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Leaf size as a key determinant of contrasting growth patterns in closely related *Limonium* (Plumbaginaceae) species

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ABSTRACT

This study aims to analyze the importance of leaf size on plant growth capacity among an array of closely related *Limonium* species, and its impact on the underlying determinants of growth reduction under extreme water deficit conditions. To do so, thirteen Balearic *Limonium* species with contrasting leaf size were grown under long-term well-watered (WW) and severe water-deficit (WD) conditions in a common garden experiment. Fundamental growth traits were measured, including relative growth rate (*RGR*), net assimilation rate (*NAR*), leaf area ratio (*LAR*), leaf mass area (*LMA*) and leaf mass ratio (*LMR*). WD promoted small changes in leaf size, and species with larger leaves had higher *RGR* than species with smaller leaves, irrespective of the water treatment. Most *RGR* variation across species and treatments was explained by *NAR*, with comparatively much lower importance of *LAR*. The factorization of *LAR* underlying components denoted the importance of *LMA* in explaining *RGR*, whereas the impact of *LMR* on *RGR* was negligible in *Limonium*. Further, species with larger leaves had higher water consumption but also higher water use efficiency, especially under WD. Therefore, contrary to general trends in species from dry environments, increased leaf size is linked to increased growth capacity and also increased water use efficiency across closely related *Limonium* species.

1. Introduction

Water deficit is one of the most frequent stresses reducing plant growth capacity in arid and semi-arid environments. At the short-term, plants respond to water deficit by closing stomata to minimise water loss. However, stomatal closure increases the resistance to atmosperic CO_2 intake, which means lower photosynthetic CO_2 fixation and ultimately reduced growth (Chaves et al., 2002, 2009; Lambers et al., 2008). Sustained water deficit conditions have further critical effects on plant functioning and survivorship, because of increased risk of hydraulic failure and carbon starvation (McDowell, 2011; Rowland et al., 2015). Consequently, plants success in water-limited environments depends on a plethora of adaptive responses allowing the maintenance of a positive carbon balance under long-term water deficit. These responses require a tight coordination at the whole plant level, are very dependent on the plant habit and growth form, and include both physiological and morphological components that govern plant's relative growth rate (*RGR*), (Galmés et al., 2005; Lambers et al., 2008). On one side, the physiological component of *RGR* is the net assimilation rate (*NAR*) and represents the plant's net photosynthetic effectiveness in capturing light, assimilating CO_2 and storing photoassimilates. On the other side, the proportion of a plant biomass invested in leaf area, defined as the leaf area ratio (*LAR*), represents the morphological component of *RGR*. In turn, *LAR* is factorised in the leaf mass area (*LMA*), related to leaf thickness and density, and the leaf mass ratio (*LMR*), a relative measure of biomass allocation to the leaves (Lambers et al., 2008).

There is no general agreement on the relative importance of physiological and morphological components in explaining the whole-plant growth, with studies attributing highest importance to *NAR* (Van der Werf et al., 1998; Reich et al., 2003; Shipley, 2006; Lambers et al., 2008), and also to *LAR* (Poorter and Remkes, 1990; Poorter and van der Werf, 1998; Wright and Westoby, 2000; Villar et al., 2005; Tomlinson et al., 2014). In fact, the physiological and morphological components of *RGR* vary depending on the species and the environment and respond to different plant adaptive strategies (Lambers and Poorter, 1992;

Abbreviations: B_T, total plant biomass; g_s, stomatal conductance; LA_1, leaf area per leaf; *LAR*, leaf area ratio; *LMA*, leaf mass area; *LMR*, leaf mass ratio; *NAR*, net assimilation rate; *RGR*, relative growth rate; *RWC*, leaf relative water content; *WC*, water consumption; *WUE*, plant water use efficiency; WW, WD, well-watered and water-deficit treatments

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Galmés et al., 2005; Shipley, 2006; Medek et al., 2007). Regarding *LAR*, *LMA* is often the best *RGR* predictor, with a central role in the resource acquisitive-conservative aixs of the global leaf economics spectrum (e.g., Westoby et al., 2002; Wright et al., 2004; Reich, 2014; Díaz et al., 2016).

Contrary to *LMA*, trait relationships across the global spectrum attributed poor importance to leaf size in explaining *RGR* and general adaptive patterns in plants (e.g., Wright et al., 2004; Reich, 2014). A recent meta-analysis, including a wide array of plant clades, environments and growth forms, showed that *LMA* and leaf size are largely independent (Díaz et al., 2016). However, species from dry environments frequently have small leaves with high *LMA*, which is considered adaptive in such habitats and related to longer leaf lifespan (Wright et al., 2004). Actually, in some plant groups it has been described that small leaves with high *LMA* provide higher chances to survive in dry environments (e.g., Lambers and Poorter, 1992; Wright et al., 2004; Ramírez-Valiente et al., 2010). Variations in leaf size have also been related to plant growth, with a deep impact on the adaptive strategies to stressful conditions (e.g., Ackerly and Reich, 1999; McDonald et al., 2003; Niinemets et al., 2007; Kröber et al., 2014). Given the above, the importance of leaf size in determining growth capacity is not clear. The apparent incongruences may come to a large extent from the comparison of different environments, growth forms and plant groups. In fact, current evidence suggests that many of the global trait relationships reported can be weak when the plant species compared are within narrow phylogenetic or environmental ranges or at intra-specific level (see Messier et al., 2017 for a review). Therefore, the influence of leaf size on growth capacity may be idealy tested in a group of closely related species in which i) leaf size and growth patterns are particularly variable, thus minimizing the effects of considering too different plant groups and growth forms, and ii) inhabiting a very similar environment. All such conditions are met in the Balearic *Limonium*.

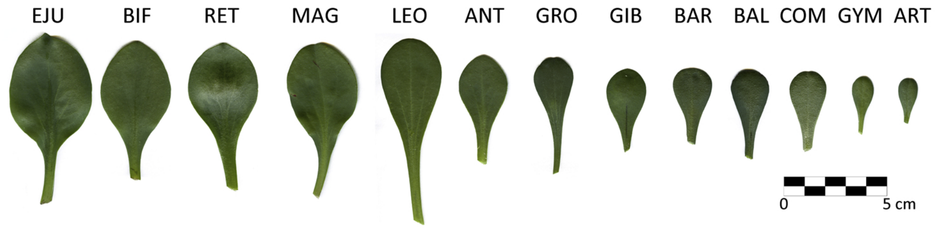
The genus *Limonium* Mill. (Plumbaginaceae) underwent an important diversification in the Balearic Islands, with up to 47 species (Erben, 1993; Rossellö and Sáez, 2001). In the archipelago, the species inhabit highly stressful habitats in the coast margin, and are notoriously drought adapted (Galmés et al., 2005, 2017). All the perennial species have a similar plant habit, being small, cushion-like evergreen shrubs with high leaf density and annual scapi. Despite this similarity, one of the most intriguingly variable traits across *Limonium* species is the leaf size (Erben, 1993; Sáez, 2005). Since the narrow differences in habitat among species cannot explain that large variation in leaf size (e.g., many species coexist; Sáez, 2005), we expect that leaf size may be involved in the adaptive response to their harsh environment, resulting in contrasting growth capacity. Specifically, we hypothesize that leaf size variation may govern different growth underlying parameters in *Limonium*, such as *LAR* and *NAR*, and hence explain different growth strategies to face severe drought.

Thus, in this study we selected an array of *Limonium* species with contrasting leaf size and grew them under well-watered (WW) and severe water deficit (WD) conditions in order to determine i) the impact of WD on growth across *Limonium* species with different leaf size, ii) the importance of leaf size in determining growth capacity across closely related *Limonium* species, and iii) the relative importance of the *RGR* underlying components in explaining differences in the growth capacity across species under WW and WD conditions.

2. Materials and methods

2.1. Species, water treatments, water consumption, and climatic conditions

From an extensive *Limonium* sampling across the Balearic Islands (Galmés et al., 2014), 13 species with contrasting leaf size (leaf area per leaf, LA_1) were selected for this study (see Fig. 1 for the species included in the study and the code used). After seed germination in nurseries, 15 plantlets per species were transplanted individually to 3 L pots and grown outdoors in spring (31st May to 28th June) at the University of the Balearic Islands, maintaining pots irrigated at field capacity. During the following months (29th June to 13th September), five plants per species were still maintained at field capacity (well-watered treatment,



Species	Code	WW (mm^2)		WD (mm^2)		% WD vs. WW		
L. ejulabilis Rosselló, Mus & Soler	EJU	1320.0 ± 115.2	h	1308.6 ± 73.4	f	99.1 ± 5.6	bcd	
L. biflorum (Pignatti) Pignatti	BIF	1097.4 ± 48.7	gh	994.4 ± 60.5	de	90.6 ± 5.5	abcd	
L. retusum L. Llorens	RET	990.8 ± 148.8	fg	1242.2 ± 89.7	ef	125.4 ± 9.1	de	
L. magallufianum L. Llorens	MAG	821.2 ± 42.0	efg	954.3 ± 21.9	d	116.2 ± 2.7	cde	*
L. leonardi-llorensii L. Sáez, Carvalho & Rosselló	LEO	787.5 ± 78.2	defg	1198.5 ± 88.8	def	152.2 ± 11.3	e	*
L. antonii-llorensii L. Llorens	ANT	701.0 ± 71.9	cdef	596.8 ± 106.6	c	85.1 ± 15.2	abc	
L. grosii L. Llorens	GRO	527.3 ± 26.1	bcde	466.8 ± 50.2	bc	88.5 ± 9.5	abcd	
L. gibertii (Sennen) Sennen	GIB	488.0 ± 47.6	abcd	381.8 ± 47.3	abc	78.2 ± 9.7	abc	
L. barceloi Gil and L. Llorens	BAR	486.3 ± 29.5	abcd	279.3 ± 31.7	ab	57.4 ± 6.5	a	*
L. balearicum (Pignatti) Brullo	BAL	390.2 ± 54.0	abc	263.3 ± 15.7	ab	67.5 ± 4.0	ab	
L. companyonis (Gren. & Billot) Kuntze	COM	377.0 ± 24.0	ab	327.8 ± 20.5	abc	86.9 ± 5.4	abc	
L. gymnesicum Erben	GYM	229.8 ± 22.5	ab	143.6 ± 13.8	a	62.5 ± 6.0	ab	*
L. artruchium Erben	ART	184.8 ± 20.7	a	142.3 ± 8.0	a	77.0 ± 4.3	ab	

Fig. 1. *Limonium* species included in the study, ordinated by decreasing leaf size (LA_1) in the well-watered treatment (WW). Scaled images of the leaf are shown for each species. Species names following Erben (1993) and Rosselló and Sáez (2001). For each species, the LA_1 (mm^2) is shown for the WW and severe water-deficit treatment (WD). The treatment effect on LA_1 is indicated as the % of LA_1 in WD with respect to WW. Values are averages with standard error (n = 4–5 plants per species). Within each column, letters denote significant differences among species by ANOVA-Tukey. For each species, ANOVA differences between treatments are indicated with an asterisk in the %WD *vs.* WW column.

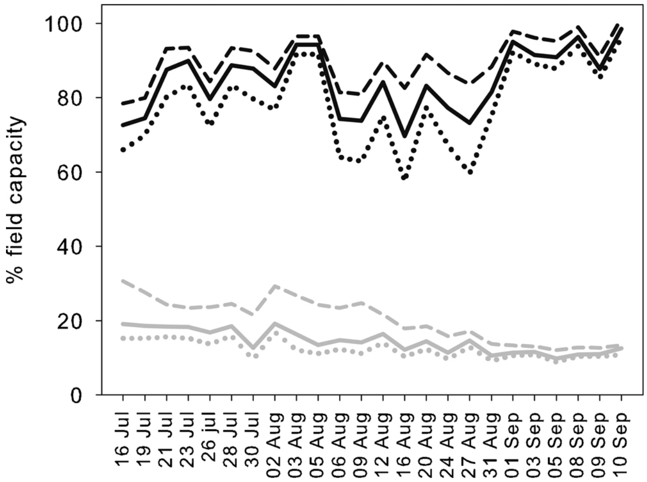


Fig. 2. Average water available in the pot as percent of field capacity (in 3 L pots it was 2251 mL of water) across all the *Limonium* species in the well-watered (WW; black) and severe water deficit (WD; grey) treatments. Solid line is average for all the species, and dashed and dotted lines are maximum and minimum values among all the species, respectively. The period shown corresponds to the days with water treatments completely established.

WW), and in five other plants irrigation was gradually reduced for two weeks to reach 30% field capacity (severe water deficit treatment, WD). Water content in a pot at field capacity was 2.25 L. Water loss and reposition was controlled gravimetrically every 2–3 days, and water consumed (*WC*) for each plant was obtained from the sum of all replaced water volumes during the WD period. Plants were initially fertilized with slow-release fertilizer and, once a week, water reposition was performed in all pots with 50% Hoagland's solution to prevent nutrient deficiencies. On average for all the species and replicates, in WW the pot water content ranged between 70% (just before irrigation) and 100% field capacity, while in WD ranged between 9% (just before irrigation) and 30% field capacity (Fig. 2). Hence, species' differences within each treatment were very small for the average and for the minimum (just before irrigation) values of field capacity (Table S1).

The remaining five plant replicates per species were cut just before the WD treatment application and used to measure initial plant biomass (iB_T) and initial plant leaf area (*iLA*), in order to calculate growth parameters in the WW and WD plants (see next section). The *iLA* was obtained after scanning all existing leaves per plant and calculating the area with Image J (1.49v, National Institute of Health, USA). The iB_T was obtained after drying all plant tissue into paper envelopes in an air-forced oven to constant weight.

Pot water content (as % of field capacity) denoted the severity of the WD treatment at the soil level. In order to evaluate the effect of water deficit at leaf level, the leaf relative water content (*RWC*) and the stomatal conductance to water (g_s) were measured two months after WD treatment application, in leaves completely developed during WD period. The *RWC* was determined at mid-morning as in Galmés et al. (2007) in the youngest fully developed leaves. The stomatal conductance was measured in similar leaves with a Li-6400-40 (Li-Cor Inc., Lincoln, NE, USA) from 0900 to 1200 h. Gas flow was set at 250 μmol mol^{-1}, and conditions in the leaf chamber consisted of a photosynthetic photon flux density of 1500 μmol m^{-2} s^{-1}, a vapor pressure deficit of 1.2–2.5 kPa and a leaf temperature of 25 °C. The stomatal conductance was measured after steady state for at least 30 min at an ambient CO_2 concentration of 400 μmol mol^{-1}. The leaf size in *L. gymnesicum* (GYM) and *L. balearicum* (BAL) was too small to fit in the Li-6400-40 leaf chamber and, consequently, g_s was not measured in these species.

The climatic conditions during the water treatment application period were those typical of the Mediterranean summer, with average daily temperature range (minimum-maximum) of 23.6–30.6 °C in July, 21.6–27.2 °C in August and 17.6–23.4 °C in September; and average air relative humidity range of 36–65% in July, 45–74% in August and 48–83% in September.

2.2. Growth-related measurements

At mid-September, three months after WD treatment application, all WW and WD plants were cut to obtain total dry biomass (B_T), handling all plant fractions separately to measure the leaf mass ratio with respect to B_T for leaves (*LMR*), stem (*SMR*) and root (*RMR*). It is worth indicating that all the *Limonium* species studied are perennial and showed no symptoms of leaf senescence at the time of measuring B_T. The plant water use efficiency (*WUE*, g L^{-1}) was calculated per each plant as the ratio between B_T and *WC*.

The LA_1 was obtained from five well-developed leaves per plant, and four - five plants per species and treatment. Leaves were scanned and leaf area was obtained with Image J (1.49v, National Institute of Health, USA). The dry weight of the scanned leaves was used to calculate the leaf mass area (*LMA*). The total leaf area per plant (*LA*) was estimated from the weight of the green leaves biomass fraction and the *LMA*.

The leaf area ratio (*LAR*, m^2 kg^{-1}) was calculated from *LA* and B_T as in Eq. (1). The net assimilation rate (*NAR*, g m^{-2} day^{-1}) was calculated as in Eq. (2), where parameters indicated with an *i* correspond to the plants cut just before the treatment application, and *t* is the number of days of the treatment period. The relative growth rate (*RGR*, mg g^{-1} day^{-1}) was calculated as in Eq. (3).

$$LAR = LA / B_T = LMR / LMA \tag{1}$$

$$NAR = (B_T - iB_T) / t * (\ln LA - \ln iLA) * (LA - iLA) \tag{2}$$

$$RGR = (\ln (B_T) - \ln (iB_T)) / t \tag{3}$$

2.3. Statistical analyses

Two-way analysis of variance (ANOVA) was performed to test for the overall effects of the treatment and the species, and their possible interaction. Further, one-way ANOVA analyses were performed to test for differences between water treatments for each species, and for species differences within each treatment. In the latter case, Tukey post-hoc test was used. The univariate general linear model for unbalanced data (Proc. GLM) was applied, with type III sum of squares. Parameter relationships were tested with Pearson correlation analyses, and the square of the correlation coefficient was indicated in the plots. In all cases, the ANOVAs and correlations were performed including all the plant replicates. All analyses were performed in IBM SPSS STATISTICS 20.0 software package (SPSS Inc., Chicago, IL, USA). All differences considered significant at $P < 0.05$.

3. Results

3.1. Effect of the severe water deficit on plant water status and stomatal conductance

The leaf relative water content (*RWC*) ranged between 81% and 92% across species and treatments. Differences between the well-watered (WW) and the severe water deficit (WD) treatments were significant only in three of the 13 species, with lower *RWC* under WD (Table 1). In turn, the stomatal conductance (g_s) ranged among the species from 181 to 456 mmol H_2O m^{-2} s^{-1} under WW, and from 51 to 306 mmol H_2O m^{-2} s^{-1} under WD. Differences between treatments were significant in nine species, with the percent of g_s in WD with respect to WW ranging between 27.8% (RET) and 86.9% (LEO), (Table 1).

The two-way ANOVA showed a significant effect of the treatment and the species on both *RWC* and g_s (Table 2). However, the interaction

Table 1

Absolute values for the well-watered (WW) treatment, and % in the severe water deficit treatment (WD) with respect to WW, for the measured parameters, as defined in Table 1. See Fig. 1 for species' codes and for LA_1 values. Values are averages with standard error (n = 4–5). Asterisks denote ANOVA significant differences between treatments (P < 0.05). The g_s could not be measured in GYM and ART due to the small leaf size (see Methods section).

			EJU	BIF	RET	MAG	LEO	ANT	GRO	GIB	BAR	BAL	COM	GYM	ART
RWC	WW	%	91.3 ± 1.5	92.4 ± 1.0	85.8 ± 3.8	88.1 ± 2.6	91.3 ± 0.5	89.3 ± 2.6	85.9 ± 3.4	84.4 ± 2.9	83.9 ± 5.6	91.8 ± 0.5	88.5 ± 1.6	81.8 ± 0.8	83 ± 3.3
	WD	%WW	91.5 ± 3.3	92.5 ± 2.5 *	100.2 ± 0.9	98.6 ± 2.2	100.1 ± 1.3	95.2 ± 2.6	94.1 ± 0.5	102.6 ± 2.5	100.8 ± 2.5	92.0 ± 1.3 *	93.2 ± 1.6 *	100.9 ± 0.6	104 ± 2.6
g_s	WW	mmol H_2O m^{-2} s^{-1}	235.0 ± 25.3	268.9 ± 17.1	183.5 ± 12.1	243.0 ± 27.2	180.5 ± 18.6	210.3 ± 21.4	206.7 ± 36.7	306.0 ± 37.7	399.0 ± 67.9	455.7 ± 62.6	401.8 ± 45.3	–	–
	WD	%WW	43.9 ± 9.7 *	61.4 ± 12.2 *	27.8 ± 8.3 *	48.5 ± 9.4 *	86.9 ± 19.3	28.3 ± 0.3 *	50.0 ± 14.6	31.2 ± 4.7 *	63.8 ± 14.1	67.1 ± 14.6	56.7 ± 5.8 *	–	–
LMA	WW	g m^{-2}	125.3 ± 5.3	117.0 ± 9.1	135.5 ± 17.2	123.6 ± 5.8	131.7 ± 4.6	103.2 ± 7	97.3 ± 3.8	93.7 ± 2.3	92.5 ± 2.8	93.7 ± 6.3	79.0 ± 4.6	81.0 ± 3.3	76.0 ± 6.4
	WD	%WW	85.1 ± 3.3 *	83.7 ± 5.7	88.0 ± 5.2	73.7 ± 2.9 *	79.4 ± 2.8 *	97.6 ± 8	90.1 ± 6.5	100.6 ± 2.5	126.8 ± 15.1	103.2 ± 7.0	90.2 ± 5.2	93.9 ± 2.8	95.2 ± 6.3
B_T	WW	g	14.5 ± 0.8	14.9 ± 2	13.6 ± 1.6	12.2 ± 1.2	10.0 ± 1.5	9.1 ± 0.9	11.7 ± 2.0	8.5 ± 1.0	10.9 ± 0.8	7.5 ± 0.7	9.0 ± 0.3	3.0 ± 0.6	3.8 ± 0.6
	WD	%WW	65.2 ± 3.8 *	54.0 ± 3.2 *	64.2 ± 2.7 *	80.4 ± 5.2	79.8 ± 4.9	47.5 ± 5.6 *	69.0 ± 4.4	57.5 ± 7.6 *	52.2 ± 5.5 *	77.5 ± 6.1	70.2 ± 4.4 *	51.1 ± 8.6	83.2 ± 6.9
LMR	WW	%	68.2 ± 3.5	61.4 ± 1.6	65.0 ± 2.0	56.9 ± 0.9	62.0 ± 1.4	70.0 ± 1.6	56.3 ± 2.7	60.6 ± 1.1	70.3 ± 0.7	66.9 ± 1.1	69.6 ± 1.3	58.8 ± 1.9	56.8 ± 5.6
	WD	%WW	96.0 ± 2.6	99.1 ± 1.5	106.3 ± 1.9	98.8 ± 2.3	112.1 ± 2.5 *	87.6 ± 5.2 *	120.5 ± 4.7 *	99.2 ± 5.6	93.9 ± 3.5	91.2 ± 2.1 *	94.7 ± 4.3	103.0 ± 3.3	116.2 ± 2.5
SMR	WW	%	10.4 ± 0.7	8.2 ± 1.9	7.8 ± 0.5	8.9 ± 0.5	10.9 ± 1.2	6.0 ± 1.7	9.6 ± 2.6	12.5 ± 2.0	6.9 ± 1.3	6.3 ± 0.8	9.7 ± 0.5	6.5 ± 0.9	12.3 ± 1.6
	WD	%WW	59.3 ± 7.0 *	108.1 ± 7.7	92.9 ± 10.2	83.3 ± 2.3	81.7 ± 11.8	114.3 ± 21.2	106.9 ± 5.5	68.7 ± 12.2	114.8 ± 13.9	126.4 ± 15.7	68.6 ± 12.2	99.1 ± 16.6	80.8 ± 6.5
RMR	WW	%	23.5 ± 1.4	30.4 ± 0.6	27.2 ± 2.2	36.0 ± 1.7	27.0 ± 2.1	25.2 ± 0.9	36.5 ± 2.7	29.4 ± 2.2	24.1 ± 1.5	28.0 ± 0.9	22.6 ± 1.7	36.1 ± 3.1	30.9 ± 5.8
	WD	%WW	120.8 ± 9.1	99.7 ± 2.9	87.1 ± 4.9	104.5 ± 2.3	87.9 ± 5.3	126.3 ± 11.6 *	65.6 ± 4.8 *	118.3 ± 11.9	107.9 ± 11.4	110.5 ± 7.8	127.1 ± 11.5	95.3 ± 6.0	77.9 ± 3.4
WC	WW	L	10.3 ± 0.2	10.6 ± 0.2	10.6 ± 0.5	6.9 ± 0.4	8.3 ± 0.2	8.3 ± 0.9	9.2 ± 1.7	8.5 ± 1.0	6.6 ± 0.5	7.3 ± 0.1	8.1 ± 1.0	7.2 ± 0.6	7.5 ± 0.9
	WD	%WW	48.8 ± 2.3 *	44.2 ± 2.3 *	47.5 ± 0.9 *	72.4 ± 2.4 *	54.6 ± 1.9 *	42.7 ± 3.0 *	51.2 ± 1.6 *	40.6 ± 3.2 *	61.2 ± 1.8 *	54.9 ± 1.3 *	57.5 ± 2.2 *	24.2 ± 1.3 *	40.1 ± 3.0 *
WUE	WW	g L^{-1}	1.42 ± 0.15	1.49 ± 0.52	1.10 ± 0.25	1.71 ± 0.30	0.97 ± 0.28	1.09 ± 0.04	1.15 ± 0.02	0.75 ± 0.14	1.44 ± 0.07	1.09 ± 0.04	1.10 ± 0.25	0.57 ± 0.05	0.53 ± 0.03
	WD	%WW	129.0 ± 5.8 *	113.2 ± 2.3	155.5 ± 4.5 *	112.8 ± 4.7	180.3 ± 6.4 *	108.1 ± 8.7	146.2 ± 7.7 *	182.8 ± 12.3 *	93.6 ± 8.0	129.2 ± 8.4	119.0 ± 6.0	150.6 ± 18.1	189.5 ± 2.3 *
RGR	WW	mg g^{-1} day^{-1}	40.4 ± 0.5	46.1 ± 1.3	48.2 ± 1.3	46.6 ± 0.9	51.0 ± 1.6	45.8 ± 1.1	41.6 ± 1.6	43.6 ± 1.4	37.8 ± 0.7	35.2 ± 1.0	36.8 ± 0.3	40.7 ± 2.3	32.1 ± 1.7
	WD	%WW	90.0 ± 1.4 *	88.0 ± 1.2 *	91.8 ± 0.8 *	95.8 ± 1.4	96.4 ± 1.2	84.7 ± 2.6 *	92.4 ± 1.4	88.0 ± 3.1 *	83.7 ± 2.5 *	93.5 ± 2.2	90.8 ± 1.5 *	85.7 ± 3.6 *	95.6 ± 2.5
NAR	WW	g m^{-2} d^{-1}	7.2 ± 0.5	8.1 ± 0.4	9.8 ± 1.4	9.8 ± 0.5	9.9 ± 0.2	7.0 ± 0.5	7.0 ± 0.3	6.9 ± 0.3	5.2 ± 0.2	5.1 ± 0.2	4.5 ± 0.2	5.4 ± 0.5	4.0 ± 0.5
	WD	%WW	80.8 ± 2.3 *	77.0 ± 3.9 *	78.0 ± 3.5	75.9 ± 3.2 *	73.2 ± 3.3 *	92.9 ± 8.7	73.8 ± 5.6 *	91.3 ± 7.2	105.6 ± 13.8	103.4 ± 6.8	88.3 ± 5.5	78.6 ± 4.1	79.9 ± 3.1
LAR	WW	m^2 kg^{-1}	5.5 ± 0.5	5.4 ± 0.5	5.0 ± 0.6	4.7 ± 0.2	4.7 ± 0.2	6.9 ± 0.5	5.8 ± 0.2	6.5 ± 0.2	6.1 ± 1.5	7.3 ± 0.5	8.9 ± 0.5	7.3 ± 0.5	7.6 ± 0.8
	WD	%WW	111.9 ± 2.9	117.1 ± 7.2	116.3 ± 5.5	133.2 ± 5.5 *	141.2 ± 6.4 *	91.3 ± 13.4	137.1 ± 12.4 *	100.3 ± 9.4	97.5 ± 15	87.8 ± 5.6	104.7 ± 6.0	108.8 ± 4.0	122.6 ± 9.5

Table 2
Two-way ANOVA results to test for the global effects of the treatment, the species and their interaction for leaf relative water content (*RWC*), stomatal conductance to water (g_s), leaf area per leaf (LA_1), leaf mass area (*LMA*), total plant dry biomass (B_T), mass ratio for leaves (*LMR*), stems (*SMR*) and root (*RMR*), total water consumed during the treatment application (*WC*), plant water use efficiency (*WUE*), relative growth rate (*RGR*), net assimilation rate (*NAR*), and leaf area ratio (*LAR*). The *F*-value and *P*-value are indicated for the differences between treatments, among species, and their interaction, respectively.

	Treatment		Species		Treatment x species	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
RWC	7.488	0.007	2.383	0.010	1.549	0.121
g_s	60.528	< 0.001	9.469	< 0.001	0.788	0.640
LA_1	0.101	0.751	77.468	< 0.001	3.532	< 0.001
LMA	11.552	0.001	13.024	< 0.001	2.372	0.010
B_T	93.898	< 0.001	22.646	< 0.001	2.015	0.031
LMR	0.374	0.542	5.393	< 0.001	3.541	< 0.001
SMR	4.391	0.039	3.122	0.001	1.418	0.176
RMR	0.017	0.897	5.685	< 0.001	3.146	0.001
WC	861.182	< 0.001	15.324	< 0.001	5.359	< 0.001
WUE	53.008	< 0.001	13.072	< 0.001	1.846	0.061
RGR	82.642	< 0.001	45.111	< 0.001	1.310	0.226
NAR	44.150	< 0.001	26.443	< 0.001	2.106	0.024
LAR	9.255	0.003	7.717	< 0.001	1.245	0.265

between treatment and species was not significant, denoting that the differences in *RWC* and g_s among the *Limonium* were in general not depending on the water treatment, but due on variable responses to WD among the species (Table 2). Consequently, both traits indicated that leaf water status was not impaired and thus, that the *Limonium* species are very adapted to long-term severe water-deficit, as that in the WD treatment (see Fig. 2).

3.2. Leaf morphology differences among the studied *Limonium*

The two-way ANOVA showed no significant effect of the water treatment on leaf size (leaf area per leaf, LA_1), (Table 2). This important finding denotes the interest of *Limonium* for testing the effect of leaf size on growth and on the response to severe water deficit, since LA_1 is in this case a trait independent of the water treatment.

LA_1 varied more than 7-fold across the studied *Limonium* species (Fig. 1). The variation was very similar in both treatments, ranging from (average ± standard error) 185 ± 21 mm^2 (ART) to 1320 ± 115 mm^2 (EJU) under WW, and from 142 ± 8 mm^2 (ART) to 1309 ± 73 mm^2 (EJU) under the severe water deficit treatment (WD), (Table 1). Four *Limonium* species displayed significant differences in LA_1 between treatments, although with opposite effects. Thus, under WD, LA_1 was lower in BAR and GYM, but higher in MAG and LEO (Fig. 1).

The *LMA* ranged from 76 ± 6 g m^{-2} (ART) to 135 ± 17 g m^{-2} (RET) under WW, and from 71 ± 4 g m^{-2} (COM) to 117 ± 14 g m^{-2} (BAR) under WD (Table 1). The two-way ANOVA denoted a general reduction of *LMA* due to the water deficit treatment (Table 2), although the differences between treatments were significant only in three species (Table 1).

3.3. Variation in relative growth rate and its relationship with plant biomass, water consumption, plant water use efficiency and leaf size

There was a tight correspondence between total plant biomass (B_T) and relative growth rate (*RGR*), (Fig. 3A,B), with a positive correlation between both traits ($R^2 = 0.58$ in WW and $R^2 = 0.60$ in WD; $P < 0.001$; Table S2). *RGR* ranged from 32.1 ± 1.7 (ART) to 51.0 ± 1.6 mg g^{-1} d^{-1} (LEO) under WW, and from 30.7 ± 0.8 (ART) to 49.2 ± 0.6 mg g^{-1} d^{-1} (LEO) under WD, while the percent of B_T under WD as compared to WW varied between 48% (ANT) and 83% (ART), (Table 1). The two-way ANOVA showed a significant effect of the treatment on *RGR*, without interaction between species and treatment (Table 2). The water deficit treatment reduced *RGR* in eight of the 13 species (Fig. 3B).

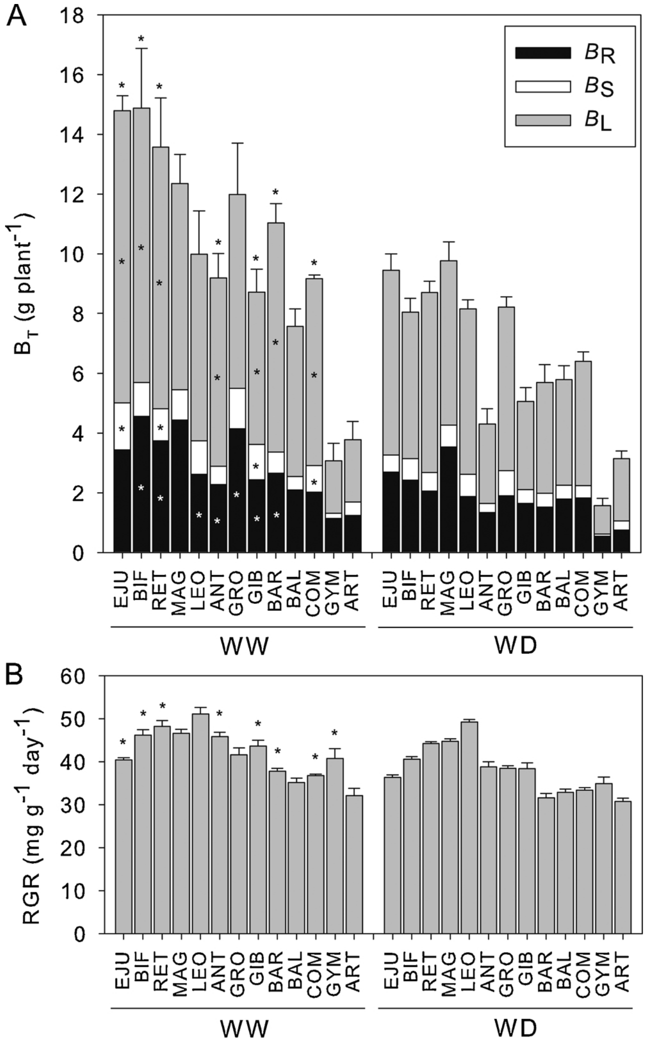


Fig. 3. (A) Biomass allocation in the *Limonium* species under the well-watered (WW) and the severe water deficit (WD) treatments. Species ordination as in Fig. 1. Bars correspond to total dry biomass (B_T) and the error bars represent its standard error (n = 4–5). The asterisk on the WW bars indicate significantly different B_T between treatments ($P < 0.05$). The different biomass fractions are indicated within each bar, thus leaf biomass (B_L), stem biomass (B_S) and root biomass (B_R). Asterisks within each biomass fraction in the WW bars indicate significant differences ($P < 0.05$) between treatments in the biomass ratios (i.e., the proportion of each biomass fraction with respect to B_T). (B) Average relative growth rate (*RGR*) per species and treatment. Error bars correspond to standard error (n = 4–5). Asterisks on the WW bars indicate significantly different *RGR* between treatments ($P < 0.05$).

The leaf mass ratio (*LMR*) was the most important plant fraction, ranging between 56.3% and 70.3% under WW, and between 56.2% and 69.6% under WD. In turn, the stem mass ratio (*SMR*) varied between 6.0% and 12.5% under WW, and between 6.1% and 10.2% under WD, while the root mass ratio (*RMR*) ranged between 22.6% and 36.5% under WW, and between 23.7% and 37.6% under WD. According to the two-way ANOVA results (Table 2), differences between treatments were non-significant in most species for *LMR* (Table 1).

Water consumed per plant (*WC*) during the WD period ranged from 6.6 L to 10.6 L under WW, and from 1.7 L to 5.0 L under WD (Table 1). There were significant differences between treatments and among

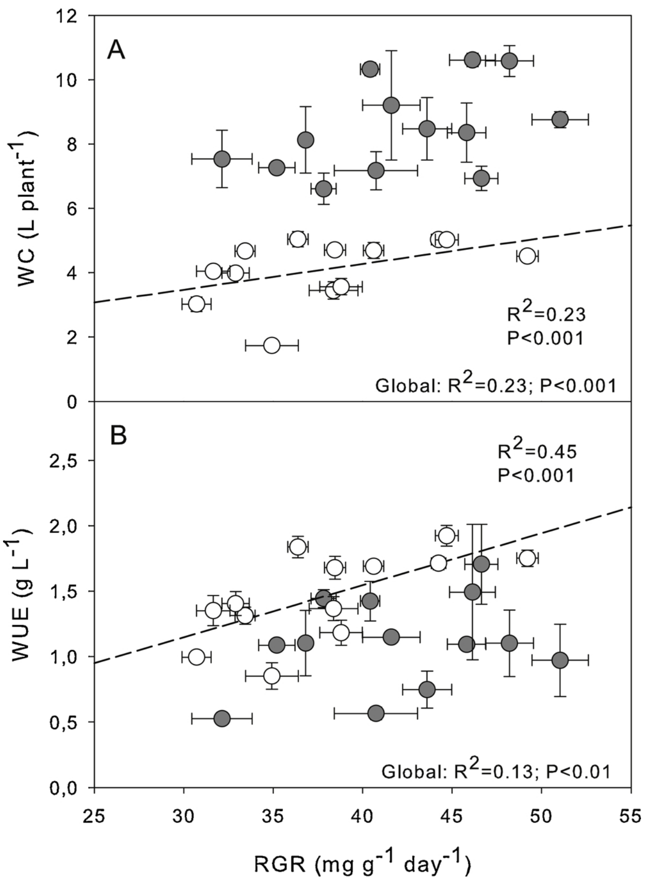


Fig. 4. Relationship of the relative growth rate (*RGR*) with (A) the water consumed during the stress treatment period (*WC*), and (B) the plant water use efficiency (*WUE*; total biomass / water consumed). Regression lines and corresponding R^2 and *P* values indicated when significant, and consider all the replicates per species. Grey dots and solid line correspond to the well-watered treatment (WW), and open dots with dashed line to the severe water deficit treatment (WD). Error bars correspond to standard error (n = 4–5).

species as resulted from the two-way ANOVA (Table 2). Accordingly, *WC* was significantly lower in all the species under WD as compared to WW (Table 1). In turn, the plant water use efficiency (*WUE*) ranged from 0.53 ± 0.03 (ART) to 1.71 ± 0.30 g L^{-1} (MAG) in WW, and from 0.85 ± 0.10 (GYM) to 1.92 ± 0.08 g L^{-1} (MAG) in WD. The overall *WUE* differences between treatments were significant (Table 2). Although *WUE* is calculated from *WC*, the correlation between these two parameters was non-significant under WW ($P > 0.05$; Table S2), and was positive and highly significant under WD ($R^2 = 0.67$; $P < 0.001$; Table S2).

LA_1 positively correlated with *WC* under both treatments, although the correlation was rather low under WW ($R^2 = 0.28$, $P < 0.01$ under WW, and $R^2 = 0.46$, $P < 0.001$ under WD; Fig. S1A) and with *WUE* ($R^2 = 0.40$, $P < 0.001$ under WW, and $R^2 = 0.51$, $P < 0.001$ under WD; Fig. S1B), while *RGR* correlated positively with *WC* and *WUE* only under WD (Fig. 4A,B). Further, *RGR* correlated positively with LA_1 in both treatments (Fig. 5).

3.4. Variation in the physiological and morphological components of RGR

The net assimilation rate (*NAR*), ranged from 5.5 ± 0.8 (ART) to 12.1 ± 1.8 g m^{-2} d^{-1} (LEO) under WW, and from 4.1 ± 0.2 (ART) to 8.5 ± 0.3 g m^{-2} d^{-1} (MAG) under WD (Table 1). In turn, variation in the leaf area ratio (*LAR*) was between 3.8 ± 0.2 (LEO) and 6.9 ± 0.6 m^2 kg^{-1} (COM) under WW, and between 5.0 ± 0.3 (EJU) and 7.0 ± 0.4 m^2 kg^{-1} (COM) under WD. For both *NAR* and *LAR*, there were overall differences between treatments and among species (Table 2). However, the differences between the two treatments were significant only in five species for *NAR* and three species for *LAR* (Table 1).

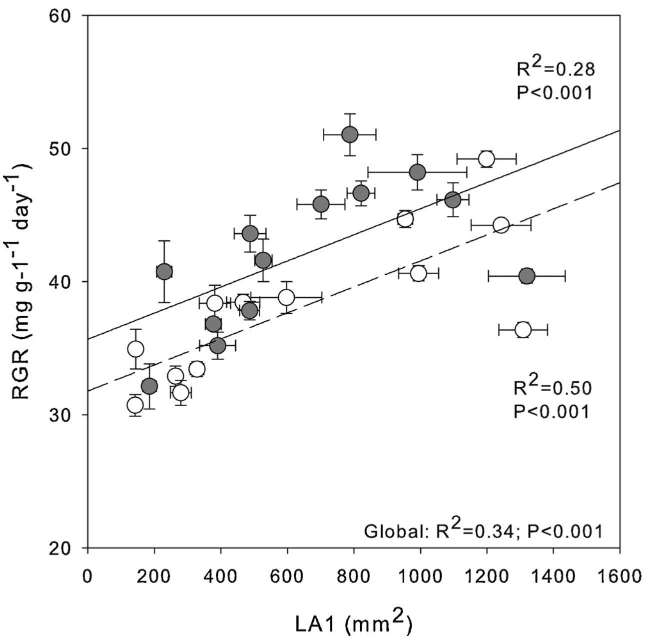


Fig. 5. Correlation of the relative growth rate (*RGR*) with leaf size (leaf area per leaf, LA_1) for the 13 *Limonium* species studied. Legend as in Fig. 4.

Under both WW and WD, there was no significant correlation between LA_1 and *LMR* (Fig. 6A), denoting that the proportion of leaf biomass *vs.* total plant biomass in *Limonium* is independent of leaf size. On the contrary, LA_1 correlated positively with *LMA* under both treatments (Fig. 6B), although the correlation was rather low under WD. Since *LAR* calculation results from *LMR* and *LMA*, the LA_1 to *LMA* correlation explained to a high extent the negative correlation between LA_1 and *LAR* occurring in both treatments (Fig. 6C). There was also a significant correlation between LA_1 and *NAR* in both treatments (Fig. 6D), albeit the correlation was positive and thus, there was a tradeoff between *LAR* and *NAR* across species and treatments ($P < 0.001$; $R^2 = 0.80$ under WW, and $R^2 = 0.70$ under WD; Fig. S2).

RGR did not correlate with *LMR* (Fig. 7A) and correlated positively with *LMA* in WD and especially in WW (Fig. 7B). There was a negative correlation between *RGR* and *LAR* in both treatments (Fig. 7C), and also a positive correlation between *RGR* and *NAR* (Fig. 7D). Hence, in both WW and WD, *NAR* showed higher correlation with *RGR* than with LA_1 (Figs. 6D, 7 D), which indicated that *NAR* was not fully determined by leaf size. Moreover, for both LA_1 and *RGR*, and irrespective of the water treatment, the correlation was higher with *NAR* than with *LAR*, confirming a higher impact of *NAR* over *LAR* in explaining differences in leaf size and growth in *Limonium*.

4. Discussion

4.1. Severe water deficit has a low impact on leaf morphology in Limonium

The Balearic *Limonium* is a group of closely related species inhabiting the same, harsh environment, and in which leaf size (leaf area per leaf, LA_1) is one of the most variable traits, showing a ca. 7-fold variation among the species included in this study (Fig. 1). This, together with the non-significant effect of the water treatments on LA_1 (Table 2) makes this an ideal case study to understand the importance of leaf size in explaining growth capacity under harsh conditions. This fact is not common in literature, with many examples showing leaf size reduction under water stress in species with different growth forms and from different habitats (e.g., Westoby

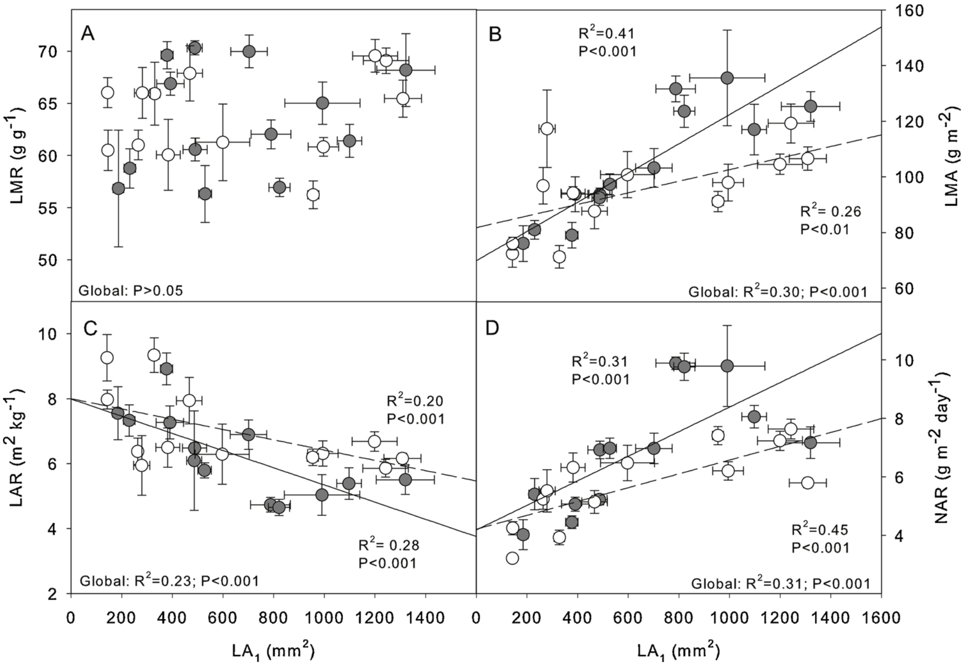


Fig. 6. The relationship between the leaf size (LA_1) and (A) the leaf mass ratio (*LMR*), (B) the leaf mass area (*LMA*), (C) the leaf area ratio (*LAR*), and (D) the net assimilation rate (*NAR*), for the 13 *Limonium* species. Legend as in Fig. 4.

et al., 2002; McDonald et al., 2003; Ramírez-Valiente et al., 2010; Yates et al., 2010; Carins-Murphy et al., 2014).

Modifications responding to stress adaptation in arid and semi-arid environments are also frequently related to increases in leaf mass area (*LMA*), (e.g., Ackerly, 2004; Galmés et al., 2005; Gratani and Varone, 2006; Poorter et al., 2009; Wellstein et al., 2017). However, variation of *LMA* across *Limonium* species and water treatments was less than 2-fold (Table 1), and with a low effect due to severe water deficit (WD), (Table 2). Altogether, this denotes a limited importance of *LMA* in the adaptation to harsh conditions in *Limonium* which, added to the positive correlation with LA_1 (Fig. 6B), suggests a surrogate role of *LMA* on LA_1 in this group.

4.2. Limonium species with larger leaves have higher growth capacity and higher water use efficiency

As compared to the well-watered treatment (WW), the severity of WD (Fig. 2) resulted in lower water consumption (*WC*) in all the species. However, the important water shortage had a low effect on total biomass (B_T), water use efficicency (*WUE*) and relative growth rate (*RGR*) in most *Limonium* species (Table 1). This agrees with the capacity of *Limonium* species to colonize and survive under extreme harsh conditions in coast environments. Hence, the important *WC* differences between treatments indicated that *Limonium* species are in general

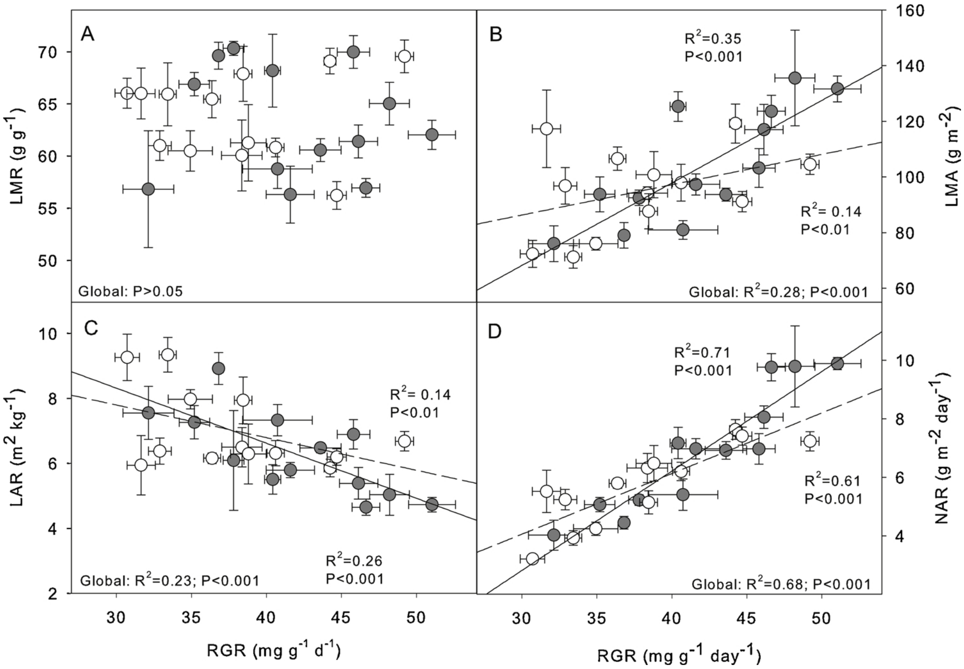


Fig. 7. The relationship between the relative growth rate (*RGR*) and (A) the leaf mass ratio (*LMR*), (B) the leaf mass area (*LMA*), (C) the leaf area ratio (*LAR*), and (D) the net assimilation rate (*NAR*), for the 13 *Limonium* species. Legend as in Fig. 4.

consuming more water when available, albeit this was not resulting in proportional increases in *RGR*. In fact, the correlation between *RGR* and *WC* was significant under WD but not under WW (Fig. 4A), denoting luxury water spendage in the latter treatment. Accordingly, for the same *RGR, Limonium* plants under WW consumed almost double amount of water than plants under WD (Fig. 4A).

On the other hand, under WD, the higher *WC* in high *RGR* species responded to those species having also larger plants (Fig. 3A,B). However, high *RGR* species had also higher *WUE* (Fig. 4B) and thus, may have adaptations conferring better performance under water deficit conditions. Interestingly, there was a positive correlation of LA_1 with *RGR* in both treatments (Fig. 5) pointing to LA_1 as a key trait explaining higher *WC* but also higher *WUE* (Fig. S1A,B) in high *RGR* species.

The higher *WUE* in large-leaved species denoted lower *WC* per unit leaf surface, which agrees with LA_1 correlating positivey with *LMA* (Fig. 6B) and negatively with *LAR* (Fig. 6C). Conversely, there was no relationship between LA_1 and the leaf mass ratio (*LMR*), probably due to the concomitant adjustments in *LMA*, (Fig. 6A,B). These results are in agreement with a previous comparison of diverse Mediterranean species with different growth forms, in which the two *Limonium* species considered were among those that did not decrease *LMR* under water deficit (Galmés et al., 2005).

The root mass ratio (*RMR*) showed no correlation with *RGR* in *Limonium* under any treatment, and correlated with B_T only under WD (Table S2). Thus, the root proportion did not have an impact on growth capacity in *Limonium*. Contrarily, *RMR* negatively correlated with *LMR* which denotes that, irrespective of plant size, species with higher root proportion are less foliose. Since this occurred under both treatments, results suggest a rather species' constitutive trait of the root fraction, with little response to the different water treatments imposed.

Finally, the positive correlation of LA_1 with *NAR* (Fig. 6D) suggests that the morphological differences (i.e, *LMA*, *LAR*) among species with different leaf size have an impact on the elemental determinants of growth in *Limonium*. Thus, as compared to small LA_1 species, the lower *LAR* and higher *LMA* result in higher *NAR* (Fig. 6D) and *RGR* (Fig. 5) in the large LA_1 species.

4.3. Growth capacity in Limonium is governed by NAR and reductions of LAR in large-leaved species results in increased RGR

The dissection of the *RGR* in its underlying components, *NAR* and *LAR*, showed that most *RGR* variation in *Limonium* was explained by *NAR*, irrespective of the water treatment (Fig. 7D). A previous report across Mediterranean species with contrasting growth forms showed that the largest importance of *NAR* in explaining the drought-driven decrease in *RGR* corresponded to *Limonium* species (Galmés et al., 2005). Furthermore, the higher importance of *NAR* than *LAR* in explaining *RGR* has been related to plants grown under high light (reviewed in Shipley, 2006), as it is the case of *Limonium*.

The opposite relation of *NAR* and *LAR* with *RGR* (Fig. 7C,D) indicates the existence of a tradeoff between both components in *Limonium* ($R^2 = 0.74$, $P < 0.001$; Fig S2). This commonly described tradeoff (Konings, 1989; Poorter and Remkes, 1990; Poorter and Van der Werf, 1998; Villar et al., 2005; Lambers et al., 2008) has been attributed to the strong positive relationship between photosynthesis (A_N, as a component of *NAR*) and *LMA* (as a component of *LAR*), (e.g., Poorter and Van der Werf, 1998). Agreeing with the tradeoff, *Limonium* species with higher *RGR* achieved higher *NAR* through reductions in *LAR* (Figs. 6C,D and 7 C,D), mostly resulting from higher *LMA* (Figs. 6B and 7 B), and corresponded to species with higher LA_1 (Fig. 5). Since the positive correlation between LA_1 and *LMA* (Fig. 6B), higher *RGR* could result from higher *LMA* instead of higher LA_1, because of the impact of *LMA* on *NAR* (i.e., A_N). The correlation of A_N with *LMA* occurred in *Limonium* only under WW and was negative (Table S2). Diverse meta-analyses have shown a negative correlation between A_N and *LMA* (Westoby et al., 2002; Wright et al., 2004; Osnas et al., 2013) as a consequence of tight correlations between A_N and N content (positive) and between N content and *LMA* (negative), which depicts contrasting strategies in the resource acquisitive-conservative axis of plant economics spectrum, rather than a direct role of *LMA* in driving A_N (e.g., Reich, 2014; Diaz et al., 2016). Under WW, higher *NAR* and *RGR* was achieved by large-leaved *Limonium* with lower A_N and higher *LMA* (i.e., resource-conservative).

In *Limonium*, leaves are disposed in crowns on branches with short internodes, with cushion-like plant habit. As compared to small leaves, larger leaves reduce self-shading because are less aggregated, which is further boosted by a longer petiole (Fig. 1). This adaptation has been described in other species (Niklas, 1988; Takenaka, 1994; Pearcy et al., 2005; Poorter and Rozendaal, 2008), whereas self-shading differences have been found as the most determining factor in explaining carbon gain differences among species from Australian dry woodlands (Falster and Westoby, 2003). Moreover, *Limonium* species with large LA_1 have lower number of leaves (i.e., proportional to plant size) as resulting from lower *LAR*, allowing less dense leaf packing in the shrub and less self-shading. Therefore, species with larger leaves, may be on the long-term more efficient than small-leaved species, which agrees with the higher *NAR* and *RGR* in those species (Figs. 5, 6D).

Leaf size negatively correlated with stomatal conductance (g_s) under both treatments (Table S2). Stomatal traits are frequently coordinated with leaf venation and hydraulics (Brodribb et al., 2007; Nardini et al., 2014; Scoffoni et al., 2016). If this was the case in *Limonium*, lower g_s in large-leaved species would also suggest lower vein density and hydraulic conductance due to a vein "dilution" in larger leaves (Dunbar-Co et al., 2009; Sack et al., 2012; Nardini et al., 2014), and thus would be associated to higher growth capacity.

4.4. The Limonium case study endorses the importance of leaf size in the adaptation to harsh conditions when comparing among closely related species

In explaining growth capacity across the global leaf economics spectrum, *LMA* has higher predictive power than leaf size, despite the latter is much more variable than the former (e.g., Wright et al., 2004; Díaz et al., 2016). However, when considering groups of closely related species like here in *Limonium*, phylogenetic constraints may limit variation in some traits, like *LMA*, promoting higher adaptive importance to the traits still able to have norotious variation, like leaf size and shape (e.g., McDonald et al., 2003; Dunbar-Co et al., 2009; Yates et al., 2010; Blonder et al., 2016).

A common assertion in leaf adaptation to drought conditions is the trend to have small leaves, providing advantages related to higher water use efficiency, heat dissipation, excess light interception, etc. (Westoby et al., 2002; McDonald et al., 2003; Ramírez-Valiente et al., 2010; Yates et al., 2010; Carins-Murphy et al., 2014). However, this did not occur in *Limonium*, showing no differences in leaf size between water treatments, and with higher water use efficiency and growth capacity for the species with larger leaves. This indicates that adaptations in large-leaved *Limonium* overcome commonly described constraints to a large leaf size under harsh conditions.

5. Conclusions

The Balearic *Limonium*, with a large number of closely related species inhabiting a very similar, harsh environment, and with an exceptional variation in leaf size, is a very interesting case study to test for the impact of leaf traits on the adaptation to stressful conditions. Contrary to most literature reports, severe water deficit had no impact on leaf size in *Limonium*, and larger-leaved species had higher growth capacity and also higher water use efficiency. Moreover, *RGR* was highly determined by its physiological component (*NAR*), that positively correlated with leaf size. On the contrary, the positive correlation of leaf size with leaf mass area largely determined a negative correlation of leaf size with the morphological component of *RGR* (i.e., *LAR*). Altogether, results in this study suggest the existence of a coordination between leaf physiological and morphological factors governing growth capacity in *Limonium*.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2019.05.011.

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