Soriano, P.; Moruno Manchón, JF.; Boscaiu Neagu, MT.; Vicente Meana, Ó.; Hurtado, A.; Llinares Palacios, JV.; Estrelles, E. (2014). Is salinity the main ecologic factor that shapes the distribution of two endemic Mediterranean plant species of the genus Gypsophila?. Plant and Soil. 384(1-2):363-379. doi:10.1007/s11104-014-2218-2.

Is salinity the main ecologic factor that shapes the distribution of two endemic Mediterranean plant species of the genus *Gypsophila*?

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Abstract

Aims Responses to salt stress of two *Gypsophila* species that share territory, but with different ecological optima and distribution ranges, were analysed. *G. struthium* is a regionally dominant Iberian endemic gypsophyte, whereas *G. tomentosa* is a narrow endemic reported as halophyte. The working hypothesis is that salt tolerance shapes the presence of these species in their specific habitats.

Methods Taking a multidisciplinary approach, we assessed the soil characteristics and vegetation structure at the sampling site, seed germination and seedling development, growth and flowering, synthesis of proline and cation accumulation under artificial conditions of increasing salt stress.

Results Soil salinity was low at the all sampling points where the two species grow, but moisture was higher in the area of *G. tomentosa*. No considerable differences were found in the species' salt tolerance. The different responses observed in the studied parameters did not show a clear pattern indicating that one of them was more tolerant to salinity.

Conclusions G. tomentosa cannot be considered a true halophyte as previously reported because it is unable to complete its life cycle under salinity. The presence of *G. tomentosa* in habitats bordering salt marshes is a strategy to avoid plant competition and extreme water stress.

Keywords *Gypsophila*, salt germination, reproductive success, soil paterns, proline, cation accumulation.

Introduction

Soil endemics are plants present in diverse territories and climates whose distribution is limited by their specificity to different soil types. Gypsophytes and halophytes, confined in gypsum and salty soils, respectively, are excellent examples. Diverse anatomical and physiological mechanisms enable these species to colonise extreme habitats where they find less competition. Furthermore, restricted adaptation to the environmental conditions of soil endemics limits their presence to specific plant communities in these habitats.

Saline soils contain diverse types of salts, such as NaCl, $CaCl_2$, gypsum ($CaSO_4$), $MgSO_4$, KCl, etc. Erosion, water flow and topography are responsible for salt distribution. Therefore, the most soluble ones are accumulated by lixiviation in the lowest areas in small endorheic hollows (saline depressions), while the least soluble ones, like gypsum, remain on hills. This characteristic behaviour has been described for diverse territories (Peinado and Martínez-Parras 1982; Breckle 1999).

One clear example of natural stressful environments is the gypsum habitat, which often shelters rare, threatened and endemic plants. Gypsum soils cover more than 100 million ha around the world and they have certain physical constraints, such as limited water retention, presence of a hard soil surface crust that can restrict seedling establishment, mechanical instability and lack of plasticity and cohesion, structural deterioration and low porosity that interfere with root growth (Palacio et al. 2007). In addition, such soils also have some unsuitable chemical characteristics for plant development: deficiency of some macronutrients, ionic antagonisms (Ca/Mg), unbalanced ion concentration, with excess sulphur and calcium, and toxicity due to a high concentration of sulphate ions (Mota et al. 2004; Palacios et al. 2007).

Soil salinity is usually related to presence of sodium chloride. A high NaCl concentration in soil is one of the most restrictive environmental factors (osmotic and ionic stress), and only a small category of plants, halophytes, have adapted to survive and complete their biological cycle under such conditions. The exact definition of halophytes is ambiguous and controversial (Grigore et al. 2012a, and references therein). Halophytes are generally considered to be plants that can grow and complete their life cycle in habitats with soil salinity above 200 mM NaCl (Flowers et al. 1986; Flowers and Colmer 2008). This is a broad operational definition since, obviously, the concentration threshold largely varies among species, and there is a continuous spectrum of salt tolerance among plant species, ranging from typical glycophytes (salt-sensitive plants) to extreme halophytes. Natural saline habitats range from wet maritime environments, such as salt marshes and mangrove swamps, to arid salt deserts (Flowers et al. 1986). The estimated area of salt-affected

soils comes close to 1 billion ha, which represents about 7% of the earth's continental extent (Ghassemi et al. 1995).

For our study, we selected two Iberian endemic species of the genus *Gypsophila* L., that coexist in the same territory, but whose scale of distribution and ecological optimum considerably differ. Taxonomically, the two species are closely related, and are included in the subgenus *Gypsophila* (López Gonzalez 1990). Both are perennial, and have some morphological and phenological differences. *Gypsophila struthium* L. subsp. *struthium* is one of the most abundant gypsophytes in Spain, and is exclusive of gypsum soils. It is specifically adapted to gypsum, which even has a positive effect on the germination of its seeds (Cañadas et al. 2013). This species is endemic in the SE Iberian Peninsula, with a wide distribution in the C, E and S, in the Murcian-Almerian, Balearic-Catalonian-Provençal, Baetic and Mediterranean Central Iberian biogeographic provinces. *G. tomentosa* L., an Iberian endemism from C, E and S Spain, is less frequent, has much smaller populations and is considered as a halophyte, specific for saline environments (Peinado and Martínez-Parras 1982; García Fuentes et al. 2001; Marchal et al. 2008), but is also regarded as a subgypsophyte (Mota et al. 2009). The two species can share the same geographic area, but usually appear in different plant communities. *G. struthium* subsp. *struthium* is frequent in several associations of the vegetation order *Gypsophiletalia* Bellot and Rivas Goday in Rivas Goday et al. 1957 (Rivas Martínez et al 2001; Ferrandis et al. 2005; Marchal et al. 2008), whereas *G. tomentosa* is characteristic of three associations, all of which belong to the order *Limonietalia* Br.-Bl. & O. Bolòs 1958 (Peinado and Martínez-Parras 1982; Rivas Martínez et al. 2001).

There is some evidence that Mediterranean restricted endemics are more ecologically specialised than their widespread congeners (Médail and Verlaque 1997; Debussche and Thompson 2003), but detailed case studies are still scarce. Indeed, there are still relatively few papers that deal with either stress tolerance in endemics that include characteristics of their habitats (Lidón et al. 2009; Boscaiu et al. 2013a) or response to stress in congeners with different scales of distribution (Ishikawa and Kachi 2000).

Two main questions were posed in the present study. Firstly, what are the most important edaphic differences between the habitats of the two species? Secondly, does their tolerance to NaCl differ, and if so, does soil salinity shape their distribution pattern? Such questions can be approached only from a multidisciplinary perspective. Therefore, a detailed field study in the selected area was carried out, followed by an analysis of the two species' response to salt stress in different developmental stages to assess: (a) soil characteristics in relation with the two species' distribution pattern; (b) the phytosociological characterisation of the study area; (c) the two species' seed germination and seedling growth responses at different salt concentrations; (d) the two species'

growth and flowering under salt stress conditions; (e) synthesis of proline, one of the commonest osmolytes in plants; and (f) levels of mono- and bivalent cation accumulation.

Material and methods

Origin of plant material

Seeds of *G. struthium* were collected from a protected area, Los Cabecicos, and seeds of *G. tomentosa* were taken from an adjoining area, Salinas de la Redonda, with halophytic vegetation dominated by *Sarcocornia fruticosa* and *Arthrocnemum macrostachyum*. Both localities are situated at an altitude of roughly 500 m, in the Valley of Villena in the Vinalopó basin, a river that is dry for most of the year; this valley is located in the Alicantine sector of the Murcian-Almerian biogeographic province (SE Spain), and is surrounded by small mountains of the Baetic range. The substrate is formed mainly by a gypsicolous Keuper Triassic formation with saline facies alternating with Jurassic dolomites (Alonso 1996). The climate is of an upper Mesomediterranean thermotype, continental, with accentuated temperature contrast (m_1=-0.4; M_8=31). Rainfall is very low (less than 400 mm/year) due to the rain shadow effect of the mountains, and the ombrotype is semi-arid in the valley and dry in the neighbouring mountains (Rivas-Martínez and Rivas-Saenz 1996-2009).

Vegetation analysis

Plant communities in the two species' sampling area were analysed by following the phytosociological methods of the Sigmatist School of Zurich-Montpellier, which were successively integrated (Rivas-Martínez 2005; Géhu 2006; Biondi 2011; Géhu 2011).

Soil analysis

Soil analyses were carried out on the samples collected in November. This month is optimal for seed germination in both species as it is part of the wet season, when temperatures are still mild and seeds show no dormancy after dispersal, as we previously determined (Moruno et al. 2011). Soil sampling was performed along a linear transect from the zone where *G. struthium* inhabits up to the saline depression, including the area where *G. tomentosa* grows (Fig. 1). In all, 28 samples were taken every 5 m at a depth of 20 cm using a 100-cm^3 cylinder to determine bulk density for the chemical analysis. Three soil profiles were characterised by identifying the horizons, texture, colour

and content in chlorides, carbonates and organic matter by a qualitative valuation (FAO 2006). Soil salinity was characterised in a saturation extract by measuring electrical conductivity (EC) with a Hanna Instruments HI98312 portable conductimeter, and soil pH was measured using a Hanna Instruments HI98107 portable pH-meter. Ca^{2+} and Mg^{2+} were analysed by complexometric methods. Anions were analysed by standard procedures: HCO_3^- by titration with H_2SO_4; $SO4^{2-}$ by the Versenate method; Cl^- by the silver nitrate method. Na^+ and K^+ were estimated as the difference between the sum of the measured anions and cations on the basis of ionic balance. Limestone was measured by the Bernard calcimeter method. Finally, the following physical parameters were determined for each sample: field moisture, humidity, saturation density.

Germination experiments

In a first experiment, seeds were germinated in Petri dishes (four replicates of 25 seeds each) on 0.6% agar with NaCl solutions (0, 100, 200, 300, 400 and 500 mM). Germination was carried out at 15°C in the darkness, which is considered the optimal condition for these species (Moruno et al. 2011). Seed germination was monitored over 20 days and the germinated seeds were removed from the dishes. Germination was expressed as the final mean percentages ± standard deviation (s.d.). Additionally, reduction of germination percentage was calculated as RGP = [1-(number of germinated seeds in the salt/germinated seeds in the control)] x 100. Velocity of germination (MGT) was expressed as the mean germination time (Brenchley and Probert 1998). The germination rate was also expressed using a modified Timson's index (TI) according to Ungar (1996).

All the seeds that did not germinate in the previous experiment were thoroughly washed in distilled water and were then transferred to new Petri dishes with 0.6% agar. They remained in the germination chamber under the aforementioned conditions in order to check their recovery capacity. The recovery germination percentage was determined by applying the equation described by Khan et al. (2000). Furthermore, the total germination percentage (germinated seeds in salt solution + germinated seeds after being transferred to distilled water) was calculated.

The osmotic potential (Ψ) at each NaCl concentration was assessed by the van't Hoff's equation. As the linear relation between the inverse of MGT and Ψ (Bradford 1990) is accepted, the base potential (Ψ_b) of each species was calculated by extrapolating the least-squares regression line on the 1/MGT plot against Ψ to the x-axis intercept. The hydrotime (Θ) was also estimated as the inverse of the slope of this regression line (Kebreab and Murdoch 1999).

Effects of salinity on seedling development

To test the effect of salinity on seedling development, radicle and plumule growth were measured. For this purpose, seeds were sown in 14-cm diameter Petri dishes with 1% agar supplemented with 0, 50, 100, 150 and 200 mM NaCl at 15ºC. Seeds were placed in a line and arranged vertically in the incubator to allow radicle growth on the agar surface to facilitate measurements. The seedlings´ radicle and plumule lengths were determined after post-sowing day 9 using the ImageJ software (Rasband 1997-2012). The reduction percentage of radicle and plumule development was calculated as RPR and RPP, respectively [1-(length in salt/length in control)] x 100 (Madidi et al. 2004). At the end of the experiment, the seedling survival (SS) percentage was calculated.

Effects of salinity on plant growth and flowering

Plants (n=20) were obtained by directly sowing seeds in pots with a mixture of peat, coconut fibre and sand (appreciatively 3:2:1). They were kept in a greenhouse with controlled maximal and minimal temperatures. When plants were 2-months-old, treatments with aqueous NaCl solutions (100, 200, 300, 400 and 500 mM) and a control without salt were applied. Plants were watered weekly by applying the corresponding salt solutions or distilled water on the trays where the pots were placed. After 90 days, four plants from each treatment were harvested and fresh weight was measured. Leaf material was partially stored at -80ºC until used for the analysis described below and was partially dried in an oven at 60ºC until constant weight to be then ground to a moderately coarse powder and stored at room temperature.

The saline treatments were continued with the remaining plants (n=16) until flowering. The number of flowers was recorded weekly. After anthesis, all the flowers were enclosed in paper bags to avoid loss of seeds, which were harvested after capsules had ripened. Fresh plant weight decrease (RPW) and reduction of flower production (RPF) were expressed as percentages in relation to the values obtained in the controls.

Proline determination

Proline content was determined from the frozen plants (n=4) following the method of Bates et al. (1972), but with the modifications by Vicente et al. (2004). Extraction was carried out with 3% sulphosalicylic acid, and cell debris were removed by filtration. One filtrate volume was mixed with one volume of freshly prepared acid ninhydrin and one volume of glacial acetic acid to be incubated at 95ºC for 1 h. The reaction was stopped by cooling on ice and samples extracted with two

volumes of toluene. The absorbance of the organic phase was determined at 520 nm using toluene as a blank.

Cation accumulation

Na^+, K^+, Ca^{2+} and Mg^{2+} were quantified in the dry leaves obtained from the same individuals for proline determination. Dry leaf material was digested in a microwave digestor (Model: Ethos One, Milestone Microwave Laboratory Systems) as detailed by Grigore et al. (2012b). Quantification of Na^+ and K^+ was performed by a flame photometry Jenway PFP7, and by an atomic absorption spectrometry (Model: Varian SpectrAA 220) for the bivalent cations, at 239.9 nm for Ca^{2+} and at 202.6 nm for Mg^{2+}.

Data analysis

The statistical analysis was performed using the SPSS 16.0 statistical software. Germination percentages were arcsine-transformed prior to the analysis. The significance of the differences among treatments was tested by applying a one-way ANOVA because this test is a very robust method that provides good approximations for small samples when model assumptions are not fully satisfied (Khan and Rayner 2003). When the ANOVA null hypothesis was rejected ($p<0.05$), a *post hoc* Tukey test was used to estimate homogeneous groups when more than two samples were compared.

Results

Vegetation analysis

The selected species are characteristic of two associations included in the different vegetation orders: *Gypsophiletalia* (*G. struthium*) and *Limonietalia* (*G. tomentosa*), belonging to the classes *Rosmarinetea officinalis* Rivas-Martínez, T.E. Díaz, F. Prieto, Loidi & Penas 2002 and *Sarcocornietea fruticosae* Br.-Bl. and Tüxen ex A. and O. Bolòs 1950, respectively.

G. struthium characterises the community *Helianthemo thibaudii-Teucrietum libanitidis* Rivas Goday & Rigual in Rivas Goday, Borja, Monasterio, Galiano, Rigual & Rivas-Martínez 1957 corr. Díez Garretas, Fernández-González & Asensi 1996 nom. mut. This type of vegetation corresponds to a priority habitat according the Nature 2000 Network, 1520* Iberian gypsum steppes (*Gypsophiletalia*).

G. tomentosa is characteristic of the association *Limonio delicatuli-Gypsophiletum tomentosae* Peinado *et* Mart. Parras 1982, an endemic community of subsaline soils from the SE area of the Iberian Peninsula with hyperhalophilous vegetation, including *Sarcocornia fruticosa* and *Arthrocnemum macrostachyum* (*Frankenio corymbosae-Arthrocnemetum macrostachyi*, *Limonio cossoniani-Sarcocornietum fruticosae*). An idealised catenal schema of the vegetation communities along the analysed transect is represented in Fig. 1.

Soil analysis

Three sections with distinctive features were identified along the transect, which corresponded to the three different profiles analysed (Fig. 2). The first section (soil samples 1 to 14) corresponds to the area of *G. struthium*, the second one (samples 15 to 25) to that of *G. tomentosa*, whereas the third one (samples 26-28) is situated in the more depressed area, in the salt marsh, where neither species grows (see Fig. 1). Regarding physical characteristics, the samples of the first two sections mainly presented a sandy texture, whereas the third section had a finer texture that varied from sandy-silty to silty-clayey the deeper the soil depth. This texture type is specific for saline lands with temporary flooding. However, the first section was characterised by more marked stoniness and compaction, as shallow soil depth hampers plant rooting, and low water retention capacity and humidity (<15%). In the second section, soil was deeper in the upper zone, but became shallower towards the lowest area, where organic matter content was very low. In these areas, the gypsum crust is formed increasing bulk density from 0.7 to 1.3 g cm^{-3}. As reflected in Fig. 3, field moisture was higher in the second transect (15-22%) and reached the maximum values in the third section (20-30%).

Regarding EC, both the first sections showed relatively low values, ranging from 2.4 to 3.0 dS/m. However, some peaks of 5.0-6.0 dS/m were observed in the second section as a result of the formation of small depressions in the basal area of the transect. The third section (26 to 28) is situated in the lower area and is identified by a high EC (33-73 dS/m) (Fig. 3).

Along the whole transect, pH was comprised between 6.6 and 7.9, and the percentage of limestone varied between 5-25%, with smaller values towards the lower points. The chemical composition (Fig. 4) of samples from the first section showed saturation of $CaSO_4$ and $MgSO_4$ (16-29 mmol/L), with Ca^{2+} being the predominant cation. In the second section, chlorides were already present, which increased when approaching the third section of the transect, where they became dominant (Cl^->600 mmol/L). This establishes a close relationship between EC and chloride concentration (Fig. 5).

Germination assays

The germination responses under different salinities for both species are shown in Table 1. All the considered parameters showed statistically significant differences according to the variation of osmotic potential in both species. Maximum germination percentage values were obtained in the absence of salt stress in the control treatments. Increasingly negative water potentials lowered the germination percentage in both species. No significant differences were observed up to a salinity value corresponding to 100 mM, but the effects on the germination percentages became evident at 150 mM, and seeds did not germinate at 300 mM of NaCl.

Significant differences in the reduction of germination percentage (RGP) between both species were found at 150 mM and 200 mM NaCl. This reduction was more marked for *G. tomentosa*, with germination declining from 95.0% to 10.4% from 0 to 200 mM NaCl, whereas the germination percentage of *G. struthium* only lowered from 86.6% at 0 mM to 49.4% at 200 mM. These values imply an RGP at 200 mM of 89.0% and 42.9% for each species, respectively (Table 1). Velocity parameters MGT and TI were significantly lower in *G. tomentosa* at 100 mM (Table 1; Fig. 6). The statistical analysis of the velocity indices for both two species gave F values of 39.9 and 118.4 for MGT, and of F=49.7 and F=637.3 for TI. Larger differences in the response of *G. tomentosa* to increasing salt levels in the medium were found.

The linear regression of the germination rates at the different osmotic potentials tested provide a Ψ_b of -1.80 MPa for *G. struthium* and of -1.37 MPa for *G. tomentosa*. The hydrotime (Θ) calculated for *G. struthium* was 8.81 MPa day and was 6.74 MPa day for *G. tomentosa*. The Ψ_b values should be treated with caution as they were obtained from extrapolation which went beyond the range of the experimental conditions. We considered these calculated values, Ψ_b and Θ, as theoretical figures. The regression followed the same pattern in both species. At all the osmotic potential values, *G. struthium* was above *G. tomentosa* (Fig. 6), indicating fiercer competitiveness for *G. struthium*.

The percentages (R) and the mean germination time (MGTR) in the recovery experiments are also presented in Table 1. The one-way ANOVA indicated that these two parameters were not significant for *G. struthium* (R: P=0.415, F=1.050; MGTR: P=0.378, F=1.135) and for *G. tomentosa* (R: P=0.454, F=0.967; MGTR: P=0.595, F=0.715).

In *G. struthium*, slightly lower recovery percentages were obtained at 150, 200 and 500 mM, but total germination (seeds germinated in salt solution and seeds germinated during recovery) for both species reached similar values to those in control (Gs: P=0.139, F=1.774; Gt: P=0.423, F=1.052) for all the NaCl treatments (Fig. 7).

The mean germination time for the recovery tests in both species was around 3 days less than the value calculated for the controls (Table 1).

When analysing the development of seedlings, we found high sensitivity to the salt environment (Fig. 8). Radicle length showed a significant reduction for both species, even at 50 mM NaCl (Fig. 8a), with a mean elongation of 8.99 mm for *G. struthium* and of 3.09 mm for *G. tomentosa*. This reduction was more marked in *G. tomentosa* with an RPR value of 85.2% than in *G. struthium* with a reduction of 56.5% at this concentration (Table 2) (Gs: P=0.000, F=112.611; Gt: P=0.000, F=484.612). Plumule growth also reduced at increasing salt concentrations: the plumule development of *G. tomentosa* was maintained with an RPP from 47.0 to 75.6% at between 50 and 150 mM NaCl, while it reduced for *G. struthium* at 50 mM with an RPP of 23.4%, and no growth was detected at higher concentrations (Gs: P=0.000, F=40.943; Gt: P=0.000, F=210.378).

In *G. struthium*, the percentage of surviving seedlings after 9 days lowered at 100 mM NaCl, although a portion of the sample remained alive even at 200 mM. Conversely in *G. tomentosa*, seedling survival was maintained at up to 150 mM NaCl, but growth was affected by salt (Table 2).

Effects of salinity on plant growth and reproductive success

Some salt treatments induced significant differences in the biomasses of the two species, as shown by the one-way ANOVA (denoted by an asterisk in Fig. 9). A lower fresh plant weight percentage (RPW) of *G. tomentosa*, if compared to the control, was observed even at 100 mM NaCl, although *G. struthium* continued to develop normally under this condition. Weight in *G. tomentosa* decreased progressively at increasing salt concentrations. However the *G. struthium* plants were severely affected at 300 mM NaCl, and their weight sharply dropped. The means of fresh weight and the s.d. for each treatment and species are shown in Figure 9, while reduced growth if compared to the control is presented in Table 2.

G. tomentosa flowered in all the treatments, but the number of flowers produced per plant was strongly affected by salt stress (see Table 2). Moreover, only the control plants produced viable seeds, whereas seeds were aborted in all the plants used in the saline treatments. In *G. struthium*, only the plants in the control treatment produced flowers.

Proline determination

An increase in proline, one of the commonest osmolytes in plants, was recorded in both species, as depicted in Figure 10. In *G. struthium*, the mean proline content increased from the control to the high saline treatment at 500 mM NaCl by 135-fold. In *G. tomentosa*, the plants from the control

treatment showed higher proline values than *G. struthium*. For this reason, although the values recorded at 500 mM NaCl were strikingly similar for both species, the difference between this treatment and the control was only 37-fold. The differences among treatments were significant for each species, but starting with the concentration of 200 mM NaCl the ANOVA was unable to detect differences between the two species.

Effects of NaCl treatments on cation accumulation

In *G. struthium*, the sodium levels in the control plants were low, but gradually increased in the 100 and 200 mM NaCl treatments to reach maximal values at 300 and 500 mM NaCl. *G. tomentosa* gave higher Na^+ values in all the treatments, except that of 500 mM NaCl, where the level of this cation suddenly dropped. Interestingly, this species is characterised by high Na^+ levels in the control (6-fold more than *C. struthium*), therefore the sodium increment in the saline treatments was far more accentuated in *G. struthium*. In this latter species, an increase of up to 8.5-fold in the 300 and 500 mM NaCl treatments was recorded, whereas in *G. tomentosa* the maximal Na^+ values found in the plants of the 300 mM NaCl treatment were only 2.15-fold if compared to the control plants (Fig. 11a). The K^+ accumulation pattern was similar for both species, with significant differences for the control and the 100 mM NaCl treatments as compared to the others, but the K^+ values were always higher in *G. tomentosa*. As expected, the K^+ levels lowered in both species in comparison to the control (Fig. 11b). Regarding calcium content, *G. struthium* gave the largest amounts in the control, and lowest ones in the 200 and 300 mM NaCl treatments. In *G. tomentosa*, Ca^{2+} decreased from the control to the 500 mM NaCl treatment, and a slight increment was recorded in the 300 and 400 mM treatments. When comparing the Ca^{2+} levels in the two species, considerably higher levels were detected in *G. struthium*, ranging from the double amount in the control to 6-fold in the 200 and 500 mM treatments *vs. G. tomentosa* (Fig. 11c). The mean magnesium values in *G. struthium* did not vastly vary, and were significantly lower only in the 400 and 500 mM treatments than in the control. Variation was greater in *G. tomentosa* with higher values in the control plants and the 100 mM NaCl treatment, while the recorded Mg^{2+} values were always higher for *G. struthium* (Fig. 11d).

Discussion

Distribution of soil endemics is related with plant specialisation, stress-tolerance and competitiveness. Two different behavioural strategies have been defined for the plants growing in gypsum soils in an attempt to justify the different distribution observed in regionally dominant gypsophiles and narrow-gypsophile endemics. Several authors (Meyer 1986; Palacio et al. 2007)

have proposed that these groups can fit two models, the 'specialist' model and the 'refuge' model, respectively. The target species in this study, *G. struthium* subsp. *struthium*, a regionally dominant gypsophyte, and *G. tomentosa*, restricted to the border of salt marshes in lower areas of gypsum habitats, correspond to these two different distribution patterns.

The soil requirements of *G. tomentosa* are not clearly established in the literature; in general, it has been reported as a halophyte and also as a subgypsophyte (Peinado and Martínez-Parras 1982; Mota et al. 2009). Our findings help clarify this issue. The analysis carried out on the vegetation structure in the studied communities indicated that it corresponds to the typical configuration and floristic composition observed in habitats of a complex geological composition that combines salt and gypsum soils (Peinado and Martínez-Parras 1982; Breckle 1999). Soil texture and composition did not reveal major differences between the two species areas. However, the area where *G. struthium* grows is characterised by low water retention capacity and humidity, greater stoniness and compaction, while soils where *G. tomentosa* inhabits are deeper and with higher humidity levels. In general terms, the ion concentration increases towards lower areas, and the increase in Na^+ and Cl^- is especially significant at the last points of the studied transect. In contrast to what might be expected, EC is relatively low in both areas: along the transect analysed and only at two sampling points, where *G. tomentosa* grows, EC slightly surpassed 4 dS/m, the value at which soil is considered saline (USDA-ARS, 2008). High EC was recorded only in the lowest area, in the central part of the lagoon, where neither species is able to grow.

The seed germination percentage in both species drastically lowered with increasing salt concentrations (at 150 mM in *G. tomentosa* and at 200 mM in *G. struthium*). Velocity of germination is a more sensitive parameter; it was already affected at 100 mM in *G. tomentosa* and at 150 mM in *G. struthium*. Likewise, the calculated hydrotime values show that *G. struthium* is a more competitive species than *G. tomentosa* when the osmotic potential decreases. The obtained results indicate that *G. tomentosa* is more sensitive to salt than *G. struthium* in the germination phase.

The recovery results demonstrate that those seeds exposed to high salinity showed equal germination as those from the control after transference to water. High recovery germination percentages indicate that previous seed germination was inhibited by an osmotic effect, whereas low germination indicates specific ion toxicity (Khan 2002). The seeds of both species obtained high recovery of germination when transferred to distilled water from hypersaline conditions after 20 d of exposure to all the salinity concentrations studied. The recovery germination experiments indicate no specific ion toxicity and that the osmotic effect limited germination.

The priming effects observed in other halophylous species of this genus, e.g., *Gypsophila oblanceolata* (Sekmen et al. 2012), were not observed in either of the species studied. Although

germination velocity increased as compared to the control, this is not a consequence of salt stimulation, but is due to the fact that the imbibition phase of germination had already finished.

These data indicate that seeds can remain in soil under field conditions when salinity levels go beyond their tolerance limits and germinate during the rainy period, in autumn, when salinity levels lower. In order to gain a complete understanding of the behaviour of *G. tomentosa*, it should be added that this species has adapted its phenology so that seeds are dispersed in autumn, this being the rainfall period. This, along with lack of primary dormancy, allows fresh seeds to be ready to germinate during the period in which salinity is almost alleviated.

Greatly reduced seedling development is considered to be the result of osmotic pressure, the ion toxic effect of salt and unbalanced nutrient uptake (Eskandari and Kazemi 2011). The seedlings of *G. tomentosa* survived at a concentration of up to 150 mM NaCl and maintained their viability. *G. struthium* obtained less viable seedlings when starting at 100 mM NaCl, although some seedlings survived during the test even at 200 mM. The measures and observations made on the reduction of seedling development and subsequent survival indicate a significant difference between both species. The seedlings of *G. tomentosa* displayed better physiological tolerance. Their growth reduced dramatically when the NaCl concentration increased, but they survived, which means that their further development is feasible when salinity is alleviated. This behaviour enables growth to continue after exposure to salt, thus species may colonise temporary saline soils.

Fresh weight progressively diminished in a concentration-dependent manner only in *G. tomentosa* as growth at the 100 mM NaCl concentration was not affected in *G. struthium*. The response at the reproduction stage proved more conclusive than the fresh weight analysis. *G. tomentosa* flowered in all the treatments, although the numbers of flowers significantly lowered with increasing salinity. In *G. struthium* however, only the plants from the control treatment flowered. Yet even in *G. tomentosa,* only these control plants proved reproductively successful since the plants from the saline treatments produced only aborted seeds. Apparently, even low saline concentration affects this species' reproductive success, which has implications for the floral phenology. Therefore we consider that this species is not a "*sensu stricto*" halophyte. This correlates with the soil analysis data: even though *G. tomentosa* is cited as a halophyte, our results indicate that it grows only on the borders of high saline areas, where it shelters from more competitive species. At the collection site, *G. tomentosa* flowers at the beginning of autumn when soil salinity is alleviated by the typical intense rainfalls during this period. On the contrary, *G. struthium* starts flowering at the beginning of summer because soil salinity does not play an ecological role in its habitat.

One of the major effects of saline stress is the osmotic component, which induces physiological drought. Plants compensate for this high osmotic pressure in the rizosphere by

synthesising the so-called osmolytes, diverse chemical compounds which, in large concentrations, play a major role in osmotic adjustment. Thanks to their specific hydrophilic structure, they act as osmoprotector substances by protecting thylakoids, and thus maintaining plasma membrane integrity (stabilising proteins under dehydration conditions and protecting cells from oxidative stress) and cause no negative effects on the metabolism of plants (Flowers et al. 1986; Flowers and Colmer 2009; Cushman 2001; Ashraf 2009). One of the commonest osmolytes in plants is proline, an amino acid that accumulates in the cytosol under stress conditions induced by salinity and drought, but also by high temperature, nutritional deficiencies, presence of heavy metals, air pollution, high UV radiation, and some biotic stress such as pathogen infection (Saradhi et al. 1995; Hare and Cress 1997). The synthesis of proline has been found to be significant in relation to the environmental factors in the *G. struthium* plants sampled in natural environments (Alvarado et al. 2000; Boscaiu et al. 2013a). Nonetheless, this is the first report on proline accumulation under experimental artificial stress conditions in *Gypsophila.* The proline levels recorded in the plants treated with salt (from 200 to 500 mM NaCl) were up to 10-fold higher than in those plants collected in the field (Boscaiu et al. 2013a). This may be explained by the accumulation of salt in the pots, which resulted in a high EC of the substrate at the end of the 3-month treatments. The EC reported in similar experiments by far surpasses that we recorded at the sampling site, with values reaching almost 100 dS/m in the plants treated for 3 months with 500 mM NaCl (Boscaiu et al. 2013b).

Although all the plants, including glycophytes, can synthesise proline in response to stress, many studies have indicated that proline accumulation represents a general response in halophytes (Flowers and Hall 1978; Tipirdamaz et al. 2006; Grigore et al. 2011). Higher proline levels have been correlated with higher tolerance to salinity when comparing two related species or varieties (e.g., Chutipaijit et al. 2009; Boscaiu et al. 2013a), but there are also many examples that show no positive correlation between Pro contents and salt-tolerance (e.g., Lutts et al. 1996; Ashraf and Foolad 2007; Chen et al. 2007). Both the studied species accumulated proline under salt stress, but *G. tomentosa* presented higher levels of proline in the control treatment. This pattern suggests that the synthesis of this compound is constitutive in *G. tomentosa*, this being the species that is exposed much more to salinity in its natural environments.

Apart from osmolyte synthesis, another basic salt tolerance mechanism in halophytes is the accumulation of inorganic ions to lower the osmotic potential, unlike glycophytes, which limit sodium uptake. Halophytes' ability to maintain a low cytosolic sodium concentration by compartmenting toxic ions in vacuoles is essential to avoid the inhibition of enzymatic activities and metabolic processes (Flowers et al. 1986). This strategy is advantageous since the accumulation of inorganic ions is more economical than the synthesis of compatible organic solutes. In the two

species under study, Na^+ increased under saline treatments, but highest values obtained with the salt treatments were less than double those in the control treatment. A significant reduction was also noted in potassium content with increasing salinity. The maximal Na:K ratio was around 2 in both species, which is much lower than that in extreme halophytes where it can exceed 10 (Flowers et al. 1986). Both the Na^+ and K^+ values were generally higher in *G. tomentosa* than in *G. struthium*. Regarding bivalent cations, *G. struthium* gave significantly higher values of the Ca^{2+} levels than *G. tomentosa*. Such differences in the chemical composition of wide and narrow gypsohytes have also been reported by Palacio et al. (2007), who found larger amounts of Ca^{2+} among other elements in the first category of plants.

It is difficult to assess whether one of the two species is more salt-tolerant than the other because their responses largely differ at different stages. Germination is apparently more affected by salinity in *G. tomentosa* but, conversely, the seedlings in this species better survive salt stress. However, *G. struthium* growth is not affected by 100 mM NaCl and may, therefore, be considered more stress-tolerant. This species is never naturally present in soils with high sodium chloride content, rather in dry gypsum habitats. Since early responses to saline and water stress are practically identical (Munns 2002), we considered that the behaviour of the studied species might be explained by their tolerance to water stress. Thus, the adaptation to these stressful environments may relate more to general adaptation to arid environments than to chemical soil composition, as Salmeron-Sánchez et al. (2014) also indicated. In this sense, our results agree with the interpretation of Pueyo et al. (2007) on the correlation of the distribution of gypsophile plant communities with the strictness of soil conditions due to a different topography.

Conclusions

After analysing and discussing the results, we consider that the reduced distribution of the *G. tomentosa* populations is related not only to salinity, but also to other factors. The hypothesis of specific NaCl tolerance as the main control factor conferring the advantage to *G. tomentosa* in salty soils is refuted here. Although this species is less competitive than *G. struthium* in the germinative phase, it takes full advantage of autumnal flowering and of seedlings' capacity to survive in the presence of salt, and it refuges in the peripheral zone of salt marshes where it finds less competition and more humidity due to soil type and topography. In conclusion, soil NaCl concentration is not the only key factor in the distribution of the two analysed species. Our data reveal that, on the one hand, in the studied population, *G. tomentosa* should not be considered a strict halophyte as previously reported. Presence of *G. tomentosa* in habitats bordering salt marshes is a strategy to avoid plant competition and extreme water stress. On the other hand, even when not confronted to

salinity in its natural habitats, *G. struthium* proves more stress-tolerant than *G. tomentosa;* in fact in natural environments, it grows under harsher conditions with less soil humidity.

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Table 1 Germination parameters (mean ± s.d.) for *G. struthium* (Gs) and *G. tomentosa* (Gt): germination percentage (GP), reduction of germination percentage (RGP), mean germination time (MGT), Timson Index (TI), recovery (R) after 20 d of transfer to distilled water from the studied NaCl solutions expressed as mM concentrations or the osmotic potential in MPa, and the mean germination time of recovery (MGTR). Letters indicate homogeneous groups ($p<0.05$) for each species.

	NaCl (mM)	**Ψ (MPa)**	**GP (%)**	**RGP (%)**	**MGT (days)**	**TI (%)**	**R (%)**	**MGTR (days)**
Gs	0	0	86.6±4.0 **a**	0 **a**	5.1±0.4 **a**	71.3±4.9 **a**	-	-
	50	-0.21	83.5±3.8 **a**	3.6±4.4 **a**	5.4±0.5 **a**	67.2±3.8 **a**	-	-
	100	-0.43	85.8±5.5 **a**	3.3±4.1 **a**	6.2±0.6 **a**	65.8±5.4 **a**	-	-
	150	-0.64	73.8±3.2 **b**	14.9±3.7 **b**	8.1±0.1 **b**	49.1±2.2 **b**	74.6±10.3	3.0±0.2
	200	-0.85	49.4±7.7 **c**	42.8±9.6 **c**	9.5±1.0 **c**	29.8±6.4 **c**	72.1 ± 6.2	2.9±0.3
	300	-1.28	0 **d**	100 **d**	-	-	83.0±10.5	2.9±0.2
	400	-1.70	0 **d**	100 **d**	-	-	80.0 ± 8.6	3.0±0.1
	500	-2.13	0 **d**	100 **d**	-	-	74.0 ± 8.3	3.2±0.2
Gt	0	0	95.0±2.0 **e**	0 **e**	5.0±0.3 **e**	78.4±3.1 **e**	-	-
	50	-0.21	92.8±3.3 **e**	3.2±3.5 **e**	5.5±0.3 **e**	73.2±1.5 **e**	-	-
	100	-0.43	93.9±5.4 **e**	2.3±2.6 **e**	7.5±0.6 **f**	65.5±3.1 **f**	-	-
	150	-0.64	58.5±4.0 **f**	38.4±5.1 **f**	9.4±0.1 **g**	29.3±3.2 **g**	91.3±5.8	3.0±0.2
	200	-0.85	10.4±4.2 **g**	89.0±4.4 **g**	13.2±1.1 **h**	4.4±1.9 **h**	95.6±0.2	3.0±0.1
	300	-1.28	0 **h**	100 **h**	-	-	94.0±5.2	3.1±0.3
	400	-1.70	0 **h**	100 **h**	-	-	96.0±3.3	3.2±0.3
	500	-2.13	0 **h**	100 **h**	-	-	93.0±2.0	3.0±0.3

Table 2 Effect of salt concentration on the considered developing parameters for *Gypsophila struthium* (Gs) and *G. tomentosa* (Gt): reduction of radicle length percentage (RPR), reduction of plumule length percentage (RPP), seedling survival (SS), reduction of fresh plant weight percentage (RPW) and reduction of flower production percentage (RPF); nt indicates not tested salt concentrations and a dash denotes insufficient number of seedlings

		NaCl concentration (mM)							
	parameters	0	50	100	150	200	300	400	500
Gs	RPR (%)	0	56.5	92.4	94.4	96.1	-	-	-
	RPP (%)	0	23.4	100	100	100	-	-	-
	SS	100	100	37.5	66.7	66.7	-	-	-
	RPW (%)	0	nt	0	nt	37.0	72.2	78.0	80.8
	RPF (%)	0	nt	100	nt	100	100	100	100
Gt	RPR (%)	0	85.2	94.7	96.3	-	-	-	-
	RPP (%)	0	47.0	72.1	75.6	-	-	-	-
	SS	100	100	100	100	-	-	-	-
	RPW (%)	0	nt	27.0	nt	43.6	54.2	65.4	76.5
	RPF (%)	0	nt	31.0	nt	64.3	56.1	84.3	90.8

Fig. 1 Idealised catenal schema of vegetation communities (schematic diagrams of the transect showing topography, plant zonation, and the soil sampling points). 1. *Helianthemo thibaudii-Teucrietum libanitidis*, 2. *Limonio delicatuli-Gypsophiletum tomentosae*, 3. *Frankenio corymbosae. -Arthrocnemetum macrostachyi*, 4. *Limonio cossoniani-Sarcocornietum fruticosae*, 5. Salt pan. The first half of the samples taken in the transect (1-14) corresponds to the habitat of *G. struthium* and the second half (15-25) corresponds to that of *G. tomentosa*

Fig. 2 Profile 1 corresponds to the top transect of *Gypsophila struthium*, profile 2 to the top of transect of *G. tomentosa*, and profile 3 to the central part of the lagoon

Fig. 3 Soil humidity (Hw), pH and EC in a saturated extract of the samples from the studied transect. a. *Gypsophila struthium* (1-14), b. *G. tomentosa* (15-25)

Fig. 4 Soil chemical composition: total concentration of ions (Ca^{2+}, Mg^{2+}, SO_4^{2-}, Na^+, K^+, Cl^- and HCO_3^-) expressed as mmol/L, in a soil-saturation extract of the localities under study. a. *Gypsophila struthium* (1-14), b. *G. tomentosa* (15-25)

Fig. 5 Relation between soil EC and chloride levels in the soil samples from points 15 to 28 of the transect, corresponding to communities with *Gypsophila tomentosa*

Fig. 6 The effect of the tested osmotic potentials on the germination rate for the *Gypsophila struthium* (Gs) and *G. tomentosa* (Gt) seeds at 15ºC

Fig. 7 Total germination of seeds (%): seed germination in salt solution (grey bars) after adding those germinated after been transferred to distilled water (white bars). The same letters indicate homogeneous groups of results ($p<0.05$). a. *Gypsophila struthium*, b. *G. tomentosa*

Fig. 8 Development of seedlings of *Gypsophila struthium* (Gs) and *G. tomentosa* (Gt). a. Radicle length, b. Plumule length in millimetres, after post-sowing day 9. Error bars express standard deviation. The same letters indicate homogeneous groups of results ($p<0.05$)

Fig. 9 Mean fresh weight ± s.d. in the *G. struthium* (Gs) and *G. tomentosa* (Gt) plants grown in the presence of the indicated NaCl concentrations. Asterisks indicate significant differences ($p<0.05$) between species. Error bars express standard deviation

Fig. 10 Mean proline ± s.d levels in the *G. struthium* (Gs) and *G. tomentosa* (Gt) plants treated with increasing salt concentrations and their exponential fitting. Asterisks indicate significant differences ($p<0.05$) between species. Error bars express standard deviation

Fig. 11 Changes in the cation levels of the salt-treated *G. struthium* (Gs) and *G. tomentosa* (Gt) plants. a. Sodium, b. Potassium, c. Calcium and d. Magnesium levels at the indicated NaCl concentrations. The values shown are means (± s.d.) of the samples from four independent plants per treatment