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Effect of single and combined Cu, NaCl and water stresses on three *Atriplex* species with phytostabilization potential

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ABSTRACT

Phytostabilization of metal enriched substrates in arid and semiarid areas depends on the use of metal-tolerant or metallophyte plants in order to decrease metal mobilization and dispersion. However, the co-occurrence of other abiotic restrictions on plant growth, such as drought and salinity calls for a new perspective for plant selection. Since components of salt and drought tolerance traits of halophytes are also present in metal stress, *Atriplex* species, typical of dry and salty soils, emerge as good candidates for phytostabilization. But in order to confirm this potential, it is necessary to explore specific responses to increasing copper, salt and water stress.
In this study, we compared the effect of single and combined copper, NaCl and PEG- induced water stresses on growth parameters of the Chilean *Atriplex atacamensis*, European *A. halimus* and Australian *A. nummularia* under controlled hydroponic assays. Results showed that increasing copper had severe effects on root development of all three species, with subsequent effects on their shoot biomass. Salt stress effects were mostly osmotic, with a decrease in shoot fresh weight and water content, and PEG- induced water stress had no clear effect on growth of roots and shoots. Combination of copper with NaCl and PEG further decreased plant growth, but this effect varied among *Atriplex* species. This shows that growth of *Atriplex* species responds differently to each individual stress and that stressor combination causes an overall negative effects in their growth parameters.

1. Introduction

Anthropogenic enrichment of metals due to industrial activities, is a worldwide issue that causes major environmental consequences (De Gregori et al., 2003; Gidhagen et al., 2002). Among them, the accumulation of potentially toxic metals in groundwater and soils do not only could pose direct effects on organisms and affect the development of ecological communities, but could also potentially threaten human health by the transport of metals through food chains (Martínez-Domínguez et al., 2008; Montenegro et al., 2009). In particular, northern and central Chile presents a wide gradient of metal enrichment from natural and anthropogenic sources (De Gregori et al., 2003). Among them, copper (Cu) stands out because of its high constitutive presence in several mineral formations and historical enrichment as a result of unregulated mining operations during the XIX and XX century (Ginocchio, 2000; Lam et al., 2016).

Copper is considered a micronutrient for plants, but the range between nutritious and phytotoxic concentrations is narrow. Copper tissue concentration among 5 and 10 mg kg^{-1} is necessary for physiological processes such as photosynthesis, cell wall metabolism and ethylene sensing (Yruela, 2005), but tissue accumulation above this threshold is considered phytotoxic (Jordan et al., 2002; Verdejo et al., 2016). Typical Cu phytotoxicity symptoms are growth inhibition, browning of roots and leaf interveinal chlorosis and reddening (Reichman, 2002). Copper phytotoxicity depends on its total concentration and other soil physicochemical parameters that determine its bioavailability, such as soil organic matter, dissolved organic carbon and pH (Ginocchio et al., 2002; Zeng et al., 2011). Therefore, in places where the presence of Cu is consistently high, such as chemically degraded soils and hard-rock mine wastes, a detrimental effect in plant communities and ecosystems may be expected (Ginocchio, 2000; Ginocchio et al., 2002; Ortiz-Calderón et al., 2008).

One alternative for the ecological rehabilitation of metal enriched areas is phytostabilization. Here, metal-tolerant plants are selected to

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sequester metals at a rhizosphere level in order to restrict wind and groundwater dispersion and therefore, in situ stabilization of metals (Alford et al., 2010; Mendez and Maier, 2008). However, a number of Cu-enriched areas occur in arid and semiarid ecosystems that, as a result of low precipitation and high temperatures, also present variable levels of salinization (Casanova et al., 2013). Therefore, plant research in a phytostabilization context, must consider not only the effects of a primary metal stressor, but also the co-occurrence of other abiotic restrictions, such as drought and salinity (Ginocchio et al., 2017). Under this conditions, halophytes or salt tolerant species have been proposed as good candidates for phytostabilization due to the expression of salt and drought tolerance traits that are also present in metal stress response (Mittler, 2006; Nikalje and Suprasanna, 2018).

Atriplex is a globally distributed genus of halophyte herbs and shrubs present in arid areas with varying levels of salinity (Manousaki and Kalogerakis, 2009; Walker et al., 2014). Its colonization success in deserts, Mediterranean and coastal areas is based on their ability to tolerate variable conditions of salt and water stresses (Brignone et al., 2016; Lutts et al., 2004). The genus *Atriplex* has been proposed for phytostabilization, because some species have been found growing near mining areas and metal enriched sites (Mendez and Maier, 2007). Further research on these species has shown that they not only survive in these areas, but can cope with high concentrations of different metals and metalloids (Manousaki and Kalogerakis, 2009; Mateos-Naranjo et al., 2013; Nedjimi and Daoud, 2009; Vromman and Paternostre, 2016).

Atriplex atacamensis is a shrub endemic to the Atacama Desert that grows near mining areas and has been described as an arsenic tolerant species (Tapia et al., 2013; Vromman et al., 2016). Further south, in the semiarid Coquimbo Region, *Atriplex nummularia* and *Atriplex halimus* are two exotic species that were introduced in the seventies due to its livestock potential (Lailhacar et al., 1995). *Atriplex nummularia,* a fast growing shrub from Australia, has been proposed for the remediation of salt enriched sites, and also tested for its metal tolerance (Jordan et al., 2002). *Triplex halimus*, native to the Mediterranean basin, has been profusely studied in the last 15 years for its potential to resist the effect of several metals (El-Bakatoushi et al., 2015; Walker et al., 2014). Although there is evidence about the potential of these species to tolerate metal stress, there is scarce knowledge on their tolerance to copper and the presence of other abiotic stressors, such as drought and salinity occurring at the same time. Therefore, the aim of the present study was to compare, at a laboratory level, growth responses of *A. atacamensis, A. halimus* and *A. nummularia* when subjected to single and co-ocurring copper, salt and water stresses.

2. Materials and methods

2.1. Plant material

Fruits of *A. atacamensis* were collected in February 2017 along the Loa River (UTM 19 K: 510307 E 7516731 N), Antofagasta Region of Chile. *Atriplex halimus* fruits were obtained commercially from Spain in 2017 (Weberseeds), and *A. nummularia* fruits were obtained in 2015 from the Corporación Nacional Forestal (CONAF) at the Coquimbo Region, Chile. Fruits were kept dry and in darkness until used. After removal of the bracts, seeds were rinsed several times and submerged in distilled water for three hours to dissolve adhered salts. Then, seeds were left to germinate in separate trays filled with perlite and distilled water under controlled laboratory conditions. Temperature was set at 22 ± 1 °C, and relative humidity was about 40%. Natural light was supplemented by Phillips lamps in order to maintain an irradiance of 60 μmol m^{-2} s^{-2} under a 12 h photoperiod.

After five weeks, eight seedlings at a two-leaf stage were transplanted into polystyrene plates floating on 1 L polypropylene boxes with an aerated Hoagland solution containing 0.2 mM of $Mg(SO_4)_2 \cdot 7H_2O$; 0.5 mM $Ca(NO_3)_2 \cdot 4H_2O$; 0.5 mM KNO_3; 0.1 mM of K_2HPO_4; 0.2 μM $Cu(SO_4) \cdot 5H_2O$; 0.2 μM of $Zn(SO_4) \cdot 7H_2O$; 2 μM of $MnCl_2 \cdot 4H_2O$; 10 μM of H_3BO_3; 0.1 μM of MoO_3; and 10.7 μM EDTA chelated Fe (Harper et al., 1998). Before transplant, all roots were cut to 3 cm to promote homogeneity among naturally heterogeneous individuals. Plants were allowed to acclimate for seven days under these conditions, and then individual tolerance essays for Cu, drought and salinity were performed.

2.2. Single-stressor assays

In total, nine experiments were carried out; each one lasted seven days under the laboratory conditions described above. For Cu treatments, $Cu(SO_4) \cdot 5H_2O$ was added to the nutrient solution in order to achieve nominal concentrations of 0, 10 or 20 μmol L^{-1}. For drought treatments, polyethylene glycol (PEG), a polymer that reduces water availability for the plant without entering to its tissues, was used. In each treatment, PEG 6000 flakes were diluted into the nutrient solution in order to reach 0, -0.1 and -0.25 MPa according to Michel and Kaufmann (1973). For salinity treatments, NaCl was added in order to reach 0, 1% and 2% concentrations. Reagents used for nutrient solution and treatments were analytical grade obtained from Merck (Germany). Each experiment had four replicates, represented on polystyrene boxes. The nutrient solution was replaced mid-experiment and the pH was adjusted every two days to 5.3 ± 0.05 with KOH or HCl to favor Cu and nutrient availability in the solution.

At the end of each experiment, five plants per treatment were randomly harvested. Roots were rinsed with deionized water and gently blotted dry. Roots and shoots were then separated for total length and fresh weight measurements. Root increment was calculated as the difference between the root length average of four plants measured at the beginning and the end of the experiments. Dry weight was determined after two days of incubation in an oven at 50 °C. Plant water content was calculated using fresh and dry weight measurements according to Eq. (1) (Vromman et al., 2011):

$$WC = \frac{DW}{(DW + FW)} \tag{1}$$

2.3. Combined stressors assay

After single stress experiments, a combined assay was performed on *A. atacamensis* and *A. halimus* seedlings in order to assess combined stress responses. Six seedlings of each species were transplanted into an aireated modified Hoagland solution that 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 1 week of acclimation under these conditions, single Cu (10 μM), and its combination with NaCl (0.5%) or PEG (-0.1 MPa) were applied for ten days. Room temperature and humidity were the same as the previous experiment and nutrient solution was replaced once mid-treatment. At the end of the experiment, root length and shoot fresh weight was measured on five seedlings per species.

2.4. Statistical analysis

Two independent single stress experiments were performed earlier, with similar trends. Experiments were carried out under a completely randomized design, and significances per species were tested by a one-way ANOVA test with significance levels determined at $P < 0.05$. If results were significant, a Tukey's test was used to identify differences among groups. In case of non-normality or homogeneity of variances, data sets were compared with a Kruskal–Wallis non-parametric test. On combined stress assays, one-way ANOVA was performed in order to compare treatments on each species, and differences among groups were also identified through Tukey tests. All parametric and non-parametric analysis were performed with the statistical package INFOSTAT (Di Rienzo et al., 2016).

3. Results

3.1. Effect of Cu on plant growth

The effect of available Cu on growth parameters varied among species and treatments. Although mortality was only observed in 11% of *A. halimus* seedlings exposed to 20 μM Cu (data not shown), a general detrimental effect was observed in all three species at this Cu concentration. Also, a reddish color was observed on the abaxial side of leaves of the three species, especially when subjected to 20 μM Cu.

In control conditions, root growth of *A. nummularia* was greater than *A. atacamensis* and *A. halimus*; yet, this increase was significantly affected by available Cu. *Atriplex atacamensis* and *A. nummularia* were the most affected species, with a 49% and 45% decrease in response to 10 uM Cu, respectively. This magnitude was lower in *A. halimus* (18%), but still significant. In all cases, no further variation was observed in response to 20 μM Cu (Fig. 1).

Root dry weight of *A. halimus* and *A. nummularia* was not significantly affected by Cu. Yet, a significant decrease (27%) was found in *A. atacamensis* when subjected to 10 μM Cu (Table 1). Interestingly, shoot dry weight of *A. atacamensis* and *A. nummularia* had a non-significant decrease under Cu treatments, while *A. halimus* had a significant decrease ($P < 0.05$, H = 4.7) in response to 20 μM Cu (Table 1).

Plant water content was also affected by copper treatments (Fig. 2), but only *A. halimus* and *A. atacamensis* had a significant reduction at 10 μM Cu which continued under 20 μM Cu treatments (Fig. 2). At the end of the experiment, total reduction of plant water content of *A. halimus* and *A. atacamensis* subjected to 20 μM Cu was 58% and 36%, respectively. In contrast, water content of *A. nummularia* significantly decreased under 10 μM Cu treatments, with no further significant decrease in response to 20 μM Cu (Fig. 2).

3.2. Impact of salinity on plant growth

Seven percent of *A. nummularia* seedlings treated with 2% NaCl died during the experiment. The rest of the plants remained alive, but with different levels of growth disruption. Although an increase in root length was observed on seddlings of the three species of *Atriplex* treated with 1% NaCl, only root length of *A. atacamensis* had no significant decrease when compared to control conditions (Fig. 1). Two percent NaCl treatments did cause a significant decrease (31%) of *A. atacamensis* root growth compared with the 42% and 43% decrease of *A. halimus* and *A. nummularia* under the same conditions (Fig. 1).

Root dry weight of the species was not affected by 1% NaCl, but under 2% NaCl a significant decrease of this parameter was found on *A. halimus* (17%) and *A. nummularia* (30%) seedlings. Shoot dry weight of the species did not decrease in response to NaCl, but *A. atacamensis* and *A. nummularia* had a non-significant 15% and 21% increase of shoot dry weight in response to 1% NaCl. Plant water content of all three *Atriplex* species decreased in response to 2% NaCl, but only *A. atacamensis* was also significantly affected by 1% NaCl (Fig. 2). Wilting and a decrease in leaf growth was visible, especially on *A. halimus*, that showed a 38% decrease of water content at 2% NaCl treatments.

3.3. Effect of solute potential reduction on plant growth

A reduction of solute potential by the addition of PEG did not cause evident growth impairments in *Atriplex* species. Still, 5% of *A. atacamensis* seedlings subjected to -0.25 MPa died during the experiment (data not shown). Concurrently, the decrease of solute potential did not cause a significant effect on root length in any of the *Atriplex* species (Fig. 1); on the contrary, growth followed a similar pattern to control conditions. Dry weight of *Atriplex* species also remained unaffected whatever the treatment (Table 1), but the reduction of solute potential did cause a significant decrease of plant water content in *A. nummularia* and *A. halimus,* that reached 20% under -0.25 MPa for both species. *Atriplex atacamensis* was not affected by the applied treatments (Fig. 2).

3.4. Effect of combined stresses on A. halimus and A. atacamensis

As it was observed on single-stress assays (Fig. 1), roots of *A. atacamensis* were bigger than *A. halimus* in control conditions. When exposed to 10 μM Cu, root growth of *A. atacamensis* had a significant decrease of 53%, but *A. halimus* was not affected (Fig. 3). Combination of Cu with NaCl or PEG caused a further decrease in root length of *A. atacamensis*, and while the difference was not significant, plants subjected to the combination of Cu and NaCl had the lowest growth.

Similar to root length, shoot fresh weight of *A. atacamensis* significantly decreased in response to single Cu and its combination with NaCl and PEG. This decrease was more marked under the Cu + PEG treatment, where it reached 55% of control conditions (Fig. 3). The response of *A. halimus* was slightly different; unlike single stress assays, shoot fresh weight did not significantly vary when subjected to 10 μM Cu. Interestingly, when Cu was combined with NaCl, shoot

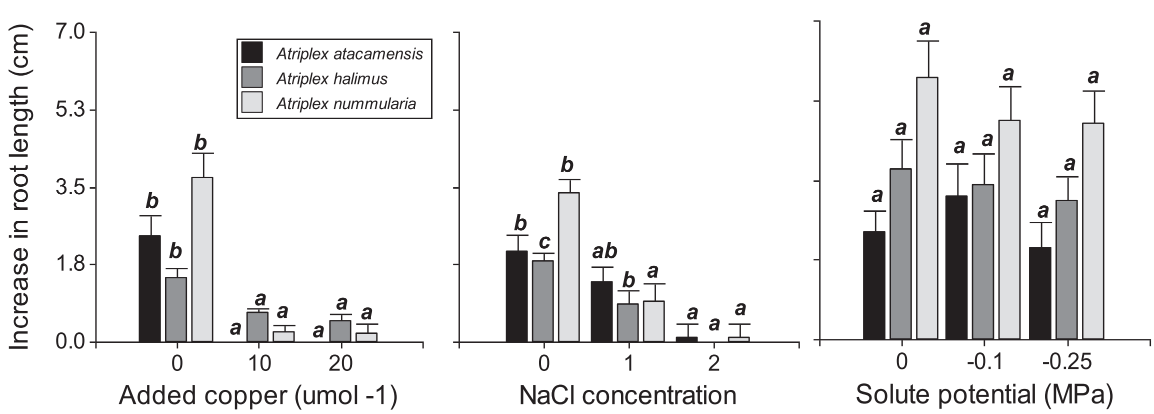

Fig. 1. Root length increase of *Atriplex atacamensis, A. halimus* and *A. nummularia* seedlings subjected to increasing Cu, NaCl and a decrease of solute potential. Bars represent the mean of four replicates ± SE and letters denote significant differences among treatments on each species, according to a one-way ANOVA or Kruskal Wallis and Tukey test ($P \leq 0.05$).

Table 1
Shoot length (SL), root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW) and shoot dry weight (SDW), of *Atriplex atacamensis* (AA), *A. halimus* (AH) and *A. nummularia* (AN) seedlings subjected to available Cu, NaCl and a PEG-induced water stress for seven days. Data are the means of four replicates ± SE. Considering the same lines, different letters denote significant difference between treatment groups according to a one-way ANOVA or Kruskal Wallis and Tukey test ($P \leq 0.05$).

Treatments		Copper addition (μmol L^{-1})			Salinity (% NaCl)			Solute potential (-MPa)		
Growth parameters	Species	0	10	20	0	1	2	0	0.1	0.25
SL (cm)	AA	5.7 ± 0.15^{a}	5.6 ± 0.2^{a}	5.5 ± 0.25^{a}	6.3 ± 0.15^{b}	5.8 ± 0.1^{b}	5.1 ± 0.1^{a}	2.4 ± 0.1^{a}	2.2 ± 0.01^{a}	2.9 ± 0.2^{b}
	AH	3.9 ± 0.2^{b}	2.9 ± 0.1^{a}	2.9 ± 0.1^{a}	3.9 ± 0.1^{b}	3.7 ± 0.15^{ab}	3.4 ± 0.1^{a}	2.9 ± 0.01^{a}	3 ± 0.1^{a}	2.8 ± 0.1^{a}
	AN	3.9 ± 0.2^{b}	3.1 ± 0.1^{a}	3.3 ± 0.1^{a}	3.6 ± 0.2^{a}	3.4 ± 0.05^{a}	3.1 ± 0.05^{a}	3 ± 0.1^{a}	3.1 ± 0.1^{a}	2.9 ± 0.1^{a}
RFW (mg)	AA	15.8 ± 1.64^{a}	11.9 ± 3.4^{a}	7.3 ± 0.2^{a}	15.5 ± 3.9^{b}	10.6 ± 0.5^{ab}	5.6 ± 0.3^{a}	3.1 ± 0.3^{a}	3 ± 0.1^{a}	4.5 ± 0.8^{a}
	AH	5.8 ± 0.8^{b}	2.2 ± 0.1^{a}	1.9 ± 0.3^{a}	3.7 ± 0.3^{b}	3.2 ± 0.2^{b}	2 ± 0.2^{a}	0.7 ± 0.2^{a}	1.4 ± 0.1^{a}	1.2 ± 0.4^{a}
	AN	10.4 ± 1.2^{b}	6.4 ± 0.6^{a}	7.1 ± 0.7^{a}	10.1 ± 0.7^{b}	9.3 ± 0.4^{b}	5.5 ± 0.3^{a}	4.6 ± 1.3^{a}	5.9 ± 0.95^{a}	4.1 ± 0.95^{a}
SFW (mg)	AA	81.7 ± 6.7^{b}	57.8 ± 4.5^{a}	46.2 ± 1.6^{a}	91 ± 9.5^{ab}	101.9 ± 4.4^{b}	70.5 ± 5.0^{a}	18.5 ± 0.4^{a}	15.8 ± 1.5^{a}	15.2 ± 1.5^{a}
	AH	33.8 ± 5.0^{b}	15.7 ± 0.3^{a}	11.4 ± 0.7^{a}	34.7 ± 2.8^{b}	32.5 ± 3.8^{ab}	22.6 ± 1^{a}	23.9 ± 2.5^{a}	22.7 ± 1.7^{a}	19.2 ± 0.6^{a}
	AN	64.6 ± 3^{b}	36.3 ± 2.1^{a}	37.4 ± 3.1^{a}	51.9 ± 4.4^{ab}	63.1 ± 4.8^{b}	37.2 ± 6.2^{a}	50.1 ± 3.5^{b}	45.5 ± 5.5^{ab}	29.4 ± 3.5^{a}
RDW (mg)	AA	1.1 ± 0.1^{b}	0.8 ± 0.1^{a}	0.8 ± 0.04^{ab}	1 ± 0.05^{a}	1 ± 0.05^{a}	0.8 ± 0.1^{a}	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0.6 ± 0.04^{a}
	AH	0.3 ± 0.02^{a}	0.4 ± 0.1^{a}	0.4 ± 0.2^{a}	0.3 ± 0.03^{b}	0.2 ± 0.02^{ab}	0.2 ± 0.02^{a}	0.1 ± 0.04^{a}	0.2 ± 0.05^{a}	0.2 ± 0.03^{a}
	AN	1.0 ± 0.1^{a}	0.8 ± 0.1^{a}	0.7 ± 0.1^{a}	0.9 ± 0.1^{b}	0.8 ± 0.01^{ab}	0.6 ± 0.07^{a}	0.7 ± 0.1^{a}	0.7 ± 0.1^{a}	0.5 ± 0.1^{a}
SDW (mg)	AA	7.4 ± 0.6^{a}	5.8 ± 0.5^{a}	5.9 ± 0.2^{a}	8.0 ± 0.5^{a}	9.2 ± 0.3^{a}	8.1 ± 0.6^{a}	2.1 ± 0.1^{a}	1.8 ± 0.2^{a}	1.8 ± 0.2^{a}
	AH	2.8 ± 0.4^{b}	1.9 ± 0.1^{ab}	1.8 ± 0.1^{a}	3.3 ± 0.3^{a}	3.2 ± 0.4^{a}	3.2 ± 0.2^{a}	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	1.2 ± 0.01^{a}
	AN	5.6 ± 0.3^{a}	4.6 ± 0.05^{a}	5 ± 0.5^{a}	4.4 ± 0.3^{a}	5.2 ± 0.4^{a}	4.4 ± 0.5^{a}	3.9 ± 0.2^{a}	3.7 ± 0.3^{a}	2.8 ± 0.3^{a}

fresh weight had a 40% non-significant increase. Finally, the combination between Cu and PEG had a negative effect on *A. halimus*, with a significant 65% decrease (Fig. 3).

4. Discussion

4.1. Growth parameters of Atriplex species vary under control conditions

In this work, nine experiments were performed on three Atriplex species, either native to Chile or from the European Mediterranean and Australia. As expected, plant growth parameters of these species greatly varied under control conditions; whereas *A. nummularia* showed high shoot biomass production, *A. atacamensis* had high root elongation. In contrast, *A. halimus* seedlings had the lowest root and shoot growth. These results differ from other studies, that reported a similar biomass production and height between *A. halimus* and *A. atacamensis* cuttings (Tapia et al., 2013), or a higher biomass production of two *A. halimus* clones over *A. nummularia* seedlings (Silveira et al., 2009). Growth differences among species are to be expected, especially in wild varieties. High variation and differentiation could reflec growth potential of each species and its underlying strategies when subjected to different conditions. Therefore, it is important to consider growth under control conditions in order to better understand their response range when subjected to single and combined stress conditions.

4.2. Effect of intermediate Cu and NaCl concentrations on growth

As halophytes, *Atriplex* species would be expected to show a positive response to salinity prior to the appearance of detrimental effects caused either by osmotic or toxicity conditions (Flowers et al., 2015). Yet, on this study, NaCl beneficial effects were not observed. Our data shows that 1% NaCl had no effect on *A. halimus* shoot dry and fresh biomass, while a non-significant increase was found *on A. nummularia* and *A. atacamensis*. Other studies show that beneficial effect of NaCl varies among *Atriplex* species and experimental conditions. For example, Nemat Alla et al. (2011) found an increase in shoot dry and fresh weight of *A. halimus* seedlings grown in pots in response to 50 mM (0.3%) NaCl, similar to the results found by Bendaly et al. (2016) on *A. halimus* cuttings grown in hydroponics. Vromman et al. (2016a) found no significant variation on growth parameters of *A. atacamensis* seedlings subjected to 100 (1.7%) mM NaCl and Silveira et al. (2009) found that NaCl concentration between 100 (0.58%) and 300 (1.75%) mM promoted growth on *A. nummularia* seedlings. Most of these studies show that the transition between beneficial effects and the expression of stress signals seems to occur around 100 mM (0.6%) NaCl. Above this point, there is an onset of detrimental effects, related to an early decrease of substrate water potential and a late response of Na toxicity and nutrient imbalance (Munns, 2002; Verslues et al., 2006).

Salt concentrations used on this study were equivalent to 170–340 mM, higher than the suggested threshold; also, direct exposition to NaCl provided by the use of an hydroponic design could have

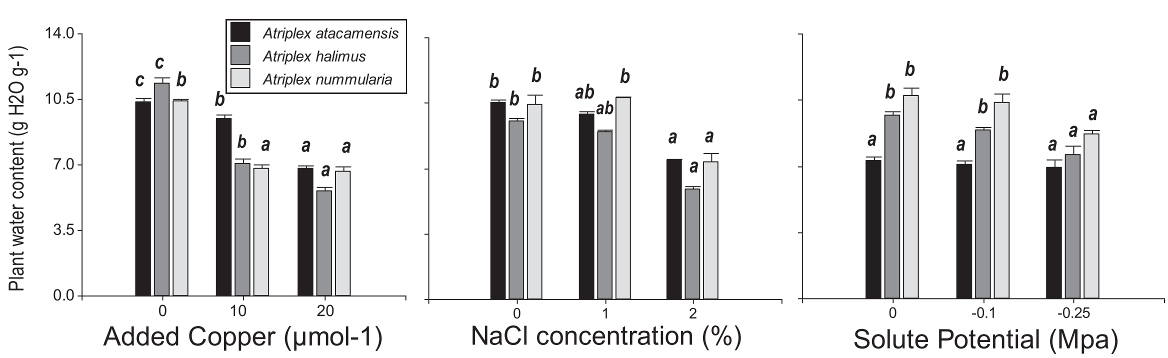

Fig. 2. Plant water content (g H2O g^{-1}) of Atriplex atacamensis, A. halimus and A. nummularia seedlings subjected to increasing available Cu, NaCl and a decrease of solute potential. Bars represent the mean of four replicates ± SE and letters denote significant differences among groups of each species when compared to control, according to a one-way ANOVA or Kruskal Wallis and Tukey test ($P \leq 0.05$).

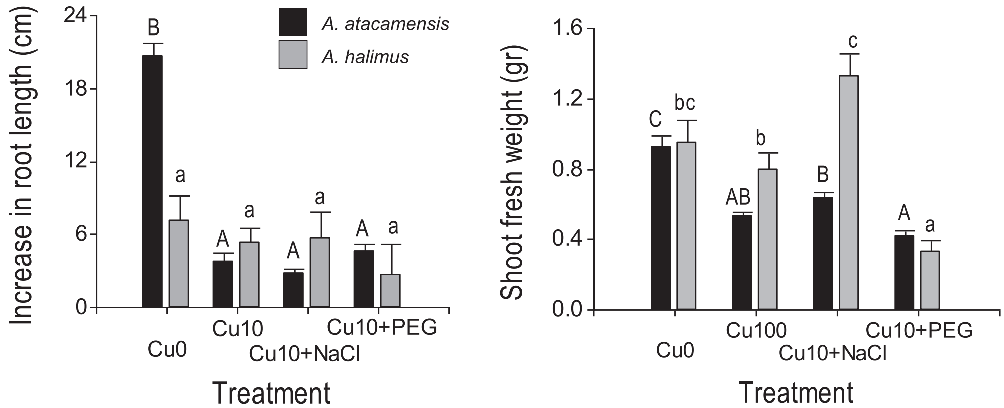

Fig. 3. Increase in root length (cm) and Shoot fresh weight (gr) of *Atriplex atacamensis* and *A. halimus* subjected to 10 μM Cu and its combination with 0.5% NaCl and 7.85 mM PEG. Bars represent the mean of five replicates $\pm$ SE. Letters denote differences among groups of *Atriplex atacamensis* (uppercase) and *Atriplex halimus* (lowercase) when compared to control, according to a one-way ANOVA or Kruskal Wallis and Tukey test ($P \leq 0.05$).

increase contact between roots and this stressor, which could explain the absence of a positive effect on growth. In this scenario, growth on halophyte species could be sustained by the expression of tolerance strategies that aim to avoid toxic effects of NaCl ions, and osmotic imbalance. One of these strategies refers to Na accumulation in leaf vacuoles, a response that has been described both as a detoxifying mechanism to avoid Na toxicity in the cytosol and as inexpensive osmolyte for water regulation. On this subject, Belkheiri and Mulas (2013) found that Na accumulation in *A. halimus* shoots was two times higher than *A. nummularia.* In fact, Na levels of *A. nummularia* under salinity treatments were not significantly different from control conditions. On the other side, Vromman et al. (2016a) found an increase in Na concentration in roots and leaves of *A. atacamensis* under NaCl treatments. Selective strategies among Atriplex species, and its possible co-occurrence of other morphological and physiological mechanisms are still a research subject on this group. Therefore, further observations are needed to understand the role of Na and other molecules on the osmotic and toxicity component of NaCl stress on these species.

Similar to salinity, no significant negative effects on plant growth were found for PEG-induced water stress treatments. Although root length and biomass production were not significantly affected, a decrease tendency was found on shoot fresh biomass on *A. atacamensis* and *A. halimus* that was only significant for *A. nummularia*. Even though no clear negative effect of PEG was found on *Atriplex*, other studies have found that this component can cause metabolic irruptions and has a double effect on water acquisition by the plant: it decreases water availability to roots by changing solute potential of nutrient solution and creating a physical layer that decreases root access to water (Shi et al., 2015). Therefore, it is possible that the magnitude of the effect of PEG on plant growth was hidden under the slight biomass variation in roots, and small seedling size, which did not allow to observe the full magnitude of the effect.

4.3. *Detrimental effect of Cu and NaCl on growth parameters of* Atriplex *species*

Our findings indicate that Cu treatments had a negative effect on growth parameters of all three species. However, when species-specific response was compared, we found that *A. halimus* and *A. nummularia* were less affected by Cu tan *A. atacamensis.* One of the first responses of plants to Cu addition into the media is to avoid Cu transport to leaves in order to avoid its oxidative effects on leaf biomolecules (Mateos-Naranjo et al., 2013). Most plants achieve this by root sequestration (Lange et al., 2017), where early Cu toxicity effects, such as root growth inhibition and suberization, can manifest (Marques et al., 2018). Decrease in functional root surface would restrict water and nutrient acquisition and transport to shoots, as observed on *A. atacamensis.* These species registered severe root damage under Cu treatments, and a significant decrease of shoot biomass and plant water content in response to 10 μM Cu. Since *A. atacamensis* seeds were collected in a heavily Cu enriched site (62.8 μM available Cu), it is pertinent to ask whether those populations were able to tolerate Cu due to a decrease of soil Cu bioavailability, the expression of other strategies (i.e., avoidance or exclusion) or its association with soil microorganisms, as it has been described for these species and *A. nummularia* in arid regions of Chile (Aguilera et al., 1998).

In order to avoid the mismatch between field and laboratory conditions, assessment of toxicity symptoms in plants should be closely connected to understandable and replicable metal concentrations. Regretfully, the array of concentrations used on Cu toxicity assays is large (Han et al., 2012; Marques et al., 2018), making proper interpretation and comparison difficult, even among species of the same genus. It is known that Cu bioavailability is mostly governed by substrate pH and organic matter content (Cataldo and Wildung, 1978), therefore, we suggest to consider these parameters on the selection of experimental conditions and ecological interpretation.

Root growth of *A. nummularia* and *A. halimus* was significantly affected by Cu treatments, but unlike *A. atacamensis,* positive growth occurred even under the highest Cu concentration. Since root elongation measurements were made against day one, we believe that these two species underwent residual growth before Cu toxicity symptoms appeared. The fact that these two species were able to sustain growth under those circumstances, and that overall growth parameters were less affected than *A. atacamensis,* could indicate a broader tolerance range to Cu, or the presence of evasion strategies in order to limit Cu transport and assimilation. In fact, Mateos-Naranjo et al. (2013) showed that *A. halimus* is able to tolerate intermediate Cu concentrations, without accumulating Cu in roots. Once a threshold is passed, Cu causes a decrease in photosynthetic rate and growth. More studies on the effect of Cu on growth, ion accumulation and physiological indicators of *Atriplex* species could shed some light into different strategies among species to evade or tolerate high concentration of metals in experimental settings and natural conditions.

High NaCl concentrations also caused a significant decrease in *Atriplex* growth parameters. We propose that they were mostly related to the osmotic component of NaCl stress, because shoot fresh weight and water content decreased in response to 2% NaCl, but root and shoot dry weight did not. This could mean that physiological mechanisms that allow biomass production were not affected. Studies performed by Nemat Alla et al. (2011) and Boughalleb and Denden (2011) revealed that *A. halimus* had optimal growth from 50 up to 300 mM NaCl. Silveira et al. (2009) found similar values for *A. nummularia* cuttings, and Vromman et al. (2016a) found that *A.*

atacamensis was able to grow under at least 100 mM NaCl. In this study, we used NaCl concentration ranging from 171 to 342 mM, and the most negative results were found only on the higher treatment. Unlike *A. halimus* and *A. nummularia*, whose salt tolerance has been explored under a soil remediation context, there are no studies that explore *A. atacamensis* tolerance to salinity. A greater understanding of *A. atacamensis* halophytism could give clues to understand how it deals with the osmotic and ionic component of salt stress, and how that can help to understand its response to metal stress.

4.4. *Effect of combined stresses on* Atriplex

Numerous studies have investigated metal, drought and salt stress independently, but few have examined the consequences of its interaction. Gathered evidence on combined stress research show that the combination of two or more stresses creates a new state, different than the effect of each individual stressor (Mittler, 2006), and that on most cases, this combination has a greater negative impact than single stress conditions (Choudhury et al., 2017). In our experiments, we found that combined stresses caused different responses on root elongation and shoot fresh weight of *A. atacamensis* and *A. halimus*. As it was observed on the first assay, root elongation of *A. atacamensis* was severely affected by single Cu treatments. Here, we found that the combination of Cu and PEG caused a further significant decrease on *A. atacamensis* but had no effect on *A. halimus*. Visual observations confirmed that root morphology of *A. halimus* was less affected than *A. atacamensis* under single Cu conditions (Fig. 4). However, on a combined stress scenario, root browning and thickening was observed on both species. More research is needed to confirm whether this response was linked to single PEG or its combination with Cu, but a prior study performed by Chazen et al. (1995) found that, beside the osmotic effect of PEG on nutrient solution, it also caused an additional water flow inhibition to roots that was not observed on isosmotic treatments of NaCl and KCl on *Zea mays* seedlings. If this is the case, it could explain why Cu and PEG combined treatments caused root impairments and a significant decrease in shoot fresh weight of both species. Therefore, it would be interesting to explore the effect of single and combined PEG effect on root anatomy and function, and how it may impact the on whole plant water relations.

Single salinity assays established that intermediate concentrations of NaCl could have beneficial effect on Atriplex growth, namely because halophyte species display strategies to tolerate its ionic and osmotic components (Flowers and Colmer, 2008). Therefore, it could be expected that NaCl treatments would either improve tolerance conditions of Atriplex subjected to Cu, or cause an additive negative effect. Results showed that, once again *A. atacamensis* and *A. halimus* had different responses under combined treatments. Shoot fresh weight of *A. atacamensis* significantly decreased in response to the combination of Cu and NaCl, but *A. halimus* was not affected, and even had an increase tendency. The fact that the effect of Cu and NaCl combination on *A. atacamensis* was not significantly different than single Cu, suggests that the toxicity component of NaCl stress was not operating on *A. atacamensis* seedlings. The fact that an increase tendency was found on fresh weight of *A. halimus* shoots could imply that NaCl was either not being perceived as a stress, or it was actively being used to maintain biomass production.

On this context, studies that address the effect of NaCl on metal stress show contrasting results. Ghnaya et al. (2007) found that 400 mM NaCl improved growth rate and biomass of *Sesuvium portulacastrum* cuttings subjected to Cd, but Sghaier et al. (2015) observed no effects of added NaCl on growth parameters of the shrub *Tamarix gallica* subjected to As. The combination of As and NaCl was also studied on *A. atacamensis* with similar results. But here, it was also found that the lack of a negative effect of As could be related to a decrease of As accumulation in roots when NaCl is present. Bankaji et al. (2014) compared the response of *A. halimus* and *Suaeda fruticosa* to the combination of Cu and Cd and the effect of its combination with NaCl, and found that addition of 200 mM NaCl with either 400 μM Cu or 400 μM Cd had no beneficial effect on biomass or chlorophyll content of *S. fruticosa*, but it did alleviated the detrimental effects of Cu on *A. halimus*. Overall, it seems that NaCl effect on metal tolerance of halophytes is not a generalized trait, and it depends on their specific metal tolerances. On Atriplex species, metal tolerance has been explored mostly on *A. halimus* (Bankaji et al., 2016; Lutts et al., 2004; Manousaki and Kalogerakis, 2009) and only recently on *A. atacamensis* (Tapia et al., 2013). While this does not respond if halophyte species are more tolerant to metals than glycophytes, it does offer a window to further study the performance of multi-tolerant species on challenging environmental scenarios.

5. Conclusion

In the present study we found that three *Atriplex* species candidate for phytostabilization in arid and metal enriched areas had different responses when subjected to varying levels of single Cu, NaCl

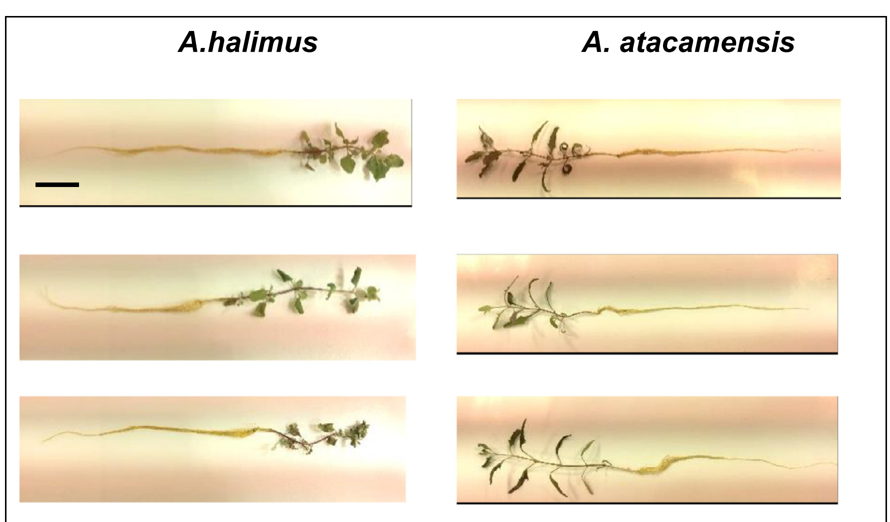

Fig. 4. Picture of *A. halimus* and *A. atacamensis* seedlings under control, Cu and Cu + PEG treatments of combined stress assay. The bar represent 10 cm in length.

and PEG-induced water stresses. Intermediate concentrations of PEG and NaCl did not cause significant growth impairments, but Cu caused a significant decrease in growth, even at its lowest concentration. Although single Cu had a negative effect on growth of all three *Atriplex* species, a combined assay performed on *A. halimus* and *A. atacamensis* showed that *A. halimus* was able not only to tolerate a higher concentration of Cu, but could also maintain growth when combined with NaCl, suggesting that salinity conditions could alleviate some of the negative effects caused by Cu.

It is clear that more studies are needed to understand specific responses of salt, water and particularly metal stress in *Atriplex* species, as well as the effect of their combination, in order to obtain a better understanding their tolerance strategies and the role of these species in the rehabilitation of saline, degraded and metal enriched soils.

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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