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Phytochelatins and monothiols in salt marsh plants and their relation with metal tolerance

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ABSTRACT

Phytochelatins (PCs) and monothiols and their relation with trace element concentrations were studied in three plant species from two Portuguese salt marshes. Belowground tissues showed always higher element concentrations, while enhanced values of monothiols were found in aboveground biomass. Glutathione was usually the most abundant monothiol. The concentration of total PCs was higher in leaves or stems than in roots of *Halimione portulacoides* and *Sarcocornia perennis*, while in *Spartina maritima* the highest concentrations were reported in large roots. PC_2 was synthesized by all tissues and species and was higher in large roots of *S. maritima*. PC_4 and PC_5 were in high levels in small roots of *S. maritima*. PC_2 was positively correlated with As, Zn and Pb. Although being the first evidence of PCs and monothiols in these species under natural conditions, our results do not point to a simple relationship with elements concentrations, suggesting a complex mechanism involved.

1. Introduction

Salt marshes provide valuable ecosystem services, namely nutrient cycling, natural treatment to wastewaters discharged in the area, carbon sequestration in sediments and plants and shelter to wild life (Costanza et al., 1997; Gedan et al., 2009). Salt marsh sediments have long been recognized as sink for metals, and hence salt marsh plants are viewed as potential remediation agents due to their capacity for accumulating high metal concentrations in their tissues (e.g., Windham et al., 2003; Quan et al., 2007; Caetano et al., 2008; Válega et al., 2008; Caçador et al., 2009; Duarte et al., 2010; Song and Sun, 2014; Idaszkin et al., 2015; Negrin et al., 2016). Salt marsh plants appear to exhibit a unique adaption capacity to cope with metal stress, despite the general deleterious effect of these elements at various biological organization levels (Nagajyoti et al., 2010). Adaptation includes element partitioning in non-photosynthetic organs and so restricted movement towards aboveground parts, higher metal concentration in the cell wall rather than in vacuoles or cytoplasm, and binding metals to specific peptides to inactivate them (Anjum et al., 2014 and references therein). Most salt marsh species exhibited higher trace element concentration in roots than in leaves and stems (e.g., Windham et al., 2003; Caetano et al., 2008; Válega et al., 2008; Caçador et al., 2009; Duarte et al., 2010; Idaszkin et al., 2015; Negrin et al., 2016) and immobilization of trace elements in cell walls was reported in *Halimione portulacoides* and *Sarcocornia perennis* (Sousa et al., 2008; Castro et al., 2009; Válega et al., 2009; Reboredo, 2012). These adaptations allow the accumulation of metals in plant tissues without major detrimental effects, depending on the type and concentration of the contaminant, the time of exposure and the plant species (Nagajyoti et al., 2010). For example, *H. portulacoides*, *Phragmites australis* and *Juncus maritimus* showed no reduction of biomass or photosynthetic efficiency (parameters usually used as indicators of physiological stress) in experiments with Cd or Cu solutions (Almeida et al., 2009; Da Silva et al., 2015). In addition, roots of *P. australis* did not show anatomical alterations after being treated with high Cd concentrations (Ederli et al., 2004).

Plants exposed to high concentrations of trace elements may respond rapidly by the induction of the synthesis of peptides known as phytochelatins (PCs) (Rauser, 1995; Cobbett and Goldsbrough, 2002). PCs are cysteine-rich peptides with the general structure (γ -Glu-Cys)n-Gly, where n is generally between 2 and 5, that are capable of binding trace element ions via thiolate coordination. PCs are synthesized enzymatically using glutathione (GSH) as the substrate (Cobbett and Goldsbrough, 2002 and references therein). In vivo and in vitro experiments showed that this reaction is activated by Pb, Cu, Zn, Hg, Ag, and particularly by Cd and As (e.g., Mehra et al., 1996; Gupta

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et al., 1998; Srivastava et al., 2004; Mishra et al., 2006; Srivastava et al., 2006; Thangavel et al., 2007; Srivastava et al., 2011; Machado-Estrada et al., 2013). PCs bind to metals and metalloids through the sulfhydryl group of the cysteine unit forming element–PC complexes which are then accumulated in the vacuole. Those complexes are then transformed into more stable compounds (Cobbett and Goldsbrough, 2002). Apart from PCs, GSH could also be associated with trace element tolerance in plants. Together with ascorbate, GSH eliminates efficiently reactive oxygen species, which interfere in cellular metabolism and are induced by a variety of stressful conditions, including those imposed by metal contamination (Alscher, 1989; Anjum et al., 2014).

Despite the numerous works on trace element accumulation in above and belowground biomass of salt marsh species, mechanisms of tolerance are poorly understood. To the best of our knowledge, only a few works have explored the role of thiol-containing compounds in similar species. Padinha et al. (2000) found high values of total thiol-containing compounds in *Spartina maritima* of contaminated salt marshes, although PCs could not be differentiated. Ederli et al. (2004) and Da Silva et al. (2015), working with *P. australis* and/or *J. maritimus* under laboratory conditions, reported an enhanced production of thiol-containing compounds in response to Cd exposure. Válega et al. (2009) found PC production in tissues of *H. portulacoides* but did not study their precursors. Most of the studies about the production of PCs by higher plants have been done under laboratory conditions in species other than those of salt marshes (e.g., Mehra et al., 1996; Gupta et al., 1998; Srivastava et al., 2004; Mishra et al., 2006; Gadapati and Macfie, 2006; Srivastava et al., 2006; Thangavel et al., 2007; Srivastava et al., 2011). The objective of this work was to examine whether PCs and other thiol-containing compounds are synthesized by *S. maritima*, *H. portulacoides* and *S. perennis* from two salt marshes of different degree of metal contamination history (Rosário-Tagus estuary and Óbidos, Portugal). Relationships between PCs and metal concentrations in above and belowground parts of those plants were also examined.

2. Study areas

Rosário salt marsh is located in the Tagus estuary and extents for an area of 2 km^2 (Crespo, 1993) (Fig. 1). The area is fully inundated twice a day by the tides (2–4 m of tidal amplitude) through a highly branched system of channels that cross the elevation transect. Salinity varied seasonally with the river discharge, although pore water salinity showed a broader variation related with the location of the freshwater discharge, giving lower salinity values in the upper marsh; in general, salinity fluctuates between 20 and 36 (Madureira et al., 1997; Caetano et al., 2012). This salt marsh is characterized by a typical zonation with homogeneous stands of *S. maritima* as a pioneer species, colonizing bare mud in the lower marsh area. Across the elevation (20–50 cm) transect, pure stands of *H. portulacoides* follow *S. maritima*, while *Sarcocornia fruticosa* and *S. perennis* are found in the upper salt marsh.

The Óbidos lagoon is a shallow coastal ecosystem that covers a wet area of 7 km^2 and is permanently connected to the sea through a narrow inlet (Fig. 1). Tidal energy dissipates in the entire lagoon with tidal amplitudes ranging between 1 and 2 m (Oliveira et al., 2006). Water circulation is mostly driven by tides, despite the small tributary (Cal River), which drains agriculture fields, ends in the upper part of the lagoon. The dominance of evaporation coupled with negligible freshwater discharges in spring and summer leads to salinity values up to 37 all over the lagoon, although values in the study area may decrease to 26 in winter rainy periods (Pereira et al., 2009a). The abundant macroalgae *Ulva* sp. and *Enteromorpha* sp. in the upper part reflects the nutrient discharges from the watershed (Pereira et al., 2009a). The margins of the upper part of the lagoon are covered by *H. portulacoides* and *S. perennis*.

Due to their location in relation to cities and chemical industries, both salt marshes have different degree of metal contamination. Óbidos lagoon is located near a small town (Caldas da Rainha, 50,000 inhabitants) and apart from industries; hence, this system is impacted by moderate metal contamination (Pereira et al., 2009b; Table 1). There are no previous reports of concentration of metals neither in vascular plants nor vegetated sediments in Óbidos. Rosário salt marsh

Fig. 1. Location of Rosário and Óbidos salt marshes in the Tagus estuary and Óbidos lagoon, respectively.

Table 1
Trace element concentrations in water (nM) and the upper (5 cm) subtidal sediments (μg g^{-1} dry weight) in Óbidos and Rosário.

	Óbidos		Rosário	
	Water (nM)	Sediments (μg g^{-1} d.w.)	Water (nM)	Sediments (μg g^{-1} d.w.)*
Cu	3.5–6.8	41–83	0.90–45	593
Cr	3.3–8.7	72–90	nd	592
Ni	5.9–10	30–39	1.90–15.0	60
Cd	0.07–0.17	0.15–0.29	0.01–0.89	11
Pb	0.42	39–45	0.01–0.15	2858
Zn	nd	128–144	1.40–62.0	2854
As	nd	22.2	nd	1022
References	c, d, e	d, e	a	b

(a) Santos-Echeandía et al., 2012; (b) Vale et al., 2008; (c) Carvalho et al., 2006; (d) Pereira et al., 2009b; (e) Pereira et al., 2009c; nd = no data; *highest reported values.

has received wastewaters enriched in several contaminants during the past decades due to its proximity to the city of Lisbon and the surrounding industrial area (Vale et al., 2008), with the consequent increase in the levels reported in the pre-industrial times (Vale, 1990). Therefore, Rosário presents high concentration of metals in sediments and waters (Table 1), being the concentrations usually higher in colonized sediments than in non-vegetated ones (Caçador et al., 1996) due to complex interactions between roots and sediments (Godinho et al., 2014). The values in upper (5 cm) vegetated sediments (dry weight) are up to: 500 μg g^{-1} for Zn, 400 μg g^{-1} for Pb, 70 μg g^{-1} for Cu, 70 μg g^{-1} for Cr, 30 μg g^{-1} for Ni, 50 μg g^{-1} for As and 1 μg g^{-1} for Cd (Caçador et al., 1996; Caetano et al., 2008). Trace element concentration in plant tissues differs between species and organs, being higher in roots than in aboveground tissues (Caetano et al., 2008; Caçador et al., 2009; Duarte et al., 2010).

3. Materials and methods

3.1. Chemicals

All chemicals used in the determination of thiol-containing compounds were of the highest purity available. Trifluroacetic acid (TFA), diethylenetriamine-pentaacetic acid (DTPA), methanesulfonic acid (MSA), 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), monobromobimane (mBBr), *N*-acetyl-l-cysteine (NAC), glutathione, γ-glutamylcysteine and l-cysteine (Cys) were purchased from Sigma (St. Louis, MO, USA). Custom ordered PCs (PC_2, PC_3, PC_4 and PC_5) [PCn, (γ-Glu-Cys)n-Gly, where n = 2–5] were obtained from Anaspec (San Jose, CA, USA) and HPLC-grade acetonitrile (ACN) and methanol from Merck Millipore (Darmstadt, Germany). All solvents were filtered with 0.2 μm Acrodisc syringe nylon filter (Pall Life Sciences, Port Washington, NY, USA). Water was purified by a Merck Millipore Milli-Q system (Darmstadt, Germany). Ultrapure grade acids were used for digestion of samples for trace element determination.

3.2. Sampling and sample preparation

Salt marsh plants from Rosário and Óbidos were sampled once in October 2014. The whole plants of *S. maritima*, *H. portulacoides* and *S. perennis* were collected in Rosário, and of *H. portulacoides* and *S. perennis* in Óbidos. Twelve samples of each plant species were collected in Rosário, and three of each in Óbidos. After sampling, the plants were stored in plastic bags and quickly transported to the laboratory in refrigerated boxes. In the laboratory, above and belowground parts of plants were immediately washed with tap water and rinsed with Milli-Q water to remove dust and sediment particles. Leaves of *H. portulacoides* and *S. maritima* and fleshy stems for *S. perennis* were used as aboveground parts. Roots were washed over a sieve with 212 μm mesh size. In the case of *S. maritima*, which presented roots of different thicknesses, they were separated in small (< 1 mm) and large roots (> 1 mm). For the determination of thiol-containing compounds, plant material was dried with tissue paper and frozen at −80 °C until the analysis. From each sample, a portion was separated to determine the water content by weight difference before and after drying at 60 °C. From each type of tissue, a portion was also taken for metal determination, which was oven dried at 60 °C until constant weight and powdered in a grinding ball mill.

3.3. Determination of thiol-containing compounds

The analyzed thiol-containing compounds were the monothiols glutathione (GSH), γ-glutamylcysteine (γ-EC) and cysteine (Cys) and the phytochelatins PC_2, PC_3, PC_4 and PC_5. All thiol-containing compounds were extracted following Akhter et al. (2012) and preparation of standards and solutions, derivatization and HPLC conditions were made following Minocha et al. (2008). The frozen plant samples were ground in liquid nitrogen using a mortar and pestle, and 0.10 g of each sample was mixed with 1.5 mL of 6.3 mM DTPA, 0.1% (v/v) TFA and 25 μL of 20 mM TCEP. The mixture was sonicated in iced water for 25 min and the supernatant was collected after centrifugation at 15,000 × *g* for 60 min at 4 °C. After that, 615 μL of 200 mM HEPPS buffer (6.3 mM DTPA, pH 8.2) was mixed with 25 μL of 20 mM TCEP that was used as a disulfur reductant. To this mixture, 250 μL mix of standards or sample extract was added. Ten microliters of 0.5 mM NAC was also added as an internal standard. This reaction mix was pre-incubated at 45 °C for 10 min in a water bath in order to fully convert the disulfide bonds to sulfhydryls so that the thiols were present in a reduced state before mBBr derivatization. The derivatization was then carried out by incubating the samples in the dark in a water bath for 30 min at 45 °C after the addition of 10 μL of 50 mM mBBr. The reaction was terminated by the addition of 100 μL of 1 M MSA. The derivatized samples were filtered with 0.2 μm nylon syringe filters and a 10 μL injection volume was analyzed by HPLC.

The HPLC system consisted of an Agilent 1100 Series chromatograph (Agilent Technologies, Santa Clara, CA, USA) with fluorescence detector, degasser and autosampler. The excitation and emission wavelengths were set at 380 and 470 nm, respectively. Thiol-containing compounds were separated using solvents (A) 99.9% ACN and (B) 89.9% water + 10% ACN, both containing 0.1% TFA by volume. A linear gradient of mobile phase A from 0 to 10.6% was run for 11.2 min at 1 mL min^{-1} to elute monothiols. Further, the linear gradient of solvent A was raised from 10.6 to 21.1% in 13.6 min at 1 mL min^{-1}. Thiol-containing compounds were identified based on their retention times compared with the corresponding standards and quantified from a calibration curve. All measurements were carried out at least in triplicate. Concentrations are expressed per g dry weight, estimated from fresh weight to dry weight ratios.

3.4. Determination of trace elements

Approximately 0.10 g of ground dry samples were placed in teflon bombs and digested with 2 mL of HNO_3/$HClO_4$ (7:1 v/v) at 110 °C in an oven during 3 h. Later, the samples were filled until 25 mL with Milli-Q water. All laboratory ware was cleaned with HNO_3 (20%) for two days and rinsed with Milli-Q water to avoid contamination. Three procedural blanks were prepared using the same reagents and analytical procedure of samples. The blanks accounted for < 1% of the total element concentration in the samples. Concentrations of Zn, Pb, Cu, As, Ni, Cr, Co and Cd were determined by a quadropole ICP-MS (Thermo Elemental, X-Series) equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebulizer (Caetano et al., 2013). The quality control of the results was obtained through the use of the certified reference materials (CRM): BCR60 (*Lagarosiphon major*) and

BCR62 (*Olea europaea*). Analytical precision expressed as relative standard deviation (RSD in %) of CRM analysis varied between 1 and 2%. Instrumental limits of detection expressed on $\mu g\ g^{-1}$ were: 0.028 (Cr), 0.0020 (Co), 0.0080 (Ni), 0.020 (Cu), 0.23 (Zn), 0.74 (As), 0.0080 (Pb) and 0.0060 (Cd).

3.5. Data analysis

Data was first carefully examined to eliminate outliers. First, the differences in the concentration of total monothiols (GSH + Cys + γ-EC) and total PCs ($PC_2 + PC_3 + PC_4 + PC_5$) between above and belowground tissues within each species were evaluated with Student *t*-tests. Later, the content of each monothiol and each PC was compared between each type of tissue, species and site with one-way ANOVA and Bonferroni tests or nonparametric tests according to the characteristics of the set of data. Data were transformed if necessary to meet the required assumptions (homogeneity and normality) for the parametric tests.

Spearman correlation coefficients were calculated between the concentrations of the elements that presumably may induce the PCs synthesis (Cu, Zn, As, Cd and Pb) and values of total PCs, PC_2, PC_3 ($n = 10$), and PC_4 and PC_5 ($n = 5$). All statistical analyses were carried out with STATISTICA 7.0 (StatSoft, Inc., Tulsa, OK, USA), following Legendre and Legendre (1998). The acceptable level of statistical significance was 5%. Error values represent ± SD.

4. Results

4.1. Trace elements in below and aboveground tissues

Fig. 2 shows the concentrations of Pb, Zn, As, Cu, Cd, Cr, Co and Ni in roots and leaves or stems of *H. portulacoides* and *S. perennis* from Óbidos and of *S. maritima*, *H. portulacoides* and *S. perennis* from Rosário. The element partitioning between above and belowground parts showed a pattern characterized by much higher concentrations in roots than in leaves or stems of the analyzed plant species. For example, *H. portulacoides* and *S. perennis* from Rosário exhibited the highest differences between below and above parts for Zn, whereas in Óbidos those were found for Ni. *S. maritima* showed the highest differences between roots and leaves for As, and concentrations of all determined elements were higher in small than in large roots.

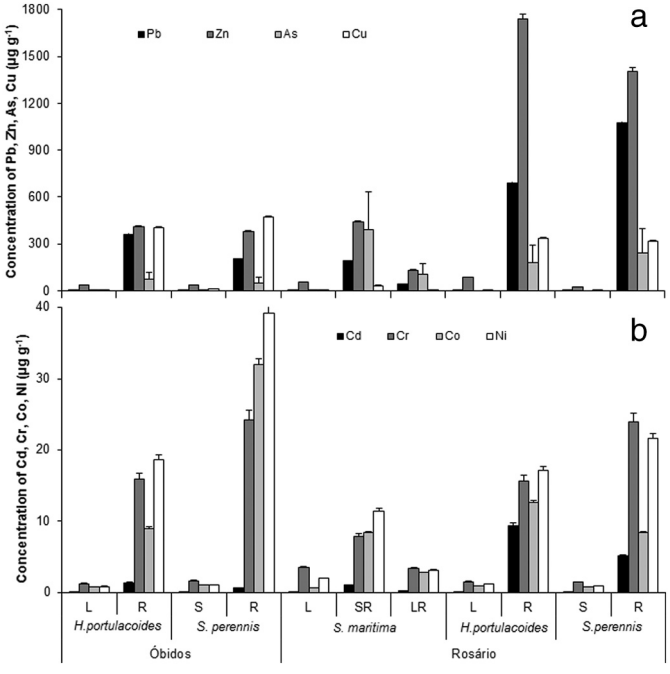

Fig. 2. Concentrations ($\mu g\ g^{-1}$ dry weight) of (a) Pb, Zn, As and Cu and (b) Cd, Cr, Co and Ni in aboveground parts (leaves or stems) and belowground parts (roots) of *Halimione portulacoides*, *Sarcocornia perennis* and *Spartina maritima*, from Óbidos and Rosário salt marshes. L = leaves; S = stems; R = roots; SR = small roots; LR = large roots. Data are means ± standard deviation.

4.2. Total monothiols and total phytochelatins

Table 2 gives the mean concentrations of total monothiols and total PCs quantified in the analyzed parts of the three plants from the two salt marshes. Concentration of total monothiols and total PCs were always significantly higher ($p < 0.01$) in aerial parts than in roots for *H. portulacoides* and *S. perennis* in both sites, except the content of total PCs in *H. portulacoides* in Óbidos, where the differences were non-significant ($p = 0.23$). In *S. maritima*, the concentration of total monothiols was significantly ($p < 0.01$) higher in leaves than in roots but the content of total PCs was significantly ($p < 0.01$) higher in large roots than in small roots or leaves.

4.3. Monothiols

Fig. 3 shows the mean concentrations of GSH, γ-EC and Cys quantified in each type of tissue analyzed. GSH accounted to approximately 80% of total monothiols in aboveground parts and to 40–50% in roots, although it was not found in belowground parts of *S. perennis* in either salt marsh. γ-EC was only quantified in above and belowground tissues of *S. maritima* and *H. portulacoides* from Rosário, being below the limit of detection in samples of *H. portulacoides* from Óbidos and of *S. perennis* from Óbidos and Rosário. Concentrations of Cys presented a narrower interval than GSH and were quantified in all samples.

Values of GSH were significantly ($p < 0.05$) higher in aboveground tissues than in roots for all species, the highest and lowest concentrations being found in leaves of *S. maritima* and in roots of *H. portulacoides* in Óbidos, respectively. The values of γ-EC were significantly ($p < 0.05$) higher in *S. maritima* than in *H. portulacoides* for all the plant parts analyzed. The concentration of Cys in large roots of *S. maritima* was significantly ($p < 0.05$) higher than in all other plant parts analyzed, with the exception of leaves of *H. portulacoides*.

4.4. Phytochelatins

PC_2 was quantified in all the analyzed samples. PC_3, PC_4 and PC_5 were found in all aboveground parts of the plants from the two salt marshes, except PC_5 in leaves of *H. portulacoides* from Rosário. When PC_4 and PC_5 were quantified in aboveground tissues, they accounted for 65–80% of the total PCs. In belowground tissues, PC_4 and PC_5 were only found in roots of *S. maritima*, whereas PC_3 was found in all tissues, except in roots of *S. perennis* in Óbidos (Fig. 4).

The concentration of PC_2 in large roots of *S. maritima* ($0.15 \pm 0.06\ \mu mol\ g^{-1}$) was significantly ($p < 0.05$) higher than in the other types of tissues, species and sites. The concentration of PC_2 in the rest of the tissues was below $0.04\ \mu mol\ g^{-1}$, meaning that values of PC_2 in large roots of *S. maritima* exceeded in more than 3 times the concentrations in the rest of the analyzed tissues. Reported values of PC_3 were always higher than $0.03\ \mu mol\ g^{-1}$, with few significant differences between the plant parts and species analyzed. The values of PC_4 in leaves of *S. maritima* was significantly ($p < 0.01$) lower than in the rest of the types of tissues, species and sites. The highest concentration of PC_5 were found in stems of *S. perennis* in either salt marsh ($\sim 0.16\ \mu mol\ g^{-1}$) and in small roots of *S. maritima* ($\sim 0.15\ \mu mol\ g^{-1}$), which were significantly ($p < 0.05$) higher than in the rest of analyzed tissues.

4.5. Relationship between phytochelatins and trace element

PC_2 was positively and significantly ($p < 0.05$) correlated with the

Table 2
Concentrations (μmol g^{-1} dry weight) of total monothiols (glutathione + γ-glutamylcystein + cysteine) and totals phytochelatins (PC_2 + PC_3 + PC4 + PC_5) in all the studied tissues, plants and sites Data are means ± standard deviation.

Salt marsh	Species	Tissues	Total monothiols (μmol g^{-1} d.w.)	Total phytochelatins (μmol g^{-1} d.w.)
Óbidos	*H. portulacoides*	Leaves	0.86 ± 0.047	0.18 ± 0.084
		Roots	0.15 ± 0.0060	0.083 ± 0.0080
	S. perennis	Stems	0.52 ± 0.10	0.31 ± 0.011
		Roots	0.055 ± 0.0090	0.028 ± 0.0030
Rosário	*S. maritima*	Leaves	2.3 ± 0.026	0.38 ± 0.081
		Small roots	0.68 ± 0.15	0.35 ± 0.072
		Large roots	0.78 ± 0.098	0.62 ± 0.047
	H. portulacoides	Leaves	1.5 ± 0.13	0.21 ± 0.034
		Roots	0.32 ± 0.28	0.13 ± 0.044
	S. perennis	Stems	0.18 ± 0.061	0.19 ± 0.098
		Roots	0.094 ± 0.020	0.079 ± 0.023

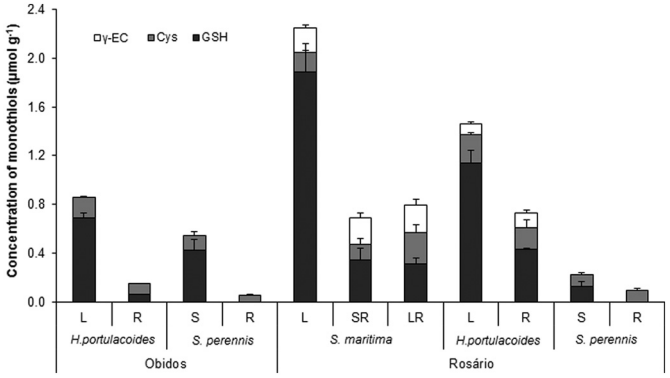

Fig. 3. Concentrations (μmol g^{-1} dry weight) of monothiols (glutathione, γ-glutamylcystein and cysteine) in aboveground parts (leaves or stems) and belowground parts (roots) of *Halimione portulacoides*, *Sarcocornia perennis* and *Spartina maritima*, from Óbidos and Rosário. L = leaves; S = stems; R = roots; SR = small roots; LR = large roots. GSH = glutathione; γ-EC = γ-glutamylcysteine; Cys = cysteine. Data are means ± standard deviation.

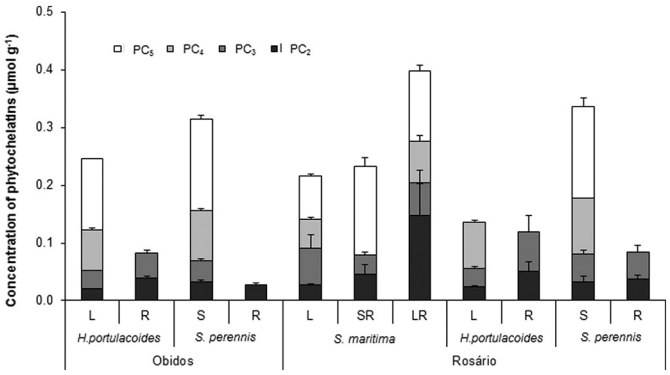

Fig. 4. Concentrations (μmol g^{-1} dry weight) of phytochelatins (PC_2, PC_3, PC_4 and PC_5) in aboveground parts (leaves or stems) and belowground parts (roots) of *Halimione portulacoides*, *Sarcocornia perennis* and *Spartina maritima* from Óbidos and Rosário L = leaves; S = stems; R = roots; SR = small roots; LR = large roots. Data are means ± standard deviation.

concentration of As, Zn and Pb (r_S of 0.68, 0.63 and 0.61, respectively). The concentration of total PCs was significantly ($p < 0.05$) correlated with Cu, Zn and Pb, although the values were negative (r_S of −0.65, −0.61 and −0.64, respectively). The rest to the tested correlations were non-significant.

5. Discussion

To the best of our knowledge the present work evidences, for the first time, the synthesis of phytochelatins and monothiols in above and belowground parts of *S. maritima*, *H. portulacoides* and *S. perennis* in salt marshes, and examines the complex relations with tolerance to metals and metalloids. These species has been pointed out as potential tools for environmental remediation and understanding the mechanisms behind metal tolerance is crucial to move forward on environmental protection.

5.1. Retention of trace elements in roots

The present study, by showing higher concentrations of elements in below than in aboveground parts of the studied plants, is in line with numerous works that proved the ability of *S. maritima*, *H. portulacoides* and *S. perennis* to prevent the migration of undesirable elements from pore water to aboveground tissues (e.g., Caetano et al., 2008; Válega et al., 2008; Duarte et al., 2010). To assess the ability of belowground parts to retain elements, retentions factors (ratio between element concentration in roots and in leaves or stems) were calculated. This decreasing sequence of retention factors was found: As, Pb, Cd, Cu, Zn, Ni, Co and Cr. It is noticeable that elements showing higher retention factors (Pb, Cd and As) are those whose role in biological systems have not been identified (Nagajyoti et al., 2010) and are good inducers of PCs synthesis (e.g., Srivastava et al., 2004; Gadapati and Macfie, 2006; Thangavel et al., 2007; Machado-Estrada et al., 2013). Our results are in agreement with the already mentioned capacity of these species as accumulators of elements, and the lack of signs of physiological stress in the studied plants emphasizes the potential use of these species as valuable remediation agents. Despite no specific measurement of biomass or photosynthetic capacity were performed during this work, plants in the study area did not show visual signs of stress (chlorosis, reduction of size, etc.).

5.2. Predominance of glutathione in aboveground parts

Concentration of total monothiols was higher in stems and leaves than in roots of all studied species and sites. Regardless the type of tissue, GSH, if present, was the most abundant monothiol, mainly in aboveground parts. GSH is usually in high cellular concentration in plants due to the diverse functions it exerts, and since it is synthesized in both the cytosol and the chloroplast (Alscher, 1989) it is commonly found in higher levels in photosynthetic tissues (e.g., Gadapati and Macfie, 2006; Ju et al., 2011; Akhter et al., 2012; Da Silva et al., 2015). GSH is efficient in the elimination of reactive oxygen species that interfere in the cellular metabolism, which in plants are formed as a consequence of oxidative stresses such as extreme temperature, drought, herbicides or contaminants (Alscher, 1989; Anjum et al., 2014). Although GSH might bind metals, its major role in trace element homeostasis seems to be providing the substrate for the synthesis of PCs (e.g., Xiang et al., 2001; Srivastava et al., 2004; Sghayar et al., 2015).

Total monothiols concentrations were within the range observed in

vascular plants and seaweeds under natural conditions (Pawlik-Skowrońska et al., 2007; Machado-Estrada et al., 2013). Most research on synthesis of thiol-containing compounds in response to metal stress has been performed under laboratory conditions. The current study provides a good approach to the homeostasis of GSH and other thiol-containing compounds under salt marsh conditions. The enhancement of GSH and their precursors, Cys and γ-EC, in *S. maritima* relatively to *H. portulacoides* and *S. perennis*, is in line with other studies indicating that thiol metabolism is species-specific (Gadapati and Macfie, 2006; Akhter et al., 2012; Machado-Estrada et al., 2013). The higher concentration of monothiols found in *S. maritima* might reflect the higher exposure of this species to environmental stress in result of its position in the lower marsh subjected to the harsh action of tides and flooding (Mitsch and Gosselink, 2007).

5.3. Production of phytochelatins and its relationship with metal tolerance

Phytochelatins has been proposed as biomarkers of metal and metalloid exposure, constituting an early and specific warming for this kind of stress (e.g., Padinha et al., 2000; Thangavel et al., 2007; Pawlik-Skowrońska et al., 2007). At least one type of PC was produced by all the analyzed tissues and species from the two studied sites, which might be considered a response to the accumulation of trace elements. However, the concentration of total PCs (PC_2 + PC_3 + PC_4 + PC_5) was higher in leaves or stems than in roots of *H. portulacoides* and *S. perennis*, which differs from the trace elements distribution. *S. maritima* presented higher values of total PCs in roots than in leaves, although being more abundant in large roots while elements were more concentrated in small roots. These results are consistent with the lack of positive correlations between element and total PCs concentrations and point to the complexity of this relationship.

Lower molecular weight PCs (PC_2 and PC_3) were found in almost all the analyzed tissues, with the only exception of roots of *S. perennis* in Óbidos where PC_3 was below the detection limit. In addition, PC_2 concentrations were several times higher in large roots of *S. maritima* than in the other plant parts from the two salt marshes, and slightly higher in roots than in leaves of *H. portulacoides* and *S. perennis* (Fig. 4). Moreover, there was a positive correlation between PC_2 and As, Pb and Zn. It appears that accumulation of these elements triggered the synthesis of PC_2 in these species, as previously proposed (e.g., Mishra et al., 2006; Thangavel et al., 2007; Srivastava et al., 2011; Machado-Estrada et al., 2013). Although high Cd concentrations have been proved to induce PCs production (e.g., Cobbett and Goldsbrough, 2002; Thangavel et al., 2007; Akhter et al., 2012), no relation was obtained in the present study. Possibly, Cd levels found in the current work were below the activation value for that mechanism.

The synthesis of higher molecular weight PCs (PC_4 and PC_5) could represent an advantage for metal inactivation due to their higher number of cysteine groups (Rauser, 1995). Although PC_4 and PC_5 were not found in some tissues, if present, they were at higher concentrations than others PCs. In addition, in belowground tissues they were only found in *S. maritima*, and the concentration of PC_5 was significantly ($p < 0.05$) higher in small than in large roots, as observed for trace elements. In this case trace element tolerance might be better expressed by PC_5 than PC_2.

The complexity of PC induction is manifested by the diverse interactions between PC and metals reported in the literature. Positive relationships (Gupta et al., 1998; Pawlik-Skowrońska et al., 2007; Thangavel et al., 2007; Akhter et al., 2012; Machado-Estrada et al., 2013) contrasts with negative (Sghayar et al., 2015) and neutral ones (Pawlik-Skowrońska et al., 2007; Akhter et al., 2012; Sghayar et al., 2015). The rate of degradation of PCs (Gupta et al., 1998) or the efficient elimination of metals in tissues with higher thiol content (Padinha et al., 2000) may explain the observed differences. Moreover, since metals can affect differently specific plant organs (Nagajyoti et al., 2010), some elements may be stressful and trigger the production of PCs in some tissues but not in others. In *H. portulacoides* from Rosário salt marsh, several elements are retained in the epidermis of roots (Godinho et al., 2014), which may not trigger a response in terms of PCs. PC production may be influenced by element distribution in epidermis and internal tissues and consequently distort the PC-element relationships.

Comparing the analyzed species, *S. maritima* stands out regarding PCs production. *S. maritima* synthesized higher levels of PCs, especially the heavier and more efficient ones. That might be related with higher exposure of this species to contaminants transported by the tide than *H. portulacoides* and *S. perennis* that are located in the upper marsh. However, it should not be excluded that other mechanisms are involved in metal tolerance in *H. portulacoides* and *S. perennis*, such as element immobilization in cell walls (e.g., Castro et al., 2009; Reboredo, 2012; Godinho et al., 2014).

6. Conclusions

Phytochelatins and their precursors were detected in *H. portulacoides*, *S. perennis* and *S. maritima* in above and belowground parts. Arsenic, zinc and lead were pointed out as inducers of PC_2 and *S. maritima* as the main species to synthesize PCs. Although being the first evidence of PCs and their precursors in tissues of these salt marsh plants under natural conditions, our results do not point to simple interactions between PC production and trace elements concentrations. Difficulties in finding a clear relationship between PCs and trace elements may be related to the element distribution in plant organs and in tissues within an organ and to the existence of other mechanism of metal tolerance that may be working together with PCs production.

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