Responses to Environmental Stress in Plants Adapted to Mediterranean Gypsum Habitats

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Abstract

Gypsum areas are stressful environments inhabited by gypsophytes, plants that are exclusive for such habitats, and by plants that grow on gypsum but also on other soil types, the so-called gypsovags. To investigate possible differences between gypsovags and gypsophytes with respect to basic stress response mechanisms, two common osmolytes, glycine betaine and total soluble sugars, as well as monovalent (Na^+ and K^+) and bivalent (Ca^{2+} and Mg^{2+}) cations, were quantified, under field conditions, in two Iberian endemic gypsophytes (*Gypsophila struthium* subsp. *hispanica* and *Ononis tridentata*) and two common Mediterranean gypsovags (*Rosmarinus officinalis* and *Helianthemum syriacum*). Their spatial variation according to a topographic gradient and their temporal variation over a period of three successive seasons were correlated with climatic data and soil characteristics. This analysis confirmed that water stress is the main environmental stress factor in gypsum habitats, whereas the percentage of gypsum in the soil does not seem to play any relevant role in the activation of stress responses in plants. Glycine betaine may contribute to stress tolerance in the gypsophytes, but not in the gypsovags, according to the close correlation found between the level of this osmolyte and the gypsophily of the investigated taxa. Cation contents in the plants did not correlate with those present in the soil, but the gypsophytes have higher levels of Ca^{2+} and Mg^{2+} than the gypsovags, under all environmental conditions, which may represent an adaptation mechanism to their specific habitat

Keywords: cations, gypsophytes, gypsovags, glycine betaine, total soluble sugars

Introduction

Soils with gypsum accumulation extend to over 100 million ha in the world, and are wide-spread in Africa and Asia (Verheye and Boyadgiev, 1997). In Europe, some of the largest deposits of gypsum are found in Spain (over 30 000 km²), mostly in the dry South-Eastern part of the country (Mota *et al.*, 2004). The vascular flora colonising these gypsum habitats is extremely diverse and includes many endemic and rare taxa. In the Iberian Peninsula alone, there are about 30 endemic gypsophyte species (Merlo *et al.,* 1998) and Iberian gypsophile plant communities are considered as priority habitats, being among the most threatened in Europe (Gómez Campo, 1987; Pueyo *et al.*, 2007). This high rate of endemicity is explained by the stressful environmental conditions affecting these habitats, which constitute a selective force for the evolution of endemics (Meyer, 1986). The environmental stress is caused by both physical and chemical constraints. Among the first, the limited water retention of the gypsum soils, combined with the arid climatic conditions present in gypsum zones, makes such habitat adverse for the establishment of flora, as it has been discussed by several authors (Martínez-Duro *et al.,* 2010; Palacio *et al.,* 2007; Pueyo e*t al.,* 2007). In addition, gypsum soils are characterised by mechanic instability and lack of plasticity and cohesion, structural deterioration and low porosity, which interfere with root growth (Verheye and Boyadgiev, 1997; Palacio *et al.,* 2007). The formation of a hard soil surface crust restricts seedling establishment and limits the presence of many species in those areas (Escudero *et al.,* 1997; Meyer, 1986; Meyer and García-Moya, 1989; Romao and Escudero, 2005). On the other hand, such soils

have some chemical characteristics unsuitable for development of plants: deficiency of nutrients, ionic antagonisms and unbalanced ion concentration, with an excess of sulphur and calcium, and toxicity due to the high concentration of sulphate ions (Mota *et al.*, 2004; Palacio *et al.*, 2007).

The high rate of edaphic endemisms in anomalous habitats was explained by the 'refuge' (Gankin and Major, 1964) and the 'specialist' models (Meyer, 1986). Plants that fit to the refuge model are stress-tolerant, but show little competitiveness in the absence of stress. This model has been tested in several examples, such as serpentine endemics, which in experimental conditions developed better on normal soils in the absence of interspecific competition. In the specialist model, the endemic species is excluded from neighbouring areas, due to its adaptation to its own soil. Such specialist species always grow better in experimental conditions on their particular type of soils than on normal soils, even in the absence of competitors.

Plants that grow on gypsum soils are called gypsophytes when they are exclusive to such soils, and gypsovags when they appear on gypsum but also grow on other soil types. Palacio *et al.* (2007) considered that gypsovags are stress tolerant species that fit to the refugee model and grow on gypsum soils due to the reduced competition, whereas gypsophytes could be either specialists (in the case of regional dominant gypsophytes), but also refuges (as some narrow endemics).

Gypsicolous flora is of great scientific and conservationist interest, but responses to stress of plants from gypsum soils have not received much attention. Specifically, very little is known regarding one of the fundamental and conserved response mechanisms, based on the maintenance of cellular osmotic balance under stress conditions, through the control of ion transport and the biosynthesis and accumulation of different osmolytes in the cytoplasm. The mineral composition of plants present on gypsum habitats was analysed in early studies (Duvigneaud, 1968; Duvigneaud and Denaeyer-De Smet, 1966), while Alvarado *et al.* (2000) studied nitrogen metabolism in five species growing on gypsum. Palacio *et al.* (2007, 2014) reported differences in the chemical composition of ash, and in Ca, S, N, Mg P and Na concentrations between gypsophytes and gypsovags. However, the patterns of variation of stress markers – ions and osmolytes – according to spatial or temporal (seasonal) gradients in plants from gypsum areas have never been studied, except for our previous work describing environmentally-induced changes in proline contents in seven species from this habitat (Boscaiu *et al.*, 2013).

The aim of the present work was to investigate possible differences in the responses of gypsophytes and gypsovags to abiotic stress, under field conditions in a natural gypsum zone. For this, we have analysed the accumulation of glycine betaine and soluble sugars – the most common osmolytes in plants besides proline, which was previously measured in the same plant material – as well as monovalent and bivalent cation contents, in two gypsophytes (*Gypsophila struthium* and *Ononis tridentata*) and two gypsovags (*Rosmarinus officinalis* and *Helianthemum syriacum*) sharing the same habitat; changes in the levels of these compounds were then correlated with differences regarding soil properties and

meteorological data, in the selected experimental plots and in three successive seasons.

Materials and methods

Selected taxa

In the region of 'Los Serranos', where the gypsum study area is located, 1 427 species of vascular plants have been described (Torres, 2007), of which only 37 live in the selected experimental plots. This is a very low figure, in agreement with the idea that gypsum represents a very stressful habitat for the establishment of flora. The area of study is characterised by the presence of two gypsum indicator plants: *Ononis tridentata* subsp. *angustifolia* (Lange.) Devesa López and *Gypsophila struthium* subsp. *hispanica* (Willk.) G. López, both included in the checklist of Iberian gypsophytes, ranking as '5' in the scale, which means species exclusive of gypsum soils. *G. struthium*, an Iberian endemic of the family Caryophyllaceae, which appears on most of the Iberian gypsum outcrops, is considered as an excellent gypsum indicator (Martínez-Nieto, 2013). The subspecies *G. struthium hispanica* is restricted to the North-Eastern part of the Iberian Peninsula (López-Gonzalez, 1990). *Ononis tridentata* is a leguminous shrub of the western Mediterranean region, common in regions rich in gypsum and marl soils with dry climate. *O. tridentata* subsp. *angustifolia* is endemic to the Eastern Iberian Peninsula (Devesa, 2000). It grows on sunny and degraded slopes, related to the pathways of livestock.

The other two species are gypsovags, plants that often grow on gypsum soils, but are abundant also on different types of soils. *Rosmarinus officinalis* L. is a wide-spread Mediterranean labiates species frequent in dry areas, mostly on limestone. *Helianthemum syriacum* (Jacq.) Dum. Course (Cistaceae) is common throughout the Mediterranean region, on limestone, gypsiferous marls, dolomites and sandy soils.

Study area and experimental design

Samples were collected in summer (13 of July 2009), late autumn (11 of December 2009) and spring (26 of April 2010) near the village of Tuéjar, in the Province of Valencia (SE Spain) (39°47'28"N, 1°04'25"W) at 603 m.a.s.l. Three plots of 10 x 10 m were selected on a hillside with a SW orientation and 11.5° and 19° slopes. Five individuals of each species were collected in the three plots, with exception of *Gypsophila struthium* that grew only in plot 3, which is the flattest and is situated at the bottom of the slope.

Soil and climatic analysis

For each sampling date, three random soil samples were taken from all experimental plots at a depth of 0-15 cm. In the laboratory the samples were air-dried and passed through a 2 mm sieve and analysed to determine gypsum content, organic C, available P and assimilable K (Boscaiu *et al.* 2013). A 1:1 soil:water extract was prepared to determine electrical conductivity ($EC_{1:1}$) and pH.

Several multiple sensors (ECH2OT, Decagon®) for salinity, humidity and temperature measurements were installed in April 2009, at depths of 10 cm and 20 cm, and were connected to a datalogger (EM50, Decagon®) in each plot; additionally, sensors for air temperature and rainfall were also connected to the datalogger in plot 2. Climatic data

for the month previous to the first sampling were obtained from the nearest agro-meteorological station, located in Chulilla.

Plant material sampling

Young shoots were sampled separately from five individuals of each taxon, cooled on ice and transported to the laboratory, where leaves were separated from branches. Part of the leaf material was frozen and stored at -75 ºC, while the rest was dried in the oven at 65 ºC for 3-4 days until constant weight, to obtain the percentage of dry weight (DW) of each individual. Biological samples were the same as those we used previously for the analysis of proline (Boscaiu *et al.,* 2013).

Osmolyte quantification

For glycine betaine determination, frozen plant material (250 mg) was ground to a fine powder in a mortar, in the presence of liquid nitrogen. The extraction and quantification of glycine betaine was performed following the method of Grieve and Grattan (1983) with the modifications proposed by Nawaz and Ashraf (2010). For total soluble sugar (TSS) analyses, 50 mg of fresh material was homogenised in 80% ethanol as described by Wankhade and Sanz (2013). TSS contents were estimated in aliquots of the extract by reaction with the anthrone-H_2SO_4 reagent, according to Fairbairn (1953), using glucose as standard.

Cation contents

Dry leaf material (300 mg) was digested in 8 ml of 65% HNO_3 and 2 ml of 30% H_2O_2 in a microwave digestor (Model Ethos One) as described by Grigore *et al.* (2012). The quantification of Na^+ and K^+ was performed by flame spectrometry, in a Jenway PFP 7 spectrophotometer, and atomic absorption spectrometry (in a Varian SpectrAA 220) was used for Ca^{2+} (at 239.9 nm) and for Mg^{2+} (at 202.6 nm) determinations.

Statistical analysis

Data were analysed by the StatGraphic Centurion 16 and SPSS v.15 programmes. Significance of seasonal differences for soil and plant parameters in each plot were analysed by one-way ANOVA. Prior to ANOVA, the normality and homogeneity of variance were checked. When the ANOVA null hypothesis was rejected, post-hoc comparisons were performed using the Tukey test. A two-way ANOVA was applied to test the effect of season and plot on the osmolyte and cation contents in plants. Ecological factors were correlated with variations in osmolyte and ion contents of the four studied species by the multivariate approach of principal component analysis (PCA).

Results and discussions

General soil properties were characterized at the beginning of the study and were reported in a previous publication (Boscaiu *et al.,* 2013). The soil in the studied area is gypsiferous, but gypsum content varied among plots. The two upper plots (1 and 2) had significantly lower values of gypsum content than the lower plot 3 (Table 1). A high gypsum content means less available K, organic C and water holding capacity showing that such habitats have low soil fertility and water retention capacity.

The climate is typical Mediterranean, with hot and dry summers. The higher reference evapotranspiration in correlation with the absence of rainfall during almost the entire summer resulted in a pronounced water deficit prior to the first sampling, in the summer of 2009. On the contrary, due to abundant rainfall the next spring, there was practically no water deficit in the month previous to this sampling (Fig. 1). Seasonal variability of climate may influence many soil properties. Indeed, several soil parameters showed not only spatial differences among plots, but also a seasonal variation within the same plot. Due to the topographic position of the plots, soil humidity throughout the studied period was higher in plot 3, situated at the bottom of the slope, whereas plots 1 and 2 were much drier. In summer, the sensors located in the three plots detected significantly lower soil humidity in relation to the dryness of the climate (data not shown).

Soil salinity determined as $EC_{1:1}$ (in 1 to 1 soil-water extracts) was moderate, steady in time and similar in the three plots. The variation in soil pH and available K could not be

Table 1. Soil properties (mean values ± standard deviation, n = 9) of the three plots under study; values followed by the same letter do not significantly differ at probability level less than 5%. $EC_{1:1}$: electric conductivity in 1-to-1 soil-water extracts; WHC: water holding capacity; OC: organic carbon

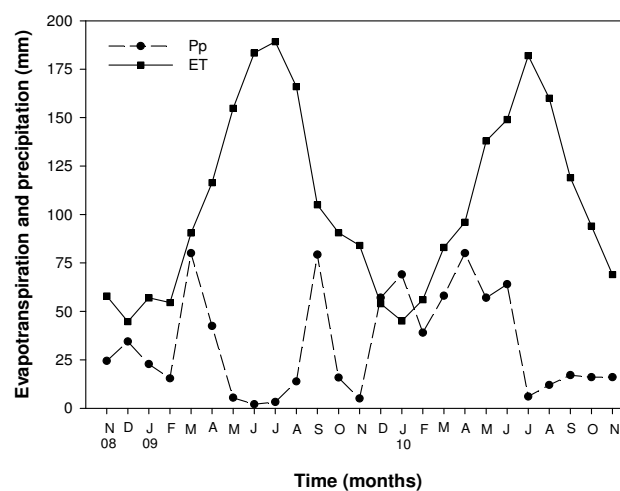| Characteristic	Plot 1	Plot 2	Plot 3
Gypsum (%)	33.50 ± 15.40a	49.80 ± 14.60a	74.10 ± 23.00b
$EC_{1:1}$ (dS m^{-1})	2.52 ± 0.21a	2.40 ± 0.14a	2.40 ± 0.15[a]
pH	7.93 ± 0.05a	7.87 ± 0.06a	7.90 ± 0.10[a]
WHC (%)	21.60 ± 5.30ab	23.40 ± 3.70b	18.10 ± 5.50a
OC (%)	1.49 ± 0.72b	1.38 ± 0.89ab	0.76 ± 0.51a
P Burriel (mg kg^{-1})	1.26 ± 0.35a	1.39 ± 0.36a	2.20 ± 0.66b
Available K (mg kg^{-1})	229.00 ± 87.20b	164.20 ± 80.70ab	132.00 ± 82.30a

Fig. 1. Water deficit represented in function of monthly precipitation and evapotranspiration data registered by the nearest meteorological station, located in Chulilla

related to a seasonal pattern, but the latter was lower in plot 3, which has the highest amount of gypsum (Table 1).

As mentioned above, Mediterranean climate is characterized by a peak of water deficit in summer, which is restrictive for plants. Therefore, contrary to plants in milder temperate climates, which suffer the rigor of winter, Mediterranean plants endure the most stressful conditions in

summer. In conditions of drought most plants lose water from their tissues. The percentages of dry weight detected in leaves were generally higher in summer (Fig. 2), except in *O. tridentata*, which has succulent leaves; thus, changes in water content do not necessarily follow a seasonal pattern. Anatomic studies by Grigore *et al.* (2011) showed that *O. tridentata* leaves do not have typical aquiferous tissue, but long palisade cells, occupying almost all leaf lamina, which confers succulence.

A basic general response to drought is the synthesis and cytoplasmic accumulation of osmolytes, a conserved phenomenon observed in all plants, tolerant as well as sensitive to stress (Munns and Tester, 2008). Osmolytes are compatible solutes, diverse low-molecular-weight organic compounds that do not interfere with normal metabolism even when present at high concentrations. Their primary function in plants is to prevent water loss, maintaining cell turgor and thus the gradient for water uptake into the cell.

Proline (Pro) and glycine betaine (GB) are two of the most common plant osmolytes, synthesised in response to different abiotic stresses that cause cellular dehydration (Ashraf and Foolad, 2007; Chen and Murata, 2008; Verbruggen and Hermans, 2008). Besides their primary role
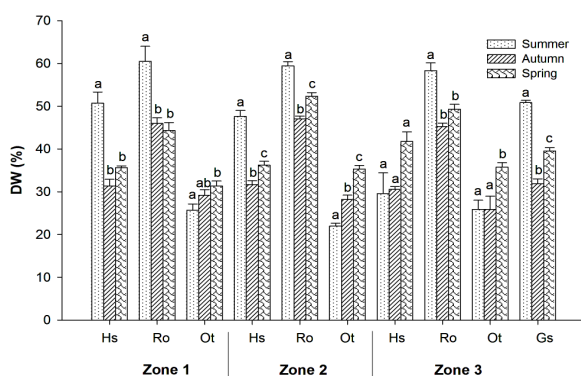

Fig. 2. Dry weight percentages (with respect to fresh weight) of the investigated taxa, in the three plots under study in summer 2009, autumn 2009 and spring 2010. Different letters indicate seasonal significant differences within each species and plot. Species abbreviations: Ro, *Rosmarinus officinalis*; Hs, *Helianthemum syriacum*; Ot, *Ononis tridentata*; Gs, *Gypsophila struthium*

small spatial and seasonal variation of this compound in the four species (Tables 2 and 3, Fig. 3), which was generally not significant or did not correlate with soil water availability or other environmental factors that could affect the synthesis of osmolytes. Similar results, regarding the absence of significant variations of GB levels in response to strong seasonal fluctuations in the degree of environmental stress, have been previously reported in GB accumulators from other habitats (Gil *et al.,* 2014). Therefore, it is not clear whether this osmolyte plays a relevant role in the stress tolerance mechanisms of the studied species but, in any case, its contribution to osmotic adjustment should be more important in *O. tridentata* than in the other taxa.

Soluble carbohydrates, such as sugars (e.g. sucrose, glucose,

in osmotic adjustment, Pro and GB have other functions in plants, such as scavenging free oxygen radicals and stabilizing cellular membrane structures. They also play a role in the regulation of gene expression as signalling molecules, and serve as metabolites for the cellular storage of carbon and nitrogen during stress, which would be used by the cell once stress has ceased (Munns and Tester, 2008; Szabados and Savouré, 2010). We reported variation in Pro contents in the four species under study, in a previous publication (Boscaiu *et al.,* 2013). Pro levels were found to closely correlate with climate parameters in the gyspovags *H. syriacum* and *R. officinalis*, but not in the gysphytes *G. struthium* and *O. tridentate*.

In the present work, we have detected low GB contents in all taxa except *O. tridentata*, which contains double concentration of this osmolyte, in comparison with the other species (Fig. 3). Yet these levels are still lower than the values measured in halophytes that are typical GB accumulators (Khan *et al.,* 2000; Tipirdamaz *et al.,* 2006). There was only a

Table 2. Significant effect of spatial (plot) and seasonal variations of plant osmolytes and cations for the three species present in the three plots at 99.99% (*), at 99% (**) and 95% (*) confidence levels, according to two-way ANOVA. NS: not significant. Species abbreviations as in Fig. 1

Property	Species	Plot	Season	Interaction
DW	Ro	NS	***	NS
	Hs	*	***	***
	Ot	NS	***	*
Proline	Ro	NS	***	NS
	Hs	**	***	***
	Ot	NS	***	NS
Glicyne betaine	Ro	**	***	NS
	Hs	NS	*	*
	Ot	NS	***	NS
TSS	Ro	NS	***	NS
	Hs	NS	***	**
	Ot	***	***	***
Na	Ro	**	***	***
	Hs	NS	***	*
	Ot	***	*	NS
K	Ro	***	***	**
	Hs	***	***	***
	Ot	***	***	NS
Ca	Ro	***	***	***
	Hs	*	***	*
	Ot	*	***	*
Mg?	Ro	***	***	***
	Hs	NS	***	*
	Ot	NS	***	NS

Table 3. Significant effect of seasonal variations on osmolytes and cations in *G. struthium* present only in plot 3, according to one-way ANOVA

Property	Season
DW	***
Proline	*
GB	NS
TSS	***
Na	*
K	***
Ca	***
Mg	**

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fructose or trehalose) or polyalcoholss (sorbitol, mannitol, different inositol derivates, etc.) also play a role as functional osmolytes in several species; however, their participation in specific stress tolerance mechanisms are often difficult to assess, due to their multiple additional functions as primary energy sources, metabolic precursors and regulatory signalling molecules (Gil *et al.*, 2013). Total soluble sugars (TSS) have been reported by many authors to increase under salt stress (Gil *et al.*, 2013, and references therein) and drought (Rodrigues *et al.*, 2010; Souza *et al.*, 2004) conditions; yet many other reports indicated the opposite: a stress-dependent reduction of TSS contents (Alves and Setter, 2004). In the four species analysed here, TSS levels were similar in the three plots, but a seasonal pattern of variation was observed, with higher TSS concentrations in autumn (Tables 2 and 3, Fig. 4). Since TSS levels did not correlate with environmental stress factors, this increase is probably related to phenological changes in the plants, and do not support a role for TSS in osmotic adjustment or, in general, stress tolerance.

Salinity and drought also affect plant mineral nutrition and disrupt ion homeostasis. An increase of Na^+ concentration in plants has been generally observed under saline stress, but obviously not in plants affected by water stress. The levels of Na^+ registered in the four selected species were extremely low (Fig. 5), not at all comparable to those reported in halophytes, or plants adapted to saline environments (Gil *et al.*, 2014); this was to be expected since salt stress in gypsum soils is not due to sodium salts, the most abundant in most soil types, but to calcium sulphate. Potassium, on the other hand, is an essential element in plant nutrition and it is involved in many metabolic processes. A decrease of K^+ levels in water-stressed plants has been observed in many species, mainly due to membrane damage and disruption in ion homeostasis (Wang *et al.*, 2013); yet K^+ has also been reported to increase under drought conditions, contributing significantly, as the 'physiological' cation, to cellular osmotic adjustment (Silva *et al.*, 2010). In the four species included in the present study, and in the three experimental plots, a significant increase in K^+ levels was registered in autumn, coinciding with increased

Fig. 4. Variation of monovalent cation (Na^+ and K^+) contents in the investigated taxa, in the three plots under study in summer 2009, autumn 2009 and spring 2010. Different letters indicate seasonal significant differences within each species and plot. Species abbreviations as in Fig. 1

Fig. 5. Variation of bivalent cation (Ca^{2+} and Mg^{2+}) contents in the investigated taxa, in the three plots under study in summer 2009, autumn 2009 and spring 2010. Different letters indicate seasonal significant differences within each species and plot. Species abbreviations as in Fig. 1
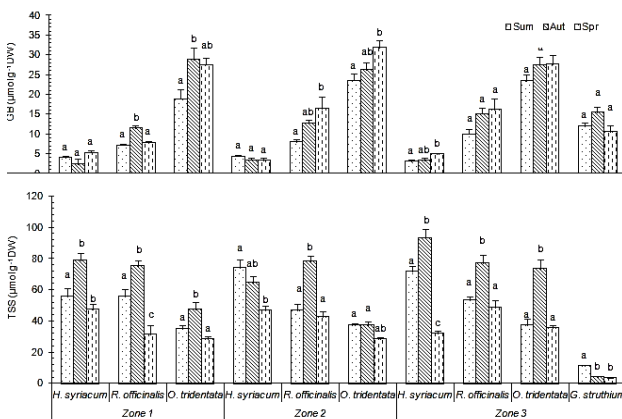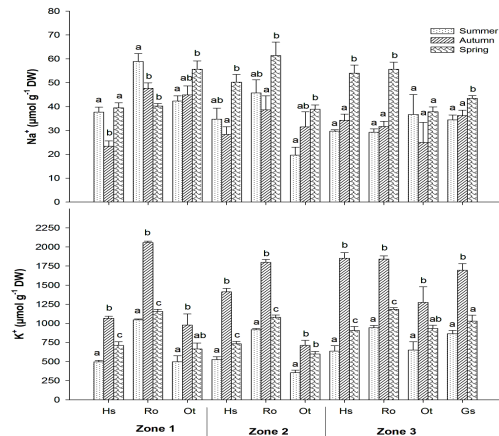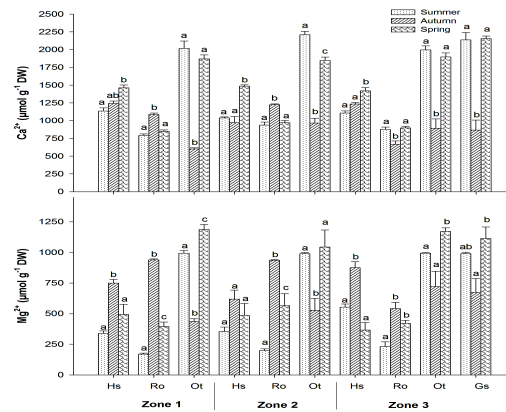

Fig. 3. Variation of glycine betaine (GB) and total soluble sugars (TSS) contents in the investigated taxa, in the three plots under study in summer 2009, autumn 2009 and spring 2010. Different letters indicate seasonal significant differences within each species and plot
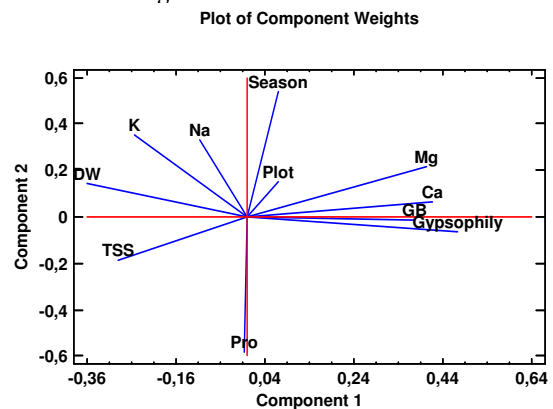

Fig. 6. Biplot from the principal component analysis (PCA) showing the relationships among proline (Pro), glycine betaine (GB), total soluble sugars (TSS), Na, K, Ca and Mg in plants, and environmental factors summarised as the 'plot' and 'season' variables. Gyspophily is a variable introduced to indicate whether the species are gysophytes or gyspovags

concentration of total sugars (Fig. 4). This pattern may be correlated with an intensification of metabolic processes after the more stressful summer period or with phenological stages of the plants, but apparently not with stress tolerance.

Another essential mineral nutrient is the divalent cation Ca^{2+}, involved in a wide array of cellular processes, such as cell wall stabilisation, maintenance of cell membrane integrity and control of membrane permeability and selectivity, cation-anion balance and osmoregulation (Marschner, 1995). Mg^{2+}, the other major divalent cation, also plays an important structural and functional role in plants. It is part of the ring structure of the chlorophyll molecule and is a cofactor of many enzymes involved in respiration and photosynthesis, or in the synthesis of DNA and RNA (Marschner, 1995). The concentration of Ca^{2+} may fluctuate according to the type and intensity of stress, but also with the species or even the tissue type within a species (Kader and Lindberg, 2010). Similarly, Mg^{2+} contents largely vary in plants in response to stress but, here again, many reports indicate an increase (Joshi 1984), while others showed a decrease (Loupassaki *et al.*, 2002) in its levels. In the analysed taxa, there was no clear pattern of variation in the bivalent cation levels (Fig. 5) and no correlation with soil characteristics (Table 1).

A 'principal component analysis' (PCA) was performed in order to establish possible correlations of changes in osmolytes and cation contents in the plants and environmental variables (Fig. 6). Proline levels in the plant samples, determined previously (Boscaiu *et al.*, 2013) were also included in the analysis. Considering climatic data (the previous month rainfall and mean air temperature) and soil properties (soil moisture at 10 cm depth, electric conductivity and gypsum content), environmental variables were defined as 'season' and 'plot' and assigned arbitrary values according to the degree of abiotic stress affecting the plants in each season, and to the gypsum content in each plot, respectively. Thus, spring was assigned a value of '1', as the less stressful season, followed by autumn ('2') and summer ('3'); similarly, plots 1, 2 and 3, were assigned '1', '2' and '3' values, respectively, according to an increasing level of gypsum in the soil. An additional variable was included in the PCA, grouping the investigated taxa according to their degree of gyspophily, with a lower value ('1') assigned to gyspsovags and a higher one ('2') associated with gypsophytes. About 74% of the variance was explained by the cumulative percentage of three components, with Eigenvalues greater than 1. The first component (X-axis in Fig. 6), which explained 32.5% of variance, was defined by the character of gypsophily of the plants, showing a strong positive correlation with GB, Ca^{2+} and Mg^{2+} contents and a negative correlation with the percentage of dry weight, TSS and K contents. The second component (Y-axis), which explained a further 16% of the variance, was closely associated to the variable 'season' – that is, mostly with the degree of water stress, since water deficit is maximum in summer (3) and minimum in spring (1) – and, to a lesser extent, with the variable 'plot': due to its topographic position at the bottom of the hill, plot 3 contained the highest gypsum level and was the most humid throughout the year (value 3). This component was negatively correlated only with the amount of proline, but not with other osmolytes or cations.

The PCA highlighted the statistically significant correlation of Ca^{2+} and Mg^{2+} levels with the gypsophilous character of the plants, while cation contents were not associated to environmental parameters. These data are in agreement with the absolute values measured in the four species. Mean Ca^{2+} concentrations of 1 588 and 1 719 $\mu mol\ g^{-1}$ DW were determined in *O. tridentata* and in *G. struthium*, respectively, which were higher than in the two gypsovags (1 236 $\mu molg^{-1}DW$ in *H. syriacum* and 922 $\mu molg^{-1}DW$ in *R. officinalis*). The same was found for Mg^{2+}, significantly higher in the gypsophytes (895 $\mu molg^{-1}DW$ in *O. tridentata* and 925 $\mu molg^{-1}DW$ in *G. struthium*) than in the gypsovags (538 and 419 $\mu molg^{-1}DW$ in *H. syriacum* and *R. officinalis*, respectively). Palacio *et al.* (2014) have also reported recently higher levels of Ca and Mg in *G. struthium* and *O. tridentata* than in gypsovag taxa. The higher water content – succulence – observed in many gypsophiles may have a diluting effect on toxic sulphate levels in the cell, as discussed by Parsons *et al.* (1976), in addition to being an adaptation to dry habitats.

Conclusions

The present work confirms that water stress is the main environmental stress factor in gypsum habitats, whereas the percentage of gypsum in the soil does not seem to play any relevant role in the activation of stress responses in the plants. Proline accumulation appears to be an excellent indicator of stress in many species, including the two gypsovags analysed in the present work, but not in the two gypsophytes. Glycine betaine, on the contrary, may contribute to the stress tolerance mechanisms in the latter taxa or, at least, in *O. tridentata*, which accumulated this osmolyte to higher levels than the other species. Cation contents in the plants did not correlate with those present in the soil, but the current study indicates that the gypsophytes have higher levels of Ca^{2+} and Mg^{2+} than the gypsovags, under all environmental conditions, which may represent an adaptation mechanism to their specific habitat.

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