Physiological and morphological characterisation of *Limonium* species in their natural habitats: Insights into their abiotic stress responses

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Abstract

Background and aims Morphological and biochemical traits of four halophytes of the genus *Limonium* were analysed in plants sampled from salt marshes in SE Spain. This work aimed to explore the mechanism(s) behind the adaptation of these species to stressful habitats, with particular emphasis on responses to drought.

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Methods Plants of each species together with soil samples were collected in summer, which is the most stressful season in the Mediterranean. Soil parameters and plant morphological traits were determined, and the levels of several biochemical stress markers in plants were measured using spectrophotometric assays. A multivariate analysis was performed to correlate soil and plant data.

Results Morphological characteristics regarding the underground system topology and several biochemical traits (higher foliar Ca^{2+} , sucrose and glucose, and lower proline, glycine-betaine and fructose) clearly separate *L. santapolense* individuals from plants of the other three species.

Conclusions Drought tolerance of *L. santapolense* in the field is mostly dependent on morphological adaptations: when growing in an arid location, plants of this species develop long taproots that can extract water from the deep, moist layers of the soil.

Keywords Antioxidants · Climate change · Drought · Endemics · Osmolytes · Salt marshes · Soil analysis

Abbreviations

ETo	potential evapotranspiration
S	sand
Sl	silt
C	clay
OM	organic matter
ECsat	electric conductivity in the saturation extract
NS	number of apical shoots
NL	number of leaves

SFW	Shoot fresh weigh
RFW	Root fresh weight
LA	total leaf area
RL	total root length
LRL	lateral root length
PRL	taproot or principal root length
RSA	root surface area
D	average diameter of the roots
R/S	root to shoot ratio
SRL	specific root length
M	root magnitude
a	root altitude
Nd	numbers of root nodes
TI	topological index
Pro	proline
GB	glycine betaine
TSS	total soluble sugars
Fru	fructose
Suc	sucrose
Glu	glucose
MDA	malondialdehyde
DPPH	2,2-diphenyl-1-picrylhydrazyl
TPC	total phenolic compounds
TF	total flavonoids
PCA	Principal component analysis

Introduction

Salt marshes, like many other coastal habitats, are considered as highly threatened ecosystems, intensively modified by anthropogenic actions (Barbier et al. 2011). In the Iberian Peninsula, as in many other regions of the world, these habitats have suffered numerous threats. Considered as insalubrious in the past, they were eliminated when located near human settlements; expansion of agriculture and touristic pressure also contributed to their reduction. In addition, effects of climate change represent another threat for salt marshes in the Mediterranean area. Not only rises of temperature and the risk of longer and more intense drought periods but also the sudden alteration of seasonal weather patterns may modify the existing conditions in these ecosystems (Thorne et al. 2012). The characteristic vegetation of the salt marshes is represented mainly by halophytic plants, which are tolerant to soil salinity in a greater or lesser degree. There is a wide range of halophytes, from plants present at the borders of salt marshes and adapted to

only (relatively) low salinity levels, to plants that show optimal growth under moderate saline conditions and tolerate salt concentrations even higher than that of seawater. It is not possible to define a precise salinity threshold to separate halophytes from glycophytes, as plant species show a continuous range of sensitivity to salt stress (Grigore and Toma 2017). Nevertheless, a generally accepted, operational definition is that halophytes are plants of natural saline environments, which are able to complete their life cycle at soil salinities equivalent to, at least, 200 mM NaCl (Flowers and Colmer 2008). However, salinity is not the only limiting factor for plants in salt marshes, where they are simultaneously affected by other additional stressful conditions. For example, plants growing in such habitats under Mediterranean climatic conditions have to switch from waterlogging, after heavy rains in spring, to extremely dry conditions in summer, when the soil surface is covered by a crust of salt due to intense evapotranspiration (Álvarez-Rogel et al. 1997, 2000). Responses to one or the other type of stress broadly vary among different genera of plants and often even between congener species.

A useful approach for unravelling the mechanisms underlying plant tolerance to salt – and other abiotic stresses – is to study the responses to stress of taxonomically (implicitly, also genetically) related taxa. A good candidate for this type of comparative studies is the genus *Limonium* L. of the Plumbaginaceae family. This genus includes more than 400 species, many of which are halophytes, and are well represented in the Mediterranean (Greuter et al. 1989) with numerous endemics in the area of study (Mateo and Crespo 2014).

Four species of *Limonium* have been selected for our ongoing research on this genus: *L. santapolense* Erben, *L. girardianum* (Guss.) Fourr., *L. virgatum* (Willd.) Fourr. and *L. narbonense* Mill. The four species flower in summer, *L. santapolense* from May to July and the other three from July to September. Regarding their geographic distribution, *L. santapolense* is a local endemic, present only on littoral sandy substrates in a small area in the province of Alicante, whereas *L. girardianum* is endemic to S France, E Spain and Balearic Isles, growing on sandy coasts and cliffs. *L. virgatum* and *L. narbonense* have a broader distribution throughout the Mediterranean region, the first on sandy beaches and rocky coasts, reaching the Middle East and North of Africa, and the second in salt marshes throughout the Mediterranean, in Spain also on the

Atlantic coast (Erben 1993). Besides the conservation value of the two endemic species, all four are important elements of the salt marsh ecosystems as their presence and frequency in plant communities increase the diversity and the degree of differentiation between the local habitats in the area of study. Populations of the four species growing in the wild in SE Spain have not been studied in depth so far, and their morphological and biochemical traits may reflect local adaptations. Thus, their analysis is important not only for obtaining a broader knowledge of the four species, but also to predict their possible future response to the challenge of climate change.

We have previously analysed the germination patterns of these species (Monllor et al. 2018), and their responses to salt stress (Al Hassan et al. 2017) and water stress (González-Orenga et al. 2019) under controlled greenhouse conditions. Tolerance to salinity was similar in the four species and was mainly based on the active transport and accumulation of ions in the leaves, with the concomitant synthesis of soluble sugars and proline as compatible solutes for osmotic adjustment (Al Hassan et al. 2017). In contrast, water stress, induced by withholding irrigation of the plants, affected mostly *L. santapolense*, which appeared to be highly sensitive to dehydration – plants lost about one-third of their fresh weight after the water deficit treatment (González-Orenga et al. 2019). This finding was somewhat surprising since the natural habitat of this species is drier than those of the plants of the other three taxa analysed. Our working hypothesis to explain this apparent discrepancy is that *L. santapolense* plants possess some specific mechanisms that enable their survival in the field, under harsh natural conditions with very little water availability, but that cannot be mimicked in pot experiments in the artificial environment of a greenhouse. The analysis of plants sampled in the wild, in correlation with the climatic and edaphic conditions at the sampling sites, represents a useful complementary approach to study the stress response mechanisms of the four *Limonium* species, considering that the specific distribution of each taxon, within the same general habitat, may depend on local variations of soil characteristics.

With these ideas in mind, we undertook the present study on the mechanisms of stress tolerance in the selected *Limonium* species, with the following specific aims: (i) to analyse the climatic and soil conditions at the sampling sites of each species; (ii) to study the growth patterns of plants of the four species in the wild; (iii) to

study stress response mechanisms based on the regulation of ion transport and osmolytes accumulation; and (iv) to determine the levels of oxidative stress affecting the plants, and the concentrations of representative non-enzymatic antioxidants.

Material and methods

Sampling sites and material sampling

Mature plants of *L. santapolense*, a rare endemic restricted to the province of Alicante (SE Spain), were collected from Clot de Galvany, a saltmarsh located near the city of Elche (39° 15' N/0° 31' W). Plants of the other three species were collected from 'La Albufera' Natural Park, near the city of Valencia (39° 20' N/0° 19' W). *Limonium* At the sampling sites of the four species, four whole plants of each species and three soil samples were collected in July 2018.

Climatic analysis

Climatic data were obtained from the nearest meteorological stations (Elche for 'Clot de Galvany' and Benifaió for El Saler), provided by the Agroclimatic Information System for Irrigation (SIAR 2018), of the Spanish Ministry of Environment, Rural and Marine Affairs (MARM). The following bioclimatic indexes were calculated using available meteorological data of the last 16 years:

- TI: Thermicity index, $TI = 10 * (T + M + m)$
- CI: Continentality index, $CI = T_{max} - T_{min}$
- OI: Ombrothermic index, $OI = (P/12) * 10 / \sum T_m$
- Ppv: Summer precipitation in mm of the three consecutive warmest months in the year.
- Ttv: Value in tenths of degree resulting from the sum of the monthly average temperatures of the three consecutive warmest months in the year.
- ETo: Potential evapotranspiration, calculated according to Penman-Monteith equation (Allen et al. 1998).
- GI: Giacobbe index, $GI = (P_{June} + P_{July} + P_{August}) / T^a$ of the warmest month

where: T, yearly average temperature; m, average temperature of the minima of the coldest month of the year; M, average temperature of the maxima of the

coldest month of the year; T max, average temperature of the warmest month; T min, average temperature of the coldest month; Tm, average temperature of each month; P, total yearly precipitation.

All indexes were calculated according to Rivas-Martínez and Rivas-Saenz (1996–2018), except for GI calculated according to Giacobbe (1938, 1959).

These specific indexes were chosen as they are the most suitable for local differentiations within the Mediterranean climate type (Ferriol et al. 2006).

Meteorological data (mean, maximum and minimum temperatures, rainfall, air humidity and evapotranspiration) of the previous month to sampling were obtained from the same source.

Soil analysis

Soil samples were taken at a depth of 0–15 cm. Once the samples were air dried at room temperature (approx. 25 °C), they were crushed with a roller to break aggregates and then 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 content (OM%) was determined as described by Walkley and Black (1934), and carbonates were measured with a Bernard Calcimeter.

The following parameters were determined in soil saturation extracts: pH, electric conductivity (EC), and concentrations of cations (Na^+ , K^+ , Ca^{2+} and Mg^{2+}) and chlorides. A Crison pH-meter Basic 20 and a Crison Conductimeter Basic 30 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), and divalent cations of calcium and magnesium were measured with an atomic absorption spectrometer SpectrA 220 (Varian, Inc., CA, USA). Cation exchange capacity (CEC) was determined following Rhoades (1982).

Plant sampling in the wild

Four plants were selected from distant areas of their natural location and then uprooted as described in Fita et al. (2013), trying to recover intact roots systems. Roots were excavated digging a 30 cm depth-pit 50 cm away from the plant shoot without breaking any

roots that grew horizontally further than those 50 cm and then removing carefully the soil, as if it was in a pot. If roots grew deeper, the same procedure was repeated until reaching the end of the root. The number of shoots per plant (NS), number of leaves (NL), shoot fresh weight (SFW), and root fresh weight (RFW) were recorded. The roots were scanned (Epson LA 1600+, Epson America Inc. Long Beach, CA, USA), and the pictures were analysed with the WinRhizo Pro software (WinRhizo Pro 2003b, Reagent Instruments Inc. Quebec Canada) to obtain the total root length (RL, cm), the lateral root length (LRL, cm), the primary root length (PRL, cm), the root surface area (RSA, cm^2) and the average diameter of the roots (D, mm).

To better assess the root architecture, the root topological parameters defined by Fitter (1987) were evaluated. Root magnitude (M) was evaluated as the number of external links of a root, root altitude (a), as the maximum external path length of the root, numbers of root nodes (Nd) were counted, and the topological index (TI) was calculated as the ratio of log altitude over log magnitude (Magalhães and Seifert 2015). Other composite parameters were calculated, such as root to shoot ratio (R/S) as RFW/SFW , and the specific root length (SRL, cm/g) as RL/RFW .

A fraction of the plant material was stored at -20°C , and the remaining material was dried for several days in an oven at 65°C until constant weight. Water contents (WC%) were expressed as percentages of fresh weight, in roots and leaves.

Ion concentration measurements

Ion concentrations were determined in dry roots and leaves, after being eluted in aqueous extracts according to Weimberg (1987), by heating the samples (0.05 g of dried, ground plant material in 15 mL of water) for 15 min at 95°C , followed by filtration through a $0.45\ \mu\text{m}$ filter (Gelman Laboratory, PALL Corporation).

Ion (Na^+ , K^+ , Cl^- , Ca^{2+} , Mg^{2+}) concentrations in plant extracts were measured using the same instruments as for their determination in soil samples.

Osmolyte quantification

Proline (Pro) was extracted with 2 mL of 3% (w/v) sulfosalicylic acid, from 0.05 g of dry leaf material, and was quantified in toluene according to the acid-ninhydrin method of Bates et al. (1973). The extract,

mixed with acid ninhydrin, was heated at 95 °C for one h, cooled on ice and extracted with toluene. The absorbance of the organic phase was measured at 520 nm, using toluene as a blank. Pro concentrations were expressed as $\mu\text{mol g}^{-1}$ DW.

Glycine betaine (GB) was extracted from 0.05 g dry leaf material with 1 mL water, according to Grieve and Grattan (1983) with the modifications proposed by Nawaz and Ashraf (2010). The extract was supplemented with potassium iodide, kept on ice for 90 min and then extracted with 1, 2-dichloroethane (pre-cooled at -20 °C); finally, the absorbance of the sample was measured at 365 nm. GB concentration was expressed as $\mu\text{mol g}^{-1}$ DW.

Total soluble sugars (TSS), were measured in 0.05 g dry plant material extracted with 2 mL of 80% (v/v) methanol, following the method described by Dubois et al. (1956). The sample was mixed on a rocker shaker for 24 h; the extract was then centrifuged, concentrated sulfuric acid and 5% phenol was added to the supernatant, and the absorbance was measured at 490 nm. TSS concentrations were expressed as ‘mg equivalent of glucose’ (used as the standard) per g DW.

HPLC analysis of soluble carbohydrates

Plant dry material (0.05 g) was boiled in 2 mL Milli-Q water for 10 min and then filtered through 0.22 μm nylon filters. The soluble sugar fraction was analysed using a Waters 1525 high performance liquid chromatography (HPLC) coupled to a 2424 evaporative light scattering detector (ELSD), according to Al Hassan et al. (2016). The source parameters of ELSD were the following: gain 75, data rate 1 point per second, nebulizer heating 60%, drift tube 50 °C, and gas pressure 2.8 Kg/cm^2 . The analysis was carried out injecting 20 μL aliquots with a Waters 717 auto-sampler into a Prontosil 120–3-amino column (4.6×125 mm; 3 μm particle size) maintained at room temperature. An isocratic flux (1 mL/min) of 85% acetonitrile (J.T.Baker) was applied for 25 min in each run. Standards of glucose, fructose, and sucrose were employed to identify peaks by co-injection. Sugars were quantified with peak integration using the Waters Empower software and comparison with glucose, fructose, and sucrose standard calibration curves.

Malondialdehyde and total antioxidant activity

Malondialdehyde (MDA, a reliable oxidative stress marker) concentrations were determined in the same 80% methanol leaf extracts used to quantify TSS, according to the method of Hodges et al. (1999). The extracts were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% TCA (or with 20% TCA without TBA for the controls), and then incubated at 95 °C for 15 min. After the reaction was stopped by placing the tubes on ice for a few minutes, absorbance was measured at 600 and 532 nm, and the concentration of MDA was determined using the equation described by Hodges et al. (1999).

The antioxidant activity was evaluated according to Falchi et al. (2006), by measuring the ability of the samples to quench the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), a synthetic and stable free radical product, whose quenching by a scavenger substrate could be followed spectrophotometrically at 517 nm. Leaf dry material (0.05 g) was extracted using 2 mL of 90% methanol by sonication during 10 min. The sample was centrifuged at 14000 rpm for 15 min, and the supernatant was collected. Then, 50 μL of this methanol-soluble phenolic fraction was diluted with 2 mL of 96% ethanol, 0.5 mL of the resulting solution was added to 1.5 mL 96% ethanol and 0.5 mL of an ethanolic solution containing 0.5 mM DPPH. To check the radical stability, a blank sample was prepared without the plant extract. Mixtures were then incubated at 25 °C for 10 min, and the absorbance was measured at 517 nm.

The radical scavenging activity (S) of each extract was expressed in percentage and calculated as $S = 100 - [(A_x/A_0) \times 100]$; A_x is the optical density of the DPPH solution in the presence of the extract, and A_0 in its absence.

Non-enzymatic antioxidants

Total phenolic compounds (TPC) and total flavonoid (TF) concentrations were determined in 80% methanol extracts, as for TSS. TPC were measured by its reaction with the Folin-Ciocalteu reagent and sodium bicarbonate, according to Blainski et al. (2013). Absorbance measurements were taken at 765 nm, and TPC concentrations were expressed as equivalents of gallic acid (mg eq. GA g^{-1} DW). TF was determined according to Zhishen et al. (1999); the extracts were mixed with

sodium nitrite, and then aluminium chloride was added under alkaline conditions before absorbance was measured at 510 nm. TF concentrations were expressed as equivalents of catechin (mg eq. C. g⁻¹ DW).

Statistical analysis

Statistical analyses were performed using the programme Statgraphics Centurion XVI. Before the analysis of variance, the Shapiro-Wilk test was used to check for validity of normality assumption and the Levene test for the homogeneity of variance. The significance of the effects of stress was evaluated by one way ANOVA. Tukey's HSD test was applied to identify the homogeneous groups when significant differences were found between the studied species. Correlations between soil and biochemical plant parameters were performed by the Pearson product-moment coefficient. Parameters that showed significant correlations were used for a principal component analysis (PCA). A cluster analysis was applied to discriminate the four species based on their growth and biochemical responses to water stress, using Squared Euclidean distances for the proximity procedure. All means throughout the text include the standard error (SE). A dendrogram based on the nearest neighbour method, using squared Euclidean distances between biochemical parameters and ion concentrations, was also performed with Statgraphics Centurion XVI.

Results

Climatic analysis

Individuals of three of the four *Limonium* species under study were collected from salt marshes in El Saler, near the city of Valencia, and those of the fourth, *L. santapolense*, from a more southern location (Clot de Galvany) near Elche, in the province of Alicante. Both areas have a similar climate with the highest temperatures in summer, coinciding with a drastic reduction of rainfall, which is characteristic of the Mediterranean climate. However, the amount of precipitation differs in the two areas; the average annual rainfall is much higher in El Saler than in the Clot area. The mean rainfall calculated for the last 18 years is 240.02 mm in Clot and 441.66 mm in El Saler, although ETo is similar in the two zones. The two areas are located near the beach,

and therefore have similar Continentality indices, and both are classified within the Thermomediterranean thermotype, characterised by warm temperatures (a yearly mean of 16–18 °C) and mild winters, and have very similar thermicity indices (TI) (Table 1). Based on their ombrothermic index calculated according to Rivas-Martínez and Rivas-Saenz (1996–2018), Clot is classified as arid, and El Saler as semi-arid.

Meteorological data for the four weeks previous to sampling (from 15th of June to 15th of July, 2018) in the two areas, summarised in Table 2, indicate that they differed mainly in the amount of rainfall in the last two weeks of June, which were extremely dry in Clot, but more than 100 mm were registered in El Saler. As the evapotranspiration was similar in both locations, the water deficit was obviously much more intense at Clot, the sampling area of *L. santapolense*. During the first two weeks of July, the meteorological conditions were similar in the two areas, with a pronounced water deficit, as is characteristic for the Mediterranean climate in summer.

Soil analysis

The textural classes of the soils were determined according to their corresponding percentages of sand, silt and clay, based on the USDA classification (Soil Survey Division Staff 1993). As shown in Table 3, all

Table 1 Values of the climate variables in the collection sites of *Limonium santapolense* (Clot) and *L. giradianum*, *L. narbonense* and *L. virgatum* (El Saler)

Bioclimatic Indexes	Clot	El Saler
TI	406.1	389.3
CI	15.2	15.1
OI	0.9	1.6
Ppv	18.8	38.2
Ttv	74.1	73.9
ETo	97.1	98.9
GI	0.6	1.4

Data were obtained from the nearest meteorological stations (Elche for 'Clot de Galvany' and Benifaió for El Saler) and calculated for the period 1999–2018)

TI, Thermicity index; CI, Continentality index; OI, Ombrothermic index; Ppv, Summer precipitation in mm of the three consecutive warmest months in the year; Tt, Value in tenths of degree resulting from the sum of the monthly average temperatures of the three consecutive warmest months in the year; ETo, Evapotranspiration; IG, Giacobbe index

Table 2 Meteorological data in the period previous to the sampling (from 15th of June to 15th of July, 2018) in the collection sites of *Limonium santapolense* (Clot) and *L. girardianum*, *L. narbonense* and *L. virgatum* (El Saler)

Meteorological Data	Clot		El Saler	
	June	July	June	July
Mean T (°C)	23.6	26.8	22.7	26.1
T max (°C)	32.4	37.4	32.6	37.1
T min (°C)	12.4	19.6	12.7	27.8
Mean H (%)	61.9	64.8	65.4	69.7
H max (%)	96.4	95.2	95.2	97.8
H min (%)	23.5	17.2	25.0	17.6
Pp (mm)	9.6	0.0	136.9	5.8
Eto (mm)	163.1	187.8	163.0	184.6

T, temperature; H, atmospheric humidity; Pp, Precipitation; Eto, Evapotranspiration. Data were obtained from the nearest meteorological stations (Elche for ‘Clot de Galvany’ and Benifaió for El Saler)

soils in El Saler area contain a high percentage of sand, between 88% and 94%, belonging therefore to the ‘sandy’ textural class. The texture is sandy loam at the collection site of *L. santapolense* (Clot de Galvany), as the percentage of sand in the soil is lower (55%), and the percentage of silt is higher (35%), whereas differences in % of clay are small in respect to the other areas.

All analysed soils had a basic pH, with values ranging from 8.25 to 9.05, being slightly higher in the growth area of *L. girardianum* and lower for that of *L. santapolense*. Organic matter content commonly ranges between ≤ 0.5 and 2.0%, for surface soils from arid regions (Bresler et al. 1982). Very low and similar values have been determined in the soils of Clot and El Saler, except in the growing area of *L. narbonense*, where the soil is not so poor in organic matter, although it is still below 2% (Table 3).

Statistically significant differences have been found in the carbonate content of the soils from Clot, where *L. santapolense* grows, and those of El Saler, the area where the remaining species were sampled. According to the scale of Yáñez (1989), the soils of El Saler area should be classified as soils with a high carbonate content, whereas that of Clot as soil with a very high carbonate content.

The results obtained for the cation exchange capacity (CEC) indicate similar values for *L. santapolense*,

L. girardianum and *L. virgatum*, but a higher mean value for soil samples from the area of *L. narbonense*.

Sodium and chloride concentrations in the saturation extract of all tested soils were much higher than those of the other ions measured, K^+ , Ca^{2+} and Mg^{2+} . Significantly higher levels of sodium were found in the sampling areas of *L. virgatum* and *L. narbonense*, as compared to that of *L. santapolense*, with intermediate values measured for the *L. girardianum* location, whereas chlorides contents were similar in all soil samples. Magnesium was the next chemical in concentration, with higher values in the areas of the species sampled from El Saler, significantly higher than in Clot, especially in the collection zone of *L. narbonense*. Higher values in El Saler than in Clot were also registered for potassium. Finally, calcium values were significantly lower in the area of *L. girardianum* than in the sampling areas of the other three species.

Morphology of plants growing in the wild

Limonium virgatum and *L. girardianum* were small (less than 10 g per plant), with small leaves and thin shallow underground structures. Plants of these two species were similar, except for the higher number of shoots and leaves and longer roots of *L. virgatum*, and the higher specific length of *L. girardianum*. According to the topological measurements, both had a herringbone development in which the primary root predominates among others, and penetrates deeply in the soil without extensive branching (Magalhães and Seifert 2015) (Table 4). *L. santapolense* and *L. narbonense* both had an average biomass above 75 g but showed very different morphology (Table 4, Fig. 1). *L. santapolense* had an average of five shoots per plant and showed a larger leaf area in comparison with the other three selected species. It also showed a long underground system (averaging 187 cm) that penetrated deep in the soil, reaching the proximity of the soil water table, as observed in the field.

However, it must be noticed that differences were found between *L. santapolense* plants growing in areas with a shallow water table and those growing in other areas, within the same saltmarsh but with deeper groundwater. In the first case, the plants had a short taproot with nodes evenly distributed over it (Fig. 1a, right), whereas the plants present in the second type of location develop a very long taproot (more than 1 m), which only ramified when the root reaches deep moist

Table 3 Soil variables at the collection sites of the four analysed *Limonium* species. Values shown are means $\pm$ SD ($n = 3$); different lower case letters in each row indicate statistically significant differences between the different locations, according to Tukey test ($\alpha = 0.05$)

Soil variable	<i>L. santapolense</i> Clot de Galvany	<i>L. virgatum</i> El Saler	<i>L. girardianum</i> El Saler	<i>L. narbonense</i> El Saler
Texture	Sandy loam (55 S, 35 SI, 10 C)	Sandy (94 S, 2 SI, 4C)	Loamy-sandy (88 S, 6 SI, 8 C)	Sandy (92 S, 2 SI, 6 C)
pH	8.2 $\pm$ 0.0 a	8.7 $\pm$ 0.1 b	9.1 $\pm$ 0.0 c	8.5 $\pm$ 0.1 d
OM (%)	0.5 $\pm$ 0.0 a	0.4 $\pm$ 0.0 a	0.3 $\pm$ 0.0 a	1.7 $\pm$ 0.2 b
CaCO ₃ (%)	42.3 $\pm$ 3.6 b	26.3 $\pm$ 0.3 a	24.3 $\pm$ 0.8 a	22.0 $\pm$ 0.9 a
EC _{sat} (dS/m)	31.4 $\pm$ 2.7 a	53.1 $\pm$ 1.0 b	33.9 $\pm$ 4.4 a	50.3 $\pm$ 9.3 b
CEC (cmol+/kg)	2.9 $\pm$ 0.9 b	1.4 $\pm$ 0.06 ab	0.9 $\pm$ 0.2 a	8.8 $\pm$ 0.7c
Na ⁺ _{sat} (meq/L)t	183.7 $\pm$ 17.0 a	321.2 $\pm$ 1.0 b	239.1 $\pm$ 33.3 ab	291.5 $\pm$ 54.1 b
Cl ⁻ _{sat} (meq/L)	352.6 $\pm$ 39.6 a	339.9 $\pm$ 22.8 a	327.2 $\pm$ 45.3 a	361.7 $\pm$ 116.8 a
K ⁺ _{sat} (meq/L)	3.6 $\pm$ 0.3 a	10.0 $\pm$ 1.2 b	10.2 $\pm$ 1.8 b	10.4 $\pm$ 2.4 b
Ca ²⁺ _{sat} (meq/L)	17.1 $\pm$ 2.4 b	14.9 $\pm$ 4.3 b	6.0 $\pm$ 0.8 a	15.7 $\pm$ 1.7 b
Mg ²⁺ _{sat} (meq/L)	16.6 $\pm$ 1.5 a	23.6 $\pm$ 0.1 b	30.7 $\pm$ 0.3 c	37.9 $\pm$ 4.3 d

S, % sand; SI, % silt; C, % clay; OM, organic matter; EC_{sat}, electric conductivity in saturation extract

areas in the soil (Fig. 1a, left). Regarding the topological indexes, it was a moderate herringbone root even though, as we have pointed out above, it can grow very deep as a single root (Fig. 1a). In terms of underground systems, *L. narbonense* was very different from the rest due to its rhizomatous root system. It has a rhizomatous

structure of 2–3 cm in diameter, which penetrates the soil up to 40 cm and then starts to branch. This structure was covered by fine roots of 1–2 mm in diameter and up to 10 cm in length. The topological index of *L. narbonense* corroborates its herringbone structure (Fig. 1b).

Table 4 Means and standard deviations of shoot and root traits evaluated from wild plants of the four *Limonium* species ($n = 4$)

Traits	<i>L. santapolense</i>	<i>L. virgatum</i>	<i>L. girardianum</i>	<i>L. narbonense</i>
NS	5.0 $\pm$ 0.7 b	11.5 $\pm$ 1.8 c	1.0 $\pm$ 0.0 a	2.8 $\pm$ 0.7 ab
NL	43.8 $\pm$ 7.5 bc	63.3 $\pm$ 8.3 c	23.8 $\pm$ 3.7 ab	15.2 $\pm$ 8.6 a
SFW (g)	70.0 $\pm$ 21.4 a	6.6 $\pm$ 0.8 a	2.0 $\pm$ 0.30 a	47.3 $\pm$ 22.4 a
RFW (g)	5.2 $\pm$ 0.7 a	1.0 $\pm$ 0.2 a	0.2 $\pm$ 0.1 a	50.2 $\pm$ 18.1 b
LA (cm ²)	552.3 $\pm$ 150.3 b	33.6 $\pm$ 5.6 a	23.9 $\pm$ 4.7 a	98.2 $\pm$ 47.4 a
RL (cm)	187.4 $\pm$ 49.2 a	120.4 $\pm$ 43.2 a	57.4 $\pm$ 14.6 a	83.7 $\pm$ 28.4 a
LRL (cm)	152.1 $\pm$ 55.7 a	113.7 $\pm$ 42.7 a	54.7 $\pm$ 13.7 a	45.0 $\pm$ 21.9 a
PRL (cm)	35.3 $\pm$ 10.6 b	6.5 $\pm$ 1.1 a	2.6 $\pm$ 1.2 a	38.7 $\pm$ 10.5 b
RSA (cm ²)	85.0 $\pm$ 14.3 ab	35.1 $\pm$ 10.9 a	15.6 $\pm$ 4.1 a	162.5 $\pm$ 49.1 b
D (mm)	1.6 $\pm$ 0.3 a	1.0 $\pm$ 0.0 a	0.9 $\pm$ 0.1 a	6.9 $\pm$ 1.2 b
R/S	0.1 $\pm$ 0.0 a	0.1 $\pm$ 0.0 a	0.1 $\pm$ 0.0 a	2.2 $\pm$ 1.4 a
SRL (cm/g)	37.9 $\pm$ 9.6 a	113.5 $\pm$ 19.5 ab	273.9 $\pm$ 68.3 b	2.0 $\pm$ 0.5 a
M	27.7 $\pm$ 11.6 ab	56.7 $\pm$ 8.7 c	40.7 $\pm$ 10.4 bc	5.5 $\pm$ 1.2 a
A	9.7 $\pm$ 2.9 ab	22.2 $\pm$ 4.8 c	20.7 $\pm$ 5.7 bc	5.2 $\pm$ 0.6 a
Nd	21.0 $\pm$ 8.1 ab	53.0 $\pm$ 9.8 c	34.7 $\pm$ 9.5 bc	4.5 $\pm$ 0.9 a
TI	0.7 $\pm$ 0.1 a	0.8 $\pm$ 0.0 a	0.8 $\pm$ 0.0 a	1.0 $\pm$ 0.1 b

NS, number of apical shoots; NL, number of leaves; SFW, shoot fresh weight (g); RFW, root fresh weight (g); LA, total leaf area (cm²); RL, total root length (cm); LRL, lateral root length (cm); PRL, taproot or principal root length (cm); RSA, root surface area (cm²); D, average diameter of the roots (mm); R/S, root to shoot ratio; SRL, specific root length (cm/g); M, root magnitude; a, root altitude; Nd, numbers of root nodes; TI, topological index. Numbers of the same row followed by different letter differ significantly in the ANOVA test at $P < 0.05$

Fig. 1 Examples of the four *Limonium* species sampled in the wild: *L. santapolense* (a), *L. narbonense* (b), *L. girardianum* (c) and *L. virgatum* (d)

Ion accumulation

Levels of ions and their interspecific variation were generally higher in leaves than in roots, and these differences were, in most cases, statistically significant (Fig. 2). Mean Na^+ concentrations were higher in *L. girardianum* and *L. narbonense* than in *L. santapolense* and *L. virgatum*, ranging in leaves from $631 \mu\text{mol g}^{-1}$ DW in *L. santapolense* to $1993 \mu\text{mol g}^{-1}$ DW in *L. narbonense*, but the differences were significant only in roots, due to the wide individual variation within each species in the leaves (Fig. 2a). Cl^- concentrations in roots were significantly lower than in leaves in all four species and did not vary between species, whereas in leaves significantly higher Cl^- levels of $467 \mu\text{mol g}^{-1}$ DW were found in *L. narbonense*, as compared to around $200 \mu\text{mol g}^{-1}$ DW in leaves of the other three taxa (Fig. 2b). Also, root K^+ concentrations did not differ in the four species, whereas K^+ concentrations in leaves were significantly higher in *L. santapolense* and *L. virgatum* than in *L. girardianum*, whereas *L. narbonense* showed intermediate values (Fig. 2c). The most considerable differences between root and leaf Ca^{2+} concentrations were observed in *L. santapolense*, which showed very low levels of this cation in the roots but stood out by its high levels of foliar Ca^{2+} concentrations, up to $319 \mu\text{mol g}^{-1}$

DW, much higher than those measured in the other three species (Fig. 2d).

Osmolytes

The most common osmolytes in halophytes, proline (Pro), glycine betaine (GB) and total soluble sugars (TSS) were quantified in plant leaves of the four *Limonium* species (Table 5). Levels of Pro in *L. santapolense* and *L. virgatum* were lower – below $20 \mu\text{mol g}^{-1}$ DW – than those measured in *L. girardianum* and *L. narbonense* – ca. 50 and $75 \mu\text{mol g}^{-1}$ DW, respectively. Regarding leaf GB concentrations, *L. santapolense* showed the lowest value, about $14 \mu\text{mol g}^{-1}$ DW, whereas similar concentrations, ca. $40 \mu\text{mol g}^{-1}$ DW, were measured in the other three species. TSS ranged from the lowest value (around $40 \text{ mg eq. glucose g}^{-1}$ DW) in *L. virgatum* to the highest ($\sim 70 \text{ mg eq. glucose g}^{-1}$ DW) in *L. santapolense* (Table 5).

In addition to the spectrophotometric determination of TSS, individual sugars in the leaf water-soluble fraction were separated, identified and quantified by HPLC. Three peaks were detected in the chromatograms, corresponding to fructose (Fru), sucrose (Suc), and glucose (Glu). Fru concentrations were very low in *L. santapolense* ($25.5 \mu\text{mol g}^{-1}$ DW) in comparison with the other taxa ($656 \mu\text{mol}$

Fig. 2 Ions levels in roots and leaves of the four *Limonium* species sampled in the field. Na^+ (a); Cl^- (b); K^+ (c); Ca^{2+} (d). Bars represent means $\pm$ SD ($n=4$). Asterisks indicate significant

differences between roots and leaves for each species, and letters significant differences between species (lower-case letters for roots and capital letters for leaves) at $P<0.05$

g^{-1} DW in *L. giradianum*, for example), whereas the reverse pattern was observed in the case of Suc, which showed very low levels in all species (below 1 in *L. girardainum* and *L. narbonense*) except in

L. santapolense ($75.5 \mu\text{mol g}^{-1}$ DW). Finally, Glu levels were also low in all species, somewhat higher in *L. santapolense* ($13.5 \mu\text{mol g}^{-1}$ DW) and not detectable in *L. girardianum* (Table 5).

Table 5 Biochemical parameters quantified in the leaves of plants sampled in the wild of four *Limonium* species. Mean followed by SE, $n=4$

Biochemical Parameters	<i>L. santapolense</i>	<i>L. virgatum</i>	<i>L. giradianum</i>	<i>L. narbonense</i>
Pro ($\mu\text{mol g}^{-1}$ DW)	19.3 $\pm$ 1.8 a	14.7 $\pm$ 0.4 a	47.7 $\pm$ 2.2 b	75.6 $\pm$ 25.5 c
GB ($\mu\text{mol g}^{-1}$ DW)	14.2 $\pm$ 1.3 a	42.6 $\pm$ 6.8 b	40.3 $\pm$ 3.2 b	42.0 $\pm$ 3.2 b
TSS (mg eq. G g^{-1} DW)	70.2 $\pm$ 5.2 c	38.7 $\pm$ 2.7 a	67.0 $\pm$ 0.7 c	54.7 $\pm$ 4.8 b
Fru ($\mu\text{mol g}^{-1}$ DW)	27.6 $\pm$ 2.1 a	632.0 $\pm$ 22.1 c	656.5 $\pm$ 244.2 d	291.0 $\pm$ 91.6 b
Suc ($\mu\text{mol g}^{-1}$ DW)	73.5 $\pm$ 14.6 b	9.0 $\pm$ 1.4 a	0.7 $\pm$ 0.1 a	0.3 $\pm$ 0.0 a
Glu ($\mu\text{mol g}^{-1}$ DW)	13.6 $\pm$ 0.2 c	8.2 $\pm$ 2.2 b	0.0 a	6.5 $\pm$ 0.4 b
MDA (nmol g^{-1} DW)	171.4 $\pm$ 5.3 b	97.4 $\pm$ 8.7 a	210.3 $\pm$ 25.4 b	191.6 $\pm$ 35.5 b
DPPH (%)	84.2 $\pm$ 0.3 b	67.6 $\pm$ 7.3 a	82.8 $\pm$ 1.1 b	74.5 $\pm$ 4.0 ab
TPC (mg eq. GA g^{-1} DW)	37.1 $\pm$ 3.7 a	23.9 $\pm$ 6.2 a	39.2 $\pm$ 4.8 a	24.9 $\pm$ 5.2 a
TF (mg eq. C g^{-1} DW)	5.9 $\pm$ 0.3 b	6.9 $\pm$ 0.5 b	4.0 $\pm$ 0.4 a	2.9 $\pm$ 0.6 a

Pro, proline; GB, glycine betaine; TSS, total soluble sugars; Fru, fructose; Suc, sucrose; Glu, glucose; MDA, malondialdehyde; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPC, total phenolic compounds; TF, total flavonoids

Oxidative stress and antioxidant compounds

Malondialdehyde (MDA) is a product of peroxidation of unsaturated fatty acids, used as a reliable marker of free radical damage to cell membranes in plants and animals (Del Rio et al. 2005; Suzuki and Mittler 2006). The DPPH-free radical scavenging assay is a useful method for quantifying the ability of compounds in an extract to act as free radical scavengers or hydrogen donors, indicating the overall antioxidant capacity of the sample (Sagar et al. 2011). Both biochemical markers were determined in plants of the four *Limonium* taxa. No significant differences were found between *L. santapolense*, *L. girardianum* and *L. narbonense*, and somewhat lower values were measured in *L. virgatum* (Table 5), indicating that the degree of oxidative stress affecting the plants in the field and the total antioxidant activity of the leaf extracts were roughly the same in all cases.

In response to oxidative stress, plants activate enzymatic and non-enzymatic antioxidant mechanisms. Synthesis of phenolic compounds, including flavonoids, many of them possessing strong antioxidant activities, is one of the most frequent and most efficient strategies used by plants to reduce oxidative stress. Total phenolic compounds (TPC) were measured in leaves of plants of the four *Limonium* species, and ranged from 23.9 mg eq. GA g⁻¹ DW in *L. virgatum* to 39.2 in *L. girardianum* with no significant interspecific differences observed. Flavonoid concentrations were significantly lower in *L. girardianum* (4.0 mg eq. C g⁻¹ DW) and *L. narbonense* (2.9) than in *L. santapolense* (5.9) and *L. virgatum* (6.9) but these differences are probably irrelevant in terms of antioxidant capacity since absolute TF values were very low in all cases (Table 5).

PCA and cluster analysis

A Pearson Moment Correlation was performed with all analysed parameters and those plant and soil variables that showed a significant correlation were further subjected to a Principal Component Analysis (PCA) (Table 6 and Fig. 3). Climatic data could not be included since three of the species analysed, *L. virgatum*, *L. girardianum* and *L. narbonense* were sampled from the same area, so plants grew in the same climatic conditions. The PCA extracted four components with an eigenvalue higher than one out of the total 25 parameters considered. Together, the four components account

for 88.6% of the total variability. The first component, explaining 45.2% of the variability, was mostly related to soil parameters (positively with EC, K⁺, Mg²⁺ and Na⁺ levels in the soil, and negatively with the percentage of CaCO₃), and also correlated with osmolytes, positively with glycine betaine (GB) and negatively with sucrose (Suc). The second component, which explained an additional 23.8% of the total variability, was positively related to the levels of Ca in soil and with glucose (Glu), and negatively related to the concentrations of the oxidative stress marker (MDA), total antioxidant activity (DPPH), total phenolics (TPC) and total soluble sugars (TSS) in the plants (Table 6).

The loading plot indicates that the concentrations of Na⁺ and Cl⁻ in the soil correlated positively with those in the leaves of plants, and with the osmolytes Pro, GB and Fru. Growth parameters [fresh weight of shoots (SFW), leaf area (LA) and length of the roots (LR)] are grouped together (Fig. 3). The projection of the four individuals of the four species in the PCA score plot shows a clear separation along the first component of *L. santapolense*, due to the particularity of its habitat with higher soil level of CaCO₃ (Fig. 3); this taxon is also separated from the other species based on its high levels of foliar sucrose (Suc) and low values of fructose (Fru). *L. narbonense* and *L. virgatum* were situated on the opposite side, as they grow in soils with higher concentrations of Na⁺, K⁺ and Mg²⁺, and therefore higher EC. *Limonium girardianum* was separated along the second component due to its higher values of MDA.

A cluster analysis based on the nearest neighbour method, including only morphological traits, biochemical parameters and ion concentrations of the plants, also separated the four species and supported the results of the PCA (Fig. 4). The more distant species was again *L. santapolense*, and the most related, based on the analysed characteristics, were *L. narbonense* and *L. girardianum*.

Discussion

In previous studies performed in our laboratory, it was found that once the bottleneck of germination was overcome, the four studied *Limonium* species tolerated high salinity levels, up to 800 mM NaCl in the irrigation solution (Al Hassan et al. 2017), but they differed in their responses to water deficit. Plants of three taxa, *L. virgatum*, *L. girardianum* and *L. narbonense*, grown

Table 6 Weights of the main four principal components extracted by the PCA in the four *Limonium* species

Component	Component 1	Component 2	Component 3	Component 4
Eigenvalue	11.30	5.96	3.75	1.13
Variance (%)	45.20	23.86	15.00	4.53
pH	0.16	−0.29	−0.18	−0.09
EC	0.23	0.24	−0.00	0.11
CaCO ₃	−0.28	0.07	−0.06	0.14
OM	0.09	0.17	0.38	−0.17
Na ⁺ s	0.27	0.14	−0.06	0.084
K ⁺ s	0.28	−0.07	0.01	−0.11
Mg ²⁺ s	0.25	0.16	0.12	−0.02
Ca ²⁺ s	−0.08	0.37	0.10	0.18
Cl [−] s	0.19	0.27	−0.08	0.19
Na ⁺ l	0.08	0.05	0.45	0.08
Cl [−] l	0.13	0.13	0.29	−0.07
Ca ²⁺ l	−0.19	0.07	−0.14	0.33
Ca ²⁺ r	0.21	0.25	−0.01	0.18
MDA	−0.03	−0.24	0.36	0.11
GB	0.25	−0.08	0.01	0.16
TSS	−0.18	−0.21	0.22	−0.05
TPC	−0.11	−0.26	0.12	0.44
Pro	0.10	−0.10	0.41	0.05
DPPH	−0.14	−0.22	0.192	0.22
Fru	0.20	−0.23	−0.16	−0.07
Glu	−0.18	0.30	−0.00	0.17
Suc	−0.27	0.09	−0.02	0.20
LA	−0.24	0.12	0.062	−0.29
RL	−0.19	0.14	0.01	−0.29
SFW	−0.21	0.15	0.12	−0.35

EC, soil electric conductivity; CaCO₃, calcium carbonate in soil; OM, soil organic matter; Na⁺s, sodium in soil; K⁺s, potassium in soil; Mg²⁺s, magnesium in soil; Ca²⁺s, calcium in soil; Cl[−]s, chloride in soil; Na⁺l, sodium in leaves; Cl[−]l, chloride in leaves; Ca²⁺l, calcium in leaves; Ca²⁺r, calcium in roots; MDA, malondialdehyde; GB, glycine betaine; TSS, total soluble sugars; TPC, total phenolic compounds; Pro, proline; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Fru, fructose; Glu, glucose; Suc, sucrose; LA, total leaf area; RL, total root length; SFW, shoot fresh weight

in pots did not show a drastic reduction of growth after one month of lack of irrigation, whereas those of *L. santapolense* lost one-third of its fresh weight (González-Orenga et al. 2019).

Climate analysis of the sampling zones of *L. santapolense* at Clot de Galvany and the other three species at El Saler revealed differences between the two areas in the amount of rainfall, which lead to different climate types: arid for the first and semi-arid for the second. Although dry summers represent a characteristic trait of the Mediterranean climate, during the month previous to sampling the water deficit was much more

pronounced in the area of *L. santapolense* at Clot than in El Saler, where in the last two weeks of June the rainfall was over 130 mm. Soil analyses did not reveal big differences between the sampling sites, except the texture of the soil, which was sandy-loam for the *L. santapolense*'s collection area and sandy in the other three sites. This difference is related to the geology of the two zones. The Clot area belongs to the undifferentiated quaternary and combines a series of more recent formations such as colluvia, alluvium, brackish deposits and debris in general (IGME 1973) that increased the levels of carbonates, whereas the salt marshes in El Saler

climate more humid and intensifies the cryptoprecipitation. Therefore, despite the differences in the texture of the soil, Clot de Galvany represents a drier habitat than El Saler. In fact, soil humidity registered by a WET 2 sensor simultaneously with plant uprooting did not show apparent differences between the areas of the four species and varied in surface (10 cm depth) from 5 to 15%. The cation exchange capacity

Dendrogram
Nearest Neighbor Method, Squared Euclidean

(CEC) is an essential trait of soils, influencing their stability and nutrient availability (Hazelton and Murphy 2007). CEC depends on the organic matter and clay proportion in soils. The more elevated CEC in the area of *L. narbonense* is explained by its higher content in organic matter. However, although soil from Clot had a higher proportion of clay, due to its very low OM %, CEC is only slightly higher in this area than in those of *L. girardianum* and *L. virgatum*.

Since studies conducted in the greenhouse (González-Orenga et al. 2019) indicated that *L. santapolense* is the species most sensitive to water deficit, it was clear that plants growing in their natural environments possess some specific mechanisms of defence against drought, which are not effective under artificial conditions in potted plants. The root system of plants has an essential function in their adaptation to drought, because roots serve as the interphase between plants and soil, and play a key role in plant nutrition and development. As they are directly sensing the physico-chemical parameters of the soil, roots are essential in the adaptation of plants to different environments (Franco et al. 2011; Fry et al. 2018). According to databases such as PLANTATT, there are several main categories of roots, such as tap-rooted, rhizomatous, stoloniferous or fibrous (Hill et al. 2004). Three of the *Limonium* species analysed in the present work (*L. santapolense*, *L. girardianum* and *L. virgatum*) have a simple morphology consisting of a central primary underground system with few lateral roots and a low surface area to volume ratio. This type of roots are poor foragers for resources in shallow soils and are not optimal for microbial symbiosis (Fry et al. 2018, and references therein), but can reach deep soil layers (Alvarez-Flores et al. 2018). Some tap-rooted species in arid and semi-arid areas have the ability to produce a hydraulic lift (an upwards transport of water from the deeper, moister layers of soil to the shallow, drier zone), and act as ‘nurse plants’, beneficial for other plant species by redistributing water from deeper soil layers (Prieto et al. 2011). The fourth species under study, *L. narbonense*, presented a rhizomatous root as described in other *Limonium* species (Eber and Veenhuis 1991; Antonelli-Ushirobira et al. 2015). Such structures are adapted for storing high amounts of carbohydrates and nitrogenous reserves (Suzuki and Stuefer 1999; Schmidt and Gaudin 2017).

Although the main morphological types are stable within species, underground system development

shows high plasticity in relation to different types of abiotic stresses. Substantial variations in their length, branching and other morphologic and structural aspects may appear even within the same species when environmental conditions are different (Franco et al. 2011, and references therein). In the case of *L. santapolense*, collected from the arid area at Clot de Galvany, a substantial variation in root length was noticed. Plants growing in more humid soils near the waterlogged depressed part of the salt marsh had short taproot with nodes evenly distributed over it, whereas the plants growing distantly from the water develop a very long taproot and the main root is branched only in deeper, more humid layers of the soil. In the case of the species sampled from El Saler, roots did not show such a strong morphological variation. The development of long roots able to explore deeper moist layers of the soil may explain the fact that *L. santapolense* grows in arid areas, although under controlled conditions was the species most affected by water stress. When growing in a standard pot of 9 cm diameter, plants of this species were affected after one month of imposed water stress while in the wild they tolerate much longer periods of drought, sometimes extending to more than three months without precipitations.

In addition to the root type, many ecophysiological traits may have an adaptive value, enabling plants to inhabit stressful natural environments. *Limonium* species are well-known recretohalophytes, plants that have the ability to secrete salts through salt glands (Leng et al. 2018), but they also accumulate salts in their leaves like many other dicotyledonous halophytes (Wyn-Jones and Gorham 2002; Flowers and Colmer 2008). By sequestration of toxic ions in their vacuoles, these plants achieve a cheap osmoticum (Flowers and Yeo 1986) and also require little K^+ for cytosolic metabolism (Zia et al. 2008; Hameed et al. 2015). When examining Na^+ in roots and leaves, its level was higher in leaves, as it was already reported in these species (Al Hassan et al. 2017); differences between species were not significant. Cl^- followed a similar pattern, accumulating considerably larger concentrations in leaves than in roots. For both ions, the most significant difference between roots and leaves was detected in *L. narbonense*, sampled in a strongly saline area. The active transport and sequestration in leaf vacuoles of toxic ions was reported in other *Limonium*

species (Hameed et al. 2015) and may also represent a mechanism of avoidance of toxic ions at the underground level, where osmoregulation is achieved by the accumulation of free osmotic solutes (Alarcon et al. 1999). In addition to Na^+ and Cl^- transport to the aerial part of the plants, Ca^{2+} accumulation in the leaves can also contribute to salt tolerance mechanisms in the analysed *Limonium* taxa, especially in *L. santapolense*, the species showing highest leaf concentrations of this cation, as the essential role of Ca^{2+} in alleviating the deleterious effects of salinity is well established (Hasegawa et al. 2000; Hadi and Karimi 2012). *Limonium santapolense* also showed a striking difference from the other three selected species, regarding the major osmolytes synthesised for osmotic balance under the stressful conditions of their natural habitats. Plants of this species showed much lower leaf concentrations of Fru and also, in general, significantly lower concentrations of Pro and GB than those of *L. virgatum*, *L. giradianum* and *L. narbonense*. Osmotic adjustment in *L. santapolense* seems to be achieved by the accumulation of Suc – and, to a lesser extent, Glu – which is present at much higher levels than in the other three taxa. In contrast, under experimental conditions in the greenhouse, when plants of this species were strongly affected by drought, levels of Pro, Fru and Suc were much higher than those measured in the other species (González-Orenga et al. 2019). These data, together with published reports on other *Limonium* species (e.g., Hanson et al. 1991; Liu and Grieve 2009), support the notion that osmolyte biosynthesis is extremely variable in this genus, in contrast to other genera that use a single compound as the primary functional osmolyte, for example, sorbitol in *Plantago* (Flowers and Colmer 2008).

In the present work, measurements in the four selected *Limonium* species of leaf concentrations of MDA – which is routinely used to assess the oxidative damage induced in plants by different stress treatments (e.g. Aghaleh et al. 2009; Demiral and Türkan 2005) – did not reveal, in general, significant interspecific differences. Similarly, the total antioxidant activity of leaf extracts – determined by the DPPH-free radical scavenging assay – or the total phenolic compounds concentrations – as relevant non-enzymatic antioxidants – varied little between the four taxa, non-significantly in most cases. This indicated that *L. santapolense* plants were affected in the field roughly by the same degree of

oxidative stress than plants of the other three taxa and that the antioxidant responses were also similar in all cases, despite the different aridity of the corresponding habitats.

The analysis of the responses to environmental stress of the four studied *Limonium* species using field-collected material complements previous work in which plants of the same species were subjected to salt stress (Al Hassan et al. 2017) or water stress (González-Orenga et al. 2019) treatments under controlled greenhouse conditions. Although the general physiological and biochemical responses to stress may be qualitatively similar, comparisons between the two types of experiments should be taken with caution. First, in the greenhouse plants are likely affected by much higher levels of salt or water stress, as their root systems are constrained in the pots to closed and limited environments of relatively homogeneous salinity and moisture. On the contrary, plants in the wild may develop longer roots, with different morphology better adapted to a more heterogeneous environment, where salinity and soil moisture largely vary in different locations and in time, as we show here for *L. santapolense*. Second, the developmental stage was different between greenhouse and field, young plants grown from seeds were used in the greenhouse experiments versus adult plants of unknown age (the four species are perennial) grown in the field. Moreover, the salt and water stress treatments applied in the greenhouse cannot mimic the conditions in nature – specifically, in this case, in Mediterranean salt marshes in summer – where plants are simultaneously affected by different types and varying degrees of environmental stress, including drought, soil salinity, elevated temperatures, and UV irradiation.

Conclusions

The analysis of the biochemical responses to environmental stress of four *Limonium* species in littoral salt marshes in SE Spain, suggested that their mechanisms of stress tolerance are mostly based on the active transport of different ions (Na^+ , Cl^- , K^+ and Ca^{2+}) to the leaves, where they contribute to osmotic adjustment, together with the synthesis and accumulation of specific compatible solutes. Our data clearly separated *L. santapolense* from the other three taxa,

L. virgatum, *L. giradianum* and *L. narbonense*, as *L. santapolense* contains higher leaf Ca^{2+} concentrations and uses different compounds as functional osmolytes, namely sucrose and, to a lesser extent, glucose, which are present at very low levels in the other species. Specific morphological features of the *L. santapolense* root system – development of a very long taproot to reach deeper, more humid layers of the soil – explains the adaptation of this species to a drier environment than the habitats of the other three selected congeners and the apparent contradiction that *L. santapolense* was found to be the taxon most sensitive to water deficit in the greenhouse. Root morphology is a trait that should be more often considered in the studies on responses of plants to drought and salinity, as root growth is an essential functional mechanism of adaptation of plants to their natural environments.

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