experiments, similar values were recorded for Zn stem concentration in all plants. A similar trend was not observed in the leaves (Fig. 2H). Moreover, as previously mentioned for Pb, NaCl had no impact on Zn accumulation in stems and leaves.

Table 1. Leaf concentration of essential elements (Ca, Mg, K, P and S estimated in mg g^{-1} DW ; Fe, Mn and Cu estimated in mg kg^{-1} DW) and Na (in mg g^{-1} DW) in plants of *Kosteletzkya pentacarpos* maintained during 12 weeks on a soil substrate containing contaminated by arsenic and heavy metals (HM), NaCl (6 g kg^{-1}) or both treatment (HM + NaCl). Each value is the mean of 5 replicates ± S.E. Means followed by the same letter are not statistically different according to the Tukey test ($P > 0.05$).

	Ca (mg g^{-1})	Mg (mg g^{-1})	K (mg g^{-1})	Na (mg g^{-1})	P (mg g^{-1})	S (mg g^{-1})	Fe (mg g^{-1})	Mn (mg kg^{-1})	Cu (mg kg^{-1})
Control	5.3 ± 0.4 a	2.9 ± 0.1 a	26 ± 2 a	0.48 ± 0.14 a	3.0 ± 0.4 b	9.5 ± 1.3 a	137 ± 17 b	80 ± 6 c	11.8 ± 1.1 a
NaCl	5.5 ± 0.7 a	2.7 ± 0.2 a	14 ± 3 b	4.20 ± 0.99 b	2.7 ± 0.2 ab	9.0 ± 1.3 a	150 ± 17 b	81 ± 7 c	13.5 ± 1.1 a
HM	5.0 ± 0.4 a	3.1 ± 0.3 a	24 ± 3 a	0.71 ± 0.11 a	2.2 ± 0.3 a	13.4 ± 1.6 b	74 ± 7 a	52 ± 4 a	19.5 ± 4 b
HM+NaCl	5.1 ± 0.2 a	2.9 ± 0.3 a	10 ± 1 c	6.2 ± 1.0 c	2.5 ± 0.3 ab	8.5 ± 1.1 a	160 ± 11 b	68 ± 7 b	10.8 ± 0.8 a

Table 1 indicated that salinity induced a decrease in the leaf K content and an increase in leaf Na which was higher for plants exposed to NaCl in the presence of HM. Heavy metals reduced P, Fe and Mn content and in all cases, the deleterious impact of HM was reduced by additional NaCl. Conversely, HM increased the S and Cu concentrations in the leaves in the absence but not in the presence of NaCl.

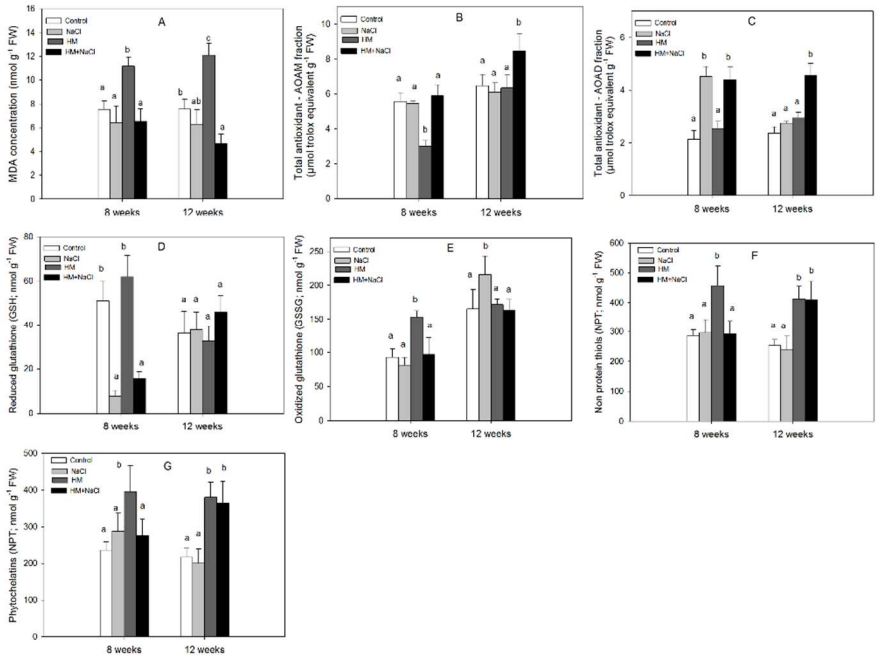

Fig. 3. Leaf malondialdehyde (MDA) concentration (A), total antioxidant activity (FRAP; hydrophilic AOAM (B) and lipophilic AOAD (C) faction), reduced glutathione (GSH; D) oxidized glutathione (GSSG; E), total non-protein thiols (F) and phytochelatins (G) in plants of *Kosteletzkya pentacarpos* cultivated during 8 and 12 weeks on a non-polluted soil (control), the same soil added with NaCl (Na), with a mixture of heavy metals (HM) or with heavy metals and NaCl (HM+Na). Each value is the mean of 5 replicates and vertical bars are standard errors. Mean sharing a common letter are not significantly different according to the Tukey test ($P > 0.05$).

Malondialdehyde (MDA) concentration significantly increased in plants exposed to HM stress (Fig. 3A). Salinity in the absence of HM had no impact on the leaf MDA content and it abolished to HM-induced increase in leaf MDA. Leaves of plants exposed to HM+NaCl treatment for 12 weeks even exhibited lower MDA concentration than control plants. Data for total antioxidant capacity are provided by Fig. 3B and 3C for the hydrophilic (AOAM) and hydrophobic (AOAD) fractions, respectively. After 8 weeks of treatment, AOAM was reduced in plants exposed to HM but remained unchanged in other treatments. After 12 weeks of treatment, the leaf AOAM fraction was slightly higher in plants exposed to HM+NaCl treatment. After 8 weeks, The AOAD fraction was significantly increased in plants exposed to NaCl, both in presence and absence of HM. After 12 weeks, the highest FRAP-AOAD values were recorded for plants exposed to HM+NaCl treatments.

After 8 weeks of treatment, reduced glutathione (GSH; Fig. 3D) dropped in leaves of plants exposed to NaCl while heavy metal treatment had no significant impact on this parameter. After 12 weeks of treatment no difference was recorded for GSH between treatments. Oxidized glutathione (GSSG; Fig. 3E) was significantly higher in HM-treated plants after 8 weeks and in NaCl-treated ones after 12 weeks. Heavy metal also increased non-protein thiols (NPT; Fig. 3F) and phytochelatin content (PC; Fig. 3G) after 8 weeks but no increase was recorded at this time for plants exposed to HM+NaCl treatment. After 12 weeks, however, a similar increase of NPT and PC was recorded in HM- and in HM+NaCl-treated plants.

3.2. Soil and leachate-related parameters

Samples from contaminated soils were collected at each harvest to assess the pollutant bioavailability. Data are presented in Table 2 for 4 and 12 weeks of treatment and also presents the data for soil issued from column maintained in the absence of plants. Addition of NaCl did not impact pH values after 4 weeks and the presence of plants had no effect on this parameter. The mean EC increased as a consequence

of salinization but remained the same in the presence and in the absence of plants. Pollutant bioavailability was the lowest for Pb and the highest for Cd. Salinity significantly reduced As bioavailability but increased Cd bioavailability. Cadmium bioavailability increased from 4 to 12 weeks of treatment and was still the highest in soil treated with NaCl in the presence of *K. pentacarpos*. Such an impact was however not recorded in the absence of *K. pentacarpos*. Zinc bioavailability also decreased in columns cultivated with *K. pentacarpos* after 12 weeks comparatively to 4 weeks.

Table 2. The pH, electrical conductivity (EC) and pollutant bioavailability in polluted soils cultivated in column with *Kosteletzkya pentacarpos* during 4 or 12 weeks. Contaminated soils were exposed or not to additional NaCl (6 g kg^{-1}). Soil issued for the same duration in a column free of plants was used as control. Biovailability was estimated through selective extraction with $CaCl_2$ 0.01 M and was expressed as a percentage of corresponding elements estimated by total analysis through acid attack (see material and Methods). For a given parameter and a given period of treatment, values followed by the same letters are not significantly different according to the Tukey test ($P > 0.05$).

	Column with plants		Column without plants	
	0 NaCl	+NaCl	0 NaCl	+NaCl
4 Weeks				
pH	7.07 a	7.12 a	7.24 a	6.99 a
EC (mS cm^{-1})	0.78 a	5.04 b	0.91 a	5.22 b
As (%)	0.37 c	0.21 a	0.29 b	0.23 a
Cd (%)	1.23 a	2.77 c	1.25 a	2.01 b
Pb (%)	0.002 a	0.003 a	0.002 a	0.005 a
Zn (%)	0.66 a	0.70 b	0.81 c	0.75 bc
12 Weeks				
pH	6.92 a	7.0 a	6.98 a	7.13 b
EC (mS cm^{-1})	0.81 a	4.99 b	0.83 a	5.05 b
As (%)	0.30 a	0.28 a	0.34 ab	0.37 b
Cd (%)	2.89 b	3.26 c	2.74 b	2.53 a
Pb (%)	0.004 a	0.004 a	0.002 a	0.003 a
Zn (%)	0.34 a	0.39 a	0.78 b	0.83 b

Cumulative values for leakage volume are provided for the four treatments in Fig. 4A. It is interesting to notice that the total volume of leachate collected only slightly increased between week 8 and week 12. Nevertheless, the leakages were highly concentrated in pollutants comparatively to those collected at the beginning of the experiment: as a consequence, the total amount of pollutant recovered in the leachates mainly increased at the end of the experiment as indicated in Fig. 4B

for As, Fig. 4C for Cd and Fig. 4D for Zn. Considering its low mobility, only small amounts of Pb were quantified in the leachate and data are not presented. In all cases, the presence of NaCl strongly reduced leaching of pollutant, salt impact being especially important at the end of the experiment.

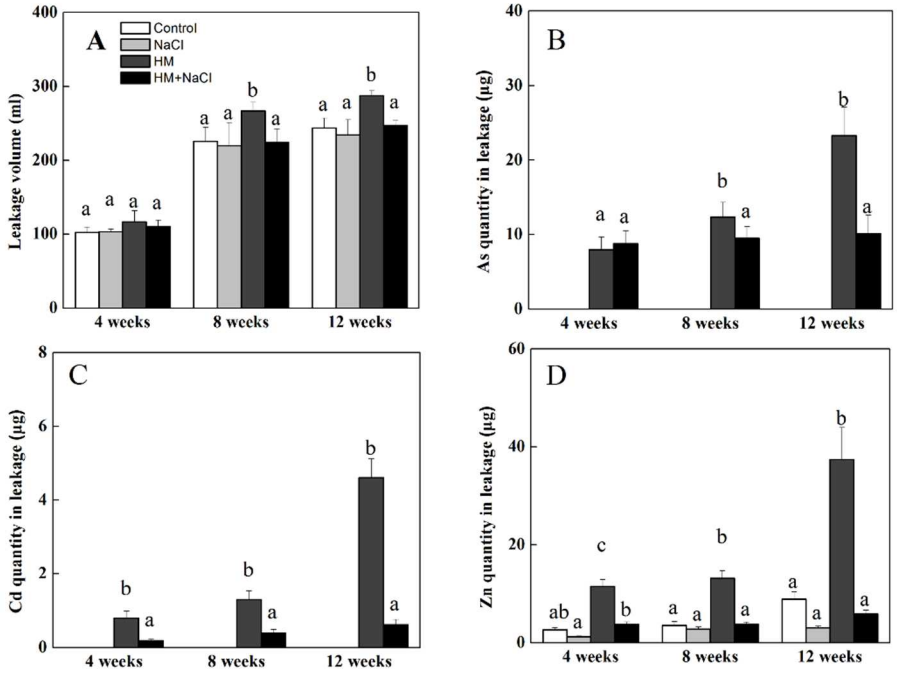

Fig. 4. Cumulative volume of leachate (A) and amounts of arsenic (B), cadmium (C) and zinc (D) recovered at the bottom parts of the columns after 4, 8 and 12 weeks of culture of *Kosteletzkya pentacarpos* cultivated on a non-polluted soil (control), the same soil added with NaCl (Na), with a mixture of heavy metals (HM) or with heavy metals and NaCl (MH+Na). Each value is the mean of 5 replicates and vertical bars are standard errors. Mean sharing a common letter are not significantly different according to the Tukey test ($P > 0.05$).

In column maintained in the absence of plants, leakage volume was 118 mL, 395 mL and 420 mL after 4, 8 and 12 weeks respectively. The analysis of leachate in terms of pollutant concentration allowed us to

compare pollutant leaching in the absence and in the presence of plants and to quantify for each element, the percentage of mobilized pollutant which accumulated in the plant shoot of *K. pentacarpos* (Table 3). In columns cultivated with *K. pentacarpos*, salinity reduced the total amount of As removed from the system, while a slight increase was recorded for Cd, Zn and Pb. From a relative point of view, removal of Pb was especially low. In the presence or in the absence of NaCl, most of the extracted As was removed by the leaching process (> 90%). The addition of NaCl however strongly increased the proportion of Cd and Zn taken up by the plants and drastically reduced the proportion of pollutant removed by the leachates. Only a slight proportion of removed Pb was obtained in the leachates. In the specific cases of columns maintained in the absence of plants, the total amounts of pollutants removed by the system were higher for all considered elements. Salinity has a minor impact on HM leaching, except for Cd which significantly increased. For columns maintained in the absence of plants, all removed pollutants were of course recovered in the leachates. Plants exposed to NaCl and to NaCl+HM removed 10.71 and 12.78 mg of Na in the shoot part (detailed data not shown) but for the two situations, Na content removed by leachate was close to 250 mg and constituted more than 95% of the removed Na.

Table 3. Total amounts (in µg) of As, Cd, Zn and Pb removed from the columns systems. Contaminated soils were cultivated or not by the halophyte plant species *Kosteletzkya pentacarpos* during 12 weeks while some column remained free of plants. Half of the columns were irrigated by NaCl solution. The relative proportion of the pollutant removal by the plants with subsequent storage in the shoot part on the one hand, and the proportion of pollutants removed by leaching on the other hand are given as percentages.

	Total amount removed (in µg)	% removed by plant	% removed by leaching
Plant + 0 NaCl			
As	24.16 ± 2.13	8.94	91.06
Cd	7.62 ± 0.84	44.88	55.12
Zn	117.4 ± 9.8	65.93	34.07
Pb	11.8 ± 1.4	94.07	5.93
Plant + NaCl			
As	12.17 ± 0.95	9.6	90.4
Cd	10.28 ± 1.02	95.14	4.86
Zn	126.7 ± 15.1	97.32	2.68
Pb	15.05 ± 2.31	95.35	4.65
No Plant + 0 NaCl			
As	35.91 ± 0.98	0	100
Cd	22.94 ± 2.06	0	100
Zn	164.95 ± 1.45	0	100
Pb	15.72 ± 1.13	0	100
No Plant + NaCl			
As	27.68 ± 2.03	0	100
Cd	33.56 ± 2.56	0	100
Zn	159.4 ± 13.2	0	100
Pb	18.37 ± 3.0	0	100

4. Discussion

Halophyte plant species have been recommended as promising material for phytoremediation of heavy-metal contaminated sites (Lutts and Lefèvre, 2015; Van Oosten and Magio, 2015). However, most studies dealing with heavy metal resistance in halophyte are performed in nutrient solution under hydroponic culture and do not consider the impact of plant on pollutant mobility and availability. The present study demonstrates that the use of the wetland halophyte plant species *Kosteletzkya pentacarpos* allows to extract toxic elements and to reduce pollutant leaching, and that the extent of these properties depends on the nature of the considered element and the presence of NaCl. Salinity indeed increased Cd bioavailability and decreased As bioavailability while it had a limited (Zn) or no (Pb) impacts on other elements bioavailability. Nevertheless, salinity increased the proportion of mobilized Cd, Zn and Pb removed by the plant and strongly decreased the proportion of those elements removed by leaching process. A salt-induced increase in Cd mobility has been reported in other studies and may lead to ground water contamination (Du Laing et al., 2008; Wahla and Kirkham, 2008; Zhao et al., 2013). In our experimental system, cultivating *K. pentacarpos* allows to reduce considerably from 33.56 to less than 1 µg the amounts of Cd removed by the leachate. A decrease was also recorded for Pb and Zn, although NaCl had no or very low impact on these elements bioavailability which suggest that NaCl was acting on the plant absorbing capacities rather than on element availability in the substrate.

The 0.01 M $CaCl_2$ extraction provided information about exchangeable metal pools and has also been used to study the impact of soil ageing on bioavailability (Schreck et al., 2011). The spiked soil used in this study was allowed to equilibrate for a limited duration and was therefore not fully relevant from the behavior of a soil contaminated since a long time. This could, at least partly, explain the high bioavailability recorded in this study (Lambrechts et al., 2011a,

2011b, Houben, 2013). The advantage of the system is that a control soil with similar physical properties may be used and allow comparison. In all treatments, *K. pentacarpos* reduced the volume of leachate comparatively to column maintained in the absence of plants. This contrast with the data obtained by Lambrechts et al. (2011b) who demonstrated that cultivating the grass species *Lolium perenne* on a contaminated soil increased percolation and lead to a higher leachate volume in relation to an increase in infiltration capacity caused by the root development. Those discrepancies may be related to the contrasting morphophysiological properties on the Malvaceae *K. pentacarpos* comparatively to the Gramineae *L. perenne*. Indeed, *K. pentacarpos* is a wetland species exhibiting a high transpiration rate (He et al., 2003; Qin et al. 2015), which implies an important flow of solution to the roots and to the shoot. The process should increase with the plant development in relation to an increase in the total leaf area, which may thus reduce the volume of leachate recovered from a fixed volume of soil, even if this soil is regularly moistened to 80 % of field capacity. In contrast to *L. perenne*, *K. pentacarpos* also develops a strong taproot system and a dense network of fibrous roots which facilitate nutrient absorption. Some previous reports clearly demonstrated that *K. pentacarpos* is not a hyperaccumulating plant and thus sequester high amounts of pollutant in the root system (Han et al., 2012a, 2012b).

Beside its quantitative importance, root system of *K. pentacarpos* also produces high amounts of mucilage that forms a gel-like structure (Ghanem et al., 2010). Pectin is a major constituent of mucilage and this ionic polysaccharide exhibits a high metal binding capacity. Lutts et al. (2016) demonstrated that salinity increased mucilage accumulation at the root surface and strongly increased Zn and, to a lesser extent, Cd binding capacities. Binding of pollutant on the root surface of salt-treated plants may thus explain a decrease of HM in the leachate. In the present study, however, amounts of As, Cd and Zn recovered in the leachate were especially important between 8 and 12 weeks for plants not exposed to NaCl, while during this period, the volume of leakage was rather low. A possible explanation could be

that after 8 weeks, binding sites of root mucilage were saturated on plants cultivated in the absence of salt but those plants still absorbed important volume of water for transpiration, the overall consequence being a low volume of percolates with a high concentration of pollutant. Since roots were not analyzed in the present study considering the difficulties to accurately separate roots from adhering soil particles, additional work is required to test this hypothesis.

In order to efficiently contribute to phytomanagement of polluted soils, *K. pentacarpos* needs also to display tolerance mechanisms at the shoot level. Heavy metals reduced stem and leaf dry weight, confirming that accumulated elements may, to some extent, affect plant growth. It is however noteworthy that Cd accumulation increased in response to NaCl (Fig. 2) but that NaCl paradoxically suppressed the heavy metal-induced growth inhibition. This suggests that NaCl may efficiently trigger heavy metal tolerance processes on this plant or that growth inhibition recorded in the present experiment may be due to other accumulated elements, such as As whose absorption was reduced by the presence of NaCl. The Cd concentration recorded in the present study was by far lower than values recorded for Cd accumulation in *K. pentacarpos* (syn. *K. virginica*) using nutrient solution in hydroponic culture. Zhou et al. (2018b) reported values close to 300 mg Kg^{-1} DW in plants exposed to 10 µM $CdCl_2$ while Han et al. (2013a) mentioned a leaf Cd concentration of 160 mg Kg^{-1} DW in plants exposed to 5 µM $CdCl_2$. Although growth was more drastically affected in those studies than in this one, most plants remained alive at these high doses and this leads us to consider that in the present study, Cd did not reach a lethal level. It is also interesting to notice that in nutrient solution, NaCl was reducing the Cd accumulation (Han et al., 2012b; chapter 1 and 2) while NaCl increased Cd accumulation the shoots of plants maintained on a contaminated solid substrate (see Fig. 2). It might be argued that in nutrient solution, formation of chlorocomplexes (especially $CdCl^{+}$) hampered Cd absorption while on a solid substrate, the recorded NaCl-induced increase in Cd solubility (see Table 2) may be sufficient to lead to an increased Cd accumulation. According to

Filipović et al. (2018), formation of $CdCl_n^{2-n}$ chlorocomplexes may also occur in the soil solution, but a higher shoot Cd concentration observed in our study suggests that for *K. pentacarpos*, an increase in Cd solubility compensates for a decrease in availability resulting from a modified speciation.

Zinc was also reported to accumulate in *K. pentacarpos* cultivated in nutrient solution under Zn excess (Han et al., 2012b, 2013b; chapter 1) and, as previously reported for Cd, Zn concentration in plants cultivated on polluted solid substrate (Fig. 2) was lower than values reported for hydroponic culture. Salinity had a limited impact on Zn bioavailability and had no influence on Zn accumulation by the shoots. Moreover, bioavailability decreased with the duration of the experiment which could partly explain the recorded decrease in stem Zn content (Fig. 2G). Stem was reported to act as an accumulating organ, especially for those elements bound to organic acids (Lutts et al., 2004).

To the best of our knowledge, As accumulation in *K. pentacarpos* has never been considered until now. The present study demonstrates i) that NaCl decreased As bioavailability on a short term basis ii) that NaCl reduced As accumulation in the shoot iii) that NaCl reduced the proportion of As removed by the plant and iv) that the amount of As removed from the columns decreased with NaCl. Vromman et al. (2016) reported that salinity decreased As absorption in the roots of the halophyte plant species *Atriplex atacamensis* but that it also increased As translocation from the roots to the shoots in this species. In the present study, As was the only element whose mobility was reduced by NaCl both in the absence and in the presence of NaCl (Table 3). This specific behavior might be related to the fact that it is present in the soil mainly as anionic form (arsenate (As(V)) in aerobic conditions and arsenite (As (III)) in anaerobic soils). Arsenate is absorbed through phosphate transporters and a decrease in P induced by HM treatment was recorded in the present study, although the concentration of P was 1000 order of magnitude higher than the

endogenous accumulated As which hardly explain P decrease as a result of direct As competition for P transporters.

Lead was the less mobile element in our experimental conditions and salinity had no impact on Pb bioavailability, as previously reported by Zhao et al. (2013) for multicontaminated estuaries sediments. Despite its low mobility, the major portion of removed lead was absorbed by the plants and Pb content in plant tissues was higher than in the leachates. Li et al. (2019) recently confirmed that in the halophyte *Suaeda salsa*, salinity reduces phytotoxicity induced by low doses of Pb but, according to these authors, the involved process may be related to an hormesis effect leading to Pb dilution occurring as a consequence of NaCl-induced growth stimulation. In our experiment, 50 mM NaCl however did not stimulate plant growth in *K. pentacarpos.*

Remediation of multiple heavy metal-contaminated soils remains difficult and it is thus interesting to pay attention to physiological properties which are reported to be affected in a similar way by various pollutants, as it is the case for management of plant antioxidative status. The present study shows that *K. pentacarpos* exposed to a polycontaminated substrate encountered oxidative stress (as indicated by an increase in MDA content) but salinity completely abolished this oxidative stress (Fig. 3A). After 12 weeks, MDA content was even lower in HM+Na-treated plants than in controls. This reinforces the hypothesis that salinity helped the plants to cope with heavy metals even if NaCl had no effect (Zn and Pb) or even increased (Cd) toxic element accumulation in the shoot part. MDA accumulation after 8 weeks in HM-treated plants may be correlated with a global decrease in total antioxidant activity since both AOAM (Fig. 3B) and AOAD (Fig. 3C) fractions decreased. This, however, was not observed after 12 weeks. Since HM concentration did not strongly vary between 8 and 12 weeks, this suggests that the physiological consequences of toxic element accumulation and the strategy adopted by the plant to cope with them vary not only with endogenous stress intensity, but also with the duration of the stress

exposure, as reported elsewhere (Quinet et al., 2012; Vromman et al., 2016).

Glutathione assumes dual functions in HM-stressed plants: it first acts as a major antioxidant but it is also the precursor of phytochelatins involved in HM complexation and vacuolar sequestration (Yadav, 2010). As a major antioxidant, GSH should be recovered in the AOAM fraction and it is therefore puzzling that salinity reduced GSH after 8 weeks with no impact on the total AOAM antioxidant activity. A similar trend was however reported at each repetition of the experiment, and this suggests that other antioxidant such as ascorbate may compensate the GSH decrease (Han et al., 2013a). Similarly, a salt-induced increase of the AOAM fraction may be related to lipophilic antioxidant such as α-tocopherol which was already showed to play an important function in *K. pentacarpos* exposed to Cd (Han et al., 2013a). Phytochelatins are known to efficiently bind Cd and As (Yadav, 2010) but in the present study, the NaCl-induced increase in Cd accumulation did not lead to higher PC content in HM+Na-treated plants, and PC content was even lower after 8 weeks than in plants exposed to heavy metals in the absence of NaCl (Fig. 3G), suggesting that other strategies may be adopted by the plant to cope with a higher Cd content. Cell wall sequestration may be an alternative strategy to maintain Cd away from the cytosolic metabolic activities but in chapter 1, it was recently reported that in *K. pentacarpos*, NaCl reduces the proportion of Cd bound to the cell wall and increased the proportion of intracellular Cd.

5. Conclusions

Kosteletzkya pentacarpos appears as a suitable halophyte plant species for phytoremediation of polycontaminated salt-affected soils. It drastically reduced Pb, Cd, Zn and As percolation but also displayed tolerance mechanisms to accumulated pollutants. Salinity increased the proportion of Cd and Zn removed by the plants, improved plant tolerance to heavy metals in *K. pentacarpos* and reduced heavy metal

percolation in polluted soils cultivated with this species. Additional work is however required to precisely identify the physiological properties involved in heavy metal resistance in *K. pentacarpos*.

Reference

Acosta, J.A., Jansen, B., Kalbitz, K., Faz, A., Martínez-Martínez, S., 2011. Salinity increases mobility of heavy metals in soils. Chemosphere 85, 1318-1324.

Bai, J., Zhao, Q., Wang, W., Wang, X., Jia, J., Cui, B., Liu, X., 2019. Arsenic and heavy metals pollution along a salinity gradient in drained coastal wetland soils: depth distributions, sources and toxic risks. Ecol. Ind. 96, 91-98.

Benzie, I.F., Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239, 70-76.

Cereser, C., Guichard, J., Drai, J., Bannier, E., Garcia, I., Boget, S., Parvaz, P., Revol, A. 2001. Quantitation of reduced and total glutathione at the fentomole level by high-performance liquid chromatography with fluorescence detection: application to red blood cells and cultured fibroblasts J Chrom B: Biomed Sci Appl 752, 123-132

Cheng, H., Wang, Y.S., Ye, Z.H., Chen, D.T., Wang, Y.T., Peng, Y.L., Wang, L.Y., 2012. Influence of N deficiency and salinity on meral (Pb, Zn, and Cu) accumulation and tolerance by Rhizophora stylosa in relation to root anatomy and permeability. Environ. Poll. 164, 110-117.

Cheng, M., Kopittke, P.M., Wang, A., Sale, P.W.G., Tang, C., 2018a. Cadmium reduces zinc uptake but enhances its translocation in the cadmium-accumulator, *Carpobrotus rossii* without affecting speciation. Plant Soil 430, 219-231.

Cheng, M., Wang, A., Liu, Z., Gendall, A.R., Rochfort, S., Tang, C. 2018b. Sodium chloride decreases cadmium accumulation and changes the response of metabolites to cadmium stress in the halophyte *Carpobrotus rossii*. Ann. Bot. 122, 373-385.

Cheng, M., Kopittke, P.M., Wang, A., Tang, C., 2019. Salinity decreases Cd translocation by altering Cd speciation in the halophytic Cd-accumulator *Carpobrotus rossii*. Ann. Bot. 123, 121-132.

Clemente, R., Walker, D.J., Pardo, T., Martínez-Fernández, D., Bernal, P.L., 2012. The use of halophytic plant species and organic amendments for the remediation of a trace elements-contaminated soil under semi-arid conditions. J. Hazard. Mat. 223/224, 63-71.

Degryse, F., Broos, K., Smolders, E., Merckx, R., 2003. Soil solution concentration of Cd and Zn can be predicted with $CaCl_2$ soil extract. Eur. J. Soil Sci. 54, 149-157.

De Vos, C.R., Vonk, M.J., Vooijs, R., Schat, H. 1992. Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus* Plant Physiol 98, 853-858.

Dufey, I., Hakizimana, P., Draye, X., Lutts, S., Bertin P., 2009. QTL mapping for biomass and physiological parameters linked to resistance mechanisms to ferrous iron toxicity in rice. Euphytica 167, 143-160.

Du Laing, G., De Vos, R., Vandecasteele, B., Lesage, E., Tack, F.M.G., Verloo, M.G., 2008. Effect of salinity on heavy metal mobility and availability in intertidial sediments of the Scheldt estuary. Est. Coast. Shelf Sci. 77, 589-602.

Filipović, L., Romić, M., Romić, D., Filipović, V., Ondraš ek, G. 2018. Organic matter and salinity modify cadmium soil (phyto)availability. Ecotox. Environ. Saf. 147, 824-831.

Ghanem, M.E., Han, R.M., Classen, B., Leclerq, J., Mahy, G., Ruan. C.J., Pérez-Alfocea, F., Lutts, S. 2010. Mucilages and polysaccharides in the halophyte plant species *Kosteletzkya virginica* (L.) Presl.: localization and composition in relation to salt-induced physiological modifications. J. Plant Physiol. 167, 382-392

Han, R.M., Lefevre, I., Albacete, A., Pérez-Alfocea, F., Barba-Espín, G., Díaz-Vivancos, P., Quinet, M., Ryan, C.J., Hernández, J.A., Cantero-Navarro, E., Lutts, S., 2013a. Antioxidant enzyme activities and hormonal status in response to Cd in the wetland halophyte *Kosteletzkya virginica* (L.) Presl. under saline conditions. Physiol. Plant. 147, 352-368.

Han, R.M., Lefevre, I., Ruan, C.J., Beukelaers, N., Qin, P., Lutts, S., 2012a. Effects of salinity on the response of the wetland halophyte *Kosteletzkya virginica* (L.) Presl. to copper toxicity. Water, Air, Soil Pollut. 223, 1137-1150.

Han, R.M., Lefèvre, I., Ruan, C.J., Qin, P., Lutts, S., 2012b. NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regul 68, 97-109.

Han, R.M., Quinet, M., André, E., Teun van Elteren, J., Destrebecq, F., Vogel-Mikuš, K., Cui, G., Debeljak, M., Lefèvre, I., Lutts, S., 2013b. Accumulation and distribution of Zn in the shoots and reproductive

structures of the halophyte plant species *Kosteletzkya virginica* as a function of salinity. Planta 238, 441-457

He, Z.X., Ruan, C.J., Qin, P., Seliskar, D.M., Gallagher, J.L., 2003. *Kosteletzkya virginica*, a halophytic species with potential for agroecotechnology in Jiangsu provine, China. Ecol. Engin. 210, 271-276

Heath, R.L., Packer, L. 1968. Photoperoxidation in isolated chloroplasts. II. Role of electron transfer. Arch. Biochem. Biophys. 125, 850-857.

Houba, V.J.G., Temminghoff, E.J.M., Gaikhorst G.A., van Wark, W., 2000. Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. Commun. Soil Sci. Plant Anal. 31, 1299-1396.

Houben, D. 2013. Heavy Metal Mobolity in Contaminated Soils as Affected by Plants, Amendments and Biochar: Implications for Phytostabilization. PhD Thesis, Université caatholique de Louvain, Louvain-la-Neuve, Belgium, 286 p.

Lambrechts, T., Couder, E., Bernal, P., Faz, A., Iserentant, A., Lutts, S. 2011a. Assessment of heavy metal bioavailability in contaminated soils from a former mining area (La Union, Spain) using a rhizospheric test. Water Air and Soil Poll. 217, 333-346

Lambrechts, T., Gusto, Q., Couder, E., Houben, D., Iserentant, A., Lutts, S. 2011b. Comparison of EDTA-enhanced phytoextraction and phytostabilisation strategies with *Lolium perenne* on a heavy metal contaminated soil. Chemosphere 85, 1290-1298.

Lefèvre, I., Marchal, G., Meerts, P., Corréal, E., Lutts, S. 2009. Chloride salinity reduces cadmium accumulation by the Mediterranean halophyte species *Atriplex halimus* L. Environ Exp Bot 65, 142-152.

Lefèvre, I., Vogel-Mikuš, K., Jeromel, J., Vavpetič, P., Planchon, S., Arčon, I., van Elteren, J., Lepoint, G., Gobert, S., Renaut, J., Pelicon, P., Lutts, S. 2014. Cadmium and zinc distribution in relation to their physiological impact in the leaves of the accumulating *Zygophyllum fabago* L. Plant Cell Environ 37, 1299-1320.

Li, X., Zhang, X., Wang, X., Yang, X., Cui, Z., 2019. Bioaugmentation-assisted phytoremediation of lead and salinity co-contaminated soil by *Suaeda salsa* and *Trichoderma asperellum*. Chemosphere 224, 716-725.

Liu, J.L., Diao, Z.H., Xu, X.R., Xie, Q., 2019. Effects of dissolved oxygen, salinity, nitrogen and phosphorus on the release of heavy metals from coastal sediments. Sci. Total Environ. 666, 894-901.Lutts S., Lefèvre I.,

Delpérée C., Kivits S., Dechamps C., Robledo A. & E. Correal. 2004. Heavy metal accumulation by the halophyte species *Atriplex halimus*. J. Environ. Qual. 33, 1271-1279.

Lutts, S., Lefèvre, I. 2015. How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? Ann Bot 115, 509-528.

Lutts, S., Qin, P., Han, R., 2016. Salinity influences biosorption of heavy metals by the roots of the halophyte plant species *Kosteltzkya pentacarpos*. Ecol. Eng. 95, 682-689.

McLaughlin, M.J., Tiler K/G., Smart, M.K., 1997. Speciation of cadmium in soil solutions of saline/sodic soils and relationships with cadmium concentrations in potato tubers (*Solanum tuberosum* L.). Aust. J. Soil Res. 35, 1083-198.

Page, A.L., Miller, R.H., Keeney, D.R., 1982. Methods in Soil Analysis, Part 2 – Chemical and Microbiological Properties, 2nd Ed, American Society of Agronomy, Madison.

Qin, P., Han, R.M., Zhou, M.X., Zhang, H.S., Fan, L.S., Seliskar, D.M., Gallagher, J.L., 2015. Ecological engineering through the biosecure introduction of *Kosteletzkya virginica* (seashore mallow) to saline lands in China: a review of 20 years of activity. Ecol. Eng. 74, 174-186.

Quinet, M., Vromman, D., Clippe, A., Bertin, P., Lequeux, H., Dufey, I., Lutts, S., Lef è vre, I. 2012. Combined transcriptomic and physiological approaches reveal strong differences between short and long term response of rice (*Oryza sativa*) to iron toxicity. Plant Cell Environ. 35, 1839-1857.

Riaz, M., Yan, L., Wu, X., Hussain, S., Aziz, O., Jiang, C., 2018. Mechanisms of organic acids and boron induced tolerance of aluminium toxicity: a review. Ecotoxicol. Environ. Saf. 165, 25-35.

Schäfer, H.J., Greiner, S., Rausch, T., Haag-Kerwer, A. 1997. In seedlings of the heavy metal accumulator *Brassica juncea* Cu^{2+} differentially affects transcript amounts for γ-glutamylcysteine synthetase (γ-ECS) and metallothionein (MT2) FEBS lett 404:216-220.

Schreck, E., Foucault, Y., Geret, F., Pradere, P., Dumat, C., 2011. Influence of soil ageing on bioavailability and ecotoxicity of lead carried by process waste metallic ultrafine particles. Chemosphere 85, 1555-1562.

Singh, M., Singh, A., Prasad, S.M., Singh, R.K., 2017. Regulation of plants metabolism in response to salt stress: an omics approach. Acta Physiol. Plant. 39, 48.

Tandon, K., John, M., Heusse-Aβbchiler, Schaller, V., 2018. Influence of salinity and Pb on the precipitation of Zn in a model system. Minerals 8, 43.

Van Oosten, M.J., Maggio, A., 2015. Functional biology of halophytes in phytoremediation of heavy metal contaminated soils. Environ. Exp. Bot. 111, 135-146.

Vromman, D., Lefèvre, I., Šlejkovec, Z., Martínez, J.P., Vanhecke, N., Briceño, M., Kumar, M., Lutts S. 2016. Salinity influences arsenic resistance in the xerohalophyte *Atriplex atacamensis Phil*. Environ. Exp. Bot. 126, 32-43.

Wahla, I.H., Kirkhgam, M.B., 2008. Heavy metal displacement in salt-water-irrigated soil during phytoremediation. Environ. Poll. 155, 271-283.Wang, S.L., Xu, X.R., Sun, Y.X., Liu, J.L., Li, H.B., 2013. Heavy metal pollution in coastal areas of South China: A review. Marine Poll. Bull. 76, 7-15

Wen, X., Lu, J., Wu, J., Lin, Y., Luo, Y., 2019. Influence of coastal groundwater salinization on the distribution and risks of heavy metals. Sci. Total Environ. 652, 267-277.

Yadav, S.K., 2010. Heavy metals toxicity in plants: an overview on the role of glutathione and phyytochelatins in heavy metal stress tolerance of plants. South Afr. J. Bot. 76, 167-179.

Yang, Q., Li, Z., Lu, X., Duan, Q., Huang, L., Bi, J. 2018. A review of soil heavy metal pollution from industrial and agricultural regions in China: Pollution and risk assessment. Sci. Total Environ. 642:690-700.

Zhao, S., Feng, C., Wang, D., Liu, Y., Shen, Z., 2013. Salinity increases the mobility of Cd, Cu, Mn, and Pb in the sediments of Yangtze Estuary: relative role of sediments properties and metal speciation. Chemosphere 91, 977-984.

Interchapter 6-7

What do we know?

- Pollutants induce a decrease in shoot dry weight and an increase in MDA. Both symptoms were alleviated by the additional presence of NaCl but this positive impact is not related to any increase in protecting phytochelatins synthesis.
- Salinity increases Cd bioavailability in the soil and Cd accumulation in the shoots while it has an opposite effect on As accumulation. Salinity did not modify Pb and Zn bioavailability and accumulation.
- Cultivating plants on the polluted soil drastically reduce the volume of leachate. In all cases, salinity reduces the total amounts of heavy metals removed by the leachate and significantly decreases the proportion of Cd and Zn removed by the plants.

Salinity decreases heavy metal percolation, especially in soils cultivated with *K. pentacarpos*. Salinity also improves plant tolerance to heavy metals in *K. pentacarpos* and this species thus appears as promising plant material for phytoremediation of polycontaminated soils.

What are the questions?

- All the works performed until now mainly consider the vegetative stage of the plant and duration of stress remain limited to a few days (Chapter 4) to a few weeks (Chapter 6). However, plants susceptible to be involved in phytoremediation strategy must be able to cope with heavy

metal stress at all developmental stage, including the reproductive stage.

- A previous work (Han et al., 2013; Planta 238:441-457) demonstrated that Zn-treated plants prevent Zn accumulation in the seeds and that NaCl may modify Zn distribution in the reproductive organs. This work, however, did not consider mixed pollution and was performed using a nutrient film technic and not on solid substrate.
- The use of chelating agent may help the plant to absorb pollutants through a direct increase in their bioavailability. Ethylenediamine-*N*,*N*′-disuccinic acid (EDDS) is an environment-friendly chelate that has never been previously tested on *K. pentacarpos*. The effect of salt on EDDS-induced phytoextraction remains unknown.

What is our strategy?

Agricultural soil will be artificially contaminated with As, Cd, Pb and Zn, treated with moderate does of salinity and EDDS. The considered morphological parameters will be recorded every two weeks from March to September. The number of flower as well as fruit will be counted every 10 day after their appearance of the first flower and fruit appear respectively.

In addition, the effect of NaCl and EDDS on heavy metal accumulation in *K. pentacarpos* as well as pollutants bioavailability in soil will also be investigated.

CHAPTER 7 Effect of NaCl and EDDS on heavy metal accumulation in *Kosteletzkya pentacarpos* in polymetallic polluted soil

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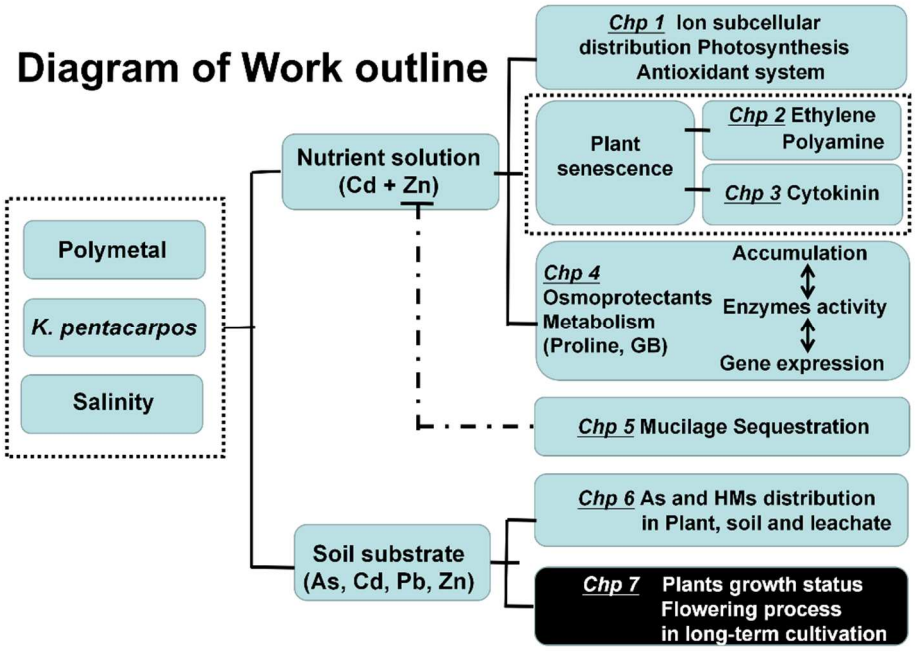
Diagram of Work outline
Chp 1 Ion subcellular distribution Photosynthesis Antioxidant system
Plant senescence
Chp 2 Ethylene Polyamine
Chp 3 Cytokinin
Nutrient solution (Cd + Zn)
Polymetal
K. pentacarpos
Salinity
Chp 4 Osmoprotectants Metabolism (Proline, GB)
Accumulation
Enzymes activity
Gene expression
Chp 5 Mucilage Sequestration
Chp 6 As and HMs distribution in Plant, soil and leachate
Soil substrate (As, Cd, Pb, Zn)
Chp 7 Plants growth status Flowering process in long-term cultivation

Chapter 7

ABSTRACT

The capability of heavy metal bioaccumulation in plant is one of the most important parameters for phytoremediation. In the present study, the effect of NaCl and S, S-ethylenediaminesuccinic acid (EDDS) on heavy metal accumulation in *Kosteletzkya pentacarpos* in polymetallic (arsenic, cadmium, lead and zinc) polluted soil was investigated. The addition of NaCl decreased the bioavailability of As and Cd, while EDDS increased it in As and Zn in soil. Polymetallic toxicity inhibited plant growth and reproduction, while NaCl and EDDS had no obvious effect. In addition to As, NaCl reduced accumulation of all heavy metal in root. In contrast, EDDS enhanced the accumulation of all heavy metals. NaCl reduced As accumulation in both main stem (MS) and lateral branch (LB), associated with decrease of Cd in leaf of main stem (LMS) and Zn in leaf of lateral branch. Conversely, EDDS increased the accumulation of all four heavy metals in LB associated with increase of As and Cd in LMS and LLB. Salinity significantly decreased heavy metal bioaccumulation factor (BF) of all four heavy metals, while EDDS significantly increased it. In terms of translocation factor (TF_c), NaCl had different influence on heavy metal, which is increased in Cd and decreased in As and Pb in the presence or absence of EDDS. EDDS reduced all heavy metals in addition to Zn in the presence of NaCl in polluted soil. Polymetallic pollutant also made a modification of the constituent of cell wall. NaCl increased the cellulose content in MS and LB, with little impact by EDDS. It is concluded that salinity and EDDS have different effects on heavy metal bioaccumulation in *Kosteletzkya pentacarpos* and this species could be a potential candidate for phytoremediaton in saline.

Keywords : *K. pentacarpos*, polymetal, accumulation, phytoremediation

1. Introduction

Approximately 830 million hectares of land world, accounting for more than 10% of the world's total land area, are affected by salinity. In the world, coastal land is one of the most important part of salt-affected areas. As developed areas during the past decades, the economic growth has resulted in the excessive release of heavy metals to the coastal waters and land, which caused severe heavy metal contamination in these soils (Arfaeinia et al. 2016). The heavy metal toxicity greatly impairs plant growth and development and thus induces reduction in biomass accumulation and grain yield of cultivated crops. Furthermore, heavy metal uptake by crops is especially risky due to their toxicity to human being (Bhat et al. 2018, Zhang et al. 2018).

One of the promising methods to restore heavy metal contaminated soils is phytoremediation. However, the heavy metal bioavailability in soil is frequently quite low. Chelating agents such as ethylene diamine tetraacetic acid (EDTA) may be used to increase heavy metal bioavailability in soil (Eshraghi &Nezamzadeh-Ejhieh 2018). The principle is that EDTA forms complexes with the heavy metal, which allows their detachment from soil particles and subsequent absorption by plant roots. However, EDTA is a persistent agent because it resists to degradation by micro-organisms. Moreover, because of the high mobility of EDTA-heavy metal complexes, there is a risk of leaching and groundwater contamination if the plant is not able to absorb sufficient amounts of solubilized heavy metals. This substance may also cause damage to soil bacteria and fungi, as well as to the plant itself. To avoid these major drawbacks, another chelatant has been proposed for the remediation of polluted soils, namely S, S-ethylenediaminesuccinic acid (EDDS). Unlike EDTA, this substance is biodegradable within 7-32 days and it appears to be quite less damaging to the environment as the risk of leaching heavy metal is reduced (Yoo et al. 2018).

Phytoremediation of heavy metal-contaminated saline lands,

especially coastal saline areas, requires the use of halophyte plant species. *Kosteletzkya pentacarpos* (L.) Presl. (formerly designed as *Kosteletzkya virginica*) is a perennial dicot halophyte species of Malvaceae family, native to the brackish marshes of mid-Atlantic and southeastern United States. It has been recommended as a potential resource for food, feed, biodiesel as well as health care (Halchak et al. 2011; Qin et al. 2015; Vaughn et al. 2013). *Kosteletzkya pentacarpos* is able to cope with a high level of salinity in its natural environment (up to 420 mM NaCl), exhibiting a high selectivity for K over Na (Blits and Gallagher 1990).

Kosteletzkya pentacarpos is also, to some extent, able to cope with heavy metal pollution in salt marsh conditions and it could therefore be recommended as an interesting tool for phytomanagement of heavy metal polluted coastal areas (Han et al. 2012). Han et al. (2013a) demonstrated that NaCl differently interfered with Cd and Zn toxicities in this wetland species. Cadmium increased the leaf K concentration while Zn had an opposite effect. Salinity reduced both Cd and Zn accumulation. Distribution of heavy metals among plant organs also appeared differently affected by salinity since Cd was reduced mainly in the leaves while Zn was reduced in the roots. Management of heavy metal oxidative stress by *K. pentacarpos* appeared as a crucial component of resistance to Cd (Han et al. 2013b) or Zn (Han et al. 2013a). However, there are three limitation in these experiments : 1) These data were obtained for plants exposed to one single pollutant (Cd, Zn and Cu) while this species in field conditions are frequently exposed simultaneously to several pollutants 2) Most experiments are tested in hydroponic culture using nutrient solutions which is far from real conditions and does not consider the impact of heavy metal bioavailability 3) all of the experiments are in short-term and there is no information reflecting the response of *Kosteletzkya pentacarpos* to combination of heavy metal toxicity in long-term in relation to the whole life span.

In present study, we made an artificial contaminated (spiked) soil with moderate concentration of four heavy metals/metalloids reflecting

high level of pollution encountered by the plants in contaminated salt marshes (arsenic (As), cadmium (Cd), lead (Pd) and zinc (Zn) respectively). In addition, NaCl and EDDS (S, S-ethylene diamine succinic acid) were provided to the soil. Plants were cultivated in contaminated soil for nearly half a year until they produce fruit. The purpose of the experiment is to compare the behavior of plants growing on polluted soil and those maintained on unpolluted soil as well as to specify the impact of salinity and / or EDDS on this response.

2. Materials and methods

2.1. Culture condition and plant material

The soil used for the experiments has a silty texture and came from an agricultural plot located in Louvain-la-Neuve (Avenue Baudouin I). An aqueous solution containing 4 heavy metals/metalloids was sprayed on a thin layer of soil on a total amount of 700 kg soil (Fig 7.1 left). The level of pollution reached was 155.5, 6.5, 312.5 and 187.5 mg / kg for As, Cd, Zn and Pb, respectively. The salts used to prepare the solution consist in As_2O_3, $CdCl_2$ 2 ½ $2H_2O$, $PbCl_2$ and $ZnCl_2$.



Fig 1. A quantity of 300 kg soil is humidified homogeneously by polymetallic solution (left). *Kosteletzkya pentacarpos* seedlings were transferred to big columns (right).

Each column (50 cm depth) received 20 kg soil with pH 8.20 ± 0.01 and the average organic matter 13.96 g / kg soil. Eight treatments were then considered with 3 columns per treatment, as detailed in Table 7.1. For salt treatments, 60 g NaCl (3 g / kg soil) were added while for EDDS treatment, 16 g of EDDS molecule (0.8 g / kg of soil) was used. All chemicals were purchased for SigmaChemical Belgium.

Table 1 Code and treatment of the experiment on *Kosteletzkya pentacarpos* subjected to pollution in heavy metal and the presence and absence of EDDS or / and NaCl.

Code	Treatment
SNPK	No-polluted soil + plant
SNPKA	No-polluted soil + plant + NaCl
SNPKE	No-polluted soil + plant + EDDS
SNPKEA	No-polluted soil + plant + NaCl + EDDS
SPK	Polluted soil + plant
SPKA	Polluted soil + plant + NaCl
SPKE	Polluted soil + plant + EDDS
SPKEA	Polluted soil + plant + NaCl + EDDS

Germination of *Kosteletzkya pentacarpos* was performed in trays filled with a perlite and vermiculite mix (1:3 v/v) and moistened regularly with water from 20th February 2017. Seedlings were grown in a phytotron under a 12h photoperiod. Eighteen days after sowing, 5 seedlings were transferred in each column (50 cm high and 24 cm in diameter, Fig 1 right) into a greenhouse in 10th March 2017. Natural light was supplemented by Philips lamps. After five months (first September 2017) all plants were harvested. The main stem and lateral branches were separately collected as well as leaves on main stem and on lateral branches.



Fig 7. 2 The cultivation of *Kosteletzkya pentacarpos* in big columns after three months

2.2. Growth and reproduction assessment

Stem height, number of lateral branches (LBs), leaf number on the main stem (LMS), number of leaves on LBs (LLB) were recorded every two weeks (totally 9 times during g the whole experiment) from 25th March, 2017 until the end of the study. The first flower and fruit appeared in 5th June and 21th June, respectively, therefore, from that time, the number of flower bud (NF) (Fig 3) as well as fruit (NFR) were counted every 10 day.



Fig 3 The flowers of *Kosteletzkya pentacarpos* which grew in SNPK treatment (left) and SPK (right) after 5 months.

Plants were then harvested, separately 7 plants were selected randomly, and leaves in main stem and lateral branches in these 7 plants were immediately frozen in liquid nitrogen. Fresh material of main stem, lateral branches, leaves on main stem and leaves on lateral branches from the remaining 8 plants were weighed then incubated in an oven for 72h at 70 °C for dry weight determination. Meanwhile, the soil in the upper, middle and bottom level was collected and pooled from each column of a same treatment.

2.3. Evaluation of ion concentration in plants

Dried samples were ground to a fine powder using a porcelain mortar and a pestle, digested in 35% HNO_3 and evaporated to dryness on a sand bath at 80 °C. The minerals were incubated with a mix of 37% HCl and 68% HNO_3 (3:1) and the mixture was slightly evaporated. Minerals were finally dissolved in HCl 0.1N. Ion concentrations were

determined by SOLAAR S4 atomic absorption spectrometry (Thermo Scientific, Cambridge, UK).

Translocation factor was estimated on the basis of ion concentration (TF_c) according to:

TF_c Concentration in the shoot ($mg.g^{-1}$ DW) / Concentration in the roots ($mg.g^{-1}$ DW)

The bioaccumulation factor (BF) calculation is according to:

BF [Shoot concentration of heavy metal (mg g^{-1} DW) / concentration in soil ($mg.g^{-1}$ soil)] * 100

2.4. Ion concentration and bioavailability in soil

Approximately 0.3 g of mixed soil (collected from 5 cm and 20 cm depth) sample was weighed and digested with 10 mL of HCl on an electric hot plate at 190 °C until the solution was reduced to 3 mL. Then, 5 mL of HF (40%, w/w), 5 mL of HNO_3 (63%, w/w), and 3 mL of HClO4 (70%, w/w) were added and the solution was digested until no black material remained. The digestion was continued further with 3 mL of HNO_3, 3 mL of HF, and 1 mL of $HClO_4$ until the silicate minerals had completely disappeared. Finally, the digestion solution was transferred to a 25 mL volumetric polypropylene tube, and 1% HNO_3 was added to bring the sample up to a fixed volume for the metal determinations. After filtering the digested samples through a syringe filter (0.45 μm), the concentrations of heavy metals were measured by using inductively coupled plasma mass spectrometry (ICP-MS, Thermo Electron Corporation).

For heavy metal bioavailability analysis in soil, sample was extracted by $CaCl_2$ solution: 1 g dry soil was weighed into 15 falcon tubes and 10 ml 0.01M $CaCl_2$ was added to each sample. After 24h of incubation at room temperature, samples were centrifuged in 20 min in 3,000 *g*. The filtered supernatant was added to 20 μl HNO_3. The concentrations

of bioavailable heavy metals were measured by using inductively coupled plasma mass spectrometry (ICP-MS, Thermo Electron Corporation).

Heavy metal bioavailability concentration of bioavailable heavy metal (mg kg^{-1} soil) / total concentration of heavy metal (mg kg^{-1} soil)

2.5. Structural polysaccharides analysis by the Van Soest method

The structural polysaccharides were determined by the detergent fiber assay which is based on the Van Soest (VST) method (Van Soest and Wine 1967; Van Soest 1973). Briefly, the neutral detergent fiber residue (NDF) was determined by the use of extraction 1: 0.1 mmol/L phosphate buffer at pH 7 for 15 min at 90℃and the addition of an analytical thermostable α-amylase for samples which contained starch; and extraction 2: Van Soest neutral detergent at 100℃ for 1 h and the addition of sodium sulfite. The acid detergent fiber residue (ADF) was determined by two successive extractions. The first extraction was performed with the Van Soest neutral detergent, as described above, without the addition of sodium to the Van Soest neutral detergent. Then, it is followed by the extraction with the Van Soest acid detergent at 100℃ for 1 h. The acid detergent lignin residue (ADL) was determined from the acid detergent fiber residue by extracting it by sulfuric acid 12.2 mol/L at room temperature for 3 h. Sodium sulfite was added to the Van Soest neutral detergent extraction and was not added to the Van Soest neutral detergent extraction prior to the ADF extractions because it is a standard recommendation. The cellulose VST, hemicelluloses VST and lignin VST contents are calculated as difference between ADF and ADL, difference between NDF and ADF, and ADL, respectively and are expressed in g.100 g^{-1} OM (organic matter).

2.6. Statistical analysis

Parts of tissue materials were dried in 70 ℃ oven, which were used to analyze for growth parameters, ionic determination and structural

polysaccharides analysis. Data obtained were subjected to an analysis of variance, one - way ANOVA and two - way ANOVA (treatment and duration of stress as level of classification), using SPSS software. The statistical significance of the results was analyzed by Student-Newman-Keuls test at 5% level ($P < 0.05$).

3. Result and discussion

3.1. Plant growth and reproduction

All tested plants remained alive until the end of treatment. Because the root of each plant was entwined together and fibrous roots were quite developed after 16 weeks cultivation, it is impossible to get accurate results of root dry weight. In this case, the dry weight of plants upper parts, stem (main stem and lateral branch) as well as leaf (leaf on main stem and on lateral branch) were presented in figure 4. Except SPKEA treatment, the MS dry weight and LB dry weight of plants growing in polluted soil significantly decreased ($P < 0.05$, Fig. 4 a and b), compared to plants growing in non-polluted soil. The LB dry weight of plants in SPK treatment sharply decreased by 86%. However, polymetallic treatment seemed to have no significant impact on LMS dry weight ($P > 0.05$), except for SPKEA treatment (Fig. 4 c). In contrast, polymetallic significantly decrease the LLB dry weight in the presence and absence of NaCl or/and EDDS ($P < 0.05$, Fig. 4 d). On the other hand, NaCl and EDDS had slightly positive impact on stem and leaf dry weight, suggesting partial polymetallic toxicity alleviation.

In a global view, in figure 4, it is indicated that heavy metal toxicity has more negative impact on the shoot ramification (the branches and leaves in branches development) than the main stem development in *K. pentacarpos.* In Han's study (2012), heavy metal such as cadmium indeed strongly inhibited axillary bud development. Besides, the addition of salinity or/and EDDS also had obviously positive effect on the axillary bud development, especially in the EDDS treatment in the

present or absence of the salinity. It is suggested that EDDS significantly promoted sunflower growth, resulting from Zn and Pb uptake reduction (Fässler et al. 2010).

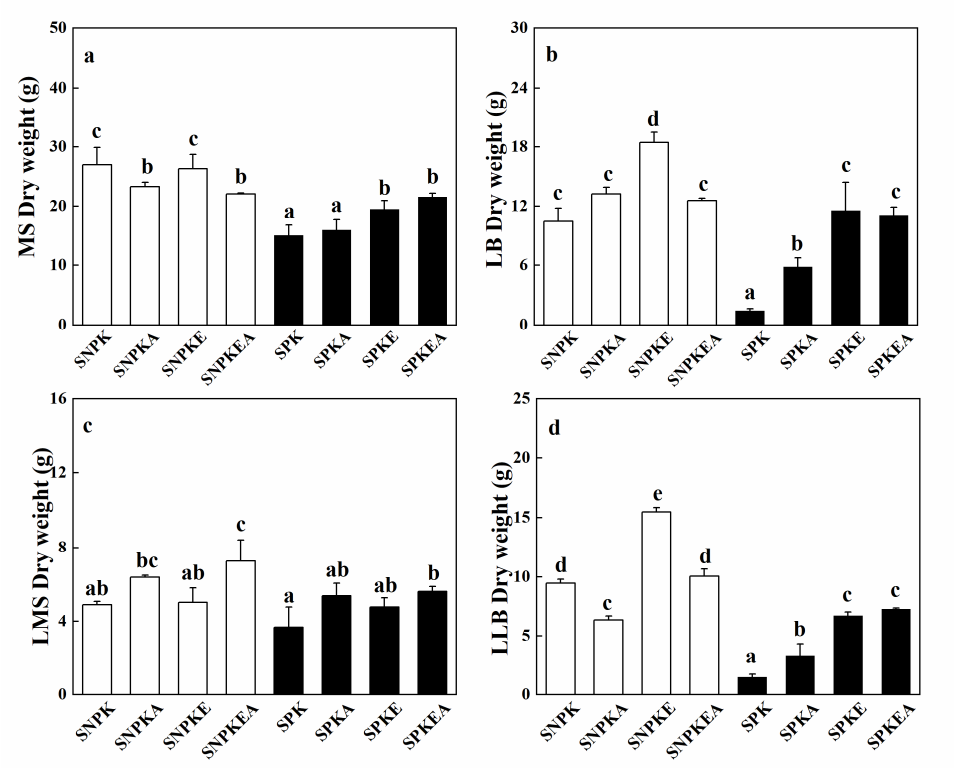

Fig 4 Dry weight of MS (main stem, a), LB (lateral branch, b), LMS (leaf on main stem, c) and LLB (leaf on lateral branch, d) in *Kosteletzkya pentacarpos* cultivated in non-polluted or polluted soil during five months in the presence or absence of NaCl or / and EDDS. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

Morphological parameters were measured when plants were transferred to columns and included stem height, number of lateral branches, number of leaves on the main stem (LMS), number of leaf on lateral branches (LLB), total number of flower as well as total number of fruit (Fig. 5). The addition of NaCl or/and EDDS had no effect on morphology of plant in the presence or absence of heavy

metal. After 16 weeks growth in soil, the stem elongation of *K. pentacarpos* growing in column with heavy metal polluted treatment in the presence and absence of NaCl or/and EDDS was slightly but significantly inhibited ($P < 0.05$). The number of LN had exhibited a similar tendency as a consequence of heavy metal toxicities (Fig. 5 b). After 8 weeks, the number of LMS and number of LLN were significantly different between non-polluted and polluted treatments (Fig. 5 c and d). At 16 weeks, the number of LMS and LLB of plants growing in polluted soil significantly decreased ($P < 0.05$), especially number of LLN, which decreased by 68%. In the present study, a drastic effect of polymetals on lateral branches growth, especially in the number of LLN was observed, which demonstrated once again that heavy metals deeply affect lateral ramification.

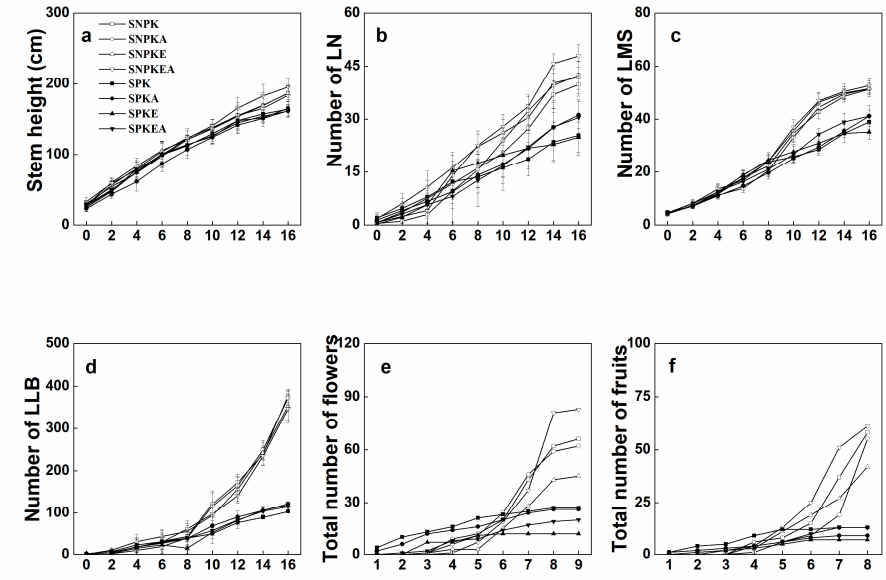

Fig 5. Growth parameters of *Kosteletzkya pentacarpos* in non-polluted or polluted soil during five months in the presence or absence of NaCl or / and EDDS. The stem height (a), number of lateral branch (LB, b), number of leaf on main stem (LMS, c) and number of leaf on lateral branch (LLB, d) were record every two week. Total number of flowers (e) and fruits (f) were recorded every ten day from the first day that they appeared, totally 9 and 8 time, respectively. Each value is the mean of 15 replicates and vertical bars are S.E. in firgure 1

(a-d).

For reproductive parameters, plant had more flowers and fruits growing in non-polluted soil in the last record (Fig. 5 e and f). An interesting observation was that plants growing in polluted soil initiated the reproductive stage at least 10 days earlier than plants growing in non-polluted soil. The first flower bud and fruit appeared in plants growing in polluted soil. Ryser and Sauder (2006) reported that even if the concentration of heavy metals were very low, flowering phenology of *Hieracium pilosella* was still very sensitive to metals. It delayed and reduced *Hieracium pilosella* reproduction. In contrast, it seems opposite in *K. pentacarpos*, which the explanation maybe that plants exposed to heavy metals tried to enter the flowering phase in advance in order to use available strategy for precocious reproduction rather than vegetative growth.

3.2. **Heavy metal concentration and bioavailability in soil**

After the experiment, the total concentration of heavy metal in soil was measured (Table 2). In this study, total concentration and bioavailability of pollutants for each treatment were only analyzed once. All heavy metals were detected in all treatment. The As, Cd, Pb and Zn content in non-polluted soil was lower than the threshold values for agricultural uses (30, 1.0, 200, 155 mg/kg, respectively). In our results, the total concentration of all heavy metal and As was higher in EDDS treatment than without EDDS in polluted soil. This was not our expectation. In terms of the EDDS function, it is well known that the EDDS application could increase the soil heavy metals' bioavailability, leading to promoting heavy metals uptake by plant, eventually improving phytoextraction efficiency. However, the total concentration of heavy metals and As decreased in the absence of EDDS, which is suggested that EDDS did not have a positive impact on heavy metal and As uptake in *Kosteletzkya pentacarpos* In contrast, the concentration of heavy metal and As was lower in the presence than in the absence of salt. Because the total concentrations and total

amount of pollutants were fixed in this experiment. It could be explained that the reduction part of heavy metals and As was transferred to the plants tissue. It is suggested by Weggler-Beaton (2000) that cadmium concentrations in soil solution and shoots of wheat (*Triticum aestivum* cv. Halberd) and Swiss chard (*Beta vulgaris* cv. Foodhook Giant) plants increased linearly with increasing Cl concentration in soil solution of the biosolids-amended soil. The additional Cl^- could form $CdCl^+$ complex with $Cd2^+$, which the activity of $CdCl^+$ complex correlated best with the Cd uptake of both plant species. However, Manousaki (2009) pointed out that although the increasing salinity increased cadmium uptake by *A. halimus* L., in the case of lead, there was not a clear effect of the presence of salt on lead accumulation in plant tissues.

Table 2 Heavy metal (As, Cd Pb and Zn) concentration ($mg.kg^{-1}$ soil) in different layer mixed soil after cultivation of *Kosteletzkya pentacarpos* in the presence and absence of NaCl or / and EDDS for 5 months. The sample was tested once.

Concentration	As	Cd	Pb	Zn
SNPK	5.4	0.92	22.1	83.2
SNPKA	8.2	0.28	24.2	73.7
SNPKE	9.6	0.41	44	67.2
SNPKEA	7.6	0.28	23.4	67.6
SPK	173	6.1	347	216
SPKA	127	4.7	230	184
SPKE	179	6.2	360	218
SPKEA	146	5.5	253	215

The bioavailable fractions of pollutants in the soil have also been determined. Only polluted soil values are above the detection limit (LD) except for Pb which remained extremely low. The percentage of the corresponding total fraction are shown in Table 3. EDDS increased the bioavailability of As and Zn, while NaCl decreased the bioavailability of As and Cd in polluted soil.

Table 3 The percentage of bioavailable fraction ($CaCl_2$ extraction) of heavy metal (As, Cd, Pb and Zn) in the total fraction in different layer mixed soil after cultivation of *Kosteletzkya pentacarpos* in the presence and absence of NaCl or / and EDDS for 5 months. The sample was tested once.

Bioavailability (%)	As	Pb	Cd	Zn
SPK	0.47	<0.25	0.53	0.078
SPKA	0.39	<0.25	0.25	0.088
SPKE	0.69	<0.25	0.46	0.16
SPKEA	0.42	<0.25	0.42	0.16

3.3. Heavy metal concentration in the vegetative organs

All heavy metals were detected in root of plants growing on non - polluted and polluted treatments (Tab 4). The concentration of As in root from non - polluted treatment remained lower than the limit of quantification (LQ, 8 ppb) and close to LD (2 ppb). The highest concentration of As in root was found in SPKEA. Both EDDS and salinity increased the accumulation of As in root exposed to polluted soil. NaCl induced the strongest increase by 181% and 139% in the presence and absence of EDDS, respectively. The concentrations of Pb in non-polluted treatment were always lower than 2 ppb. In polluted soil, EDDS significantly increased Pb accumulation in root by 30% in the absence of NaCl. The addition of salinity had no significant effect on Pb levels in roots. The concentrations of Cd in root of plants grown on unpolluted soils were lower or equal to 0.9 mg / kg DW and therefore remained very close to LQ (0.8 mg / kg DW). EDDS induced a significant increase in Cd concentration in root in polluted soil ($P < 0.05$). In contrast, salinity significantly decreased the Cd accumulation in root ($p < 0.05$). The highest concentration of Zn was found in SPKE (99.2 ± 1.2 mg / kg DW). The addition of NaCl significantly decreased the accumulation of Zn in roots growing in polluted soil ($P < 0.05$) while EDDS significantly increased the level of Zn in the absence of NaCl ($P < 0.05$).

In summary, EDDS enhanced the concentration of all pollutants in

root of *Kosteletzkya pentacarpos* growing in polluted soil. In contrast, NaCl reduced accumulation of all pollutants in root: only As increased, although its bioavailablity in the presence of NaCl in polluted soils decreased. It has been widely reported (Panda et al. 2017; Vromman et al. 2016) that the additional salinity could enhance plant resistance to As resulting from decreasing As uptake in root. However, our study showed the opposite result, which could explain that the relatively low concentration of As was not too toxic for *Kosteletzkya pentacarpos.* At the same time, in long-term clutivation, the additional salinity increased plants tolerance to As toxicity.

Table 4 Total concentration (mg kg^{-1} DW) of heavy metal (As, Pb, Cd and Zn) in root of *Kosteletzkya pentacarpos* exposed to non - polluted or polluted soil in the presence or in the absence of NaCl or / and EDDS for five months. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

Treatment	As	Pb	Cd	Zn
SNPK	2.5 ± 0.8 a	1.7 ± 0.4 a	0.60 ± 0.09 a	20.6 ± 1.3 a
SNPKA	1.7 ± 0.2 a	1.8 ± 0.2 a	0.90 ± 0.16 a	28.0 ± 5.6 a
SNPKE	2.0 ± 0.5 a	1.5 ± 0.3 a	0.55 ± 0.07 a	25.0 ± 4.8 a
SNPKEA	2.1 ± 0.2 a	1.9 ± 1.1 a	0.73 ± 0.21 a	21.4 ± 0.8 a
SPK	43.0 ± 4.6 b	132 ± 20 b	6.5 ± 0.3 c	79.0 ± 6.7 c
SPKA	121 ± 4.4 d	126 ± 12 b	4.3 ± 0.8 b	66.2 ± 2.0 b
SPKE	53.9 ± 7.4 c	171 ± 16 c	10.4 ± 0.7 d	99.2 ± 1.2 d
SPKEA	129 ± 2.4 e	153 ± 27 bc	7.2 ± 2.9 c	72.4 ± 10.1 bc

All heavy metals were detected in main stem (MS) and lateral branches (LB) of plants (Tab 5). As concentration in MS and LB remained lower than the limit of quantification (LQ, 8 ppb). The highest concentration of As was found in SPKE in LB. In polluted soil, NaCl significantly decreased the concentration of As in both MS and LB. The addition of EDDS reduced As concentration in MS, while it increased As in LB when plants grown in polluted soil. The stem

concentrations of Pb in non - polluted treatment were always lower than 2 ppb. The addition of NaCl significantly decreased the Pb level in LB of plants grown on polluted soil except in the absence of EDDS (p <0.05). EDDS had limited impact on Pb accumulation in the shoot. The concentrations of Cd found in MS and LB of plants grown on unpolluted soils are all less than LQ (0.8 mg / kg DM).

Table 5 Total concentration (mg kg^{-1} DW) of heavy metal (As, Pb, Cd and Zn) in stem (MS: main stem; LB: lateral branch) of *Kosteletzkya pentacarpos* exposed to non - polluted or polluted soil in the presence or in the absence of NaCl or / and EDDS for five months. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

Treatment	As (mg kg^{-1} DW)		Pb (mg kg^{-1} DW)		Cd (mg kg^{-1} DW)		Zn (mg kg^{-1} DW)	
	MS	LB	MS	LB	MS	LB	MS	LB
SNPK	0.63 ± 0.12 a	0.59 ± 0.08 a	0.25 ± 0.02 a	0.33 ± 0.05 a	0.34 ± 0.04 a	0.46 ± 0.09 a	13 ± 2.9 a	21 ± 1.8 a
SNPKA	0.56 ± 0.12 a	0.63 ± 0.11 a	0.12 ± 0.02 a	0.28 ± 0.11 a	0.52 ± 0.1 a	0.43 ± 0.07 a	17 ± 1.0 abc	22 ± 0.47 a
SNPKE	0.53 ± 0.16 a	0.59 ± 0.15 a	0.20 ± 0.02 a	0.36 ± 0.15 a	0.29 ± 0.03 a	0.46 ± 0.04 a	14 ± 0.6 ab	22 ± 2.0 a
SNPKEA	0.53 ± 0.12 a	0.57 ± 0.15 a	0.19 ± 0.08 a	0.25 ± 0.08 a	0.62 ± 0.12 a	0.63 ± 0.19 a	17 ± 1.4 abc	23 ± 2.2 a
SPK	2.5 ± 0.2 d	2.2 ± 0.17 d	5.7 ± 0.2 c	5.4 ± 0.8 d	2.0 ± 0.2 b	3.7 ± 0.15 b	22 ± 5.6 cd	45 ± 2.6 c
SPKA	1.8 ± 0.3 c	1.1 ± 0.02 b	5.8 ± 0.4 c	2.6 ± 0.1 b	2.7 ± 0.1 c	3.5 ± 0.4 b	20 ± 1.7 bc	37 ± 1.7 b
SPKE	2.3 ± 0.3 d	2.8 ± 0.3 e	6.6 ± 0.5 d	5.9 ± 0.6 d	2.2 ± 0.5 b	5.2 ± 0.2 c	30 ± 4.0 e	62 ± 4.3 e
SPKEA	1.0 ± 0.2 b	1.7 ± 0.3 c	4.7 ± 0.2 b	4.0 ± 0.5 c	2.1 ± 0.1 b	3.6 ± 0.4 b	27 ± 1.4 de	52 ± 3.4 d

Table 6 Total concentration (mg kg^{-1} DW) of heavy metal (As, Pb, Cd and Zn) in leaf (LMS: leaf on main stem; LLB: leaf on lateral branch) of *Kosteletzkya pentacarpos* exposed to non - polluted or polluted soil in the presence or in the absence of NaCl or / and EDDS for five months. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

Treatment	As concentration (mg / kg DW)		Pb concentration (mg / kg DW)		Cd concentration (mg / kg DW)		Zn concentration (mg / kg DW)	
	LMS	LLB	LMS	LLB	LMS	LLB	LMS	LLB
SNPK	0.46 ± 0.14 a	0.40 ± 0.09 a	1.1 ± 0.1 a	1.0 ± 0.2 a	1.2 ± 0.4 a	0.95 ± 0.11 a	44 ± 10.9 a	48 ± 6.3 a
SNPKA	0.39 ± 0.07 a	0.27 ± 0.04 a	1.0 ± 0.1 a	1.3 ± 0.2 a	1.8 ± 0.01 a	1.6 ± 0.3 a	55 ± 6.3 a	55 ± 3.7 a
SNPKE	0.41 ± 0.04 a	0.41 ± 0.07 a	1.0 ± 0.2 a	1.4 ± 0.2 a	0.92 ± 0.1 a	0.88 ± 0.05 a	47 ± 8.3 a	51 ± 6.9 a
SNPKEA	0.46 ± 0.04 a	0.33 ± 0.08 a	1.0 ± 0.1 a	1.2 ± 0.2 a	1.5 ± 0.2 a	1.6 ± 0.3 a	67 ± 1.8 b	60 ± 6.7 a
SPK	2.3 ± 0.3 b	3.7 ± 0.6 bc	9.4 ± 2.1 c	10.6 ± 1.9 c	5.3 ± 0.5 c	5.8 ± 0.6 bc	101 ± 3.2 d	115 ± 12 c
SPKA	2.0 ± 0.3 b	3.2 ± 0.2 b	4.4 ± 1.0 b	6.3 ± 0.6 b	4.3 ± 0.5 b	5.0 ± 0.2 b	92 ± 4.1 d	78 ± 13 b
SPKE	2.8 ± 0.3 c	5.1 ± 0.4 d	5.7 ± 1.5 b	3.7 ± 0.9 a	7.2 ± 1.2 d	6.4 ± 0.8 c	101 ± 1.4 d	102 ± 9.1 c
SPKEA	2.8 ± 0.1 c	4.0 ± 0.6 c	7.4 ± 1.0 bc	3.6 ± 0.4 a	5.7 ± 0.5 c	6.4 ± 0.9 c	81 ± 2.7 c	78 ± 1.5 b

EDDS significantly decreased the Cd concentration in MS in the presence of NaCl ($P < 0.05$) in polluted soil while it increased it in LB in the absence of salt ($P < 0.05$). On the other hand, the additional NaCl significantly increased the Cd concentration in MS in the absence of EDDS ($p < 0.05$) while decreased it in LB in the presence of EDDS ($P < 0.05$). For zinc, the addition of NaCl significantly decreased the accumulation of Zn in LB in polluted soil ($P <0.05$) and had no impact on it in MS. EDDS significantly increased the Zn in the presence and absence of NaCl in MS as well as in LB ($P < 0.05$). In summary, NaCl reduced As accumulation in both MS and LB, and Pb as well as Zn accumulation in LB. Conversely, EDDS increased the accumulation of all four heavy metals in LB and Zn in MS.

The concentration of As in LMS and LLB from plants growing in non-polluted soil was by far lower than for plants growing on the polluted substrate (table 6). In polluted treatment, NaCl significantly decreased the concentration of As in the presence of EDDS in LLB. In contrast, EDDS increased As concentration in LMS as well as LLB. NaCl significantly decreased Pb content in LMS and LLB of plants grown in polluted soil in the absence of EDDS ($P <0.05$). EDDS also significantly decreased Pb concentration in the presence or absence of NaCl in LLB ($p <0.05$). NaCl significantly decreased Cd concentration in LMS ($P < 0.05$) but had no impact on LLB. EDDS significantly increased Cd in LMS and LLB in the presence or absence of NaCl in polluted soil ($P <0.05$). NaCl significantly decreased Zn accumulation in LMS and LLB in polluted soil ($P <0.05$). EDDS significantly decreased the level of Zn in LMS in the presence of NaCl ($P <0.05$), but had no impact on Zn in LLB.

In our result, the salinity application differently affected different pollutants species. Unlike our results, Vromman (2016) indicated that NaCl increased As translocation from the root to the shoot of *Atriplex atacamensis* Phil. but had no impact on As distribution between apoplasm and symplasm. For the lead, the salinity decreased its translocation to LMB and LMB. Li et al (2019) demonstrated that the addition salinity induced growth stimulation to dilute Pb in *Suaeda*

salsa. In case of the cadmium, although the salinity did not obviously affect the concentration of Cd in *Kosteletzkya pentacarpos*, the amount of Cd in plant do increased, which has the similar results with Ghnaya's research on *Sesuvium portulacastrum* (Ghnaya et al. 2007).

The EDDS application had limited impact on As accumulation in plants shoots, while it increased Cd concentration in LMS and LB in our study. Lan (2013) indicated that the biodegradable EDDS had the potential for enhancing the efficiency of remediation by *S. orientalis* in Cd polluted soil as a consequence of Cd accumulation enhancement in plant tissue. For lead, EDDS differently affects it accumulation in shoot in different plant species. It is also suggested that the application of EDDS had less impact on Pb accumulation (only 310 mg kg^{-1}DW) in the shoot of *Cynara cardunculus,* than it in EDTA trratment (1332 mg kg^{-1} DW) (Epelde et al. 2008). However, in Attinti's study (2017), concentration of lead in shoots of vetiver grass increased dramatically after EDDS applications, indicating chelation occurred. In our study, the additional EDD strongly decreased Pb concentration in leaves in *K. pentacarpos.* In marques' study (2008), the EDDS application enhanced the accumulation in leaves, stems and roots of *Solanum nigrum* L. grew in Zn contaminated soil up to 140, 124 and 104%. The same results for Zn in our study.

Addition of salinity significantly decreased the bioaccumulation factor (BF) of all pollutants in polluted soil, except for Cd in the absence of EDDS. On the other hand, EDDS significantly increased the BF value of As, Cd and Zn, but decreased it for Pb. Together with the results in the total concentration of all pollutant in polluted soil, EDDS reduced Pb accumulation in *K. pentacarpos* (total Pb concentration of Pb in non-EDDS treated soil decreased). In terms of translocation factor (TF_c), NaCl increased TF_c of Cd and decreased it for As and Pb in the presence or absence of EDDS. Furthermore, the addition of EDDS reduced TF_c for all heavy metals except Zn in the presence of NaCl in polluted soil.

In a global view, the salinity or EDDS differently affect pollutants

accumulation in *K. pentacarpos* depending on the pollutant species. Furthermore, from the result in Table 4, 5 and 6, it is suggested that these two applications had opposite influences on pollutants accumulation in *K. pentacarpos*, which is also confirmed in most results from EDDS+salinity treatment (the value in EDDS+salinity treatment is always between the EDDS treatment and the salinity treatment). It is easy to understand that in general the EDDS application should increase heavy metals bioavailability in soil. More ion is released and then absorbed by plants. In contrast, it is widely studied that the salinity application always alleviates pollutants toxicity in halophyte plant species, with one of the most important reasons that, it could reduce pollutants accumulation in plants tissue (chapter 1 and 2).

3.4. Structural polysaccharides analysis

Polymetallic pollution induced a slight modification in the cell wall composition. In main stem, the content of lignin and hemicellulose in plants growing in polluted soil in the presence and absence of NaCl or/and EDDS decreased. Similarly, the content of hemicellulose in lateral branch of plants growing in polluted soil in the presence and absence of NaCl or/and EDDS also decreased significantly while slightly. Secondly, NaCl increased the cellulose content in the main stem and lateral branches, when plants grew in the presence and absence of EDDS in polluted soil. EDDS had little impact on the content of lignin, hemicellulose as well as cellulose.

Table 7 Bioaccumulation factor (BF) and translocation factor which is estimated on the basis of concentration (TF_c) of *K. pentacarpos* seedlings grown in polluted soil in the presence or absence of NaCl or / and EDDS for five months. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

	As		Pb		Cd		Zn	
	BF	TF_C	BF	TF_C	BF	TF_C	BF	TF_C
SPK	1.7 ± 0.01 b	6.0 ± 0.04 d	2.1 ± 0.04 d	5.0 ± 0.10 d	61 ± 2.0 a	49 ± 1.6 b	26 ± 1.4 b	61 ± 3.2 a
SPKA	1.2 ± 0.01 a	1.5 ± 0.01 b	1.7 ± 0.01 b	4.3 ± 0.01 c	61 ± 0.24 a	75 ± 0.30 c	22 ± 0.21 a	61 ± 0.58 a
SPKE	1.9 ± 0.02 c	5.5 ± 0.06 c	1.9 ± 0.01 c	3.4 ± 0.02 b	81 ± 0.63 c	41 ± 0.32 a	31 ± 0.58 c	58 ± 1.1 a
SPKEA	1.2 ± 0.31 a	1.4 ± 0.02 a	1.5 ± 0.01 a	3.1 ± 0.02 a	69 ± 0.71 b	50 ± 0.52 b	25 ± 0.24 b	66 ± 3.2 b

Due to the electrical neutral and the relative stability, the cellulose as well as the lignin have a limited effect on heavy metal and As fixation, while the hemicellulose has an ability to absorb and chelate pollutants. The synthesis of hemicellulose may be stimulated by some kinds of messager molecular or plant hormones, when plants are stress by pollutants. Xiong (2009) demonstrated that exogenous nitric oxide enhances cadmium tolerance as well Cd accumulation of rice by increasing pectin and hemicellulose contents in root cell wall. Besides, Zhu (2013) reported that exogenous auxin alleviates cadmium toxicity in *Arabidopsis thaliana* by stimulating synthesis of hemicellulose 1 and increasing the cadmium fixation capacity of root cell walls.

Table 8 Contents of lignin, hemicellulose and cellulose in main stem and lateral branches of *K. pentacarpos* seedlings grown in polluted soil in the presence or absence of NaCl or / and EDDS for five months. Each value is the mean of 3 replicates and vertical bars are S.E. Values exhibiting different letters are significantly different at $P < 0.05$ according to SNK test.

Treatment	Main Stem (MS)			Lateral Branch (LB)		
	Lignin (g / 100 g OM)	Hemicellulose (g / 100 g OM)	Cellulose (g / 100 g OM)	Lignin (g / 100 g OM)	Hemicellulose (g / 100 g OM)	Cellulose (g / 100 g OM)
SNPK	15.2 ± 0.84 cd	10.9 ± 0.48 b	51.0 ± 0.19 bc	11.3 ± 1.2 a	10.9 ± 0.41 a	44.9 ± 1.1 bc
SNPKA	14.5 ± 0.50 acd	11.6 ± 0.41 bc	52.1 ± 3.5 c	10.6 ± 0.75 a	11.6 ± 0.25 b	43.8 ± 0.74 ac
SNPKE	15.2 ± 0.56 cd	12.7 ± 1.1 c	50.3 ± 0.38 ac	11.6 ± 0.66 ab	11.7 ± 0.31 b	44.6 ± 1.6 bc
SNPKEA	15.4 ± 0.84 cd	12.8 ± 1.2 c	52.4 ± 0.27 c	10.7 ± 0.28 a	13.2 ± 0.12 d	44.5 ± 0.64 bc
SPK	13.9 ± 0.12 ab	9.5 ± 0.19 a	48.9 ± 0.19 ab	13.7 ± 1.5 bc	10.4 ± 0.63 a	42.3 ± 0.08 a
SPKA	14.7 ± 0.46 bd	10.6 ± 0.07 ab	55.8 ± 0.47 d	11.0 ± 2.4 a	10.8 ± 0.20 a	46.8 ± 1.6 d
SPKE	13.4 ± 0.67 a	11.0 ± 0.66 b	48.4 ± 0.53 a	11.8 ± 0.94 ac	10.4 ± 0.26 a	43.1 ± 0.19 ab
SPKEA	14.6 ± 0.80 bc	11.5 ± 0.59 b	52.0 ± 0.67 c	11.1 ± 0.70 a	12.3 ± 0.42 c	46.9 ± 1.5 d

Reference

Arfaeinia H, Nabipour I, Ostovar A, Asadgol Z, Abuee E, Keshtkar M, Dobaradaran S (2016): Assessment of sediment quality based on acid-volatile sulfide and simultaneously extracted metals in heavily industrialized area of Asaluyeh, Persian Gulf: concentrations, spatial distributions, and sediment bioavailability/toxicity. Environ Sci Pollut Res Int 23, 9871-90

Attinti R, Barrett KR, Datta R, Sarkar D (2017): Ethylenediaminedisuccinic acid (EDDS) enhances phytoextraction of lead by vetiver grass from contaminated residential soils in a panel study in the field. Environmental Pollution 225: 524-533

Bhat SA, Singh S, Singh J, Kumar S, Bhawana, Vig AP (2018): Bioremediation and detoxification of industrial wastes by earthworms: Vermicompost as powerful crop nutrient in sustainable agriculture. Bioresour Technol 252, 172-179

Vromman D, Lefèvre I, Šlejkovec Z, Martínez JP, Vanhecke N, Briceño M, Kumar M, Lutts S (2016): Salinity influences arsenic resistance in the xerohalophyte *Atriplex atacamensis* Phil. Environmental and Experimental Botany 126: 32-43.

Epelde L, Hernández-Allica J, Becerril JM, Blanco F, Garbisu C (2008): Effects of chelates on plants and soil microbial community: Comparison of EDTA and EDDS for lead phytoextraction. Science of The Total Environment 401 (1-3): 21-28

Eshraghi F, Nezamzadeh-Ejhieh A (2018): EDTA-functionalized clinoptilolite nanoparticles as an effective adsorbent for Pb (II) removal. Environ Sci Pollut Res Int

Halchak JL, Seliskar DM, Gallagher JL (2011): Root system architecture of Kosteletzkya pentacarpos (Malvaceae) and belowground environmental influences on root and aerial growth dynamics. Am J Bot 98, 163-174

Han RM, Lefèvre I, Ruan CJ, Qin P, Lutts S (2012) NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regul 68:97-109

Han RM, Quinet M, André E, Van Elteren J, Destrebecq F, Vogel-Mikus K, Cui G, Debeljak, Lefèvre I, Lutts S (2013a) Accumulation and distribution of Zn in the shoots and reproductive structures of the halophyte plant species

Kosteletzkya virginica as a function of salinity Planta 238:441-457

Han RM, Lefèvre I, Albacete A, Pérez-Alfocéa F, Baeba-Espín G, Díaz-Vivancos P, Quinet M, Ruan CJ, Hernández JA, Cantero-Navarro E, Lutts S (2013b) Antioxidant enzyme activities and hormonal status in response to Cd stress in the wetland halophyte *Kosteletzkya virginica* under saline conditions. Physiol Plant 147:352-368

Ghnaya T, Slama I, Messedi D, Grignon C, Ghorbel MH, Abdelly C (2007): Cd-induced growth reduction in the halophyte *Sesuvium portulacastrum* is significantly improved by NaCl. Journal of Plant Research 120(2: 309-316.

Lan JC, Zhang SR, Lin HC, Li T, Xu XX, Li Y, Jia YX, Gong GS (2013): Efficiency of biodegradable EDDS, NTA and APAM on enhancing the phytoextraction of cadmium by *Siegesbeckia orientalis* L. grown in Cd-contaminated soils. Chemosphere 91 (9): 1362-1367.

Li, X., Zhang, X., Wang, X., Yang, X., Cui, Z., 2019. Bioaugmentation-assisted phytoremediation of lead and salinity co-contaminated soil by *Suaeda salsa* and *Trichoderma asperellum*. Chemosphere 224, 716-725.

Manousaki E and Kalogerakis N (2009): Phytoextraction of Pb and Cd by the Mediterranean saltbush (*Atriplex halimus* L.): metal uptake in relation to salinity. Environmental science and pollution research 16 (7): 844-854

Marques APGC, Oliveira RS, Smardjieva KA, Pissarra J, Rangel AOOS, Castro PML (2008): EDDS and EDTA-enhanced zinc accumulation by *solanum nigrum* inoculated with arbuscular mycorrhizal fungi grown in contaminated soil. Chemosphere 70 (6): 1002-1014

Panda A, Rangani J, Kumari A, Paride AK (2017): Efficient regulation of arsenic translocation to shoot tissue and modulation of phytochelatin levels and antioxidative defense system confers salinity and arsenic tolerance in the Halophyte *Suaeda maritima.* Environmental and Experimental Botany 143: 149-171.

Qin P, Han R-m, Zhou M-x, Zhang H-s, Fan L-s, Seliskar DM, Gallagher JL (2015): Ecological engineering through the biosecure introduction of Kosteletzkya virginica (seashore mallow) to saline lands in China: A review of 20 years of activity. Ecological Engineering 74, 174-186

Ryser P, Sauder WR (2006): Effects of heavy-metal-contaminated soil on growth, phenology and biomass turnover of Hieracium piloselloides. Environmental Pollution 140 (1): 52-61

Xiong J, An LY, Lu H, Zhu C (2009): Exogenous nitric oxide enhances cadmium

tolerance of rice by increasing pectin and hemicellulose contents in root cell wall. Plants 230 (4): 755-765.

Yoo JC, Beiyuan J, Wang L, Tsang DCW, Baek K, Bolan NS, Ok YS, Li XD (2018): A combination of ferric nitrate / EDDS-enhanced washing and sludge-derived biochar stabilization of metal-contaminated soils. Sci Total Environ 616-617, 572-582

Van Soest P (1973): Collaborative study of acid-detergent fiber and lignin. J AOAC 56: 781 - 784.

Van Soest P, Wine P (1967): Use of detergents in the analysis of fibrous feeds. IV. Determine of plant cell wall constitutes. J AOAC 50: 50 - 55.

Vaughn SF, Moser BR, Dien BS, Iten LB, Thompson AR, Seliskar DM, Gallagher JL (2013): Seashore mallow (Kosteletzkya pentacarpos) stems as a feedstock for biodegradable absorbents. Biomass and Bioenergy 59, 300-305

Weggler-Beaton K, McLaughlin MJ, Graham RD (2000): Salinity increases cadmium uptake by wheat and Swiss chard from soil amended with biosolids. Australian Journal of Soil Research 38(1): 37-46.

Zhu XF, Wang ZW, Dong F, Lei GJ, Shi YZ, Li GX, Zheng SJ (2013): Exogenous auxin alleviates cadmium toxicity in *Arabidopsis thaliana* by stimulating synthesis of hemicellulose 1 and increasing the cadmium fixation capacity of root cell walls. Journal of Hazardous Materials 263: 398-403.

General Discussion and Perspective

1. The specific response in *K. pentacarpos* exposed to simultaneously Cd and Zn toxicity

Data is commonly found in previous study to analyze the impact of excessive cadmium or excessive zinc on the plant behavior (Han et al. 2012, Han et al. 2013, Hu et al. 2019). However, it is far from reality, and does not reflect the truth to some extent. It has been mentioned that in most heavy metal polluted sites, high concentration of Cd always co-exists with excessive Zn, which inhibits plant growth and affects microbial community, leading to destroying the ecosystem in soil and eventually threatening animals and human health through food chain contamination (Dresler et al. 2017, Vaseem et al. 2017). In the present work, the physiological and biochemical responses to the interaction of Cd and Zn toxicity in the halophyte species *K. pentacarpos* in hydroponic were determined, and we provided evidences that the simultaneously presence of Cd and Zn induces a specific stress at the plant level which is not only the sum of the stresses induced by each heavy metal. This suggests that plant perceive a complex stressing environment "as a whole" and do not necessarily discriminate among individual stress components.

1.1. The antagonistic relationship between excessive Cd and Zn in *K. pentacarpos*

Most physiological plant responses directly depend on heavy metal absorption, translocation and eventually accumulation in plant. It is obvious that heavy metal toxicity is the consequence of internal accumulated elements, while external ions are not directly toxic by themselves, although they might indirectly influence the plant through an impact on soil microbial activity or modification in the soil structure. Hence, interactions between the studied elements in terms of

absorption by the roots and distribution within the plant is a crucial aspect.

In recent years, the mutual impact of Cd and Zn in plant uptake was widely studied, but until now, there is no clear conclusion in the relationship between Cd and Zn when they simultaneously exist in substrate. In the aspect of absorption by *Ricinus communis* L., the interaction pattern of Cd and Zn showed an antagonistic behavior under low concentration of Cd and Zn, while they exhibited a synergistic behavior under high concentration of combination of Cd and Zn (Wang et al. 2016). In contrast, it was found that Zn had mostly positive effect on Cd uptake in root and seedlings of tobacco, while Cd had antagonistic effect on Zn uptake by roots and accumulation in leaves (Tkalec et al. 2014). In *K. pentacarpos*, no matter it was exposed to short-term combination of Cd and Zn toxicity for 2 days or chronic stress for 3 weeks in aquatic condition, the excessive Zn had a negative effect on Cd accumulation in root of plants simultaneously exposed to both heavy metals. It is well established that Cd and Zn share numerous biochemical properties and that Cd is mainly absorbed by poorly-selective Zn transporters. An excess of Zn would therefore logically lead to a decrease in Cd absorption and our data tend to confirm this hypothesis.

We found that in *K. pentacarpos*, most Cd was fixed in cell debris mainly containing the cell walls (74%), while for Zn, it was equally accumulated in cytoplasm (45%) and cell debris (50%). According to literature, cell walls play a vital role in Cd fixation and this allow to maintain Cd away from the most important metabolic places within the cells (Astier et al. 2014, Douchiche et al. 2010). In Fu's study (2011), the subcellular fractionation of Cd-containing tissues indicated that both in root and leaves of *Phytolacca americana* L., the majority of the element was located in soluble fraction and cell walls. Ramos et al. (2002) and Weng et al. (2012) also verified that most of Cd was localized in the cell wall in *Lactuca sativa* cv. Grandes Lagos and *Kandelia obovata* (S., L.), respectively. Furthermore, hemicellullose is a major component of the cell wall. It has been reported (Lutts et al.

2016) that an increase in hemicellulose content of in response to Cd as well as an increase in mucilage which is mainly composed of pectin were record. The present study demonstrated that the hemicellulose content in the stem of *K. pentacarpos* increased when it was exposed to the excessive Zn alone (chapter 5). Hence, it could be hypothesized that the combination of Cd and Zn may modify the structure of polysaccharide as well to accumulate heavy metals in *K. pentacarpos*. This should be tested in future.

Root sequestration of heavy metals is a strategy to avoid accumulation of toxic ions in photosynthetic tissues. In the present study, such a process was clearly demonstrated and heavy metal accumulation was always higher in the root than in the shoot (Chapter 1 to 4). In order to avoid perturbation of root metabolism, heavy metal accumulation could however not exceed a threshold level and requires detoxifying mechanisms such as PC oversynthesis and vacuolar sequestration of the PC-HM complexes. Our data demonstrate that *K. pentacarpos* is able to trigger such a type of adaptative response, as suggested by the recorded increase in root PC observed in HM-treated plants. However, the fact that PC content may be higher in the leaves than in the roots (Chapter 1, Table 4) while Cd and Zn concentration were lower in the former than in the latter leads us to hypothesize that HM sequestration by non-protein thiol may be, to some extent, overcame in the below-ground part of the plant. Beside root sequestration, stem retention may be regarded as a complementary strategy to reduce heavy metal accumulation in photosynthetic leaves. In another halophyte plant species (*Atriplex halimus*), Lutts et al. (2004) demonstrated that precipitation of Cd in oxalate crystals specifically occur in the stem. The presence of mucilage within the xylem vessels (Ghanem et al. 2010) may offer to *K. pentacarpos* a convenient opportunity to retain heavy metals, as suggested by the recorded increase in uronic acid of the stem mucilage in Zn-treated plants (Chapter 5), although interaction between Cd and Zn for the absorption site still remains an open question.

Previous data suggested that Cd or Zn could be xylem-loaded by

either symplastic or apoplastic pathway in *K. pentacarpos* (Han et al. 2012). In dwarf polish wheat, Wang et al. (2017) demonstrated that several metal transporters such as *cadmium-transporting ATPase* and *plant cadmium resistance 4* were specifically regulated by Cd+Zn. Meanwhile, it has been identified that many metal transporters are able to transport both Cd and Zn, including AtNRAMP3 and AtNRAMP4 (Guerinot 2000). Another group is known as heavy metal ATPases (HMAs), which has two subgroups based on their metal-substrate specificity: a copper (Cu)/silver (Ag) group and a zinc (Zn)/cobalt (Co)/Cd/lead (Pb) group (Axelsen et al, 2001; Williams et al, 2005). Previous results suggested that OsHMA2 plays a role in Zn and Cd loading to the xylem and participates in root-to-shoot translocation of these metals in rice (Takahashi et al, 2012). It could be assumed that in our study, Cd and Zn mutually affect each other's translocation in *K. pentacarpos* when plants are simultaneously exposed to both heavy metal stress, they compete to bind to the fixed quantity of the specified transporters.

Although nowadays, more and more heavy metal transporters are recognized with the understanding of their structure and function (Nakanishi-Masuno et al, 2018; Zhang et al, 2018; Hicks and Yang, 2019), there are still several issued should be studied in future: 1) when halophyte plant species is simultaneously exposed multiple heavy metals excess, how does the plant perceive and select the different heavy metal ions to bind? 2) How does the plant distribute transporters to target different heavy metal ion to minimize the damage?

1.2. The oxidative status and antioxidant system

Is the combination of Cd and Zn toxicity able to more drastically impair oxidative status compared single metal toxicity, leading to activating specific antioxidant system to response to oxidative stress?

In the aquatic plant *Lemna minor* L., mixed treatment showed lower oxidative stress, as suggested by lower values of lipid peroxidation,

protein oxidation, peroxidase and DNA damages, which suggests an alleviating effect of Zn on oxidative stress in Cd-treated plants (Balen et al. 2011). The same results were found in tobacco: supplementation of excessive Zn reduced oxidative stress. However, DNA damage, low glutathione reductase (GR) as well as superoxide dismutase (SOD) were noticed, suggesting that although excessive Zn diminished the negative oxidative impact of Cd, Zn accumulation could still impose toxic effects on plant. In another study, the effect of different concentration of Zn in combination treatment on antioxidative system in tomato was presented (Cherif et al. 2012). At low level of Zn, it restored and enhanced the functional activity of SOD, CAT, APX and GR. In contrast, Zn excess induced higher oxidative stress than that for Cd or Zn alone treatment in tomato. In previous work in our lab, the Cd-induced oxidative stress which was quantified by the accumulation of MDA, H_2O_2, carbonyl and O_2^-, was paralleled with decreased GSH, α-tocopherol and increased reduced ascorbate (AsA) as well as SOD and POX (Han et al. 2013).

To the best of our knowledge, this is the first time that the oxidative stress and antioxidant system in *K. pentacarpos* is described in response to a combination of Cd and Zn toxicity. We have already discussed that although Cd did not directly participate in redox reaction, such as Fenton reaction, it is still capable to disturb the electronic transmission chain in photosynthesis in *K. pentacarpos*. In our results, combination of Cd and Zn strongly inhibits photosynthesis process, with triggering NPQ (photoprotection) to dissipate excessive energy (Houri et al, 2019). It has been indicated that excessive Zn could exacerbate the negative effects of Cd, which induces a decrease of variable chlorophyll fluorescence intensity ratio (690 and 730) and in the stress adaption index (Ap), suggesting a reduction of potential photosynthetic activity (Cherif et al. 2012).

Although Cd accumulation was reduced in root and leave in the combined treatment, the oxidative stress was clearly induced as indicated by the generation of MDA, carbonyl and H_2O_2. However, interaction between Cd and Zn in terms of oxidative stress may

depend on the considered parameter. For example, no difference was found between Cd alone and Cd+Zn treatment for relative leakage ratio, which indicates that the membrane was damaged mainly by Cd, resulting in an outflow of ions. Nevertheless, MDA increased in response to Zn alone (Chapter 2): since MDA is the results of membrane lipid peroxidation, this indicates that biological membrane may be, to some extent, affected by Zn but that this deleterious impact is somewhat masked by the highest toxicity of Cd in response to mixed treatment.

To cope with oxidative stress induced by mixed toxicity, high global antioxidant activity was observed. Glutathione is one of the most important antioxidants, which assumes a dual function in the presence of heavy metal since it acts both as an antioxidant and as a precursor of PCs. In *K. pentacarpos*, the accumulation of reduced GSH in root and its depletion in the leaf was recorded in the mixed treatment. It has been found that in a short-term basis (few hours) in *Arabidopsis*, root GSH was preferentially allocated to synthesis of phytochelatin (PC) involved in Cd chelation, which led to decreased GSH levels, without alternative pathways activated to complement GSH's antioxidative functions (Jozefczak et al, 2014). After one day adjustment, multiple antioxidative pathways increased including ascorbate-glutathione cycle. In relation to our result of PC content in leaf, the depletion of reduced GSH might mainly be attributed to the synthesis of phytochelatins. It can be concluded that *K. pentacarpos* tries to find a compromise to balance the glutathione activity as a free radical scavenger and the synthesis of PC as an efficient metal chelator under combination of Cd and Zn toxicity. However, we have no experimental proof that PCs are equally involved in Cd- and in Zn chelation. Although it is sometimes considered that Cd chelation by NPT is more efficient than Zn chelation, it could also depend on the type of synthesized PCs, which was not considered in our experimental work since we were unable to discriminate among the various PCs. The impact of heavy metals on enzyme activities involved in glutathione and PCs synthesis would also be an interesting aspect to analyze in future works.

In addition to non-enzymatic antioxidant, enzymatic antioxidants such as SOD, CAT etc also acts important function for detoxification under heavy metal stress (Han et al, 2012; Tair et al, 2019). Han et al (2013) indicated that the excessive Cd alone enhanced DHAR, GR and POX activity to detoxify ROS induced by Cd exposure. However, when plants are under multiple HMs stress, how specified enzymatic antioxidants acts are still unknown. Besides, antioxidant system status in plants differs from heavy metals exposure duration. The mechanism regarding the dynamic changes of the antioxidant system with plants acclimation to multiple HMs stress should be deeply discovered in the future. Tang et al. (2006) recently demonstrated that Cd and Zn differently regulate the expression of numerous genes involved in photosynthesis but also in the management of oxidative status, especially in the chloroplast compartment. Hydrogen peroxide was the only ROS quantified in our study: we therefore could not assume that oxidative stresses induced by Cd and Zn are similar and the precise quantification of the ROS species (especially O_2^-) could provide interesting information in this area.

1.3. Plant hormones status and osmoprotectants

Plant hormones are produced in plant at low concentration, but control all aspects of plant growth, development and reproduction (Kucera et al, 2005; Verma et al, 2016; Wilkinson et al, 2012; León and Sheen, 2003). When plants suffer from abiotic stress, phytohormones play vital roles in stress perception, signal transduction and stress resistance (Peleg and Blumwald, 2011; Colebrook et al, 2014; Mauch-Mani and Mauch, 2005; Bakshi et al, 2019).

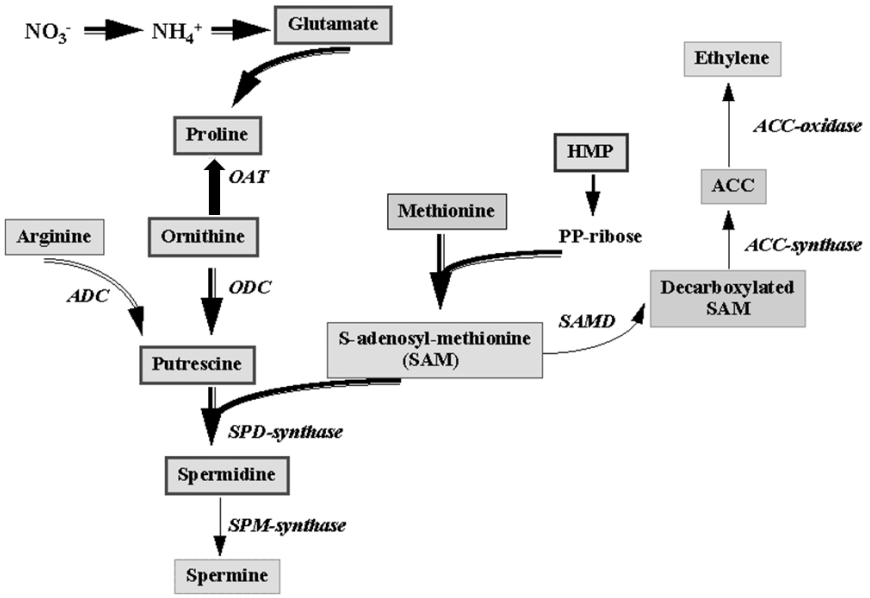

Figure 1 Ethylene, polyamine, proline metabolism pathway

Beside the "classical" phytohormones, polyamines mainly including Put, Spd and Spd, plays critical multiple functions in plants, such as protein protection, free radical scavenging (Minocha et al, 2014), stabilization of biological membrane, regulation of mineral nutrition and ion homeostasis, interaction with cell wall components and involvement in lignification process, interaction with DNA and regulation of the cell cycle (Tiburcio et al, 2014), involvement in abiotic stress signaling (Benavides et al, 2018), and anti-senescing properties (Sengupta et al. 2016). Starting from arginine, two pathways are known to form Put via ornithine by arginase and ornithine decarboxylase and via arginine through arginine decarboxylase (Fig 1). In another side, ornithine is also an important substrate for proline biosynthesis, although this amino acid can also be synthesized from glutamate. Spd and Spm are also important members of the polyamine family, and are synthesized from Put associated with SAM. Furthermore, SAM is the shared precursor in ethylene and Spd as well as Spm synthesis. Hence, the metabolisms of these compounds are deeply connected and often considered as competitive.

It is well known that all kinds of abiotic stresses induce premature leaf senescence which is directly related to a modification of hormonal status. One of the specific objectives of our study was to decipher the Cd- and the Zn-induced senescence processes in *K. pentacarpos* focusing mainly on polyamines and cytokinins content as well as ethylene production. We pinpoint that modification of the hormonal status induced by the two heavy metals drastically differ.

The concentration of Put was increased and the concentration of Spd was reduced in Cd alone treatment and mixed treatment, while they were not modified by Zn alone treatment. It is indicated that Cd could induce polyamines modification, including when *K. pentacarpos* was exposed to a combination of Cd and Zn. Vromman et al. (2011) hypothesized that polycationic molecules may assist in arsenate sequestration in the stressed tissues of *A. atacamensis* related to an increase in free soluble PAs (Spd and Spm increase) in As-treated plants. Such a process should not be involved here since, in contrast to arsenate, Cd and Zn are both accumulating as divalent cations. However, exogenous application of PAs indirectly indicates the capacity of PAs to enhance plant tolerance to heavy metals. Considering the multiple role of PAs in plant metabolism, these approaches frequently remain descriptive and the real underlying cause(s) of the recorded improvement remain elusive. Tang et al (2018) found that exogenous spermidine could alleviate the accumulation ROS (superoxide anion, hydrogen peroxide, malondialdehyde), in relation to an increase in soluble protein and antioxidant in *S. matsudana* leaves under the corresponding cadmium stress. Moreover, in the present experimental approach, we quantified the free soluble form of polyamine while protective functions assumed by these compounds may result from the conjugated or bound fractions that were not quantified in our study (Quinet et al., 2010).

Meanwhile, the increased concentration of Put was accompanied by a concomitant increase of ethylene concentration in leaf in *K. pentacarpos*; consequently, this may lead to a decrease in SAM available for Spd and Spm synthesis. Hence, quantifying SAM is

absolutely required in the future to test this hypothesis. Besides, there are a lot of puzzling issue between PAs and ethylene metabolism. It would be imperative to unveil biochemical as well as molecular insights into combined ethylene and PA-mediated control of HM-specific toxicity in plants. If explored, we will better understand the deeply inner relation between PAs and ethylene and how plants make signal and response to the multiple heavy metals stress. It has also to be mentioned that *K. pentacarpos* is a wetland semi-aquatic plant species and that ethylene may assume specific functions in these types of plants. Lutts et al. (1996) for example demonstrated that ethylene is involved in cell elongation in rice, a quite contrasting situation comparatively to classical terrestrial plant species.

Although Zn excess does not modify the polyamines and ethylene metabolism, it has a possible impact on other kind of phytohormone. Cytokinin-dependent regulatory module underlies the maintenance of zinc nutrition in rice (Gao et al., 2019). These authors used cytokinin-related mutants and transgenic lines to provide unequivocal evidence that cytokinins have a key role in controlling Zn status in plants. Our work was performed under Zn in excess condition in the presence and in the absence of Cd. In *K. pentacarpos*, the additional Zn drastically reduced CK concentration but Cd had no impact on it. What is interesting is that excessive Zn strongly decreased total cytokinin content in plant leaves, even in combination of Cd and Zn treatment. According to Atici et al. (2005) and Xu et al. (2013) high Zn concentration can have strong effects on CK metabolism and induced a decrease in CK content in plants, while optimum Zn concentration increased their content. In addition to cytokinin, auxin, ABA, SA as well as JA were more significantly affected in response to mixed treatment than in the case of exposure to one single heavy metal. It is suggested (Atici et al, 2005) that the high concentrations of Zn (1.0 and 10 mM) decreased contents of Z, ZR and GA_3 in germinating chickpea seeds in relation with the enhanced ABA content. Wu et al (2015) related plant hormone status with AsA-GSH cycle and hypothesized that 6-benzylaminopurine (BAP, one synthetic CK)-regulated AsA-GSH cycle is mediated by CK signal pathway via IAA and ABA,

in the leaves of eggplant (*Solanum melongena* L.) seedlings under 10 mM zinc stress. The specific hormonal status of mixed treatment corroborates our hypothesis that this treatment induces a specific physiological modification in the plant and it seems highly probable that, for some phytohormones, Cd and Zn act on common target, but not necessarily in the same way and not necessarily at the same step of the biochemical pathway. The lack of information regarding *K. pentacarpos* genome and the fact that mutants are consequently not available hamper a more sophisticated experimental approach.

In addition to phytohormones, the osmoprotectant proline widespread exists in almost all kinds of plant species as an essential amino acid, while glycinebetaine accumulates in a limited number of plant species, such as halophyte plants (Ashraf and Foolad, 2007). Amount of literatures have reported that both accumulate under abiotic stress, such as heavy metal toxicity, drought and salinity (Ashraf and Foolad, 2007; Apse and Blumwald, 2002). In our work, the combination of Cd and Zn toxicities significantly reduce proline biosynthesis compared to Cd alone. Tripathi et al (2013) found that the activities of pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) were increased immediately in *Triticum aestivum* (Wheat) exposed to excessive Cu and Cd and remained higher through the end of the experiment, whereas ornithine amino transferase (OAT) activity of the treated plants was lower than P5CS and P5CR enzymes. The activity of proline dehydrogenase (PDH) was decreased sharply in the early phase of metal exposure (up to 12 h) but remained unchanged thereafter until the end of the experiment. In our study, glutamate pathway and ornithine pathway play an equivalent role in proline synthesis in *K. pentacarpos* exposed to multiple heavy metal stress. On the other hand, it is speculated that the shared precursor, ornithine, could also participated in Put synthesis under mixed treatments. What is specific in the mixed treatment is that the concentration of Spd decreased in relation to an increased concentration of Spm compared to Cd or Zn alone, and this suggests that Spm synthesis from Spd may be specifically stimulated to cope with mixed toxicity since Spm harboring 4 positive charges at cellular

pH is often thought to be the most efficient polyamine for protection of cellular structures. After the AVG (ethylene synthesis inhibitor) was added, the accumulation of Cd was reduced in mixed treatment, associated with a decrease in Put concentration and a sharp increase in Spd and Spm. For GB, the previous studies shown that it exits mainly in chloroplast to protect photosynthetic machinery (Chen and Murata, 2008). Here, it is accumulating not only in leaves but also in roots of *K. pentacarpos*. Meanwhile, BADH expression was activated in response to all treatments in roots. Exogenous Cd strongly increased GB accumulation in leaves while Zn reduces it and the clear correlation between *KvBADH* gene expression and BADH activity suggests that Cd stress could induce GB synthesis so as to make protective function. Hence, it may be assumed that Cd and Zn differ not only for their phytohormonal impact, but also for the type of osmoprotectant they induce.

Until now, the involvement of several plant hormones and plant growth regulators is found to be associated with heavy metal stress responses. However, the clear link between hormonal pathways and metal-binding ligands in plants, either due to certain signaling pathway or common synthesis pathway, still needs to be explained. Further investigations on hormone synthesis mutants or transgenic plants may help to identify clear interrelations.

2. NaCl improves the plant resistance to combination of Cd and Zn toxicities while it is not completely same with the effect under single metal toxicity

2.1. NaCl reduces heavy metal accumulation and improves plant antioxidant system in mixed of Cd and Zn treatment.

Although it is well-known as a halophyte plant species, *K. pentacarpos* is confirmed to be able to grow very well in the nutrient

solution without salinity (Han et al. 2011). Moderate doses of salinity may stimulate plants growth in halophyte plant species: this has been confirmed in *K. pentacarpos* exposed to 100 mM NaCl. At this NaCl dose, leaf water content remained unaffected which maybe as a consequence of leaf mucilage enhancement and modification in its composition to some extent (Ghanem et al., 2010). In the present research, 50 mM NaCl was added in the substrate and it did not significantly improve plant growth in the absence of heavy metal no matter in the acute treatment (48 h) or long-term treatment (3 weeks). Growth stimulation by itself should not be regarded as an absolute criterion for definition of halophyte plant species (Van Oosten and Maggio 2015; Sruthi et al., 2017) and the plant physiology could be modified in a positive way independently of growth stimulation.

Indeed, 50 mM NaCl was clearly able to improve plant behavior in *K. pentacarpos* when it was exposed to Cd and/or Zn stress, which may be partly explained by a decrease in heavy metal accumulation. It is therefore puzzling to observe that both E and g_s increased in Cd+Zn+NaCl treatment which is not on line with a reduced transpiration (Chapter 1). Salinity may modify metal speciation in the nutrient solution (such as formation of less available chlorocomplexes) but Lefèvre et al. (2009) who used a nutrient solution similar to ours found using speciation programs (Geochem and VisualMinteq) that only a minor part of heavy metals is forming this type of complexes in our experimental conditions. An alternative could be that salinity interacts with transporters putatively involved in Cd and Zn absorption (Herce-Sesa, et al, 2019). Furthermore, from a relative point of view, the recorded NaCl-induced decrease in Cd accumulation in mixed treatment (74%) was higher than it in Cd alone treatment (44%), which indicates that NaCl had stronger impact on heavy metal, especially on Cd, in combination of Cd and Zn treatment. It could be assumed that the accumulated Cd, Zn as well as moderate NaCl could complicatedly interact and that NaCl could inhibit Cd translocation under Zn accumulation condition. This should be proved in the future. In Chapter 1, only roots and leaves were analyzed for heavy metal accumulation. An increased mucilage content in the stem

together with an increase in the proportion of uronic acids as indicated in Chapter 5 for Zn-treated plants, could not be ruled out.,

In addition to reduce heavy metal accumulation, salinity also modifies the subcellular distribution of Cd. In both roots and leaves of plants exposed to combination of Cd and Zn in the presence and absence of salinity, Cd is mainly fixed in cell debris (more than 50%), which is mostly composed of cell wall. It has been demonstrated that salt could induce an increased cell wall thickening by reinforcement of the secondary wall with hemicellulose and lignin deposition, which improves the HM binding capacity (Le Gall, 2015). It is noteworthy that when plants are stressed by Cd+Zn, the hemicellulose proportion is significantly increasing, which is one of the most important constituents for heavy metal sequestration outside of the cell (Hu et al, 2010). In addition, NaCl increases the Cd proportion in metal-rich granule (MRG), which is probably related to the increase of cysteine-rich phytochelatin (PC). MRG is regarded as high molecular weight insoluble complexes, which is formed by polymerization of low molecular weight of PC in vacuole. It is widely known that the vacuole plays a critical role in plant cell turgor regulation, especially in halophyte species (Zhang et al, 2017). It is indicated that the volume fraction of vacuole was greater in the halophyte *Suaeda maritima* (L.) Dum grown under saline conditions when compared with those under non-saline conditions (Hajibagheri et al, 1984). It could be hypothesized that NaCl stimulate a larger volume fraction of vacuole allowing a higher storage of HM-binding PCs. Since S concentration increased in the stressed tissue, it is also confirmed that *K. pentacarpos* triggers this PC protective mechanism, especially under Cd stress. It also illustrates an extremely important (and frequently neglected point) implying that organ response to heavy metals is not only a matter of total amount of accumulated ions but also directly depends on ion distribution. It still remains to explain 1) what is the signal transduction regulating heavy metal subcellular distribution? 2) How does salinity affect heavy metals distribution and complementation in molecular mechanism? should be discovered in the future.

The disturbance of photosynthesis process induced by heavy metal accumulation directly leads to excessive electron generation (Arena et al., 2017). In our work, NaCl increased all fluorescence-related parameter (except NPQ) and pigment concentration, suggesting that low doses of NaCl unexpectedly contributes to protect the chloroplast structure as well as photosynthesis process. Meanwhile, the direct consequence from improvement of photosynthesis is the reduced oxidative damage, which is indicated by the decrease concentration of carbonyl, H_2O_2, and MDA. The additional NaCl also trigger the non-enzymatic protection and more especially increases the AsA and GSH concentration to alleviate heavy metal toxicity (Han et al. 2013; Lokhande et al., 2011). In addition, we also found that salinity addition significantly increased GB synthesis in *K. pentacarpos* exposed to Cd toxicity, which is thought to protect chloroplasts. It has indeed been reported that higher accumulation of GB is able protect photosynthetic machinery under stress conditions (Park et al. 2007).

2.2. NaCl regulates plant hormones status allowing the plant to cope with a combination of Cd and Zn toxicities

In this work, we investigated the modification of the phytohormones status in plants exposed to mixed Cd and Zn treatment in the presence and absence of salinity. At first, salinity significantly reduced ethylene biosynthesis and increased polyamine concentration, which was especially the case for protecting spermidine and spermine (Chapter 2).

In Radyukina's study (2007), the constitutive level of free Spd and Spm and the expression of their genes (*SPDS*, *SPMS*) in *Thelungiela halophila* were significantly higher as compared with that of glycophytes, for example *Plantago major* L. Under combination of excessive of Cd and Zn in *K. pentacarpos*, the recorded concentration of ethylene was quite high, but salinity strongly reduced it. Ethylene being mainly involved in senescence process in plants, this implies that moderate doses of NaCl reduces senescence in the halophyte *K. pentacarpos* through a decrease in ethylene production. This is an

unusual behavior in the plant kingdom and additional work must be undertaken to explain the impact of NaCl on ethylene synthesis.

For PAs, with the decrease of ethylene, the concentration of Put was expected to decrease, but this, obviously, was not the case. We may assume that the combination treatment activates another pathway (from ornithine) for Put biosynthesis (Yoshida, 1969; Dalton et al, 2016), which could be related to the proline synthesis (see Fig. 1).

In addition to ethylene and PAs, in the absence of exogenous trans-zeatin riboside, salinity significantly increases total cytokinin and total gibberellins. Numerous literatures introduced the effect of exogenous cytokinin as well as salinity on plant behaviors: exogenous kinetin (KN) application reduced the inhibitory effects of NaCl on K^+ and Ca^{2+} uptake, improved the antioxidant system assess by the determination of ascorbate-glutathione cycle, reduced oxidative damage in *Solanum lycopersicum* (Ahanger et al, 2018); exogenous cytokinins (CPPU and 6-BA) also increased photosynthesis process and reduced ROS generation induced by salinity (Luo et al, 2010). However, in all these studies, the additional salinity was regarded as a stressed factor for plant. In our study, 50 mM NaCl is more likely a positive stimulus. However, the exogenous *t*-zeatin riboside assumes key function in heavy metal resistance especially in Zn-treated plants but its efficiency was lower in the presence of NaCl in our halophyte *K. pentacarpos*, which may be attributed to a fact that heavy metal reduced the endogenous CK concentration while NaCl increased it.

3. *K. pentacarpos* is a promising candidate for phytoremediation of polymetal-contaminated salt areas

3.1. Exogenous NaCl in nutrient solution compromises the use of *K. pentacarpos* for phytoextraction and heavy metal removal.

Phytoremediation is an attracting alternative to expensive industrial

approaches of decontamination but require the use of heavy-metal tolerant plants (Ali et al. 2013). Hyperaccumulators are not suitable for heavy metals removal since they usually produce a very low amounts of biomass. Moreover, cleaning salt-affected sites contaminated by heavy metals require the use of halophyte plant species (Clemente et al. 2012, Cheng et al. 2019, Lefèvre et al., 2009, Lutts and Lefèvre, 2015).

It has been confirmed that our studied halophyte species, *K. pentacarpos*, has a potential to accumulate various kinds of heavy metal (Zn, Cd and Cu) in the absence of salinity (Han et al. 2011, Han et al. 2012). Although the level of accumulation is far below the level fixed for hyperaccumulating plants, its high amounts of biomass compensate the consequences of this medium concentration in terms of quantity of pollutants removed by the plant: in optimal conditions, the plant may produce more than 30 T DM $year^{-1}$ (Qin et al., 2015).

However, without the consideration of heavy metal bioavailability (under nutrient solution), moderate doses of salinity (50 mM) had a negative impact on heavy metal absorption and accumulation, protecting plant from ionic toxicity while it compromises the use of *K. pentacarpos* for phytoremediation. The same results have been confirmed in present study: under the combination of Cd and Zn, the Cd concentration decreased even more strongly than it in Cd alone treatment. It must however be considered that salinity alleviates oxidative damage induced by excessive heavy metals through activation of antioxidant system as well as modification of different plant hormones status, resulting in the improvement of heavy metal resistance and plant growth, the extension of life span, and eventually, enhancement of total amount of toxic ion accumulation. In the first part of the present work, we did not recorded a strong impact of the treatment on the survival rate but the plants were exposed to heavy metals at the young stage for a limited periods from 2 days (Chapter 4) to 2 weeks (Chapter 1-3) and it might be argues that this duration was not sufficient to fully apprehend all the long-term consequences of heavy metal accumulation. Hence, it could not be excluded that plants

which are still living after 2 weeks could encounter strong problems after a longer time of stress exposure and the beneficial impact of NaCl would then be more useful for a phytoextracting approach if it helps the plant to remain alive and grow for a longer period. It could thus be of primary importance to consider longer term experiments with this type of material to clearly identify the plant response at each phenological stage (Van Oosten and Magio, 2015).

3.2. Salinity differently impacts the bioavailability of different heavy metal as well as As and it reduces the heavy metal percolation in soil cultivated with *K. pentacarpos*.

Although the bioavailability of heavy metals was not considered in nutrient solution (even if it could be, to some extent, modified by speciation), it is a critical factor in polluted soil, which directly influence microbial community, plant roots system as well as the whole soil ecosystem (Kim et al. 2015, Zhang et al. 2018). In our study, the bioavailability of As and three kinds of heavy metal (Cd, Pb and Zn) in 3 months *K. pentacarpos* cultivation was slightly different comparatively to the 6 months trials using *K. pentacarpos*. Salinity increased Cd bioavailability in polluted soil in small columns after 3 months cultivation (Chapter 6), while it decreased Cd bioavailability in big columns after 6 months cultivation (Chapter 7). For the other heavy metals and As, it shows the same tendency: NaCl reduces As bioavailability while it had a limited (Zn) or no (Pb) on other elements bioavailability. This aspect illustrates that bioavailability is not a fixed property but varies depending on the interaction between root system and surrounding soil (Zhao et al., 2013; Kumar et al. 2019; Li et al. 2019). Accordingly, interaction between solid substrate and root system should be more deeply analyzed in the future in *K. pentacarpos* but a sound approach will require an adapted specific experimental system.

Arsenic and heavy metal bioavailability has high correlation with the accumulation of all pollutants in *K. pentacarpos* in present work. To the best of our knowledge, this is the first study analyzing As

accumulation in *K. pentacarpos* while it has been reported as an important environmental constraint in coastal wetland soils (Yang et al., 2018; Bai et al., 2019). Salinity reduced As bioavailability in relation to a decrease in its accumulation in plant in both short or long term studies. In Vromman's study (Vromman et al., 2016), salinity strongly increased As translocation from the root to the shoot of *Atriplex atacamensis* Phil. Although salinity differently impacts the bioavailability of Cd in short or long terms basis, salinity has a positive influence on Cd accumulation in plant tissue on the whole (only decrease in leaf in long term basis). It is an interesting point that in nutrient solution, NaCl always decreased Cd absorption and translocation to protect plants from heavy metal toxicity, while in soil in the presence of salinity, plants accumulated Cd, illustrating that data may strongly differ between hydroponic and soil approaches.

One possible theory to explain these observations is that Cd absorption is hampered in nutrient solution, resulting from formation of chlorocomplexes (mainly $CdCl^{+}$) (Lutts and Lefèvre 2015), while in contrast, the increasing Cd solubility induced by NaCl lead to accumulation enhancement in soil substrate. The presence of an excess on Na^{+} could indeed help in the detachment of Cd^{2+} retained at the surface of clay particles, thus inducing an increase in Cd bioavailability. In this case, Cd bioavailability had a critical effect on absorption and accumulation in plant. In a long-term experiment, however, it was shown that salinity decreased rather than increase the Cd bioavailability, which might be due to the soil properties (low level of clay and high level of silt). For Zn, accumulation concentration in plants growing on polluted soil was lower than it in solution. It may be attributed to different Zn solubility in soil/solution substrate. Salinity had a limited impact on Zn bioavailability while it induced a decrease in accumulation on both short- or long- term time basis. The present work is also the first one to consider lead accumulation in *K. pentacarpos.* Although the Pb accumulation in *K. pentacarpos* is approximately 10 mg/kg dry leaf or stem material in both short and long-term experiments, such a low concentration should be regarded as the ultimate consequence of a low bioavailability of Pb in polluted.

However, considering the high biomass production of *K. pentac*arpos, it is still believed that it is able to absorb and accumulate this heavy metal, especially if adapted strategies are used to increase bioavailability without increasing leaching processes.

The present study demonstrated that this wetland halophyte species allows to reduce pollutant leaching and meanwhile, the additional salinity also strongly reduces leaching of pollutant. It has been verified that in the surface of developed root of *K. pentacarpos*, there exits abundant hydroxyl-group-rich mucilage, which could store huge reserves of water (Ghanem et al, 2010). Another interesting point is that in columns with plants, salinity reduces total amount of As removed from column system, while slightly increases Cd, Zn, Pb amount. Most of the extracted As is removed by leaching process in the presence or absence of NaCl. This phenomenon may depend on the properties of considered element as well as the plant species. Overall, in long-term time basis, the growth and reproductive cycle of *K. pentacarpos* is disturbed after heavy metal and As accumulation in plants, which induced plants to enter flowering stage in advance. It is thought to be one strategy to cope with pollutant constraint.

Chelating agent (EDDS) was tested in order to mitigate the impact of NaCl on heavy metal bioavailability; it clearly increased the bioavailability of As and Zn while NaCl decreased the bioavailability of As and Cd on polluted soil. Data are not so clear regarding the impact of EDDS on heavy metal accumulation in the plant: it is supposed to increase as a result of a chelate-induced increase in bioavailability but no uniform trend was reported in this respect. Accumulation varies between roots, stems and leaves but also depending on the location on the shoot (main stem versus lateral branches) thus confirming that the plant behavior is not uniform and that long terms experiments needs to integrate the impact of plant morphogenesis in relation to heavy metal distribution (Lefèvre et al., 2010). Considering the toxicity of EDTA (Lambrecht et al., 2011), EDDS was chosen as a non-toxic chelating agent but we apply one single dose of EDDS at the beginning of the experiment at the time of

spiked-soil contamination and it cannot be excluded that this biodegradable EDDS was not efficient anymore after 16 weeks of treatment.

In summary, *K. pentacarpos* is expected not only to have economic value (several production (medicine; fodder; biofuels) made of clean *K. pentacarpos* (cultivated in non-polluted area) has been put on the market), but also to be a potential candidate able to cope with environmental constrains such as heavy metal stress, due to its relative toleranced to metal toxicity. However, the research on phytoremediation with *K. pentacarpos* is only in its initial phase. What is the clear and detailed mechanism (physiological, biochemical and genetic) to deal with the relationship between salt absorption and metal accumulation in *K. pentacarpos* should be taken into consideration. In addition, the efficiency of using halophyte to remove pollutants from contaminated saline is relatively low, compared to hyperaccumulating plant species. Hence, genetic engineering approach to develop transgenic plants with characters of high biomass production, more metal translocation and accumulation, tolerance against metal toxicity might be more beneficial in this respect.

Reference

Ahanger MA, Alyemeni MN, Wijaya L, Alamri SA, Alam P, Ashraf M, Ahmad P (2018): Potential of exogenously sourced kinetin in protecting *Solanum lycopersicum* from NaCl-induced oxidative stress through up-regulation of the antioxidant system, ascorbate-glutathione cycle and glyoxalase system. PloS One, https://doi.org/10.1371/journal.pone.0202175

Ali H, Khan E, Sajad MA (2013): Phytoremediation of heavy metals--concepts and applications. Chemosphere 91: 869-81

Apse MP, Blumwald E (2002): Engineering salt tolerance in plants. Current Opinion in Biotechnology, 13: 146-150

Arena C, Figlioli F, Sorrentino MC, Izzo LG, Capozzi F, Girodano S, Spagnuolo V (2017): Ultrastructural, protein and photosynthetic alterations induced by Pb and Cd in *Cynara cardunculus* L., and its potential for phytoremediation. Ecotoxicology and Environmental Safety, 145: 83-89

Ashraf M, Foolad MR (2007): Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany, 59: 206-216

Astier C, Gloaguen V, Faugeron C (2014): Phytoremediation of cadmium-contaminated soils by young Douglas fir trees: effects of cadmium exposure on cell wall composition. International Journal of Phytoremediation 16:790-803

Atici Ö, Ağar G, Battal P (2005): Changes in phytohormone contents in chickpea seeds germinating under lead or zinc stress Biologia Plantarum 49: 215-222.

Axelsen KB, Palmgren MG. Inventory of the superfamily of P-type ion pumps in Arabidopsis. Plant Physiol 2001; 126:696 - 706

Bai J, Zhao Q, Wang W, Wang X, Jia J, Cui B, Liu X (2019): Arsenic and heavy metals pollution along a salinity gradient in drained coastal wetland soils: Depth distributions, sources and toxic risks. Ecological Indicators 96: 91-98.

Bakshi P, Handa N, Gautam V, Kaur P, Sareen S, Mir BA, Bhardwaj R (2019): Chapter 19 - Role and Regulation of Plant Hormones as a Signal Molecule in Response to Abiotic Stresses. Plant Signaling Molecular: 303-317

Balen B, Tkalec M, Sikic S, Tolic S, Cvjetko P, Pavlica M, Vidakovic-Cifrek Z (2011): Biochemical responses of *Lemna minor* experimentally exposed to cadmium and zinc. Ecotoxicology 20: 815-26

Benavides MP, Groppa MD, Recalde L, Verstraeten SV (2018): Effects of polyamines on cadmium- and copper-mediated alterations in wheat (*Triticum aestivum L*) and sunflower (*Helianthus annuus L*) seedling membrane fluidity. Archives of Biochemistry and Biophysics, 654: 27-39.

Bera S, De AK, Adak MK (2019): Modulation of glycine betaine accumulation with oxidative stress induced by aluminium toxicity in rice. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 89: 291-301

Chen THH, Murata N (2008); Glycinebetaine: an effective protectant against abiotic stress in plants. Trends Plant Science 23:9

Cheng M, Kopittke PM, Wang A, Tang C, 2019. Salinity decreases Cd translocation by altering Cd speciation in the halophytic Cd-accumulator *Carpobrotus rossii*. Annals of Botany 123:121-132.

Cherif J, Derbel N, Nakkach M, von Bergmann H, Jemal F, Lakhdar ZB (2012): Spectroscopic studies of photosynthetic responses of tomato plants to the interaction of zinc and cadmium toxicity. J Photochemistry and Photobiology B 111: 9-16.

Clemente R, Walker DJ, Pardo T, Martínez-Fernández D, Bernal PL, 2012. The use of halophytic plant species and organic amendments for the remediation of a trace elements-contaminated soil under semi-arid conditions. Journal of Hazardous Material 223/224: 63-71.

Colebrook EH, Thomas SG, Phillips AL, Hedden P (2014): The role of gibberellin signalling in plant responses to abiotic stress. Journal of Experimental Biology, 217: 67-75

Dalton HL, Blomstedt CK, Neale AD, Gleadow R, DeBoer KD, Hamill JD (2016): Effects of down-regulating ornithine decarboxylase upon putrescine-associated metabolism and growth in *Nicotiana tabacum* L. Journal of Experimental Botany, 67: 3367–3381.

Douchiche O, Soret-Morvan O, Chaibi W, Morvan C, Paynel F (2010): Characteristics of cadmium tolerance in 'Hermes' flax seedlings: contribution

of cell walls. Chemosphere 81, 1430-6

Dresler S, Wojciak-Kosior M, Sowa I, Stanislawski G, Bany I, Wojcik M (2017): Effect of short-term Zn/Pb or long-term multi-metal stress on physiological and morphological parameters of metallicolous and nonmetallicolous *Echium vulgare* L. populations. Plant Physiology and Biochemistry 115: 380-389.

Fu XP, Dou CM, Chen YX, Chen XC, Shi JY, Ye MG, Xu J (2011): Subcellular distribution and chemical forms of cadmium in *Phytolacca americana L*. Plant Science, 186(1): 103-107

Gao SP, Xiao YH, Xu F, Gao XK, Cao SY, Zhang FX, Wang GD, Sanders D, Chu CC (2019): Cytokinin‐dependent regulatory module underlies the maintenance of zinc nutrition in rice. New phytologist, https://doi.org/10.1111/nph.15962

Gerhardt KE, Gerwing PD, Greenberg BM (2017) Opinion: taking phytoremediation from proven technology to accepted practice. Plant Science 256: 170-185

Ghanem M, Han RM, Classen B, Quetin-Leclerq J, Mahy G, Ruan CJ, Qin P, Perez-Alfocea F, Lutts S (2010): Mucilage and polysaccharides in the halophyte plant species *Kosteletzkya virginica*: localization and composition in relation to salt stress. Journal of Plant Physiology 167: 382-92

Guerinot ML (2000): The ZIP family of metal transporters. Biochimica & Biophysica Acta 1465: 190-8.

Hajibagheri MA, Hall JL, Flowers TJ (1984): Stereological analysis of leaf cells of the halophyte *Suaeda maritima* (L.) Dum. Journal of Experimental Botany 35: 1547–1557

Han R-M, Lefèvre I, Ruan C-J, Beukelaers N, Qin P, Lutts S (2011): Effects of salinity on the response of the wetland halophyte *Kosteletzkya virginica* (L.) Presl. to copper toxicity. Water, Air, & Soil Pollution 223: 1137-1150

Han R-M, Lefèvre I, Ruan C-J, Qin P, Lutts S (2012): NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regulation 68: 97-109

Han RM, Lefevre I, Albacete A, Pérez-Alfocea F, Barba-Espin G, Diaz-Vivancos P, Quinet M, Ruan CJ, Hernandez JA, Cantero-Navarro E, Lutts S (2013): Antioxidant enzyme activities and hormonal status in

response to Cd stress in the wetland halophyte *Kosteletzkya virginica* under saline conditions. Physiologia Plantarum 147: 352-68

Herce-sesa B, López-López L, Moreno C (2019): Multi-elemental ionic liquid-based solvent bar micro-extraction of priority and emerging trace metallic pollutants (Cd, Ag, Pd) in natural waters. Journal of Hazardous Materials, 370: 63-69

Hicks D, Yang J (2019): NaAtm1: Studying a Heavy-Metal ABC Transporter System. The FASEB Journal, 33: Experimental Biology 2019 Meeting

Houri T, Khairallah Y, Zahab AA, Osta B, Romanos D, Haddad G (2019): Heavy Metals Accumulation Effects on The Photosynthetic Performance of Geophytes in Mediterranean Reserve. Journal of King Saud University - Science, in press

Hu GH, Huang SH, Chen H, Wang Fei (2010): Binding of four heavy metals to hemicelluloses from rice bran. Food Research International, 43: 203-206

Hu Y, Lu L, Tian S, Li S, Liu X, Gao X, Zhou W, Lin X (2019): Cadmium-induced nitric oxide burst enhances Cd tolerance at early stage in roots of a hyperaccumulator *Sedum alfredii* partially by altering glutathione metabolism. Science of Total Environment 650: 2761-2770

Jozefczak M, Keunen E, Schat H, Bliek M, Hernandez LE, Carleer R, Remans T, Bohler S, Vangronsveld J, Cuypers A (2014): Differential response of *Arabidopsis* leaves and roots to cadmium: Glutathione-related chelating capacity vs antioxidant capacity. Plant Physiology and Biochemistry 83: 1-9

Kim RY, Yoon JK, Kim TS, Yang JE, Owens G, Kim KR (2015): Bioavailability of heavy metals in soils: definitions and practical implementation--a critical review. Environment Geochemistry & Health 37, 1041-61

Kucera B, Cohn MA and Leubner-Metzger G (2005): Plant hormone interactions during seed dormancy release and germination. Seed science research, 15: 281-307

Kumar V, Sharma A, Kaur P, Sidhu GPS, Bali AS, Bhardwaj R, Thukral AK, Cerda A (2019): Pollution assessment of heavy metals in soils of India and ecological risk assessment: A state of the art. Chemosphere 216: 449-462.

Le Gall H, Phillipe F, Domon JM, Gillet F, Pelloux J, Rayon C (2015): Cell wall metabolism in response to abiotic stress. Plants: 112-166

Lambrechts T, Gusto Q, Couder E, Houben D, Iserentant A, Lutts S. 2011. Comparison of EDTA-enhanced phytoextraction and phytostabilisation strategies with *Lolium perenne* on a heavy metal contaminated soil. Chemosphere 85: 1290-1298.

Lefèvre I, Marchal G, Meerts P, Corréal E, Lutts S (2009): Chloride salinity reduces cadmium accumulation by the Mediterranean halophyte species *Atriplex halimus* L. Environmental Experimental Botany 65: 142-152.

Lefèvre I, Corréal E, Faz-Cano A, Zanuzzi A, Lutts S. 2009. Structural development, water status, pigment concentrations and oxidative stress of *Zygophyllum fabago* seedlings in relation to cadmium distribution in the shoot organs. International Journal of Plant Science 170: 226-236.

León P and Sheen J (2003): Sugar and hormone connections. Trends in Plant Science 8: 110-116.

Li X, Wang X, Yang X, Cui Z (2019): Bioaugmentation assisted phytoremediation of lead and salinity co-contaminated soil by *Suaeda salsa* and *Trichoderma asperellum*. Chemosphere 224: 716-725

Lokhande VH, Srivastava S, Patade YY, Dwivedi S, Tripathi RD, Nikam TD, Suprasanna P (2011) Investigation of arsenic accumulation and tolerance potential of *Sesuvium portulacastrum* (L.) L. Chemosphere, 82: 529-534

Luo HY, Gao HB, Gao ZK, Xia QP, Wang M, Li XJ (2010): Reactive oxygen species metabolism and chlorophyll fluorescence parameters in tomato seedlings under NaCl stress with exogenous cytokinins treatment. Acta Botanica Boreali-Occidentalia Sinica 30:1852-1858

Lutts S, Hausman JF, Quinet M, Lefevre I. 2013. Polyamines and their roles in the alleviation of ion toxicities in plants. In: P Ahmad, MM Azooz, MNV Prasad, eds. Ecophysiology and responses of plants under salt stress. New York: Springer, Science þ business media, 315–353.

Lutts S, Lefèvre I, Delpérée C, Kivits S, Dechamps C, Robledo A, Correal E (2004): Heavy metal accumulation by the halophyte species Mediterranean saltbush. Journal of Environmental Quality 33: 1271-1279.

Lutts S, Lefèvre I. 2015. How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? Annals of Botany 115: 509-528.

Mauch-Mani B and Mauch F (2005): The role of abscisic acid in plant–pathogen interactions. Current Opinion in Plant Biology, 8: 409-414

Minocha R, Majumdar R, Minocha SC (2014): Polyamines and abiotic stress in plants: a complex relationship. Frontiers in Plant Science 5:175.

Nakanishi-Masuno T, Shitan N, Sugiyama A, Takanashi K, Inaba S, Kaneko S, Yazaki K (2018): The *Crotalaria juncea* metal transporter CjNRAMP1 has a high Fe uptake activity, even in an environment with high Cd contamination. International Jouranl of Phytoremediation, 20: 14

Peleg Z and Blumwald E (2011): Hormone balance and abiotic stress tolerance in crop plants. Current Opinion in Plant Biology, 14: 290-295

Qin P, Han RM, Zhou MX, Zhang HS, Fan LS, Seliskar DM, Gallagher JL (2015) Ecological engineering through the biosecure introduction of *Kosteletzkya virginica* (seashore mallow) to saline lands in China: a review of 20 years of activity. Ecological Engineering 74: 174-186.

Quinet M, Ndayirajige A, Lefèvre I, Lambillotte B, Dupont-Gillain CC, Lutts S (2010.) Putrescine differently influences the effect of salt stress on polyamine metabolism and ethylene synthesis in rice cultivars differing in salt-resistance. Journal of Experimental Botany 61: 2719-2733

Radyukina NL, Kartashov AV, Ivanov YuV, Shevyakova NI and Kuznetsov VlV (2007b) Functioning of defence systems in halophytes and glycophytes under progressing salinity. Russian Journal Plant Physiology 54:806–815

Ramos I, Esteban E, Lucena JJ, Garate A (2002): Cadmium uptake and subcellular distribution in plants of *Lactuca* sp. Cd–Mn interaction. Plant Science, 162:761-767

Sengupta A, Chakraborty M, Saha J, Gupta B, Gupta K (2016): Polyamines: Osmoprotectants in Plant Abiotic Stress Adaptation. 97-127

Sruthi P, Shackira AM, Puthur JT (2017) Heavy metal detoxification mechanisms in halophytes: an overview. Wetlands Ecological Management 25: 129-148.

Tair HAA, El-Yazal MAS, Ahmed SMA, Rady MM (2019): Polyamines modulate growth, antioxidant activity, and genomic DNA in heavy metal–stressed wheat plant. Environmental Science and Pollution Research, 26: 22338–22350

Takahashi R, Ishimaru Y, Shimo H, Ogo Y, Senoura T, Nishizawa NK, et al.

(2012) The OsHMA2 transporter is involved in root-to-shoot translocation of Zn and Cd in rice. Plant Cell Environment 35: 1948-57

Tang L, Yao A, Yuan M, Tang Y, Liu J, Liu X, Qiu R (2016): transcriptional upregulation of genes involved in photosynthesis of the Zn/Cd hyperaccumulator *Sedum alfredii* in response to zinc and cadmium. Chemosphere 164: 190-200.

Tiburcio AF, Altabemlla T, Bitrián M, Alcázar R (2014): The roles of polyamines during lifespan of plants: from development to stress. Planta 240:1-18.

Tkalec M, Stefanic PP, Cvjetko P, Sikic S, Pavlica M, Balen B (2014): The effects of cadmium-zinc interactions on biochemical responses in tobacco seedlings and adult plants. PLoS One 9, e87582

Tang CF, Zhang RQ, Hu XJ, Song JS, Li B, Ou DL, Hu X, Zhao YL (2018): Exogenous spermidine elevating cadmium tolerance in *Salix matsudana* involves cadmium detoxification and antioxidant defense. International journal of phytoremediation, https://doi.org/10.1080/15226514.2018.1524829

Van Oosten and Maggio A (2015): Functional biology of halophytes in the phytoremediation of heavy metal contaminated soils. Environmental and Experimental Botany, 11: 135-146

Vaseem H, Singh VK, Singh MP (2017): Heavy metal pollution due to coal washery effluent and its decontamination using a macrofungus, *Pleurotus ostreatus*. Ecotoxicology and Environmental Safety 145: 42-49

Verma V, Ravindran P, Kumar PP (2016): Plant hormone-mediated regulation of stress responses. BMC plant biology, https://doi.org/10.1186/s12870-016-0771-y

Vromman D, Lefèvre I, Šlejkovec Z, Martínez JP, Vanhecke N, Briceño M, Kumar M, Lutts S (2016): Salinity influences arsenic resistance in the xerohalophyte *Atriplex atacamensis Phil.* Environmental and Experimental Botany, 126: 32-43

Wang SL, Xu XR, Sun YX, Liu JL, Li HB (2013): Heavy metal pollution in coastal areas of South China: a review. Marine Pollution Bulletin 76; 7-15

Williams LE, Mills RF. (2005) $P_{(1B)}$-ATPases--an ancient family of transition

metal pumps with diverse functions in plants. Trends in Plant Science 10:491 - 502

Wilkinson S, Kudoyarova GR, Veselov DS, Arkhipova TN, Davies WJ (2012): Plant hormone interactions: innovative targets for crop breeding and management. Journal of Experimental Botany, 63: 3499-3509

Wang SF, Zhao Y, Guo JH, Zhou JL (2016): Effects of Cd, Cu and Zn on *Ricinus communis* L. Growth in single element or co-contaminated soils: Pot experiments. Ecological Engineering, 90: 347-351

Wang Y, Wang XL, Wang C, Peng F, Wang RJ, Xiao X, Zeng J, Kang HY, Fan X, Sha J, Zhang HQ, Zhou YD (2017): Transcriptomic profiles reveal the interactions of Cd/Zn in dwarf polish wheat (*Triticum polonicum* L.) roots. Front Physiol, 8: 168

Weng BS, Xie XY, Weiss DJ, Liu JC, Lu HL, Yan CL (2012): *Kandelia obovata* (S., L.) Yong tolerance mechanisms to cadmium: subcellular distribution, chemical forms and thiol pools. Marine Pollution Bulletin 64: 2453-2460.

Wu XX, He J, Ding HD, Zhu ZW, Chen JL, Xu S, Zha DS (2015): Modulation of zinc-induced oxidative damage in *Solanum melongena* by 6-benzylaminopurine involves ascorbate–glutathione cycle metabolism. Environmental and Experimental Botany, 116: 1-11

Xu QS, Chu WY, Qiu H, Fu YY, Cai SJ, Sha S (2013): Responses of *Hydrilla verticillata* (L.f.) Role to zinc: in situ localization, subcellular distribution and physiological and ultrastructural modifications. Plant Physiology and Biochemistry 69: 43-48

Yang Q, Li Z, Lu X, Duan Q, Huang L, Bi J (2018) A review of soil heavy metal pollution from industrial and agricultural regions in China: Pollution and risk assessment. Science of the Total Environment 642: 690-700

Yashi D. Formation of putrescine from ornithine and arginine in tobacco plants. Plant and Cell Physiology, 10: 393–397

Zhang HW, Zhao FG, Tang RJ, Yu YX, Song JL, Wang Y, Li LG, Luan S (2016): Two tonoplast MATE proteins function as turgor-regulating chloride channels in *Arabidopsis*. PNAS, 114: 2036-2045

Zhang J, Li H, Zhou Y, Dou L, Cai L, Mo L, You J (2018): Bioavailability and soil-to-crop transfer of heavy metals in farmland soils: A case study in the

Pearl River Delta, South China. Environmental Pollution 235: 710-719

Zhang XD, Meng JG, Zhao KX, Chen X, Yang ZM (2018): Annotation and characterization of Cd-responsive metal transporter genes in rapeseed (*Brassica napus*). BioMetals 3:107-121

Conclusion

Our study confirms the initial hypothesis that a « mixed » toxicity is inducing a specific physiological status, which should not be regarded as the simple additive impacts of each pollutant. Salinity improves the capacity of the plant to cope with a mixture of Cd and Zn and this improvement is not only due to a decrease in heavy metals absorption or growth stimulation but also to a modification of the plant physiological properties sustaining heavy metal resistance. This conclusion is verified by our results referring to the plant physiological and biochemical response at individual plant organ and whole plant level, during short-term HMs combined exposure at young seedling stage in nutrient solution and long-term exposure till reproductive stage in combined HM-contaminated soil, which include the following facts:

1. All plants survive and grow in the simultaneous presence of Cd and Zn. Cadmium mainly accumulates in the cell walls, while Zn is evenly distributed in cytoplasm and cell walls. Both accumulations interfere with photosynthesis resulting in oxidative stress. Salinity reduces Cd and Zn bioaccumulation and translocation, mitigating the deleterious impact of heavy metals on photosynthetic process and oxidative status.
2. Mixed exposure increases ethylene synthesis and induces a specific polyamines pattern in relation to increase in senescence processes. Exogenous *trans*-zeatin riboside increases plant growth and reduces oxidative stress in Zn-treated plants maintained in the absence of NaCl. Plants exposed to the mixed treatment exhibit a specific hormonal status in relation to accumulation of ABA and depletion of SA.
3. *K. pentacarpos* triggers an adaptative response in terms of osmotic adjustment through osmocompatible solutes (proline and GB) metabolism when exposed to NaCl and mixed

pollution.

4. *K. pentacarpos* increased mucilage production in plant organs in response to Zn excess with a rise in uronic acids in roots and stems. Changes are also observed in neutral monosaccharides components and polysaccharide structure in different plant organs.
5. Cultivating plants on the polluted soil reduce the volume of leachate. Salinity differently affects the heavy metals' bioavailability. It also reduces the total amounts of heavy metals removed by the leachate and decreases the proportion of Cd and Zn removed by the plants.
6. Polymetallic and As toxicity in soil inhibit plant growth and reproductive process during long-term exposure. NaCl and chelating agent EDDS differently affect pollutant accumulation in stem and leaf, respectively. NaCl makes a modification of the cell wall constituent, while EDDS has limited impact on it.

K. pentacarpos is not regarded as a hyperaccumulating plant species since it is not able to accumulate pollutants in plant tissue up to the hyperaccumulator level. At the same time, salinity reduces heavy metals absorption, translocation and accumulation. However, salinity improves the capacity of the plant to cope with a mixture of heavy metals with specific plant physiological properties sustaining heavy metal resistance. Therefore, *K. pentacarpos* is recommended for the management of saline contaminated by simultaneous multiple heavy metal pollutants.

Scientific Achievements

Peer-reviewed journal papers

- **Zhou MX**, Classen B, Agneessens R, Godin B, Lutts S. **2019**. Influence of salinity on mucilage and polysaccharides in *Kosteletzkya pentacarpos* under zinc stress condition**. Reviewing in International Journal of Environmental Research**

- **Zhou MX**, Renard M-E, Quinet M, Lutts S. **2019**. Effect of NaCl on proline and glycinebetaine metabolism in *Kosteletzkya pentacarpos* simultaneously exposed to Cd and Zn toxicities. **Plant and Soil 441(1-2), 525-542. IF: 3.259 (2019)**

- **Zhou MX**, Ghnaya T, Dailly H, Cui GL, Vanpee B, Han RM, Lutts S. **2019**. The cytokinin *trans*-zeatin riboside increased resistance to heavy metal in the halophyte plant in the absence but not in the presence of NaCl. **Chemosphere 233, 954-965. IF: 5.108 (2019)**

- **Zhou MX**, Thibaut E, Lutts S. **2019**. Salinity modifies heavy metals and arsenic absorption by the halophyte plant species *Kosteletzkya pentacarpos* and pollutant leaching from a polycontaminated substrate. **Ecotoxicology and Environmental Safety 182, 109460. IF: 4.527 (2019)**

- **Zhou MX**, Han RM, Ghnaya T, Lutts S. **2018**. Salinity influences the interactive effects of cadmium and zinc on ethylene and polyamine synthesis in the halophyte plant species *Kosteletzkya pentacarpos*. **Chemosphere 209, 892-900. IF: 5.108 (2019)**

- **Zhou MX**, Dailly H, Renard M-E, Han RM, Lutts S. **2018**. NaCl impact on *Kosteletzkya pentacarpos* seedlings simultaneously exposed to cadmium and zinc toxicities. **Environmental Science and Pollution Research 25(18), 17444-17456. IF: 2.901 (2019)**