Journal of
Plant Ecology

Research Article

Adaptive responses to drought of two *Retama raetam* subspecies from Tunisia

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Handling Editor: Jianming Deng

Received: 23 August 2019, **First Decision:** 22 December 2019, **Accepted:** 2 January 2021, **Online Publication:** 31 January 2021

Abstract

Aims The survival and ecological distribution of plants in arid habitats are mainly conditioned by water availability and physiological adaptations to withstand drought. In the present study, we have compared the physiological responses to drought of two *Retama raetam* (retama) subspecies from Tunisia, one of them living under the desert climate (subsp. *raetam*) and the other one growing on the coast (subsp. *bovei*).

Methods To physiologically characterize the two *R. raetam* subspecies, and to elucidate their main mechanisms underlying their tolerance to drought stress, parameters related to seed germination, growth, photosynthesis (net photosynthetic rate, intracellular CO_2 concentration, transpiration rate, stomatal conductance and water-use efficiency) and accumulation of osmolytes (proline, glycine betaine [GB] and soluble sugars) were determined in 4-month-old plants subjected to stress for up to 1 month.

Important Findings Drought significantly inhibited germination, growth and all the evaluated photosynthetic parameters. Plants of *R. raetam* subsp. *bovei* were severely affected by drought after 3 weeks of treatment when photosynthesis rates were up to 7-fold lower than in the controls. At the same time, proline and GB significantly accumulated compared with the irrigated controls, but much less than in *R. raetam* subsp. *raetam*; in the latter subspecies, proline and GB increased to levels 24- and 6-fold higher, respectively, than in the corresponding controls. In summary, the population living in the desert region exhibited stronger tolerance to drought stress than that adapted to the semiarid littoral climate, suggesting that tolerance in *R. raetam* is dependent on accumulation of osmolytes.

Keywords germination, leaf gas exchange, osmolytes, *Retama raetam*, water stress, xerophytes

两个突尼斯细枝豆属植物亚种对干旱的适应性响应

摘要：干旱生境植物的生存和生态分布主要取决于水的可获得性以及植物抵御干旱的生理适应能力。在本研究中，我们比较了来自突尼斯的细枝豆属植物*Retama raetam*的两个亚种对干旱的生理响应，其中一种生长在沙漠气候下(*raetam*亚种)，另一种生长于海岸带(*bovei*亚种)。为了对这两个亚种进行生理表征

并揭示它们耐旱性的主要机制，从受干旱胁迫最长达一个月的四月龄植株中获取了多项参数，涉及植株的萌发、生长、光合作用(净光合速率、细胞内CO_2浓度、蒸腾速率、气孔导度和水分利用效率)和渗透物(脯氨酸、甜菜碱和总可溶性糖)积累等。研究结果表明，干旱会显著抑制植株的萌发、生长，对所研究的各项光合参数也都产生很大的负面影响。经过3周的处理后，*bovei*亚种受到了干旱条件的显著影响，其光合作用速率与对照相比最高下降了7倍。与此同时，该亚种中出现了对脯氨酸和甜菜碱的显著积累(相较于灌溉条件下的对照)，但远低于*raetam*亚种；在*raetam*亚种中，脯氨酸和甜菜碱分别增加至相应对照的24和6倍。综上所述，相较于生长在半干旱海岸气候下的细枝豆属植物种群，生长于沙漠地区的种群对干旱胁迫表现出更强的耐性，表明这种耐性在很大程度上取决于渗透物质在体内的累积。

关键词：萌发，叶气交换，渗透物，*Retama raetam*，水分胁迫，旱生植物

INTRODUCTION

Water shortage limits plant growth and crop productivity, particularly in arid regions located typically at 15–30° N and S latitudes. These zones are characterized by the presence of a desert or a (semi-) arid climate, with very low rainfall (Boyer 1982). In these habitats, growth of plants and their distribution depend to a large extent on their morphological and physiological adaptations to avoid water loss (Wood 2005). A ramified root system and a small leaf area are common strategies found in many xerophytes, which confer them different degrees of drought tolerance (Bechtold 2018; Shao *et al.* 2008; Younis *et al.* 2017).

Water stress affects fundamental physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrate and nutrient metabolism and, consequently, reduces plant growth (Farooq *et al.* 2009). Drought stress drastically limits the seed germination of plants living in extreme habitats and causes either germination inhibition or irreversible damage to seedlings (Gorai *et al.* 2006). For example, germination in the desert species *Diplotaxis harra* (harra or charra) and *Reaumuria vermiculata* (reaumeria) is completely inhibited at high temperatures or low water potentials (Gorai and Neffati 2007; Tlig *et al.* 2008). At the whole-plant level, drought stress effects are usually perceived as a drop in the photosynthesis rate, which is associated with alterations in the metabolism of C and N (Cornic and Massacci 1996). The photosynthetic reduction is brought about by several coordinated events, such as stomatal closure, which affects leaf water content (WC) and diminishes the activity of photosynthetic enzymes (Chaves *et al.* 2003; Lawlor and Cornic 2002). However, under severe stress, photosynthesis may be mostly controlled by the chloroplast's capacity to fix CO_2, e.g. by the Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) enzyme activity, rather than by increased diffusive resistance (Bota *et al.* 2004). Therefore, plant responses to water stress can differ significantly at various organizational levels, depending on the intensity and duration of stress, and also on the plant species and growth stage (Chaves *et al.* 2002).Osmotic adjustment is also considered one of the crucial processes in plant adaptation to salt and drought stress (Hmidi *et al.* 2018; Lansac *et al.* 1994). It involves the synthesis and accumulation of small compatible solutes (osmolytes), such as proline, glycine betaine (GB) or sugars, as well as some inorganic ions (Chaves *et al.* 2003; Hare *et al.* 1998). Several xerophytic genera, including *Atriplex* (Amaranthaceae), *Calligonum* (Polygonaceae), *Retama* (Fabaceae) or *Tamarix* (Tamaricaceae), are ecologically important since they act as reservoirs by stabilizing the dunes in these areas (Dhief *et al.* 2011; Kawada *et al.* 2012). *Retama raetam* is frequently present in NE Mediterranean regions and the Sinai Peninsula (Mittler *et al.* 2001). In Northern Africa, this genus is represented by three species: *Retama monosperma* (L.) Boiss, *R. sphaerocarpa* (L.) Boiss and *R. raetam* (Forssk.) Webb in Webb and Berthel. These three species possess important medicinal properties, showing hypotensive, hypoglycaemic, antibacterial and antioxidant activities (Maghrani *et al.* 2003; Saada *et al.* 2018).

The present study analyses the responses to water stress of two subspecies of *R. raetam* (*R. raetam* subsp. *raetam* and *R. raetam* subsp. *bovei*) collected from two different areas in Tunisia, Bir el Haj (BH), in a desert zone, and Sidi Makhlouf (SM), near the Mediterranean coast. We hypothesized that it would be possible to find differential physiological and biochemical adaptations to drought between the two subspecies, based on their different ecological habitats. These comparative analyses are particularly

interesting as one of the subspecies can grow under extreme conditions and none has previously been studied. The specific aims of this study were to: (i) characterize seed germination in different temperature regimes, and at various concentrations of polyethylene glycol (PEG; to mimic drought stress); (ii) analyse vegetative growth and photosynthetic responses at different water stress levels; (iii) correlate osmolyte accumulation under stress with tolerance to drought in *R. raetam*.

MATERIALS AND METHODS

Plant material and growth conditions

Retama raetam (Forssk.) Webb in Webb and Berthel (fam. Fabaceae) seeds were collected from wild populations of *R. raetam* subsp. *raetam* (*RRr*) and *R. raetam* subsp. *bovei* (Spach) Talavera and Gibbs (*RRb*), located in different regions of SE Tunisia (Le Floc'h *et al.* 2010): *RRr*—BH (33°21.26′ N, 9°15.18′ E; Douz) and *RRb*—SM (33°14.96′ N, 10°49.05′ E; Medenine). According to Noy-Meir (1973), BH is a desert area subjected to arid conditions (Saharan climate) which are highly restrictive for plant survival, whereas SM is closer (ca. 30 km) to the coast and is subjected to milder littoral conditions (semiarid climate). Average temperatures are similar in both locations, from minimum temperatures below 10 °C in January to maximum ones above 35 °C in July/August. Precipitations are very scarce in both habitats, although higher levels are recorded every year in SM (359 mm) than in BH (220 mm). Seeds of both subspecies were collected from selected shrubs before being cleaned and stored under stable conditions at 20 °C and 30% relative humidity in the seed bank of the Laboratoire d'Ecologie Pastorale at the Institut des Régions Arides (IRA) (Médenine, Tunisia) until they were used in this study.

Germination assays

Seeds were pre-treated with concentrated sulphuric acid (96%) for 2 h to encourage emergence before performing the germination studies (Teketay 1998). Five replicates of 20 seeds per treatment were sown in plastic Petri dishes (Ø 90 mm) on the surface of two layers of moistened filter paper before being placed inside a germination chamber in the dark. The number of germinated seeds was counted every 2 days for 20 days, and the adopted germination criterion was the visual presence of an emerged radicle. Besides the final seed germination percentage, the germination rate was calculated according to the modified Timson's index (Timson 1965): $\sum G/t$, where G is the percentage of seeds germinated after 2 days intervals and t is the total germination time (Khan and Ungar 1984). Firstly, the effect of temperature on germination in water was tested by applying regimes from 5 to 35 °C at 5 °C intervals. Then, to estimate the effect of water stress, seeds were germinated at 15 °C at different PEG (PEG 6000) concentrations, 0, 28, 52.5, 67, 76 and 84 g/L, which corresponded to 0, −0.3, −0.7, −1, −1.2 and −1.4 MPa of osmotic potential, respectively, according to Michel and Kaufmann's equation (1973).

Water stress treatments

Plant growth and osmolyte quantification were determined in plants obtained by seed germination, maintained in a phytotron under controlled conditions of a 16/8 h (light/darkness) photoperiod, 130 µmol m^{-2} s^{-1} photosynthetic active radiation, 80% relative humidity and 25 °C average temperature. Sowing was done in pots 15 cm Ø × 18 cm high (2 L vol.) filled with a mixture of blonde peat, perlite and vermiculite at 2:1:1 ratio. Plants were watered with full strength Hoagland's nutrient solution (Hoagland and Arnon 1950), until irrigation was suppressed. Leaf gas exchange and water potentials were measured from plants grown in pots filled with a mixture of sand and commercial peat at 2:1 ratio in a plastic greenhouse under ambient temperature, relative humidity and rainwater-irrigation conditions. In both cases, plants were grown for 4 months, and water stress treatments were performed by preventing irrigation altogether. Treatments were extended until plants reached irreversible wilting over 4 weeks (from 0 to 28 days). On each occasion, five plants subjected to drought stress and five controls were sampled weekly (after 7, 14, 21 and 28 days of treatment) for the biochemical analyses. After 3 weeks of withholding irrigation, five stressed plants were rehydrated and the same physiological parameters as those measured in the untreated plants were quantified 7 days later.

Growth parameters

At the end of each water stress period, five plants per treatment were selected to determine the total leaf number, primary stem length (cm), maximum root length (cm), primary stem diameter (cm), fresh weight (FW) and WC of the total aerial part

and total roots (expressed in g and %, respectively). To calculate both the dry weight (DW) and WC percentages of the whole aerial part and roots, fresh samples were weighed (FW) before being dried for 4 days at 65 °C until constant weight (DW): WC was calculated as: WC (%) = [(FW − DW)/FW] × 100.

Leaf gas exchange and water potential

Gas exchange measurements were taken in five plants per treatment by a portable infrared gas analyser, model LCpro-SD (ADC BioScientific Ltd, Hoddesdon, UK). Subapical ends of shoots below 2 cm of tips were placed in a conifer leaf chamber to determine the net photosynthetic rate (A), intercellular CO_2 concentration (C_i), transpiration (E) and stomatal conductance (g_s) in a total photosynthetic area of 58 mm^2 under ambient CO_2, temperature and relative humidity conditions. Water-use efficiency (WUE) of the leaves was calculated as the A/g_s ratio expressed in mmol (CO_2 assimilated) mol^{-1} (H_2O transpired). The water potential (Ψ_w) was measured by cutting plants at the height of 1 cm from the basal part of shoots in five replicates per treatment in a pressure chamber according to the method described by Scholander *et al.* (1965).

Osmolyte quantification

Samples of the aerial parts of five stressed plants and five controls were taken every week during 1 month to determine the levels of proline (Pro), GB and total soluble sugars (TSS). Pro extractions were performed by grinding 0.1 g of fresh cuttings in a mortar with 3% (v/v) sulphosalicylic acid solution at room temperature. Pro contents were calculated by the acid ninhydrin method according to Bates *et al.* (1973), with the minor modifications described by Vicente *et al.* (2004). Pro concentration was expressed as µmol g^{-1} of DW.

GB was determined according to Grieve and Grattan (1983), with the modifications indicated in Nawaz and Ashraf (2010), from the aqueous extracts prepared from fresh samples. GB levels were expressed as µmol g^{-1} DW.

TSS were quantified according to the phenol–sulphuric acid reaction protocol described by Robyt and White (1987). Extracts were obtained from the same plant material described for Pro, but extracted with 80% (v/v) methanol and incubated at room temperature for 24 h with oscillating agitation. The absorbance of samples was determined at 490 nm. TSS were calculated and expressed as milligrams equivalent of glucose (mg eq. glucose g^{-1} DW).

Statistical analyses

The statistical differences between the means of the different measured parameters in *RRr* and *RRb* were determined by an analysis of variance (ANOVA) at the 95% confidence level using 'time of water stress' and 'subspecies' as grouping factors. Before the ANOVA, the data requirements of the normality and homogeneity of variances were checked by Levene's and Shapiro–Wilk tests. When the null ANOVA hypothesis was rejected, *post hoc* comparisons were made to establish possible statistical differences between the different treatments applied using Tukey's test. The statistical SPSS v.16 software was used for these analyses.

RESULTS

Germination behaviour

The germination responses of the *RRr* and *RRb* seeds to the different applied temperatures and water potentials are shown in Fig. 1. The seeds from both subspecies were able to germinate at all tested temperatures within the 5–30 °C range. The highest germination percentages lay between 10 and 20 °C with an optimum at 15 °C—96% and 93% for the *RRr* and *RRb* seeds, respectively (Fig. 1a). Both, the germination percentages and the germination rates decreased significantly with increasing temperatures above 15 °C, and were completely inhibited at 35 °C (Fig. 1a and c). The *RRr* seeds showed higher average germination percentages and rates than *RRb* at all the studied temperatures, but differences were only significant at 25 °C (1.3-fold). Increasing PEG concentrations also reduced the germination percentages and rates (Fig. 1b and d). Similar germination levels to those obtained at the optimal temperature were found using distilled water, followed by those treated at 28 g/L PEG, without significant differences between seeds of the two subspecies, but germination was completely inhibited at 84 g/L PEG in both cases. Intermediate PEG concentrations, from 52.5 to 76 g/L, progressively reduced the germination percentages, in a concentration-dependent manner, from 80% to 20%, approximately, with the *RRr* seeds showing slightly (but statistically significant) higher values than the *RRb* seeds at each PEG concentration (Fig. 1b). A similar pattern was observed for the decrease in germination rates in the presence of PEG, for both subspecies (Fig. 1d).

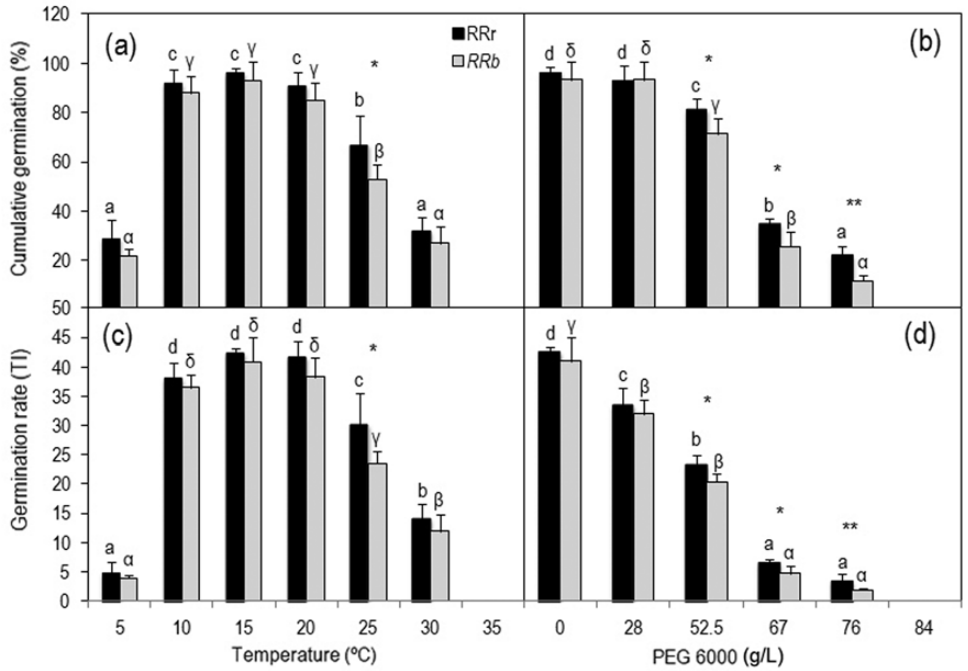

Figure 1: Changes in the germination percentage (%) (**a**, **b**) and rate (Timson's index, modified) (**c**, **d**) of *Retama raetam* subsp. *raetam* (*RRr*) and subsp. *bovei* (*RRb*) at the indicated temperatures (a, c) and PEG concentrations (b, d) (means ± standard deviations, $n = 5$). Different letters (Latin for *RRr*, Greek for *RRb*) indicate significant differences between treatments for each subspecies, according to ANOVA ($P < 0.05$, Tukey's test). Asterisks denote significant differences between subspecies for a specific treatment, according to ANOVA ($*P < 0.05$, $**P < 0.01$).

Plant growth

Ceasing irrigation inhibited the development of both studied subspecies (Table 1). Total leaf number dropped significantly after 3 weeks of treatment in the stressed plants compared with the respective controls. After 4 weeks, the *RRr* plants showed a significant reduction in the total number of leaves and *RRb* had lost them all by senescence. Primary stem length did not show any significant variations in the treated plants and only slight increases were observed in the controls. Maximum root length did not show significant differences in either the treated or untreated plants. Primary stem diameter significantly decreased from day 28 in the *RRr* treated plants and from day 21 in *RRb*, compared with the controls. The measurements of the growth parameters agreed with the variations observed in the FW of the whole aerial plant parts and that of roots, which significantly decreased in the water-stressed plants of both subspecies, compared with the corresponding controls. The most significant differences were found in the WC of the aerial part and the roots of the plants, which were reduced to ca. 60% and 40% of the corresponding control values in *RRr* and *RRb*, respectively, after 4 weeks of treatment. Rehydration for 7 days of the plants previously stressed for 3 weeks improved all the studied growth parameters, as compared with the non-rehydrated plants, but significant differences were observed only in the primary stem diameter of *RRb* plants. Therefore, although rehydration of plants can restore their normal water status, the values of the major growth parameters remained low after 7 days of rewatering, probably because the recovery period was not long enough to observe stronger effects.

Leaf gas exchange and water potential

All the studied photosynthetic parameters decreased continuously and significantly with time in the water-stressed plants, and their levels were close to zero at day 28 of treatment (Fig. 2). Around 30% reduction in the net photosynthetic rate was observed after 1 week of water stress, which decreased to around 60% in the second week for both subspecies. The observed tendency was similar for g_s and E, with reduced percentages of ~40%–70% in *RRr* and 60%–80% in *RRb*, in the second week of treatment, respectively. C_i varied only slightly, between ~10 and 25 µmol

Table 1: Growth parameters comparison between *Retama raetam* subsp. *raetam* (*RRr*) and subsp. *bovei* (*RRb*) at the indicated times during the water-stress treatment (WS) and for untreated plants (Control)

		Treatment time (days)					
Retama raetam* subsp. *raetam		**0**	**7**	**14**	**21**	**28**	**Rehydration**
Total leaf number	Control	24.0 ± 3.94ab	37.6 ± 6.66b	29.2 ± 7.69b	29 ± 11.31b*	28 ± 2.6b**	9.4 ± 2.61a**/α
	WS	—	28.6 ± 8.93β	18.4 ± 7.54αβ	16 ± 3.67α*	7.4 ± 6.73α**	
Primary stem length (cm)	Control	58.2 ± 11.23ab	52.8 ± 11.48a	70.2 ± 2.39bcd	78.2 ± 6.46cd	81.8 ± 5.67d**	66.4 ± 4.39abc**/α
	WS	—	62.8 ± 11.54α	64 ± 5.66α	62 ± 10.79α	61.4 ± 8.29α	
Maximum root length (cm)	Control	32.6 ± 10.36a	33.1 ± 8.22a	38.6 ± 8.29a	39 ± 7.21a	41.4 ± 15.98a	45.2 ± 14.68a/α
	WS	—	34.6 ± 5.68α	42 ± 12.9α	40.2 ± 7.66α	31.2 ± 14.82α	
Primary stem diameter (mm)	Control	1.94 ± 0.2a	2.04 ± 0.13a	2.04 ± 0.07a	2.08 ± 0.22a	2.26 ± 0.27a*	2.13 ± 0.25a/α
	WS	—	1.85 ± 0.2α	1.93 ± 0.11α	1.87 ± 0.25α	1.85 ± 0.21α*	
FW (g) (total aerial part)	Control	1.69 ± 0.31a	1.93 ± 0.67ab	1.98 ± 0.33ab	2.57 ± 0.75ab*	3.3 ± 1.29b**	1.43 ± 0.31a**/α
	WS	—	1.95 ± 0.72α	1.87 ± 0.46α	1.59 ± 0.54α*	1.27 ± 0.34α**	
WC (%) (total aerial part)	Control	44.92 ± 8.82ab	43.71 ± 5.47a	64.72 ± 4.99b*	52.76 ± 22.06ab	58.32 ± 5.1ab***	52.08 ± 2.22ab/α***
	WS	—	32.92 ± 18.6α	33.99 ± 22.6α*	51.25 ± 20.63α	34.9 ± 3.27α***	
FW (g) (total roots)	Control	0.59 ± 0.28a	0.92 ± 0.63a	0.77 ± 0.12a**	0.88 ± 0.3a	0.83 ± 0.28a*	0.46 ± 0.11a*/α
	WS	—	0.96 ± 0.43β	0.44 ± 0.11α**	0.65 ± 0.27αβ	0.43 ± 0.1α*	
WC (%) (total roots)	Control	54.79 ± 9.8ab	57.31 ± 15.18ab	73.14 ± 3.84b**	70.38 ± 10.71b	51.2 ± 6.87a**	51.45 ± 7.44a/βγ**
	WS	—	61.85 ± 8.54γ	**42.04 ± 15.31**αβ**	57.61 ± 8.01βγ	**31.33 ± 9.91**α**	

Table 1: Continued

Retama raetam subsp. *bovei*		Treatment time (days)					
		0	**7**	**14**	**21**	**28**	**Rehydration**
Total leaf number	Control	35.2 ± 5.63ab	37.6 ± 6.66ab	42.8 ± 7.91b	42.2 ± 7.73b***	57 ± 24.16b***	17.6 ± 5.03a**/αβ
	WS	—	44.4 ± 22.19γ	32 ± 7.07βγ	21.4 ± 4.39β***	0α***	
Primary stem length (cm)	Control	49.2 ± 9.86a	52.8 ± 11.47a	61.4 ± 4.16a	55.2 ± 5.26a	60 ± 5.52a	62.2 ± 7.36a/α
	WS	—	62.5 ± 7.04α	58 ± 5.83α	55.4 ± 5.46α	60.2 ± 3.11α	
Maximum roots length (cm)	Control	22.8 ± 2.05a	30.4 ± 5.18ab	31.4 ± 4.04ab	30.2 ± 2.95ab	35.2 ± 8.41b	33.4 ± 8.68ab/α
	WS	—	32.4 ± 5.45α	32.2 ± 7.22α	30.2 ± 4.32α	30.4 ± 3.51α	
Primary stem diameter (mm)	Control	1.89 ± 0.23ab	1.97 ± 0.22ab	1.8 ± 0.16a	2.15 ± 0.2ab***	2.24 ± 0.17b***	2.06 ± 0.23ab/β**
	WS	—	1.88 ± 0.22αβ	1.38 ± 0.53α	1.54 ± 0.14αβ***	1.52 ± 0.16αβ***	
FW (g) (total aerial part)	Control	1.66 ± 0.45a	1.83 ± 0.53a	1.86 ± 0.63ab	2.91 ± 0.29b***	2.77 ± 0.83ab*	1.95 ± 0.26ab/α
	WS	—	2.09 ± 0.73α	1.36 ± 0.17α	1.57 ± 0.06α***	1.42 ± 0.5α*	
WC (%) (total aerial part)	Control	49.27 ± 13.39a	39.43 ± 12.61a	49.31 ± 9.64a	54.42 ± 11.71a	51.28 ± 7.82a***	53.01 ± 3.59a/β***
	WS	—	36.05 ± 19.16αβ	44.55 ± 11.61β	51.47 ± 5.69β	21.31 ± 7.95α***	
FW (g) (total roots)	Control	0.41 ± 0.12a	0.73 ± 0.13ab	0.96 ± 0.32b*	0.92 ± 0.24b	0.54 ± 0.09a**	0.5 ± 0.12a/α
	Water stress	—	0.9 ± 0.18γ	0.56 ± 0.04αβ*	0.64 ± 0.2β	0.31 ± 0.03α**	
WC (%) (total roots)	Control	53.89 ± 15.95a	57.58 ± 9.24a	66.07 ± 12.39a	61.53 ± 14.67a	54.01 ± 4.26a***	56.53 ± 1.97a/β***
	Water stress	—	62.58 ± 11.4β	58.16 ± 6.65β	57.87 ± 2.61β	18.07 ± 9.17α***	

Results are expressed as means ± standard deviations, $n = 5$. Values in the same row followed by different letters (Latin for control and Greek for stressed plants) are significantly different according to ANOVA ($P < 0.05$, Tukey's test). Bold data within the same column and parameter studied, including Rehydration data (compared with Control and WS plants at 28 days of treatment), denote the existence of significant differences according to ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

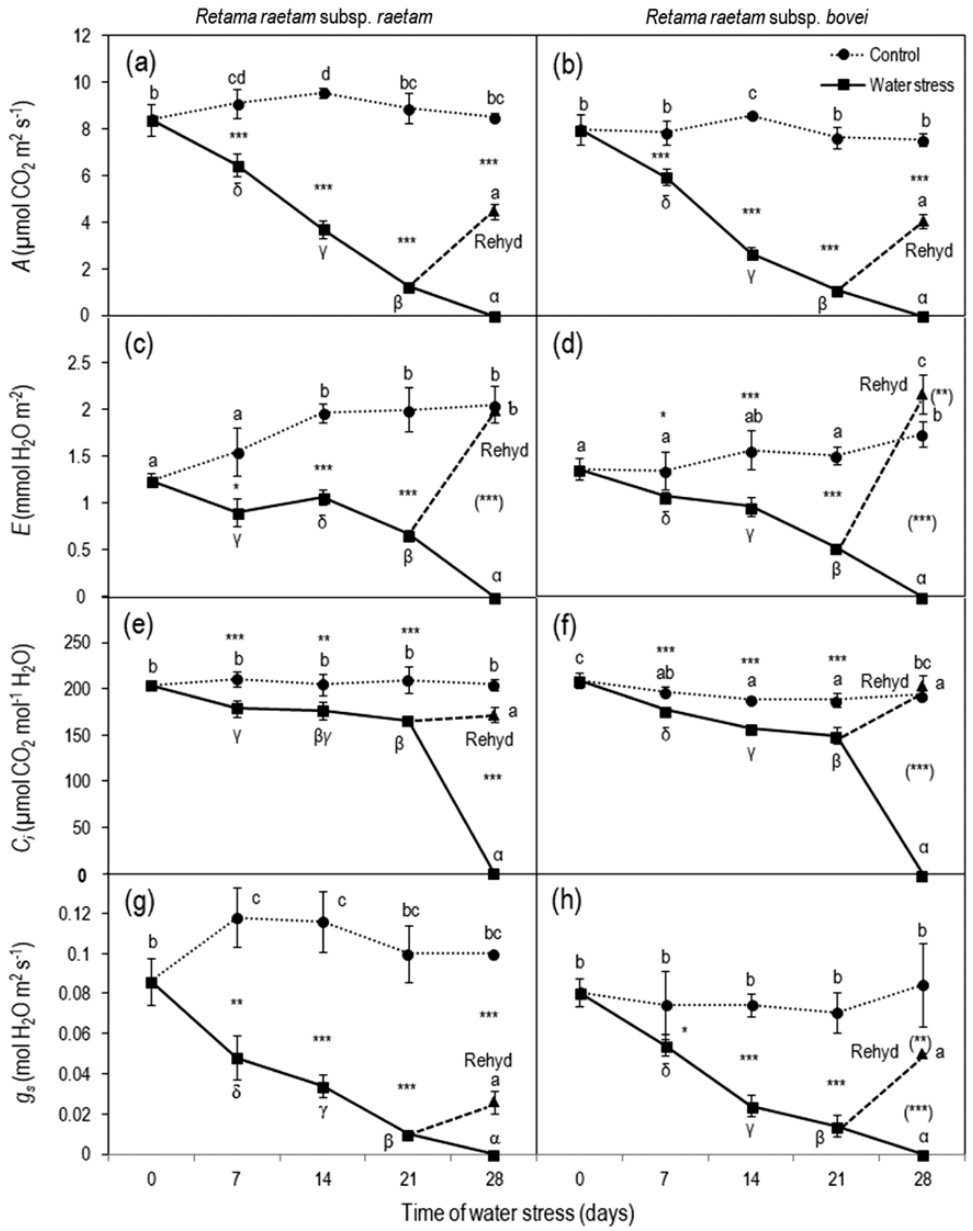

Figure 2: Changes in the levels of net photosynthesis assimilation—*A* (µmol CO_2 m^2 s^{-1}) (**a**, **b**), transpiration—*E* (mmol H_2O m^{-2}) (**c**, **d**), intercellular CO_2 concentration—C_i (µmol CO_2 mol^{-1} H_2O) (**e**, **f**) and stomatal conductance—g_s (mol H_2O m^{-2} s^{-1}) (**g**, **h**) of the water-stressed *Retama raetam* subsp. *raetam* (*RRr*) (a, c, e, g) and subsp. *bovei* (*RRb*) (b, d, f, h) compared with the untreated controls (means ± standard deviations, $n = 5$). Values after rehydrating the plants treated for 3 weeks are indicated as 'Rehyd'. Different letters (Latin for control plants, Greek for water-stressed plants) indicate significant differences between treatments, according to ANOVA ($P < 0.05$, Tukey's test). Asterisks denote significant differences between control and water-stressed plants, at each specific time of treatment, according to ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

CO_2 mol^{-1} H_2O until treatment day 21. Later, these levels drastically dropped to zero in both subspecies. The leaf WUE levels also significantly dropped from week 2 and were zero by the end of week 4 (Fig. 3a and b). The Ψ_w levels confirmed that the *RRb* plants were more stressed than the *RRr* ones, despite being subjected to the same drought conditions. From the second week of treatment onwards, the water potentials in the plants were around −8 to −14 mbar lower in those from *RRb* compared with *RRr* (Fig. 3c and d). The irrigated plants presented no significant differences between treatments for both subspecies for the C_i, g_s and Ψ_w levels, and slight variations were found in *A* for the controls. The *E* levels increased by a

maximum of 1.6-fold in the untreated controls being quantitatively higher in *RRr* than in *RRb*. Although the leaf WUE levels also decreased in the controls, they were always significantly higher than those found in the stressed plants, for both subspecies. Rehydration of plants after 3 weeks of water stress treatment significantly improved all the analysed photosynthetic parameters, even reaching the same values as the untreated control plants in the case of E and C_i (Figs 3 and 4).

Osmolytes content

The Pro levels in the stressed plants remained low (<1 µmol g^{-1} DW) in *RRr* during the first week, and until the second week in *RRb* (Fig. 4a and b). Later, the Pro concentrations progressively and significantly increased until they reached the highest levels at the end of the treatments, and were 24- and 16-fold (18.3 and 11.5 µmol Pro g^{-1} DW) higher than in the untreated controls of *RRr* and *RRb*, respectively. A similar pattern was recorded for the GB contents, which increased by ~6- and 3-fold (55.29 and 26.9 µmol GB g^{-1} DW) in the same BH and SM populations, respectively (Fig. 4c and d). TSS also rose significantly with increasing daily water stress in *RRr*, but levels were similar to those found in the control plants. The highest TSS levels detected in the treated plants were 36.5 and 18.7 mg eq. glucose g^{-1} DW for *RRr* and *RRb*, respectively (Fig. 4e and f).

DISCUSSION

In the present study, the germination and growth responses of two *R. raetam* subspecies collected from two regions in South Tunisia were investigated. Plant establishment success in these arid zones depends mainly on germination success. Seed emergence is strongly influenced by temperature and soil water availability. The results showed that both studied *R. raetam* subspecies could germinate at a wide range of temperatures (5–30 °C) with a maximum germination capacity at 15 °C when considering the optimal temperature defined by Probert (1992). Their germination percentages and speed gradually

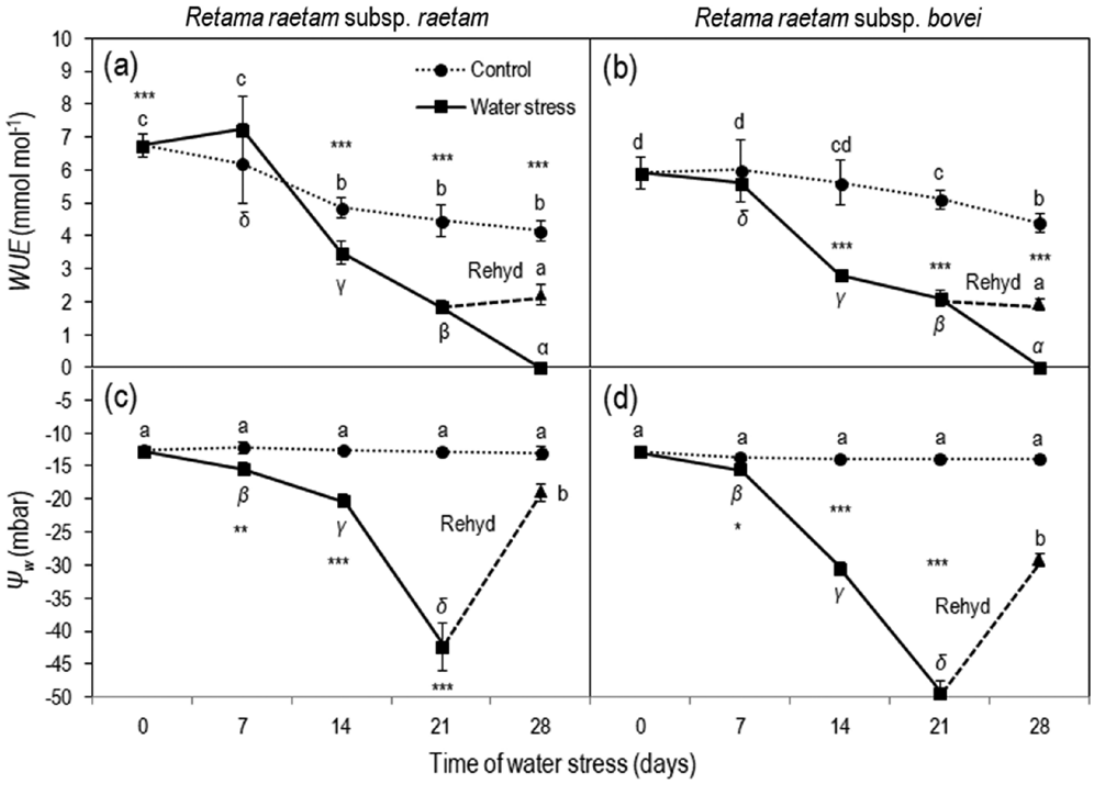

Figure 3: Changes in the levels of leaf water-use efficiency—WUE (mmol mol^{-1}) (**a**, **b**) and water potentials—Ψ_w (mbar) (**c**, **d**) of the water-stressed *Retama raetam* subsp. *raetam* (*RRr*) (a, c) and subsp. *bovei* (*RRb*) (b, d) compared with the untreated controls (means ± standard deviations, $n = 5$). Values after rehydrating the plants treated for 3 weeks are indicated as 'Rehyd'. Different letters (Latin for control plants, Greek for water-stressed plants) indicate significant differences between treatments, according to ANOVA ($P < 0.05$, Tukey's test). Asterisks denote significant differences between control and water-stressed plants, for each specific time of treatment, according to ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

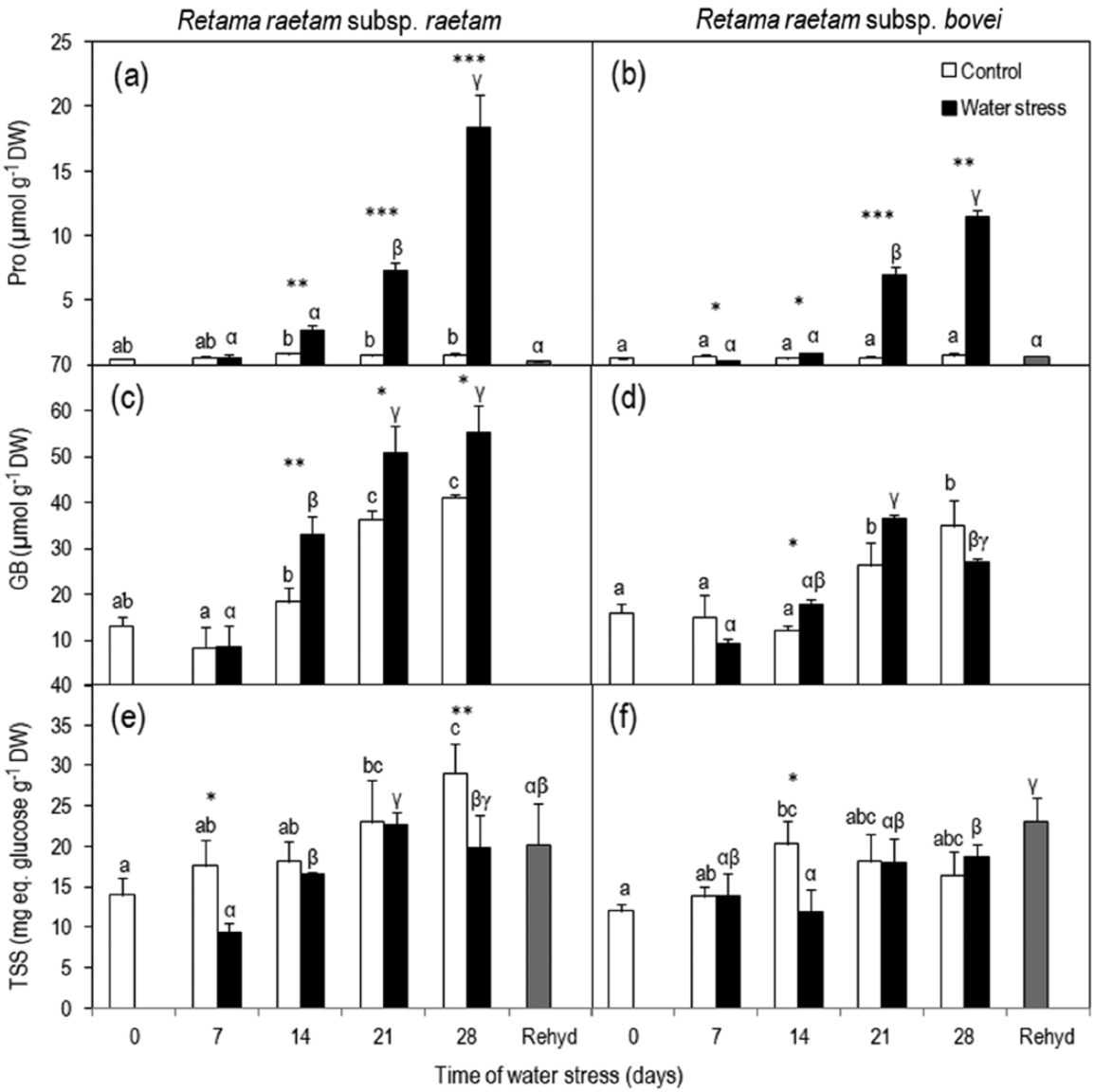

Figure 4: Changes in the concentrations of Pro (µmol g^{-1} DW) (**a**, **b**), GB (µmol g^{-1} DW) (**c**, **d**) and TSS (mg eq. glucose g^{-1} DW) (**e**, **f**) of the water-stressed *Retama raetam* subsp. *raetam* (*RRr*) (a, c, e) and subsp. *bovei* (*RRb*) (b, d, f), compared with the untreated controls (mean ± standard deviations, $n = 5$). Values after rehydrating the plants treated for 3 weeks are indicated as 'Rehyd'. Different letters (Latin for control plants, Greek for water-stressed plants) indicate significant differences between treatments, according to ANOVA ($P < 0.05$, Tukey's test). Asterisks denote significant differences between control and water-stressed plants, at each specific time of treatment, according to ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

lowered as temperatures rose from the optimum one, and were inhibited at 35 °C. These results coincide with those found by Abdellaoui *et al.* (2019). A study of the germination of three desert species from Egypt (*R. raetam*, *Ononis serrata* and *Mesembryanthemum crystallinum*) indicated similar maximum germination temperatures, which ranged between 15 and 20 °C, whereas germination was inhibited at 30 °C (Youssef 2009). The germination of many other desert plants, such as *D. harra* or *R. vermiculata*, has been reported to decrease with a rise or fall in temperatures from 15 °C (Gorai and Neffati 2007; Tlig *et al.* 2008). Mediterranean plants adopt a typical germination strategy of low optimum temperatures of 15–20 °C and a high germination rate (Baskin and Baskin 1998). For these plants, soil moisture conditions usually lead to germination early in spring, when winter and spring rainfall supplies moisture requirements (Tlig *et al.* 2008). When comparing both populations, *RRr* displayed a higher germination capacity and a faster

germination, compared with those collected from *RRb* plants within the studied temperature range and was thus more tolerant to non-optimal temperatures.

Drought stress is one of the most important environmental stresses to affect the germination process (Gorai *et al.* 2009). Our findings showed that moderate osmotic stress (−0.3 MPa) did not reduce the germination percentage in both studied *R. raetam* populations. However, with severe stress (−0.7 and −1 MPa), this parameter significantly decreased compared with the control plants. Studies into *RRb* from Southern Tunisia have also reported that seed germination is inhibited when water stress severity falls within the same range (Abdellaoui *et al.* 2019). The application of water potentials below −0.6 MPa to *R. raetam* seeds from Cairo-Suez (Egypt) rapidly reduced germination, whereas a potential of −1 MPa led to a complete inhibition (Youssef 2009). These results also coincide with reports of studies on other species, such as *Ziziphus lotus*, with seed germination percentages of 95% and ~5% for −0.4 and −1 MPa, respectively (Maraghni *et al.* 2010).

Plant responses to drought stress depend on the species, water shortage severity, age and development stage of the plants (Bray 1997). Drought strongly inhibited plant growth in both studied *R. raetam* subspecies and led to a marked reduction in DW, stem length, basal stem diameter and number of leaves in the stressed plants, as compared with the non-stressed controls. Growth inhibition was lower in *RRr* compared with the controls, with longer stem lengths from week 2 in the treated plants compared with those of *RRb*. Nevertheless, these results coincide with the different morphologies of these subspecies in the adult state. Plants from BH (*RRr*) are higher and show an upward growth tendency by emitting only a few secondary branches, unlike those from SM (*RRb*), which are characterized as being short shrubs that colonize broad areas by growing laterally. This different morphology can be explained as the result of an adaptation process to extreme drought conditions as *RRr* plants are subjected to more restrictive water stress conditions. Plants may escape drought stress by cutting short their growth period, or by avoiding stress with high water tissue potentials; i.e. by reducing water loss, by improving water uptake or by a combination of both. As an example, some plants may reduce their surface area by shedding leaves or by producing smaller leaves to withstand drought, but this results in lower growth and biomass production rates (Farooq *et al.* 2009).

Photosynthesis, along with cell growth, is one of the main primary processes to be affected by drought (Chaves *et al.* 2002). The photosynthesis results showed that drought significantly decreased gas exchange parameters like A, E and g_s in both *R. raetam* subspecies (Fig. 2). These reductions were more marked in the *R. raetam* plants than live on the coast in a semiarid habitat (*RRb*) than in those from a desert climate habitat (*RRr*), indicating that the former had slightly lower photosynthesis rates than the latter *RRr*. Despite living in more restrictive conditions, the water potentials of the plants from BH were more positive and indicated higher WC. Plants were less stressed under the water shortage conditions of our experiments, as compared with those from SM, which indicates higher water deficit tolerance as they are better adapted to drought in nature. Lawlor and Cornic (2002) reported that the A of higher plants substantially decreased as the leaf water potential and relative WC lowered. In *R. raetam*, seasonal variations have been described in the daily photosynthetic responses, which suggest that there are acclimation processes for drought resistance (Merquiol *et al.* 2002; Mittler *et al.* 2001). In fact, the extent to which the photosynthesis rate of an individual species is depressed in summer may depend on each species' specific adaptations, and also on the particular climate conditions of each living habitat (Flexas *et al.* 2013). CO_2-exchange measurements have been regarded as reliable indicators of plant growth rates given their direct implication in photosynthesis and net productivity (Ashraf 2004). The effect of water stress on reduced photosynthesis can be caused by either stomatal or non-stomatal factors (Athar and Ashraf 2005). There are reports on the effects of diffusion limitations through the stomata and mesophyll, and photosynthetic metabolism alterations, which usually lead to direct reductions in A levels (Flexas *et al.* 2004; Lawlor and Cornic 2002). Our results confirmed that significant reductions in g_s produced major reductions in A, and also in C_i, but to a lesser extent. Recently, Huang *et al.* (2020a, 2020b) used a general model, based on biochemical kinetics, to show how some parameters like WC, body mass and temperature affect plant metabolic rates and growth, implicitly including plant respiration and photosynthesis. Therefore, the decreasing leaf WUE of the untreated controls could be explained by the increasing body mass during growth.

Accumulation of compatible solutes, such as Pro, GB and TSS, in plants benefits stressed cells

by protecting or stabilizing macromolecules and structures from damage induced by abiotic stress (Bohnert and Jensen 1996). In this study, the levels of free Pro and GB significantly increased under drought for both subspecies (Fig. 4). This suggests that these osmolytes play an essential role in the responses of *R. raetam* to drought, as previously found for other shrub species (Lansac *et al.* 1994). Osmotic adjustment is considered one of the crucial mechanisms in plant adaptation to various stresses, but it vastly varies among species, and even among subspecies or varieties within a single species (Chaves *et al.* 2003). TSS, GB and Pro are major constituents of osmotic regulation in many plants (Alhaithloul 2019; Szabados and Savouré 2010). Besides its role in osmotic adjustment, Pro protects plasma membrane integrity, prevents protein denaturation, acts as an energy sink and carbon and nitrogen source, and also as a hydroxyl radical scavenger (Bartels and Sunkar 2005; Hare *et al.* 1998; Szabados and Savouré 2010). GB plays a key role as an osmoprotectant in cells subjected to stress (Ashraf and Harris 2004). TSS perform different functions in plants ranging from energy storage to signalling and their accumulation in cells has been associated with stress tolerance (Bartels and Sunkar 2005; Chaves *et al.* 2003). In fact, increased levels of these osmolytes have been directly related to resistance to stress of plants living in extreme environments (Szabados *et al.* 2011). Our work shows that, in response to water stress, *RRr* plants synthesize and accumulate higher levels of osmoprotectant compatible solutes, specifically Pro and GB, than *RRb* plants, which correlates with the better adaptation to drought of *R. raetam* subsp. *raetam*.

CONCLUSIONS

Morphological adaptations and physiological responses to drought in the studied subspecies confirmed that *R. raetam* can adapt to different habitats. In evolutionary terms, this must be crucial for determining actual phenotypic variability in this species. Resistance to water stress agreed with the relative levels of the quantified osmolytes, which likely acted in cellular osmotic adjustment and also as osmoprotectants. In fact, the greater drought tolerance of subsp. *raetam* (*RRr*) correlated with higher intracellular levels of GB and Pro, compared with subsp. *bovei*. The measured photosynthetic and growth parameters confirmed that *RRr* possesses more efficient biological mechanisms of resistance to drought than *RRb*, which allows these plants to develop in extreme habitats, as shown by their geographical distribution.

Funding

Dhikra Zayoud's stay in Valencia was supported by a grant from the Tunisian Ministry of Higher Education and Scientific Research.

Acknowledgements

The experiments concerning seed germination and photosynthetic parameters were performed at the Institut des Régions Arides (Tunisia). The growth characterization and biochemical assays were conducted at the Institute for Plant Molecular and Cell Biology (Spain).

Conflict of interest statement. The authors declare that they have no conflict of interest.

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