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REGULAR ARTICLE

Multidisciplinary studies supporting conservation programmes of two rare, endangered *Limonium* species from Spain

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Abstract

Background and aims Two local threatened endemics from Valencian salt marshes were analysed from a multidisciplinary perspective combining field studies with experiments performed under greenhouse-controlled conditions. The work aimed to investigate the habitat of the two species but also to explore their limits of tolerance to severe drought and salinity and the mechanisms behind their stress responses.

Methods The number of individuals in several populations, climatic conditions, soil characteristics and accompanying vegetation in the natural habitats were analysed in the field study. Plants obtained by seed germination were grown in the greenhouse and subjected to one month of water and salt stress treatments. Growth and biochemical parameters were analysed after the treatments were finalised.

Results No correlation between climatic parameters and the number of individuals censed of the two *Limonium* species could be established. Although *L. dufourii* was found in more saline soils in the natural habitats, under controlled greenhouse conditions, this species was more severely affected by salt treatment than *L. albuferae*, which is more susceptible to water stress. A common biochemical response was the increase of proline under all stress treatments, but mostly in water-stressed plants. Oxidative stress markers, MDA and H_2O_2, did not indicate significant differences between the treatments. The differences in

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the two species' responses to the two kinds of stress were correlated with the activation of the antioxidant enzymes, more pronounced in conditions of salt stress in *L. albuferae* and of water stress in *L. dufourii*.

Conclusions Although *L. albuferae* is found in sites with lower salinity in the natural habitats, the greenhouse experiment indicated that it tolerates higher concentrations of salt than *L. dufouri*, which is more resistant to drought. The two species efficiently mitigate oxidative stress by activation of antioxidant enzymes. The results obtained may be helpful for the conservation management of the two species: whereas salinity is not problematic, as the two species tolerated under controlled conditions salinities far beyond those in their natural environments, water scarcity may be a problem for *L. albuferae*, which proved to be more susceptible to water deficit.

Keywords Salt marshes · Salinity · Water stress · Endemics · Osmolyes · Antioxidants

Introduction

Coastal salt marshes represent ecosystems of great biodiversity and great ecological value (Gardner et al. 2015; Mitsch et al. 2015; Sutton-Grier and Sandifer 2019; Wolanski et al. 2009). In the region of Valencia (E Spain), they often appear as depressions integrated into dune systems, with the most saline areas located in the centre and the least saline at the edges of the salt marsh. The distribution of the different plant species in these saline areas is mainly determined by their relative tolerance to salinity, so that the plant communities are installed in concentric rings, depending on the salinity of the soil – although other factors, such as competition between species, can contribute significantly to the distribution of plants in the salt marsh (Grigore and Toma 2020). The complex of salt marshes developed in the Albufera Natural Park territory, located a few kilometres south of the city of Valencia, is of particular floristic and environmental interest (Ballester et al. 2003; Soria 2006). It shelters the unique populations of endemic *Limonium albuferae*, only found in this area (Ferrer-Gallego et al. 2016), and *Limonium dufourii*, which is present also in a few other salt marshes outside the Natural Park (Aguilella et al. 2010).

The genus *Limonium* Mill. (Plumbaginaceae) is outstanding in the region of Valencia; of the 28 species present, 20 are Iberian endemics, and 12 grow exclusively in this region (Mateo and Crespo 2014). One of the most threatened endemic species of *Limonium* of the Valencian territory is *L. dufourii* (Girard) Kuntze (Aguilella et al. 2010). Historically, this species was more widely distributed along the coast and salt marshes in the region of Valencia, but today it is represented only by five natural populations restricted to small coastal areas in the provinces of Castellón (Torreblanca) and Valencia: Marjal dels Moros (with three populations), El Saler (Albufera Natural Park) and Cullera (Aguilella et al. 2010). Most of these populations have a very low number of individuals, and molecular analyses show that substantial genetic variability and differentiation exist within and between populations (Palacios and González-Candelas 1997; Palacios et al. 1999). All the populations of *L. dufourii* are included in the Plant Microreserve network or Natural Parks (L'Albufera, Prat de Cabanes-Torreblanca) of the Valencian Community and also, additionally, in the European Union's Natura 2000 network of protected sites (as Site of Community Importance, SCI). The species is strictly protected in the Valencian region at the highest legal category, "In danger of extinction", included in the Valencian Catalogue of Threatened Plant Species (Aguilella et al. 2010).

Limonium albuferae P.P. Ferrer et al. is known only from a small site in the Albufera Natural Park, Racó de l'Olla (Ferrer-Gallego et al. 2016). At the beginning of 2020, 255 plants were counted, covering an area of about 160 m^2. Therefore, this species will be included in the "In danger of extinction" category in the next edition of the Valencian Catalogue of Threatened Plant Species.

In a previous study on the two species (*L. dufourii* and *L. albuferae*), based on metabolites profiling and the analysis of ion transport and accumulation, *L. albuferae* was found to be more salt-tolerant than *L. dufourii*, primarily due to its ability to accumulate fructose as a specific osmolyte (González-Orenga et al. 2019a). However, there is no information on the responses of the two species to drought, which can also affect their natural populations, especially in the changing climatic conditions of the global warming scenario, and neither on their ability to activate antioxidant mechanisms. Salinity and drought, like

all other types of abiotic stress, are associated with an increase in reactive oxygen species (ROS) production, leading to cellular damage by oxidising unsaturated fatty acids in cell membranes, amino acid residues in proteins, and DNA molecules (Apel and Hirt 2004; Choudhary et al. 2019; Das and Roychoudhury 2014). Different biomarkers can be used for assessing the extent of the oxidative stress affecting the plants; for example, malondialdehyde (MDA), a lipid peroxidation product employed as a reliable oxidative stress marker in both animals and plants (Del Río et al. 1996) or hydrogen peroxide (Sofo et al. 2015). In response to increased ROS production, two main antioxidant categories are activated by plants. One is represented by non-enzymatic antioxidants, including phenolic compounds, especially the subclass of flavonoids, carotenoids, ascorbic acid, or glutathione. To the second category belong antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) (and other peroxidases), or glutathione reductase (GR), which are activated in conditions of oxidative stress (Dumanović et al. 2021; Ozgur et al. 2013).

This study has been performed from a multidisciplinary perspective, including an analysis of soil and vegetation in the natural environments of the two species, completed with climatic information, and an analysis of physiological and biochemical responses of plants grown under controlled stress conditions in the greenhouse. Several questions concerning the habitats occupied by the two species were posed. Are there any differences between the two species in the soil characteristics and composition of plant communities? Is the decline of the populations of *L. dufourii* related to reduced water availability or increased salinity enhanced by changes in the climatic conditions due to global warming? On the other hand, considering that water scarcity and increased soil salinity may be restrictive factors, the study aims to analyse the responses of plants to induced water stress and salinity under controlled greenhouse conditions, with a special emphasis on their antioxidant mechanisms not investigated before in these species. Based on our previous knowledge, the starting hypothesis was that the decline of some populations of *L. dufourii* is related to changes in its habitat and a lesser efficiency of stress tolerance mechanisms than in the recently described *L. albuferae*.

Material and methods

Area of study

The field study was conducted in several salt marshes from the Albufera Natural Park, located in the "Devesa de l'Albufera" (Valencia province, Spain). The area belongs to Wetlands of International Importance of the Ramsar Convention since 1990, and in 1991 it was declared as Special Protection Area under the EU Directive on the Conservation of Wild Birds (79/409/EEC). It also contains habitats and refuges of species included in the EU Habitats Directive (92/43/EEC), and it is also classified within the Special Protection Areas in the Mediterranean, according to the Geneva Protocol (Soria 2006). The populations of the two *Limonium* species (*L. dufourii* and *L. albuferae*) are located in small salt marshes, locally named 'malladas', which are inter-dune depressions, often inundated during the winter period.

Climatic analysis

To establish a correlation with the evolution of the number of individuals in the censed populations, climate data were retrieved from SIAR, the Agroclimatic Information System for Irrigation (SIAR 2020) of the Spanish Ministry of Agriculture, Fisheries and Food. Data on the mean, maximum and minimum temperatures, rainfall and reference evapotranspiration (ETo) were collected for the past 19 years, on a monthly basis, from the agroclimatological station Benifaio (Valencia province), located 11 km from the area of study.

Population censuses

In each monitoring unit, censuses were made following the methodology of the Spanish Atlas and Red Data Book of Vascular Plants (Iriondo et al. 2003, 2009), adapted for the monitoring of endangered Valencian plant species by Navarro et al. (2010). Censuses were made from late July to late August, coinciding with the blooming period.

For *L. dufourii*, five natural population monitoring units were established, referred to as Devesa A (monitored since 2004), B (since 2005), C (since 2005) and D (since 2006); a new E unit has been established

in 2020, as a result of the tracking made for the present work. Additionally, Devesa 1 and 2 units were established for two new artificial populations, planted in winter 2013–2014. Although the species vanished in monitoring units A, C and D in 2008–2009, their sites have been revisited every year, corroborating the absence of the species.

Vegetation analysis

Vegetation inventories were carried out in the areas where the populations of the two species are located in the study territory. The study was conducted according to the phytosociological method (Braun-Blanquet 1964), adopting the International Code of Phytosociological Nomenclature (Weber et al. 2000). Braun-Blanquet values were transformed according to van der Maarel (1979). The nomenclature of the taxa follows EuroMed (2006) and the syntaxonomic nomenclature, according to Rivas-Martínez et al. (2001, 2002). Three measurements of soil electrical conductivity (EC, dS m^{-1}) were performed with a WET sensor (Delta Devices, Cambridge, England) at 10 cm depth in each inventory. The inventories were carried out mainly from mid-June to mid-November 2019.

Soil characteristics

Soil sampling was performed in July 2019. Samples were taken at 0–10 cm and 10–20 cm depth in the vicinity of specimens of the two species, from one salt marsh where the present unique population of *L. albuferae* is located, and from three salt marshes for *L. dufourii*. From each salt marsh, three soil samples were taken (n = 3). The samples were air-dried at room temperature and then crushed with a roller to break aggregates and passed through a 2-mm sieve. Analyses were performed on fine soil (diameter < 2 mm). Soil texture was analysed by the hydrometer method (Bouyoucos 1962). Organic matter was determined by the Walkley and Black method (1934) and carbonates by the technique of Bernard calcimeter (Loeppert and Suarez 1996). The following parameters were analysed in a saturation extract: pH, electrical conductivity (EC), Cl^-, Na^+, K^+, Ca^{2+}, and Mg^{2+}. A Crison pH-meter Basic 20 and a Crison conductivity-meter Basic 30 (Crison, Barcelona, Spain) were used to measure pH and EC, respectively. Sodium and potassium were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), chlorides were measured in a MKII Chloride Analyzer 92 6 (Sherwood, Inc., Cambridge, UK). Divalent cations (Ca^{2+} and Mg^{2+}) were measured with an atomic absorption spectrometer SpectrA 220 (Varian, Inc., CA, USA).

Plant growth under greenhouse conditions

Seeds of *L. albuferae* and *L. dufourii* provided by the Centre for Forest Research and Experimentation of the Valencian Region (CIEF, Valencia) were sown on a mixture of commercial peat and vermiculite (3:1) and watered with Hoagland nutrient solution (Hoagland and Arnon1950). After three weeks, plantlets were transferred to individual 1 L pots placed in plastic trays, with five pots per tray, and watered one further week with Hoagland solution. One week later, when plants had achieved a sufficient size, stress treatments were started. Plants subjected to the salt treatments were watered with aqueous solutions of 200, 400, 600, and 800 mM NaCl; those for the controls with distilled water, and those for the water stress treatment were not irrigated at all. Watering was performed by adding 1 L of the corresponding salt solution or water to each tray every five days. Five replicas (individual plants) were used per species and per treatment. All experiments were conducted in a controlled environment chamber in the greenhouse under the following conditions: long-day photoperiod (16 h of light), temperature of 23 °C during the day and 17 °C at night, and 50–80% relative humidity.

Moisture and EC in the pots were measured with the WET sensor (Delta Devices, Cambridge, England) at the beginning and during the treatments, as long as it was permitted by the device's limitations. Pot substrates were collected at the end of the treatments, and moisture and EC were determined in the laboratory. Moisture was determined by the gravimetric method. The samples were dried in an oven at 105 °C until they reached constant weight and then weighed again to calculate the water content as WC% = [(FW-DW)/FW] × 100, where FW and DW are the fresh and dry weights of the substrate samples.

For EC measurements, samples were collected from each pot, air-dried and then passed through a 2 mm sieve. A soil: water suspension (1: 5) was prepared in deionised water and mixed for one hour at

600 rpm and 21 °C before being filtered. Electrical conductivity was measured with a Crison 522 conductivity-meter and expressed in dS m^{-1}.

After one month of treatment, the aerial parts and the roots of the plants were harvested and weighed separately, and several growth parameters were measured: Fresh weight of leaves (FWL) and roots (FWR), water content percentage of leaves (WCL) and roots (WCR), and leaf number (LN). Water content percentage in leaves was calculated as indicated above for the soil samples, except that the plant material was dried at 65 °C.

Photosynthetic pigments

Chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids (Caro) were quantified according to the method reported by Lichtenthaler and Wellburn (1983), from 0.1 g of fresh leaves ground in 30 mL of ice-cold 80% acetone, mixed by vortexing and then centrifuged. The absorbance of the supernatant was measured at 663, 646 and 470 nm, and the concentration of each group of compounds was calculated according to equations previously described (Lichtenthaler and Wellburn 1983). Pigment concentrations were expressed in mg g^{-1} DW.

Osmolytes

Proline (Pro) content was quantified using fresh leaf material, according to the ninhydrin-acetic acid method of Bates et al. (1973). Pro was extracted in 3% aqueous sulphosalicylic acid, the extract was mixed with acid ninhydrin solution, incubated for one h at 95ºC, cooled on ice and then extracted with two volumes of toluene. The absorbance of the supernatant was read at 520 nm, using toluene as a blank. Pro concentration was expressed as µmol g^{-1} DW.

Total soluble sugars (TSS) were measured according to a previously published procedure (Dubois et al. 1956). Fresh leaf material was ground in liquid N_2 and extracted with 80% (v/v) methanol. After mixing in a rocker shaker for 24 h., the samples were centrifuged at 12,000 rpm for 10 min; supernatants were collected, appropriately diluted with water and supplemented with concentrated sulphuric acid and 5% phenol. After 20 min incubation at room temperature, the absorbance was measured at 490 nm. TSS concentrations were expressed as equivalents of glucose, used as the standard (mg eq. glucose g^{-1} DW).

Oxidative stress markers and non-enzymatic antioxidants

Leaf hydrogen peroxide contents in both, control and salt-treated plants were quantified as previously described (Loreto and Velikova 2002). Fresh leaf material (0.05 g) was extracted with a 0.1% (w/v) trichloroacetic acid (TCA) solution, followed by centrifugation of the extract. The supernatant was thoroughly mixed with one volume of 10 mM potassium phosphate buffer (pH 7.0) and two volumes of 1 M potassium iodide. The absorbance of the sample was determined at 390 nm. Hydrogen peroxide concentrations were calculated against an H_2O_2 standard calibration curve and expressed as µmol g^{-1} DW.

Malondialdehyde (MDA), total phenolic compounds (TPC), and total flavonoids (TF) were quantified in the same methanol extracts of fresh leaf material used for TSS measurements. MDA was determined according to the method of Hodges et al. (1999), with some modifications (Taulavuori et al. 2001). The extracts were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% TCA and then incubated at 95 °C for 20 min. After subtracting the non-specific absorbance at 440 and 600 nm, the MDA contents were calculated using the equation included in Taulavuori et al. (2001), based on the extinction coefficient at 532 nm of the MDA-TBA adduct (155 mM^{-1} cm^{-1}). Control samples (extracts mixed with 20% TCA without TBA) were processed in parallel. The concentration of MDA was finally expressed as nmol g^{-1} DW.

TPC were quantified, according to Blainski et al. (2013), by reaction with the Folin–Ciocalteu reagent. The methanol extracts were mixed with sodium bicarbonate and the reagent, incubated at room temperature in the dark for 90 min and the absorbance was recorded at 765 nm. Gallic acid (GA) was used as standard, and the measured TPC concentrations were expressed as GA equivalents (mg eq. GA g^{-1} DW).

Total 'antioxidant flavonoids' (TF) were determined by a previously described method (Zhishen et al. 1999), based on the nitration of aromatic rings containing a catechol group, by incubation with $NaNO_2$, followed by reaction with $AlCl_3$ at alkaline pH. After the reaction, the absorbance of the samples

was determined at 510 nm, and TF contents were expressed as equivalents of the catechin standard (mg eq. C g^{-1} DW).

Antioxidant enzymatic activity

Antioxidant enzyme activities were determined, at room temperature (25 °C), in crude protein extracts prepared from fresh plant material as described by Gil et al. (2014). Samples were ground in the presence of liquid N_2 and then mixed with extraction buffer [20 mM Hepes, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.1% (v/v) Triton X-100, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) polyvinylpolypyrrolidone and 5% (v/v) glycerol]. A 1/10 volume of 'high salt buffer' (225 mM Hepes, pH 7.5, 1.5 M KCl and 22.5 mM $MgCl_2$) was added to each sample, and the homogenates were centrifuged for 20 min at 20,000 g and 4 ºC. Supernatants were collected, concentrated in U-Tube TM concentrators (Novagen, Madison, WI, USA), and centrifuged to remove precipitated material. The final samples (referred to as 'protein extracts') were divided into aliquots, flash-frozen in liquid N_2 and stored at -75 ºC until used for enzyme assays. Protein concentration in the extracts was determined by the Bradford's (1976) method, using the Bio-Rad commercial reagent and bovine serum albumin (BSA) as the standard.

Superoxide dismutase (SOD) activity in the protein extracts was determined according to Beyer and Fridovich (1987) by following spectrophotometrically (at 560 nm) the inhibition of nitroblue tetrazolium (NBT) photoreduction; the reaction mixtures contained riboflavin as the source of superoxide radicals. One SOD unit was defined as the amount of enzyme causing 50% inhibition of NBT photoreduction under the assay conditions.

Catalase (CAT) activity was determined, according to Aebi (1984), following the decrease in absorbance at 240 nm due to the consumption of H_2O_2 added to the protein extracts. One CAT unit was defined as the amount of enzyme that will decompose one mmol of H_2O_2 per minute at 25 ºC.

Ascorbate peroxidase (APX) activity was determined as described by Nakano and Asada (1981) by measuring the decrease in absorbance at 290 nm, which accompanies ascorbate oxidation as the reaction progresses. One APX unit was defined as the amount of enzyme required to consume one mmol of ascorbate per minute at 25 ºC.

Glutathione reductase (GR) activity was determined according to Connell and Mullet (1986), following the oxidation of NADPH [the cofactor in the GR-catalysed reduction of oxidised glutathione (GSSG)] by the decrease in absorbance at 340 nm. One GR unit was defined as the amount of enzyme that will oxidise one mmol of NADPH per minute at 25 ºC.

Statistical analysis

Data were analysed using the programme Statgraphics Centurion XVII (Statgraphics Technologies, The Plains, VA, USA). All mean values throughout the text are based on five biological replicates. Significant differences between treatments were tested by one-way analysis of variance (ANOVA) at the 95% confidence level, and post hoc comparisons were made using Tukey's HSD test at $p < 0.05$. A two-way analysis of variance (ANOVA) was performed for all traits analysed to check the interaction between the species and the treatments. A principal component analysis (PCA) was used to check the similarity in the responses to water and salt stress between the two species.

Results

Climatic analysis

As it can be observed in the climatic diagram calculated for the period 2001–2019 (Fig. 1a), there is a strong water deficit in summer in the area of study, which belongs to the thermo-Mediterranean climate belt, specific for coastal and low-altitude zones, according to the Worldwide Bioclimatic Classification System (1996–2020). The evapotranspiration surpasses the rainfall amount in all the years analysed (Fig. 1b).

Evapotranspiration did not vary much during the last two decades, although a slight increase can be noticed in the last few years. On the contrary, both mean temperatures and evapotranspiration curves showed a substantial variation from one year to another.

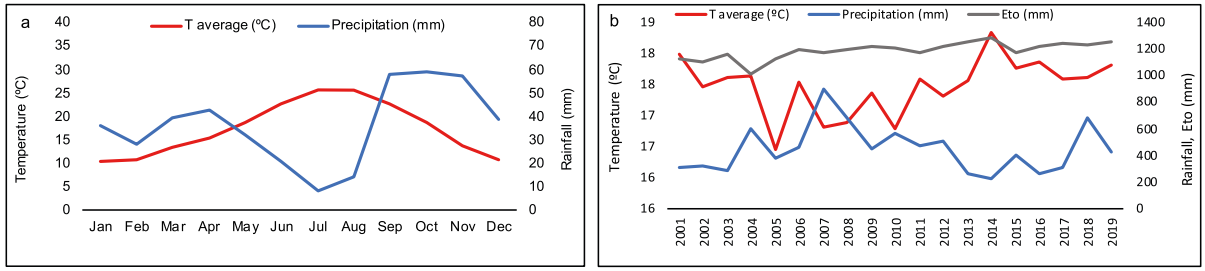

Fig. 1 Climatic characteristics in the area of study. Climatic diagram calculated for the period 2001–2019 (**a**); Evolution of mean temperature, rainfall and evapotranspiration in the study area (**b**). Data from the meteorological station of Beinfaio (Valencia), located near the site of the field study, obtained from SIAR (2020)

Trimestral variation of the main climatic parameters (mean, maximal and minimal temperatures, rainfall and evapotranspiration) is presented as supplementary material in Suppl. Table 1. A strong variation of the trimestral rainfall was detected, with minimal values in the third trimester and maximal in the fourth, coinciding with the general Mediterranean climate pattern, characterised by dry summers and rainfall mainly in autumn.

Population censuses

Results of censuses for the two species are shown in Table 1. During the period of population monitoring (2004–2020), three of the four previously known populations of *L. dufourii* disappeared: A (in 2007–2008), C (in 2008–2009) and D (in 2007–2008).

Table 1 Censuses of the *L. albuferae* (LA) and *L. dufourii* (LD) populations, performed in the Albufera Natural Park

X	731,022	731,395	731,469	732,091	730,987	731,592	731,386	730,217	730,543
Y	4,357,733	4,358,286	4,358,185	4,356,743	4,357,713	4,357,785	4,358,355	4,362,094	4,361,434
Species	LA	LD	LD	LD	LD	LD	LD	LD	LD
MU	Racó de l'Olla 1	Devesa B	Devesa E	Devesa 2	Racó de l'Olla	Devesa A	Devesa C	Devesa D	Devesa 1
2004						10			
2005		13				10	28		
2006		15				7	31	21	
2007		9				3	98	11	
2008		12				0	31	0	
2009		34				0	0	0	
2010		37				0	0	0	
2011		44				-	0	0	
2012		50				0	0	-	
2013		45				0	0	-	
2014		40		328		0	0	0	170
2015		241		648		-	0	0	175
2016		263		1822		0	0	0	89
2017	238	133		120	38	0	0	0	0
2018	243	374		809	38	-	0	-	11
2019	39	94		27	0	-	0	-	3
2020	255	77	17	3	0	0	0	0	17

MU monitoring units, - not censed

The extinction of populations A, C and D may be closely related to the high rainfall amount recorded in 2007 and 2008 (see Suppl. Table 1 and Fig. 1b).

A similar situation could occur for *L. dufourii* plants in the Devesa B monitoring unit, situated at a slightly higher level than the surrounding saline basins, unlike the previous ones. After several years with a low number (50 or less) of registered individuals, this monitoring unit experienced a notable increase between 2014 and 2015, from 40 to 241 specimens. This increase occurred after the succession of two very dry years, 2013 (only 263.8 mm) and 2014 (224.40 mm). Population levels remained high in subsequent years, with a minimum of 133 individuals (in 2017) and a maximum of 374 (in 2018). However, after the intense rainfall recorded in 2018 (684.02 mm), the population showed a sharp decline again, with only 94 specimens registered in 2019.

Vegetation analysis

Vegetation inventories of plants communities were performed in 22 locations, corresponding to different salt marshes in the Albufera Natural Park. Each inventory was accompanied by the collection of soil data obtained with a portable sensor. Suppl. Table 2 summarises the habitat characteristics of each site (the extension and coverage of the plant community), soil moisture and electrical conductivity, the list of species present in the community and their coverage. Two species had a higher presence in some of the inventories, equal to 4 in the Braun-Blanquet scale: *Sarcocornia fruticosa*, a structural shrubby species of the Mediterranean salt marshes, but also *Spartina patens*, an invasive with an increasing presence in recent years in the area of study. The soil electrical conductivity measured by the WET sensor near the plants of the two studied species in all inventories was higher in the case of *L. dufourii,* but it is necessary to take into consideration that *L. albuferae* was found in one unique location; thus, this finding does not demonstrate a better salt tolerance of *L. dufourii.*

Soil characteristics

Soil samples, collected in the summer of 2019 from the unique location of *L. albuferae* and three salt marshes with *L. dufourii*, all located in the Albufera Natural Park, were analysed. From each location, three soil samples were taken in the vicinity of the plants at two depths, 0 – 10 cm and 10- 20 cm. Their physical and chemical properties are summarised in Table 2.

All soils had a sandy texture. The percentage of sand represented the primary component, with low amounts of silt and clays. The soil pH is neutral, and the salinity in the superficial layer (0–10 cm) was higher than at 10–20 cm depth – which is the area

Table 2 Soil characteristics in the salt marshes with *Limonium albuferae* and *L. dufourii* in the Albufera Natural Park

Parameter	0–10 cm depth		10–20 cm depth	
	L. albuferae	*L. dufourii*	*L. albuferae*	*L. dufourii*
Sand (%)	99.50 ± 0.07^{b}	92.69 ± 1.79^{a}	94.90 ± 0.61^{b}	91.11 ± 0.63^{a}
Silt (%)	0.35 ± 0.05^{a}	5.11 ± 1.25^{b}	3.57 ± 0.43^{ab}	6.23 ± 0.22^{b}
Clay (%)	0.15 ± 0.02^{a}	2.19 ± 0.53^{b}	1.53 ± 0.18^{a}	2.67 ± 0.19^{b}
Bulk density (g cm^{-3})	1.10 ± 0.07^{a}	1.30 ± 0.05^{a}	1.17 ± 0.09^{a}	1.31 ± 0.06^{a}
Porosity (%)	58.46 ± 2.73^{b}	51.00 ± 2.14^{aA}	55.80 ± 3.45^{a}	50.57 ± 3.73^{a}
Carbonates (%)	23.21 ± 0.64^{a}	22.07 ± 1.63^{a}	24.56 ± 1.56^{a}	26.05 ± 4.09^{a}
Organic Matter (%)	1.66 ± 0.38^{a}	0.97 ± 0.37^{a}	0.71 ± 0.08^{a}	0.61 ± 0.25^{a}
pH	7.86 ± 0.21^{a}	7.42 ± 0.18^{a}	7.87 ± 0.03^{a}	7.79 ± 0.14^{a}
EC (dS m^{-1})	12.75 ± 4.93^{a}	37.91 ± 6.24^{b}	10.33 ± 2.38^{a}	15.73 ± 1.98^{a}
Na^{+} (meq L^{-1})	97.87 ± 28.63^{a}	295.52 ± 51.05^{b}	74.02 ± 22.53^{a}	107.63 ± 12.95^{a}
K^{+} (meq L^{-1})	2.68 ± 0.61^{a}	7.26 ± 2.13^{b}	2.32 ± 0.36^{a}	2.78 ± 0.15^{a}
Cl^{-} (meq L^{-1})	54.85 ± 13.02^{a}	252.64 ± 49.15^{b}	44.51 ± 9.28^{a}	76.49 ± 15.89^{a}
Ca^{2+} (meq L^{-1})	11.04 ± 1.78^{a}	18.62 ± 1.86^{b}	11.24 ± 0.72^{a}	12.10 ± 0.76^{a}
Mg^{2+} (meq L^{-1})	8.80 ± 2.75^{a}	15.62 ± 5.37^{a}	7.24 ± 1.14^{a}	12.97 ± 5.78^{a}

Values represent means followed by SD ($n = 3$). CEC, cationic exchange capacity, EC, Electric conductivity in saturated paste. Same letters indicate homogenous groups according to the Tukey test ($p \leq 0.05$)

explored by the roots of the plants−, especially in the areas with *L. dufourii*. When comparing the two species, higher EC values were obtained at the two depths for *L. dufourii*, confirming the measurement performed by the WET sensor in the natural habitats. The most abundant ion in the soil was Na^+, found at a higher concentration than that of Cl^-. The soil samples are also characterised by a high percentage of carbonates and divalent cations, Ca^{2+} and Mg^{2+}.

Plant growth under greenhouse conditions

Substrate EC was measured with the WET Sensor at the beginning and after one week of treatment, but further measurements were not possible due to the high EC reached in the salt treatments of 400–800 mM NaCl, which were beyond the capacity of the device. Therefore, the final EC was measured in an extract 1:5. EC in the pots gradually increased in parallel to the concentration applied, reaching values over tenfold higher than in the control in those watered with 800 mM NaCl, for the two species (Fig. 2a). Substrate moisture decreased drastically in the WS treatment already after one week and even more after 17 days in the two species; no further measurements were possible with the WET sensor. Thus, the final moisture determination was carried out using the gravimetric method. The results indicated a similar reduction in soil moisture in the two species at the end of the WS treatments (down to around 3%), whereas only a slight decrease was found in the presence of 400, 600 and 800 mM NaCl, with respect to the control and 200 mM NaCl treatments (Fig. 2b).

Table 3 Two-way analysis of variance (ANOVA) of treatment, species and their interactions, for the measured growth parameters

Parameter	Treatment	Species	Interaction	Residual
RFW	57.48***	0.43	1.35	40.73
RWC	87.19***	0.12	1.51	11.17
Lno	56.79***	1.14	13.21**	28.81
LA	34.10***	1.31	46.12***	18.54
LFW	72.24***	2.84**	11.68***	13.22
LWC	88.98***	1.91***	5.21***	3.88
Chl a	37.45***	6.04**	5.54	50.96
Chl b	48.85***	2.52	5.50	43.11
Caro	45.13**	2.53	9.34	42.98

Numbers represent percentages of the sum of squares (SS) at the 5% confidence level

RFW root fresh weight, *RWC* root water content, *LA* leaf surface area, *Lno* increment in the number of leaves, *LFW* leaf fresh weight, *LWC* leaf water content percentage, *Chl a* chlorophyll a, *Chl b* chlorophyll b, *Caro* carotenoids

Analysis of the growth parameters

Stress treatments had a strong effect on all analysed growth parameters and also on the photosynthetic pigments contents, whereas the effect of species was significant only for the leaf fresh weight (LFW) and leaf water content (LWC), as well as for Chl a content. The interaction of the two factors was also significant only for leaf traits: number of leaves (Lno), leaf area (LA), mean fresh weight (LFW) and mean water content (LWC) (Table 3). Chlorophyll a showed a predominantly uncontrolled variation, as shown by the higher SS percentage of the residual.

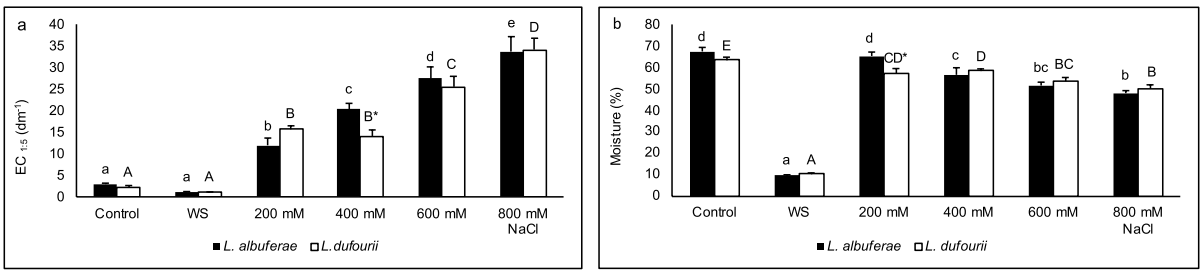

Fig. 2 Substrate electrical conductivity in a soil: water suspension (1:5) (**a**), and moisture (**b**), measured in pots with *L. albuferae* or *L. dufourii* at the end of 30 d of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p \leq 0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$)

Figure 3 shows the variation of leaf traits according to the various applied treatments. Leaf area decreased mainly in *L. dufourii*, whereas *L. albuferae* showed only a smaller reduction under water stress (Fig. 3a). The leaf number strongly decreased under the highest salt concentration, but the formation of new leaves was also reduced under water stress and salt treatments in the two species (Fig. 3b). Leaf fresh weight suffered a reduction under water stress in both species and under all salt concentrations in *L. dufourii*, but only under the higher salt concentrations in *L. albuferae* (Fig. 3c). Leaf water content showed a similar variation in the two species being affected only by the water stress (Fig. 3d).

Osmolytes, oxidative stress markers and antioxidant systems

Several biochemical parameters, such as osmolytes (proline and total soluble sugars), oxidative stress markers (MDA and H_2O_2), non-enzymatic antioxidants (total phenolic compounds and flavonoids) and the activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase), were determined in leaves of plants sampled at the end of the water stress and salt treatments.

The two-way ANOVA showed that, except MDA, all other analysed biochemical traits were significantly influenced by the treatments and, with the exception of MDA and total phenolic compounds (TPC), also by the species. The interaction between the two factors was highly significant for total soluble sugars contents (TSS) and the antioxidant enzymes activities, but not significant for Pro and TPC (Table 4). The most significant contribution to variation of MDA, TPC and TF is accounted for by the residual source of variation.

As indicated above, the variation of proline (Pro) leaf contents followed a similar pattern in the two species, increasing in parallel to the external concentration of NaCl, and especially under water stress. The relative increase over control values in plants subjected to water deficit was 29-fold for *L. albuferae* and 2.7-fold for *L. dufourii*; the corresponding values in the presence of the highest salt concentration applied, 800 mM NaCl, were 17-fold and 1.7-fold, respectively. However, it should be noted that Pro concentrations differed in the two species, being

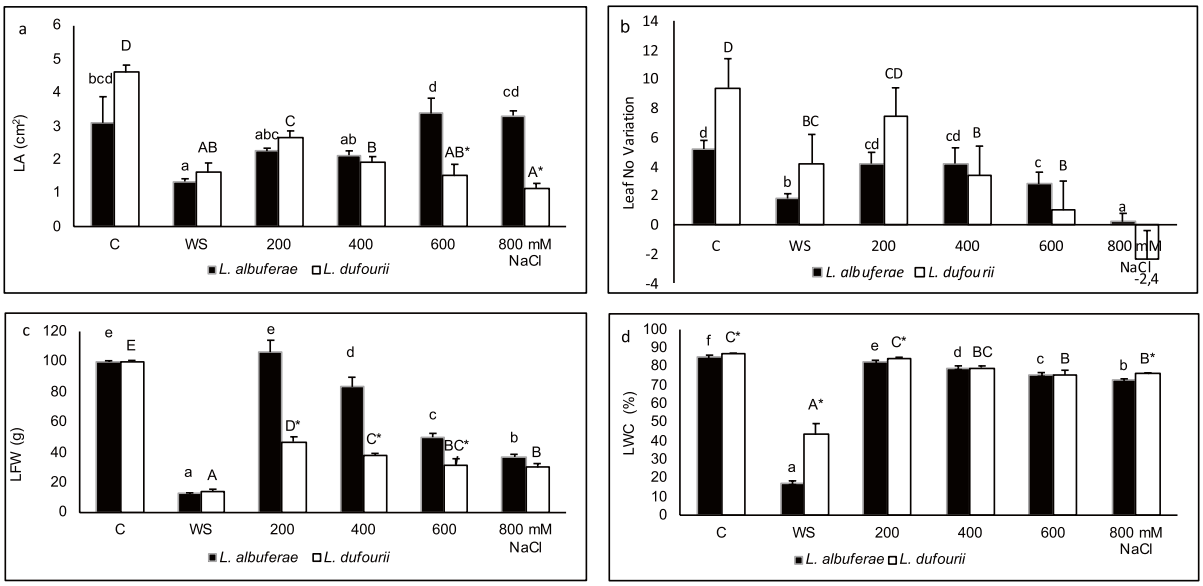

Fig. 3 Growth parameters in the two *Limonium* species after 30 days of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Leaf area (**a**), variation in the number of leaves (**b**), leaf fresh weight (**c**) and leaf water content (**d**). Mean ± SE values are shown ($n = 5$). Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p \leq 0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$)

Table 4 Two-way analysis of variance (ANOVA) of treatment, species and their interactions for the biochemical parameters considered

Parameter	Treatment	Species	Interaction	Residual
Pro	61.32***	21.35***	1.07	16.25
TSS	36.55***	6.97**	20.53***	36.03
MDA	6.09	5.72	18.28*	69.91
H_2O_2	57.95***	4.71**	9.22*	28.10
TPC	33.80***	0.004	8.48	57.71
TF	17.85*	10.86**	17.06*	54.21
SOD	39.90***	7.60***	24.90***	27.51
CAT	14.85***	50.01***	13.19***	21.95
GR	64.90***	4.27**	8.59**	22.30

Numbers represent percentages of the sum of squares at the 5% confidence level

Pro proline, *TSS* total soluble sugars, *MDA* malondialdehyde, H_2O_2 hydrogen peroxide, *SOD* superoxide dismutase, *CAT* catalase, *GR* glutathione reductase

significantly higher in *L. dufourii* than in *L. albuferae* in the control plants and under all tested stress conditions (Fig. 4a).

Under water stress, total soluble sugars (TSS) significantly increased in *L. dufourii* and decreased in *L. albuferae*, although the levels in non-stressed, control plants were more than four-fold higher in the latter species. Watering the plants with 800 mM NaCl induced a significant increase of TSS contents in both species. When comparing the two species, apart from the controls, significant differences in TSS levels were found under water deficit and moderate (200 mM) salinity conditions, but not in the presence of 400 mM or higher NaCl concentrations (Fig. 4b).

Malondialdehyde (MDA) contents did not vary in *L. albuferae* under any of the applied stress treatments and showed a significant (albeit small) increment only in water-stressed *L. dufourii* plants (Fig. 5a). In contrast, hydrogen peroxide decreased with respect to the corresponding controls in both species (Fig. 5b). Total phenolic compounds (TPC) showed a similar pattern of variation in response to stress in the two species, with small, in most cases non-significant changes as compared to the controls (Fig. 5c), whereas total flavonoids (TF) contents increased significantly only in plants of *L. albuferae* treated with 400 mM or higher NaCl concentrations (Fig. 5d).

Activity of antioxidant enzymes

The specific activities of the three tested antioxidant enzymes (SOD, CAT, and GR) showed different qualitative and quantitative patterns of variation in the two species in response to the applied stress treatment (Fig. 6). In *L. albuferae*, compared to the basal levels in non-stressed plants, the activity of the three tested enzymes increased significantly at very high salinities (600–800 mM NaCl) but not at lower NaCl concentrations or under water deficit conditions (Fig. 6a, b, c). In *L. dufourii*, SOD increased significantly only in the presence of 800 mM NaCl and in water-stressed plants (Fig. 6a), and GR also at the highest salt concentration tested, but not under water deficit stress (Fig. 6c). In contrast, CAT activity did not show significant changes in any of the treatments (Fig. 6b). Comparing the two species, significant differences were found; higher activation of SOD and CAT under salt tress in *L. albuferae*, whereas, on the contrary,

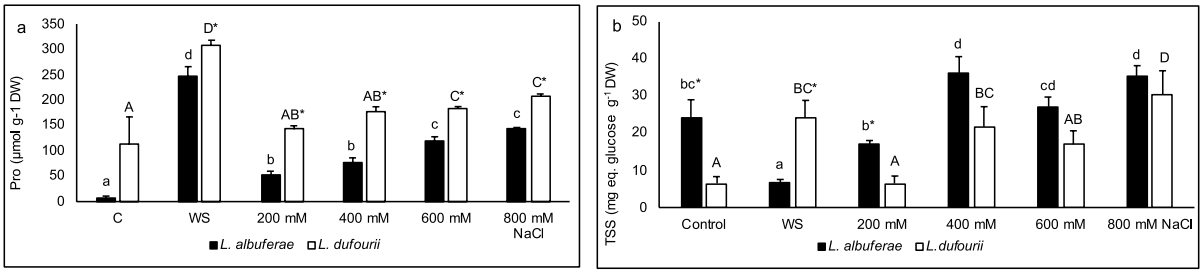

Fig. 4 Proline (**a**) and total soluble sugars (**b**) concentrations in leaves of the two *Limonium* species after 30 days of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Mean ± SE values are shown ($n = 5$). Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p \leq 0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$)

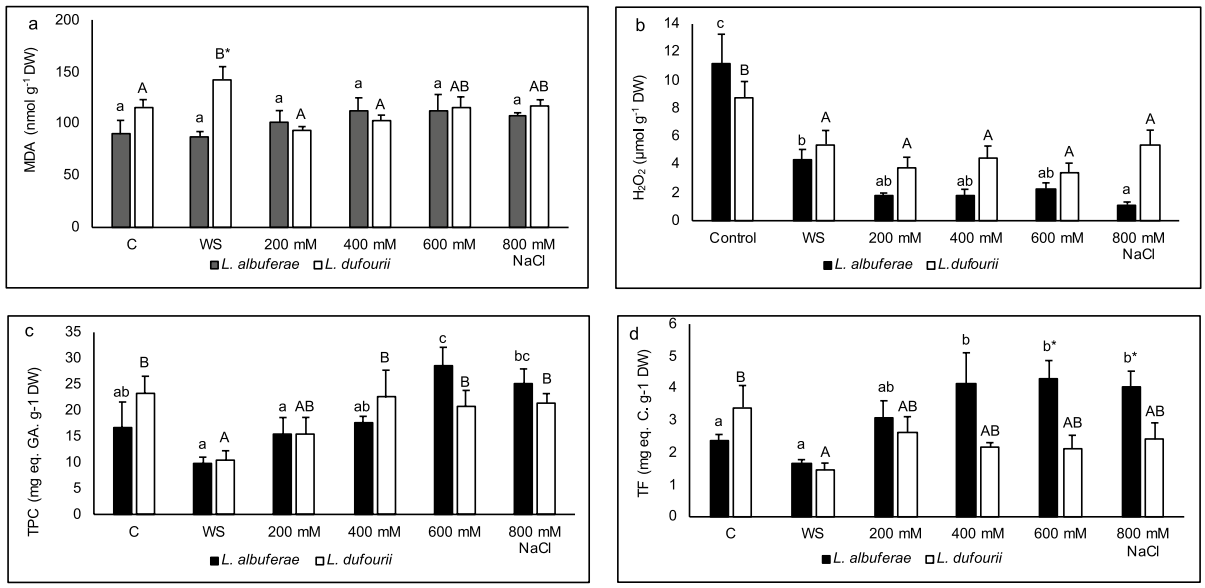

Fig. 5 Malondialdehyde (**a**), hydrogen peroxide (**b**), total phenolic compounds (**c**), and total flavonoids (**d**) concentrations in leaves of the two *Limonium* species after 30 days of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Mean ± SE values are shown ($n = 5$). Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p \leq 0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$)

higher activation of SOD and GR under water stress in *L. dufourii*.

Principal component analysis

A principal component analysis (PCA) was also performed, including all growth parameters, osmolytes, oxidative stress markers, antioxidants and enzyme activities determined in control and stressed plants. Five components had an Eigenvalue above 1. The biplot of the two main principal components, which together explained 67.96% of total variability, is shown in Fig. 7. The first component (X-axis), explaining 39.57% of variability, is related to the moisture of the substrate and, therefore, mainly to the water stress effect. The second component (Y-axis), explaining an additional 28.12% of variability, is related to the EC of the substrate and, as such, mostly to the salt treatments. Changes in substrate moisture correlated positively with changes in all growth parameters – especially the water content of root and leaves and leaf fresh weight – and photosynthetic pigments concentrations, which agrees with the inhibition of growth and the decrease in pigments contents observed under water stress (Fig. 7a). On the other hand, a strong *negative* correlation was detected between substrate water content and Pro, reflecting the large increase in Pro levels induced by water deficit (Fig. 7a). Regarding changes in the substrate EC, strong positive correlations were found with the antioxidant systems, especially with the activity of the SOD, CAT and GR enzymes, which increased with the salt treatments, at least at high salinity (Fig. 7a). The PCA also showed a clear separation of control, water stress and salt treatments but not of the two species, which responded in a similar manner to each applied stress treatment (Fig. 7b).

Discussion

As stated in the introduction, the two studied *Limonium* species are extremely interesting from the conservationist perspective. Both are endemic, with a small distribution area in Eastern Spain and highly threatened due to the scarcity of their populations (a single one was known for *L. albuferae*) and the large fluctuations in the number of their individuals. The

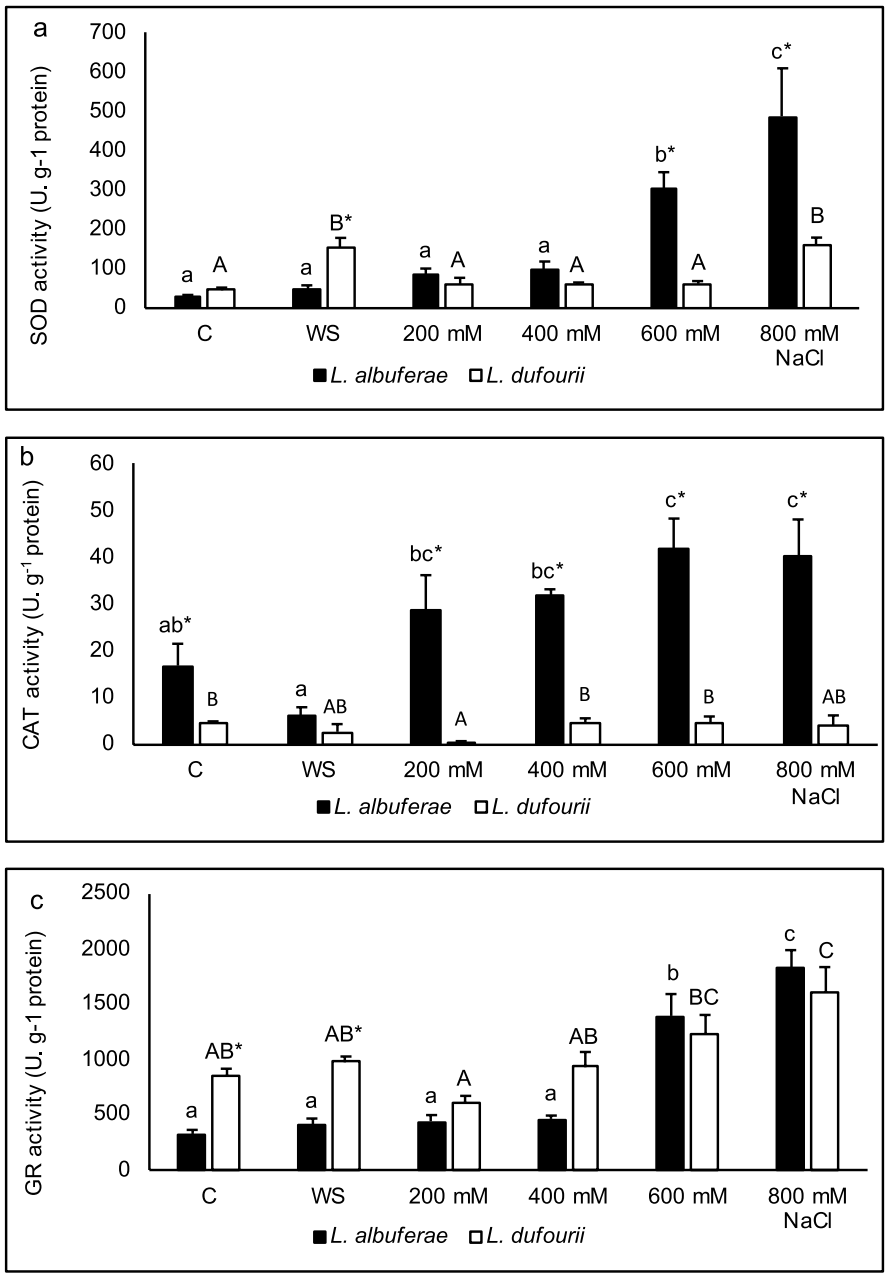

Fig. 6 Activity of the antioxidant enzymes, superoxide dismutase (SOD) (**a**), catalase (CAT) (**b**) and glutathione reductase (GR) (**c**), in the leaves of the two studied *Limonium* species after 30 days of water stress (WS; complete withholding of irrigation) or salt stress (at the indicated NaCl concentrations) treatments. Mean ± SE values are shown ($n = 5$). Same letters within each species (lower case for *L. albuferae* and upper case for *L. dufourii*) indicate homogeneous groups between treatments, according to the Tukey test ($p \leq 0.05$). Asterisks indicate significant differences between plants of the two species subjected to the same treatment ($p \leq 0.05$)

highest number of individuals of *L. dufourii* were registered in drier years, when flooding of the salt marshes did not occur or was very brief; conversely, the population declined after a year with intense rainfall. It should be taken into account that in this area of Eastern Spain, the highest concentration of precipitation occurs in autumn and, therefore, its effects on the censuses of *Limonium* species are detected when they are carried out in the summer of the following year. The possible effects of climatic conditions on the number of individuals of *L. albuferae* could not be assessed, as the species has been described only recently (Ferrer-Gallego et al. 2016). In 2019, an apparent decrease in the population of *L. albuferae* was observed; only 39 specimens were initially counted, which could be related to the intense colonisation of this site by the invasive species *Spartina patens* (Aiton) Muhl. However, after manual

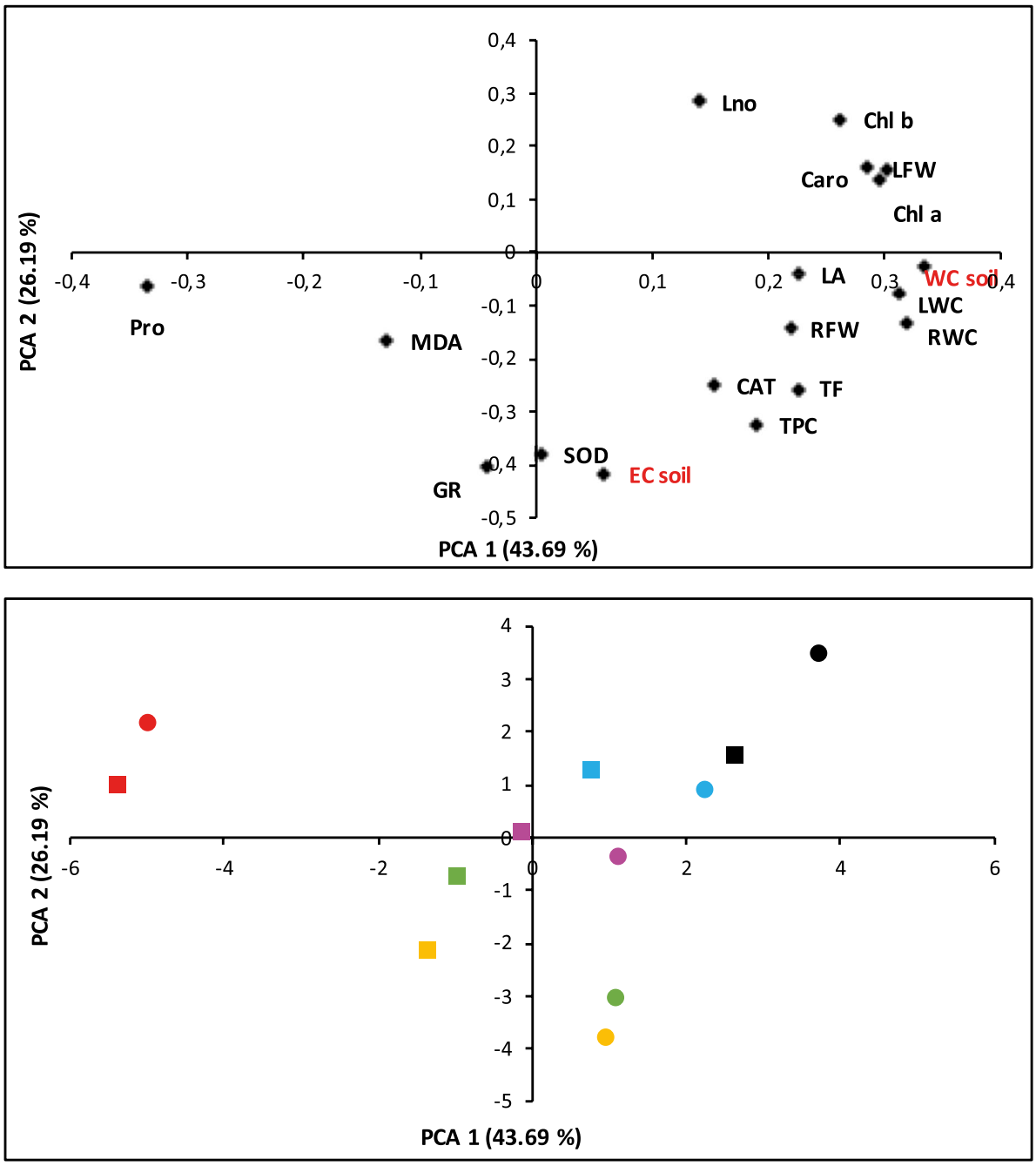

Fig. 7 Principal Component Analysis (PCA). Loading plot of the principal component analysis performed with the analysed traits (**a**): growth parameters, osmolytes, oxidative stress markers and non-enzymatic antioxidants levels, and antioxidant enzyme activities, in correlation to substrate moisture and EC. Scatter plot of the PCA scores (**b**): *L. albuferae* (circles) and *L. dufourii* (squares) plants, grown under water stress (red), and 200 (blue), 400 (purple), 600 (yellow), and 800 (green) mM NaCl, versus the corresponding non-stressed controls (black). Each symbol corresponds to the mean of the analysed plants per species and treatment ($n=5$). Abbreviations: RFW, root fresh weight; RWC, root water content; LA, leaf surface area; Lno, increment in the number of leaves; LFW, leaf fresh weight; LWC, leaf water content; Chl a, chlorophyll a; Chl b, chlorophyll b; Caro, carotenoids; Pro, proline; TSS, total soluble sugars; MDA, malondialdehyde, H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase

elimination of the invasive plants, 255 individuals of *L. albuferae* were censed in 2020.

The soil salinities in the salt marshes where the two species were found were moderate, and in the case of *L. albuferae,* significantly lower than those registered for the less salt-tolerant *L. dufourii*. Moreover, the salinity of the natural habitat of *L. albuferae* was well below the limit of tolerance of the species established in the greenhouse experiments. However, due to the extreme scarcity of this species, represented by a single population, the soil analyses were performed from only one area and are not conclusive for its ecological characterisation. The peculiar rarity of *L. albuferae* is definitely not related to edaphic conditions but most likely to evolutionary factors.

Regarding the analysed soil parameters, Na^+ and Cl^- contents in samples from 0–10 cm depth were 3 and 4.5-fold higher, respectively, in the areas of *L. dufourii* than in that of *L. albuferae* at the same depth. These differences explain the higher mean EC also detected with the WET sensor in the areas where *L. dufourii* was present. The same pattern was found for K^+, Ca^{2+} and Mg^{2+} soil concentrations, although the differences between the areas of the two species were not as marked as for Na^+ and Cl^-. Other soil properties relevant to plants' life, such as texture or pH, were similar in all soil samples.

The phytosociological analysis inventories could not be ascribed to associations as the specimens of the two species are often located in areas that have been extensively altered and have suffered a geomorphological restoration. The vegetation dynamics is very rapid, subjected to flooding and, therefore, to changes in salinity. This triggers the advance and retreat in a short time of different species, and the rainfall regime has been very variable in recent years, also greatly altering the plant communities. Nevertheless, the performance of phytosociological inventories brought relevant information by revealing the abundant presence in some inventories of the invasive species *Spartina patens*, which was recently reported as a major threat for native halophytes in this area (Martínez-Fort and Donat-Torres 2020). *Spartina patens* appears to pose a severe risk also to the two endemic *Limonium* species, as the salt marshes where *L. dufourii* disappeared (Devesa A, C, and D) are completely invaded by this species. In the remaining sites, the large yearly variation in the number of individuals is related to genetic factors of *L. dufourii*, which shows very different flowering patterns, occasionally behaving as annual or monocarpic perennial (plants die after the first reproductive stage) or flowering every year. Also, it should be noticed the high frequency of *Dittrichia viscosa* (L.) Greuter, regarded as a native invasive species, extremely competitive at low and moderate salinities in salt marshes of the region (Al Hassan et al. 2016).

Salt marsh ecosystems are highly dynamic, characterised by large variations in the salinity of the soil at the temporal and spatial scales, as reported in previous studies performed on the territory of the Albufera Natural Park (Boscaiu et al. 2013; Gil et al. 2014; González-Orenga et al. 2020). Therefore, reintroduction or reinforcement programmes for endemic and rare salt marsh species should also consider information on their limits of tolerance to stressful environmental factors. In Mediterranean salt marshes, a general increase in average temperatures and short-term 'heatwaves', due to climate change, will lead to increased evapotranspiration. Consequently, drought and soil salinity will also intensify, inflicting greater stress on plants and potentially causing the dieback of those less tolerant (Touchette et al. 2019).

The analysis of growth inhibition in response to the applied stress treatments indicated that the two species were mostly affected by water stress. In both, the strongest reduction in the most relevant growth parameters, leaf fresh weight and water content, was found in plants subjected to one month of water deficit, especially those of *L. albuferae*. This indicated that *L. albuferae* is much more susceptible to drought than other *Limonium* species growing in the study area, which were the subject of previous work (González-Orenga et al. 2019b). On the contrary, the salt-induced changes in growth parameters suggested that *L. dufourii* is more sensitive to high soil salinity than *L. albuferae*. Growth reduction under salt stress is a general trait in glycophytes but also in many halophytes (Flowers et al. 1986; Flowers and Colmer 2008). Only in some dicotyledonous halophytes, especially in those more salt-tolerant, low and moderate concentrations of NaCl stimulate growth, as we have observed in *L. albuferae*, in which foliar fresh weight was slightly higher in the presence of 200 mM NaCl than in control plants. Stimulation of growth under low and moderate salinity conditions has been reported only in a few species of the genus *Limonium,* such as *L. bicolor* (Bunge) Kuntze (Li 2008; Wang

et al. 2017), *L. delicatulum* (Girard) Kuntze (Souid et al. 2016), *L. pectinatum* (Aiton) Kuntze (Morales et al. 2001), or *L. girardianum* and *L. virgatum* (Al Hassan et al. 2017). In some others, such as *L. stocksii,* no differences with respect to the control were found up to 300 mM NaCl (Hameed et al. 2015), whereas in many species, growth was optimal in control conditions (Ben Hamed et al. 2014; Grieve et al. 2005). Contrary to the intense dehydration caused by water stress, for both species, leaf water content decreased only slightly in the plants subjected to salt stress, demonstrating the small contribution of water loss to the reduction of fresh weight.

The biochemical analyses revealed an increase of Pro contents in the two *Limonium* species, more pronounced in response to the water stress treatment than under salt stress. The relative increase was more accentuated in *L. albuferae* due to the very low Pro levels in the absence of stress, but higher absolute values were found in *L. dufourii* in all applied treatments. The accumulation of Pro to high levels under water deficit conditions agrees with its strong negative correlation with substrate water content, revealed by the PCA. Pro is also a reliable marker of salt stress, increasing in the plants in parallel with the increase in the external concentration of NaCl; however, Pro does not seem to be directly involved in the mechanisms of salt tolerance, as it accumulates to higher absolute levels in *L. dufourii*, the less salt-tolerant of the two species. Pro biosynthesis in salt-stressed plants of *Limonium* is a well-known phenomenon and was already reported in the early work of Cavalieri and Huang (1979). In general, plant species of a particular genus tend to use only one, or very few different compounds, as functional osmolytes; one representative example is *Plantago*: all investigated species of this genus accumulate predominantly sorbitol in response to various abiotic stresses (Flowers and Colmer, 2008). In *Limonium*, however, a large variety of chemical compounds with the function of compatible solutes have been reported in different species including, besides Pro, quaternary ammonium compounds like β-alanine betaine, choline-*O*-sulfate or glycine betaine, and different soluble sugars (fructose, sucrose and glucose) and polyalcohols (e.g., inositol isomers and derivatives) (Al Hassan et al. 2017; Furtana et al. 2013; Gagneul et al. 2007; González-Orenga et al. 2019b; Hanson et al. 1991; Morales et al. 2001; Rhodes and Hanson 1993; Tabot and Adams 2014; Tipirdamaz et al. 2006). Recently, in a metabolic profiling of these two species, we reported a gradual increase in Pro concentrations in parallel to increasing salinity but also a higher accumulation of fructose and glucose in *L. albuferae* (González-Orenga et al. 2019a). These data are consistent with the results presented here, indicating higher values of total soluble sugars in salt-stressed plants of this latter species.

As already mentioned, abiotic stress is associated with increased ROS production that generates oxidative stress (Das and Roychoudhury 2014; Dumanović et al. 2021). In our experiments, no significant changes in MDA levels were observed in the stressed plants, except for a slight (but significant) increase in plants of *L. albuferae* subjected to water stress; H_2O_2 levels even decreased in comparison to the non-stressed controls. Similarly, no variation in MDA and H_2O_2 under salt treatments was found in *L. latifolium* (Ben Hamed et al. 2014), but an increase was reported in some other species (Hameed et al. 2015; Souid et al. 2016). Several studies have shown that halophytes generally do not generate ROS in excess as they are perfectly adapted to the stressful environments where they live and possess efficient mechanisms to avoid or substantially reduce oxidative stress (Bose et al. 2014; Gil et al. 2014), and this seems to be also the case in the selected *Limonium* species. Phenolic compounds, especially the subgroup of flavonoids, include many secondary metabolites that are potent antioxidants and increase under stressful conditions in many plant species (Di Ferdinando et al. 2012). Many *Limonium* species contain efficient free radical scavengers and have strong antioxidant properties (Senizza et al. 2021; Souid et al. 2019; Ruiz-Riaguas et al. 2020). Field studies on several *Limonium* species from Tunisia indicated a variation in the levels of polyphenols and flavonoids in relation to seasonal constraints in their natural habitats (Souid et al. 2018, 2019). In salt-treated plants of some species, an increase in the concentration of these compounds has been reported (Wang et al. 2016), but in others, only small increases (Souid et al. 2016) or no significant variations under water stress (González-Orenga et al. 2019a, b) were observed. In the present work, the only significant increases in total phenolics or flavonoid levels were observed in salt-stressed *L. albuferae* plants.

Phenolic compounds (including flavonoids), as other non-enzymatic antioxidants, are regarded as a secondary line of defence against oxidative stress, activated only under severe stress conditions, whereas antioxidant enzymes constitute the first ROS scavenging system (Fini et al. 2011). The specific activities of three antioxidant enzymes were determined in control and stressed plants of the two investigated species since enzymatic antioxidant mechanisms have been reported to be important for counteracting oxidative stress in *Limonium* under salt (Li 2008; Souid et al. 2016; Zhang et al. 2014) and drought (Souid et al. 2018) stress conditions. SOD is the first enzyme to be activated in response to stress as it catalyses the dismutation of superoxide radicals into O_2 and H_2O_2 (Alscher et al. 2002). CAT complements the activity of SOD by decomposing the produced H_2O_2 into O_2 and H_2O and is induced by the accumulation of its substrate (Gunes et al. 2007). Glutathione reductase (GR) contributes to recover and maintain the adequate cellular redox state by reducing oxidised glutathione (GSSG) to its reduced form (GSH), using NADPH as a cofactor (Hameed et al. 2015). Changes in the activities of these enzymes in response to stress followed different qualitative and quantitative patterns regarding both the stress treatment and the species. Thus, water deficit induced SOD activity in *L. dufourii*, but no significant changes with respect to the controls were observed for the other two enzymes in this species, nor in *L. albuferae* plants for any of the three tested enzymes. Therefore, it appears that the activation of enzymatic antioxidant mechanisms against water stress is more efficient in *L. dufourii* than in *L. albuferae*, which may contribute to the relatively higher drought tolerance of the former species. Conversely, in the more salt-tolerant *L. albuferae*, the three antioxidant enzyme activities increase significantly in response to the 600 and 800 mM NaCl treatments, whereas in *L. dufourii* SOD and GR (but not CAT) activities also increased, but to lower levels and only under the presence of the highest salt concentration tested.

Conclusions

The field study did not reveal a clear correlation between the number of individuals censed in the analysed populations with the climatic conditions. The vegetation analysis underlined the presence of invasive species, mostly *Spartina patens*, with a notable presence in some inventories. Although in the natural habitats, *L. albuferae* is found in sites with lower salinity, the observed changes in several growth and biochemical variables in plants of the two selected *Limonium* species subjected to stress treatments under controlled greenhouse conditions, indicated that *L. albuferae* is more salt-tolerant than *L. dufourii* but more susceptible to drought stress. Conversely, *L. dufourii* is more drought-tolerant but more salt-sensitive than *L. albuferae*. In its natural habitat in the salt marsh, *L. dufourii* appears to be sensitive to prolonged flooding. Proline was synthesised in both species, especially under water stress, whereas MDA and H_2O_2 did not show a significant variation. The activity of antioxidant enzymes plays the most important role in the mitigation of oxidative stress in both species and both stress types. Increased accumulation of phenolic compounds in the two species, and flavonoids in the more salt-tolerant *L. albuferae*, also contribute to alleviating oxidative stress in the presence of high salt concentrations.

The results presented here may be useful in the conservation management of the two species. Salinity does not seem to threaten the future reintroduction of specimens in salt marshes, as the two species under controlled conditions tolerated salinities far beyond those in their natural environments. Water scarcity, however, may be a problem for *L. albuferae*, which proved to be more susceptible to water deficit. On the other hand, *L. dufourii* should not be introduced in sites prone to prolonged flooding. The field study also established that, besides abiotic stress factors, competition with invasive species could be a major threat to the preservation of these species in their natural habitats. These data should be considered in the design and implementation of conservation, reinforcement or reintroduction programmes and for the general management of the threatened populations of these rare and endemic *Limonium* species.

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