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Gypsum-exclusive plants accumulate more leaf S than non-exclusive species both in and off gypsum

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Gypsophiles accumulate more leaf S than gypsovags both in and off gypsum

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Abstract

Gypsum-exclusive species (gypsophiles), are restricted to gypseous soils in natural environments. However, it is unclear why gypsophiles display greater affinity to gypseous soils than other soils. These plants are edaphic endemics, growing in alkaline soils with high Ca and S. Gypsophiles tend to show higher foliar Ca and S, lower K and, sometimes, higher Mg than non-exclusive gypsum species, named gypsovags. Our aim was to test if the unique leaf elemental signature of gypsophiles could be the result of special nutritional requirements linked to their specificity to gypseous soils. These nutritional requirements could hamper the completion of their life cycle and growth in other soil types. To test this hypothesis, we cultivated five gypsophiles and five gypsovags dominant in Spanish gypsum outcrops on gypseous and calcareous (non-gypseous) field soil for 29 months. We regularly measured growth and phenology, and differences in leaf traits, final biomass, individual seed mass, seed viability, photosynthetic assimilation and leaf elemental composition. We found all the gypsophiles studied were able to complete their life cycle in non-gypseous soil, producing viable seeds, attaining greater biomass and displaying higher photosynthetic assimilation rates than in gypseous soil. The leaf elemental composition of some species (both gypsophiles and gypsovags) shifted depending on soil, although none of them showed leaf deficiency symptoms. Regardless of soil type, gypsophiles had higher leaf S, Mg, Fe, Al, Na, Mn, Cr and lower K than gypsovags. Consequently, gypsophiles have a unique leaf chemical signature compared to gypsovags of the same family, particularly due to their high leaf S regardless of soil conditions. However, these nutrient requirements are not sufficient to explain why gypsophiles are restricted to gypsum soil in natural conditions.

Key words

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- 1 gypsophile, semiarid, thiophores, leaf chemical signature, nutrient, phenology, edaphism,
- 2 gypsum
- 3

1. Introduction

The effect of soil on plant performance and distribution has been studied by ecologists and botanists for decades, particularly in relation to the restriction of plants to certain types of soils with special physicochemical features. For example, serpentines and saline soils are special substrates that support singular floras (Mota *et al.*, 2017) composed of species that tolerate the physicochemical challenges imposed by them (Kazakou *et al.*, 2008, Munns and Tester, 2008).

Gypseous soils are also atypical substrates. These soils have high gypsum content (Casby-Horton *et al.*, 2015) and normally develop in arid or semiarid environments, limiting plant life (Palacio *et al.*, 2017). High gypsum content in soil impacts the physical and chemical properties of soils and their functions (Herrero and Porta, 2000). The moderate solubility of gypsum (about 2.4 g l⁻¹) leads to highly dynamic soil environments, with dissolution-precipitation sequences altering physical properties (Casby-Horton *et al.*, 2015). Although the solubility of gypsum does not produce osmotic or ion-toxic stress for plants (Casby-Horton *et al.*, 2015), the chemical conditions of gypseous soils influence plant nutrition (Boukhris and Loissant, 1972; Palacio *et al.*, 2007; Salmerón-Sánchez *et al.*, 2014), ultimately limiting growth (FAO, 1990).

Plants living on gypseous soils have to cope with alkaline soils saturated in calcium, sulphate and magnesium ions, and reduced in N, P and K availability (Moore *et al.*, 2014). Gypseous soils have low nutrient retention (Casby-Horton *et al.*, 2015) and high Ca cation activity due to the solubility of gypsum (FAO, 1990). The combination of high Ca, jointly with high sulphate, alters plant metabolism (Meyer, 1980) and decreases the availability and uptake of macronutrients like K and P (Stout *et al.*, 1951; FAO, 1990). To overcome these

chemical restrictions, plants growing on gypseous soils may have developed special mechanisms and strategies (Moore *et al.*, 2014).

Species that thrive on special soils generally show different ecological amplitudes, ranging from tolerant species with a broad distribution, to highly specialized edaphic endemics restricted to them (Kruckeberg and Rabinowitz, 1985). In the case of gypseous soils, Meyer (1986) described mainly two types of plants living on gypsum, depending on their affinity to this substrate: 1) gypsovags, species with wide ecological amplitude, which can grow both on and off gypseous soils; and 2) gypsophiles, edaphic endemics restricted to gypseous soils. Two types of gypsophiles have been further described (Palacio *et al.*, 2007): widely distributed gypsophiles (hereafter, wide gypsophiles) considered as gypsum specialists (*sensu* Gankin and Major, 1964), and narrowly distributed gypsophiles, which, similar to gypsovags, would fit the refuge model, being stress tolerant species not specifically adapted to gypseous soils. Gypsovags seem to be stress tolerant plants that may display different mechanisms to cope with the limitations imposed by gypsum (Bolukbasi *et al.*, 2016). Gypsophiles are usually restricted to gypseous soils (Mota *et al.*, 2011), and their individual fitness may be compromised in non-gypseous soils (Ballesteros *et al.*, 2014). However, it is unclear why gypsophiles display greater affinity to gypseous soils than other soil types.

Edaphic endemics often have substrate-specific physiological mechanisms or strategies to cope with the harsh conditions of special substrates (Mota *et al.*, 2017). In soils with atypical chemical composition, the mineral nutrition of plants has been crucial to explain plant restriction or growth limitation (Rorison, 1960). The concentration of elements in leaves (hereafter, leaf elemental composition) is used to understand plant mineral nutrition, since it links plant function (Aerts and Chapin, 1999) and soil chemistry. For example, halophytes require high concentrations of NaCl (100–200 mM) for optimal growth (Flowers *et al.*, 1977)

and show high leaf Na, Mg and low Ca, K, as compared to co-occurring non-specialized species (Matinzadeh *et al.*, 2019). In serpentine soils, edaphic endemics maintain high leaf Ca:Mg molar ratios (O'Dell *et al.*, 2006), indicating that they have a high selectivity for Ca at the root surface, maintaining sufficient Ca uptake despite a very low soil Ca:Mg ratio (Kazakou *et al.*, 2008). While, consistent chemical patterns have been found in wide gypsophiles, who display a common leaf elemental composition similar to that of the gypseous soils in which they grow (Duvigneaud and Denaeyer-De Smet, 1968).

Wide gypsophiles tend to have higher foliar S and Ca, lower K and, sometimes, higher foliar Mg as compared to co-existing gypsovags (Duvigneaud and Denaeyer- De Smet, 1968; Boukhris and Loissant 1970, 1972, 1975; Alvarado, 1995; Palacio *et al.*, 2007; Muller *et al.*, 2017). This unique leaf chemical composition was observed despite phylogenetic constraints in gypsophilic species from the Chihuahuan Desert (Muller *et al.*, 2017). However, the ecological or adaptive implications of the atypical chemical composition of wide gypsophiles remain unexplored. It has been suggested that the leaf elemental composition of wide gypsophiles could be a nutritional requirement to complete their life cycle and support growth or could confer some form of protection from competition or disturbances (Meyer, 1980). However, no previous studies have evaluated the nutrient composition of wide gypsophiles growing on non-gypseous soils.

Our aim was to test if wide gypsophiles are restricted to gypseous soils because they are not able to complete their life cycle off gypsum. We focused only on wide gypsophiles (hereafter, gypsophiles), since narrowly distributed gypsophiles are a less distinctive group. We also wanted to explore the extent to which the atypical chemical composition of gypsophiles is linked to chemical conditions of the substrate. To this end, we cultivated five widespread Iberian gypsophiles and five co-occurring gypsovags (some of them closely

related phylogenetically) in gypsum and calcareous (non-gypseous) soils. The selection of calcareous soil as the non-gypsum treatment stemmed from the fact that gypsum outcrops are frequently intermingled with alternating layers of marls, limestone and clays (Quirantes, 1978). Consequently, calcareous soils are the most readily available non-gypsum alternative for plants growing on gypseous soils in the wild, showing similar physicochemical features including similar Ca content and differing mainly in the higher S content of gypseous soils (FAO, 1990). We analysed plant survival and fitness and measured leaf elemental composition as a tool to understand plant mineral nutrition and its relationship with soil chemical features. We hypothesized that: 1) Gypsophiles would have lower growth and fitness in non-gypseous soils than in gypseous soils, in accordance with Ballesteros *et al.* (2014); 2) Gypsophiles would have substrate-specific physiological mechanisms or strategies linked with chemical features of gypseous soils (i.e., nutritional requirements), and as a result, they would accumulate higher S and Mg concentrations than gypsovags, irrespective of the substrate. However, such concentrations would be lower on calcareous (non-gypseous) than on gypseous soil, owing to the lower S and Mg availability in the former.

2. Material and Methods

2.1. Study species

The selected species included a suite of five dominant gypsophile and gypsovag shrubs from gypsum environments in northeastern Spain (Table 1). Gypsophile species included *Gypsophila struthium* subsp. *hispanica* (Willk.) G.López., *Herniaria fruticosa* L., *Helianthemum squamatum* Pers., *Lepidium subulatum* L., *Ononis tridentata* L.; and gypsovag species included *Boleum asperum* Desv., *Helianthemum syriacum* (Jacq.) Dum.Cours., *Linum suffruticosum* DC., *Matthiola fruticulosa* (L.) Maire and *Rosmarinus officinalis* L. All the

gypsophile species included in the study show high affinity for gypseous soils (Mota *et al.*, 2011) and are widely distributed within the Iberian Peninsula (Palacio *et al.*, 2007).

2.2. Soil collection and analyses

Gypseous soil was collected from a gypsum outcrop in the Middle Ebro Basin (Villamayor del Gállego, Zaragoza, Spain, 41°41'44.5" N, 0°44'26.7" W) and calcareous soil (non-gypseous, hereafter calcareous) was collected from the Iberian System (Ricla, Zaragoza, Spain, 41°30'45.8"N, 1°26'47.8"W). Soil was collected by removing O horizons in unfertilized areas, sieved to 1 cm, and then thoroughly mixed and used to fill pots. Physical and chemical properties were analysed from five replicates per experimental soil type (Table A.1).

Soils were air dried for 2 months prior to physical and chemical analyses and subsequently divided into two subsamples: one to be sieved to pass a 2 mm sieve, and the other to remain non-sieved. Sieved soils were used to measure the following variables: gypsum content, measured according to Artieda *et al.* (2006); carbonate content determined by Bernard calcimetry; soil texture, estimated with a particle laser analyser (Mastersizer 2000 Hydro G, Malvern, UK); and soil pH and conductivity, measured with a pH/conductivity meter (Orio StarA215, Thermo Scientific, Waltham-MA, USA) by diluting samples with distilled water to 1:2.5 (w/v) to measure pH and then 1:5 (w/v) to measure conductivity). A subsample of each sieved soil was finely ground using a ball mill (Retsch MM200, Restch GmbH, Haan, Germany) and subsequently used to analyse elemental concentrations. N and C concentrations were measured with an elemental analyzer (TruSpec CN, LECO, St. Joseph-MI, USA), whereas the elemental composition of Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Si, Ti, V, Zn was measured by extracting samples with

HNO₃-H₂O₂ (9:3) by microwave acid digestion (Speed Ave MWS-3⁺, BERGHOF, Eningen, Germany), followed by inductively coupled plasma-optical emission spectrometry (Varian ICP 720-ES, Agilent Technologies, Santa Clara-CA, USA). All elemental analyses were performed by EEZ-CSIC Analytical Services.

2.3. Experimental design

For each species, seeds were collected from several individuals within the same population (Table 1). In April 2016, seeds were germinated in nursery trays with 0.06 L cells filled with a one-part gravel in the bottom of the cell and four-parts field soil on top of it. Half of the trays had calcareous soil and the other half had gypseous soil (see Table A.1, A.2, for soil features). In November 2016, plants with high root volume (*G. hispanica*, *R. officinalis* and *O. tridentata*) and plants with shallow roots (the rest of species) were transplanted into 7 L and 5.6 L square pots, respectively. Five months after transplantation, pots were thinned to one plant per pot, with ten replicates per species and soil treatment. All plants were kept well-watered throughout the experiment and regularly de-weeded by hand, removing any potential competition and drought stress. Each year, throughout the duration of the experiment, plants were housed in a greenhouse from November to March to avoid freezing damage. Five replicates per species and soil treatment were harvested in September 2019, 29 months after sowing.

2.4. Phenological patterns and growth

Phenological patterns were recorded for each plant every two weeks between 29 November 2017 and 7 Sept. 2019. Five phenophases were considered (adapted from Montserrat-Martí *et al.*, 2009): plant vegetative growth, flower bud formation, flowering, fruit

set and leaf shedding. The incidence of each phenophase was estimated in the canopy of each individual as the percentage of stems displaying it. Canopy height, maximum shoot length (measured from the base of plant to the distant most leaf, hereafter canopy length), and the maximum and their perpendicular canopy diameters were measured monthly using a metallic millimetre straightedge. Mature fruits were collected at seeding and stored in a dry location at room temperature until seed viability tests.

2.5. Leaf gas exchange, plant biomass, functional traits and seed traits

Leaf gas exchange, including photosynthetic assimilation and stomatal conductance, were measured with a Portable Photosynthesis System coupled with a Chlorophyll Fluorescence Module (CIRAS-2, PP Systems, Amesbury-MA, USA), a LED light unit on the leaf cuvette (PLC6 U), and a circular bead plate of 18 mm diameter. Three plants of each soil treatment and species were measured after 9 am and before 1 pm on 11 July 2017, except for *M. fruticulosa* and *B. asperum*, which did not have enough green leaves for assessment.

At harvest in September 2019, plants were lifted from their pots and rinsed with tap water to remove soil. Plants were separated into green leaves, stems, fine roots (diameter < 2 mm), coarse roots (rest of roots), and seeds (if available). All plant fractions were subsequently dried to a constant weight at 50 °C and weighed in a precision scale (42 g / 0.00001 g, MS105DU, Mettler Toledo, Columbus-OH, USA).

Specific leaf area (SLA) was measured as the one-sided area of a fresh leaf divided by its oven-dry mass. Leaf dry matter content (LDMC) was measured as the oven-dry mass (mg) of a leaf, divided by its water-saturated fresh mass (g), expressed in mg g⁻¹ (Pérez-Harguindeguy *et al.*, 2013). To measure leaf area, images of leaves were captured with a Dino-Lite Digital Microscope (AnMo Electronics, Taiwan) and processed with ImageJ (National

Institutes of Health, Bethesda-MD, USA). SLA and LDMC were calculated for the final harvest from among 4-10 individual leaves of each plant with petioles included.

Individual seed mass was weighed on a precision scale (42 g / 0.00001 g, MS105DU, Mettler Toledo, Columbus-OH, USA) as total seed weight divided by number of seeds ($N = 20$). Seed viability was assessed by monitoring the emergence of 20 seeds per species over 30 days. Seeds were sown on filter paper inside Petri dishes, kept well-watered with distilled water, and placed in a growth chamber (ASL Aparatos Científicos, Madrid, Spain) with 16 hours of light (flux= 1743-1900 lm, CCT = 4000-6500 K) at 25 °C and 8 hours of darkness at 15 °C.

2.6. Leaf chemical analyses

To assess leaf elemental composition, we collected leaf tissue from three to five individuals per species and soil type during two sampling periods: October 2017 and September-November 2018; different replicates were assessed at the two sampling periods. Leaves were dried to a constant weight at 50 °C and subsequently finely ground using a ball mill (Retsch MM200, Restch GmbH, Haan, Germany). N and C concentrations were analysed with an elemental analyzer (TruSpec CN, LECO, St. Joseph-MI, USA). The elemental composition of Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Si, Ti, V, Zn was measured by extracting samples with HNO₃-H₂O₂ (8:2) by microwave acid digestion (Speed Ave MWS-3⁺, BERGHOF, Eningen, Germany), followed by inductively coupled plasma-optical emission spectrometry (Varian ICP 720-ES, Agilent Technologies, Santa Clara-CA, USA). All elemental analyses were performed by EEZ-CSIC Analytical Services. Only elements with values above 0.025 ppm (the detection limit of the ICP-OES spectrometer) were included in the statistical analyses.

2.7. Calculations and statistics

All statistical analyses were run in R 3.6.0 (R Core Team, 2020).

To model the gradualness of growth and flowering patterns, changes in canopy length and in the percentage of shoots bearing flowers within the canopy over time were fitted to a Boltzmann sigmoid regression (Self-Starting Nls Four-Parameter Logistic Model function on R). In this analysis, the scale parameter indicates the steepness of the curve and, consequently, the gradualness of the change in growth or flowering (Palacio *et al.*, 2013). Shoot growth rate ($L\text{-day, mm day}^{-1}$) was calculated as the difference in canopy length between two consecutive monthly measurements divided by the number of days elapsed between both measurements. The maximum value of shoot growth rate, the day with the maximum shoot growth rate, the day of first flowering, the day with the maximum percentage of stems with flowers (day of maximum flowering), and the maximum flowering (maximum percentage of stems with flowers) were selected as variables to study changes in phenological patterns. Water use efficiency (WUE) was calculated by dividing the photosynthetic assimilation (A) by stomatal conductance (g_s) (Pérez-Harguindeguy *et al.*, 2013).

Differences between soils and gypsum affinity plant types (i.e. gypsophiles and gypsovags) for the variables canopy length and canopy area at harvest, gradualness of shoot growth (slope of the Boltzmann curve), maximum shoot growth rate, day of maximum shoot growth rate, photosynthetic assimilation (A), stomatal conductance (g_s), transpiration (E), instantaneous Water Use Efficiency (WUE), day of first flowering, day of maximum flowering, maximum percentage of flowering, individual seed mass, total biomass, root:shoot ratio and for each elemental concentration were analysed by generalized linear mixed models

(hereafter GLMM) with “soil” (gypseous / calcareous) and “gypsum affinity” (gypsophile / gypsovag) as fixed factors and “species” as a random factor. Species was included as a random factor to account for species-specific effects and avoid biases related to species selection. In the case of elemental concentrations, we also added taxonomic “family”, and “species” nested within “family” and “year” as random factors to avoid biases related to phylogenetic effects on elemental concentration (Neugebauer *et al.*, 2018) or different sampling dates. Analyses were assessed with function *glmm* on R (*lme4* package version 1.1-15 in R, Bates *et al.*, 2007). The models were fitted to a Gamma distribution when there was not a normal distribution of residuals since, in most cases, data had a constant coefficient of variation and variances increased with means (McCullagh and Nelder, 1989). Model link functions of the Gamma distribution were selected according to the lower AIC criterion and included in each table as sub-indexes. Similarly, differences between soil types within each gypsum affinity class and differences between soil types within each species were assessed by GLMM.

Principal Component Analysis (PCA, *vegan* package version 2.4-6 in R, Oksanen *et al.*, 2007, and *ggplot2* package in R, Wickham, 2016) was used to visualize relationships among elemental concentrations and taxa. We used elements with concentrations above the detection limit of the ICP-OES spectrometer and samples for which we also had N and C concentration data ($N = 182$). All elemental data were transformed to Center Log-Ratio coordinates (Aitchison, 1982) using CoDaPack (Comas-Cufí and Thió-Henestrosa, 2011), to maintain relationships between elements regardless of the concentration, which allows studying joint patterns among elements (Soriano-Disla *et al.*, 2013, Prater *et al.*, 2019). Redundancy Analysis (RDA, *vegan* package version 2.4-6 in R, Oksanen *et al.*, 2007) was

performed with the same data set as the PCA, including “soil” (gypseous / calcareous) and “gypsum affinity” (gypsophile / gypsovag) as fixed factors.

Differences in nutrient composition between soils and species were assessed using non-parametric contrasts based on distance (Adonis function on *vegan* package version 2.4-6 in R, Oksanen *et al.*, 2007) with “soil” (gypseous / calcareous) and “species” as fixed factors and using the Euclidean as distance from Center Log-ratio coordinates. Significant interactions between soil and species on nutrient composition was analysed by multilevel pairwise comparisons with “interaction” as a fixed factor (*pairwiseAdonis* package version 0.3 in R, Martinez-Arbizu, 2019).

A heat map (*ggplot2* package in R, Wickham, 2016) was used to visualize the distances among soils and species jointly with a cladogram from an adapted phylogenetic tree. Distances were calculated using Euclidean as distance from Center Log-Ratio coordinates (vegdist function on *vegan* package version 2.4-6 in R, Oksanen *et al.*, 2007). Distances among branches of the cladogram were extracted from Tree of Life Web Project (Maddison and Schulz, 2007).

3. Results

3.1. Life cycle, growth and phenology

Contrary to our expectations, gypsophile species had similar growth, and similar maximum percentage of stems with flowers and individual seed mass, in both substrates (Table 2, and see F-ratios of GLMMs on Tables A.3, A.4). Also, they produced fruits which rendered viable seeds. Similarly, and in agreement with our expectations, gypsovags completed their life cycle in both soil types, except for *R. officinalis*, which did not produce fruits in either substrate. Gypsovags had similar growth and lower individual seed mass in

gypseous than calcareous soils, and a similar maximum percentage of stems with flowers in both substrates ($P < 0.05$).

Gypsophiles and gypsovags differed in plant size, leaf traits, growth, and phenology (Table 2). Regardless of the soil type, gypsophiles had larger canopy areas than gypsovags, 1.4-fold lower Root:Shoot ratios and 1.5 fold lower LDMC ($P < 0.05$). Furthermore, the timing of phenological events was delayed in gypsophiles as compared to gypsovags, independent of soil type. Gypsophiles attained maximal shoot growth rate 31 days later on average than gypsovags in both soil types ($P < 0.05$, Figure 1a). Gypsophiles also initiated flowering and reached maximal bloom almost two months later than gypsovags on average ($P < 0.05$ for both traits, Figure 1b). Soil type had an effect on the flowering phenology of gypsovags: plants grown on calcareous soil initiated flowering earlier than those grown on gypsum ($P < 0.05$).

Regardless of the species or gypsum affinity, plants grown in calcareous soil had larger canopy area and total biomass ($P < 0.05$, Table 2). They also had 1.2-fold higher photosynthetic assimilation, 1.2-fold higher SLA, and started flowering ten days earlier on average than plants grown on gypseous soil ($P < 0.05$, Table 2). Considering each gypsum affinity separately, gypsophiles grown in calcareous soil had larger canopy area and higher SLA than those grown on gypsum ($P < 0.05$, Table 2). Gypsophiles reached their maximal shoot growth rate 27 days later, on average ($P < 0.05$), when growing on calcareous vs. gypseous soil. Gypsovags grown in calcareous soil had higher total biomass and leaf N at harvest and lower individual seed mass than those grown on gypsum ($P < 0.05$). Gypsovags also initiated flowering later on gypsum than calcareous soil ($P < 0.05$, Table 2).

3.2. Leaf elemental composition

Gypsophiles had a different leaf elemental composition compared to gypsovags that was independent of soil type ($P < 0.05$, Table 3), and these differences were maintained in both samplings (data not shown). Gypsophiles and gypsovags shifted their leaf elemental composition based on soil type ($P < 0.05$, Table 3). As indicated by the PCA biplot, plant leaf elemental composition was more strongly influenced by phylogenetic relationships than by soil type, with species of the same family plotting close to each other, regardless of soil type (Figure 2). The biplot of the first and second PCA axes indicated that gypsophiles showed a unique leaf elemental composition compared to gypsovags, irrespectively of the substrate. Gypsophiles growing on both soil types were located in the upper quadrants and associated with the vectors for S, Cu, Mg, Ti, Al, Fe, Mn. This pattern indicates they showed higher concentrations of these elements, regardless of soil type. In contrast, gypsovags were located in the bottom left quadrant, aligned with higher K, P, Zn and N concentrations. Furthermore, the biplot of first and second or second and third PCA axes (Figure 2, and Figure B.1) indicated that plants from different soil type were distributed along the second component. Plants grown in gypseous soils had more positive values along the second component than those grown in calcareous soils, and S vectors had positive values and K and P vectors had negative values. This pattern indicates plants grown on gypsum had high leaf S and low leaf K and P. In accordance with the PCA results, gypsum affinity and soil types were associated with different leaf elemental compositions based on the RDA analysis (F -ratio = 32.11 for gypsum affinity, $P < 0.05$; F -ratio = 8.72, $P < 0.05$, for soil type, respectively, $TVE = 18.8\%$ for RDA model, Figure B.2).

Assessing each element separately, gypsophiles had higher leaf Mg, S, Fe, Al, Na, Mn, Cu than gypsovags and lower K concentrations ($P < 0.05$, Table 4, see F -ratios of GLMMs on Tables A.5, A.6). Particularly large differences were observed for S and Mg. Leaf S of

gypsophiles was triple that of gypsovags, and leaf Mg was 2.4-fold greater in gypsophiles than gypsovags. The leaf S concentration of gypsovags increased from 5.9 mg·g⁻¹ in calcareous soil to 7.7 mg·g⁻¹ in gypsum ($P < 0.05$), whereas S concentrations in gypsophiles shifted from 15.6 mg·g⁻¹ in calcareous soil to 24.4 mg·g⁻¹ in gypsum soil ($P < 0.05$). In contrast, leaf Mg of gypsovags and gypsophiles did not differ between soil types. Leaf Ca was similar between gypsophiles and gypsovags on gypsum, but gypsophiles had almost twice the leaf Ca concentrations of gypsovags when growing on calcareous soil ($P < 0.05$). Gypsovags increased Ca concentrations up to 1.15-fold when growing on gypsum ($P < 0.05$), whereas gypsophiles had similar Ca concentrations on both substrates. For leaf Cr and Mo, gypsophiles had greater concentrations when grown on gypseous soil ($P < 0.05$), whereas gypsovags had similar concentrations in both soils. In general, plants grown on calcareous soil had higher P, K and C and lower S, Mo, Li, Mn, Cu and Mg, than those cultivated on gypseous soils ($P < 0.05$).

Despite these general trends, some species-specific trends were observed. In accordance with the PCA results, the gypsophile *H. fruticosa* was closer to gypsovags than gypsophiles in both soils, whereas the opposite was true for the gypsovag *H. syriacum* (Figure 2, and Figure B.2). Furthermore, some species of gypsophiles and gypsovags shifted their leaf elemental composition between soil types ($P < 0.05$, Table A.7), as observed in distance biplots (Figures 2 and B.1) or the heatmap of distances (Figure B.3). Shifts in leaf elemental composition were mainly related to leaf S, K, P, regardless of gypsum affinity (Tables A.8 and A.9).

4. Discussion

In contrast to our expectations, gypsophiles had equal or better growth and fitness when growing in calcareous soils. Gypsovags also had similar or higher growth and fitness in calcareous soil than gypseous soil, which is not surprising owing to their widespread occurrence on both substrates. In support of our second hypothesis, gypsophiles showed higher S and Mg concentrations than gypsovags irrespective of the soil type. However, both groups of plants shifted their leaf elemental composition according to soil nutrient availability and had higher leaf S and Mg when growing on gypseous soils. Despite these general trends, species-specific responses were observed within gypsum affinities.

4.1. Gypsophiles completed their life cycle on calcareous soil, being similarly or even more productive than on gypseous soil

Gypsum-exclusive species are restricted to gypseous soils in natural environments. However, we observed that gypsophiles were able to complete their life cycle, producing viable seeds in calcareous soils in the greenhouse. This result demonstrates that soil chemistry alone is not a factor preventing the occurrence of gypsophiles off of gypseous soils. This result is supported by field observations in Spain indicating that, even though gypsophiles are far more frequently found on gypseous soils, they are sometimes also found naturally off gypseous soils (Mota *et al.*, 2011, Luzuriaga *et al.*, 2015). Nevertheless, it is unclear if the few gypsophile individuals found growing off gypseous soils in nature could complete their life cycle, producing viable seeds and recruiting new individuals, since most data were observations of presence / absence. In any case, care should be taken when extrapolating results from experimental studies to natural conditions (Wenk and Dawson, 2007). Our experiment involved regular de-weeding and watering, removing any potential competition from neighbouring plants or water stress, conditions that are far from those in natural

environments. The combination of different stress factors (plant competition, drought and altered soil chemistry) could be the underlying mechanism explaining gypsophile restriction to gypseous soils, rather than soil chemistry alone, as demonstrated by our experiment. Further experiments on natural gypseous and calcareous soils testing for the combined effects of soil chemistry plus plant competition and water availability are needed to shed light on these issues.

In contrast to our first hypothesis, some gypsophiles were more productive on calcareous than on gypseous soil, showing higher photosynthetic assimilation rates, higher SLA and larger biomass. Ballesteros *et al.* (2014) found poorer plant performance on marls than gypseous soils. Our calcareous soil had higher pH, N, P and K and lower conductivity, S and Mg concentrations, indicating better conditions than gypseous soil for standard plant growth. Both gypsum affinity groups showed a delay in the initiation of flowering when growing on gypseous soils, probably due to the more stressful conditions of gypsum for plant growth. However, we observed that gypsophiles showed a consistent phenological delay compared to gypsovags. Such a phenological delay has been described in the literature (Escudero *et al.*, 2014), although the ecological and adaptive factors behind it remain unexplored. Furthermore, gypsophiles did not show any leaf deficiency symptoms and had similar maximum flower production and individual seed mass in both soils, similar to gypsovags. Similarly, Heiden *et al.*, (unpubl. res.) observed that gypsophiles had low germination in acidic soils but germinated equally well on alkaline calcareous and gypseous soils. Consequently, gypsophiles seem to require soils with high pH and high Ca availability to germinate and complete their life cycle, but do not have a requirement for high S or gypsum to grow and complete their life cycle under experimental conditions.

4.2. *Gypsophiles* displayed higher leaf S and Mg and lower leaf K than *gypsovags* both in and off gypsum

In accordance with our second hypothesis, *gypsophiles* had higher leaf S and Mg and lower K concentrations than *gypsovags* in both soil types. This pattern indicates a high preference of *gypsophiles* for these two elements, in accordance with previous studies of plants growing on gypseous soils, where the S and Mg concentrations of *gypsophiles* tended to be higher than those of *gypsovags* (Alvarado, 1995, Palacio *et al.*, 2007, Muller *et al.*, 2017).

The ability of *gypsophiles* to accumulate S was remarkable, reaching S foliar concentrations between $15 \text{ mg} \cdot \text{g}^{-1}$ and $25 \text{ mg} \cdot \text{g}^{-1}$, one order of magnitude higher than standard foliar S concentrations of non S-deprived plants (Kalra, 1997). Such high S-accumulation was maintained even when grown in calcareous soil, which had 55-fold less S than the gypseous soil. Despite the lower S availability, *gypsophile* species managed to grow without any signs of deficiency and accumulated S to a higher extent than closely-related *gypsovags* on calcareous soil. We cannot rule out the possibility that S-accumulation is a nutritional requirement of *gypsophiles* that may impede the completion of their life cycle or their competitive ability in natural conditions. The S content in calcareous soils under greenhouse conditions could be sufficient, but the situation may be different in the field, with lower water availability and increased plant-plant competition. Finally, *gypsophiles* showed higher leaf S than *gypsovags*, although some *gypsovags* also had relatively high leaf S in both soil types. High leaf S concentrations are not exclusive of *gypsophiles* but also related to phylogenetic effects (Neugebauer *et al.*, 2018). It has been suggested that the ability to accumulate S could be an ancient trait, evolved before the acquisition of *gypsophily*, that may serve as a pre-requisite to become a *gypsophile* (Moore *et al.*, 2014).

The low leaf K (below $8 \text{ mg}\cdot\text{g}^{-1}$) of gypsophiles could be an adaptation to gypseous soils, since low K requirements may be advantageous in soils with low K availability (Alvarado, 1995), such as gypsum (Casby-Horton *et al.*, 2015). Low K requirements are linked to high leaf Ca concentrations in the gypsophile species studied (Alvarado, 1995), since plants have a preference for using Ca ions over other cations such as K, Na or Mg as osmotic compounds (Kinzel, 1989). High leaf Ca has been considered a distinctive trait of gypsophiles (Palacio *et al.*, 2007, Muller *et al.*, 2017), although we did not observe differences between gypsum affinity types. However, gypsophiles showed high leaf Ca concentrations irrespective of the soil type, whereas gypsovags increased Ca concentrations when growing on gypseous soil. This shift can be explained by the higher Ca activity of gypsum as compared to calcareous soils (FAO, 1990). These results seem to indicate a higher ability to uptake Ca in gypsophiles than gypsovags, although further experiments are needed.

Gypsophiles species had higher leaf Mg than gypsovags, with increased Mg accumulation on gypsum, where it was highly available. However, neither group of plants shifted Mg concentrations in response to changes in the substrate. Mg accumulation is also a distinctive trait of gypsophile species (Palacio *et al.*, 2007, Merlo *et al.*, 2019). However, Mg concentrations are deeply affected by phylogenetic relationships (White *et al.*, 2018), and some gypsophiles, such as *H. squamatum* and *L. subulatum*, did not show an accumulator pattern, as described by Merlo *et al.* (2019). It has been suggested that high Mg concentrations could be advantageous in gypseous soils, favouring foliar succulence (Merlo *et al.*, 2019) or forming crystals with oxalate or sulphate (He *et al.*, 2012) to help detoxify excess S and Ca.

Finally, we observed higher leaf Fe, Al, Na, Mn, Cr and Mo concentrations in gypsophiles than gypsovags. Similar to our results, Alvarado *et al.* (1995) found higher leaf

Fe and Mn in gypsophiles compared to gypsovags. Leaf Na was analysed only in few gypsum plant surveys (Bolukbasi *et al.*, 2016, Merlo *et al.*, 2019); where significant differences between gypsum affinities were not observed. Differences in Cr, Mo and Mn between gypsophiles and gypsovags are difficult to understand, although Mo and Mn are linked to S metabolism (Maillard *et al.*, 2016, Courbet *et al.*, 2019). Despite these general trends, species responded differently to each soil and had different leaf elemental concentration, indicating species-specific responses within gypsum affinities mainly related to S, K and Mg concentrations.

4.3. Gypseous soils affect the leaf elemental composition of plants

Plants grown in gypseous soils had higher leaf S, Mg, Li and lower P and K, mirroring their soil nutrient availability, and also higher Cu and Mn (despite total leaf concentrations that were similar to those in calcareous soils). Thus, gypseous soils affect the leaf elemental composition of plants (Palacio *et al.* 2007, Salmerón-Sánchez *et al.*, 2014), leading mainly to high leaf S and low P regardless of the gypsum affinity of the species (FAO, 1990). Similarly, Boukhris and Lossaint (1970) and Robson *et al.* (2017) observed that plants had high leaf Ca when cultivated on both gypsum and calcareous soil, but less S when growing out of gypsum, according with soil nutrient availability. The mechanisms of P cycling in plants growing on gypsum deserve further study, due to the high relevance of this nutrient for plant growth and the remarkable P immobilization in gypseous soils (FAO, 1990).

5. Conclusions

Gypsophile species grew and were able to complete their life cycle in non-gypseous soils under experimental conditions and in the absence of competition, producing flowers and

fruits which rendered viable seeds. Gypsum endemics had similar or higher growth on calcareous than gypseous soil. Most species shifted their leaf elemental composition according to nutrient soil availability, displaying higher leaf S and lower P in gypseous soils. However, gypsophiles accumulated higher S and Mg and lower K concentrations than gypsovags, irrespective of the substrate. The remarkable ability of gypsophiles to accumulate S even in low S-availability conditions suggests a possible nutritional requirement for high S. However, our results indicate this nutritional requirement may not be the unique driver of the exclusion of gypsophiles from non-gypseous soils in natural environments, and the role of other biotic (plant-plant competition, herbivory) and abiotic (water stress) factors deserves further study.

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Author contributions

GM and SP designed and set up the experiment; AC, maintained the experiment, measured all variables, analysed data and led the manuscript writing; JPF measured and

discussed photosynthesis and related traits; All authors discussed the results and wrote the manuscript.

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Appendices

Appendix A. Supplementary tables

Appendix B. Supplementary figures

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1 TABLES

2 Table 1: Characteristics of study species

Species	Family	Gypsum affinity	Gypsophily*	Seed collection (Spain)
<i>Boleum asperum</i> Desv.	Brassicaceae	Gypsovag	3.03	Castelflorite
<i>Gypsophila struthium</i> subsp. <i>hispanica</i> (Willk.) G.López	Caryophyllaceae	Gypsophile	4.69	Villamayor de Gállego
<i>Helianthemum squamatum</i> Pers.	Cistaceae	Gypsophile	4.87	Villamayor de Gállego
<i>Helianthemum syriacum</i> (Jacq.) Dum.Cours.	Cistaceae	Gypsovag	-	Villamayor de Gállego
<i>Herniaria fruticosa</i> L.	Caryophyllaceae	Gypsophile	4.05	Villamayor de Gállego
<i>Lepidium subulatum</i> L.	Brassicaceae	Gypsophile	4.91	Villamayor de Gállego
<i>Linum suffruticosum</i> DC.	Linaceae	Gypsovag	-	Villamayor de Gállego
<i>Matthiola fruticulosa</i> (L.) Maire	Brassicaceae	Gypsovag	-	Sariñena
<i>Ononis tridentata</i> L.	Fabaceae	Gypsophile	4.43	Villamayor de Gállego
<i>Salvia officinalis</i> Spenn.	Lamiaceae	Gypsovag	-	Leciñena

3 *Exclusivity to gypseous soils in Spain from expert evaluation. Values of gypsophily range between 0 and 5. Extracted from Mota *et al.*,
4 (2011)

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Table 2: Means and standard deviation of leaf traits, seed traits, growth and phenological variables for each treatment. Major letters indicate significant differences between gypsophiles and gypsovags regardless of the soil type ($P < 0.05$). Minor letters indicate significant differences between soil types ($P < 0.05$) within each gypsum affinity group.

Variables	Gypsovags				Gypsophiles			
	Calcareous		Gypseous		Calcareous		Gypseous	
Final canopy area (dm ²)	10.71±7.30	A	8.73±4.57	A	18.42±11.03	Ba	15.27±9.42	Bb
Final length (mm)	189.28±64.37		200.17±68.29		176.24±88.65		165.71±76.22	
Gradualness	19.58±12.52		20.06±12.92		20.52±12.62		21.04±13.36	
Max. shoot growth rate (mm·day ⁻¹)	1.21±0.99		1.12±1.34		1.27±1.40		1.02±0.95	
Day max. shoot growth rate	372.44±49.63	A	388.54±63.54	A	425.40±44.03	Ba	398.04±56.15	Bb
E (mmol H ₂ O m ⁻² s ⁻¹)	5.77±2.37		6.12±3.33		8.07±3.56		6.47±4.08	
A (μmol CO ₂ ·m ⁻² ·s ⁻¹)	12.01±1.94		10.01±3.18		12.87±5.49		9.69±4.19	
Gs (mmol·m ⁻² ·s ⁻¹)	507.31±292.08		563.49±375.79		599.43±362.60		475.69±313.17	
WUE	2.34±0.82		2.00±0.94		1.82±0.91		2.09±1.61	
SLA(cm ² /g)	63.36±33.78		55.02±20.81		84.63±45.46	a	66.46±38.28	b
LDMC (mg·g ⁻¹)	280.99±97.34	A	297.42±78.36	A	197.87±83.18	B	194.57±69.59	B
Leaf N (%)	1.72±0.98	a	1.66±0.87	b	1.56±0.97		1.59±0.84	
Day 1st Flower	340.25±30.25	Aa	360.18±28.80	Ab	402.40±41.83	B	411.80±32.05	B
Day Max Flowering	363.63±23.08	A	373.59±25.60	A	421.73±42.99	B	428.80±36.60	B
Max. Flowering (% stems)	63.13±27.68	A	68.82±17.64	A	42.00±26.17	B	29.33±23.74	B
Individual seed mass (mg)	0.77±0.73	a	0.91±0.62	b	1.38±2.19		2.83±3.33	
Total biomass (g)	12.85±11.96	a	9.40±6.39	b	13.13±10.52		12.54±9.40	
Root:Shoot	1.66±0.62	A	1.54±0.69	A	1.12±0.44	B	1.21±0.51	B

- 1 Table 3: F-ratios, P-value and Variability ($SS_{\text{factor}}/SS_{\text{total}}$) from non-parametric contrasts based
- 2 on distances in which soil (a = 2), gypsum affinity types (b = 2) and species (c = 10) were
- 3 fixed factors. Data set of $N=180$.

Leaf elemental composition	Treatments				
	Soil	Gypsum affinity	Species	Soil*Gypsum affinity	Soil*Species
F-ratio	25.55	90.59	42.47	0.58	1.71
P-value	0.001	0.001	0.001	0.732	0.013
Variability (%)	4.04	14.33	53.75	0.09	2.17

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- 1 Table 4: Means and standard deviation of leaf elemental concentration ($\text{mg}\cdot\text{g}^{-1}$) for each
- 2 treatment. Major letters indicate significant differences between gypsophiles and gypsovags
- 3 regardless of the soil type ($P < 0.05$) Minor letters indicate significant differences between
- 4 soil types ($P < 0.05$) within each gypsum affinity group.

Element ($\text{mg}\cdot\text{g}^{-1}$)	Gypsovags				Gypsophiles			
	Calcareous		Gypseous		Calcareous		Gypseous	
Al	0.3±0.1	A	0.3±0.1	A	0.5±0.6	B	0.4±0.4	B
C	429.0±47.7		427.7±47.7		389.7±47.7	a	378.8±56.7	b
Ca	26.4±12.3	a	30.3±14.1	b	46.9±22.4		46.0±21.8	
Cr	1.3E-2±1.1E-2	A	1.5E-2±1.7E-2	A	3.4E-2±7.3E-2	Ba	2.4E-2±4.7E-2	Bb
Cu	1.0E-2±6.1E-3		1.2E-2±9.4E-3		1.2E-2±6.7E-3	a	1.3E-2±5.6E-3	b
Fe	0.3±0.1	A	0.3±0.1	A	0.5±0.7	B	0.4±0.5	B
K	10.4±3.2	Aa	8.3±3.3	Ab	8.2±4.1	Ba	6.2±2.3	Bb
Li	4.5E-3±4.5E-3	a	6.8E-3±7.3E-3	b	3.6E-3±3.0E-3	a	5.4E-3±5.5E-3	b
Mg	4.1±2.1	A	4.1±2.0	A	8.8±5.3	B	10.8±7.4	B
Mn	6.2E-2±2.8E-2	A	5.7E-2±2.8E-2	A	7.8E-2±2.8E-2	Ba	6.4E-2±2.2E-2	Bb
Mo	5.6E-3±5.6E-3		7.8E-3±7.7E-3		6.1E-3±4.4E-3	a	1.7E-2±2.0E-2	b
N	17.3±8.8		15.9±8.9		15.3±8.1		14.5±7.4	
Na	1.0E-1±6.5E-2	A	8.7E-2±3.0E-2	A	1.4E-1±6.1E-2	B	1.3E-1±6.1E-2	B
P	2.3±1.5	a	1.1±0.6	b	2.4±2.6	a	1.0±0.7	b
S	5.9±4.2	Aa	7.7±4.7	Ab	15.6±7.6	Ba	24.4±16.4	Bb
Si	1.0±0.2		1.0±0.2		1.0±0.3		1.0±0.3	
Ti	3.7E-3±1.3E-3		4.2E-3±2.1E-3		5.6E-3±5.8E-3		5.5E-3±5.6E-3	
Zn	5.1E-2±3.2E-2		5.3E-2±2.9E-2		5.1E-2±4.9E-2		5.0E-2±3.8E-2	

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FIGURE CAPTIONS

Figure 1: A) Variation in relative shoot growth (mm/mm day⁻¹) and B) percentage of flowering stems (%) from December 2017 to September 2018. Centroids of each treatment mean were drawn ($\pm$ S.E.). Circles indicate gypsophiles; squares, gypsovags. Filled symbols indicate plants grown on gypsiferous soils; empty symbols, calcareous soils

Figure 2: Biplot distance of first and second principal components based on Center Log-ratio transformation of leaf elemental composition. Centroids of each treatment mean were drawn ($\pm$ S.D.). Circles indicate gypsophiles; squares, gypsovags. Filled symbols indicate plants grown on gypsiferous soils; empty symbols, calcareous soils. BoAs: *B.asperum*; GyHi: *G.hispanica*; HeFr: *H.fruticosa*; HeSq: *H.squamatum*; HeSy: *H.syriacum*; LeSu: *L.subulatum*; LiSu: *L.suffruticosum*; MaFr: *M.fruticulosa*; OnTr: *Ononis tridentata*; RoOf: *R.officinalis*; WA: all gypsophiles on calcareous soils; VA: all gypsovags on calcareous soils; WY: all gypsophiles on gypsiferous soils; VY: all gypsovags on gypsiferous soils.

SUPPLEMENTARY CAPTIONS

Table A.1: Means and standard deviation of physicochemical features of native soils.

Table A.2: Means and standard deviation of elemental composition of native soils.

Table A.3: F-ratios of GLMMs in which soil (calcareous and gypseous) and gypsum affinity (gypsovag and gypsophile) were fixed factors and species was a random factor. Bold type indicates $P < 0.05$.

Table A.4: F-ratios of GLMMs within gypsum affinities in which soil (calcareous and gypseous) was a fixed factor and species was a random factor. Bold type indicates $P < 0.05$.

Table A.5: F-ratios of GLMMs with soil (calcareous and gypseous) and gypsum affinity (gypsovag and gypsophile) as fixed factors and time, family and species nested within family as random factors. Bold type indicates $P < 0.05$.

Table A.6: F-ratios of GLMMs within gypsum affinity types in which soil (calcareous and gypseous) was a fixed factors and time, family and species nested within family were random factors. Bold type indicates $P < 0.05$.

Table A.7: Means and standard deviation of the Euclidean distance between the chemical composition of each species in gypseous vs. calcareous soil, plus differences between soil types on each species based on Center Log-ratio transformed elements. F-ratios. P-value from

multiple comparisons of species*soil interaction. P-values were adjusted by Bonferroni. Bold type indicates the three most variable elements on each species between soil types.

Table A.8: F-ratios of GLMM within species in which soil (calcareous and gypseous) was a fixed factor and time was a random factor (b=2). Bold type indicates $P < 0.05$.

Table A.9: Means and standard deviation of elemental concentrations of study species grown on calcareous and gypseous soil.

Figure B.1: Biplot distance of second and third principal components based on Center Log-Ratio transformed elements. Centroids of each treatment mean were drawn ($\pm$ S.D.). Circles indicate gypsophiles; squares, gypsovags. Filled symbols indicate plants grown on gypsiferous soils; empty symbols, calcareous soils. BoAs: *B.asperum*; GyHi: *G.hispanica*; HeFr: *H.fruticosa*; HeSq: *H.squamatum*; HeSy: *H.syriacum*; LeSu: *L.subulatum*; LiSu: *L.suffruticosum*; MaFr: *M.fruticulosa*; OnTr: *Ononis tridentata*; RoOf: *R.officinalis*; WA: all gypsophiles on calcareous soils; VA: all gypsovags on calcareous soils; WY: all gypsophiles on gypsiferous soils; VY: all gypsovags on gypsiferous soils.

Figure B.2: Biplot distance of first and second redundancy components based on Center Log-Ratio transformed elements. Centroids of each treatment mean were drawn ($\pm$ S.D.). Circles indicate gypsophiles; squares, gypsovags. Filled symbols indicate plants grown on gypsiferous soils; empty symbols, calcareous soils. BoAs: *B.asperum*; GyHi: *G.hispanica*; HeFr: *H.fruticosa*; HeSq: *H.squamatum*; HeSy: *H.syriacum*; LeSu: *L.subulatum*; LiSu: *L.suffruticosum*; MaFr: *M.fruticulosa*; OnTr: *Ononis tridentata*; RoOf: *R.officinalis*; WA: all

gypsophiles on calcareous soils; VA: all gypsovags on calcareous soils; WY: all gypsophiles on gypsiferous soils; VY: all gypsovags on gypsiferous soils.

Figure B.3: HeatMap of Euclidean distance based on Center Log-ratio transformed foliar concentrations of elements. Centroids of each treatment mean were drawn. Greyer colours indicate furthest and whitish grey shows plants with more similar leaf elemental composition.

BoAs: *B.asperum*; GyHi: *G.hispanica*; HeFr: *H.fruticosa*; HeSq: *H.squamatum*; HeSy: *H.syriacum*; LeSu: *L.subulatum*; LiSu: *L.suffruticosum*; MaFr: *M.fruticulosa*; OnTr: *Ononis tridentata*; RoOf: *R.officinalis*; Calc: on calcareous soils; Gyp: on gypsiferous soils. Species with asterisk are gypsophiles. The cladogram is an adapted phylogenetic tree.