Growth and physiological effects of single and combined Cu, NaCl, and water stresses on *Atriplex atacamensis* and *A. halimus*

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ABSTRACT

Halophyte species have been proposed as suitable candidates for the phytostabilization of metal enriched sites in arid and semiarid ecosystems, but co-occurring conditions, such as salinity and water stress, can affect plant growth and colonization. In this work, we determined the effect of single and combined copper (Cu), salt (NaCl), and water stress on growth and tolerance strategies used by two xerohalophyte species of the genus *Atriplex*: Chilean *A. atacamensis* and European *A. halimus*. Seedlings of both species were subjected to 5 and 10 u M Cu, 0.5% NaCl, and 7.85 mM polyethylene glycol 6000 (PEG) in hydroponic cultures to create single and combined stress treatments. A single Cu stressor decreased growth parameters of both species, with a higher effect on *A. atacamensis*. Copper accumulation was higher in roots, which can be related to an increase of root non-protein thiols (NPT). Single NaCl had no negative effects on growth but increased leaf sodium (Na). Single PEG decreased shoot growth, especially in *A. halimus*, but had no effect on GSH expression. A combination of Cu, NaCl, and PEG further decreased growth parameters, but did not decrease glutathione, proline, or non-protein thiol expression. *Atriplex atacamensis* and *A. halimus* are affected differently by combined stresses, but both species show an active tolerance response to the presented conditions.

1. Introduction

Copper (Cu) is an element widely spread in soils and a biotic constituent of living organisms (Nagajyoti et al., 2010; Richardson, 1997). At the plant level, Cu is an essential micronutrient involved in photosynthetic processes, cellular respiration, cell wall metabolism, and lignin formation (Nagajyoti et al., 2010; Reichman, 2002); however, exposure to high bioavailable Cu concentrations can cause severe effects on plant growth and development, such as photosynthesis inhibition, disruption of cell membrane integrity, root browning, interveinal chlorosis, and, finally, wilting (Reichman, 2002; Ye et al., 2014).

Anthropic soil Cu enrichment, derived from long-term mining operations among other sources, has caused serious consequences to productive lands, natural ecosystems, and human health (Lam et al., 2016; Tordoff et al., 2000; Wang and Liu, 2003). In this context, phytostabilization—the use of metal tolerant plants for in situ immobilization of potential pollutants the at soil level—has been proposed as a cost-effective alternative to control metal dispersion and reduce environmental risks (Ginocchio and Baker, 2004; Heckenroth et al.,

2016). However, depending on site-specific conditions (i.e., climate), metal-enriched soils could coexist with other co-occurring stresses, such as drought and salinity, which can further restrict phytostabilization efforts (Ginocchio et al., 2017; Mendez and Maier, 2007). In these conditions, a combination of two or more abiotic stressors may result in new conditions for plant development, different from the effect of each stressor by itself (Mittler, 2006; Zandalinas et al., 2018); therefore, plant selection for phytostabilization of metal-enriched soils must consider not only specific metal stressors but also the presence of multiple co-occurring stresses and their effect on plant growth and development.

It has been proposed that halophyte species could be acceptable alternatives for phytostabilization of metal-enriched soils in arid and semiarid environments (Mendez et al., 2007; Parraga-Aguado et al., 2014). Physiological strategies developed by halophytes to cope with osmotic and ionic effects of salt stress are shared with tolerance mechanisms developed for both metal (Lutts and Lefevre, 2015) and water stresses (Hamed et al., 2013; Manousaki and Kalogerakis, 2011). These shared physiological responses correspond to augmented antioxidant

response (Ozgur et al., 2013), compatible osmolyte synthesis, ion chelation, and sequestration in intracellular compartments (Bankaji et al., 2015).

The genus *Atriplex* comprises a group of halophyte species that grow in arid and semi-arid regions of the world (Brignone et al., 2016). The ability of these species to colonize xerophytic areas with different levels of soil salinity has outlined them as candidates for the stabilization of metal-enriched soils. In particular, *Atriplex halimus*, a shrub native to the European Mediterranean, and *A. atacamensis*, endemic to the Atacama Desert, north of Chile, have shown variable levels of tolerance to metals (i.e., Cd, Zn, Pb) and metalloids (i.e., As) (Lefevre et al., 2010; Lutts et al., 2004; Tapia et al., 2013; Vromman et al., 2011; Walker et al., 2014). Further studies on these species have shown that a combination of metal and salt stresses have a different effect on plant growth and physiological status than single stress conditions (Bankaji et al., 2016; Manousaki and Kalogerakis, 2009; Vromman et al., 2017, 2016). Although work has been done to understand the physiological response of these species when exposed to combined stresses, the effect of Cu excess and its combination with water and salt stresses have not been evaluated yet; therefore, in the present study, we assessed and compared growth parameters and physiological response mechanisms of *A. atacamensis* and *A. halimus* when subjected to single and combined conditions of Cu, water, and salt stresses under controlled laboratory conditions.

2. Materials and methods

2.1. Plant material and growth conditions

Fruits of *Atriplex atacamensis* were collected in February 2017 along the Loa River near Calama (UTM 19 K: 510,307 E 7,516,731 N) of the Antofagasta Region, located in northern Chile. A composed sample from those soils showed that the total Cu concentration was 15.7 mg Kg^{-1}, with a pH of 8.1 and 28.7 meq L^{-1} soluble Na. Fruits of *Atriplex halimus* collected in Spain were obtained commercially on August 2017 from Weberseeds.

Seeds from debracted fruits of both species were germinated in 4 l polypropylene boxes with commercial compost. After 4 weeks, a total of 384 seedlings with 3 pairs of fully developed leaves were transferred into a hydroponic culture on 1.5 l polypropylene boxes with a modified Hoagland solution. The experimental nutrient solutions were changed once during the experimental assays. Nutrient solution consisted of 1.43 mM of NH_4NO_3, 323 mM of $NaH_2PO_4 \cdot 2H_2O$, 512 mM of K_2SO_4, 750 mM of $CaCl_2 \cdot 2H_2O$, 1.64 mM of $MgSO_4 \cdot 7H_2O$, 11.4 mM of $MnSO_4 \cdot H_2O$, 14 mM of $Na_2MoO_4 \cdot 2H_2O$, 57.8 mM of H_3BO_3, 0.96 mM of $ZnSO_4 \cdot 7H_2O$, 0.4 mM of $CuSO_4 \cdot 5H_2O$, and 42.7 mM of Fe-EDTA (Vromman et al., 2016).

After one week of acclimation, 12 single and combined treatments of water, metal, and salt stresses were applied to nutrient solutions for each species under a factorial design. For water stress treatments, polyethylene glycol 6000 (PEG) was applied in order to obtain 0 (Control) and -0.1 MPa of solute pressure, according to Michel and Kaufmann (1973). For metal stress, plants were exposed to 0 (control) 5, and 10 μM $CuSO_4 \cdot 5H_2O$. Finally, for salt stress, NaCl was applied in order to obtain 0% (control) and 0.5% of NaCl in the nutrient solution. Reagents used for nutrient solution and treatments were analytical grade obtained from Merck and Sigma-Aldrich (Germany). NaCl and Cu treatments were selected according to Cu measurements in natural and enriched areas of north and central Chile (Badilla-Ohlbaum et al., 2001; Díaz et al., 2011; Ortiz-Calderón et al., 2008; Santibáñez et al., 2008) and single stress assays performed previously on both *Atriplex* species (Orrego et. al in prep). Each treatment was applied to 32 plants (8 plants per box). Experimental boxes were kept in a phytotron with controlled conditions—room temperature was maintained at 24 ± 1 °C with a photoperiod of 12 h and a relative humidity of 55%. Since Cu availability strongly depends on pH, all treatments were adjusted to a pH of 5.3 ± 0.5 with HCl 0.5 M and KOH 0.5 M.

2.2. Plant growth measurements

Five plants per treatment combination were randomly harvested after ten days. At each harvest, plants were washed with distilled water and blotted dry carefully. Following this, root length was measured and fresh weight of roots and shoots were determined. Afterwards, plants were dried for 48 h at 45 °C for dry weight measurement of root and shoot biomass.

2.3. Extraction and quantification of chlorophyll and carotenoids

For total chlorophyll (Chl a and b) and total carotenoid quantification, 100 mg leaves were ground with 8 ml of 80% acetone and then centrifuged at 3000 rpm for 10 min at 4 °C. The absorbance of the samples supernatant was determined (D) at 663.2 nm, 646.8 nm for chlorophyll and 470 nm for carotenoids. The pigment concentrations were calculated according to the following equations (Lichtenthaler, 1987), shown in Eq. (1):

$$Chl_a (mgL^{-1}) = (12.25 \times D663.2) - (2.79 \times D646.8)$$
$$Chl_b (mgL^{-1}) = (21.5 \times D646.8) - (5.1 \times D663.2)$$
$$Carot (mgL^{-1}) = \frac{(1000 \times blanc) - (1.82 \times chl_a) - (85.02 \times chl_b)}{198}$$
(1)

2.4. Water status

For plant water content (WC) measurement (%), dry and fresh weight (DW and FW, respectively) of four individuals per treatment were used to calculate root and shoot WC according to Eq. (2):

$$WC = \frac{FW - DW}{DW} \times 100$$
(2)

For osmotic potential (Ψ) determination, the method of (Bajji et al., 1998) was used. Four leaves of the middle section of three plants per treatment were quickly collected, placed in three Eppendorf tubes with three bottom holes, and stored in liquid nitrogen. Frozen leaves were then subjected to three cycles of thawing and refreezing. Then, each Eppendorf tube was encased individually on another tube and centrifuged at 8000 RPM for 15 min at 4 °C. The collected sap was used to measure osmolarity with a vapor pressure osmometer (Wescor 5500, USA) and converted from mosmoles kg^{-1} to MPa, according to the Van't Hoff Eq. (3) (Zhu, 2001):

$$Cs (MPa) = -c (mosmoles \, Kg^{-1}) \times 2.58 \times 10^{-3}$$
(3)

2.5. Proline quantification

For proline quantification, 200 mg of freshly grinded leaves were extracted in a hot bath with 10 ml of 3% salicylic acid. Then, free proline was quantified, according to Bates et al. (1973), using a spectrophotometer (Beckman DU-640, USA).

2.6. Ion quantification

For Cu, Na, and K quantification, 50–100 mg of leaves and 20–30 mg of roots were digested in 35% HNO_3 (v/v) and evaporated to dryness on a sand bath at 80 °C. Minerals were then incubated with a mix of HCl 37% + HNO_3 68% (3:1 v/v) until evaporation (Vromman and Paternostre, 2016). The residue was then dissolved with distilled water and filtered. Ion measurements were made in an atomic absorption spectrometer (Thermo scientific, USA) in duplicate.

2.7. Non-protein thiols quantification

For non-protein thiols (NPT) concentration, 50–100 mg of frozen fresh tissue was grinded in 5% (w/v) sulfosalicylic acid and 6.3 mM diethylenetriaminepentaacetic acid at 0 °C. The homogenate was then centrifuged at 10,000 g for 30 min at 4 °C, and 300 μL of supernatant were mixed with 630 μL of 0.5 M K_2HPO_4 (pH 7.0). After 2 min, the absorbance at 412 nm was recorded. Then, 25 μL of 10 mM 5,5-di-thiobis 2-nitrobenzoic acid (pH = 7.0) was added to the solution, and, once again, the absorbance at 412 nm was recorded after 2 min, according to (De Vos et al., 1992). Thiol concentration was calculated as the difference between the 2 measurements, using an extinction coefficient of 13,600 M cm^{-1}.

2.8. Reduced and oxidized glutathione quantification

For reduced (GSH) and oxidized (GSSG) glutathione quantification in roots and leaves, 200 mg of frozen tissue was 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 (DTT). 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. A sample of 5 μL was injected into a Shimadzu HPLC system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a Nucleodur C18 Pyramid column (125 × 4.6-mm internal diameter; 5-μm particle size) (Macherey-Nagel, Düren, Germany). 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}. The mobile phase consisted of distilled water (Eluent A), acetonitrile (Eluent B) and 50 mM sodium acetate buffer pH 6.2 (Eluent C). The gradient program was as follows: 0 min, 100% C; 5 min, 100% C; 12 min, 30% A, 70% B; 17 min, 30% A, 70% B; 23–33 min, 100% C, re-equilibration time. Fluorimetric detection was performed with a Shimadzu RF-20A fluorescence detector (The Netherlands) at 420 nm after excitation at 340 nm. GSH (retention time of 3.02 min) was quantified using 9-point calibration curves with custom-made external standard solutions ranging from 0.0625 to 50 μM, and, every 10 injections, a check standard solution was used to confirm the calibration of the system. The recovery was determined using GSH as an internal standard.

2.9. Statistical treatment of data

Growth parameters, water balance, and ion quantification were analyzed using a three-way analysis of variance with Cu, NaCl concentration, and solute potential (PEG) as factors. The experimental design was fully factorial with fixed main effects. All data were analyzed by the InfoStat statistical package (Di Rienzo et al., 2016). Homogeneity of variance was calculated by Levene's test, and non-normal variables were transformed by root or logarithm. When the ANOVA was significant at P = 0.05, Tukey's honestly significant difference tests were performed to determine if differences were significant.

3. Results

3.1. Plant growth parameters

All plants survived until the end of the experiment, although chlorosis and serious loss of turgor was observed in combined treatments of PEG and Cu on *A. halimus* since Day 4. Other visual symptoms included browning of the roots and reddening of the abaxial side of *A. atacamensis* and *A. halimus* leaves (Fig. 1).

Root length of *A. atacamensis* significantly varied in response to single and combined effects of Cu, NaCl, and PEG (P < 0.05). Single

Cu caused a significant decrease in root length, but when PEG was spiked, a 50% decrease in root length was observed in all treatments, whether single PEG or combined with Cu and NaCl (Table 1). In contrast, root length of *A. halimus* significantly decreased in response to Cu and PEG single effects (P < 0.05). Salinity tended to increase root length, but no significant effects were observed for combined treatments (Table 1).

Root fresh weight of *A. atacamensis* greatly varied in response to Cu, NaCl, and PEG single and combined effects (P < 0.05). Single 5 μM of Cu induced a 20% increase in root fresh weight of *A. atacamensis,* but 10 μM of Cu caused a 65% average decrease, even when combined with PEG or NaCl (Table 1). Root fresh weight of *A. halimus* varied in response to the interaction between NaCl and PEG (P < 0.05). Single NaCl caused a 33% increase in root fresh weight, but its combination with PEG reversed this effect and caused a significant decrease greater than control conditions (Table 1).

The shoot fresh weight of *A. atacamensis* significantly varied in response to the interaction between Cu and PEG (P < 0.001). A significant increase of the shoot fresh weight was observed in response to 5 μM Cu, but this effect significantly reversed in response to 10 μM Cu and its combination with PEG (Table 1). In *A. halimus*, the shoot fresh weight significantly varied in response to NaCl and PEG single and combined effects (P < 0001). Notably, single PEG caused an average of 63% decrease of shoot fresh weight in *A. halimus*, an effect that did not statistically vary when combined with NaCl or Cu (Table 1).

Total chlorophyll content of *A. atacamensis* and *A. halimus* varied in response to the independent interactions between Cu and NaCl (P < 0.05) and PEG and NaCl (P < 005); in *A. halimus*, it also varied in response to the interaction among Cu and PEG (P < 0.05) (Table 2). Total chlorophyll concentration of *A. atacamensis* decreased in response to increasing Cu, and all PEG-combined treatments. In *A. halimus*, a significant decrease of chlorophyll (23%) in response to single Cu was observed; however, when combined with NaCl, this effect reversed (Table 2).

Carotenoid content showed different patterns among both *Atriplex* species. Whilst single and combined stressors caused an increase of carotenoids in *A. atacamensis*, in *A. halimus,* a significant decrease was observed (Table 2). Carotenoid content in *A. halimus* varied in response to the combination of Cu, NaCl, and PEG (P < 0.05), but, in *A. atacamensis*, it varied only in the response to the combination of Cu and PEG (P < 0.001) (Table 2).

3.2. Water status

The root and shoot water contents of *A. atacamensis* varied in response to PEG and Cu combined effects (P < 0.05) (Table 3). Root water content significantly decreased in response to single PEG and its combination with Cu (Table 3). Root water content of *A. halimus* showed no significant variation in response to the measured factors, but shoot water content did follow a similar pattern as *A. atacamensis*; the presence of PEG increased the negative effects caused by single Cu treatments (Table 3).

Leaf water potential of *A. atacamensis* varied in response to the combination of Cu and NaCl (P < 005). Single Cu treatments did not have an effect on leaf osmotic potential but, when combined with NaCl, a marked decrease was noted (Fig. 2). A similar effect was observed in *A. halimus*, in which the combination between Cu, NaCl and PEG caused a 230% average decrease in leaf water potential down to a value of -8.0 MPa (Fig. 2). Leaf osmotic potential of *A. atacamensis* also decreased significantly in response to the combination of PEG, NaCl and CU, but only to an average of -3.2 MPa (Fig. 2).

3.3. Proline quantification

Leaf proline of *A. atacamensis* greatly varied in response to the combination of Cu, NaCl, and PEG (P < 0.001). A significant increase

Fig. 1. Visual symptoms of single and combined stresses on *Atriplex* seedlings. (a) Loss of turgor and leaf reddening on *Atriplex halimus* leaves subjected to 7.85 mM PEG; (b) browning of *Atriplex atacamensis* roots subjected to 10 μM Cu; (c) leaf chlorosis in *A. atacamensis* subjected to combined 10 μM Cu and 0.5% NaCl.

Table 1
Root length, root and shoot fresh weights of *Atriplex atacamensis* and *A. halimus* seedlings exposed for ten days to single and combined Cu, NaCl, and PEG stresses (n = 5; mean ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.

Treatments	*Atriplex atacamensis*			*Atriplex halimus*		
	Root length (cm)	Root fresh weight (g)	Shoot fresh weight (g)	Root length (cm)	Root fresh weight (g)	Shoot fresh weight (g)
Control	35.8 ± 1.4e	0.3 ± 0.02f	0.92 ± 0.07e	19.6 ± 2.3ab	0.33 ± 0.1bc	0.87 ± 0.2b
Cu 5 μM	24.9 ± 1.7d	0.36 ± 0.03f	1.08 ± 0.2e	22.0 ± 1ab	0.24 ± 0.03ab	0.99 ± 0.1b
Cu 10 μM	18.9 ± 0.9bc	0.11 ± 0.002d	0.52 ± 0.03 cd	17.9 ± 1.2a	0.18 ± 0.03a	0.79 ± 0.1b
NaCl 0.5%	34.2 ± 1.2e	0.31 ± 0.01f	1.11 ± 0.02e	25.3 ± 2.6b	0.44 ± 0.03c	1.39 ± 0.1c
Cu 5 μM + NaCl 0.5%	17.7 ± 0.5bc	0.17 ± 0.01e	1.05 ± 0.03e	21.6 ± 2.1ab	0.32 ± 0.03bc	1.38 ± 0.1c
Cu 10 μM + NaCl 0.5%	18 ± 0.5bc	0.09 ± 0.002 cd	0.63 ± 0.03d	6.5 ± 2.6ab	0.23 ± 0.03ab	1.32 ± 0.1c
PEG 7.85%	18.8 ± 0.7bc	0.08 ± 0.004bcd	0.27 ± 0.02a	18.7 ± 1.9a	0.23 ± 0.1ab	0.43 ± 0.1a
Cu 5 μM + PEG 7.85%	13.7 ± 0.6a	0.06 ± 0.003a	0.28 ± 0.04ab	7.3 ± 1.9ab	0.15 ± 0.03a	0.28 ± 0.1a
Cu 10 μM + PEG 7.85%	19.8 ± 0.8c	0.1 ± 0.01 cd	0.42 ± 0.03bc	16.7 ± 2ab	0.19 ± 0.04a	0.33 ± 0.1a
PEG 7.85% + NaCl 0.5%	18.2 ± 1.3bc	0.09 ± 0.01 cd	0.36 ± 0.04ab	7.7 ± 3.9ab	0.22 ± 0.1ab	0.29 ± 0.04a
Cu 5 μM + NaCl 0.5% + PEG 7.85%	16.6 ± 0.8b	0.06 ± 0.01ab	0.36 ± 0.02ab	8.3 ± 2.7ab	0.14 ± 0.03a	0.31 ± 0.1a
Cu 10 μM + NaCl 0.5% + PEG 7.85%	19.2 ± 0.6cb	0.09 ± 0.004 cd	0.56 ± 0.01d	17.3 ± 1.7a	0.16 ± 0.01a	0.31 ± 0.04a

was found in response to the combination of PEG and Cu; however, when either of these treatments was combined with NaCl, proline decreased to control levels (Fig. 3). On the other side, leaf proline of *A. halimus* varied significantly in response to PEG and its independent interaction with NaCl (P < 0.05) and Cu (P < 0.001). PEG caused an increase in leaf proline of *A. halimus* four times larger than *A. atacamensis*, but unlike *A. atacamensis*, when NaCl was applied, proline

concentration did not decrease (Fig. 3).

3.4. Ion accumulation

Single and combined effects of Cu, NaCl, and PEG caused different patterns of ion accumulation on *A. atacamensis* and *A. halimus* (Table 4, Fig. 4). Root K of *A. atacamensis* varied in response to the interaction

Table 2
Total chlorophyll and carotenoid content of *Atriplex atacamensis* and *A. halimus* seedlings subjected to single and combined Cu, NaCl, and PEG stresses (n = 5; mean ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.

Treatments	*Atriplex atacamensis*		*Atriplex halimus*	
	Total chlorophyll (mg g^{-1})		Carotenoids (mg g^{-1})	
Control	2.0 ± 0.02f	0.45 ± 0.01a	1.8 ± 0.06de	0.31 ± 0.01efg
Cu 5 μM	1.9 ± 0.03def	0.57 ± 0.01b	1.7 ± 0.03b	0.23 ± 0.01def
Cu 10 μM	1.8 ± 0.03 cd	0.66 ± 0.01c	1.4 ± 0.02a	0.21 ± 0.01ab
NaCl 0.5%	2.0 ± 0.03ef	0.47 ± 0.02a	1.8 ± 0.04cde	0.37 ± 0.01 h
Cu 5 μM + NaCl 0.5%	1.8 ± 0.03 cde	0.69 ± 0.01cde	1.8 ± 0.03bcde	0.26 ± 0.01 cd
Cu 10 μM + NaCl 0.5%	1.8 ± 0.01c	0.76 ± 0.01f	1.7 ± 0.03bc	0.18 ± 0.01a
PEG 7.85%	1.9 ± 0.02cde	0.46 ± 0.01a	1.9 ± 0.03e	0.36 ± 0.00gh
Cu 5 μM + PEG 7.85%	1.8 ± 0.02 cd	0.56 ± 0.02b	1.6 ± 0.03b	0.29 ± 0.01def
Cu 10 μM + PEG 7.85%	1.7 ± 0.02bc	0.64 ± 0.01c	1.4 ± 0.03a	0.23 ± 0.01bc
PEG 7.85% + NaCl 0.5%	1.8 ± 0.02 cde	0.49 ± 0.01a	1.7 ± 0.03bcd	0.34 ± 0.01fgh
Cu 5 μM + NaCl 0.5% + PEG 7.85%	1.6 ± 0.03ab	0.71 ± 0.01def	1.5 ± 0.03a	0.27 ± 0.01cde
Cu 10 μM + NaCl 0.5% + PEG 7.85%	1.6 ± 0.03a	0.74 ± 0.01ef	1.4 ± 0.04a	0.16 ± 0.01a

Table 3
The root and shoot water content of *Atriplex atacamensis* and *A. halimus* seedlings exposed for ten days to single and combined Cu, NaCl, and PEG stresses (n = 4; mean ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.

Treatments	*Atriplex atacamensis*		*Atriplex halimus*	
	Root WC (%)	Shoot WC (%)	Root WC (%)	Shoot WC (%)
Control	92.9 ± 0.2 cd	83.5 ± 1.8abc	94.5 ± 0.2a	88.5 ± 1.6c
Cu 5 μM	93.1 ± 0.96 cd	84.6 ± 0.9bc	93.4 ± 0.5a	86.7 ± 0.9c
Cu 10 μM	85.7 ± 2.8a	73.8 ± 2.5ab	92.5 ± 0.1a	84.7 ± 0.3bc
NaCl 0.5%	94.3 ± 0.59d	88.2 ± 0.2c	94.5 ± 0.3a	89.5 ± 1.7c
Cu 5 μM + NaCl 0.5%	92.3 ± 1.76bcd	87.9 ± 0.6c	93.6 ± 0.4a	88.9 ± 0.4c
Cu 10 μM + NaCl 0.5%	88.7 ± 0.56abcd	82.6 ± 0.5abc	91.6 ± 0.7a	89.6 ± 2.1c
PEG 7.85%	90.5 ± 0.73abcd	73.6 ± 0.7ab	92.7 ± 0.8a	68.1 ± 8.5abc
Cu 5 μM + PEG 7.85%	88.8 ± 0.81abcd	73.2 ± 0.7ab	91.9 ± 1.3a	73.1 ± 2.2abc
Cu 10 μM + PEG 7.85%	86.7 ± 1.09ab	72.6 ± 1a	92.3 ± 0.6a	63.1 ± 4.4ab
PEG 7.85% + NaCl 0.5%	89.8 ± 0.64abcd	74 ± 0.6ab	94.2 ± 0.3a	61.6 ± 7.8a
Cu 5 μM + NaCl 0.5% + PEG 7.85%	87.6 ± 0.75abc	75.1 ± 0.7ab	93.1 ± 0.5a	80 ± 1.5abc
Cu 10 μM + NaCl 0.5% + PEG 7.85%	89.7 ± 0.46abcd	79.3 ± 0.4abc	93 ± 0.4a	74.2 ± 4.6abc

between NaCl and PEG (P < 0.05), where the combination of these factors decreased root K to nearly 50% of control values. In *A. halimus* root K significantly varied according to the interaction of Cu, NaCl, and PEG (P < 0.001). An increase in Cu concentration decreased root K only when combined with NaCl and/or PEG (Table 4). When K accumulation for both species was compared, similar K content was found on roots of both species (60 mg g^{-1} DW). Nevertheless, leaf K of *A. halimus* was almost two times larger than *A. atacamensis* (Table 4).

NaCl significantly increased Na accumulation in roots and leaves of both species (Table 4); however, in *A. atacamensis*, there was also a significant influence of Cu and PEG that was not observed in *A. halimus* (Table 4). In the presence of NaCl, Cu caused a significant decrease of leaf Na, even when combined with PEG. In *A. halimus*, only NaCl explained the variation of Na accumulation in roots (P < 0.001), but in leaves it was also explained by its interaction with Cu (P < 0.05). Overall, NaCl treatments caused a near three-fold increase in root Na and a nearly six-fold increase in leaves of both species compared to control conditions (Table 4).

Copper accumulation in roots and leaves of both *Atriplex* species significantly increased in response to Cu and was five- to seven-fold higher in roots than leaves (Fig. 4). Copper concentration in roots of *A. atacamensis* increased in response to Cu single effects but, when combined with NaCl, it decreased an average of 20%. In leaves, a different

response was noted. Single Cu caused an increase in leaf and root Cu of *A. atacamensis*, but its combination with NaCl caused an increase in its accumulation (Fig. 4). Root and leaf Cu of *A. halimus* varied significantly according to single and combined effects of Cu, NaCl, and PEG (Fig. 3). Copper treatments caused a significant increase of Cu in *A. halimus* leaves and roots, but, unlike *A. atacamensis*, an increase in Cu treatments did not increased its accumulation (Fig. 4).

3.5. Oxidative response and chelation

Leaf GSH of *A. atacamensis* and *A. halimus* followed similar patterns (Table 5). A significant increase was found in response to Cu and its combination with NaCl. Combination with PEG caused a further increase in this parameter, but only in *A. atacamensis*. Root GSH of *A. atacamensis* and *A. halimus* varied in response to the combination of Cu, NaCl and PEG (P < 0.05) and also presented a similar response to single and combined treatments (Table 5). Leaf GSSG of both species slightly increased in response to 10 μM of Cu, but the most important increase was found when combined with NaCl and PEG (Table 6); in *A. atacamensis*, this increase was about 870% compared to control conditions (P < 0.001) whilst in *A. halimus* was 440% (P < 0.05) (Table 6). Root GSSG of *A. atacamensis* and *A. halimus* increased in response to single Cu, but only in *A. halimus* its combination with PEG also caused a
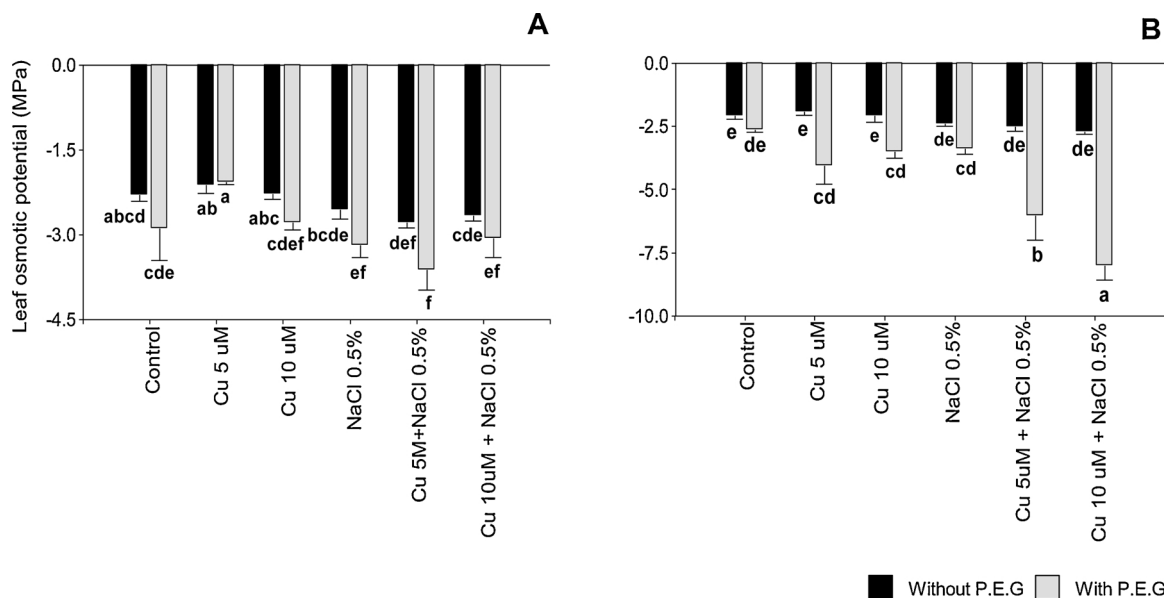

Fig. 2. Leaf osmotic potential (MPa) of *A. atacamensis* (A) and *A. halimus* (B) seedlings exposed for ten days to single and combined Cu, NaCl, and PEG stresses (n = 3; mean ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.
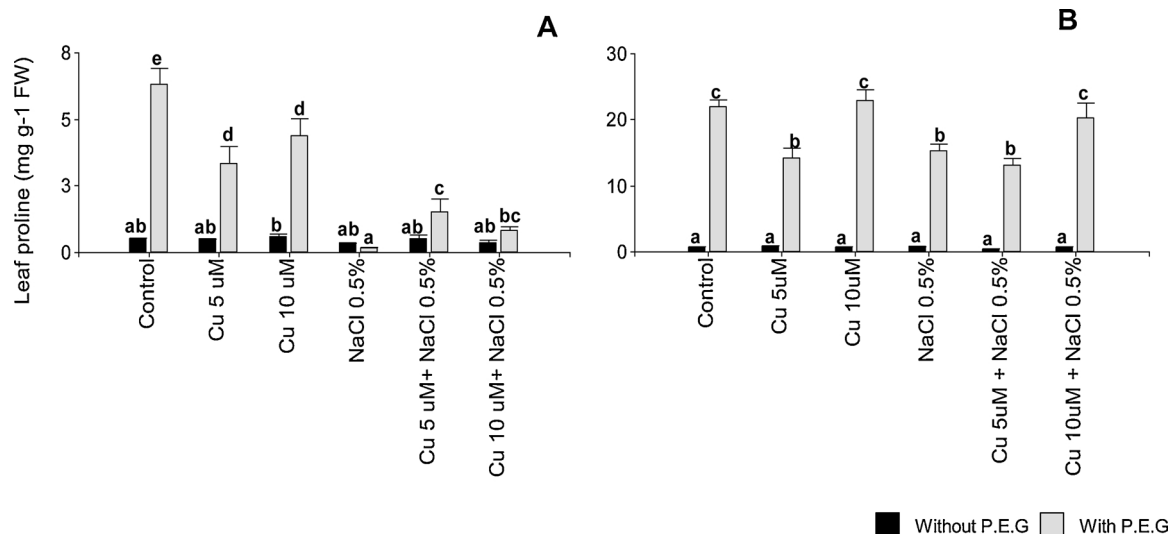
Fig. 3. Leaf proline (mg g^{-1}) of *A. atacamensis* (A) and *A. halimus* (B) seedlings exposed for ten days to single and combined Cu, NaCl, and PEG stresses (n = 4; mean ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.

further increase (Table 6).

Non-protein thiol concentration significantly increased in response to Cu in roots and leaves of both species (Fig. 5). In roots of *A. atacamensis*, this increase was explained by the interaction between Cu, NaCl, and PEG (P < 0.05), with a clear effect of Cu treatments (Fig. 5). In *A. halimus* roots, non-protein thiol concentration was also explained by the interaction among Cu, NaCl, and PEG (P < 0.001); however, the magnitude of this response was nearly half of that of *A. atacamensis* (Fig. 5).

Leaf accumulation of non-protein thiols was about one-third of root accumulation in both species (Fig. 5) and, in *A. atacamensis*, was explained by the individual interactions among Cu and NaCl (P < 0.001) and NaCl and PEG (P < 0.05). In *A. halimus*, the magnitude of the increase was lower than *A. atacamensis*, but the effect of the interaction among Cu, NaCl, and PEG remained (P < 0.001).

4. Discussion

Studies about the effect of single and combined stressors on plant species are a useful tool for understanding stress physiology but also give valuable information for defining plant uses in ecological restoration and remediation programs. In particular, discussion about the response of halophyte species to metal toxicity has multiplied in the last years (Nikalje and Suprasanna, 2018; Walker et al., 2014). In this study, growth and tolerance traits of two *Atriplex* species were measured in the presence of Cu and its combination with salinity (NaCl) and water stress (PEG).

4.1. Effect of Cu enrichment on Atriplex species

It has often been proposed that salt tolerance traits of halophytes confer them the ability to tolerate ionic stress caused by metals (Lutts and Lefevre, 2015). Therefore, it could be expected that both *Atriplex* species tolerate Cu concentrations otherwise considered toxic. Our results show that *A. halimus* and *A. atacamensis* were able to withstand 5 μM of dissolved Cu in solution without significant consequences to its growth. Fresh weight, water content, and ion acquisition of both species did not vary significantly under these conditions. Leaf chlorophyll, a known target of Cu ions and an indicator of the photosynthetic status of the plant, was also unaffected. Maintenance of near-control conditions in *Atriplex* plants subjected to intermediate Cu concentrations could be related to the deployment of physiological mechanisms that allow the plant to cope with variable levels of Cu enrichment without evident

morphological symptoms. For example, an increase of the non-enzymatic antioxidant GSH and its oxidized form (GSSG) was found, suggesting that there was an active process of reactive oxygen species (ROS) scavenging in roots and leaves (Jozefczak et al., 2012). An increase in leaf carotenoids, molecules with photoprotective, and stress signaling functions was also found but only on *A. halimus*.

On the other hand, 10 μM of Cu treatments caused significant impairments to both species. The most conspicuous effects were observed in roots, with a marked decrease in root length, root density, and fine root suberization, especially on *A. atacamensis*. Root damage is a known consequence of Cu toxicity and can be explained by cell membrane damage and alterations to the cell cycle (Pena et al., 2015) and hormone levels in root proliferation areas (Lequeux et al., 2010). Also, Cu-induced root suberization restricts water and ion permeability (Kováč et al., 2018), causing further damage to the plant. In fact, the decrease in root and shoot water content in response to 10 μM of Cu could be related to Cu-induced root damage, namely because Cu treatments did not have a significant effect on leaf osmotic potential or biomass production of none of the species. The lack of an effect of metal toxicity on water content was also described by Vromman et al. (2017) on *A. atacamensis* seedlings exposed to As, a non-nutritional metalloid.

Copper-induced root damage had variable effects on ion accumulation of both species. Potassium, an essential nutrient with numerous roles in plant metabolism and plant water balance (Marschner, 2012) decreased in both species. Although root and leaf Na was not affected by single Cu treatments, a notable increase in root and leaf Cu was found. These results are in line with several authors that describe active metal sequestration on *Atriplex* roots as a possible avoidance strategy (Kachout et al., 2012; Mateos-Naranjo et al., 2013). However, it is interesting to note that root Cu concentration and the root:leaf Cu ratio was higher in *A. atacamensis* than *A. halimus*, even when combined with other stressors. This difference was particularly evident at 10 μM of Cu, where Cu accumulation in roots of *A. atacamensis* nearly doubled *A. halimus*. Differences in Cu accumulation between organs and species could be explained by species-specific strategies or local adaptations to metal toxicity. For example, seeds of *A. atacamensis* used in this study came from a population with high levels of available Cu (15.7 mg Kg^{-1}); therefore, a certain level of tolerance could be expected. Lower Cu accumulation in *A. halimus* roots and leaves could be explained by the lack of such adaptations or a metal exclusion strategy, expressed by low metal accumulation in roots and restricted transport to shoots (Mateos-Naranjo et al., 2013). The ability to capture high concentrations of Cu in tissues without severe effects to the plant functioning can

Table 4
Sodium and K concentration in roots and leaves of *Atriplex atacamensis* and *A. halimus* seedlings exposed for ten days to single and combined Cu, NaCl, and PEG stresses (n = 4; mean ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.

Treatments	*Atriplex atacamensis*				*Atriplex halimus*			
	Root Na$^+$ (mg·g^{-1})	Leaf Na$^+$ (mg·g^{-1})	Root K$^+$ (mg·g^{-1})	Leaf K$^+$ (mg·g^{-1})	Root Na$^+$ (mg·g^{-1})	Leaf Na$^+$ (mg·g^{-1})	Root K$^+$ (mg·g^{-1})	Leaf K$^+$ (mg·g^{-1})
Control	4.5 ± 0.3a	5.4 ± 0.4a	53.7 ± 6a	69.4 ± 10.3a	6.4 ± 1.4a	6 ± 0.4a	59.2 ± 8.1cd	144.6 ± 9.7b
Cu 5 μM	5.9 ± 1.4a	7.8 ± 0.9a	42.9 ± 1.1ab	63.6 ± 2.8a	6.1 ± 2.6a	7.4 ± 1a	72.7 ± 8.4d	77.5 ± 2.1a
Cu 10 μM	4.3 ± 0.1a	28.6 ± 4.4b	42.7 ± 5.9ab	43.7 ± 5.9ab	7.6 ± 0.2a	11.7 ± 2.2a	42.3 ± 2.9abc	95.6 ± 9.8ab
NaCl 0.5%	22.7 ± 3.4c	4.9 ± 0.2d	30.1 ± 3.7abc	39.3 ± 1.5ab	29.3 ± 5.2c	70.5 ± 9.1c	40.6 ± 5.9abc	102.3 ± 20.3ab
Cu 5 μM + NaCl 0.5%	12.6 ± 0.7b	4.5 ± 0.1b	19.3 ± 1.5c	37.5 ± 2.3ab	22.1 ± 2.3c	66.4 ± 7.9bc	43.3 ± 2.9abc	104.5 ± 3.1ab
Cu 10 μM + NaCl 0.5%	18.9 ± 0.8c	9.2 ± 2.5bc	27.2 ± 1.5abc	30.9 ± 3.9b	21.7 ± 0.7bc	45.3 ± 1b	57.5 ± 1.3cd	83.4 ± 7.8a
PEG 7.85%	4.9 ± 0.2a	9.6 ± 1.2a	30.8 ± 4.5abc	41.5 ± 2.9ab	11.6 ± 2.1ab	10.2 ± 1.6a	40 ± 3.3abc	109.1 ± 14.6ab
Cu 5 μM + PEG 7.85%	4.5 ± 0.1a	31.7 ± 2.2bc	26 ± 2.1abc	30.7 ± 5.4b	9.6 ± 0.01a	6.4 ± 1.0a	47.4 ± 5.5bc	88.3 ± 11.3ab
Cu 10 μM + PEG 7.85%	9.2 ± 2.5a	11.4 ± 2.1a	29.9 ± 4.8abc	39.8 ± 5.9ab	6 ± 1.1a	9.8 ± 0.6a	31.2 ± 1.7ab	76.7 ± 11.5a
PEG 7.85% + NaCl 0.5%	20.7 ± 1.1c	63.9 ± 8.3c	27.8 ± 1.3abc	30.8 ± 3.5b	24.7 ± 0.1c	54 ± 6.9bc	51.4 ± 5.5bcd	85.8 ± 10.7a
Cu 5 μM + NaCl 0.5% + PEG 7.85%	16.4 ± 0.6bc	45.7 ± 1.7bc	24.9 ± 3.1bc	34.4 ± 1.5ab	23.2 ± 1.3c	52.4 ± 3.5bc	21.6 ± 0.3a	70.8 ± 12a
Cu 10 μM + NaCl 0.5% + PEG 7.85%	16.9 ± 1.6bc	48.1 ± 2.6bc	20.9 ± 2.7c	34.7 ± 0.8ab	23.4 ± 1.9c	51.8 ± 1.3bc	22.4 ± 1.9a	87.2 ± 8.4a

be achieved by its chelation and sequestration in a non-reactive form (Gratão et al., 2005). On this subject, we found that Cu induced non-protein thiols synthesis in roots and leaves of both species, with a similar pattern of root Cu accumulation, where *A. atacamensis* nearly doubled *A. halimus* in 10 μM of Cu treated plants.

The described effects correspond with other articles that explore Cu toxicity (Brahim and Mohamed, 2011; Dasgupta-Schubert et al., 2011; Jordan et al., 2002); however, the magnitude of the effect vary according to the concentration used. For example, Mateos-Naranjo et al. (2013) found that Cu LC$_{50}$ of *Atriplex halimus* occurred between 15 y 30 mM, a concentration three orders of magnitude higher than those used in this study. These large differences can be explained not only for the substrate used in the experiment—whether it is in vitro culture, soil, or hydroponics—but also the pH of the nutrient solution used to cultivate or irrigate the plants. Copper bioavailability decreases with rising pH (Ginocchio et al., 2009); therefore, toxic concentration of Cu can be overestimated, along with plant tolerance (Kopittke et al., 2010).

4.2. Effect of salinity on Atriplex species

No negative effects were found in growth parameters of *Atriplex* species treated with 0.5% NaCl (85.5 mM). On the contrary, shoot biomass and water content increased under these conditions. These results are in agreement with other studies that found that moderate NaCl concentrations (50–200 mM) can actually promote growth in halophyte species (Han et al., 2012; Walker et al., 2014), while higher concentrations have inhibitory effects (Nemat Alla et al., 2011).

The fact that leaf water potential and shoot dry weight were also unaffected by NaCl treatments, suggests that *Atriplex* species were actively performing osmotic adjustment in leaves in order to keep growing (Bromham and Bennett, 2014). Osmotic adjustment in halophyte species can be achieved by the accumulation of compatible osmolytes and/or inorganic ions (K, Na, Cl) in the vacuole. These strategies allow the plant to maintain a more negative leaf water potential than the substrate, favoring continuum water supply (Adolf et al., 2013). The use of each one of these strategies seems to be related to carbon economy, where ion sequestration is a less demanding mechanism than organic compatible solute synthesis (Eisa et al., 2012). In fact, proline synthesis on NaCl-treated plants was not different than control conditions in none of the species.

Our results show that Na assimilation increased under salinity treatments in both *Atriplex* species, but, unlike Cu, Na was higher in leaves than in roots. This suggests that there is active transport to upper organs in order to perform osmotic adjustment. Previous studies performed in two *Tecticornia* species (also Chenopodiaceae) showed that a group of compatible solutes, among which were Na and glycinbetaine, increased in response to 500 mM NaCl (English and Colmer, 2013); moreover, Martínez et al. (2003) found that *A. halimus* was able to accumulate Na in leaves after 22 days of withholding water, even in the absence of salinity.

The increase of Na in *Atriplex* leaves can also be explained by its use in carbon metabolism. Halophytes that also exhibit a C4 photosynthetic pathway, such as *Atriplex*, use Na ions for phosphoenolpyruvate regeneration from pyruvate in mesophyll chloroplasts (Ohnishi et al., 1990; Subbarao et al., 2003); therefore, active Na transport into leaves could also be a strategy to favor photosynthesis under salt stress or water scarcity.

4.3. Dual effect of PEG on Atriplex

PEG-induced water stress had a negative impact on root and shoot growth of both *Atriplex* species. Notably, morphological and physiological responses of *Atriplex* seedlings to PEG were similar to the most stressful treatments of this experiment, and its most visible effects were observed at the shoot level.

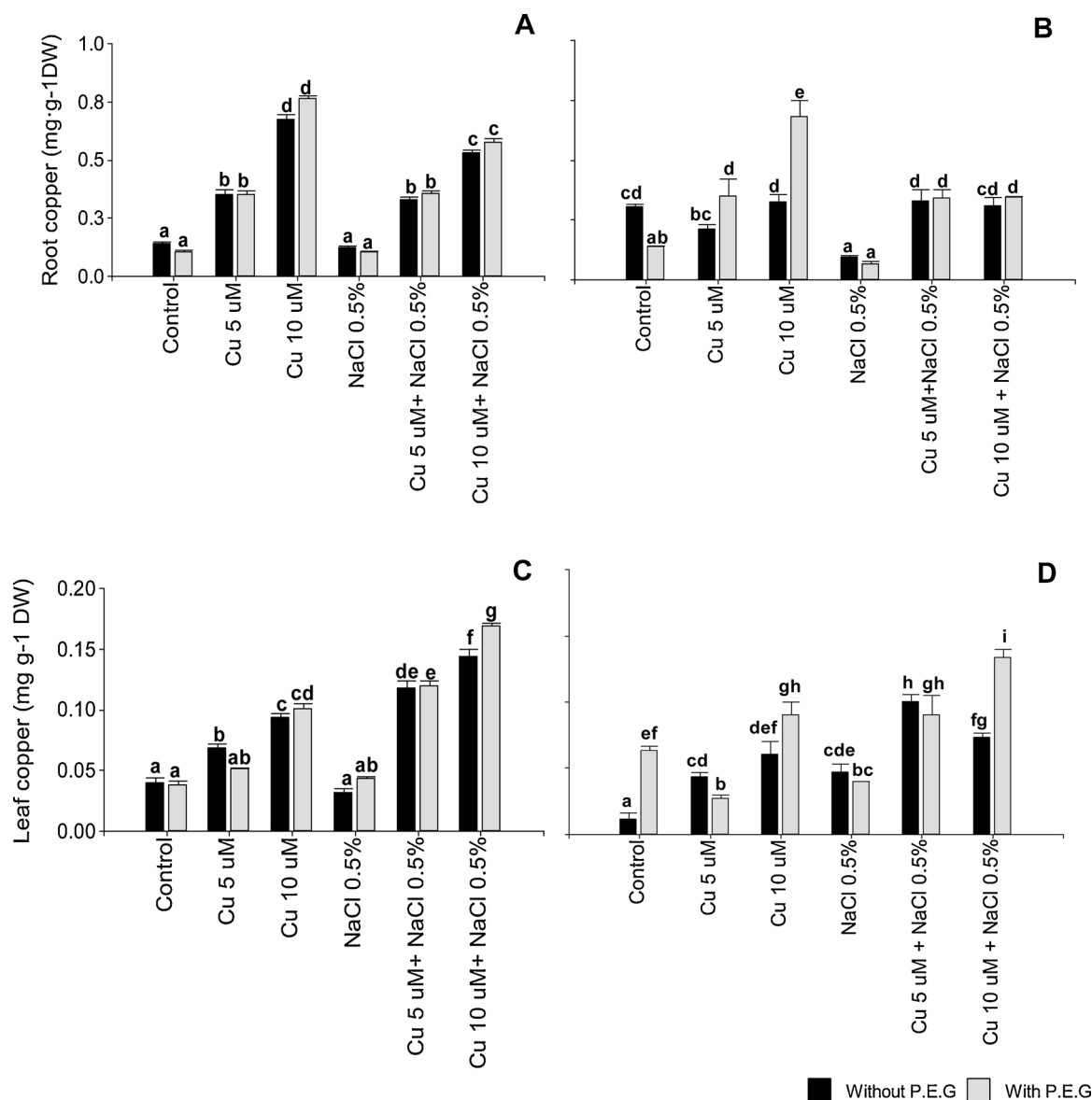Whilst PEG is considered the best component to imitate water stress

Fig. 4. Copper concentration (mg·g^{-1}) in roots (A–B) and leaves of (C–D) of *A. atacamensis* and *A. halimus*, respectively. Seedlings were exposed for ten days to single and combined Cu, NaCl, and PEG stresses (n = 5 means ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.

Table 5
Reduced glutathione (GSH) in leaves and roots of *Atriplex atacamensis* and *A. halimus* exposed for ten days to single and combined Cu, NaCl, and PEG stresses (n = 5 means ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.

Treatments	*Atriplex atacamensis*		*Atriplex halimus*	
	Root GSH (nmol g^{-1})	Leaf GSH (nmol g^{-1})	Root GSH (nmol g^{-1})	Leaf GSH (nmol g^{-1})
Control	123.4 ± 5.4a	47 ± 4.8a	151.6 ± 4.6a	52.6 ± 2.18a
Cu 5 μM	541.2 ± 33.2def	141.6 ± 7.7c	346.4 ± 24.3b	151.2 ± 18.3c
Cu 10 μM	627.6 ± 39.2f	245.4 ± 15.7ef	420.4 ± 12.7b	255.4 ± 16.9d
NaCl 0.5%	156.6 ± 17.5a	165.0 ± 6.5 cd	162.2 ± 15.1a	142.6 ± 20.9bc
Cu 5 μM + NaCl 0.5%	409.4 ± 37.4bc	251.0 ± 12.8ef	419.8 ± 37.3b	251.8 ± 15.3d
Cu 10 μM + NaCl 0.5%	338.8 ± 43.1b	282.8 ± 8.4fg	512.2 ± 34.2b	287.0 ± 9.7de
PEG 7.85%	93.6 ± 5.7a	90.6 ± 8.7b	111.4 ± 8.1a	70.6 ± 4.3ab
Cu 5 μM + PEG 7.85%	421.6 ± 27.6bcd	215.2 ± 16.8de	284.6 ± 32.5b	148 ± 16.5c
Cu 10 μM + PEG 7.85%	590.0 ± 14.9ef	285.8 ± 16.7fg	460.6 ± 10.1d	341.0 ± 11.8e
PEG 7.85% + NaCl 0.5%	99.6 ± 6.9a	178.8 ± 8.8 cd	267.0 ± 66.8a	149.2 ± 10.3c
Cu 5 μM + NaCl 0.5% + PEG 7.85%	444.2 ± 16.6bcd	337.8 ± 24.1 g	465.6 ± 28.8c	323.6 ± 26de
Cu 10 μM + NaCl 0.5% + PEG 7.85%	470 ± 22.7cde	431.6 ± 11.9 h	493.2 ± 29.0b	421.8 ± 15.1f

Table 6

Oxidized glutathione (GSSG) in leaves and roots of *Atriplex atacamensis* and *A. halimus* exposed for ten days to single and combined Cu, NaCl, and PEG stresses (n = 5 means ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.

| Treatments	*Atriplex atacamensis*		*Atriplex halimus*	
	Root GSH (nmol g^{-1})	Leaf GSH (nmol g^{-1})	Root GSH (nmol g^{-1})	Leaf GSH (nmol g^{-1})
Control	17.2 ± 1.3a	16.4 ± 1.2ab	24.2 ± 4.6a	21.4 ± 1.0a
Cu 5 μM	129.4 ± 11.7 cd	15.6 ± 1.8a	245 ± 21.7b	39.6 ± 5.2a
Cu 10 μM	257.6 ± 18.6ef	40.8 ± 4.3d	311.2 ± 27.3b	54.0 ± 7.4a
NaCl 0.5%	45.0 ± 6.1ab	17.8 ± 2.1abc	28.6 ± 5.1a	40.2 ± 7.0a
Cu 5 μM + NaCl 0.5%	255.8 ± 0.7ef	30.8 ± 1.3bcd	270.6 ± 0.7b	58.0 ± 8.6a
Cu 10 μM + NaCl 0.5%	339.6 ± 0.7 g	33.4 ± 7.1 cd	299.6 ± 0.7b	49.6 ± 4.4a
PEG 7.85%	16.2 ± 1.7a	14.2 ± 3.2a	32.4 ± 4.0a	30.8 ± 2.0a
Cu 5 μM + PEG 7.85%	114.4 ± 8.6bcd	24.4 ± 3.2abcd	226.6 ± 27.9b	42.4 ± 3.9a
Cu 10 μM + PEG 7.85%	267.6 ± 18.9fg	26.8 ± 3.2abcd	558 ± 20.5d	48.2 ± 9.6a
PEG 7.85% + NaCl 0.5%	87.8 ± 9.1abc	25.2 ± 2.1abcd	53.4 ± 9.19a	53.8 ± 6.2a
Cu 5 μM + NaCl 0.5% + PEG 7.85%	224.6 ± 10.6ef	141.0 ± 2.9e	429.2 ± 30.6c	111.8 ± 10.9b
Cu 10 μM + NaCl 0.5% + PEG 7.85%	187.8 ± 23.7de	159.2 ± 12.1e	225.0 ± 8.0b	116.0 ± 15.7b
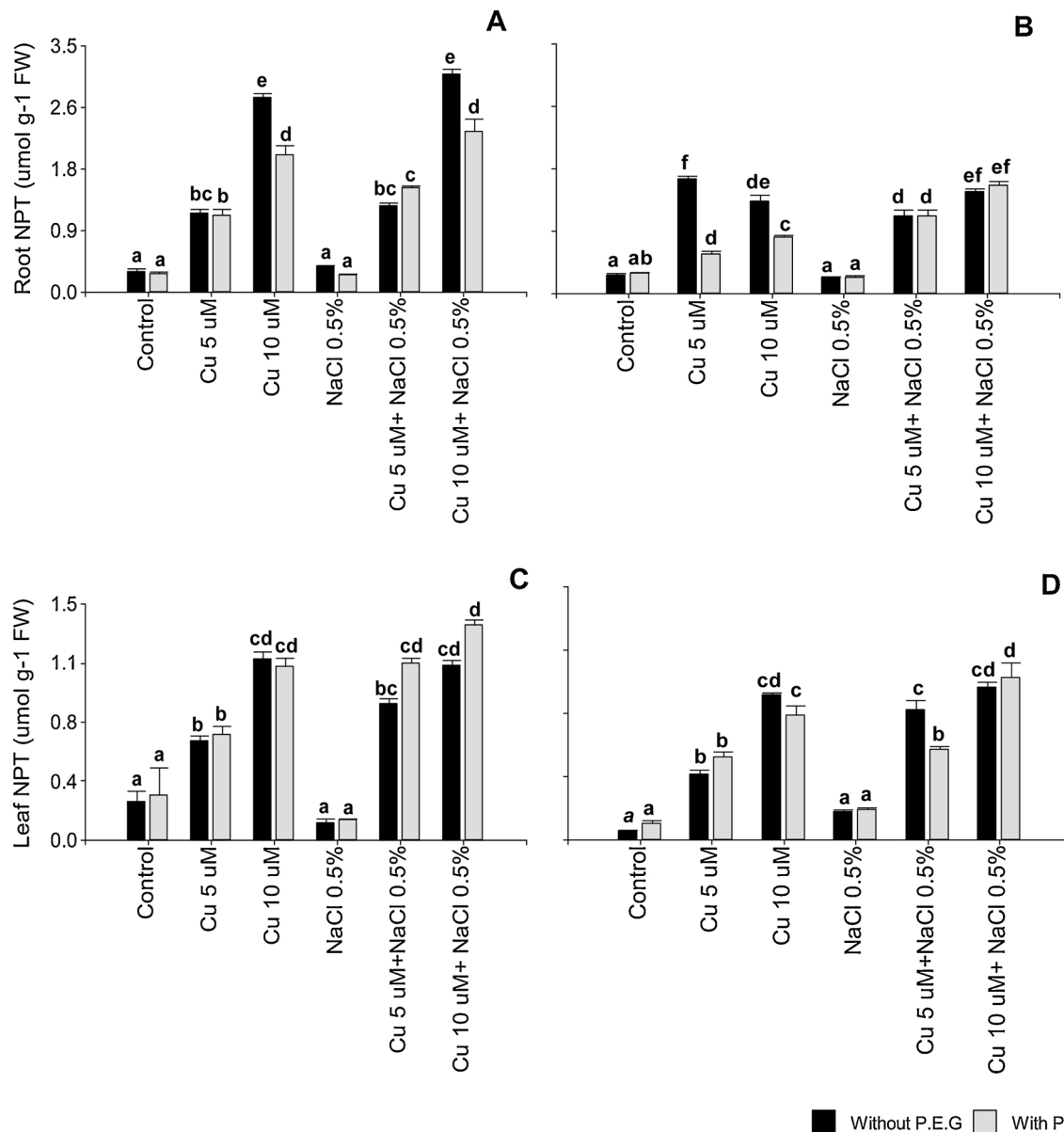
Fig. 5. Non-protein thiols (NPT) (μmol g^{-1}) in roots (A–B) and leaves of (C–D) of *A. atacamensis* and *A. halimus*, respectively. Seedlings were exposed for ten days to single and combined Cu, NaCl, and PEG stresses (n = 5 means ± EE). Different letters denote significant differences at p ≤ 0.05 among treatments.

in nutrient solutions, some studies have also suggested that, in addition to a decrease of solute potential, this molecule also has a physical effect around roots that could explain the observed effects on *Atriplex* shoots (Shi et al., 2015). On this subject, Slama *et al.* (2007) reported that growth inhibition caused by PEG was more severe than other isosmotic treatments, such as mannitol or NaCl, probably due to an increase of viscosity that restricts gas and water transport to the plant. In this line, they proposed that growth restrictions in PEG-treated plants could be better explained by a combination between osmotic stress and nutrient deficiency. In fact, our results show that K accumulation in the shoot decreased in the presence of PEG, regardless of the accompanying stressor. PEG-induced stress was particularly visible as a severe loss of turgor and wilting in *A. halimus* shoots, and, whilst no significant variation in leaf GSH or carotenoids was found, a 10–15 fold proline increase occurred, suggesting that stress sensing and response of this stressor had another timing or followed a different pathway.

4.4. Atriplex species response to combined stresses

The combination of more than one abiotic stressor caused different responses in both *Atriplex* species, but, as it was proposed by Choudhury et al. (2017) stressor combination caused a synergistic effect, where decrease in plant growth parameters had a greater magnitude when more than one stressor was present.

Of particular relevance is the effect of Cu and its individual combination with NaCl and PEG. Several studies have proposed that salinity conditions alleviate metal toxicity effects on halophyte species (Cheng et al., 2018; Wali et al., 2015). Our results show that salinity conditions did not alleviate or improve growth parameters of *A. halimus* and *A. atacamensis* subjected to Cu enrichment, particularly on 10 μM of Cu treatments; instead, the only parameters that varied in response to the combination between Cu and NaCl were carotenoids and glutathione.

Total glutathione increased in response to the combination of Cu and NaCl treatments. The GSH:GSSH ratio showed that the decrease of GSH in roots and leaves that occurred in response to Cu did not vary when combined with NaCl. This suggests that either reduced glutathione was being oxidized to GSSG at a higher rate than its recycling capacity or there was a detrimental effect on glutathione reductase synthesis (Anjum et al., 2014). It is also possible that this decrease in GSH was related to phytochelatins synthesis as Bankaji et al. (2015) observed in *Suaeda fruticosa* seedlings or for the sequestration of Cu ions in the vacuole. In fact, the observed increase in root NPT in response to Cu remained in conditions of salinity. This implies that active sequestration of Cu ions could occur in parallel to glutathione ROS scavenging activity.

Single NaCl and Cu enrichment are two abiotic disturbances that modify ion availability in the substrate. However, ion transport and assimilation also depend on the ability of the plant to acquire and transport water through the xylem (De Boer and Volkov, 2003); therefore, it could be expected that PEG-induced water stress also restricted ion assimilation, especially when combined with Cu or NaCl treatments. However, our results indicate otherwise: in conditions of salinity, PEG did not have an effect on leaf Na of none of the studied species. Similarly, it was found PEG treatments did not reduce Cu accumulation in roots or leaves of *Atriplex* species subjected to Cu; in fact, it caused an increase in Cu accumulation in leaves and roots of *A. halimus*.

Another component with an interesting response to stress combination was proline. This molecule has been described as a compatible osmolyte but also as a stress signaling molecule (Ashraf and Foolad, 2007). Our results show that proline did not vary in response to single Cu stress in any of the species, but it did in response to single PEG treatments. These results show that, in *Atriplex*, proline is synthetized in response to water stress. However, proline response to the combination of NaCl and PEG varied among the two species: whilst *A. halimus* proline levels remained high, *A. atacamensis* proline remained similar to

control levels. The difference among these responses could reflect that *A. atacamensis* uses NaCl as a substrate to decrease leaf water potential and tolerate PEG-induced water stress, as it has been described on *A. halimus* (Martínez et al., 2005). Alternatively, it could be that the detrimental effect of PEG on *A. halimus* was so severe that proline assumed more than one role in plant response to PEG.

Overall, stressor combination caused a neutral or synergistic effect in which the sum of more than one condition caused a similar or worse effect than single-stressor conditions, especially with PEG; however, further comparison among the species and a search for tolerance patterns in response to specific stress combinations were limited by the chosen factorial design. In this line, we believe that the presented results could be used as a base to perform more specific research on combined stress response at a species level or broaden the picture on *Atriplex* specific responses.

Stress combination experiments are a valid approach to describe the nature of plant responses to multiple stressors on the field (Suzuki et al., 2014). With this in mind, we observed that stressor combination on *Atriplex* species resulted in a decreased condition and an augmented synthesis and accumulation of different defense components. Yet, physiological effects and its visual expression greatly varied among *A. atacamensis* and *A. halimus* on different stressor combinations. The ability to interpret stressor magnitude and its effect on *Atriplex* species will allow us to understand further about the ecophysiology of these two species used in the phytostabilization of metal enriched sites.

5. Conclusion

Single and combined stressors had different effects between the two *Atriplex* species. Intermediate Cu treatments did not affect *Atriplex* species, but high Cu treatments did affect growth and physiological response of *A. atacamensis*. NaCl did not have detrimental effects on plant growth but did induce physiological responses, such as ion accumulation and GSH synthesis. PEG treatments affected water balance parameters of both species, especially in *A. halimus*, but did not have an effect on ion transport and accumulation. Finally, growth and physiological responses of *A. atacamensis* and *A. halimus* to combined stressors showed to be contrasting. Sodium chloride did not alleviate Cu-induced stress; PEG did not decrease ion acquisition in Cu and NaCl treatments and caused detrimental effects that depended on the presence of other stressors.

CRediT authorship contribution statement

Fabiola Orrego: Conceptualization, Data curation, Methodology, Software, Writing - original draft. **Claudia Ortiz-Calderón:** Data curation, Methodology, Writing - review & editing. **Stanley Lutts:** Methodology, Investigation, Data curation. **Rosanna Ginocchio:** Supervision, Methodology, Writing - review & editing, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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