



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

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Abstract

Background and aims 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.

Methods 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 48 h. Osmolytes concentrations, enzyme activities involved in their metabolism and expression of corresponding genes were determined in roots and leaves.

Results 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.

Conclusion 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

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

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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; Han et al. 2012b; 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 an 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 (Zhou et al. 2018b). It also increased oxidative stress and hasten senescence although additional NaCl was shown to reduce heavy metal impact on these parameters (Zhou et al. 2018a, 2019). 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 fold in 300 mM NaCl-treated plants than control plants already after 24 h 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 Malvaceae 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.

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.

Material and methods

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 12 h photoperiod [mean light intensity (PAR) = $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by Osram Sylvania (Danvers, MA) fluorescent tubes (F36 W/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 50 L tanks into a greenhouse, with 12 plants per tank. The nutrient solution contained the following chemicals (in mM): 2.0 KNO₃, 1.7 Ca(NO₃)₂, 1.0 KH₂PO₄, 0.5 NH₄NO₃, 0.5 MgSO₄ and (in μM) 17.8 Na₂SO₄, 11.3 H₃BO₃, 1.6 MnSO₄, 1 ZnSO₄, 0.3 CuSO₄, 0.03 (NH₄)₆Mo₇O₂₄ and 14.5 Fe-EDDHA. Minimum temperatures were 16–18 °C and daily maxima were 24–28 °C. Natural light was supplemented by Philips lamps (Philips Lighting S.A., Brussels, Belgium) (HPLR 400 W) in order to maintain a light irradiance of $280 \mu\text{mol m}^{-2} \text{s}^{-1}$ (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 CdCl₂ (3) 40 μM CdCl₂ (4) 200 μM ZnCl₂ (5) 400 μM ZnCl₂ (6) 20 μM CdCl₂ + 200 μM ZnCl₂ and (7) 40 μM CdCl₂ + 400 μM ZnCl₂. Heavy metal doses were chosen on the basis of our previous results (Han 2013; Han et al., 2012b, 2013a, b) 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 48 h heavy metal stress.

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 72 h to estimate dry weight and water content and to determine ion content.

For osmotic potential determination (Ψ_s), 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 9000 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).

Ion concentration

Dried samples were ground to a fine powder using a porcelain mortar and a pestle, digested in 35% HNO₃ and evaporated to dryness on a sand bath at 80 °C. The minerals were incubated with a mix of 37% HCl and 68% HNO₃ (3:1) and the mixture was slightly evaporated. Minerals were dissolved in HCl 0.1 N 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.

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 5 mL of 5% salicylic acid. After centrifugation at 5000 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 deionized water at 20 °C. Extracts were diluted with 2 N H₂SO₄ at 1:1 v/v and cooled in ice water for 1 h. Cold KI-I₂ 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 16 h 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 H₂SO₄.

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 MgCl₂, 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 (NH₄)₂SO₄ 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 Δ¹-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 Na₂CO₃ 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 (1986). Extraction was performed in 0.1 M 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.

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 4000 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 3 M 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*).

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.

Results

Plant growth and water status

Short term (48 h) treatment did not induce plant mortality but necrosis was observed in petiole and stem after 48 h 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

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
<i>EF-1α</i>	NM_001327239	<i>EF-F</i>	GGTCATTCAAGTATGCCTGG	54	32
		<i>EF-R</i>	GAACCCAACATTGTCACCAG		
<i>18SrRNA</i>	AY739080	<i>18S-F</i>	GAGTATGGTCGCAAGGCTGAA	55	32
		<i>18S-R</i>	CCTCTAAATGATAAGGTTTCAGTGG		
<i>KvP5CS1</i>	KR029088.1	<i>P5CS-F</i>	TTCAAGGGAAGCGTGTGGT	57	35
		<i>P5CS-R</i>	AACAAGAGCGTCTGGTCGAG		
<i>KvOAT</i>	KR029089.1	<i>OAT-F</i>	TTGGGTGGTGGCGTAATACC	57	31
		<i>OAT-R</i>	ATAGTGTCGTGCGTGGGTTT		
<i>KvProT</i>	KR029091.1	<i>ProT-F</i>	TGCTTGCAGCTAAAGACGGA	57	31
		<i>ProT-R</i>	ACGCGCTTCCTCTAATACCG		
<i>KvPDH</i>	KR029090.1	<i>PDH-F</i>	GCTCGTTTATGCCGTCGAAC	57	35
		<i>PDH-R</i>	CCGATTCTTCTTGTGGGGCT		
<i>KvBADH</i> ^a		<i>BADH-F</i>	GTTGTGCTGCAATACTGAAG	55	32
		<i>BADH-R</i>	TAAGACGGGATGTTGCACTG		

^a The primers design was present in figure S1

Fig. 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)

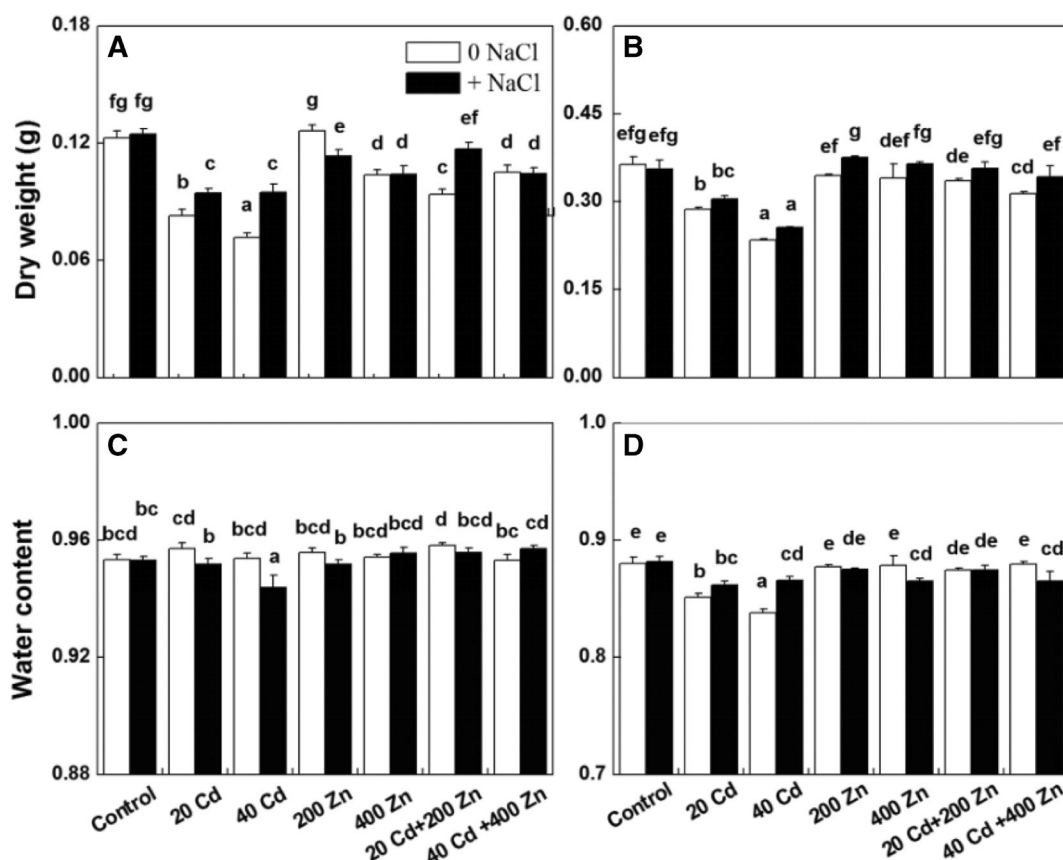
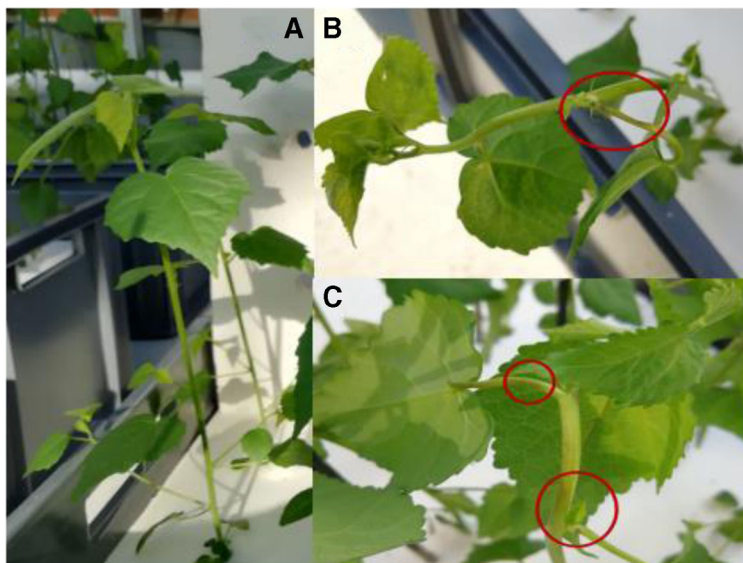


Fig. 2 Root (a) and leaf (b) dry weight and root (c) and leaf (d) water content in seedlings of *Kosteletzkya pentacarpos* exposed to 20 μM CdCl_2 , 40 μM CdCl_2 , 200 μM ZnCl_2 , 400 μM ZnCl_2 , 20 μM CdCl_2 + 200 μM ZnCl_2 and 40 μM CdCl_2 + 400 μM

ZnCl_2 in the presence or in the absence of 50 mM NaCl during 48 h. 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

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. 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.

The 48 h 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 ψ_s values in plants that were not exposed to heavy metals. Conversely, all heavy metal treatments except 200 μM Zn significantly decreased the leaf ψ_s in the absence of NaCl, the lowest value being observed for plants exposed to 40 μM Cd. Salinity slightly increased ψ_s in these plants but it led to an additional decrease in plants exposed to 200 μM Zn and to both mixed treatment.

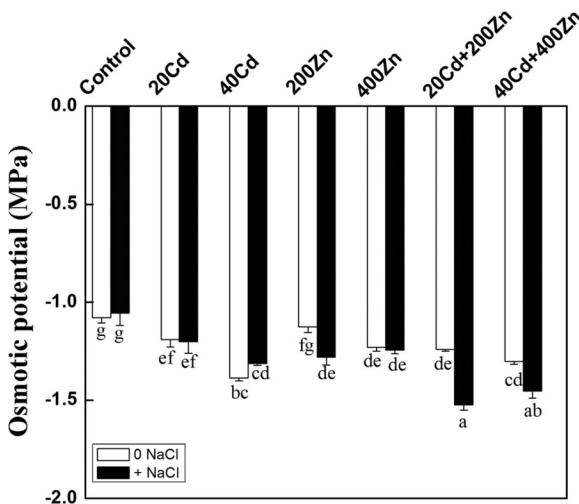


Fig. 3 Leaf osmotic potential (ψ_s) of *Kosteletzkya pentacarpos* exposed to 20 μM CdCl₂, 40 μM CdCl₂, 200 μM ZnCl₂, 400 μM ZnCl₂, 20 μM CdCl₂ + 200 μM ZnCl₂ and 40 μM CdCl₂ + 400 μM ZnCl₂ in the presence or in the absence of 50 mM NaCl during 48 h. Each value is the mean of three replicates, and vertical bars are SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test

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 ($P < 0.05$).

Cadmium had no impact on Zn accumulation in roots and leaves (Fig. 4a 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. 4c 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.

Proline and glycinebetaine accumulation in roots and leaves

After 48 h 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

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 48 h

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

Each value is the mean of three replicates $\pm$ SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test

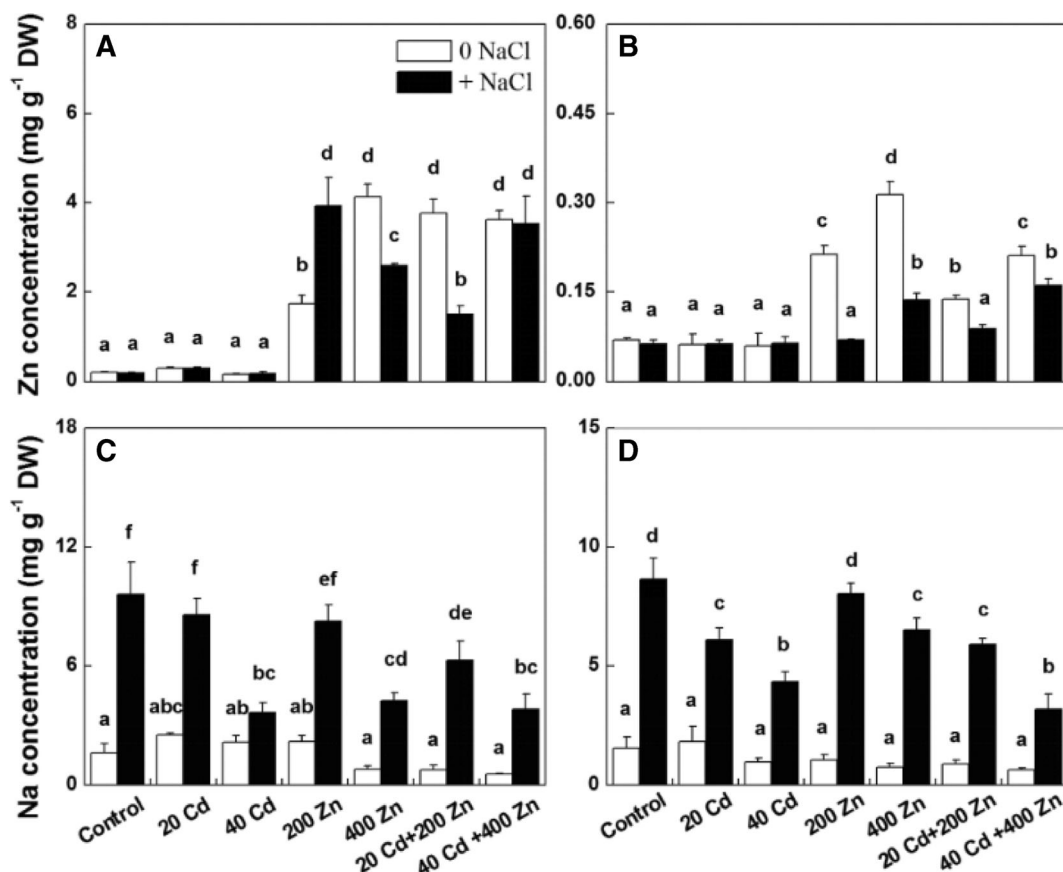
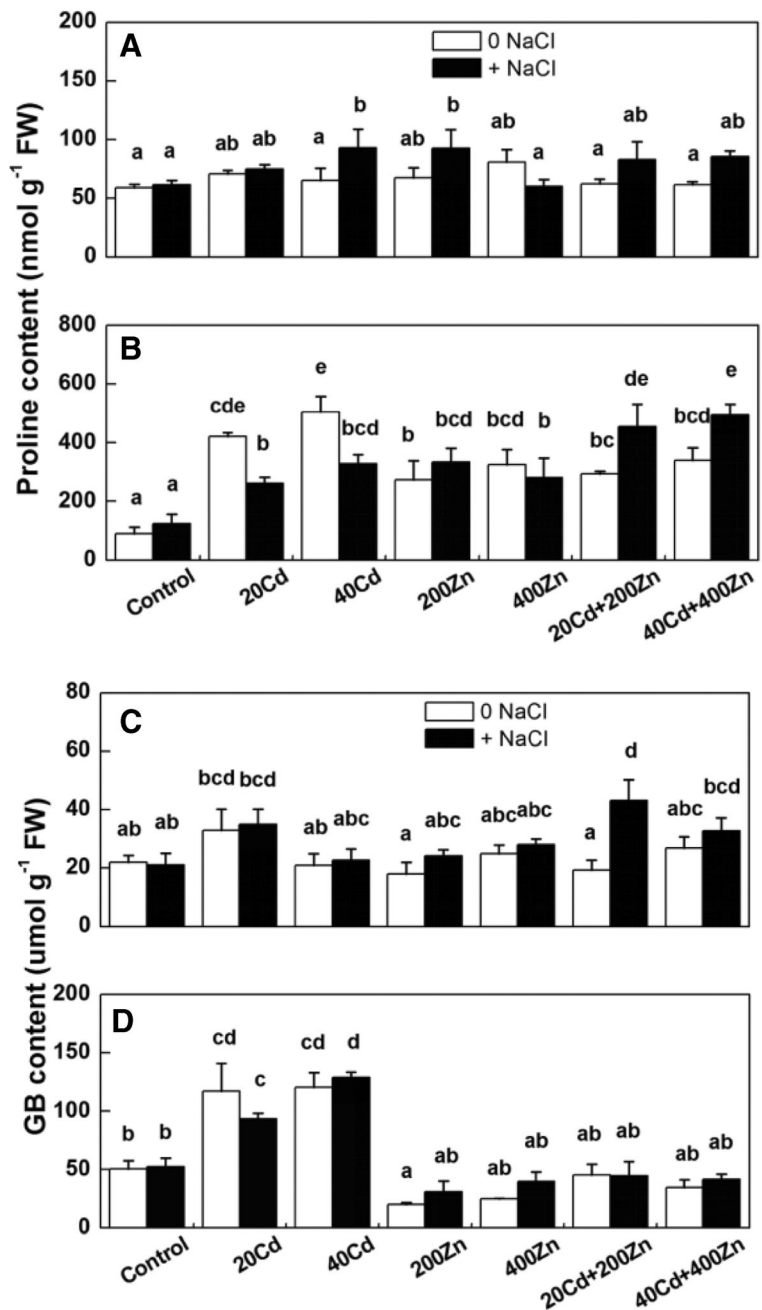


Fig. 4 Zinc accumulation and Na concentration in root (a, c) and leaves (b, d) of *Kosteletzkya pentacarpos* exposed to 20 μM CdCl_2 , 40 μM CdCl_2 , 200 μM ZnCl_2 , 400 μM ZnCl_2 , 20 μM CdCl_2 + 200 μM ZnCl_2 and 40 μM CdCl_2 + 400 μM ZnCl_2 in the

presence or in the absence of 50 mM NaCl during 48 h. 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

Fig. 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 48 h. 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



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.

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.

Enzyme activities

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 μ M 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 μ M and Cd 40 μ M, and the lowest in plants exposed

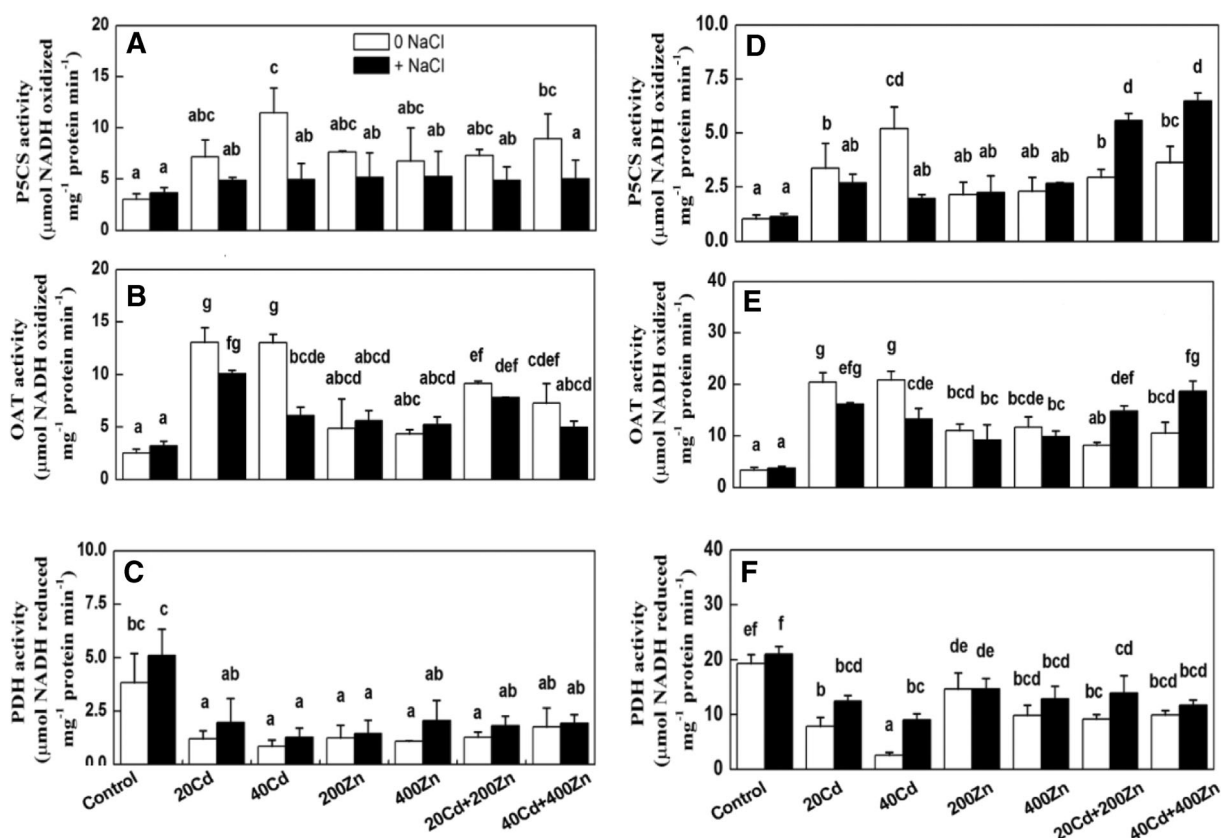


Fig. 6 Activities of Δ^1 -pyrroline-5-carboxylate synthetase (P5CS), ornithine-amino-transferase (OAT) and proline dehydrogenase (PDH) in roots (a–c) and leave (e–f) of *Kosteletzkya pentacarpus* exposed to 20 μ M CdCl₂, 40 μ M CdCl₂, 200 μ M ZnCl₂, 400 μ M ZnCl₂, 20 μ M CdCl₂ + 200 μ M ZnCl₂ and 40 μ M

CdCl₂ + 400 μ M ZnCl₂ in the presence or in the absence of 50 mM NaCl during 48 h. 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

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.

Gene expression

Gene expression after 48 h 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).

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 fold 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.

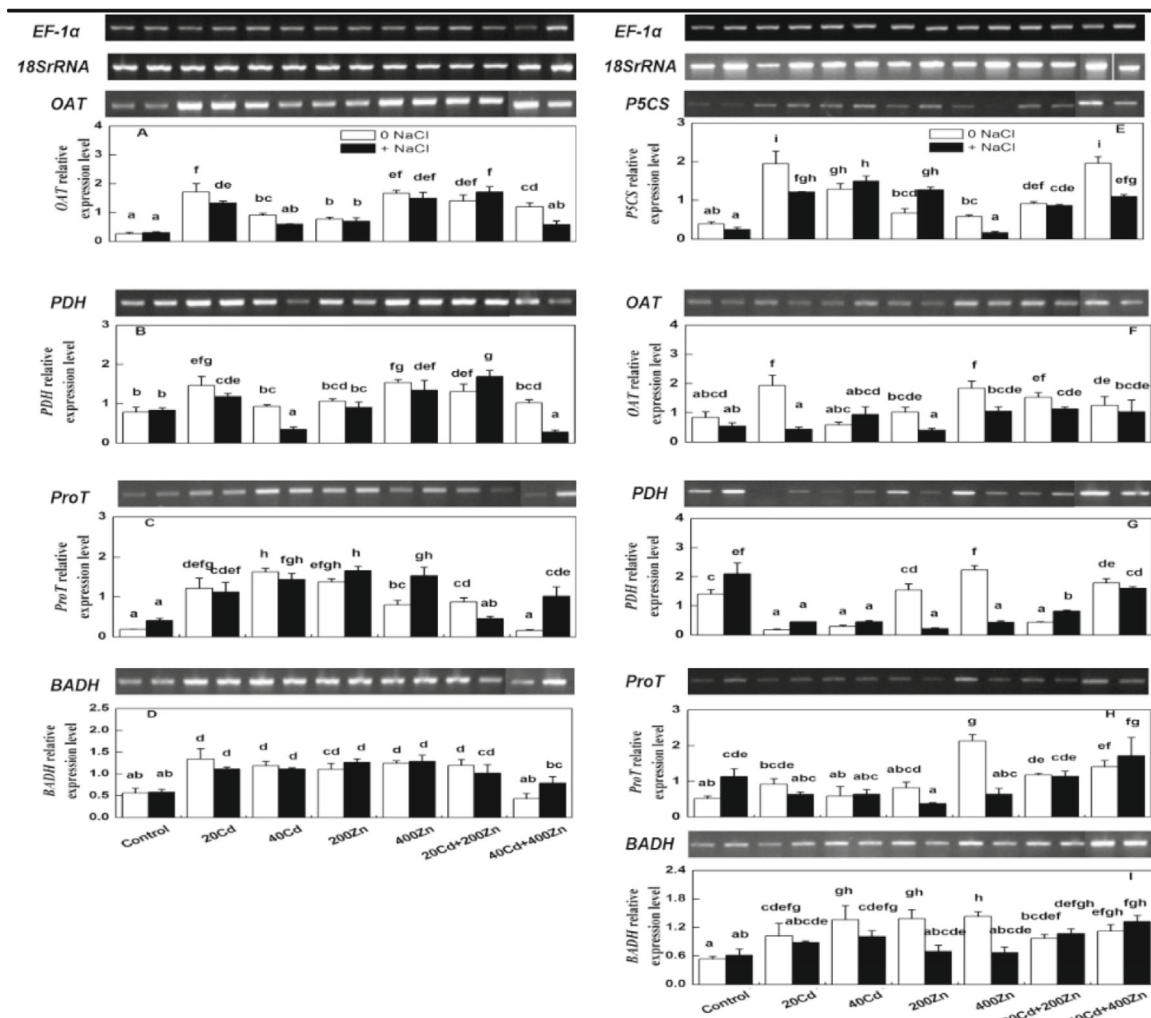
In the leaves, *KvP5CS1* was upregulated by Cd and mixed treatment (Cd + Zn) and it increased by more than 4 folds in response to Cd 20 μM and Cd 40 + Zn 400; in both cases, additional NaCl decreased *KvP5CS1* expression ($P < 0.05$). Expression of *KvOAT* was significantly increased in plants exposed 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

Table 3 Betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8) activities ($\mu\text{mol NAD}^+ \text{mg}^{-1} \text{prot min}^{-1}$) in roots and leaves 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 48 h

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

Each value is the mean of three replicates $\pm$ SE. Values exhibiting different letters are significantly different at $P < 0.05$ according to Tukey's test



ROOTS

Fig. 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 μ M CdCl_2 , 40 μ M CdCl_2 , 200 μ M ZnCl_2 , 400 μ M ZnCl_2 , 20 μ M CdCl_2 + 200 μ M ZnCl_2 and 40 μ M CdCl_2 + 400 μ M

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 μ M 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.

LEAVES

ZnCl_2 in the presence or in the absence of 50 mM NaCl during 48 h. Expression of *KvP5CS* 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

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 Saviouré 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 48 h. 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 osmotic 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.

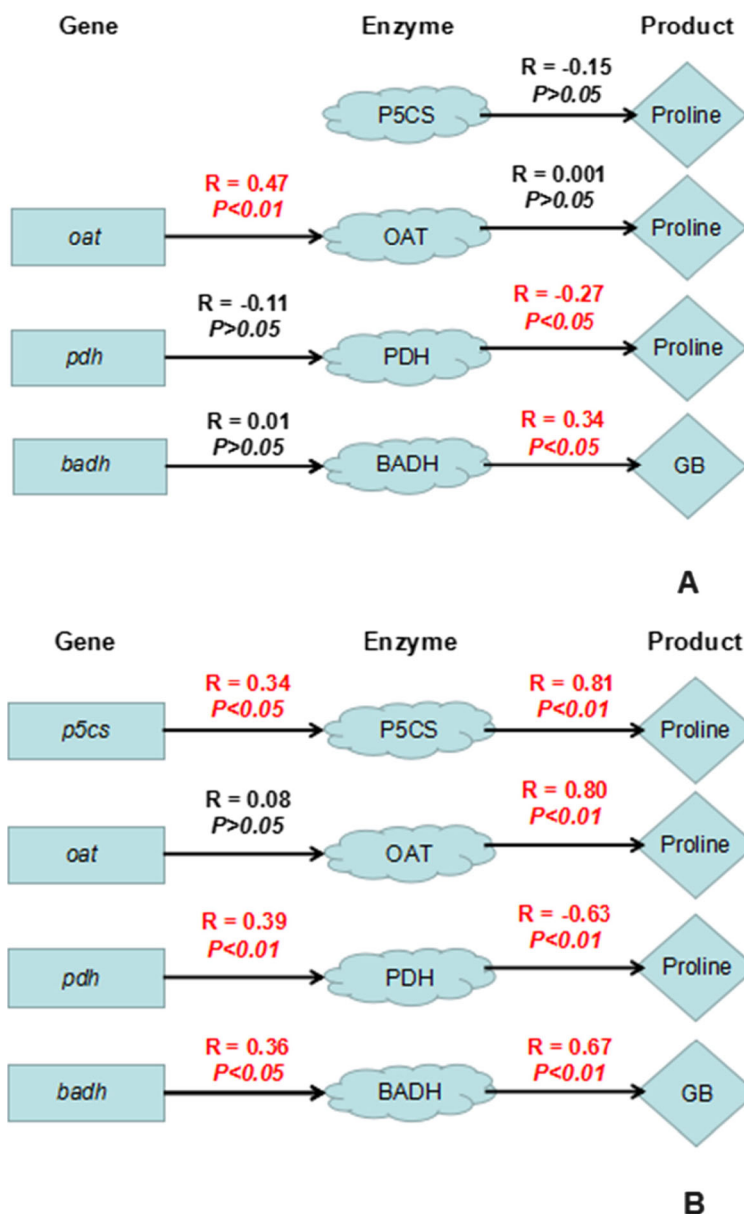
In numerous studies devoted to abiotic stresses, proline accumulation is attributed to increased P5CS activities (Szabados and Saviouré 2010; Zhang and Becker 2015) and to inhibition of PDH (Lutts et al. 1999b; Szabados and Saviouré 2010). In most cases, these modifications are, at least partly, attributed to the transcriptional regulation of the corresponding gene (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). 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 *KvP5CS1* 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 *KvP5CS1* 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 Saviouré 2010; Kavi Kishor and Sreenivasulu 2014; Zhang and Becker 2015; Kaur and Asthir 2015). In a previous work (Zhou et al. 2019 under review), 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

Fig. 8 Correlation analysis between gene expression, enzyme activities and final product in *Kosteletzkya pentacarpos*: data are pooled for all treatments (Control, 20 μ M CdCl₂, 40 μ M CdCl₂, 200 μ M ZnCl₂, 400 μ M ZnCl₂, 20 μ M CdCl₂ + 200 μ M ZnCl₂ and 40 μ M CdCl₂ + 400 μ M ZnCl₂ in the presence or in the absence of 50 mM NaCl) and are presented separately for roots (a) and leaves (b). Significant correlation are presented in red and non-significant correlation in black letters



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 *Lactuca sativa* even if proline is not clearly involved in Zn resistance in this species.

Leaf proline content was negatively correlated with Ψ_s ($r = -0.84$; $P < 0.001$) which support the involvement of proline in osmotic adjustment. In Cd-treated plants, however, NaCl decreased proline content but did not affect Ψ_s 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). Zhou et al. (2018a) reported that heavy metal induced oxidative stress in *K. pentacarpos* as indicated by an increase in malondialdehyde and H₂O₂ content while salinity reduced H₂O₂ production in these heavy metal-treated plants. Since H₂O₂ is involved in signal

transduction leading to *P5CS1* gene activation (Szabados and Saviouré 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 μ M 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. Zhou et al. (2018a) 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 48 h of treatment while Zhou et al. (2018a) studied Cd distribution after 2 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 Zhou et al. (2018a) 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 Saviouré 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 Saviouré 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 and Sreenivasulu 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 Saviouré 2010; Kavi Kishor and Sreenivasulu 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 and Sreenivasulu 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 (Fugiwara 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 are regulated by different cues, as previously mentioned for other halophyte (Ben Hassine et al. 2008) even if their transport may use the same transporters.

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-photosynthetic 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 2017). 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 Ψ_s . When data are pooled for all treatments, there was no significant correlation between Ψ_s 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. pentacarpus* 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 than Zn on proline and GB content. Zhou et al. (2018b) recently reported that Cd-induced senescence in *K. pentacarpus* was mainly due to ethylene and to putrescine (Put) accumulation. Since both of these 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 Zhou et al. (2018b), salinity reduces ethylene and Put synthesis in Cd-treated plants in *K. pentacarpus*, 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. pentacarpus* 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. pentacarpus* (Zhou et al. 2018b) but this could mainly explained by an improved conversion of Put to higher polyamines (spermidine and spermine) which assume protective functions in stressed tissues.

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