



# Nitrate modulates the physiological tolerance responses of the halophytic species *Sarcocornia fruticosa* to copper excess

Pedro Valle-Romero<sup>a</sup>, Eloy Manuel Castellanos<sup>b</sup>, Carlos J. Luque<sup>b</sup>, Noris J. Flores-Duarte<sup>c</sup>, Elena Romano-Rodríguez<sup>a</sup>, Susana Redondo-Gómez<sup>a</sup>, Ignacio D. Rodríguez-Llorente<sup>c</sup>, Eloísa Pajuelo<sup>c</sup>, Enrique Mateos-Naranjo<sup>a,\*</sup>

<sup>a</sup> Departamento de Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, 1095, 41080 Sevilla, Spain

<sup>b</sup> Departamento de Ciencias Integradas, Facultad de Ciencias Experimentales, Universidad de Huelva, 21071 Huelva, Spain

<sup>c</sup> Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, 1095, 41080 Sevilla, Spain

## ARTICLE INFO

Handling Editor: K Kees Venema

### Keywords:

Antioxidant activity  
Copper  
Halophyte  
Nitrate  
Photosynthesis  
*Sarcocornia fruticosa*  
Stress tolerance

## ABSTRACT

Coexistence impact of pollutants of different nature on halophytes tolerance to metal excess has not been thoroughly examined, and plant functional responses described so far do not follow a clear pattern. Using the Cu-tolerant halophyte *Sarcocornia fruticosa* as a model species, we conducted a greenhouse experiment to evaluate the impact of two concentration of copper (0 and 12 mM CuSO<sub>4</sub>) in combination with three nitrate levels (2, 14 and 50 mM KNO<sub>3</sub>) on plant growth, photosynthetic apparatus performance and ROS-scavenging enzymes system. The results revealed that *S. fruticosa* was able to grow adequately even when exposed to high concentrations of copper and nitrate. This response was linked to the plant capacity to uptake and retain a large amount of copper in its roots (up to 1500 mg kg<sup>-1</sup> Cu), preventing its transport to aerial parts. This control of translocation was further magnified with nitrate concentration increment. Likewise, although Cu excess impaired *S. fruticosa* carbon assimilation capacity, the plant was able to downregulate its light-harvesting complexes function, as indicated its lowers ETR values, especially at 12 mM Cu + 50 mM NO<sub>3</sub>. This downregulation would contribute to avoid excess energy absorption and transformation. In addition, this strategy of avoiding excess energy was accompanied by the upregulation of all ROS-scavenging enzymes, a response that was further enhanced by the increase in nitrate concentration. Therefore, we conclude that the coexistence of nitrate would favor *S. fruticosa* tolerance to copper excess, and this effect is mediated by the combined activation of several tolerance mechanisms.

## 1. Introduction

Many of heavy metals, such as Cu, Mn, Ni, Zn, etc., are essential for plant development, being key elements in important biological processes such as photosynthetic carbon assimilation, photochemical efficiency for light capture and transformation, and respiration process (Chen et al., 2022; Meng et al., 2018; Mishra and Tanna, 2017; Shabala, 2013). However, when present in excess, they can drastically affect these vital metabolic processes. This has led to increased attention from the scientific community in recent decades, focusing on research into metal tolerance in plants. This research aims to understand: (i) the

tolerance thresholds of a wide range of plant species against different types of metals, in order to comprehend the ecotoxicological impact of these pollutants on species communities evolution and/or determinate their potential for environmental remediation (Caparrós et al., 2022; Mateos-Naranjo et al., 2008a; Redondo-Gómez et al., 2010; Singh et al., 2023), and (ii) elucidate underlying tolerance mechanisms that would allow these species to respond to stress induced by the presence of metals (Singh et al., 2023). In this conceptual framework, the scientific community has identified several halophyte species as metal-tolerant due to their special morphological, anatomical, physiological, and biochemical features that allow them offered them to complete their life

**Abbreviations:** A<sub>N</sub>, net photosynthetic rate; CAT, catalase; ETR, electron transport rate; GPX, guaiacol peroxidase; F<sub>v</sub>/F<sub>m</sub>, maximum quantum efficiency of PSII photochemistry; Φ<sub>PSII</sub>, actual photochemical efficiency of PSII; g<sub>s</sub>, stomatal conductance; LDMC, dry mass content of branches; RDMC, dry mass content of the roots; RH, relative humidity; ROS, reactive oxygen species; SOD, superoxide dismutase; iWUE, intrinsic water use efficiency; ε, apparent carboxylation efficiency.

\* Corresponding author.

E-mail address: [emana@us.es](mailto:emana@us.es) (E. Mateos-Naranjo).

<https://doi.org/10.1016/j.plaphy.2024.108569>

Received 11 January 2024; Received in revised form 12 March 2024; Accepted 25 March 2024

Available online 26 March 2024

0981-9428/© 2024 The Authors. Published by Elsevier Masson SAS. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

cycle in environments contaminated with both salinity and heavy metals (Calone et al., 2022; Lutts and Lefèvre, 2015; Manousaki and Kalogerakis, 2011; Redondo-Gómez et al., 2006). These species have garnered attention as model organisms for the study of metal tolerance mechanisms.

Generally, the majority of halophytes tolerance mechanisms against metal excess aim to avoid the buildup of excessive levels of this element in sensitive tissues where it has toxic effects (Kabata-Pendias, 2001). This is achieved through their ability to prevent metal uptake, transport, or sequestration in tissues or cellular compartments that are sensitive to metals. This can be accomplished by metal binding to the cellular wall and extracellular root exudates or by reducing metal influx across the plasma membrane (Singh et al., 2023). Particularly, some halophytes demonstrate enhanced resistance to reactive oxygen species (ROS), such as superoxide radicals ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ), caused by heavy metals. This resistance is achieved through the synthesis of antioxidants (Sruthi et al., 2017) and the modulation of ROS-scavenging enzymes, as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), and glutathione reductase (GR), resulting in reduced damage to lipid membranes from ROS under heavy metal stress (Liang et al., 2017). Additionally, copper tolerance in halophytes has been also ascribed to the maintenance of photosynthetic functions under metal excess. This circumstance is related to the modulation of its aforementioned antioxidant apparatus and the fact that many, or even most, steps in photosynthetic pathway (light harvesting system and/or diffusional and biochemical components for  $CO_2$  assimilation) may be tolerant to the uptake and accumulation of high amounts of metal in plant tissues (Mallick and Mohn, 2003; Singh et al., 2023).

Despite the enormous progress made in determining the tolerance potential of halophytic species and disentangling their constitutive tolerance mechanisms, many studies were mostly confined to assess the tolerance against specific pollutants (Nikalje and Suprasanna, 2018; Redondo-Gómez et al., 2010), without considering the coexistence of several heavy metals and/or contaminants of different natures, which could have a different significant impact on these plant tolerance mechanisms. This impact would depend on pollutant characteristics such as concentration, nature, or their combination (Pérez-Romero et al., 2022). In this context, nitrate ( $NO_3^-$ ) contamination resulting from fertilization processes associated with intensive farming practices has been identified as a primary factor contributing to various adverse effects on ecosystem conservation (Barros et al., 2012; Martínez-Dalmau et al., 2021). Given the widespread occurrence of nitrate contamination in saline environments, like saltmarshes (Mohammadzadeh and Hajiboland, 2022), it becomes imperative to understand the potential impact of nitrate presence on the tolerance mechanisms and metal uptake capacity of halophytic plants within these ecosystems. Therefore, this study was designed and conducted to fill this gap in knowledge.

*Sarcocornia fruticosa* (L.) A. J. Scott was chosen as a model halophyte species to study in detail the impact of nitrate and metal interaction on plant performance and tolerance mechanism. Specifically, the study focused on the functionality of the photosynthetic apparatus, modulation of antioxidant enzyme machinery, and metal uptake. This species has been described as a natural phytostabilizer for several metals with distribution in coastal systems across southern and western Europe (Said et al., 2019; Silva et al., 2021). Moreover, several authors have suggested a link between heavy metal tolerance and nitrate accumulation in certain species to the Amaranthaceae family, to which this selected species belongs (Hawkesford et al., 2023; Moreira da Silva et al., 2015). Considering these factors, we hypothesize that the coexistence of nitrate in the growing medium would modulate the tolerance and metal accumulation capacity of the halophyte *S. fruticosa*, which would be linked to the activation of specific halophytic tolerance mechanisms favored by the greater availability of nitrogen resources in the environment. Taking all this into account, the main objective of this study was to investigate the ability of *S. fruticosa* photosynthesis and ROS-scavenging enzyme systems to cope with copper excess under different levels of nitrate in the

growing medium, and examine the possible role of copper and nitrogen concentration in plant tissues in explaining plant responses. Copper was selected as it attains priority pollutant status according to both the European Community and UNEP (Tóth et al., 2016; UNEP, 2013) and due to its biological importance in assessing plant tolerance mechanisms in this study.

## 2. Material and methods

### 2.1. Plant material and experimental design

*Sarcocornia fruticosa* plants completely developed and planted in 5.5 cm diameter multi-perforated pots filled with rockwool as substrate were supplied by the company 'Halófitas Onuba S.L.' (Isla Cristina, Huelva; Spain). These plants were placed in a climate-controlled, PLC-automated, software-controlled, venlo glass greenhouse module (University of Seville, Center for Research, Technology and Innovation, CITIUS II; 37°24'N, 6°0'W; Southwest Spain) under controlled conditions: 21–25 °C temperature, 40–50% RH, natural light was supplemented by fluorescent/incandescent lamps set to a photoperiod of 16 h of light (with a maximum photon density flux at leaf level of 1000  $\mu mol m^{-2} s^{-1}$ ) and 8 h of darkness, with adequate irrigation during 15 days for an acclimation period until experiment onset.

After acclimation period, plants with 10 cm height were selected and divided into six treatments of forty individuals per group and arranged in a randomized plot. For that, the pots were randomly subjected to two copper concentration treatments (0 and 12 mM  $CuSO_4$ ), in combination with three levels of nitrate (2, 14 and 50 mM  $KNO_3$ ) for 60 days ( $n = 240$ ; 40 plants per treatment). The copper concentration was chosen considering the current copper levels previously recorded in the marshes of the southwestern peninsula by Redondo-Gómez et al. (2009) and Sánchez-Gavilán et al. (2021). For nitrate, three concentrations were chosen to make our data representative of low (2 mM), medium (14 mM) and high (50 mM) levels of environmental nitrate contamination derived from chemical fertilizer application according to Mohammadzadeh and Hajiboland (2022). Copper and nitrate treatments were established by combining 20% Hoagland solution (Hoagland and Arnon, 1950) with the appropriate concentration of  $CuSO_4 \cdot 7H_2O$  and  $KNO_3$ , respectively. In addition, all treatments were supplied with a solution of 171 mM NaCl, since this is the optimal salinity within the range of natural conditions in which this species grows naturally (Pérez-Romero et al., 2023). For that, at the beginning of the experiment, 3 L of the appropriate solution was applied to the trays to a depth of 1 cm. Solution levels and pH were monitored daily, and topped up with 20% Hoagland solution and adjusted to  $6.5 \pm 7.0$  without the additional application of  $CuSO_4 \cdot 7H_2O$  and  $KNO_3$ , to avoid variations in pH and copper and nitrate concentrations due to evaporation of the solution. The control, 0 mM  $CuSO_4$  treatment, had 0.0005 mM Cu, as the Hoagland solution contains a small amount of Cu as an essential nutrient. All solutions, including  $CuSO_4 \cdot 7H_2O$  and  $KNO_3$ , were changed weekly.

### 2.2. Evaluation of plant growth and photosynthetic apparatus performance

After 60 days of treatment, the plants were harvested and divided into branches and roots. These biomass fractions were then oven dried at 60 °C for 48 h and weighed to obtain estimates of the dry mass content of the branches (LDMC) and roots (RDMC). These measurements were complemented with instantaneous leaf gas exchange and PSII photochemical efficiency measurements, obtained with an open system infrared gas analyser, equipped with a fluorescence measurement chamber (LI-6800-01, LICOR Inc., Lincoln, NE, USA) and a portable modulated fluorometer (FMS-2; Hansatech Instruments Ltd., King 's Lynn, UK) 20 and 60 days after experimental treatments imposition. Thus, net photosynthetic rate ( $A_N$ ), stomatal conductance ( $g_s$ ), apparent carboxylation efficiency ( $\epsilon: A_N/C_i$ ), intrinsic water use water use

efficiency ( $\varphi$ WUE), actual photochemical efficiency of PSII ( $\Phi_{PSII}$ ) and electron transport rate (ETR) were determined at 400 ppm CO<sub>2</sub>, 25 ± 0.5 °C, 50 ± 1% RH and 1000  $\mu$ mol photon m<sup>-2</sup> s<sup>-1</sup> (n = 7 measurements per treatment). All measurements were made between 10:00 and 13:00 h and were corrected by photosynthetic area according to (Pérez-Romero et al., 2018). Finally, the maximum photochemical quantum efficiency of PSII ( $F_v/F_m$ ) was measured in 30 min of dark-adapted branches (n = 12 measurements per treatment), according to the protocol described by Mateos-Naranjo et al. (2020).

### 2.3. Evaluation of plant antioxidant enzymes status

At the end of the experiment, 500 mg of fresh aerial tissue samples from each of experimental treatments were collected, ground and homogenized in 8 mL of a 50 mM sodium phosphate buffer solution (pH 7.6) and 0.1 mM Na-EDTA, under ice-cold conditions according to the protocol described by Duarte et al. (2015). The homogenate was then subjected to centrifugation at 9000 rpm for 20 min at 4 °C and the supernatant liquid obtained was used for several enzymatic analyses. Three samples per treatment were used and three measurements per sample and enzyme activity were registered. The protein content in the extracts was obtained according to Bradford (1976), using bovine serum albumin as standard.

Guaiacol peroxidase EC 1.11.1.7 (GPX) was calculated as Bergmeyer (1974) indicated, with a reaction mixture made of 50 mM sodium phosphate buffer (pH 7.0), 2 mM H<sub>2</sub>O<sub>2</sub>, and 20 mM guaiacol. For all these enzymatic activities, 100  $\mu$ L of vegetal extract was added to the reaction mixture to start the reaction. Superoxide dismutase EC 1.15.1.1 (SOD) activity was assayed by monitoring the reduction of pyrogallol at 325 nm, following Marklund and Marklund (1974) work. The reaction mixture was 50 mM sodium phosphate buffer (pH 7.6), 0.1 mM Na-EDTA, 3 mM pyrogallol, Mili-Q water. The reaction started with the addition of 10  $\mu$ L of enzyme extract. Catalase EC 1.11.1.6 (CAT) was prepared following the protocol of Teranishi et al. (1974) in a mixture of 890 mL of sodium phosphate buffer (50 mM, pH 7.0), 100  $\mu$ L leaf extract and 10  $\mu$ L H<sub>2</sub>O<sub>2</sub> (15%). Subsequently, to determine the enzyme activity, the decrease in absorbance at 240 nm was calculated, using the molar extinction coefficient of H<sub>2</sub>O<sub>2</sub> (39.4 mM<sup>-1</sup> cm<sup>-1</sup>). Finally, ascorbate peroxidase EC 1.11.1.11 (APX) was analyzed according to Duarte et al. (2015). The reaction mixture was composed of 50 mM of sodium phosphate buffer (pH 7.0), 12 mM  $\mu$ L of H<sub>2</sub>O<sub>2</sub> and 0.25 mM L-ascorbate, in addition to the 100  $\mu$ L of plant extract. Enzyme activity was recorded by measuring the decrease in absorbance at 290 nm due to ascorbate oxidation and calculated using the molar extinction coefficient of  $\epsilon$  (2.8 mM<sup>-1</sup> cm<sup>-1</sup>). The auto-oxidation of the substrates was evaluated by a control sample with the reaction mixture but without the enzyme extract.

### 2.4. Evaluation of copper and nitrogen content in plant tissues

At the end of the experiment, dried branch and root samples were collected and crushed to obtain 0.5 g aliquots in triplicate from each experimental treatment. Subsequently, the samples were digested with 6 mL of HNO<sub>3</sub>, 0.5 mL of HF and 1 mL H<sub>2</sub>O<sub>2</sub>; and the concentration of copper in the tissues was quantified by inductively coupled plasma spectroscopy (ICP; ARL-Fison 3410, USA). Finally, the undigested dry samples underwent total nitrogen concentration analysis using the Dumas method with an elemental autoanalyzer (Leco CHNS-932-Trumac, Spain). This technique involves sample combustion to transform nitrogen into nitrogen oxides, followed by the precise quantification of the released nitrogen gas, ensuring accurate determination of nitrogen content (López-Calderón et al., 2020).

### 2.5. Statistical analysis

Statistical analyses were carried out using RStudio statistical

software (R Development Core Team, 2008). The analysis of the effect of copper and nitrate treatments on the different parameters of *S. fruticososa* was carried out using general linear models (GLM test). Copper and nitrate concentrations were considered as grouping variables and the different quantified parameters as contrast variables. Those analyses that yielded significant results were subjected to a post hoc pairwise contrast analysis (LSD test).

## 3. Results

### 3.1. Growth and gas exchange measurements

The dry mass content of the branches and roots did not differ between the experimental treatments after 60 days of treatment (GLM

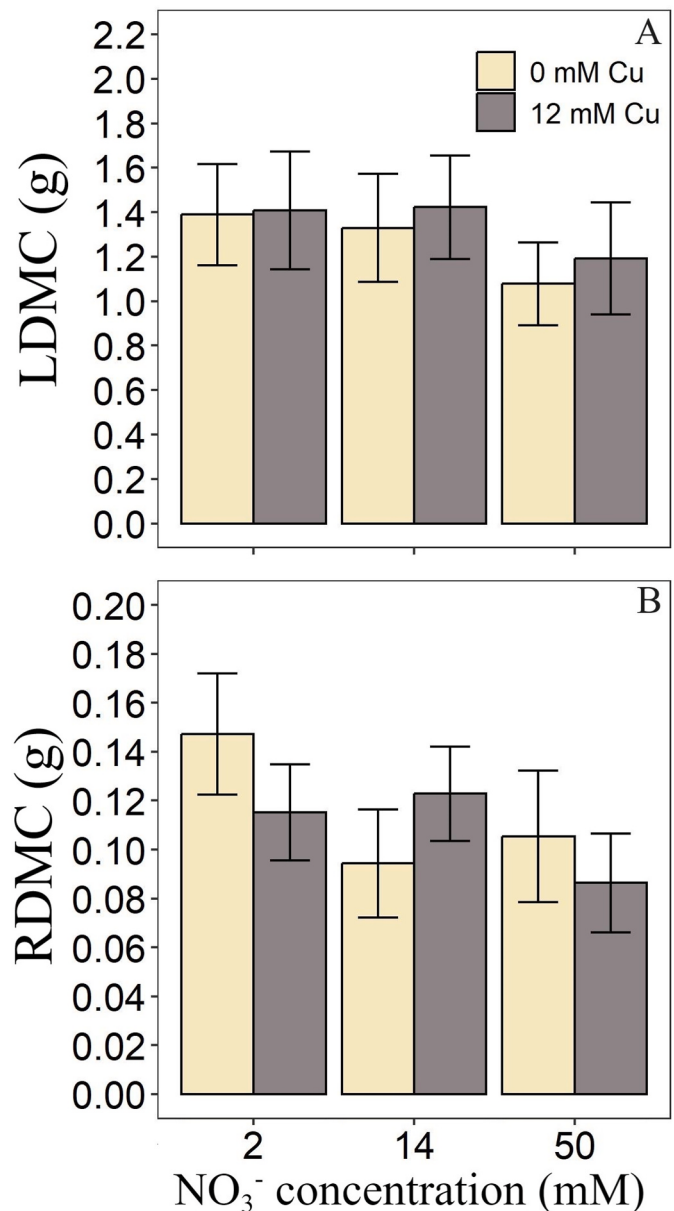


Fig. 1. (A) Dry mass content of the branches (LDMC) and (B) dry mass content of the roots (RDMC) in *Sarcocornia fruticososa* plants after 60 days of treatment, with three nitrate levels (2, 14 and 50 mM KNO<sub>3</sub>) in combination with two copper concentrations (0 and 12 mM CuSO<sub>4</sub>). Each value represents the mean ± standard error of ten replicates. Cu, NO<sub>3</sub> or Cu x NO<sub>3</sub> in the upper corner of the panel indicates significant main effects or interactions (LSD test, \*p < 0.05, \*\*p < 0.01).

Model:  $p > 0.05$ ; Fig. 1A and B). Contrarily, our results showed a significant effect of copper and nitrate treatments on the instantaneous gas exchange characteristics of *S. fruticosa* in the different sampling periods (GLM Model:  $p < 0.05$ ; Fig. 2). Overall, apparent carboxylation efficiency was lower with increased copper concentration independently of nitrate concentration in the grown medium after 20 days of treatment, and this difference was more acute 60 days after metal stress imposition and mainly in plants exposed to 50 mM nitrate (GLM Model: Cu and  $\text{NO}_3$ ,  $p < 0.01$ ; Fig. 2A and B). A very similar pattern was observed for  $A_N$  values after 20 and 60 days of treatment (GLM Model: Cu and  $\text{NO}_3$ ,  $p < 0.01$ ; Fig. 2C and D), while stomatal conductance ( $g_s$ ) showed mean values c.  $90 \text{ mmol}^{-1} \text{ H}_2\text{O m}^{-2} \text{ s}^{-1}$  in all treatments after 20 days (Fig. 2E), and decreased considerably after 60 days in presence of copper regardless of the nitrate concentration (GLM Model: Cu,  $p < 0.01$ ; Fig. 2F). Regarding intrinsic water use efficiency ( $i\text{WUE}$ ), it was observed that, in general, values were lower in the presence of copper at 20 days of treatment (GLM Model: Cu,  $p < 0.05$ ; Fig. 3A). However, at 60 days, no significant differences in treatments were observed and all treatments had a similar efficiency (Fig. 3B).

### 3.2. Chlorophyll a fluorescence measurements

Our results showed a significant effect of copper and nitrate treatments on the PSII efficiency of *S. fruticosa* in the different sampling periods (GLM Model:  $p < 0.05$ ; Fig. 4). Therefore, the maximum photochemical quantum efficiency of PSII ( $F_v/F_m$ ), did not vary significantly between experimental treatments after 20 days (Fig. 4A), while there was a negative synergistic in plants grown at 12 mM Cu + 50 mM  $\text{KNO}_3$  that showed the lowest  $F_v/F_m$  values compared to the other treatments (GLM Model: Cu x  $\text{NO}_3$ ,  $p < 0.01$ ; Fig. 4B).

However, the overall actual photochemical efficiency of the PSII ( $\Phi_{\text{PSII}}$ ) and electron transport rate (ETR) values were lower in plants grown in the presence of copper after 20 days (GLM Model: Cu,  $p < 0.01$ ; Fig. 4C and E). In addition, both copper and nitrate presence had significant effects on plants  $\Phi_{\text{PSII}}$  and ETR values after 60 days, obtaining the lowest values in the plants subjected with 12 mM Cu + 50 mM nitrate in the medium (GLM Model: Cu and  $\text{NO}_3$ ,  $p < 0.01$ ; Fig. 4D and F).

### 3.3. Antioxidant enzymes measurements

Our results showed a significant effect of copper and nitrate treatments on the antioxidant enzymes status of *S. fruticosa* at the end of the experiment (GLM Model:  $p < 0.05$ ; Fig. 5). Thus, overall ascorbate peroxidase (APX) activity was higher in plants grown in the presence of copper (GLM: Cu,  $p < 0.05$ ) and tended to increase with nitrate concentration in the growth medium with and without Cu application (GLM Model:  $\text{NO}_3$ ,  $p < 0.01$ ; Fig. 5A). Very similar patterns were recorded for catalase (CAT), superoxide dismutase (SOD), and guaiacol peroxidase (GPX), but for these enzyme activities it should be noted that the differences between both copper treatments were even more pronounced (GLM Model: Cu,  $p < 0.01$ ; Fig. 5B and D), obtaining overall the highest values in the plants subjected to 12 mM Cu + 50 mM nitrate in the medium and specially for GPX activity (GLM Model: Cu,  $\text{NO}_3$  and Cu x  $\text{NO}_3$ ,  $p < 0.01$ ; Fig. 5D).

### 3.4. Analysis of copper and nitrogen content in tissues

There were significant effects of  $\text{CuSO}_4$  and  $\text{NO}_3$  concentration treatments on Cu and N concentrations in tissues (GLM Model:  $p < 0.05$ ; Fig. 6). Therefore, the copper concentration was higher in roots than in leaves, and within the tissues a considerable increase was observed along the external exposure to Cu regardless of the concentration of nitrate in both tissues (GLM Model: Cu,  $p < 0.01$ ; Fig. 6A and B), except for the Cu concentration of leaves, which showed a reduction in plants grown at 50 mM nitrate compared to the rest of Cu treated plants (GLM Model: Cu x  $\text{NO}_3$ ,  $p < 0.01$ ; Fig. 6A). However, nitrogen concentration

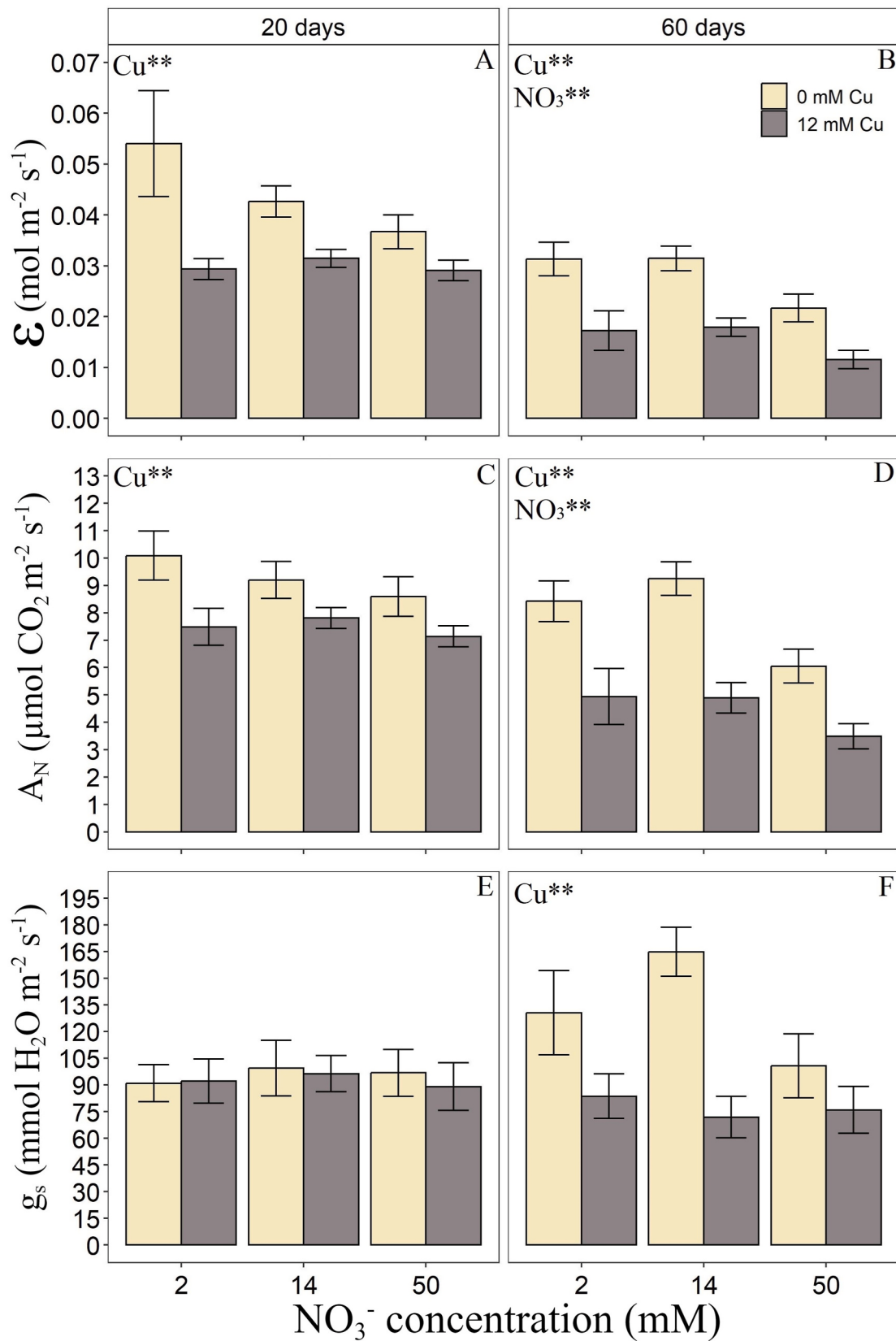
was higher in aboveground tissues than in belowground tissues and tended to increase with the concentration of nitrate in the grown medium; being this increase was even magnified in Cu-supplied plants grown at 50 mM nitrate, especially in leaves (GLM Model: Cu x  $\text{NO}_3$ ,  $p < 0.01$ ; Fig. 6C).

## 4. Discussion

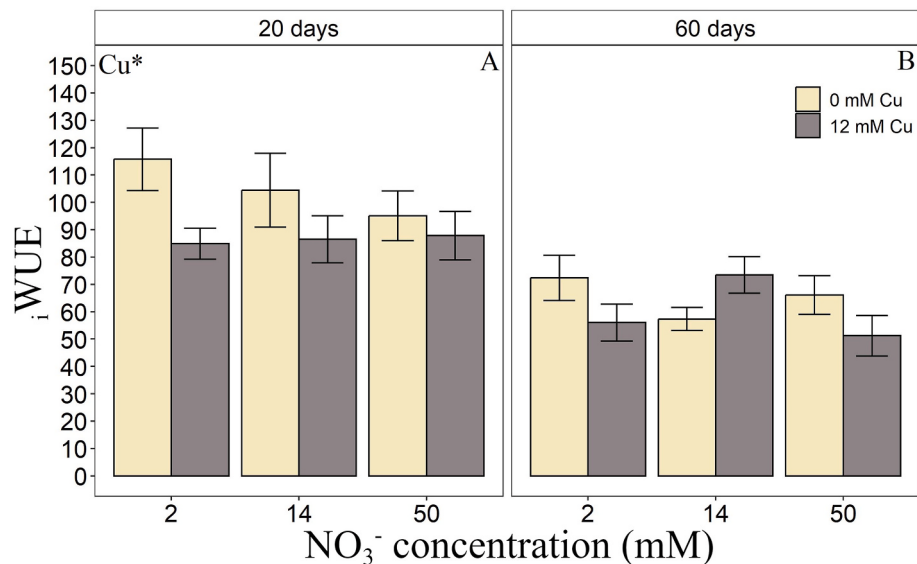
The ability of halophytes to cope with induced metal stress has been highlighted by many studies, and significant efforts have been made to unravel the mechanisms involved in this response (Flowers and Colmer, 2008; Meng et al., 2018). However, few studies have explored in depth how this innate tolerance could be modulated by the coexistence of pollutants of different nature, such as nitrate enrichment in the environment. In fact, previous studies have highlighted the overlapping effect of distinct stressors on plants tolerance capacity, resulting in the generation of integrated mechanical and chemical signals that play a pivotal role in eliciting the tolerance (Mateos-Naranjo et al., 2021, 2024; Roe and MacFarlane, 2022). In this sense, our study showed how *S. fruticosa* tolerance responses to copper excess were, to some extent, modulated by the variation in nitrate concentration in the growing medium, demonstrating a differential activation of specific tolerance mechanisms in this plant species (i.e., metal uptake and transport limitation, photosynthetic apparatus performance, and ROS-scavenging enzyme system modulation; Meng et al., 2018; Mishra and Tanna, 2017; Shabala, 2013).

Focusing on the obtained results, we found that *S. fruticosa* exhibited high tolerance to copper stress, since plants were able to sustain their growth at concentrations higher than 12 mM Cu, measured as LDMC and RDMC, compared to those grown at 0 mM Cu. This is noteworthy, especially considering the results obtained for other halophytic species belonging to the Amaranthaceae family, where the detrimental impact of copper excess negatively affects their growth (Orrego et al., 2020). The observed growth maintenance in *S. fruticosa* was attributed to the activation of different tolerance mechanisms, which could act individually or synergistically, in response to Cu excess (Caparrós et al., 2022; Liang et al., 2017). In this context, our results revealed a substantial copper uptake and retention plant capacity in the plant, particularly in its roots, with mean values around  $1500 \text{ mg kg}^{-1}$  Cu for plants grown at 12 mM Cu, regardless of the nitrate level. Consistent with this result, numerous studies have corroborated the capacity of *S. fruticosa* to retain metals in the rhizosphere (roots and rhizosediment), thereby preventing their translocation to aerial parts (Caetano et al., 2008; Moreira da Silva et al., 2015; Silva et al., 2021). This Cu accumulation pattern could be explained by well-described process in halophytes, such as Cu binding to root cell walls, chelation, and/or compartmentalization in vacuoles for non-toxic storage, avoiding ion interaction with essential cellular metabolic processes in more sensitive organs (Caparrós et al., 2022; Sheoran et al., 2010). Additionally, our results indicate that the copper accumulation pattern was altered to a certain degree by the presence of nitrate. In this regard, several authors have indicated that Cu accumulation pattern may vary depending on plant species or ecotypes, developmental stage, and environmental factors such as nitrogen supply (Yruela, 2009). Thus, our results suggested that overall  $\text{NO}_3$  supply led to a higher nitrogen content in plant tissues. This effect was even more pronounced in plants subjected to 12 mM Cu + 50 mM  $\text{NO}_3$ , followed by a concomitant reduction in the concentration of copper in their leaves compared with their Cu-supplied counterparts, which would contribute to explaining plant tolerance against excess copper. According to our results, Cui et al. (2022) indicated that Cu and N supply altered the uptake patterns of both elements through a differential regulation of the expression levels of  $\text{NO}_3$  and Cu transporters in rice plants. Hence, it is possible that something similar occurred in *S. fruticosa*, and this area deserves further research as it could contribute to explaining its high Cu tolerance.

Despite the relatively high tolerance demonstrated by *S. fruticosa* in



**Fig. 2.** (A, B) Apparent carboxylation efficiency ( $\epsilon$ ), (C, D) net photosynthetic rate ( $A_N$ ) and (E, F) stomatal conductance ( $g_s$ ) in randomly selected *Sarcocornia fruticosa* branches of plants subjected to two copper concentrations (0 and 12 mM  $\text{CuSO}_4$ ) in combination with three nitrate levels (2, 14 and 50 mM  $\text{KNO}_3$ ) after 20 and 60 days of treatment. Each value represents the mean  $\pm$  standard error of three replicates. Cu,  $\text{NO}_3$  or Cu  $\times$   $\text{NO}_3$  in the upper corner of the panel indicates significant main effects or interactions (LSD test, \*p < 0.05, \*\*p < 0.01). In case of interaction, Cu  $\times$   $\text{NO}_3$ , means that are significantly different from each other within the same sampling period are indicated by different letters (GLM, p < 0.05).

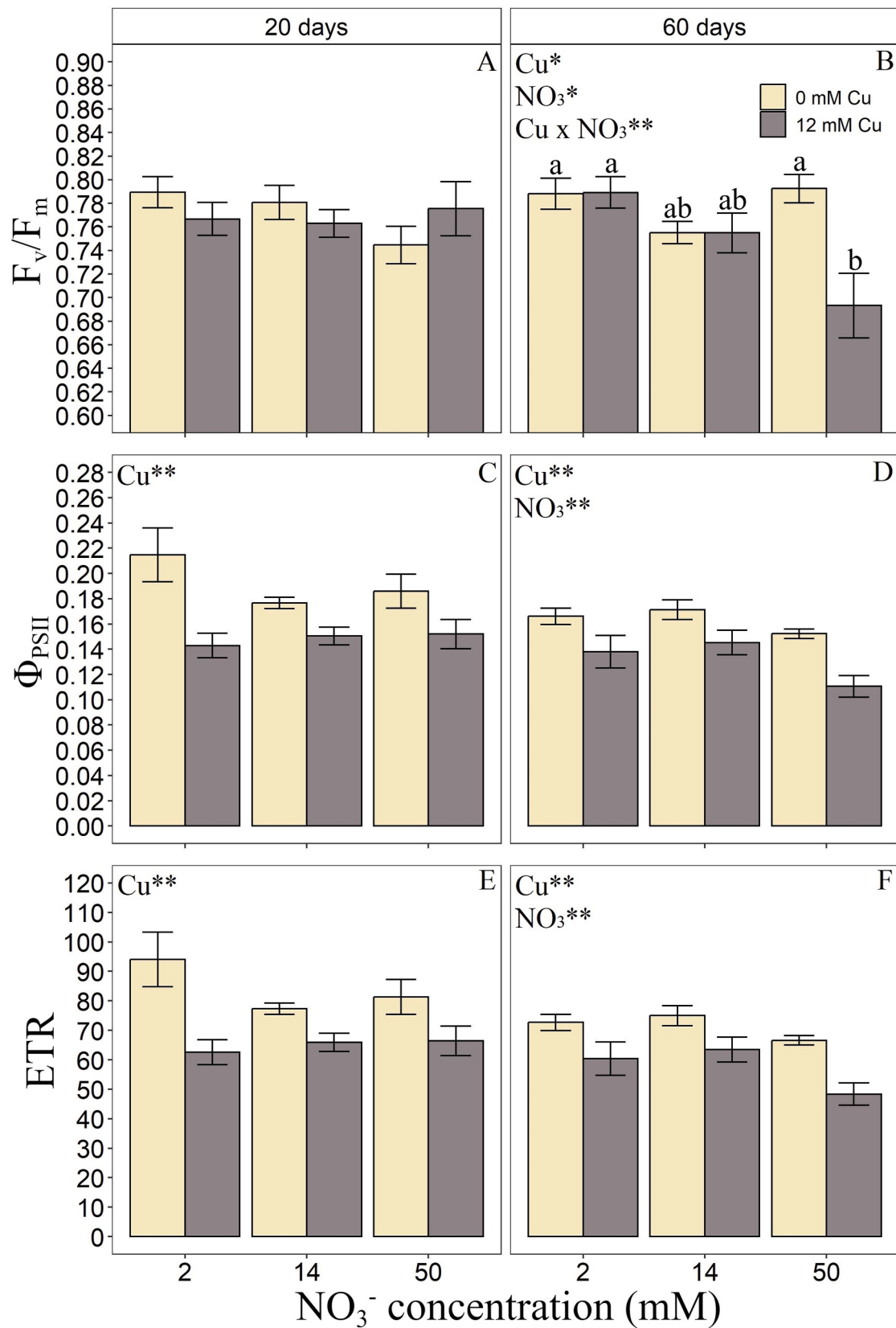


**Fig. 3.** (A, B) Intrinsic water use efficiency ( $iWUE$ ) in *Sarcocornia fruticosa* plants after 20 and 60 days of treatment, with three nitrate levels (2, 14 and 50 mM KNO<sub>3</sub>) in randomly selected *Sarcocornia fruticosa* branches of plants subjected to two copper concentrations (0 and 12 mM CuSO<sub>4</sub>) in combination with three nitrate levels (2, 14 and 50 mM KNO<sub>3</sub>) after 20 and 60 days of treatment. Each value represents the mean  $\pm$  standard error of three replicates. Cu, NO<sub>3</sub> or Cu  $\times$  NO<sub>3</sub> in the upper corner of the panel indicates significant main effects or interactions (LSD test, \* $p < 0.05$ , \*\* $p < 0.01$ ). In case of interaction, Cu  $\times$  NO<sub>3</sub>, means that are significantly different from each other within the same sampling period are indicated by different letters (GLM,  $p < 0.05$ ).

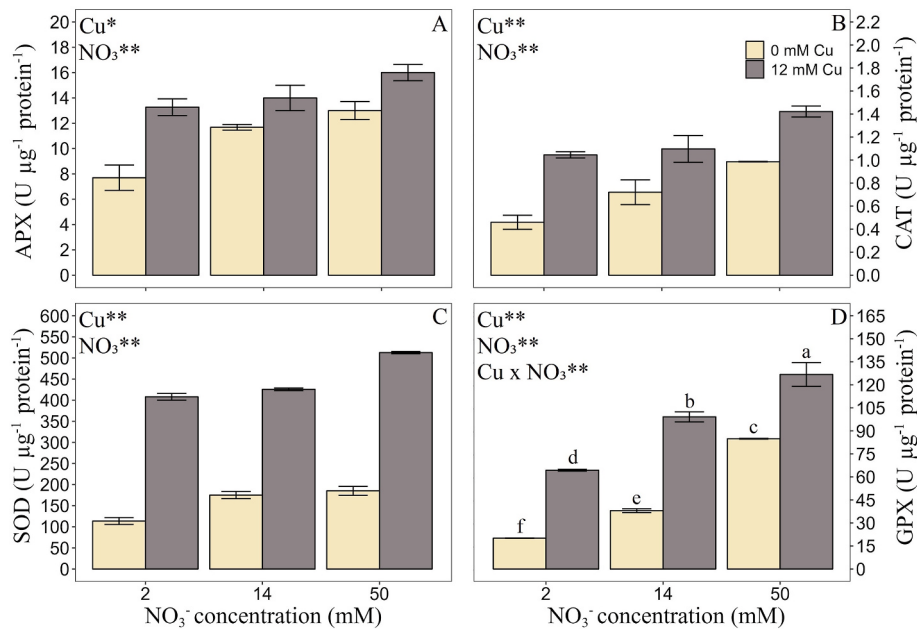
terms of growth, our photosynthetic performance analysis indicated a greater sensitivity of this metabolic process in terms of carboxylation capacity and photochemical efficiency under elevated concentrations both Cu and NO<sub>3</sub> (i.e., 12 mM Cu and 50 mM NO<sub>3</sub>). It is noteworthy that the photosynthetic apparatus of plants, including halophytes, is often highly vulnerable to heavy metal toxicity (Nikalje and Suprasanna, 2018). The effects of Cu on  $A_N$  of *S. fruticosa* were clearly discernible regardless of nitrate concentration, with this effect becoming more pronounced after 60 days of treatment. In addition, we found that although several studies have indicated that exogenous applied nitrate could favor photosynthetic activity (Agnihotri and Seth, 2016; Wei et al., 2024) as showed plants grown at 14 mM NO<sub>3</sub> and without Cu addition, when it is in excess, it can damage photosynthetic activity, as indicated by the reduction of  $A_N$  at 50 mM NO<sub>3</sub> (Han et al., 2023). These tendencies of Cu and NO<sub>3</sub> impacts on the performance of the *S. fruticosa* photosynthetic apparatus could be partially explained by a differential modulation effect of experimental factors on the down-regulation of key steps in the plant photosynthetic apparatus triggered by each stress factor in terms of apparatus integrity and functioning; i.e., CO<sub>2</sub> diffusion limitation, RuBisCO activity, and light-harvesting antenna efficiency (Flexas et al., 2012; Mateos-Naranjo et al., 2013, 2015). Our results indicated that the limitation of  $A_N$  in *S. fruticosa* was mediated by both diffusional and biochemical limitations (Amari et al., 2014; Mir et al., 2021), as indicated by the decrease in  $g_s$  and the increased in  $C_i$ , which generated a consequent drop in photosynthetic apparent carboxylation efficiency ( $\epsilon$ ) with increasing Cu concentration and in plants grown at 50 mM NO<sub>3</sub> with and without Cu supply. The reduction in instantaneous carboxylation efficiency would also suggest a metabolic impairment in *S. fruticosa*, primarily in biochemical carboxylation, which would be associated with a decrease in RuBisCO carboxylase activity (Mir et al., 2021). In this sense, several authors have indicated that enzyme activity down-regulation is really common in the presence of heavy metals (Siedlecka and Krupa, 2004) and would be attributed to the ability of Cu to compete with Mg for the formation of the RuBisCO-CO<sub>2</sub>-metal<sup>2+</sup> catalytic complex, thereby altering the affinity for CO<sub>2</sub> and potentially impairing the chlorophyll synthesis system (Mateos-Naranjo et al., 2008b; Mir et al., 2021). However, it should be noted that the reduction of  $g_s$  led to  $iWUE$  values maintenance, which would contribute to preserving the trade-off between CO<sub>2</sub> acquisition for photosynthetic process

and water losses in *S. fruticosa* in the presence of both contaminants, as other halophytes have shown in response to copper excess (Pérez-Romero et al., 2018). This circumstance would favor plant water relations, upholding a greater cell turgor and water potential gradient, and consequently a larger leaf area, compensating in part for the decline in carbon assimilation capacity per unit area and ensuring continued growth under adverse conditions (Mohammadzadeh and Hajiboland, 2022), as has been addressed in other halophytes (Redondo-Gómez et al., 2010).

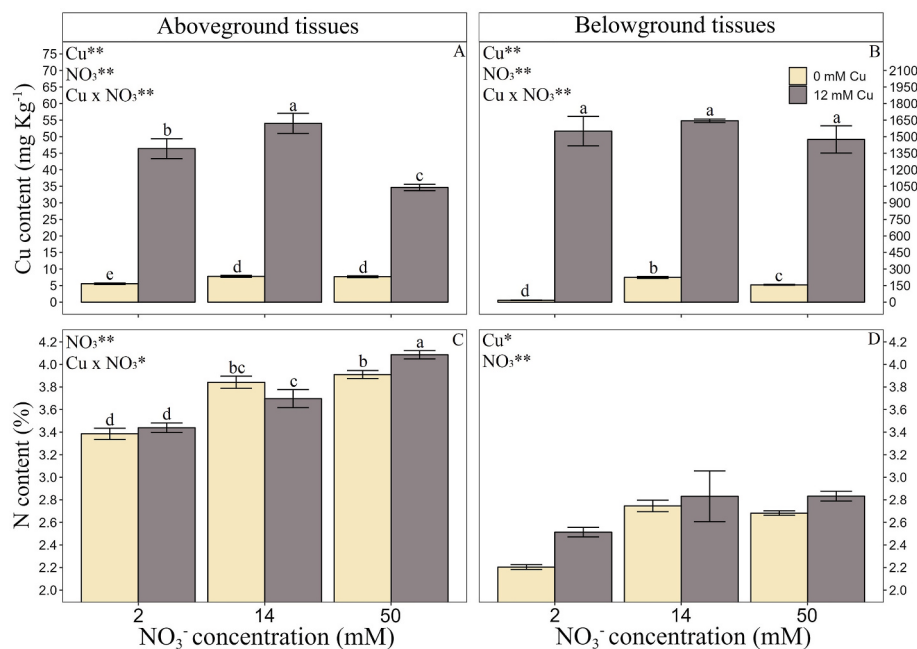
On the other hand, *S. fruticosa* impairment in carbon assimilation capacity arising from the individual or combined presence of copper and high nitrate concentration was associated with differential pollutant effects on the photochemical apparatus functionality. Thus, we observed a decrease in  $\Phi_{PSII}$  in the presence of copper, attributable to the pollutants negative impact on the PSII reaction center through the destruction of antennae pigments (Anjum et al., 2016; Khan et al., 2022). In this sense, it has been described that copper interferes with the uptake and assimilation of essential ions such as Mg, Zn, Fe, and Ni, leading to chloroplast ultrastructural alterations (Shabbir et al., 2020). Therefore, it affects the plant capacity for energy transfer from the collector antennae to reaction centers (Li et al., 2012). Additionally, our results indicated that there was a negative synergistic effect of copper supply in plants grown at 50 mM KNO<sub>3</sub>, as they presented the lowest  $F_v/F_m$  values compared with the rest of treatments. This response suggests that the combination of both pollutants tested would increase photoinhibition induced by light stress (Werner et al., 2002). Likewise, the negative impact of these pollutant factors on *S. fruticosa* photochemical apparatus was attributed to the down-regulation of its electron transport chain functionality, as indicated by the lower ETR values recorded in plants grown at 12 mM Cu, regardless of NO<sub>3</sub> concentration in the grown medium, and especially in those subjected to 12 mM Cu + 50 mM NO<sub>3</sub> after 60 days of treatment. This response indicates that the presence of Cu disrupts energy fluxes per leaf cross-section (Anjum et al., 2016; Mateos-Naranjo et al., 2008a, 2013; Nikalje and Suprasanna, 2018), being this impact more pronounced due to the synergistic effect of the presence of a high nitrate concentration. This electron transport downregulation would be an avoidance strategy used by *S. fruticosa* to protect its photosynthetic apparatus and other chloroplast structures against photoinhibition damage induce by copper excess (Meng et al.,



**Fig. 4.** (A, B) Maximum photochemical quantum efficiency of PSII ( $F_v/F_m$ ), (C, D) actual photochemical efficiency of PSII ( $\Phi_{PSII}$ ) and (E, F) electron transport rate (ETR) in randomly selected *Sarcocornia fruticosa* branches of plants subjected to two copper concentrations (0 and 12 mM  $CuSO_4$ ) in combination with three nitrate levels (2, 14 and 50 mM  $KNO_3$ ) after 20 and 60 days of treatment. Each value represents the mean  $\pm$  standard error of three replicates. Cu,  $NO_3$  or Cu x  $NO_3$  in the upper corner of the panel indicates significant main effects or interactions (LSD test, \*p < 0.05, \*\*p < 0.01). In case of interaction, Cu x  $NO_3$ , means that are significantly different from each other within the same sampling period are indicated by different letters (GLM, p < 0.05).



**Fig. 5.** (A) Ascorbate peroxidase (APX), (B) catalase (CAT), (C) superoxide dismutase (SOD) and (D) guaiacol peroxidase (GPX) in randomly selected *Sarcocornia fruticosa* plants subjected to two copper concentrations (0 and 12 mM  $\text{CuSO}_4$ ) in combination with three nitrate levels (2, 14 and 50 mM  $\text{KNO}_3$ ) after 60 days of treatment. Each value represents the mean  $\pm$  standard error of three replicates. Cu,  $\text{NO}_3$  or Cu x  $\text{NO}_3$  in the upper corner of the panel indicates significant main effects or interactions (LSD test, \* $p < 0.05$ , \*\* $p < 0.01$ ). In case of interaction, Cu x  $\text{NO}_3$ , means that are significantly different from each other are indicated by different letters (GLM,  $p < 0.05$ ).



**Fig. 6.** (A, B) Copper content (Cu) and (C, D) nitrogenous content (N) in aboveground and belowground tissues in randomly selected *Sarcocornia fruticosa* plants subjected to two copper concentrations (0 and 12 mM  $\text{CuSO}_4$ ) in combination with three nitrate levels (2, 14 and 50 mM  $\text{KNO}_3$ ) after 60 days of treatment. Each value represents the mean  $\pm$  standard error of three replicates. Cu,  $\text{NO}_3$  or Cu x  $\text{NO}_3$  in the upper corner of the panel indicates significant main effects or interactions (LSD test, \* $p < 0.05$ , \*\* $p < 0.01$ ). In case of interaction, Cu x  $\text{NO}_3$ , means that are significantly different from each other are indicated by different letters (GLM,  $p < 0.05$ ).

2018). It reduces the number of electrons being absorbed but are not fixed by carboxylation activity; therefore, they are free to create ROS (Pérez-Romero et al., 2020; Salazar-Parra et al., 2012). Congruently, the mentioned minimization of light-harvesting complexes has been addressed in other halophytes subjected to environmental stress (Koyro et al., 2013).

In contrast, our results revealed that *S. fruticosa* was able to modulate

its antioxidant system to acclimate to excess copper, as do other halophytes facing fluctuating stress environment (Bashri and Prasad, 2015; Mesnoui et al., 2016; Redondo-Gómez et al., 2011; Sruthi et al., 2017). In this sense, ROS-scavenging enzymes such as APX, CAT, SOD and GPX play an essential role in regulating ROS levels in plants (Dazy et al., 2009; Parlak and Yilmaz, 2013). In particular, SOD converts superoxide radicals to  $\text{H}_2\text{O}_2$ , while APX, GPX and CAT catalyze the decomposition

of H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O (Pirasteh-Anosheh et al., 2023). The increased of all antioxidant enzyme activities observed in this study is related to an increase in the rate of ROS production due to Cu increment, allowing the plant to cope with this stressful condition and explaining the high tolerance demonstrated by *S. fruticosa*. However, beyond this trend, the most remarkable aspect was that nitrate application increased the activity of antioxidant enzymes, being this effect more pronounced at 50 mM NO<sub>3</sub>, which was consistent with the results obtained by several authors in plants subjected to various environmental factors (De Souza et al., 2021; Moghaddam et al., 2023; Wei et al., 2015). Therefore, this upregulation ROS-scavenging enzymes, along with the avoidance of excessive light energy absorption and transformation in *S. fruticosa* photosystems aforementioned, would contribute to explaining the high tolerance demonstrated by this species under elevated concentrations of both Cu and NO<sub>3</sub>.

These physiological and biochemical findings are, by themselves, extremely important in terms of understanding the tolerance mechanisms underlying metal exposure in halophytes under the coexistence of pollutants of different natures. Nevertheless, from an ecological and application perspective, our findings are also relevant. Thus, in an ecological context, our results suggest that the development and maintenance of natural populations of *S. fruticosa* would not be adversely affected by occasional episodes of diffuse pollution by nitrates derived from fertilization practices in agricultural farms close to marshes systems dominated by this halophytic species, which are also subject to metal contamination (Redondo-Gómez et al., 2009), ensuring the maintenance of its ecosystem services. The identification of plant differential tolerance/resistance and metal uptake behaviors under the coexistence of different concentrations of copper and nitrate also provides a more realistic view of the species potential for its implementation as bio-tools in polluted estuarine ecosystems. In this sense, although numerous studies substantiate the remarkable phytoremediation capabilities exhibited by halophytes (Calone et al., 2022; Liang et al., 2017; Nikalje and Suprasanna, 2018; Roe and MacFarlane, 2022), it has been demonstrated that the uptake of metals, such as copper, when improperly managed, compromise plant growth and, consequently, the success of phytoremediation projects (Gibilisco et al., 2022; Mateos-Naranjo et al., 2008a; Orrego et al., 2020; Pérez-Romero et al., 2018). Regarding this concern, our results highlight that *S. fruticosa*, with its proficient growth maintenance and effective sequestration of copper and nitrate from the medium, emerges as an adequate species for phytoremediation applications in soils tainted with these specific pollutants.

## 5. Conclusion

*Sarcocornia fruticosa* shows high tolerance to Cu-induced stress and nitrate presence in the medium, as indicated by the capacity to maintain its growth at 12 mM Cu, even with nitrate concentration as high as 50 mM NO<sub>3</sub>. This growth maintenance was linked to the activation of different tolerance mechanisms, such as the plant ability to uptake and retain a large amount of copper in its roots, preventing its transport to the most sensitive aerial parts. This translocation limitation was further magnified with the increment nitrate concentration. Likewise, maintaining water balance, down-regulation photosystems function to avoid excess energy absorption and transformation, along with an upregulation of ROS-scavenging enzymes, contributed to explain the high tolerance demonstrated by this species under elevated concentration of both Cu and NO<sub>3</sub>. Finally, from a conservational perspective, these results suggest the resilience of *S. fruticosa* and its potential for phytoremediation applications.

## CRedit authorship contribution statement

**Pedro Valle-Romero:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Eloy Manuel Castellan:** Writing – review & editing, Resources, Project administration,

Methodology, Investigation, Funding acquisition, Conceptualization. **Carlos J. Luque:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Noris J. Flores-Duarte:** Writing – review & editing, Methodology, Investigation. **Elena Romano-Rodríguez:** Methodology, Investigation. **Susana Redondo-Gómez:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Ignacio D. Rodríguez-Llorente:** Writing – review & editing, Supervision, Resources, Methodology. **Eloísa Pajuelo:** Writing – review & editing, Supervision, Resources, Methodology. **Enrique Mateos-Naranjo:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

## 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.

## Data availability

Data will be made available on request.

## Acknowledgements

This work has been financed by the Campus of International Excellence of the Sea (CEIMAR) within the framework of the “Plan Propio 2021”, by the company “Halófitas Onuba S.L” and supported by the grant project PID2021-124750NB-I00 financiado por MICIU/AEI/10.13039/501100011033 y por FEDER, UE, and TED2021-131605B-I00, MICIU/AEI/10.13039/501100011033 y por la Unión Europea NextGenerationEU/PRTR. We also like to thank the University of Seville Greenhouse Research General Service (SGI, CITIUS) for providing facilities and equipment. We also appreciate the valuable feedback from reviewers insofar as it has helped us improve and enrich our work.

## References

- Agnihotri, A., Seth, C.S., 2016. Exogenously applied nitrate improves the photosynthetic performance and nitrogen metabolism in tomato (*Solanum lycopersicum* L. cv Pusa Rohini) under arsenic (V) toxicity. *Physiol. Mol. Biol. Plants* 22, 341–349. <https://doi.org/10.1007/s12298-016-0370-2>.
- Amari, T., Ghnaya, T., Debez, A., Taamali, M., Youssef, N.B., Lucchini, G., et al., 2014. Comparative Ni tolerance and accumulation potentials between *Mesembryanthemum crystallinum* (halophyte) and *Brassica juncea*: metal accumulation, nutrient status and photosynthetic activity. *J. Plant Physiol.* 171 (17), 1634–1644. <https://doi.org/10.1016/j.jplph.2014.06.020>.
- Anjum, N.A., Duarte, B., Caçador, I., Sleimi, N., Duarte, A.C., Pereira, E., 2016. Biophysical and biochemical markers of metal/metalloid-impacts in salt marsh halophytes and their implications. *Front. Environ. Sci.* 4, 24. <https://doi.org/10.3389/fenvs.2016.00024>.
- Barros, R., Isidoro, D., Aragüés, R., 2012. Irrigation management, nitrogen fertilization and nitrogen losses in the return flows of La Violada irrigation district (Spain). *Agric. Ecosyst. Environ.* 155, 161–171. <https://doi.org/10.1016/j.agee.2012.04.004>.
- Bashri, G., Prasad, S.M., 2015. Indole acetic acid modulates changes in growth, chlorophyll a fluorescence and antioxidant potential of *Trigonella foenum-graecum* L. grown under cadmium stress. *Acta Physiol. Plant.* 37, 1–14. <https://doi.org/10.1007/s11738-014-1745-z>.
- Bergmeyer, H.U., 1974. *Methods of Enzymatic Analysis*, vol. 4. Academic Press. <https://www.sciencedirect.com/science/book/9780120913046>.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72 (1–2), 248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
- Caetano, M., Vale, C., Cesário, R., Fonseca, N., 2008. Evidence for preferential depths of metal retention in roots of salt marsh plants. *Sci. Total Environ.* 390 (2–3), 466–474. <https://doi.org/10.1016/j.scitotenv.2007.10.015>.
- Calone, R., Mircea, D.M., González-Orenga, S., Boscaiu, M., Lambertini, C., Barbanti, L., Vicente, O., 2022. Recovery from salinity and drought stress in the perennial *Sarcocornia fruticosa* vs. the annual *Salicornia europaea* and *S. veneta*. *Plants* 11 (8), 1058. <https://doi.org/10.3390/plants11081058>.
- Caparrós, P.G., Öztürk, M., Gul, A., Batool, T.S., Pirasteh-Anosheh, H., Unal, B.T., et al., 2022. Halophytes have potential as heavy metal phytoremediators: a comprehensive

- review. *Environ. Exp. Bot.* 193, 104666 <https://doi.org/10.1016/j.envexpbot.2021.104666>.
- Chen, G., Li, J., Han, H., Du, R., Wang, X., 2022. Physiological and molecular mechanisms of plant responses to copper stress. *Int. J. Mol. Sci.* 23 (21), 12950 <https://doi.org/10.3390/ijms232112950>.
- Cui, X., He, H., Hu, S., Zhang, B., Cai, H., 2022. Synergistic interaction between copper and nitrogen - uptake, translocation, and distribution in rice plant. *Plants* 11, 2612. <https://doi.org/10.3390/plants11192612>.
- Dazy, M., Béraud, E., Cotel, S., Gréville, F., Féraud, J.F., Masfaraud, J.F., 2009. Changes in plant communities along soil pollution gradients: responses of leaf antioxidant enzyme activities and phytochelatin contents. *Chemosphere* 77 (3), 376–383. <https://doi.org/10.1016/j.chemosphere.2009.07.021>.
- De Souza, E.A.C.C., Alvarez-Pizarro, J.C., Lopes, L.D.S., Miranda, R.D.S., Gomes-Filho, E., 2021. Nitrate and ammonium nutrition modulates the photosynthetic performance and antioxidant defense in salt-stressed grass species. *J. Soil Sci. Plant Nutr.* 21 (4), 3016–3029. <https://doi.org/10.1007/s42729-021-00586-x>.
- Duarte, B., Goessling, J.W., Marques, J.C., Caçador, I., 2015. Ecophysiological constraints of *Aster tripolium* under extreme thermal events impacts: merging biophysical, biochemical and genetic insights. *Plant Physiol. Biochem.* 97, 217–228. <https://doi.org/10.1016/j.plaphy.2015.10.015>.
- Flexas, J., Barbour, M.M., Brendel, O., Cabrera, H.M., Carriqui, M., Díaz-Espejo, A., et al., 2012. Mesophyll diffusion conductance to CO<sub>2</sub>: an unappreciated central player in photosynthesis. *Plant Sci.* 193, 70–84. <https://doi.org/10.1016/j.plantsci.2012.05.009>.
- Flowers, T.J., Colmer, T.D., 2008. Salinity tolerance in halophytes. *New Phytol.* 160, 945–963. <https://doi.org/10.1111/j.1469-8137.2008.02531.x>.
- Gibilisco, P.E., Negrin, V.L., Idaszkin, Y.L., 2022. Assessing the use of two halophytes species and seaweed composting in Cu-polluted remediation strategies. *Mar. Pollut. Bull.* 176, 113413 <https://doi.org/10.1016/j.marpolbul.2022.113413>.
- Han, K., Zhang, J., Wang, C., Yang, Y., Chang, Y., Gao, Y., Liu, Y., Xie, J., 2023. Changes in growth, physiology, and photosynthetic capacity of spinach (*Spinacia oleracea* L.) under different nitrate levels. *Plots one* 18, e0283787. <https://doi.org/10.1371/journal.pone.0283787>.
- Hawkesford, M.J., Cakmak, I., Coskun, D., De Kok, L.J., Lambers, H., Schjoerring, J.K., White, P.J., 2023. Functions of macronutrients. In: Marschner's Mineral Nutrition of Plants. Academic press, pp. 201–281. <https://doi.org/10.1016/B978-0-12-819773-8.00019-8>.
- Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil. *Circular. California agricultural experiment station* 347, 2nd edit.
- Kabata-Pendias, A., 2001. Trace metals in soils—a current issue in Poland. *Acta Universitatis Wratislaviensis. Prace Botaniczne* 79, 13–20.
- Khan, E.A., Ahmed, H.M., Misra, M., Sharma, P., Misra, A.N., 2022. Nitric oxide alleviates photochemical damage induced by cadmium stress in Pea seedlings. *Phyton* 91 (5). <https://doi.org/10.32604/phyton.2022.018708>.
- Koyro, H.W., Hussain, T., Huchzermeyer, B., Khan, M.A., 2013. Photosynthetic and growth responses of a perennial halophytic grass *Panicum turgidum* to increasing NaCl concentrations. *Environ. Exp. Bot.* 91, 22–29. <https://doi.org/10.1016/j.envexpbot.2013.02.007>.
- Li, L., Huang, X., Borthakur, D., Ni, H., 2012. Photosynthetic activity and antioxidative response of seagrass *Thalassia hemprichii* to trace metal stress. *Acta Oceanol. Sin.* 31, 98–108. <https://doi.org/10.1007/s13131-012-0210-3>.
- Liang, L., Liu, W., Sun, Y., Huo, X., Li, S., Zhou, Q., 2017. Phytoremediation of heavy metal contaminated saline soils using halophytes: current progress and future perspectives. *Environ. Rev.* 25 (3), 269–281. <https://doi.org/10.1139/er-2016-0063>.
- López-Calderón, M.J., Estrada-Ávalos, J., Rodríguez-Moreno, V.M., Mauricio-Ruvalcaba, J.E., Martínez-Sifuentes, A.R., Delgado-Samir, G., Miguel-Valle, E., 2020. Estimation of total nitrogen content in forage maize (*Zea mays* L.) using spectral indices: analysis by random forest. *Agriculture* 10 (10), 451. <https://doi.org/10.3390/agriculture10100451>.
- Lutts, S., Lefèvre, I., 2015. How can we take advantage of halophyte properties to cope with heavy metal toxicity in salt-affected areas? *Ann. Bot.* 115 (3), 509–528. <https://doi.org/10.1093/aob/mcu264>.
- Mallick, N., Mohn, F.H., 2003. Use of chlorophyll fluorescence in metal-stress research: a case study with the green microalga *Scenedesmus*. *Ecotoxicol. Environ. Saf.* 55 (1), 64–69. [https://doi.org/10.1016/S0147-6513\(02\)00122-7](https://doi.org/10.1016/S0147-6513(02)00122-7).
- Manousaki, E., Kalogerakis, N., 2011. Halophytes—an emerging trend in phytoremediation. *Int. J. Phytoremediation* 13 (10), 959–969. <https://doi.org/10.1080/15226514.2010.532241>.
- Marklund, S., Marklund, G., 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* 47 (3), 469–474. <https://doi.org/10.1111/j.1432-1033.1974.tb03714.x>.
- Martínez-Dalmau, J., Berbel, J., Ordóñez-Fernández, R., 2021. Nitrogen fertilization. A review of the risks associated with the inefficiency of its use and policy responses. *Sustainability* 13 (10), 5625. <https://doi.org/10.3390/su13105625>.
- Mateos-Naranjo, E., Redondo-Gómez, S., Cambrollé, J., Figueroa, M.E., 2008a. Growth and photosynthetic responses to copper stress of an invasive cordgrass, *Spartina densiflora*. *Mar. Environ. Res.* 66 (4), 459–465. <https://doi.org/10.1016/j.marenvres.2008.07.007>.
- Mateos-Naranjo, E., Andrades-Moreno, L., Cambrollé, J., Perez-Martin, A., 2013. Assessing the effect of copper on growth, copper accumulation and physiological responses of grazing species *Atriplex halimus*: ecotoxicological implications. *Ecotoxicol. Environ. Saf.* 90, 136–142. <https://doi.org/10.1016/j.ecoenv.2012.12.020>.
- Mateos-Naranjo, E., Gallé, A., Florez-Sarasa, I., Perdomo, J.A., Galmés, J., Ribas-Carbo, M., Flexas, J., 2015. Assessment of the role of silicon in the Cu-tolerance of the C4 grass *Spartina densiflora*. *J. Plant Physiol.* 178, 74–83. <https://doi.org/10.1016/j.jplph.2015.03.001>.
- Mateos-Naranjo, E., López-Jurado, J., Redondo-Gómez, S., Pérez-Romero, J.A., Glick, B.R., Rodríguez-Llorente, I.D., Pajuelo, E., Echegoyan, A., Mesa-Marín, J., 2020. Uncovering PGPB *Vibrio spartinae* inoculation-triggered physiological mechanisms involved in the tolerance of *Halimione portulacoides* to NaCl excess. *Plant Physiol. Biochem.* 154, 151–159. <https://doi.org/10.1016/j.plaphy.2020.05.034>.
- Mateos-Naranjo, E., López-Jurado, J., Mesa-Marín, J., Luque, C.J., Castellanos, E.M., Pérez-Romero, J.A., Redondo-Gómez, S., 2021. Understanding the impact of a complex environmental matrix associated with climate change on the European marshes engineer species *Spartina maritima*. *Environ. Exp. Bot.* 182, 104304 <https://doi.org/10.1016/j.envexpbot.2020.104304>.
- Mateos-Naranjo, E., Pérez-Romero, J.A., Puglielli, G., López-Jurado, J., Mesa-Marín, J., Pajuelo, E., et al., 2024. Soil microorganisms buffer the reduction in plant growth and physiological performance under combined abiotic stress in the halophyte *Salicornia ramosissima*. *Environ. Exp. Bot.* 217, 105550 <https://doi.org/10.1016/j.envexpbot.2023.105550>.
- Mateos-Naranjo, E., Redondo-Gómez, S., Cambrollé, J., Luque, T., Figueroa, M.E., 2008b. Growth and photosynthetic responses to zinc stress of an invasive cordgrass, *Spartina densiflora*. *Plant Biol.* 10 (6), 754–762. <https://doi.org/10.1111/j.1438-8677.2008.00098.x>.
- Meng, J., Tao, M., Wang, L., Liu, X., Xu, J., 2018. Changes in heavy metal bioavailability and speciation from a Pb-Zn mining soil amended with biochars from co-pyrolysis of rice straw and swine manure. *Sci. Total Environ.* 633, 300–307. <https://doi.org/10.1016/j.scitotenv.2018.03.199>.
- Mesnoui, M., Mateos-Naranjo, E., Barcia-Piedras, J.M., Pérez-Romero, J.A., Lotmani, B., Redondo-Gómez, S., 2016. Physiological and biochemical mechanisms preventing Cd-toxicity in the hyperaccumulator *Atriplex halimus* L. *Plant Physiol. Biochem.* 106, 30–38. <https://doi.org/10.1016/j.plaphy.2016.04.041>.
- Mir, A.R., Alam, P., Hayat, S., 2021. Effect of different levels of soil applied copper on the morpho-physiological, photochemical, and antioxidant system of *Brassica juncea*. *J. Soil Sci. Plant Nutr.* 21, 3477–3492. <https://doi.org/10.1007/s42729-021-00621-x>.
- Mishra, A., Tanna, B., 2017. Halophytes: potential resources for salt stress tolerance genes and promoters. *Front. Plant Sci.* 8, 252363 <https://doi.org/10.3389/fpls.2017.00829>.
- Moghaddam, A., Larijani, H.R., Oveysi, M., Moghaddam, H.R.T., Nasri, M., 2023. Alleviating the adverse effects of salinity stress on *Salicornia persica* using sodium nitroprusside and potassium nitrate. *BMC Plant Biol.* 23 (1), 166. <https://doi.org/10.1186/s12870-023-04179-x>.
- Mohammadzadeh, P., Hajiboland, R., 2022. Phytoremediation of nitrate contamination using two halophytic species, *Portulaca oleracea* and *Salicornia europaea*. *Environ. Sci. Pollut. Control Ser.* 29 (30), 46127–46144. <https://doi.org/10.1007/s11356-022-19139-5>.
- Moreira da Silva, M., Aníbal, J., Duarte, D., Chicharro, L., 2015. *Sarcocornia frutescens* and *Spartina maritima* as heavy metals remediators in an European southwestern salt marsh (Ria Formosa, Portugal). *Journal of environmental protection and ecology* 16 (4), 1468–1477. <http://hdl.handle.net/10400.1/7938>.
- Nikalje, G.C., Suprasanna, P., 2018. Coping with metal toxicity—cues from halophytes. *Front. Plant Sci.* 9, 777. <https://doi.org/10.3389/fpls.2018.00777>.
- Orrego, F., Ortiz-Calderón, C., Lutts, S., Ginocchio, R., 2020. Growth and physiological effects of single and combined Cu, NaCl, and water stresses on *Atriplex atacamensis* and *A. halimus*. *Environ. Exp. Bot.* 169, 103919 <https://doi.org/10.1016/j.envexpbot.2019.103919>.
- Parlak, K.U., Yilmaz, D.D., 2013. Ecophysiological tolerance of *Lemna gibba* L. exposed to cadmium. *Ecotoxicol. Environ. Saf.* 91, 79–85. <https://doi.org/10.1016/j.ecoenv.2013.01.009>.
- Pérez-Romero, J.A., Idaszkin, Y.L., Duarte, B., Baeta, A., Marques, J.C., Redondo-Gómez, S., et al., 2018. Atmospheric CO<sub>2</sub> enrichment effect on the Cu-tolerance of the C4 cordgrass *Spartina densiflora*. *J. Plant Physiol.* 220, 155–166. <https://doi.org/10.1016/j.jplph.2017.11.005>.
- Pérez-Romero, J.A., Barcia-Piedras, J.M., Redondo-Gómez, S., Mateos-Naranjo, E., 2020. *Sarcocornia frutescens* photosynthetic response to short-term extreme temperature events in combination with optimal and sub-optimal salinity concentrations. *Plant Physiology and Biochemistry* 148, 45–52. <https://doi.org/10.1016/j.plaphy.2019.12.026>.
- Pérez-Romero, J.A., Barcia-Piedras, J.M., Redondo-Gómez, S., Caçador, I., Duarte, B., Mateos-Naranjo, E., 2022. Salinity modulates *Juncus acutus* L. Tolerance to Diesel Fuel Pollution. *Plants* 11 (6), 758. <https://doi.org/10.3390/plants11060758>.
- Pérez-Romero, J.A., Barcia-Piedras, J.M., Redondo-Gómez, S., Mateos-Naranjo, E., 2023. *Sarcocornia frutescens* recovery capacity after exposure to co-existed water and salinity stress. *Plant Stress* 8, 100162. <https://doi.org/10.1016/j.plstress.2023.100162>.
- Pirasteh-Anosheh, H., Samadi, M., Kazemine, S.A., Ozturk, M., Ludwiczak, A., Piernik, A., 2023. ROS homeostasis and antioxidants in the halophytic plants and seeds. *Plants* 12 (17), 3023. <https://doi.org/10.3390/plants12173023>.
- Redondo-Gómez, S., Cantos, M., Mateos-Naranjo, E., Figueroa, M.E., Troncoso, A., 2009. Heavy metals and trace element concentrations in intertidal soils of four estuaries of SW Iberian Peninsula. *Soil Sediment Contam.* 18 (3), 320–327. <https://doi.org/10.1080/15320380902772646>.
- Redondo-Gómez, S., Mateos-Naranjo, E., Andrades-Moreno, L., 2010. Accumulation and tolerance characteristics of cadmium in a halophytic Cd-hyperaccumulator, *Arthrocnemum macrostachyum*. *J. Hazard Mater.* 184 (1–3), 299–307. <https://doi.org/10.1016/j.jhazmat.2010.08.036>.
- Redondo-Gómez, S., Andrades-Moreno, L., Mateos-Naranjo, E., Parra, R., Valera-Burgos, J., Aroca, R., 2011. Synergic effect of salinity and zinc stress on growth and

- photosynthetic responses of the cordgrass, *Spartina densiflora*. J. Exp. Bot. 62 (15), 5521–5530. <https://doi.org/10.1093/jxb/err234>.
- Redondo-Gómez, S., Wharmby, C., Castillo, J.M., Mateos-Naranjo, E., Luque, C.J., De Cires, A., et al., 2006. Growth and photosynthetic responses to salinity in an extreme halophyte, *Sarcocornia fruticosa*. Physiol. Plantarum 128 (1), 116–124. <https://doi.org/10.1111/j.1399-3054.2006.00719.x>.
- Redondo-Gómez, S., Figueroa, M.E., Mateos-Naranjo, E., Davy, A.J., 2010. Salt stimulation of growth and photosynthesis in an extreme halophyte, *Arthrocnemum macrostachyum*. Plant Biol. 12, 79–87. <https://doi.org/10.1111/j.1438-8677.2009.00207.x>.
- Roe, R.A., MacFarlane, G.R., 2022. The potential of saltmarsh halophytes for phytoremediation of metals and persistent organic pollutants: an Australian perspective. Mar. Pollut. Bull. 180, 113811. <https://doi.org/10.1016/j.marpolbul.2022.113811>.
- Said, O.B., Da Silva, M.M., Hannier, F., Beyrem, H., Chicharo, L., 2019. Using *Sarcocornia fruticosa* and *Saccharomyces cerevisiae* to remediate metal contaminated sediments of the Ria Formosa lagoon (SE Portugal). Ecohydrol. Hydrobiol. 19 (4), 588–597. <https://doi.org/10.1016/j.ecohyd.2018.10.002>.
- Salazar-Parra, C., Aguirreolea, J., Sánchez-Díaz, M., Irigoyen, J.J., Morales, F., 2012. Climate change (elevated CO<sub>2</sub>, elevated temperature and moderate drought) triggers the antioxidant enzymes' response of grapevine cv. Tempranillo, avoiding oxidative damage. Physiol. Plantarum 144 (2), 99–110. <https://doi.org/10.1111/j.1399-3054.2011.01524.x>.
- UNEP, 2013. Environmental risks and challenges of anthropogenic metals flows and cycles, A report of the working group on the global metal flows to the international resource panel. van der Voet E., Salminen, R., Eckelman, M., Mudd, G., Norgate, T., Hirschier, R. <https://hdl.handle.net/1887/21807>.
- Sánchez-Gavilán, I., Rufo, L., Rodríguez, N., De la Fuente, V., 2021. On the elemental composition of the mediterranean euhalophyte *Salicornia patula* Duval-Jouve (Chenopodiaceae) from saline habitats in Spain (Huelva, Toledo and Zamora). Environ. Sci. Pollut. Control Ser. 28, 2719–2727. <https://doi.org/10.1007/s11356-020-10663-w>.
- Shabala, S., 2013. Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Ann. Bot. 112 (7), 1209–1221. <https://doi.org/10.1093/aob/mct205>.
- Shabbir, Z., Sardar, A., Shabbir, A., Abbas, G., Shamshad, S., Khalid, S., et al., 2020. Copper uptake, essentiality, toxicity, detoxification and risk assessment in soil-plant environment. Chemosphere 259, 127436. <https://doi.org/10.1016/j.chemosphere.2020.127436>.
- Sheoran, V., Sheoran, A.S., Poonia, P., 2010. Role of hyperaccumulators in phytoextraction of metals from contaminated mining sites: a review. Crit. Rev. Environ. Sci. Technol. 41 (2), 168–214. <https://doi.org/10.1080/10643380902718418>.
- Siedlecka, A., Krupa, Z., 2004. Rubisco activity maintenance in environmental stress conditions—how many strategies. Cellar and Molecular Biology Letter 9, 56–57.
- Silva, M., Anibal, J., Duarte, D.N., Veloso, N., Patrício, F., Chicharo, L., 2021. Metals from human activities in a coastal Lagoon Saltmarsh-Sediment toxicity and phytoremediation by *Sarcocornia fruticosa*. Journal of Environmental Protection and Ecology 22, 1441–1449. <http://hdl.handle.net/10400.1/17234>.
- Singh, V.K., Singh, R., Rajput, V.D., Singh, V.K., 2023. Halophytes for the sustainable remediation of heavy metal-contaminated sites: recent developments and future perspectives. Chemosphere 313, 137524. <https://doi.org/10.1016/j.chemosphere.2022.137524>.
- Struthi, P., Shackira, A.M., Puthur, J.T., 2017. Heavy metal detoxification mechanisms in halophytes: an overview. Wetl. Ecol. Manag. 25, 129–148. <https://doi.org/10.1007/s11273-016-9513-z>.
- Teranishi, Y., Tanaka, A., Osumi, M., Fukui, S., 1974. Catalase activities of hydrocarbon-utilizing *Candida* yeasts. Agric. Biol. Chem. 38 (6), 1213–1220. <https://doi.org/10.1080/00021369.1974.10861301>.
- Tóth, G., Hermann, T., Da Silva, M.R., Montanarella, L.J.E.I., 2016. Heavy metals in agricultural soils of the European Union with implications for food safety. Environ. Int. 88, 299–309. <https://doi.org/10.1016/j.envint.2015.12.017>.
- Wei, D.D., Cheng, D., Liu, W.B., Liu, T., Yang, X.H., Zheng, Y.H., 2015. Adequate potassium application enhances salt tolerance of moderate-halophyte *Sophora alopecuroides*. Plant Soil Environ. 61 (8), 364–370. <https://doi.org/10.17221/8357-PSE>.
- Wei, X., Han, L., Xu, N., Yang, X., 2024. Nitrate nitrogen enhances the efficiency of photoprotection in *Leymus chinensis* under drought stress. Front. Plant Sci. 15, 1348925. <https://doi.org/10.3389/fpls.2024.1348925>.
- Werner, C., Correia, O., Beyschlag, W., 2002. Characteristic patterns of chronic and dynamic photoinhibition of different functional groups in a Mediterranean ecosystem. Funct. Plant Biol. 29 (8), 999–1011. <https://doi.org/10.1071/FP01143>.
- Yruea, I., 2009. Copper in plants: acquisition, transport and interactions. Funct. Plant Biol. 36 (5), 409–430. <https://doi.org/10.1071/FP08288>.