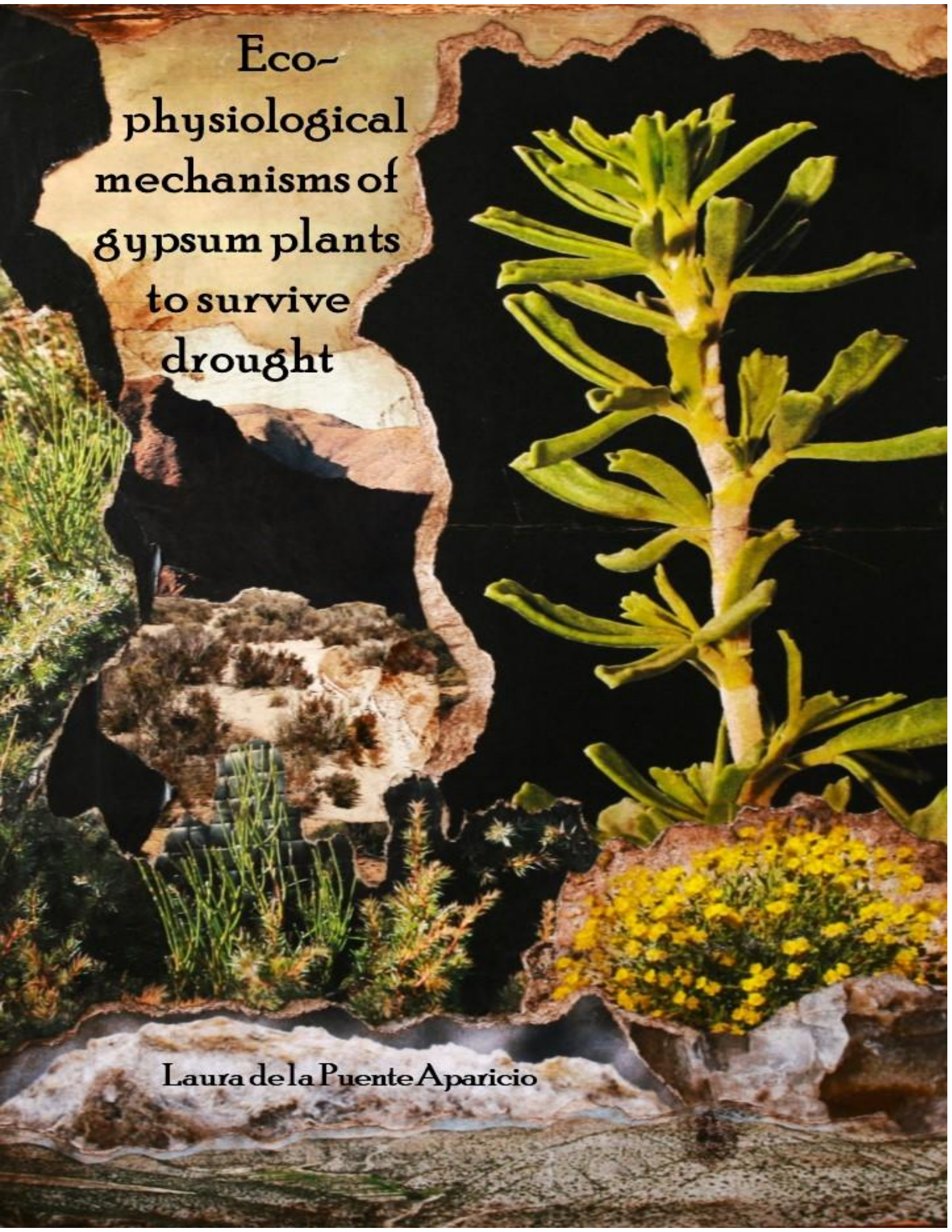
Eco-physiological mechanisms of gypsum plants to survive drought



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MECANISMOS ECOFISIOLÓGICOS PARA SOBREVIVIR A LA SEQUÍA EN PLANTAS DE SUELOS DE YESO

ECOPHYSIOLOGICAL MECHANISMS TO SURVIVE DROUGHT OF PLANTS LIVING ON GYPSUM SOILS

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SUMMARY

Soils with high gypsum ($CaSO_4 \cdot 2H_2O$) content are present in arid and semi-arid areas around the world. The adaptation of plants to gypsum soils results in communities composed of species that live exclusively on gypsum soils (gypsophyles) and other species, more generalist, that can live inside and outside these soils (gypsovags). Both groups of plants must deal with physical and chemical limitations inherent to these atypical soils, in addition to dealing with water scarcity, the most limiting factor for life in gypsum ecosystems. However, water use at the community and species level is still poorly explored in gypsum ecosystems. Gypsum crystallization water could be a critical water source for some plants to survive the dry season, but the factors that enable its utilization are still unknown. Hydroecological niche segregation, by which coexisting species partition water resources, could play a fundamental role in the maintenance of diversity in gypsum communities, and its understanding would bring us closer to the knowledge of adaptation to this atypical substrate. On the other hand, the association with soil microorganisms and the exudation of different compounds by roots could have a fundamental role for the survival and the acquisition of water and nutrients by plants dwelling in these alkaline and nutrient-poor soils.

This PhD Thesis aims to define the water sources used by gypsum plant species in two different communities (one in NE Spain and another in Iran), considering gypsum crystallization water as a potential source in addition to free water at different soil depths. Further, it aims to explain the below and above-ground strategies of two gypsophyles to survive water and nutrient limitations, including tracing gypsum crystallization water use by a labelling treatment in *Helianthemum squamatum* plants, and the *in situ* observation of rhizosphere pH during growth of *Ononis tridentata* on gypsum soils with modified fungal presence.

In the gypsum plant community of NE Spain, a clear segregation of hydroecological niches was observed during the dry season, whereas in the wet season all species used the shallow free water in the soil. In summer, deep-rooted plants were supplied by water stored at deep layers, whereas shallow-rooted plants were mainly supplied by gypsum crystallization water, implying a key role of this water source for community survival. Species gypsum affinity had no influence on the water sources used by plants.

In the plant community studied in Iran, a segregation of hydroecological niches was also observed throughout the year, but more marked during the dry season. None of the species in this community used gypsum crystallization water as the main source, but they used free water from different depths. The patterns of water depth used depended on different species-specific factors, being rooting architecture the best explanatory one. Gypsum affinity or the photosynthetic pathway did not affect the hydroecological strategies of the plant species studied.

The experimental section of this PhD Thesis showed a new water-saving response of *H. squamatum* to cope with short-term drought, reducing stomatal opening, transpiration and photosynthesis, and exuding an osmo-protector molecule to avoid stress. The plant did not use gypsum crystallization water during the short-term drought treatment or they life-time. However, labelling of gypsum crystallization water led to severe changes on the gypsum substrate that preclude comparison with previous studies run under natural conditions. The gypsum soil microbial community seems to be adapted to natural drought pulses, remaining unaltered with the drought treatment.

Soil fungi acidified the rhizosphere during the growth of *Ononis tridentata* seedlings, and thus, contributed to improve nutrient availability for plants. The exudation of organic acids and sugar alcohols increased in plants growing in the fungi-sterile soil. These exudates also favoured rhizosphere acidification and had the potential to attract soil microorganisms. The ability of gypsum plants to release root exudates could also be linked to the use of gypsum crystallization water in the field.

The water use patterns of the studied plant communities exemplify diverse adaptation mechanisms of coexisting plants to drought, a useful knowledge to apply in global change impact studies. The use of gypsum crystallization water has been revealed as a relevant water source to face drought, to be taken into account in other gypsum soil ecosystems. However, experimental demonstration of its use needs further studies. We have shown the vulnerability of *H. squamatum* to short-term experimental drought and the relevance of soil fungi for rhizosphere acidification in *O. tridentata*. Consequently, this PhD Thesis contributed with the understanding of several key aspects of plant life on gypsum soils.

RESUMEN

Los suelos con alto contenidos en yeso ($CaSO_4.2H_2O$) están presentes en zonas áridas y semiáridas de todo el mundo. La adaptación de las plantas a los suelos de yeso deriva en comunidades compuestas por especies que viven exclusivamente sobre este tipo de sustratos (gipsófitos) y otras especies, más generalistas, que pueden vivir dentro y fuera de estos suelos (gipsovags). Todas ellas deben lidiar con limitaciones físicas y químicas inherentes a los suelos ricos en yeso, además de hacer frente a la escasez de agua, el factor más limitante para la vida en estos ecosistemas. Sin embargo, el uso del agua a nivel de comunidad y a nivel de individuo ha sido poco explorado en los aljezares. Además, el agua de cristalización del yeso podría ser una fuente de agua fundamental para que algunas plantas sobrevivan la estación seca, pero los factores que permiten su utilización se desconocen. La segregación de nichos hidroecológicos, mediante la cual las especies coexistentes se reparten los recursos hídricos, podría tener un papel fundamental para el mantenimiento de la diversidad en las comunidades de yesos. Por tanto, analizar si existen procesos de segregación de nichos hidroecológicos es fundamental para comprender la adaptación vegetal a este sustrato atípico. Por otra parte, la asociación con microorganismos del suelo y la exudación radical de distintos compuestos podría tener un papel fundamental para la supervivencia y la adquisición de agua y nutrientes por parte de las plantas que habitan en estos suelos alcalinos y pobres. Esta tesis doctoral pretende definir las fuentes de agua utilizadas por las especies de plantas de yeso en dos comunidades diferentes (una en el NE de España y otra en Irán), considerando el agua de cristalización del yeso como una fuente potencial, además del agua libre, en distintas profundidades del suelo. Por otra parte, pretende explicar las estrategias de dos especies exclusivas de los suelos de yeso para sobrevivir las limitaciones de agua y nutrientes, incluyendo el seguimiento del potencial uso del agua de cristalización del yeso mediante marcaje en *Helianthemum squamatum* y la observación *in situ* del pH de la rizosfera durante el crecimiento con y sin hongos de *Ononis tridentata*.

En la comunidad de plantas de yeso del NE de España se observó una clara segregación de nichos hidroecológicos en la estación seca, de manera que en primavera las plantas se abastecieron del agua disponible en el suelo poco profundo. En verano, las plantas de raíz más profunda se abastecieron del agua almacenada en profundidad y las plantas de raíz somera utilizaron

principalmente el agua de cristalización del yeso. Este descubrimiento indica un papel clave de esta fuente de agua para la supervivencia de la comunidad. La afinidad al yeso de las distintas especies no tuvo influencia en las fuentes de agua que utilizaron.

En la comunidad de plantas estudiada en Irán también se observó una segregación de nichos hidroecológicos durante todo el año, más marcada durante la estación seca. Ninguna de las especies que habitan en esta comunidad utilizó el agua de cristalización del yeso como su fuente principal, sino que se repartieron el agua a distintas profundidades del suelo, dependiendo de distintos factores específicos de cada especie estudiada, siendo el sistema de arquitectura radical el factor más explicativo para el patrón de fuentes utilizadas. Ni la afinidad al yeso, ni la vía fotosintética afectaron a las estrategias hidroecológicas de las especies estudiadas.

La sección experimental de la tesis nos mostró una nueva respuesta de ahorro de agua por parte de *H. squamatum* para enfrentar la sequía a corto plazo, reduciendo la apertura estomática, la transpiración y la fotosíntesis, y exudando un compuesto osmo-protector para evitar el estrés. Las plantas no utilizaron el agua de cristalización durante el tratamiento de sequía ni durante todo su desarrollo. Sin embargo, el marcaje del agua de cristalización del yeso condujo a cambios severos en el sustrato que impidieron la comparación con estudios previos realizados en condiciones naturales. Las comunidades de microorganismos de los suelos de yeso parecen estar adaptadas a los pulsos naturales de sequía, permaneciendo inalteradas con el tratamiento de sequía. Los hongos del suelo acidificaron la rizosfera durante el crecimiento de las plántulas de *O. tridentata*, mejorando la disponibilidad de nutrientes para las plantas. La exudación de ácidos orgánicos y alcoholes de bajo peso molecular aumentó cuando las plantas no contaron con la colaboración de los hongos para su nutrición. Estos exudados favorecen también la acidificación y tienen, potencialmente, la función de atraer microrganismos del suelo. La capacidad de producir exudados radicales por parte de estas especies podría además estar ligada con los mecanismos de absorción del agua de cristalización del yeso en condiciones naturales.

Los patrones de uso de agua de las comunidades estudiadas nos han mostrado los mecanismos de adaptación de las plantas coexistentes a este duro ambiente, pudiendo aplicar este conocimiento en estudios de impacto del cambio global. El uso del agua de cristalización parece ser una fuente relevante a tener en cuenta en otros estudios sobre ecosistemas de suelos de yeso. Sin embargo, la demostración experimental de su uso necesita más estudios futuros. Hemos mostrado la

vulnerabilidad de *H. squamatum* frente la sequía experimental a corto plazo y la relevancia de los hongos del suelo para la acidificación de la rizosfera de *Ononis tridentata*. Por tanto, esta tesis doctoral ha colaborado con el conocimiento de varios aspectos claves para comprender la vida vegetal en suelos de yeso.

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GENERAL INTRODUCTION

Plants adapt to water limitation in dryland ecosystems

Drylands are characterized by scarce and unpredictable rainfall and high evapotranspiration caused by elevated solar radiation and temperatures (Reynolds *et al*., 2007). They occupy 41% of the global surface, and can be categorized by their aridity index, which can be defined as the ratio of annual precipitation to annual potential evapotranspiration (Figure G.I.1 taken from FAO, 2023). They can be subdivided in: hyper-arid regions, with an aridity index of less than 0.05; arid regions, between 0.05 to 0.2; semiarid regions, between 0.2 and 0.5 and dry sub-humid regions with an aridity index between 0.5 and 0.65 (Huang *et al*., 2016).

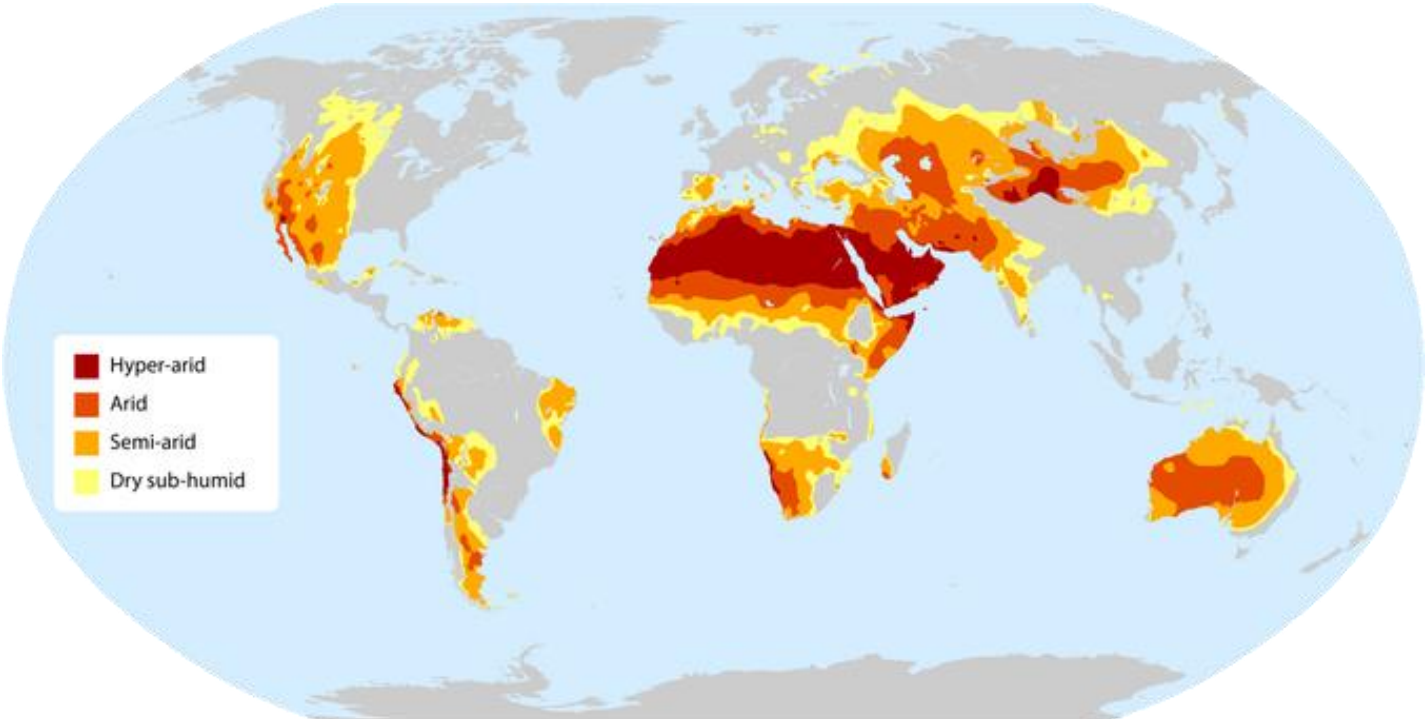


Figure G.I.1. World´s drylands divided in its subtypes (FAO, 2023)

Water is crucial for plant life. The lack of water availability (either surface, groundwater or air moisture) in arid and semiarid regions limits plant growth and survival. The intense drought periods and high temperatures typical of drylands produce significant stress on plants. Water stress has important effects on vegetation, reducing cell turgidity, stomatal conductance or carbon assimilation rates, what in turn, reduces ecosystem primary net productivity (Yin *et al*., 2019).

Thus, in a water-limited ecosystem, plants would be under a strong selective pressure to maximize soil water use and be able to grow and survive under drought. To this end, plants may show different "strategies", defined as suites of different morphological, physiological or phenological measurable features that enable plants to adjust to their environment. In ecology, plant responses have been classified along a gradient according to the ability to cope with disturbances and the ability to rapidly respond to growth opportunities (Grime, 1977; White *et al*., 2004; Loik *et al*., 2004; Voltaire, 2018). Some of the described plant adaptations include adjustments on the growth form, seed production, plant phenology and leaf and root functional traits. Many previous works showed examples of leaf size reduction, changes in leaf vertical angle (Gibson, 2012; Jubany *et al*., 2012) and narrower or more dissected leaf shapes linked to arid ecosystems. These plant characteristics avoid overheating and extremely low water potentials in the plants, and reduce water use (Stowe & Brown, 1981; Sisó, & Gil-Pelegrín, 2001). In addition, root system traits are fundamental to access and store water (Kuhn *et al*., 2022), for example, phreatophyte species have deep root systems that allow access the deep water stored in the soil. Additionally, it is usual to find species with dimorphic root systems that allow a flexible water use strategy, adapting root activity to the variations in soil water availability along the soil profile (Wang *et al*., 2021; Oliveira *et al*., 2015; Querejeta *et al*., 2021; Rempe & Dietrich, 2018). Other functional strategies to avoid drought and thermal stress include modifications on the photosynthetic pathway, e.g. CAM plants (Crassulacean Acid Metabolism) open stomata during night to avoid high transpiration; C_4 photosynthesis that spatially segregates C-fixation from decarboxylation, preventing photorespiration, and hence improving photosynthetic efficiency and reducing water loss in hot, dry environments (Lara & Andreo, 2011; Garcia *et al*., 2009).

On the other hand, there are different mechanisms to mitigate the negative impacts of drought in the short-term, adaptations that have also been the subject of many studies (Simpson and Solbring, 1977). The different strategies used by plants to carry on this purpose can be resumed as three contrasting **ecophysiological** approaches (Peguero-Pina *et al*., 2020):

1) Drought avoidance strategy, which prevents the dehydration effects at cellular and plant level, through the reduction of water loss with an early stomata closure (i.e. water saver plants) or by the increase of root water uptake, which allows the maintenance of high rates

of stomatal conductance and transpiration (i.e. water spender plants) (Smith, 1978; Monson & Smith, 1982).

2) Drought tolerance strategy, tolerating the damage associated to dehydration effects, often acting on the cell membrane stability, which can be preserved by a great number of compounds such as organic acids or polyols (Farooq *et al*., 2009; Brum *et al*., 2017; Bartlet *et al*., 2012).
3) Drought scape strategy, completing its vegetative cycle before the drought period starts.

However, most species tend to combine different strategies (Volaire, 2018; Aronson *et al*., 1992).

Unfortunately, dryland-adapted plants are part of one of the most fragile ecosystems due to the continuous increasing drought and the overexploitation of scant water resources in these areas (Malagnoux *et al*., 2008; Maestre *et al*., 2006; Seyfried *et al*., 2005) which, in addition, have been reported to be in expansion (Huang *et al*., 2016). It is, then, important to build a well-founded knowledge of arid and semiarid ecosystem functioning to improve their management and conservation, promoting their resilience to global changes (Wang *et al*., 2012).

Eco-hydrological niches affect water use in the plant community

Despite life in the arid and semiarid environments is limited by water scarcity, these ecosystems host diverse and highly adapted plant communities. The ecological strategies of plants adapted to drought can explain the structure of communities and the properties of arid ecosystems (Grime, 2002). The ecological niche is a concept that describes how organisms interact with their environment at different spatio-temporal scales (Leibold & Geddes, 2005; Chase, 2011). We could consider that species in the communities that exhibit different traits are niche-structured, minimizing overlap in resource use (Gallart *et al*., 2002; Purves & Tornbul, 2010; Levine & HilleRisLambers, 2009). In arid and semiarid ecosystems, water has a principal role in structuring communities. Therefore, we should find diverse strategies of water use by coexisting species. The partitioning of available water among coexisting species is known as ecohydrological niche segregation (Sivertown *et al*., 2015), and allows the coexistence of competing species in a big part of arid and semiarid ecosystems. Hydrological niches in plant communities can involve spatial or temporal water partitioning (Brun *et al*., 2018; Palacio *et al*., 2017; Redtfeldt & Davis, 1996; Li *et al*., 2018).

Rooting depth has been defined as a frequent significant factor for spatial hydrological niche structuring (Silvertown *et al*., 2015 and cites therein). The deepest roots constitute an investment to cope with fluctuations in water availability, reducing water stress and competition during the dry periods. Deep-rooted plants can potentially maintain gas exchange during long periods of drought, without the need to perform physiological regulation (Brum *et al*., 2017; Niinemets, 2010). However, this strategy involves a lower nutrient availability, as the shallowest layers of soil tend to be more abundant in nutrients than the deeper soil layers, i.e. the utilisation of water from subsoil/bedbrock could reduce nutrient cumulative uptake from the fertile topsoil (Querejeta *et al*., 2021, but see McCulley *et al*. 2004). In addition, the use of groundwater could lead to plant stress due to low oxygen levels (Numburg *et al*., 2005). Shallow-rooted species are more likely to become water limited during drought periods, leading to large decreases in plant water potential and requiring drought tolerance or avoiding strategies. Plant species with dimorphic root systems show both tapping roots and a dense shallow root system, potentially benefitting from deep water during the dry season, while having access to nutrients in the shallow soil (Nie *et al*.,2011; Wang *et al*., 2017). Dimorphic root systems are also particularly suitable to develop hydraulic redistribution, i.e. a passive transport of soil water along a hydraulic gradient through the rooting system (Richard & Caldwell, 1987), caused by the water potential difference between the dry shallow layers and wet deep layers (Bauerle *et al*., 2008; Prieto *et al*., 2012). This way, neighbouring species can act as "bioirrigators", fomenting other species survival and keeping plant diversity in arid and semiarid communities (Bayala & Prieto, 2020).

The temporal shifts in soil moisture typical of dryland ecosystems add a temporal dimension to niche structuring. Arid and semiarid ecosystems show remarkable fluctuations in the amount of soil water, the depth of water and the duration of inundation and of drought as the pool dries up (Silvertown *et al*., 2015). Many works showed a general trend in arid and semiarid ecosystems towards a seasonal shift of the main water source, from shallow depths in the wet season to lower depths in the dry season (Wu *et al*., 2019; Chen *et al*., 2020; Antunes *et al*., 2018; Zencich, *et al*., 2002). This variability has an impact on the structuring of plant communities. For example, in desert communities, several species are able to exploit small but frequent rainfall events, while others utilise larger but infrequent precipitations pulses (Verhulst *et al*., 2008; Angert *et al*., 2009).

Partitioning of soil moisture among coexisting species has been found repeatedly in vegetation world-wide, including desert plants (Manning & Barbour, 1988) and semiarid Mediterranean shrublands (Filella & Peñuelas, 2003). However, the evidence on the existence of hydrological niches needs to be completed with the study in other arid and semiarid ecosystems, which are comparatively understudied. Additionally, further investigation is needed to disentangle the mechanisms that underlie hydrological niche segregation and to anticipate changes in vegetation shifts in response to global warming.

Gypsum soils is a singular substrate in arid and semiarid regions

Gypsum has been acutely studied as a raw material, rock constituent, and archaeological indicator (Herrero *et al*., 2009, Chandara *et al*., 2009; Pelosi *et al*., 2013; Gazquez *et al*., 2020), but its role in nature as a soil constituent influencing life is a relative recent field of research. Gypsum soils are widespread in arid and semiarid regions of the Earth, being present in the five continents and with high relevance in Africa and Central and Western Asia, where they affect 40%, 75% and 25%, respectively, of the total land surface (Escudero *et al*., 2014; Eswaran & Gong, 1991). These soils occupy more than 100 million Ha worldwide (Figure G.I. 4, Boyadgiev, & Verheye, 1996), affecting the livelihood of millions of people (Palacio and Escudero, 2014). In Europe, gypsum

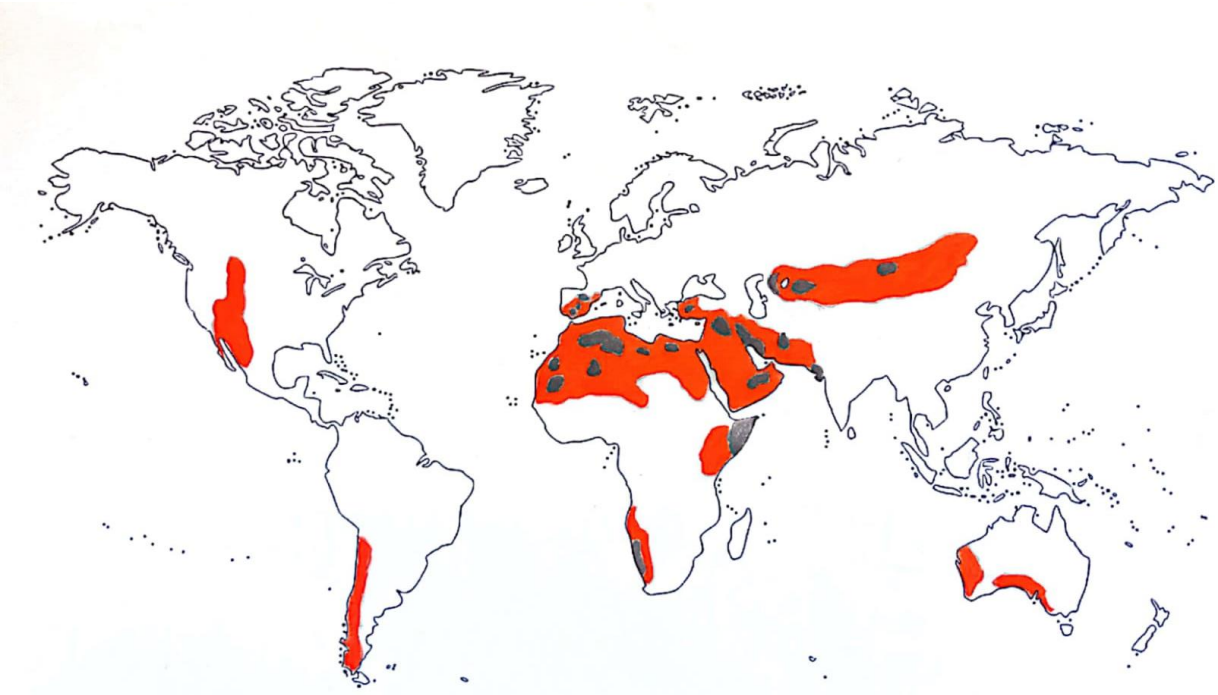


Figure G.I.2. Gypsiferous soils surface in the world. Orange indicate gypsiferous soils present but not dominant, and black indicate gypsiferous soils dominant. Figure adapted from Escudero et al., 2015 (data taken from Boyadgiev, & Verheye, 1996)

soils are predominant in Spain, where they occupy approximately 4.2% of its area (Escavy *et al*., 2012). Gypsum ($CaSO_4 \cdot 2H_2O$) occurs mainly as deposits originated by the precipitation of calcium sulphate from hypersaline lagoons, inland seas or hotsprings (Schreiber & Tabakh, 2000; Herrero *et al*., 2009), although pedogenic gypsum formation from atmospheric deposition of sea-derived sulphur oxides has also been reported in some hyperarid regions like the Namib desert (Eckardt & Spiro, 1999). Gypsiferous soils are soils that contain sufficient $CaSO_4 \cdot 2H_2O$ to influence soil physical and chemical properties and to affect plant growth (Boyadgiev, & Verheye, 1996). Gypsum presence in the soil determines an extreme and stressful environment for plant life due to its special physico-chemical features (Escudero *et al*., 2015).

The moderate solubility of gypsum (~2.4g/L) derives in dissolution-precipitation events that alter soil mechanical stability (Cashby-Horton *et al*., 2015). Further, gypsum soils show undefined microstructure and presence of low porosity horizons (Moret-Fernández & Herrero, 2015). Additionally, they present a hard surface crust formed by the uplift of dissolved gypsum that recrystallizes when water evaporates (Badía-Villas & del Moral, 2016). Such hard physical crust limits water infiltration and seedling stablishment (Romao and Escudero, 2005). However, the physical crust together with the biocrust usually present in arid regions could also lead to preservation of humidity in deeper soil layers (Meyer & García-Moya, 1989; Belnap, 2001). All these physical properties limit water availability for plants in the upper layers of the soil, and complicate seedling establishment and root penetration. The pervasive physical effects of gypsum on soil have been repeatedly reported as the main reason for the exclusion of some plant species from gypsum (e.g. Parsons, 1976; Meyer 1986, Romao and Escudero, 2005).

The chemical properties of gypsum soil can also compromise vegetation development (Merlo *et al*., 1998). These soils have an extremely high pH with high ionic concentrations of calcium and sulphate (Herrero and Porta, 2000), which can be toxic for plants (Ruiz *et al*., 2003) and saturate the cation exchange complex, leading to low nutrient retention and availability (Agriculture Organization of the United Nations, 1999). The effect of the nutrient-unbalance of gypsum soil is a reduction of plant uptake of macronutrients as nitrogen or phosphorus, with negative consequences on plant growth (Aerts &Chapin, 1999). It also reduces the uptake of potassium and iron (FAO, 1990) and increases the foliar concentrations of calcium and sulphate in most plants (Palacio *et al*., 2007, Salmeron-Sánchez *et al*., 2014). Due to the chemical constraints typical of

gypsum soils, plants adapted to live on gypsum have developed strategies and mechanisms to cope with the excess of sulphur and calcium and the low availability of nitrogen, phosphorus and potassium (Cashby-Horton *et al*., 2015; Cera *et al*., 2021; Palacio *et al*., 2022).

Water availability in gypsum ecosystems

Notwithstanding the limiting physical and chemical features of gypsum ecosystems explained above, water should be acknowledged as one of the most limiting factors for plant life in gypsum ecosystems. The mechanisms behind plant water use in arid and semiarid regions are a pending riddle to decipher, but such mechanisms are even more puzzling in gypsum soils, due to their singular characteristics and the lack of studies in comparison to other substrates. Owing to their moderate solubility, gypsum soils mainly occur in arid and semiarid areas where precipitation is scarce (Eswaran & Gong, 1991). In addition, water retention in the soil is very low (Herrero & Porta, 2000). However, water availability in these soils has been found to be higher during the dry period than in surrounding non-gypsum soils (Meyer and García-Moya, 1989). Moreover, as a hydrated salt, gypsum holds water in its crystalline structure, which may be available for plants and free-living bacteria during drought periods (Huang *et al*., 2020; Palacio *et al*., 2017).

During gypsum formation, water is preserved in the mineral making part of its crystalline structure. This property can give accurate geological and paleoclimatological information about gypsum formation (Khademi *et al*., 1997). Crystallization water of gypsum accounts for 20.8% of the mineral weight (Bock, 1961). However, these two water molecules can be lost in a two-step dehydration process turning gypsum ($CaSO_4.2H_2O$) into bassanite ($CaSO_4.\frac{1}{2}H_2O$) and, subsequently, anhydrite ($CaSO_4$). Anhydrite is typically originated by geologic processes, mainly through the dehydration of gypsum under conditions of high temperature and pressure. However, it has also been reported in shallower soil layers in very hot and dry regions (more than 42 ºC), where gypsum dehydration seems to be a slow process, independent of diurnal cycles but caused by seasonal changes (James, 1992). The temperature of gypsum dehydration is a controversial research topic that has been explored by many authors (Blount & Dickinson, 1973; references in Klimchouk, 1996). Some authors (e.g. Sonnenfeld 1984) even consider that temperature and pressure alone cannot explain the transition of gypsum into anhydrite. Most studies performed in aqueous solutions conclude that gypsum dehydration can start at 42 ºC or even at lower

temperatures if other salts, like NaCl, are present (James, 1992; Bock, 1961; Furby *et al*., 1968). Temperatures that could be easily achieved in arid regions. However, these gypsum dehydration studies were discussed by Ostroff (1964) showing that gypsum will not convert into anhydrite in a pure calcium sulphate solution under 97 ºC. In the same way, Ossorio *et al* (2014) reported that the formation of anhydrite in solution would take several months with more than 80 ºC. In dry conditions, Yechieli & Wood (2002) reported a full transformation of gypsum into anhydrite at 80 ºC within 800 hours. In the revision done by Klimchauk (1996) it is concluded that gypsum dehydration does not occur in the solid phase, but through gypsum solubilisation and subsequent precipitation as anhydrite. However, other studies such as Tang *et al*. (2019) or Badens *et al*. (1998) consider gypsum dehydration through certain conditions of water vapour pressure and ambient temperature. The gypsum dehydration process is still a matter of debate, and studies done on gypsum dissolution kinetics are controversial (Vítek *et al*., 2014) However, they agree on the modification of gypsum solubility by the presence of other dissolved salts (Ostroff, 1964; James & Lupton, 1978). Additionally, it has been reported a better solubilisation of gypsum with lower pH (Azimi & Papangelakis, 2011).

The possibility of gypsum dehydration taking place at ambient conditions gave rise to interesting investigations on the role of gypsum crystallization water as a relevant water source for life, particularly during dry conditions. For example, Palacio *et al* (2014) showed, through water stable isotopic analyses, how *Helianthemum squamatum* (L.) Dum. Cours., a gypsum specialist plant, and other shallow rooted species, used mainly gypsum crystallization water during summer. Huang *et al* (2020) showed how a cultivation of cyanobacteria on a gypsum rock in extreme xeric conditions was able to dissolve the mineral, extracting the water and leading to anhydrite deposits. Despite the huge relevance of this potential water source for plant life in arid regions, it remains largely understudied. Several aspects, such as the role of gypsum crystalline water use in shaping gypsum communities, or the identification of the underlying mechanisms that enable its uptake by plants, remain unknown.

Plants living on gypsum are adapted to the particular constraints of these atypical soils

Gypsum plant communities are highly diverse, with numerous endemic and highly specialized species that make gypsum outcrops biodiversity hotspots (Moore *et al*., 2014; Escudero *et al*.,

2015; Ortiz-Brunel *et al*., 2023). They are mainly formed by species with stress-tolerant traits (Hodgson *et al*., 1994), and dominated by shrubs, short-lived perennials and annuals (Parsons, 1976). Plant species can be categorized according to their affinity to gypsum into five categories: 1) gypsophiles, species that only live on gypsum; 2) gypsoclines, species that grow preferentially but not exclusively on gypsum; 3) gypsovags, substrate generalist species that grow on and off gypsum; 4) waif plants, rare on gypsum; and 5) gypsophobes, species that never grow on gypsum (Meyer, 1986). However, in this work we just consider two clearly differentiated categories that are common in gypsum communities: gypsophiles and gypsovags, including gypsoclines within this last group. The assembly of gypsum communities depends on plants affinity for gypsum, with gypsophile species being predominant components of plant communities on gypsum soils (Luzuriaga *et al*., 2015, 2020).

The literature on gypsum plants investigated whether there are specific traits linked to plant adaptation to these atypical soils, shared only by gypsophiles from different phylogenetic lineages, and not by gypsovags (e.g. Muller *et al*., 2017). Recent studies have shown that plant species with different affinity for gypsum soils show different nutritional strategies, which seem to be related to the adaptation of plants to the marked nutrient imbalances typical of gypsum soils (Cera *et al*., 2021, 2022). The strategies to cope with gypsum soil chemical features can be divided into accumulator plant species and species with an avoiding strategy. Accumulator plants, mainly gypsophiles, sequester calcium and sulphate across the plant tissues (Cera *et al*., 2021). Contrastingly, plant species with an avoidance strategy, mainly gypsovags, block the ionic uptake by roots and show a larger reliance on mycorrhizal fungi for nutrient uptake (Cera *et al*., 2021, Alguacil *et al*., 2012; Merlo *et al*., 2019). This is supported by studies on mychorrizal fungi that reported a higher degree of colonization in gypsovags than in gypsophiles (Palacio *et al*., 2012; Cera *et al*., 2021), and by several studies indicating increased foliar (Muller *et al*., 2017; Sanchez-Martín *et al*., 2021, Palacio *et al*., 2007, 2022) and whole-plant S, Mg and Ca accumulation in gypsophiles (Cera *et al*., 2021). Despite these nutritional strategies, plant specialization to gypsum does not seem to limit the ability of plants to deal with other non-specific constraints. Accordingly, plants with high gypsum affinity showed similar water and nutrients use efficiencies than plants with less affinity to gypsum (Sánchez-Martín *et al*., 2021). The factors underlying gypsophile restriction to gypsum soils remain a key unsolved issue in gypsum plant ecology. More information on the physiology of gypsophiles in relation to their strategies to cope with the scarcity of water

and nutrients on these atypical soils will likely help to understand the limitations that shape their ecological distribution.

Soil microbial life and root exudation could influence plant survival in gypsum

In extreme conditions, like gypsum soils, the survival of plant species depends on their ability to respond to stress. One of the known mechanisms to survive in stressful environments involves the association of plant roots with certain groups of microbes occurring in the rhizosphere, showing plant-beneficial properties (Hartman & Tringe, 2019). The larger and more diverse the groups of microorganisms are in the bulk soil, the better the starting pool from which plants can actively recruit partners (Shakya *et al*., 2013; Edwards el al, 2015). In other arid lands, soil fungi play a crucial role in the adaptation of plant species and in the regulation of different biochemical cycles (Porras-Alfaro *et al*., 2017; Alguacil *et al*., 2016). It has been demonstrated that mychorrizal fungi can promote plant growth and biomass production (Mohammadi *et al*., 2011; Miransari, 2010; Abbott & Robson, 2018) mainly through soil phosphorus solubilisation (van Der Heijden *et al*., 2015; George *et al*., 1995) and increased nitrogen uptake in the associated plants (Gage, 2004; Vergara *et al*., 2017). There are also evidences of the beneficial effect of fungi on water uptake by plants (Allen, 1982; Allen, 2007; Ruth *et al*., 2011; Xu & Zwiazek 2020; Kakouridis *et al*., 2022). In gypsum ecosystems, recent investigations reported the presence of fungal species resistant to gypsum, but not exclusive to this type of soils (Muriel *et al*., 2022). Gypsophiles and gypsovags also showed differences in the composition of Arbuscular Mycorrhizal Fungy (AMF) communities in their roots, suggesting that fungal interactions may play a role in shaping gypsum affinity (Torrecillas *et al*., 2014). However, the interactions of soil fungi with plant responses facing different abiotic stresses remains understudied in gypsum soils.

The production of root exudates to mobilize unavailable nutrients has also been reported as a mechanism to enhance nutrient acquisition in nutrient-limited soils (Lambers *et al*., 2008). Plant roots have been reported to passively and actively release sugars, organic acids, amino acids and phenolics. These compounds contribute to nutrient cycling serving as carbon and nitrogen sources for rhizosphere microorganisms (Jones *et al*., 2005) and they can be released in response to different stresses (Xia and Roberts, 1994; Kochian *et al*., 2004; Neumann and Römheld, 2000). Several studies have explored the link between shifts in root exudation and the functioning of rhizosphere-associated microbial communities, with a potential link to nutrient and carbon cycling

in the soil that may feed-back to plants (Kavamura *et al*., 2018; Hartman & Tringe, 2019). In a soil where fungi are scarce, root exudation may be a key agent for nutrient uptake through mobilization by soil acidification (Yan *et al*., 2002). However, there are no studies analysing root exudation in gypsophiles and how the presence of soil fungi may interact with exudate production in nutrient-poor, alkaline, gypsum soils.

Root exudation may also be key for the understanding of the use of gypsum crystallization water by plants (Palacio *et al*., 2014; de la Puente *et al*., 2021). Huang *et al* (2020) proposed that gypsum dissolution by the acidification caused by the exudation of organic acids was the mechanism behind its use by free-living cyanobacteria. The ability to exude different organic acids or low molecular weight alcohols by plants living on gypsum, may affect the release of gypsum crystallization water (Van Driessche *et al*., 2017; Tritschler *et al*., 2015). The study of the ability of plants to release such compounds could contribute to further support the potential role of root exudation in gypsum plants as a mechanism to obtain gypsum crystalline water.

Justification for the research

Although gypsum ecosystems represent a big part of global biodiversity and they harbour unique landscapes (Rodríguez-Sánchez *et al*., 2022), their representation in the scientific literature is scarce in comparison to ecosystems developed on other atypical soils (Escudero *et al*., 2015). Most of the research performed so far in these ecosystems has focused on the biodiversity and distribution of the gypsum flora (e.g. Braun-Blanquet & Bolós, 1957; Rivas –Martínez, 1970; Rubio & Escudero, 200; Pueyo & Alados, 2007; Castillejo *et al*., 2011). There are also many studies focused on conservation and restoration of gypsum ecosystems (e.g. Mota *et al*., 2003, 2004; Memariani, 2022) and the evolution of their flora (Moore *et al*., 2014; Martinez-Hernandez *et al*., 2015; Palacio *et al*., 2022; Blanco-Sánchez *et al*., 2023). Furthermore, the ecophysiological strategies to tolerate gypsum by plants have also been explored (e.g. Escudero *et al*., 1999; Palacio *et al*., 2007, Palacio *et al*., 2014; Cera *et al*., 2022), as well as the ecology of the plant communities that thrive on gypsum (e.g. Escudero *et al*., 1999, Romao & Escudero, 2005; Saiz *et al*., 2014; Luzuriaga *et al*., 2015; Sánchez *et al*., 2014). However, there is comparatively very limited literature about the water use by plants in these ecosystems (but see Querejeta *et al*, 2021; León-Sánchez *et al*., 2016; 2018; 2020; Palacio *et al*., 2014; Palacio *et al*., 2017). Considering this factor as one of the most life-limiting due to the arid and semiarid conditions typical of gypsum soils, we

believe it is of great importance to deepen into it, following an integrative approach that includes both the evaluation of long-term plant responses to drought and the analysis of water uptake and use by plants in the short-term. The importance of acquiring knowledge about water as an abiotic factor affecting the ecology of drylands has been imposed as a priority nowadays (Maestre *et al*., 2016). Furthermore, studying water in gypsum ecosystems implies the consideration of gypsum crystallization water as a potential water source for life (Palacio *et al*., 2014, 2017). This PhD Thesis takes over previous studies on the use of gypsum crystallization water, and tries to explain its use by gypsum plant communities and the mechanisms behind its uptake by plants.

Objectives and hypotheses

This Thesis aims to improve the understanding of plant strategies to deal with water scarcity in gypsum soils. There are two general objectives in this work. The first one is to define the different water sources used by species coexisting in two different gypsum plant communities in different seasons. The second one is to explain above and belowground strategies of gypsum specialist species to survive on gypsum. This last objective incorporates two different targets: the first one is to describe the complete physiological responses of the gypsophile *H. squamatum* to endure short-term experimental drought; and the second one is to disentangle the mechanisms that affect rhizosphere pH (with implications on nutrient and water uptake) in the gypsophile *O. tridentata*.

We can subdivide these general objectives into five more specific ones:

Related to the definition of water strategies within plant communities (1st general objective):

1. Identify potential ecohydrological niche segregation among different gypsum plant communities and its relation to different plant traits (root depth, gypsum affinity, photosynthetic pathways) (Chapters 1 and 2)
2. Evaluate the use of gypsum crystallization water by plants in the field (Chapter 1 and 2)

Related to the understanding of the physiological mechanisms involved in the response to water shortage and rhizosphere activity in two gypsum plants (2nd general objective):

3. Experimentally confirm the use of gypsum crystallization water by plants (Chapter 3)
4. Describe whole-plant responses to face short-term drought (Chapter 3)

5. Assess the effect of the soil microbiota on root exudation and rhizosphere pH of plants growing on gypsum soils (Chapter 4)

In **Chapter 1**, we analysed the distribution of water sources among 20 main dominant plant species in a hill top gypsum community in North-East Spain. We characterised the water isotopic composition along the soil profile and underneath each plant, and evaluated the effect of gypsum affinity and rooting depth of the species on the water source used in each season. Considering water uptake patterns, we hypothesized that:

- Shallow-rooted, gypsum-exclusive species will preferentially use gypsum crystallization water in summer, whereas shallow rooted, non-exclusive gypsum species will be restricted to the scarce free water available in the topsoil. Conversely, deep-rooted species, regardless of gypsum affinity, will rely mainly on the use of deep soil water and/or groundwater during summer drought.

Considering plant-soil interactions, we also hypothesized that

- Deep-rooted species will up lift water from the deeper soil layers to the shallower ones

In **Chapter 2**, we aimed to determine whether five dominant woody subshrub species coexisting in the arid Aladaghlar hills (Iran) segregated their ecohydrological niches according to the different water sources used in spring and summer. We further sought to ascertain whether species rooting architecture, gypsum affinity or their photosynthetic pathway were determining factors for the differences found in water use among species and for their ability to use gypsum crystallization water. We hypothesized that:

- Rooting depth will be a determinant factor for water use patterns in the way that, species with a deep taproot will use deep soil water throughout the year but species with a dimorphic root system will be able to change the water source depth, from shallower water in spring to deeper water in summer.
- Shallow-rooted species, without regarding species gypsum affinity, will use shallow water in spring, however, during the dry season, gypsum crystallization water will be their main water source.

- C_4 species (with higher leaf-level water use efficiency and root systems than C_3 species), should be less dependent on hydrological fluctuations, and thus, should still rely on the scarce free soil water remaining in upper soil layers during summer.

In **Chapter 3**, we aimed to perform an integrated analysis of the responses to experimental drought of the gypsophile *H. squamatum* cultivated on gypsum soil with deuterium-labelled crystallization water and on natural gypsum soil. We characterized processes in relation to water use, plant aerial status (physiology, biomass, water content and foliar nutrient composition) and the effects belowground (plant-soil microbial interactions and root exudation). We hypothesized that:

- The main water source used by this species during drought will be the crystallization water of gypsum. Moreover, if it was used any time in the plant life, we would detect the deuterium labelling not only in the xylem sap, but also in the transpired water or in its bulk organic matter, such as leaves or roots.
- Stomatal conductance and transpiration would be maintained under drought stress, due to the putative low stomatal regulation of *H. squamatum* (León Sánchez *et al*., 2017) and the potential use of gypsum crystallization water, but photosynthetic rate may be decreased.
- As a consequence, plant aerial biomass will decrease with drought, as well as leaf elemental concentrations.
- Finally, we postulated that drought will affect the soil microbiota and plant-soil interactions, leading to a reduction in the microbial biomass and an increase in the stress of the microbial communities in the soil, and promoting the exudation of certain compounds by plant roots to improve plant hydric conditions or attracting collaborative fungi.

In **Chapter 4**, we aimed to monitor changes in the rhizosphere pH of the roots of a gypsum endemic species cultivated in soils with different fungal presence (i.e. a natural gypsum soil vs a fungi sterile gypsum soil). We intended to determine the role of soil fungi on the exudation of organic acids by roots and the rhizosphere acidification in *Ononis tridentata* seedlings growing in an alkaline gypsum soil (hence with low nutrient availability). We hypothesized that:

- The presence of fungi in the soil will facilitate soil acidification, reaching lower pH in the rhizosphere.

- Root exudation will be promoted in plants grown on fungi-sterile soils, as a response to a higher nutrient stress.

General Methodology

To address the aims of this thesis and verify the postulated hypotheses, we combined field (in two different gypsum plant communities) and cultivation studies (involving two dominant Iberian gypsophiles), which results have been summarized in four chapters. Each chapter conforms an original research article either published or in revision in different international scientific journals, where methodology is described in depth. However, in this section we aimed to describe in more detail the study systems chosen for field studies, and highlight the approximations used in experimental studies. We also include preliminary data from proofs-of-concept that were not finally included in the main results of each chapter, but are relevant to understand the approaches followed.

Study areas

The study in Chapter 1 was conducted on a gypsum hill in the Middle Ebro Depression, Zaragoza Province, North-East Spain (Figure G.M.1). This region is a depression limited by mountain ranges with the Pyrenees at the north, the Iberian Mountain Range at the South-West and the Catalan Mediterranean System at the South-West (Figure G.M.1). This area is the most arid within the River Ebro Basin (López-Moreno *et al*., 2010), and is formed by low hills and flat-bottomed valleys with an average height of 300 m a.s.l (Mota *et al*., 2011) (Figure G.M.2). On top of the arid conditions that prevail in this region, our sampling was performed at the top of a gypsum hill (Figure G.M. 2), which should exacerbate water scarcity for plants, due to the higher distance to groundwater and the runoff effect of gypsum soils, which have very low water retention (Herrreo & Porta, 2000).

The Middle Ebro Depression encloses the most prominent gypsum outcrops in northern Spain and one of the most massive in Europe (Escavy *et al*., 2012). The lithology in the area is composed mainly by gypsum with several marls and clays (Quirantes, 1978). These are poorly developed soils very sensitive to erosion, characterized by high gyspum contents (more than 60 %), alkaline soil pH (7.5-8), low content of organic matter (<1.5%) and moderate salinity (EC 2-3dS/m) (Navas,

1991). Climate in this region is semi-arid and highly seasonal. Mean annual temperature is 14.9 °C, average annual rainfall is 331.5 mm, which falls mainly during spring and autumn, and evapotranspiration is around 1200 mm (Palacio *et al*., 2007), so plants experience intense drought during summer months. Dryness is accentuated by strong frequent winds dominating from NW to SE that desiccate soil and reduce air moisture (Herrero and Snyder, 1997).

Figure G.M.1. Location of the study area in the Middle Ebro Depression in NE Spain. Image provided by Google Earth (2023).

The Middle Ebro Depression is exceptional for the singularity of the flora and the richness of species, including also edaphic and local endemics (Mota-Poveda *et al*., 2011). Plant communities are predominantly composed of gypsophyle shrubs and Mediterranean widespread basophilic shrubs (gypsovags) belonging to the orders *Gypsophiletalia* and *Rosmarinetalia*, respectively (Braun-Blanquet and Bolòs, 1958). These communities include small-sized xerophytic formations of Mediterranean and gypsophilic shrubs and sub-shrubs followed by some herbaceous perennial and annual species. The most abundant species in these communities are *Rosmarinus officinalis*

L., *Thymus vulgaris* L., *Teucrium capitatum* L., *Stipa offneri* Breistr, *Helianthemum syriacum* Jacq, *Genista scorpius* L. DC, *Fumana ericifolia* Wallr, plus the gypsophytes *Helianthemum squamatum*, *Herniaria fruticosa* L., *Ononis tridentata* and *Gypsophila struthium* subsp. *hispanica* (Willk.) G.López.

Figure G.M. 2. Closer overview of the study area in Chapter 1 showing the landscape of low hills and valleys. First image provided by Google Earth (2023) and second image courtesy of Gabriel Montserrat-Martí.

These lands have been traditionally used for agro-pastoral purposes with cereal crops and livestock (Pueyo, 2005). However, according to Mota-Poveda *et al* (2011) the recent mechanization and extensiveness of the agriculture has caused intensive soil salinization and loss of plant species.

The study included in Chapter 2 was performed in the Aladaghlar hills, located in NW Iran, a field site belonging to the Irano-Turanian floristic region (see Chapter 2, Figure 1). In this region, gypsum outcrops are an integral part of the desert landscapes characteristic of the lowlands and lower mountain belt. In particular, the colourful clays and marls of the Upper Red Formation, with its varying chemical composition, contain fragmented gypsum and intercalations of crystalline gypsum layers (Ghorbani 2019). Such coloured gypsiferous formations cross the Irano−Turanian floristic region, and form an integral part of the deserts of Central Asia and Iran. In NW Iran a

particularly big area, covered by sediments composed of marls, gypsum, conglomerate and sandstone, is the Miocene Upper Red Formation, located between the villages Moshampa (Zanjan province) and Toryan Qeshlaq (East Azarbaijan Province), called locally Aladaghlar (literally from Azeri Turkish "Rainbow mountains") (Azizi *et al*. 2018). The area is located in the Zanjan basin, a remaining of the Tethys Sea until the Early Miocene, with the subsequent sedimentation of marine and continental sediments in the form of marls, fragmented gypsum, siltstone and conglomerate and intercalated evaporite layers, including thick crystalline gypsum (Alizadeh 2017, Rahimpour−Bonab *et al*. 2007).

The Aladaghlar area has a complex relief composed of hills, valleys and playas, and a very heterogeneous chemical composition, varying greatly even on single slopes. Our field sampling was performed in the Aladaghlar hill area (Figure G.M.3), in North-Western Iran, on the border of the Zanjan and Eastern Azerbaijan provinces, where the gypsum content of the slopes varies from 4% to 84% (average of 24%) (Akhani and Rahmaninia, unpublished). The climate of the region is Mediterranean xeric continental (Djamli *et al*. 2011) with severe drought during summer. The mean annual precipitation and temperature are 313 mm and 11.5°C (Min = −7.5°C, Max = 31.9°C), respectively (according to a 50-year climate data recorded in Zanjan meteorological station, east of the study area).

The harsh climate of the Aladaghlar hill area and its edaphic and topographic peculiarities result in a sparse vegetation, dominated mainly by succulent xerophytic shrubs and subshrubs as *Anabasis calcarea* (Charif & Aellen) Bokhari & Wendelbo, *Anabasis eugeniae* Iljin, *Caroxylon gemmascens* (Pall.) Tzvelev, *Kaviria aucheri* (Moq.) Akhani, *Noaea mucronata* (Forssk.) Asch. & Schweinf., *Oreosalsola montana* (Litv.) Akhani, *Salsola arbusculiformis* Drobow (Amaranthaceae), *Atraphaxis suaedifolia* Jaub. & Spach (Polygonaceae) and *Zygophyllum eurypterum* Boiss. & Buhse (Zygophyllaceae). The area is highly diverse with several endemic

species, but is also understudied, with scarce ecological information available in the scientific literature.

Figure G.M. 3. The studied hill of Aladaghlar located in NW Iran. Picture courtesy of Alexander Rudov.

Species included in experimental studies

Chapters 3 and 4 follow experimental approaches to disentangle the mechanisms used by gypsum specialist plants to cope with drought and modify rhizosphere soil conditions. To that end, they focus on two of the most conspicuous gypsophiles of the Iberian Peninsula (Rivas Goday, 1955; Bellot & Casaseca Mena, 1952).

In Chapter 3, we performed an integrated analysis of the responses to experimental drought of the gypsophile *Helianthemum squamatum* L. (Figure G.M.4), which belongs to the *Cistaceae* family. It is a chamaephyte with an Ibero-Maghrebian distribution (W-SW of the Mediterranean Region), exclusive of gypsum soils of the Iberian Peninsula, North of Morocco and Algeria. It is found in open scrubs on gypsiferous soils. It flowers from April to July and produces fruits from June to August (Castroviejo, 2020). It has flat and fleshy green-yellow leaves, covered with scales. The flowers are in a ramose inflorescence with yellow maculated petals longer than the sepals (Figure G.M.4). The root system of *H. squamatum* has been reported to reach from 50 cm to 1 m deep (Guerrero-Campo, 1998).

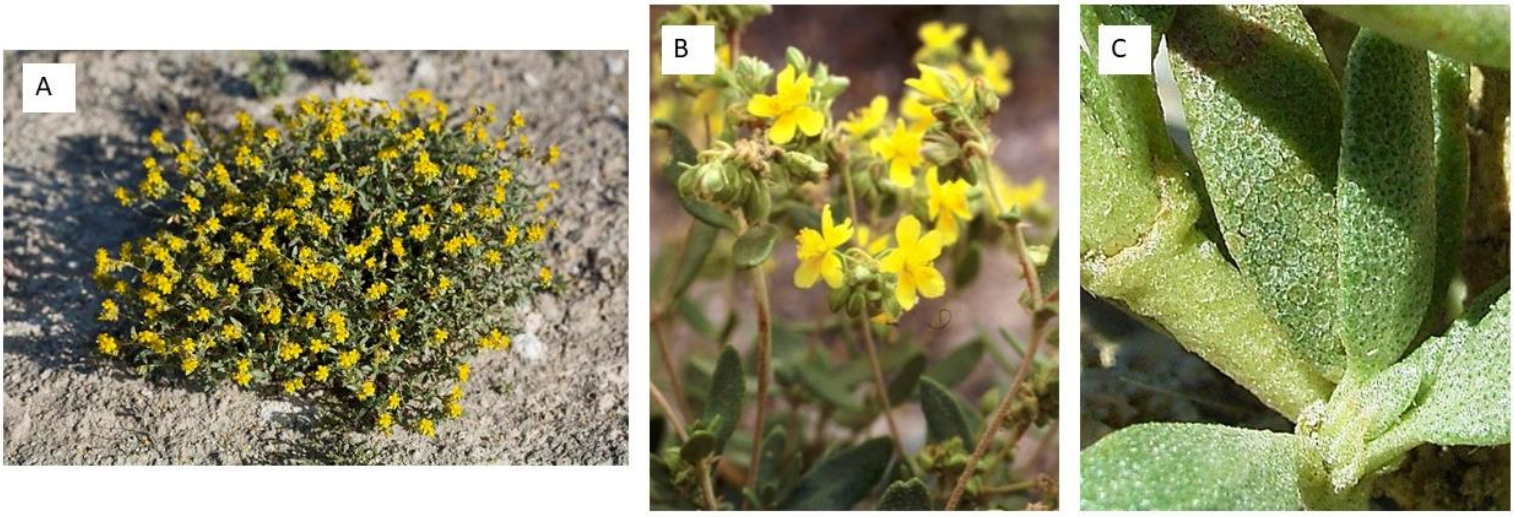


Figure G.M.4. *Helianthemum squamatum* A) Plant size and general view B) Flowers, C) Leaves. Pictures by I. Soriano, downloaded from Herbario Jaca (IPE-CSIC).

In Chapter 4, we aimed to monitor changes in rhizosphere pH of seedlings of the gypsophile *Ononis tridentata* L (Figure G.M.5), determining the role played by soil fungi and root exudation rhizosphere acidification. *O. tridentata*, from the *Fabaceae* family, is a deciduous nano-phanerophyte distributed in North Africa and Eastern Spain. It is an endemism from the Iberian Peninsula and the Maghreb. It forms low dense scrubs preferentially on gypsum soils next to other gypsophile species such as *Gypsophila hispanica* and *Herniaria fruticosa*. The flowering period goes from May to August (Castroviejo, 2020). *O. tridentata* has whitish branches and trifoliate and fleshy leaves. The flowers are light pink or whitish and its fruits are short and hairy legumes

(Figure G.M. 5). This species can reach 1.5 m high and has deep and thick taproot with a high growth rate in the seedling stage, which eases its observation in the laboratory.

Figure G.M.5. Adult individuals of *Ononis tridentata*. A) Plant general view B) Flowers C) Fruits. Pictures by Gabriel Montserrat, downloaded from Herbario Jaca (IPE-CSIC).

Rhizoboxes, windows to explore the underground

Studying the growing root *in situ* has been a scientific challenge for a long time due to its inaccessibility, however, gaining access to the roots is fundamental to unveil plant-soil-microbiota interactions, which in turn are key to understand plant and ecosystem functioning as a whole (Santner *et al*., 2015). In order to study root-soil interactions, in Chapter 4 we cultivated *O. tridentata* seedlings in purpose-built rhizoboxes, a non-destructive method to study roots growing in the soil. A rhizobox consist of a narrow rectangular container designed for the study of root growth and rhizosphere processes (Schmidt *et al*., 2018; Wenzel *et al*., 2001). To assess changes in rhizosphere pH, we used a modified design of a rhizobox from the one by Marschner and Römheld (1983) and similar to those employed in Dinkelaker *et al*. (1993). Plants were grown in soil filled into black plexiglass rhizoboxes (220 x 170 x 15 mm) with a transparent lid (Figure G.M.6), which was covered with a black acetate sheet to keep darkness and favour root growth. To promote root growth along the transparent lid, the boxes were leaned *ca*. 45º to the lid side.

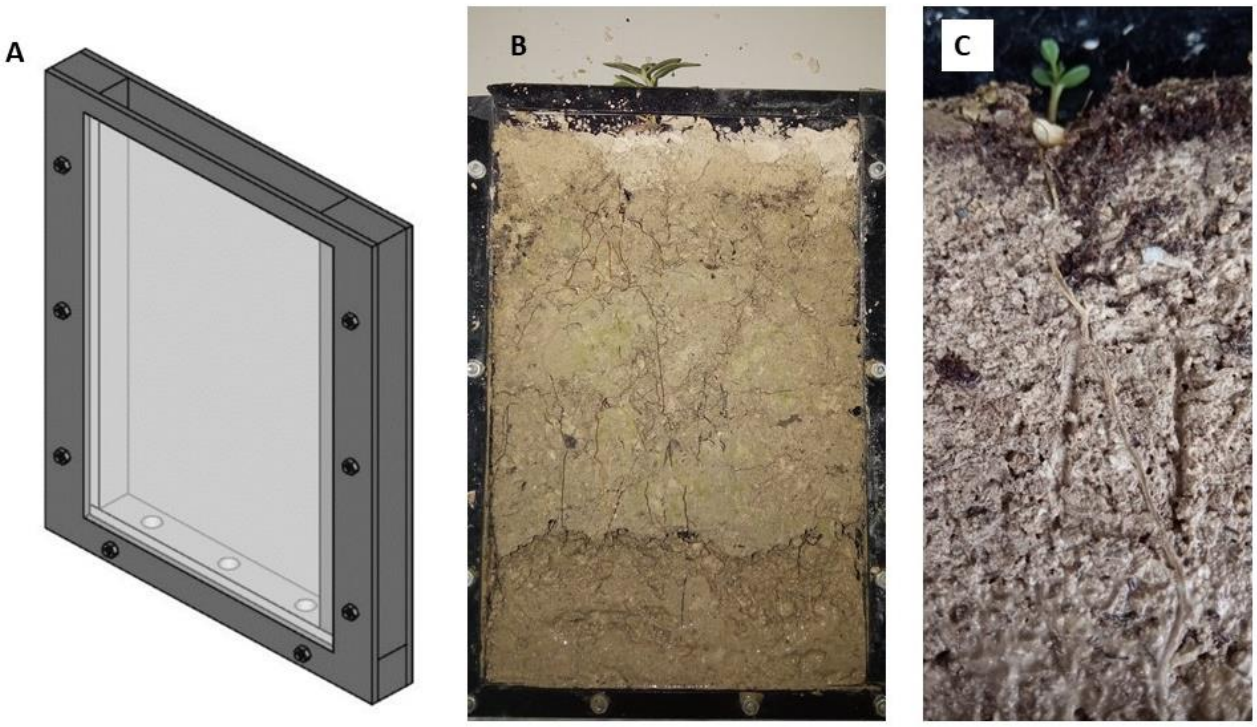


Figure G.M.6. A. Diagram of the rhizoboxes used in the experiment. Designed by Jesus Revilla (Instrument Lab, IPE-CSIC) for this Thesis and inspired by Marschner and Römheld (1983). **B**. Picture of a *H. squamatum* seedling after several weeks growing on a rhizobox on gypsum soil. **C**. *O. tridentata* seedling growing on a rhizobox on gypsum soil.

Once plants had developed sufficient visible root in the rhizobox, the transparent lid was removed to get access to the root and place optical sensors (optodes) to visualize pH changes next to the root and the surrounding soil (explained in detail in Chapter 4).

As a proof-of concept, we tried to combine plants grown in rhizoboxes with X-ray diffractometry of the root-soil system, to detect *in situ* potential changes of gypsum mineral phases (involving gypsum dehydration). To that end, we developed small stainless steel minirhizoboxes (70 mm x 70 mm x 10 mm) to fit the slide tray of an X-R diffractometer (Figure G.M.7). This methodology, however, required an exhaustive control of air moisture and microbial contamination when wild

plants were cultivated, owing to the very small quantity of soil held in the minirhizobox, which led to very low water holding capacity.

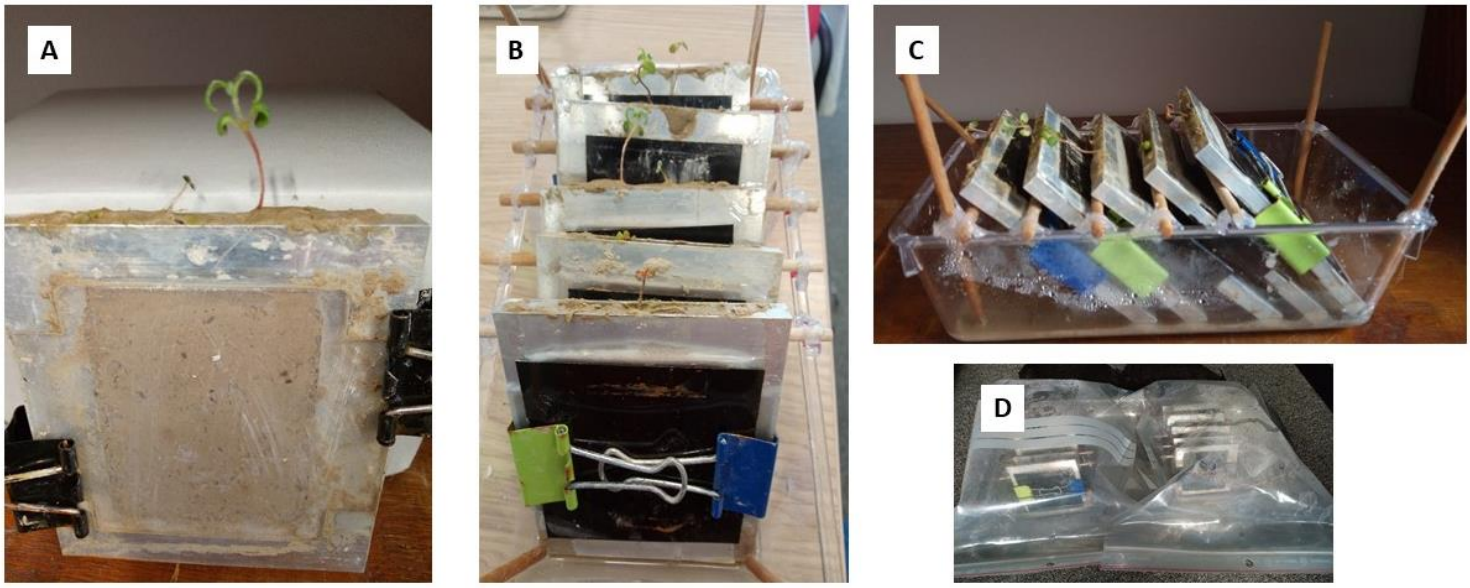


Figure G.M.7. Mini-rhizoboxes with *H. squamatum* seedlings cultivated on gypsum soil. A. Front view of the transparent lid. B. General view of the home-made installation to keep them moist and in an angle of ~45º. Black covers were installed in the lid to keep soil dark and favour root growth. C. Lateral view of the experimental system. D. Plastic zip bags to keep moisture in the mini-rhizoboxes.

Stable isotopes of water as tool for the study of plant water use in gypsum plant communities

Stable isotopes of water (^{2}H and ^{18}O) are considered ideal tracers to study water fluxes in the soil-plant-atmosphere continuum and have been used as hydrological and ecological tracers for more than fifty years (Meißner *et al*., 2014; Penna *et al*., 2018). Water phase changes explain most of the variability in water isotopic composition, as heavier isotopes have lower mobility than lighter ones (Dawson *et al*., 2002). Thus, the isotopic signature of water in the xylem sap of the species and that of the different water pools in the soil are generally analysed to infer the water sources used by plant species. These studies are highly important for water resource management in vegetated ecosystems.

To address a proper resolution of this methodology, it is important to define the isotopic composition of the available water pools for plants in the soil. Water isotopic signatures are very heterogeneous both spatially and temporally. They can change with time, soil depth and soil properties even at small scales (Troch *et al*., 2009), and they also differ according to the genesis of the water, as happens in gypsum crystallization water. In addition, recent works highlight potential isotopic fractionation effects (Martín-Gómez *et al*. 2016; Barbeta *et al*., 2020a; de la Casa *et al*., 2021; Barbeta *et al*., 2022), which question the interpretation of water source studies, based for many years in the principle of the absence of isotopic fractionation during water uptake by roots, transport through the plant, and sampling or extraction from the xylem samples. These works concluded that the isotopic offset among plant stem water and soil water is due to evaporation processes occurring in non-conductive tissues of the xylem and in the soil at the pore-scale (Martín-Gómez *et al*., 2016; Barbeta *et al*., 2020a).

Our methodology tried, as far as possible, to overcome the challenges outlined above and prevent misinterpretation in several ways. To account for soil heterogeneity, as described in Chapter 1, we first revised the bibliography about rooting depth and architecture of the studied species and, when possible, checked it in the field (as described in Chapter 2). Whenever possible, we did a thorough sampling of the soil profile, covering the most relevant depths for root location (e.g. 1 m deep soil profiles in Chapter 1). We sampled the soil water underneath each plant individual harvested at different soil depths (10 and 20 cm) in the different sampling dates, and characterized the deep water or groundwater, when technically possible, of the ecosystem under study.

To minimize the risk of stem water evaporation and to maximize the representativeness of xylem water as an indicator of the main water sources used by plants, we harvested between 6:30 and 10 h (solar time). In this time frame, we expect maximum transpiration rates and low evaporative demand to prevent stem dehydration (Grammatikopoulos, *et al.*, 1995; Martín-Gomez *et al*., 2017). In herbaceous species, the root collar was used as a proxy for non-enriched source water (Barnard *et al.*, 2006). In woody species, the bark and phloem were removed with a knife to avoid contamination with phloem water and organic compounds present in living cells and/or the bark (Ehleringer and Dawson 1992).

To obtain the isotopic signature of different water pools, water of the soil samples was carefully extracted in a cryogenic vacuum distillation (see Figure G.M.8 for assembly details of the

extraction line), the most commonly used method to extract water contained in the soil and in plant xylem (Orlowski *et al*., 2016). Cryogenic vacuum distillation was performed in two steps (with 90 min for each step according to the methodology in Meißner *et al*., 2014). The first distillation was done at 35 ºC with the aim to extract free water in the soil; and then soil samples were extracted a second time in a silicone oil bath at 130 ºC with the aim to extract gypsum crystallization water (Palacio *et al*., 2014). Soil samples were weighed before and after the extractions to ensure complete dehydration.

Deuterium offset among water sources and xylem water has been reported in cool or temperate and wet environments. In addition, this process has been usually detected in trees, which have a bigger parenchyma than shrubs. In shrubs, this effect has not been proved yet (de la Casa *et al*., 2022; Barbeta *et al*., 2020b). It is important to visualize the isotopic values of sources and plants in δ^2H-$\delta^{18}O$ plots to complement mixing models. This visualization allows reflecting the potential misinterpretation of δ^2H results, checking if xylems are considerably more negative than potential

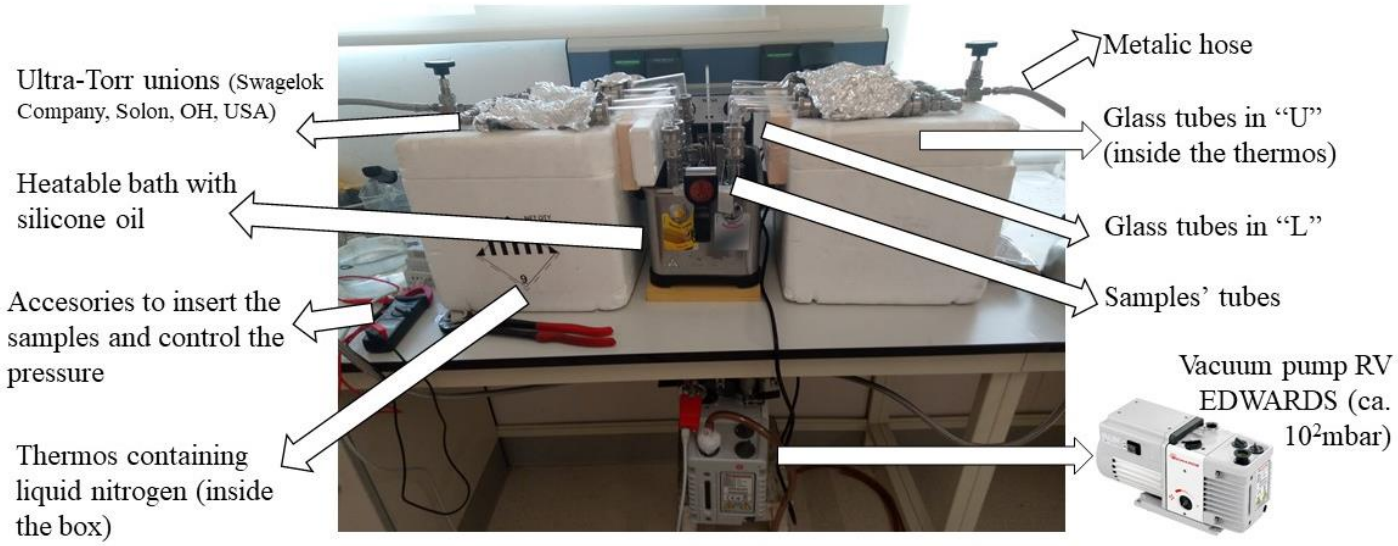
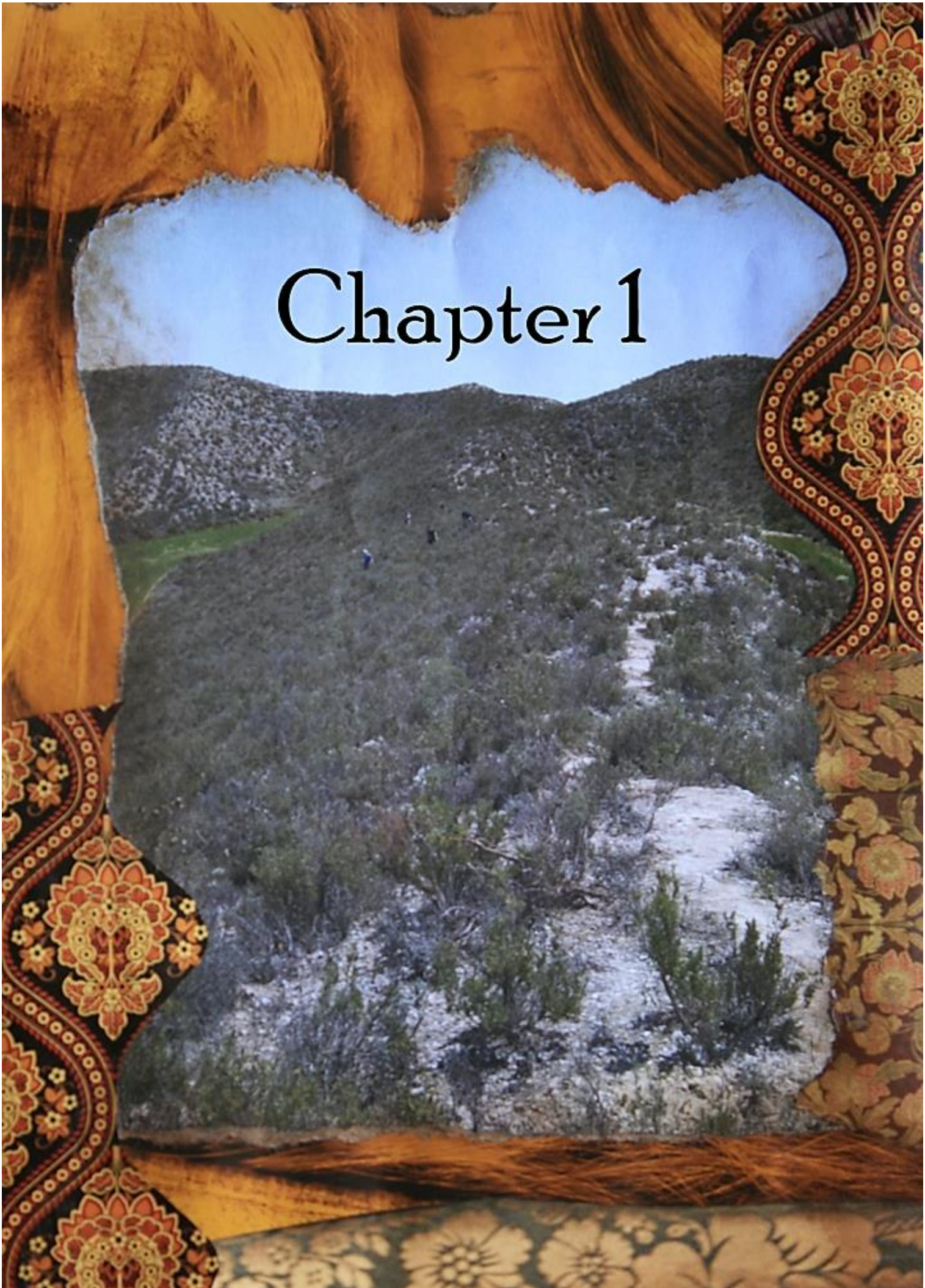


Figure GM.8. Cryogenic vacuum distillation line assembly in the Instituto Pirenaico de Ecología-CSIC (Jaca, Spain) during this thesis development with collaboration of Juan Pedrio Ferrio (CITA-CSIC). The distillation consists on a container with silicone oil that heats the tubes with the sample. These are connected to a vacuum system obtained with a pump and connected by metal hoses. The tubes are connected to other L-shaped and then, to U-shaped tubes immersed in liquid nitrogen where the water sample collected is frozen. To collect the obtained water in the U-shaped tubes, they are disconnected, covered and waited for the samples to change into liquid phase, whose is rigorously collected. The assembly line was suitable for extracting eight samples at the same time.

water sources. In addition, different slopes among plants and free water sources in $\delta^{2}H$-$\delta^{18}O$ plots could reflect potential evaporative processes in plants.


Chapter 1

Sampling day of soil and plant xylems on the top of the gypsum hill in Alfajarín, Zaragoza. Picture taken by Juan Pedro Ferrio in April 2018.

Composition of the margin by Virginia de la Iglesia and Laura de la Puente

Chapter 1

Disentangling water sources in a gypsum plant community. Gypsum crystallization water is a key source of water for shallow-rooted plants*

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ABSTRACT

- **Background and Aims**: Gypsum drylands are widespread worldwide. In these arid ecosystems, different species ability to access different water sources during drought is a key determining factor of the composition of plant communities. Gypsum crystallization water could be a relevant source of water for shallow rooted plants, but the segregation in the use of this source of water among plants remains unexplored. We analyzed the principal water sources used by 20 species living in a gypsum hilltop, the effect of rooting depth and gypsum affinity, and the interaction of the plants with the soil beneath them.
- **Methods**: We characterized water stable isotope composition, $\delta^{2}H$ and $\delta^{18}O$, of plant xylem water and related it with the free and gypsum crystallization water extracted from different depths along the soil profile and the groundwater, both in spring and summer. Bayesian isotope mixing models were used to estimate the contribution of water sources to plants xylem sap.
- **Key results**: In spring, all species used free water from the top soil as the main source. In summer, there was segregation in water sources used by different species depending on their rooting depth, but not on their gypsum affinity. Gypsum crystallization water was the main source for most shallow-rooted species, whereas free water from 50-100 cm depth was the main source for deep-rooted species. We detected plant-soil interactions in spring, and indirect evidence of possible hydraulic lift by deep-rooted species in summer.
- **Conclusions**: Plants coexisting in gypsum communities segregate their hydrological niches according to their rooting depth. Crystallization water of gypsum represents an unaccounted, vital source for most of the shallow-rooted species growing on gypsum drylands. Thus, crystallization water helps shallow-rooted species to endure arid conditions, which eventually accounts for the maintenance of high biodiversity in these specialized ecosystems.

Keywords: water sources, hydrological niche, drought, gypsum crystallization water, plant community, root depth, gypsum affinity, water stable isotopes

INTRODUCTION

Plant species from arid and semi-arid ecosystems have adapted to water scarcity by different mechanisms of water uptake and use. An important strategy is the segregation in hydrological niches, which makes possible the coexistence of different plant species in stable and diverse communities (Eherlinger *et al.,* 1991; Filella and Peñuelas, 2003; Araya *et al.*, 2011; Silvertown *et al.,* 2015, Palacio *et al* 2017). Hydrological niche segregation has often been found in several ecosystems affected by drought like Mediterranean shrublands and forests (Filella and Peñuelas, 2003; Moreno-Gutierrez *et al.*, 2012; Del Castillo *et al.,* 2016), deserts (Ehleringer *et al.,* 1991; Parks, 1997; Schachtschneider and February, 2010) or seasonal tropical forests (Ding *et al.,* 2020; Brum *et al.,* 2018; Liu *et al.,* 2010). Different traits related to changes in root architecture and rooting depth allow divergent water use strategies and the partition of this scant resource among coexisting plants (Donovan and Ehleringer, 1994; Moreno-Gutierrez *et al.*, 2012). Water from precipitation present in the topsoil favours nutrient availability and microbial processes, using this pool preferentially during the growth period (Caldwell *et al.,* 1998; Querejeta *et al.,* 2021). However, during drought, roots should access deeper soil layers, sometimes even reaching the water table, where water availability is more stable (Ehleringer *et al.,* 1991; Ryel *et al.,* 2008, 2010; Rempe and Dietrich, 2018). These deeper water pools are normally used to maintain transpiration during periods of limited growth (Voltas *et al.,* 2015). Many plants have developed dimorphic root systems with both superficial and deep roots, and the different water potential between dry shallow layers and wet deep layers can lead to hydraulic lift (Bauerle *et al.,* 2008; Prieto *et al.,* 2012). This is a widespread process in semi-arid environments consisting on the passive movement of water from deeper layers to upper layers by roots (Prieto *et al.,* 2010). Through hydraulic lift, plants can act as "bioirrigators" to neighbouring plants, hence increasing their chances of survival and, ultimately, the coexistence of diverse communities (Bayala and Prieto, 2019; Jackson *et al.,* 2000). Assessing the functional importance of contrasting soil water pools and their spatial and temporal variation is necessary to evaluate how climate change and land use may affect the ecohydrology of vegetation and the dynamics of plant communities (Ehleringer *et al.,* 1991; Dwivedi *et al.,* 2019; Oerter and Bowen, 2019). Understanding the mechanisms of different plant species to uptake and partition water resources in arid and semi-arid conditions is crucial to unravel the processes supporting plant coexistence in dryland communites (Dodd *et al.,* 1998; Peñuelas *et al.,* 1999).

Gypsiferous soils, i.e. soils with high (above 40 %) gypsum ($Ca_2SO_4.2H_2O$) content (Cashby *et al.*, 2015), are frequently present in drylands, being widespread around the world (FAO, 1990; Verheye and Boyadgiev, 1997). Together with the arid conditions, these soils have low water retention (Herrero and Porta, 2000) and, consequently, water is a major limiting factor for plants growing on gypsum soils. Some studies, however, found better water availability in summer in gypsum soils than in surrounding non-gypsum soils (Meyer and García-Moya, 1989; Escudero *et al.*, 2015). This observation is further supported by the discovery of crystalline gypsum water as a source for plants and other organisms during the dry period (Palacio *et al.*, 2014; Palacio *et al.*, 2017; Huang *et al.*, 2020). Gypsum contains water in its crystalline structure, which represents up to 20.8% of its weight. Under certain conditions of vapor pressure, temperature (from 42 ºC in pure gypsum, Marshall *et al.*, 1964), gypsum could dehydrate, changing into bassanite (the hemihydrate: $CaSO_4.0.5H_2O$) or into anhydrite ($CaSO_4$) (Van't Hoff *et al.*, 1093; Freyer & Voigt, 2003; Ossorio *et al.*, 2014). In addition, it has been demonstrated how this phase transformation can be induced by some microorganisms, leading to anhydrite re-precipitation (Huang *et al.*, 2020). There is evidence of a large use of crystallization water by the gypsum endemic plant *H. squamatum*, and it has been suggested that its use could be extended to other shallow-rooted species living in gypsum plant communities (Palacio *et al.*, 2014; Palacio *et al.*, 2017, Henschel *et al.*, 2018). However, it is still unknown up to what point plants coexisting in the same plant community show different ability to retrieve crystallization water, and thus, whether the use of this water pool is a relevant factor defining hydrological niches in gypsum plant communities.

Gypsiferous soils show also particular chemical and physical properties, which could constraint the development of plant life (Escudero *et al.*, 2015). Plant roots have to cope with a high penetration resistance (Poch and Verplancke, 1997; Moore *et al.*, 2014, Sánchez-Martín *et al.*, 2021) and morphological transitions of the soil due to dissolution-precipitation sequences of gypsum (Cashby *et al.*, 2015). In addition, most of these soils have low nutrient supply caused by their low organic matter content and cation exchange capacity and their saturation in Ca and S (Moore *et al.*, 2014; Cashby *et al.*, 2015). Despite these limitations, gypsum soils host highly diversified floras, rich in endemic and highly specialized species (Moore *et al.*, 2014) which have been the subject of deeper study from only a few years ago (Escudero *et al.*, 2015).

Plant species growing on gypsiferous soils can be classified in two groups depending on their affinity for gypsum: gypsophiles, which only grow on gypsiferous soils and often have substrate-specific physiological strategies (Palacio *et al.*, 2007; Escudero *et al.*, 2015; Cera *et al.*, 2021, ; and gypsovags, which are non-exclusive to gypsum soils (i.e. grow also off gypsum) and frequently display stress tolerant strategies (Palacio *et al.*, 2007; Bolukbasi *et al.*, 2016). Gypsophiles have shown a range of mechanisms to detoxify the excess of Ca and SO_4 considering their leaf elemental composition, whereas gypsovags would follow an avoidance strategy, reducing the absorption of these compounds (Palacio *et al*., 2007; Palacio *et al*., 2014; Merlo *et al*., 2019; Cera *et al.*, 2020). Thus, if obtaining the crystallization water from gypsum is related to its dissolution (Huang *et al.*, 2020) and, consequently, the release of Ca and sulphate ions, gypsophiles could be more prone to using this water than gypsovags.

Tracing water movement in the soil and plants is possible using the natural variations of stable isotopes of hydrogen (^{2}H) and oxygen (^{18}O) in water molecules. This widely used method, extensively applied in hydrology and ecophysiology, allows evaluating the result of several processes without disrupting the natural behaviour of the elements in the system (Meisner *et al.*, 2014; Penna *et al.*, 2018). Water phase changes (vapour-liquid-solid) explain most of the isotopic variability, as the heavier isotopes have a lower mobility (Dawson *et al.*, 2002). The water sources acquired by plants can be determined with the following premises 1) alternative water pools must be isotopically distinct and 2) there is no isotopic fractionation during water uptake. In dry environments, the first assumption is generally fulfilled: due to evaporative fractionation, upper soil layers often become enriched in the heavy isotopes ^{2}H and ^{18}O, thus being distinguishable from deeper soil layers or groundwater (Barnes and Allison, 1988; Dawson and Ehleringer, 1998). With regard to the second assumption, fractionation during water uptake is considered negligible in most plants (Dawson *et al.*, 2002 and references cited therein), with the exception of some coastal wetland species (Lin *et al.*, 1993) and certain woody xerophytes (Ellsworth and Williams, 2007). Nevertheless, different authors have reported discrepancies between source and stem water, attributed to different causes, e.g. heterogeneity in the soil (Barbeta *et al*. 2021), stem evaporation during periods of limited water flow (Dawson and Ehleringer 1993; Martín-Gómez *et al*. 2017) or sampling artefacts (Marshall *et al.*, 2020).

The purpose of this study was to analyse the distribution of water sources among the main 20 dominant plant species in a top-hill gypsum community. We characterized the variation in the isotopic composition of water along the soil profile and evaluated the effect of species rooting depth and affinity for gypsum soils on their water use both in spring and summer. We also analysed how plants interacted with the soil beneath them. Considering plant water-uptake patterns, we hypothesised that: (1) shallow-rooted, gypsum-exclusive species will preferentially use crystallization water from gypsum in summer, whereas shallow-rooted, non-exclusive species will be restricted to the (scarce) free water available in the topsoil. Conversely, deep-rooted species, regardless of gypsum affinity, will rely mainly on the use of deep soil water and/or groundwater during summer drought. Considering plant-soil interactions, we also hypothesised that (2) deep-rooted species will interact with the shallow soil, uplifting water from deeper soil layers (hence performing hydraulic lift).

MATERIALS AND METHODS

Study area and species

We conducted field sampling on a gypsum hill in the Middle Ebro Depression, Zaragoza province, NE Spain (41º37'52.5" N 0º41'23.7" W, 287 m a.s.l). The main component of the soil in this region is gypsum (63.4%), with thin outcrops of marls and clays (Quirantes, 1977). Climate is semi-arid and highly seasonal (Palacio *et al*., 2007). Mean annual temperature is 14.9 ºC, average annual rainfall is 331.5 mm, which falls mainly during spring and autumn, and evapotranspiration is around 1200 mm, so plants experience intense drought during summer months. An important proportion of the soil surface in the upper part of gypsum hill is bare or coated with biological crusts dominated by cyanobacteria, lichens and mosses (Concostrina-Zubiri *et al.*, 2014). The plant community is dominated by sub-shrubs like *Helianthemum squamatum*, with some taller shrubs, such as *Gypsophila struthium* subsp. *hispanica* or *Ononis tridentata* (Braun-Blanquet & Bolos, 1987).

We selected 20 dominant perennial plant species to represent the community living at the top of the hill, where stress conditions are most severe (Hodgson *et al*., 1994; Guerrero Campo *et al*., 1999; Cashby *et al.*, 2015). These representative species included different life forms (woody vs. herbaceous), root-depths, affinity for gypsum soils and taxonomic families. We considered species with more than one-meter-deep roots to be deep-rooted species, and the rest were considered shallow-rooted (Guerrero-Campo, 1998; Table. 1).

Plant and soil sampling

Field sampling for isotope analyses was performed in rainy spring (24 -25 April, 2018) and in the dry summer (7-8 August, 2018), after a long rainless period. On each sampling date, we harvested the main stems (including the root crown) of five individuals of each species. We selected vigorous, medium-sized individuals located at least 5 m away from each other. To minimize the risk of stem water evaporation and to maximize the representativeness of xylem water as an indicator of the main water sources used by plants, we harvested between 6:30 and 10 h (solar time). In this period, we expect maximum transpiration rates and low evaporative demand to prevent stem dehydration (Grammatikopoulos, *et al.*, 1995; Martín-Gomez *et al*., 2017). In herbaceous species, the root collar was used as a proxy for non-enriched source water (Barnard *et*

al., 2006). In woody species, the bark and phloem were removed with a knife to avoid contamination with phloem water and organic compounds present in living cells and/or the bark (Ehleringer and Dawson 1992; see Fig 1d). Two soil samples were collected underneath each plant at two different depths: 10 and 20 cm, (*ca.* ±2 cm) avoiding the intrusion of roots in the samples (see Fig. 1c). In addition, to capture variation in soil water isotopic composition along soil depth, three profiles one-meter-deep were dug underneath the bare soil on each sampling date (see Fig. 1.b). Soil samples were collected at 13 different depths (*ca.* ±2 cm): 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90 and 100 cm. In spring, we gathered two extra samples from a small temporal creek upwelling in the saline depression downhill, representative of the groundwater. At the time of sampling, the water formed a small temporal creek, easily distinguished from rain puddles. Right after harvest, water, stem and soil samples were placed in airtight sealed tubes (Duran GL18), immediately frozen with dry ice, and kept frozen until distillation in the lab.

Water extraction

Xylem and soil water were extracted by cryogenic vacuum distillation (Ehleringer and Dawson, 1992), adapted as described in Palacio *et al*. (2014). Spring samples were extracted at the Laboratory of Silvicuture of the Universitat de Lleida (Lleida, Spain) and summer samples were extracted with the same procedure at the laboratory of the Instituto Pirenaico de Ecología (IPE-CSIC, Zaragoza, Spain). Sample tubes were placed in a heated silicone oil bath, and connected with Ultra-Torr unions (Swagelok Company, Solon, OH, USA) to a vacuum system (ca. 10^{-2} mbar) including U-shaped water traps in series that were cooled with liquid Nitrogen. Eight lines were installed. After an extraction time of 90 min for plant and soil samples (West, 2006; Meisner 2014), captured water was transferred into screw-capped 2 ml vials, and stored at 4 ºC until isotope analyses. Xylem water was distilled at 130 ºC, whereas gypsum soils were distilled in two steps: first at 35 ºC, and then at 130 ºC to separate free and crystallization water and assure almost complete dehydration of gypsum (Freyer and Voigt, 2003; Palacio *et al.,* 2014). Between the first and second distillation, sample tubes were kept in a desiccator with silica gel to avoid any re-hydration with ambient moisture, which could contaminate the next extraction water. Distilled samples were completely dried in the oven for 24 h at 60 ºC. The samples were weighed before and after each distillation and after oven-drying to measure water content and confirm complete distillation.

Stable isotope analyses

Oxygen and hydrogen isotope composition ($d^{18}O$, $d^{2}H$) were determined by cavity ring-down spectroscopy (CRDS). For spring samples, the analyses were performed at the Serveis Científico-Tecnics (Universitat de Lleida), using a Picarro L2120-i with vaporizer A0211 (Picarro, Santa Clara, CA, US). Summer samples were analysed at the scientific services of the Instituto Pirenaico de Ecología (CSIC), using a Picarro L2130-i with vaporizer A0211 (Picarro, Santa Clara, CA, US). The estimated precision was 0.10% for $\delta^{18}O$ and 0.40% for $\delta^{2}H$. Deuterium excess was calculated according to Dansgaard (1964), as the divergence from the Global Meteoric Water Line: $Dex = \delta^{2}H - 8 \times \delta^{18}O$. Where appropriate, we applied the post processing correction to manage the organic contamination of the samples. After describing the magnitude of contamination with the software PostProcess ChemCorrect™ v1.2.0, the $H_2{}^{18}O$, $HD^{16}O$ and $H_2{}^{16}O$ peaks, filtered by the spectral features of organic compounds, were converted to organic-corrected $\delta^{18}O$ and $\delta^{2}H$ by applying a formula using device-specific factory calibration values (see Martín-Gómez *et al*. 2015 for details).

Statistical analyses

Changes in soil water content and in the isotopic composition of water along soil profiles, as well as $\delta^{2}H$-$\delta^{18}O$ bi-plots with soil water and xylem sap isotopic compositions were visualized using *ggplot2* in R (Wickham, 2016). Soil water content was calculated from sample weights before and after water extractions. Variation in the isotopic composition along the soil profiles was analyzed to characterize potential deep water sources for plants and locate the evaporation front in both seasons. To identify the possible sources of deep soil water for plants, we defined soil depths above 20 cm deep with homogeneous isotopic composition of free soil water that markedly differed from other depths in the soil (Fig. 2). Transition depths with intermediate and highly variable soil water isotopic composition were not included in the model, so that alternative sources could be clearly differentiated. For this reason, water isotopic values at 30 and 40 cm depth were removed from the set of sources (see Fig. 2 and Supplementary Data Fig. S1). Considering the results for the characterization of soil water along the soil profile (see Fig. 2, Fig3), we could differentiate four potential water sources for plants (see below). This characterization of sources was the simplification of a preliminary, seven-source model (see below).

Differences among study species and sampling dates in xylem water isotopic composition ($\delta^{18}O$, $\delta^{2}H$) and Deuterium excess were evaluated using residual maximum likelihood (REML) analysis with the *lmer* function from the *lme4* package in R (Bates *et al.,* 2015). Models were run separately for each water isotope: ^{2}H, ^{18}O and Deuterium-excess. General models included species nested within family as a random factor to account for potential phylogenetic bias, and gypsum affinity (two levels: gypsophile and gypsovag), water pool (two levels: free and crystallization) and root depth (two levels: shallow and deep) as fixed factors. Separate models were run for each season to explore differences between species with different gypsum affinity and root depth in each season for each isotope. Shapiro-Wilk normality test (Royston, 1995) and Levene test for homogeneity of variances (Noguchi and Gel, 2010; Gastwirth *et al.,* 2009) were used to check the normal distribution and homocedasticity of residuals. Residuals were visually checked using *DHARMa* package (Hartig, 2021). When interactions were significant, groups were analyzed with post-hoc Tukey HSD tests using *lsmeans* package (Russell, 2016).

The relative contribution of different water sources to xylem sap was estimated using Bayesian mixing models for stable isotopic data with the package *MixSIAR* (Stock *et al.,* 2018). This procedure estimates the proportion of source contributions to a mixture. The model used as 'consumers' the isotope values of xylem water in each individual ($\delta^{2}H$ and $\delta^{18}O$). For 'Sources', alternative models were run with different grouping of sources in order to select those that best described and simplified the potential water sources for plants. The Mix-SIAR model that had better accuracy and so, explained better the contribution of the sources to the xylem of plants, was run with seven different sources for each species: free soil and crystallization water from 10, 20 cm, free and crystallization water from the 'deep-soil' (50-100 cm combined), and groundwater. Values for 10 cm and 20 cm soil depth included one replicate per individual plant, whereas values from deeper soil were averaged across the three soil profiles. This model was simplified *a posteriori* by the addition of the contributions of each source into four simplified sources: 1) "crystal water", i.e. gypsum crystallization water from the soil underneath the plants and deep-soil, as they clearly departed from free water, and had a comparatively small variation along the soil profile. It was calculated by the addition of the contributions to the xylem of plants of all three crystallization water sources initially considered. 2) "shallow free": free water in the shallow soil (until *ca*. 20 cm depth), represented by free water extracted from soil collected underneath each plant owing to the better replication. It was calculated as the addition of the contribution to the

xylem of the free water at 10 cm and 20 cm. 3) "deep-soil free", free water in the deep soil (between 50 - 100 cm depth); and 4) the water table (i.e "groundwater"), not modified from the output in the Bayesian model. The contribution of the water sources to the species separated by their root depth was calculated by the addition of the contributions of the different sources to the composition of the xylem water of the different species in each rooting depth group.

The effect of plant species on the isotopic composition of the soil beneath them was considered by assessing the significance of between- and within-group variations in the isotopic composition of the soil collected under each individual. Effects were analyzed separately for each isotope (δ^2H, $\delta^{18}O$ and Deuterium excess) and season. To account for inter-specific differences in the isotopic composition of soil water, we ran lineal models using the *lm* function (Chambers, 1992). Specific models were run with REML using *lmer* function (*lme4* package) to assess differences for the fixed factors: "gypsum affinity", "root depth" and their interaction with the same random term structure as in xylem water comparisons. To assist in the interpretation of plant-soil interactions, e.g. to visually identify evidence of hydraulic lift, isotopic composition of the xylem water and the water extracted from the soil beneath the plants were visually compared with *ggplot2* package. All statistical analyses were run in R 4.0.0. (R Core Team, 2020).

RESULTS

Water source characterization along soil profiles

δ^2H and $\delta^{18}O$ composition of free soil water showed more homogeneous values in spring than in summer (Fig. 2a, b), mainly due to the spatial heterogeneity of soil water evaporative enrichment and the location of the evaporation front at slightly different positions among the three different soil profiles. In spring, water in shallow soil layers showed more negative values of both δ^2H and $\delta^{18}O$ than water in the deep soil (Fig. 2a, b), which corresponded with very negative values from a recent rain event in April 2018 (Supplementary Data Table S1). No evaporation front was observed in spring, whereas in summer, the evaporation front in the bare soil was located at *ca.* 15 cm depth, showing an abrupt change from isotopically-depleted values of δ^2H and $\delta^{18}O$ at 5-10 cm, typical of water vapour, to highly enriched values at 15-20 cm (Fig. 2 a, b). Below 20 cm, δ^2H and $\delta^{18}O$ became more negative with depth, until they stabilized from 40-50 cm to 80-90 cm depth, with a slight increase from 90 to 100 cm (Fig 2 a, b). In both seasons, the δ^2H and $\delta^{18}O$ of gypsum crystallization water showed a similar pattern with depth (between 5 and 60 cm). Values were

more positive in the upper soil layers, presumably due to the re-crystallization of gypsum with more evaporated water. In spring, this progressive depletion with depth continued until 100 cm, whereas in summer, a small increase in isotopic signatures was observed between 70-90 cm, together with larger variability among profiles.

In spring, Deuterium excess of free water was rather homogeneous along the soil profile (Fig. 2 c), becoming slightly negative in the top layer (5 cm) and in the deepest layers (60-100 cm). Conversely, Deuterium excess of free water in summer showed large variations, following an opposite pattern to that in δ^2H and $\delta^{18}O$ that indicates strong evaporative enrichment of soil water in the upper soil layers (Fig. 2 c). For crystallization water, Deuterium excess in both seasons became less negative with depth, further indicating re-crystallization of gypsum with more evaporated water in the top soil layers.

We found much higher free water content in the shallow soil layers in spring than in summer (Fig 2 d). In spring, we observed relatively uniform free water content in the soil profile until 60-70 cm, where soil water content decreased in the vicinity of the underlying bedrock. In summer, we observed severe soil desiccation in shallow soil layers and higher water content with depth, until reaching layers next to the bedrock, where the soil water content decreased again. The content of crystallization water retrieved is related with the gypsum content in the soil which was homogeneous through most of the soil profile in summer. Nevertheless, we found more variability in the upper layers in spring (Fig. 2d).

Regarding the position of the water sources and the xylem of plants in the bi-plot showing δ^2H vs $\delta^{18}O$, we observed the segregation of crystallization and free water and the clustering of the xylem sap of shallow rooted plants with crystallization water during summer. This is compatible with an important use of this water source by these species during drought (see below). Free water from the 20 first cm in the soil (collected underneath the plants) showed values typical of water vapour (Fig. 3, Supplementary Data Fig. S2). Contrastingly, free water collected underneath the bare soil, which retained more water, showed values of evaporated water (Fig. 2, see Supplementary Data Fig. S2). These could be due to the biological and physical crust formed in the bare soil that decreases evaporation (Escudero *et al.*, 2015) and/or to the more intense exploitation of the scarce free water from the soil beneath them by plants. Further, many of the isotopic values of shallow-

rooted plants with a high gypsum water contribution in their xylem sap cannot be solely explained by an eventual evaporation within the stem (see Supplementary Data Fig. S3).

Analysis of factors explaining differences among plants in their xylem isotopic composition

Both season and rooting depth had a significant effect on the isotopic composition of the xylem water of the target species. Conversely, the affinity for gypsum soils did not show a significant effect on xylem water composition, indicating that plants did not use different water sources according to this factor. Three main groups could be identified according to their xylem water composition: the first group included all species in spring, whereas the second and third groups included summer values for shallow-rooted and deep-rooted species, respectively (Fig. 4, Supplementary Data Table S2). Differences in the isotopic composition of the xylem water of plants were highly significant between seasons, as well as for the interaction between season and root depth. In spring, $\delta^{2}H$ and $\delta^{18}O$ had more negative values than in summer, and more positive D-excess, but xylem sap isotopic composition did not show significant differences due to species rooting depth. In summer, however, deep-rooted species had more negative values than shallow-rooted species (Fig. 4, Supplementary Data Table S3). Overall, these results indicate that in spring all plants in the community used similar water pools, whereas in summer plants used different water sources, depending on their rooting depth, and irrespectively of gypsum affinity.

Contribution of different water sources to the xylem water of plants

Estimation of the most likely sources of water used by plant species by Bayesian models revealed that, in spring, all plants used a large proportion of free water from the shallow soil (estimated using 10-20 cm underneath the plants). However, in summer, crystallization water from gypsum was the main source for shallow-rooted species, whereas deep-soil water (50-100 cm) was the main source for deep- rooted species (Fig. 5, Supplementary Data Fig. S4). In spring, we also detected a moderate contribution of groundwater (16 % for deep-rooted and 13 % for shallow-rooted), particularly in the deep-rooted *Ononis tridentata, Gypsophila hispanica and Genista scorpius,* and the shallow-rooted *Teucrium capitatum, Hernaria fruticosa* and *Fumana ericifolia* (Supplementary Data Fig. S4). In summer, the main source of water for shallow-rooted plants was crystallization water (59 %), irrespectively of species affinity for gypsum soils. In addition, 30 % of the water used by shallow-rooted plants was free soil water from deeper layers (50-100 cm; Fig. 5, Supplementary Data Fig. S4, Fig. S5). Deep-rooted species in summer mainly used free water

from the deeper soil layers (52 %), but crystallization water still accounted for 32 % of the water used by these plants (Fig. 5, Supplementary Data Fig S4, Fig. S5).

Soil-plant interaction

In spring, soil underneath the plants (10 - 20 cm depth) showed significant species-specific variations in δ^2H and $\delta^{18}O$ for both free and crystallization water and in deuterium-excess for crystallization water (Table 2). We also found significant differences among species in summer, for free water $\delta^{18}O$ and deuterium-excess (Table. 2). In summer, we did not find significant effects of either rooting depth, gypsum affinity or their interaction on the isotope composition of soil free water collected beneath plants (Supplementary Data Table S4). Free water isotopic composition of the shallow soil beneath some of the deep-rooted species (*G. scourpius, G. hispanica, Rosmarinus. officinalis* and *Thymelaea. tinctoria*) during summer was similar to their xylem water isotopic composition for $\delta^2 H$, and closer to that of deep-soil layers than in other species, providing an indirect evidence of hydraulic lift by these species. However, the $\delta^{18} O$ composition of the soil was consistently more negative than the xylem isotopic composition of plants (Supplementary Data Fig. S6).

DISCUSSION

Our results agree with previous studies that demonstrate the role of summer drought as a structuring factor in plant communities growing on gypsum drylands (Palacio *et al.,* 2017). Hydrological niche segregation differentiates functional strategies between shallow-rooted species, dominant in these communities, and deep-rooted plants. This spatial segregation could have consequences on plant community assembly, promoting diverse plant communities whose variable response to soil drying enhances their stability under arid conditions (Peñuelas *et al.,* 2011; Silvertown *et al.,* 1999, 2015).

We identified gypsum crystallization water as a crucial component of the water balance in gypsum drylands. Water held in the crystalline structure of gypsum was the most important water source for almost all shallow-rooted species and a highly relevant water source for deep-rooted species during summer drought. Our results demonstrate that gypsum crystallization water is widely used by plants, irrespective of their affinity for gypsum soils. Contrary to our predictions, both gypsum endemic and non-endemic species (gypsophiles and gypsovags) with shallow roots used gypsum crystallization water as the preferential water source during summer. The uptake mechanisms that make such use possible remain undescribed. The similar isotopic composition of gypsum crystallization water in both seasons agrees with the notion that continuous processes of gypsum dissolution-precipitation take place during the year, involving both precipitation and more evaporated free soil water (Fig. 2; Van Driessche *et al.,* 2012). It is known that the temperature for pure gypsum dehydration can be decreased by some ionic solutions (Gázquez *et al.,* 2017). Recent findings indicate that some microorganisms can dissolve gypsum rock by secreting organic acids, retrieving crystallization water under extreme xeric conditions (Huang *et al.,* 2020). We suggest that plant roots and their associated microorganisms could similarly be altering gypsum to mine its crystalline water. This is supported by several previous studies providing evidence on the ability of plants and their associated microorganisms to exudate organic acids and other compounds that alter the substrate where they grow (Bassan *et al.,* 2002; Chaparro *et al* 2003; Lebre *et al.,* 2017, Puente *et al.,* 2004). However, detailed analyses on the specific compounds that plants could be secreting to the gypsum soil, and their potential effect on the thermodynamic equilibrium among gypsum phases are lacking.

Other studies identified groundwater as the main water source enabling the maintenance of activity during drought for deep-rooted species (Palacio *et al.,* 2017; Koirala *et al.,* 2016; Salvucci and Entekhabi, 1995; Fan *et al.,* 2017). In contrast, our results pointed at water from 50-100 cm depth (i.e "rock moisture", Rempe and Dietrich, 2018) as the main water source in summer for deep-rooted species in the studied community. Although its dynamics and hydraulic properties have not yet been explored in detail (Dwivedi *et al.,* 2019), this crucial source of water likely came from precipitation that passed through unsaturated weathered bedrock until reaching the groundwater (Oshun *et al.,* 2019; Rempe and Dietrich, 2018). Despite the isotopic composition of groundwater and deep soil water were very similar in summer, for consistency between the spring and summer models, we kept the same water sources in the Bayesian models for both seasons. The model choice for the deep free water instead of groundwater could likely be due to its higher variability and higher probability area. Although we cannot untangle the use of these sources by plants during summer, groundwater did not outflow in the creek located under the study hill during summer (Laura de la Puente, pers.obs), being located more than 10 m deep from the top of the hill. Consequently, considering the plants position at the top of a hill and their observed (relatively limited) root length, deep soil free water seems a more plausible source of water for these plants than groundwater. Plants may also show a preference for rock moisture over groundwater, as happens with large trees that take advantage of the oxygenated conditions of the weathered bedrock (Zwieniecki, and Newton, 1996; Graham *et al.,* 2010; Liu *et al.,* 2014; Hahm *et al.,* 2020). Interestingly, our results show that not only deep-rooted species, but also some relatively shallow-rooted species (*Teucrium capitatum*, *Linum suffruticosum* and *Lithodora fruticosa),* were mainly using free water from the deeper soil during summer (Supplementary Data Fig. S2). The maximum rooting depths of these species is between 50 and 100 cm depth (Guerrero-Campo, 1998), with actual rooting depth being sensitive to reach free water (Fan *et al.,* 2017, Hodge, 2003). Nevertheless, we cannot rule out the possibility that these plants could also be using free water from slightly shallower layers, i.e. 30-40 cm deep, which had an isotopic composition similar to that from 50-100 cm deep, but was not included in the Bayesian models due to its variability and slight similitude with water from 20 cm depth. In any case, the use of free water by these species could be favoured through the segregation of water sources between coexisting shallow-rooted species to mitigate competition. Further approaches comprising experimental manipulation of

resources or models to find out the processes that stabilize community composition best, would be required to ascertain these possibilities (Silvertown *et al.*, 2015; Stoll and Weiner, 2000).

Another explanation to the use of deep soil water from relatively shallow rooted plants might be the hydraulic lift by some deep-rooted species during summer. The species *Genista scorpius*, *Gypsophila struthium* subsp. *hispanica*, *Rosmarinus officinalis* and *Thymelaea tinctoria* showed similar δ^2H isotopic values between the shallow soil beneath them and their xylem composition (Supplementary Data Fig. S4). This indicated water up lifting from the deeper soil, which could also be available to neighbouring shallow-rooted species. According to previous studies considering just one of the water stable isotopes composition (δ^2H or $\delta^{18}O$) (Dawson 1993; Ludwig 2003; Durand, 2006) to prove this phenomenon, we could have an indirect evidence of hydraulic lift in the dry season in our system. Nevertheless, further investigations including information on the water used by shallow-rooted plants located close to deep-rooted species potentially up lifting water are required to prove the influence of hydraulic lift by deep-rooted plants on neighbour shrubs (Filella and Peñuela, 2003)

We observed a significant effect of plant species on the isotopic composition of the free water from the soil beneath them in spring, when plants were using water available in the shallowest soil layers (10-20 cm). This suggests that the microenvironment created under plants is species-specific and is able to modify soil water conditions. In summer, we observed an effect of the species on the $\delta^{18}O$ isotopic composition and deuterium-excess of free shallow water, but not for free water δ^2H. This could be due to a pore scale isotope heterogeneity in the water soil caused by water surface interaction effects (Penna *et al.*, 2018) or to the differences in the relative contribution of equilibrium and kinetic effects during evaporative enrichment for $\delta^{18}O$ and δ^2H, which cause different sensitivity to environmental variables (Craig and Gordon, 1965; Cappa *et al*., 2003). Recent meta-analysis on the environmental drivers of leaf water isotopic composition revealed that δ^2H is more related to the isotopic composition of source water and atmospheric vapour, whereas $\delta^{18}O$ seems to be more responsive to air relative humidity (Cuntz *et al.*, 2020). Extrapolating these processes to the soil, it is reasonable to expect more homogeneous δ^2H isotopic values in the soil during summer, whereas $\delta^{18}O$ isotopic values would be more variable owing to the different soil micro-environment during evaporative enrichment underneath each species.

CONCLUSIONS

To conclude, our results prove that during drought there is a partitioning of water sources among co-existing species, which segregated species hydrological niche by root depth, but not by gypsum affinity. In this plant community living on the top of a gypsum hill, crystallization water of gypsum represents a vital source for most of the shallow-rooted species during summer, and allows them to survive the arid conditions, forming diverse communities. Rock moisture arises as the main water source for deep-rooted species during drought. However, our results show that all species in the community are able to use crystalline gypsum water during the summer drought period, pointing at a hidden water pool important for life in gypsum drylands. Hence, we strongly recommend that gypsum crystallization water is included as a potential source in water balance studies dealing with ecosystems developed on gypsum soils, which span over 200 million ha in all continents (Eswaran and Gong, 1991).

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TABLES AND FIGURES

Table 1. Main characteristics of study species. Max-root depth: 1: 25-50 cm, 2: 50-100 cm, 3: > 100 cm (Guerro-Campo,1998)

Id	Species	Root depht	Max-root depth	Gypsum affinity	Stem	Family
Fu.er	*Fumana ericifolia* Wallr.	Shallow	1-2	gypsovag	woody	Cistaceae
Ge.sc	*Genista scorpius* L.DC	deep	3	gypsovag	woody	Fabaceae
Gy.hi	*Gypsophila struthium* L. *subsp*. *Hispanic*a (Willk.) G. López	deep	3	gypsophile	woody	Caryophyllaceae
He.hi	*Helianthemum hirtum* (L.) Mill	shallow	1-2	gypsovag	woody	Cistaceae
He.ma	*Helianthemum marifolium* (L.) Mill.	shallow	1	gypsovag	woody	Cistaceae
He.sq	*Helianthemum squamatum* (L.) Pers.	shallow	2	gypsophile	woody	Cistaceae
He.sy	*Helianthemum syriacum* (Jacq.) Dum. Cours.	shallow	2	gypsovag	woody	Cistaceae
He.st	*Helichrysum stoechas* (L.) Moench subsp. stoechas	shallow	2	gypsovag	woody	Asteraceae
He.fr	*Herniaria fruticosa* L.	shallow	2	gypsophile	woody	Caryophyllaceae
Ko.va	*Koeleria vallesiana* (Honckeny) Gaudin subsp. *vallesiana*	shallow	1-2	gypsovag	herbaceous	Poaceae
Le.su	*Lepidium subulatum.* L	shallow	2	gypsophile	woody	Brassicaceae
Li.sf	*Linum suffruticosum* L.	shallow	2-3	gypsovag	woody	Linaceae
Li.fr	*Lithodora fruticose* (L.) Griseb.	shallow	2-3	gypsovag	woody	Boraginaceae
Ma.fr	*Matthiola fruticulosa* (Loefl. ex L.) Maire *-subsp-. fruticulosa*	shallow	1-2	gypsovag	woody	Brassicaceae
On.tr	*Ononis tridentata* L.	deep	3	gypsophile	woody	Fabaceae
Ro.of	*Rosmarinus officinalis* L.	deep	3	gypsovag	woody	Lamiaceae
St.of	*Stipa offneri* Breistr.	deep	3	gypsovag	herbaceous	Poaceae
Te.ca	*Teucrium capitatum* L. -subsp-. *capitatum*	*shallow*	1-2	gypsovag	woody	Lamiaceae
Th.ti	*Thymelaea tinctoria* (Pourr.) Endl. -subsp.- *tinctoria*	deep	3	gypsovag	woody	Thymelaeaceae
Th.vu	*Thymus vulgaris* L.	shallow	2	gypsovag	woody	Lamiaceae

Table 2. Results of linear models analyzing the effects of species on the isotopic composition ($\delta^{18}O$, $\delta^{2}H$) of soil free water collected underneath the plants (10 - 20 cm depth). *F*-values and *p-value*s are shown. Bold type indicates significant effects at $p < 0.05$.

Season	Isotope	*F*	*p-value*
Spring	$\delta^{2}H$	3.80	**<0.001**
	$\delta^{18}O$	3.54	**<0.001**
	D-ex	1.18	0.279
Summer	$\delta^{2}H$	1.33	0.173
	$\delta^{18}O$	3.05	**<0.001**
	D-ex	3.09	**<0.001**

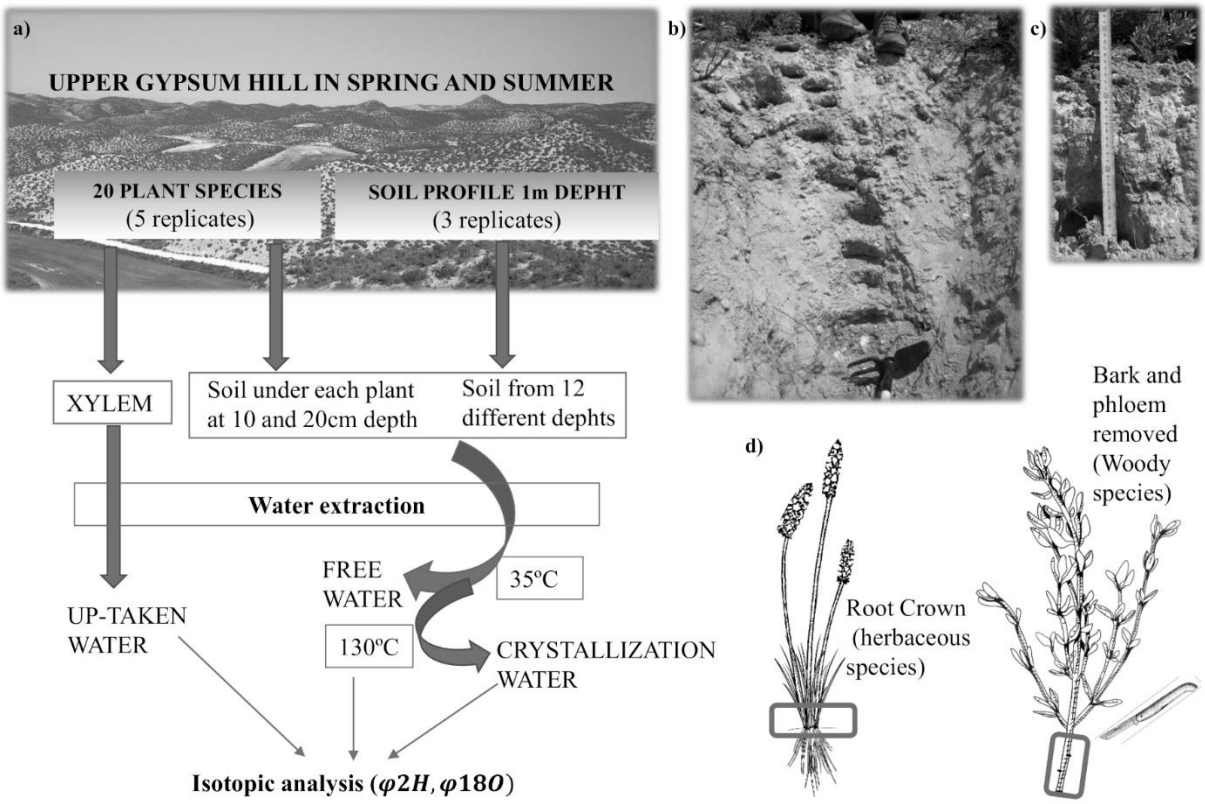


Fig.1. Overview of the sampling design. a) Diagram showing the set-up for sample collection including replicate numbers and the subsequent extraction of water at different temperatures to obtain water samples for isotopic analysis. b) Picture of one of the one-meter-deep soil profiles. c) Picture of soil collected underneath individual plants. d) Description of plant sections used for xylem sampling, both in woody (removing the bark and phloem with a knife) and herbaceous species (cutting and selecting the root crown)

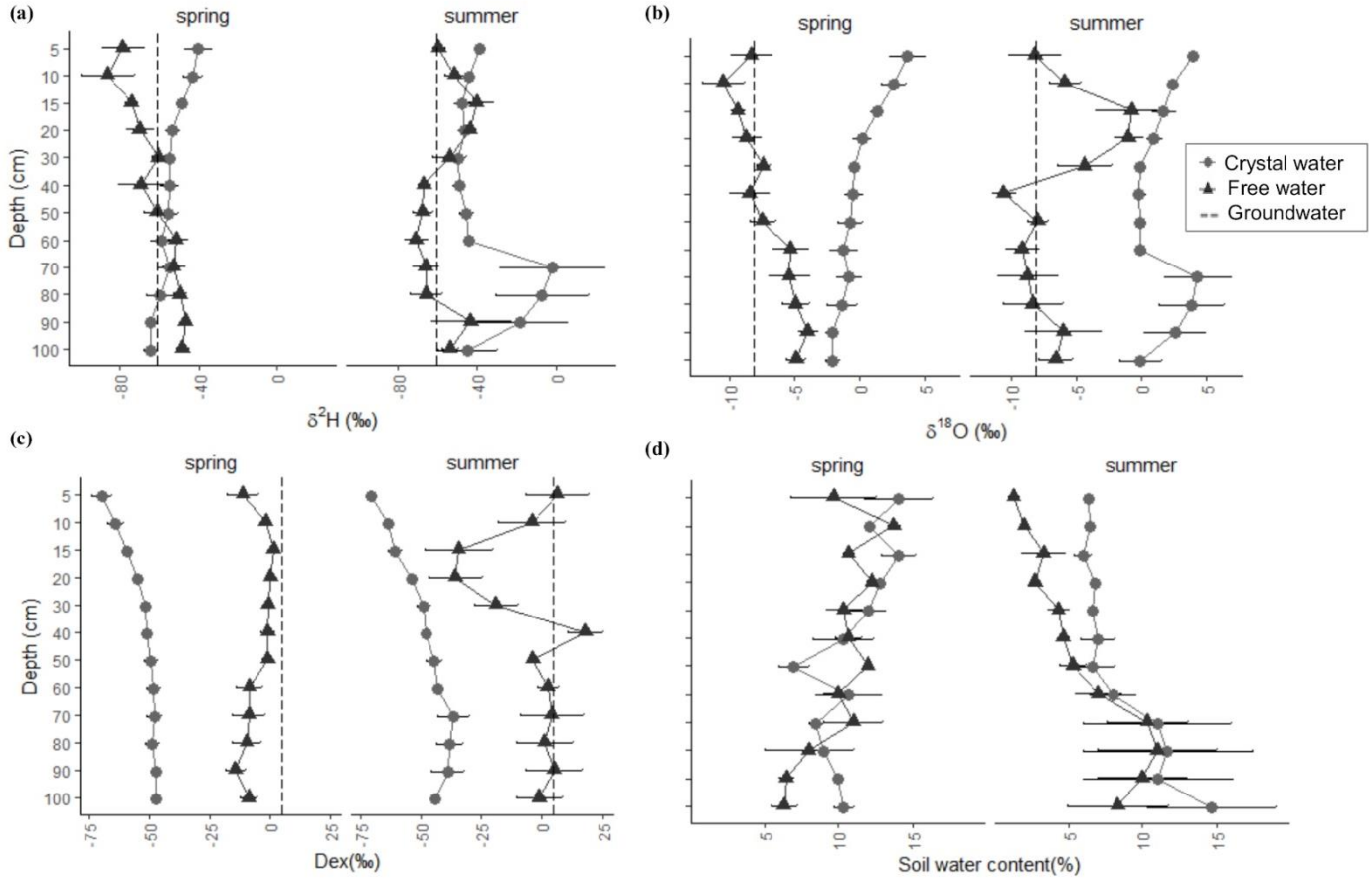


Fig 2. Changes in a) mean δ2H isotopic values, b) mean δ18O isotopic values, c) mean water Deuterium excess (Dex) values and d) water content with depth along the soil profile in spring and summer. Black triangles are for "free water", extracted at 35 ºC and grey circles are for "crystallization water" extracted at 130 ºC. Values are means ± SE of the three bare soil profiles (N = 3). (N = 3). Dashed lines in a), b) and c) indicate groundwater isotopic values.

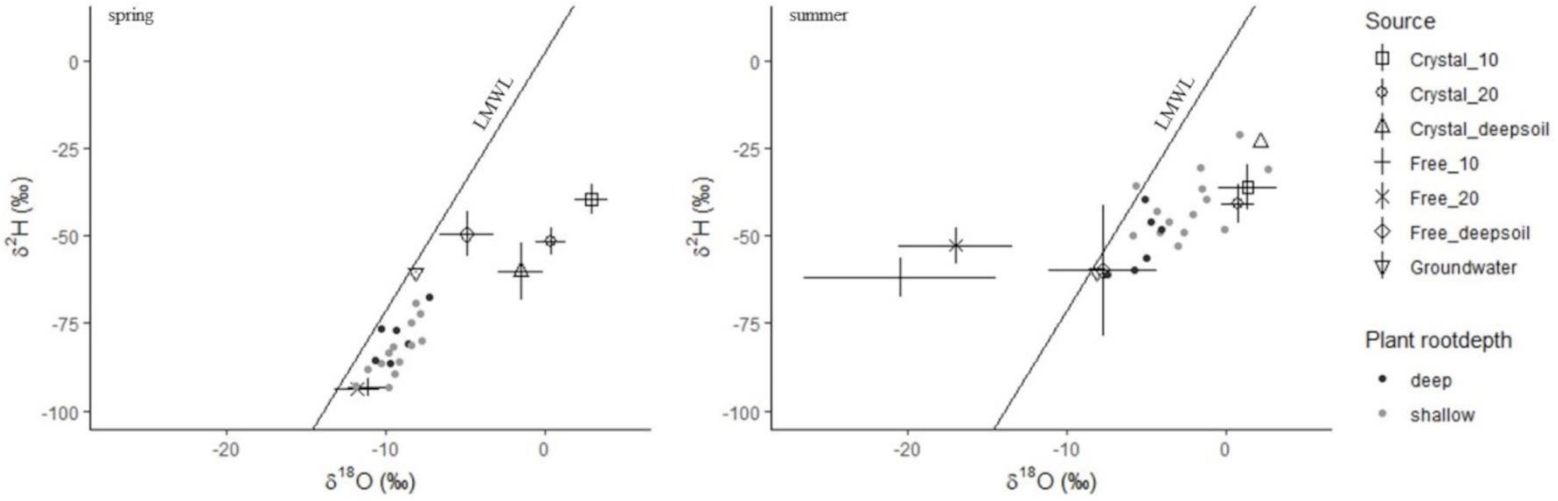


Fig. 3. δ^2 H and δ^{18} O composition of the xylem sap of the plant species and the seven different water sources used in Bayesian isotope mixing models. Water sources include: gypsum crystallization water extracted from the soil at 130ºC, free water extracted from the soil at 30ºC and groundwater. Soil from 10 and 20 cm deep was sampled underneath each plant, deep soil was sampled in the profiles and groundwater was upwelling in saline depressions in spring. Grey points are for shallow-rooted plants and black points for deep-rooted plants. LMWL: local meteoric water line

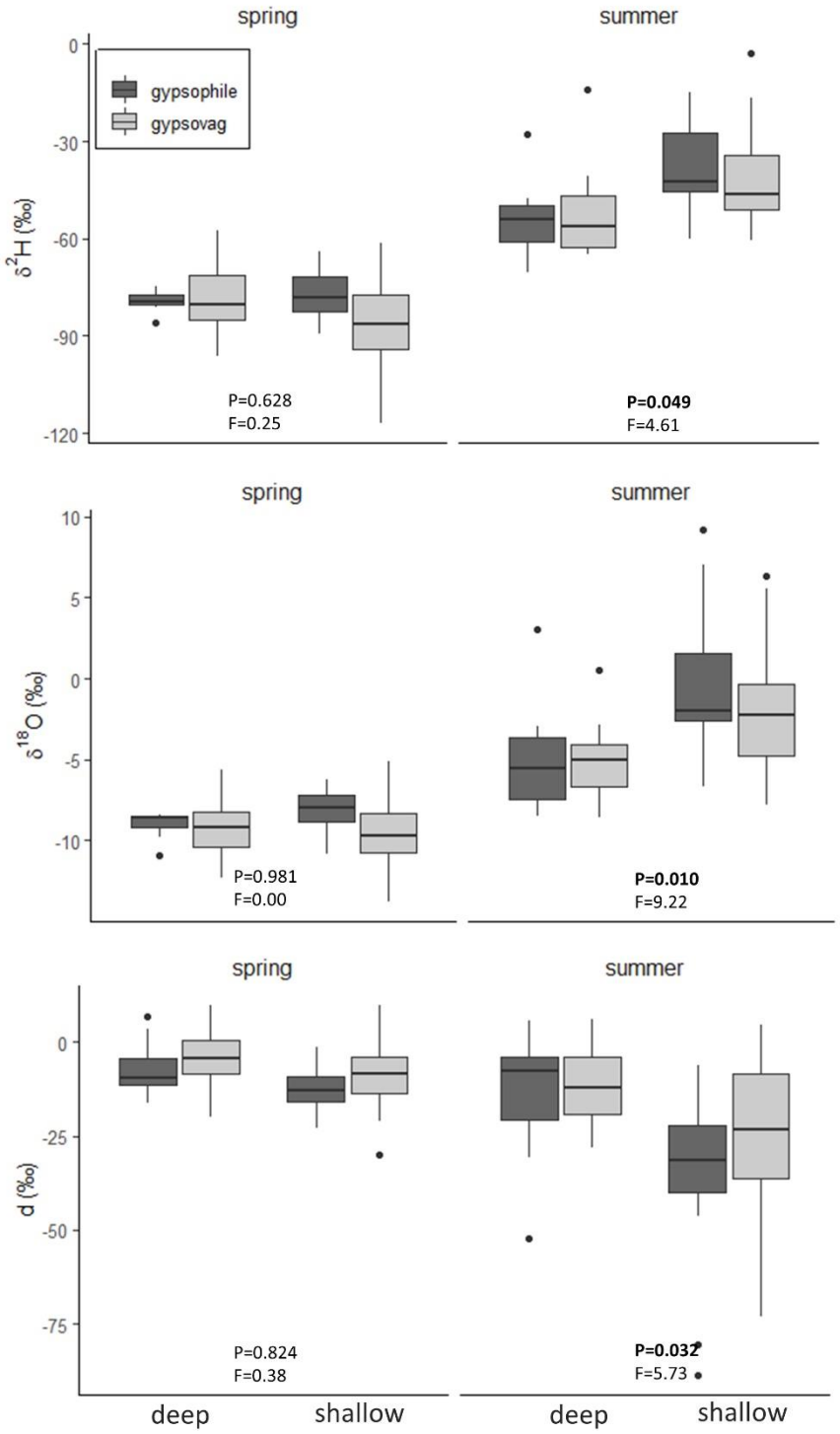


Fig. 4. Seasonal variation in the isotopic composition of xylem water, according to root depth (spring: left panels; summer: right panels). δ^2H and $\delta^{18}O$ isotope composition and Deuterium excess are shown. Different letters are for significant differences after Tukey Post-Hoc analyses across root depth and season ($p < 0.05$). *F-ratio*s and *p-value*s display differences in the xylem sap between plants with distinct root depth, in models run separately for each season Black boxes are for gypsophiles and grey boxes are for gypsovags.

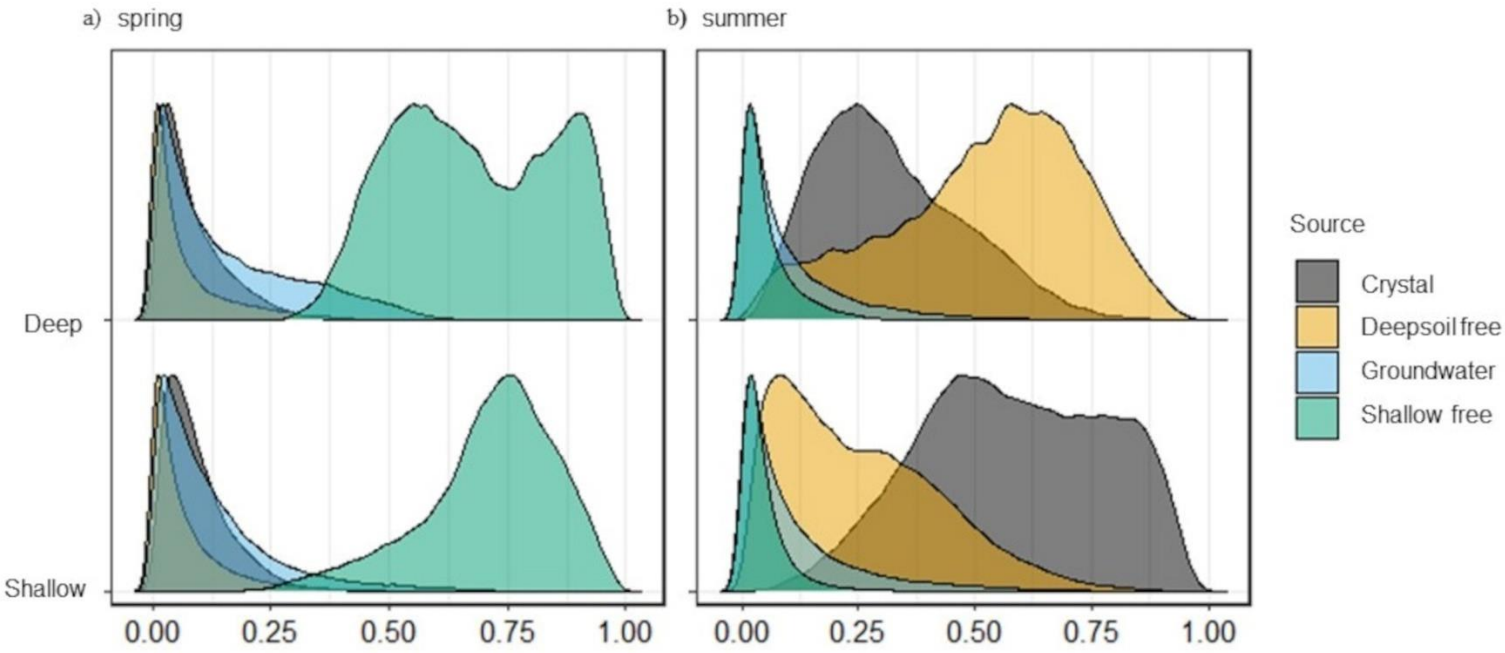
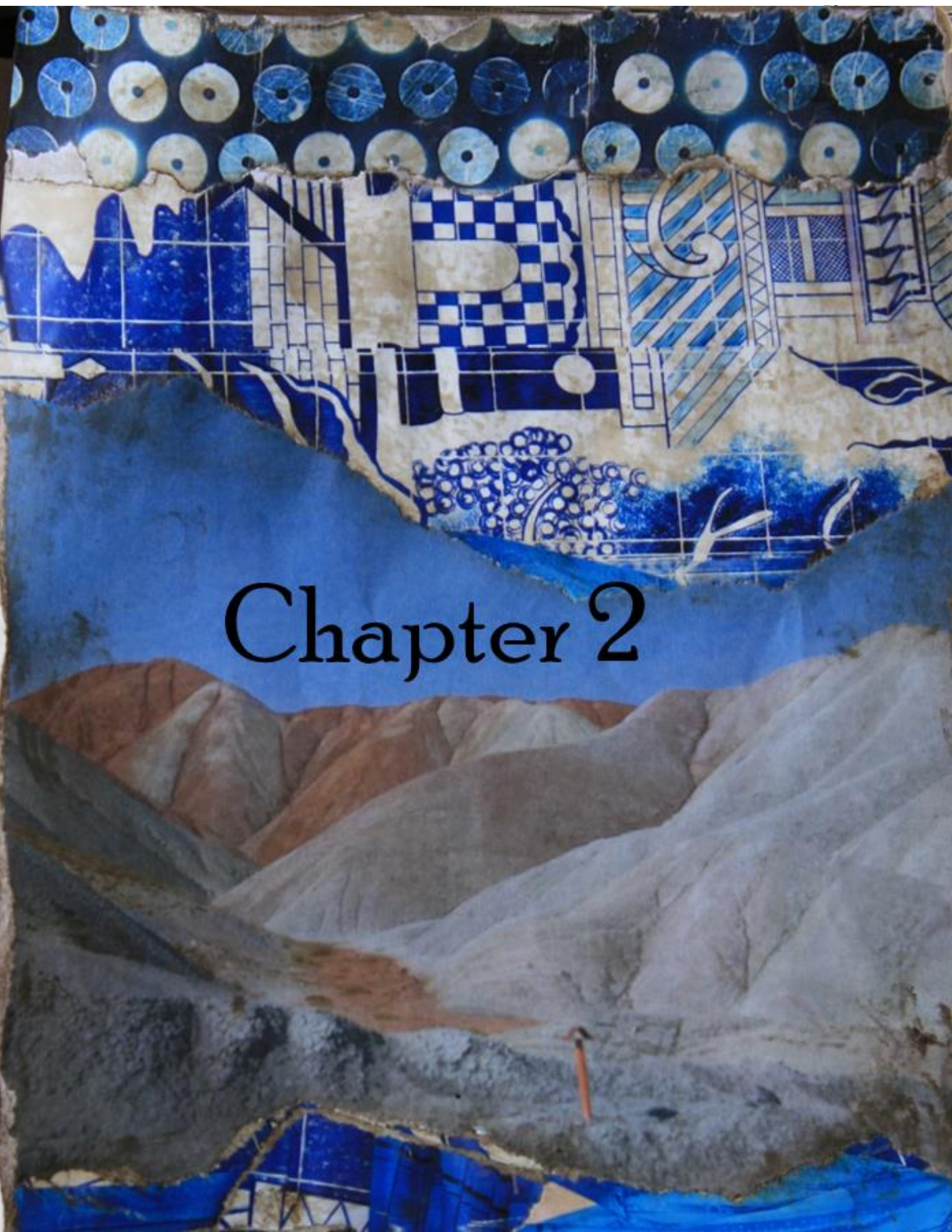


Fig.5. Results from Bayesian stable isotope mixing models showing the estimated contribution of different water sources, namely: shallow free water (10 – 20 cm), deep free water (50 – 100 cm), groundwater and gypsum crystallization water (all depths combined) to the xylem water of 20 dominant plants from a gypsum top-hill community, grouped into deep-rooted and shallow-rooted species.


Chapter 2

Data collection site in the selected Aladaghlar gypsum hill (white hill in the right). Picture taken by Alexander Rudov.

Margins designed by Virginia de la Iglesia and Laura de la Puente

Chapter 2

Ecohydrological niche segregation among desert shrubs in a gypsum-calcareous formation (NW Iran) *

*Article under revision

Alexander Rudov, Laura de la Puente, Sara Palacio, Arash Sharifi, José Ignacio Querejeta, Juan Pedro Ferrio, Hossein Akhani (2023). *Plant Ecology & Diversity*

ABSTRACT

- **Background:** Xerophilic subshrubs exhibit multiple functional types and frequently show hydrological niche segregation. In the poorly studied Irano-Turanian gypsum deserts, insights into different plant species ecohydrological strategies are crucial to understand community complexity in these vulnerable ecosystems.
- **Aim:** We aimed to unravel the ecohydrological strategies of five co-existing subshrubs members of Caryophyllales, ascertaining if their rooting architecture, gypsum affinity or photosynthetic pathway determined their water uptake, and if gypsum crystallization water could be a relevant water source for plants in different seasons.
- **Methods:** We conducted soil and xylem sampling for isotope analyses in spring and summer and extracted water by cryogenic vacuum distillation. Oxygen and hydrogen isotope composition were determined and compared with visual representation and Bayesian Mixing Models to determine species ecohydrological strategies.
- **Results:** Species-season interactions explained differences in xylem sap isotopic composition. Three basic strategies relying on contrasting utilization of free topsoil moisture and deep soil water could be detected and were in part explained by rooting architecture. Plant gypsum affinity and photosynthetic pathways did not have a significant effect on the water sources used by plants.
- **Conclusions:** Ecohydrological niche segregation was explained partly by rooting architecture and species-specific traits. Gypsum crystallization water was not used in summer by the studied species.

KEYWORDS: Caryophyllales, desert subshrubs, gypsum, Iran, niche segregation, stable isotopes, water use.

INTRODUCTION

Arid and semiarid ecosystems are located in regions that are particularly vulnerable to climate change (Hoover et al. 2015; Wertin et al. 2015; Grossiord et al. 2017; Arias et al. 2021). Different forecasted climate change scenarios predict an intensification of drought and changes in precipitation patterns (Collins et al. 2013). Temporal and spatial variation in soil water availability is a determining factor for plant distribution and community assemblage in these ecosystems (Huxman et al. 2005). To cope with scarcity and varying availability of water, plant species from arid ecosystems frequently show hydrological niche segregation (Silvertown 2004; Huxman et al. 2005; Xu et al. 2007; Dai et al. 2015; Tiemuerbieke et al. 2018; Wu et al. 2019). The partition of water resources through different mechanisms of water acquisition or by the temporal variance of water supply, allows co-existing dryland plant species to access the scant water resources, alleviating competition (Moreno-Gutiérrez et al. 2012; West et al. 2012; Silvertown et al. 2015; Brum et al. 2017; Palacio et al. 2017; Iluminati et al. 2022; Querejeta et al. 2022). Determination of water source partitioning among co-occurring plant species and functional types from arid habitats should improve our understanding of the diverse strategies used by plants in these highly adapted and diverse communities. This is crucial for the prediction of aridification impacts on desert vegetation in the context of climate change.

Precipitation pulses are a short-lived water source in desert ecosystems due to fast evaporation of soil moisture (Ehleringer and Dawson 1992). Thus, precipitation-dependent species will be more physiologically stressed during drought periods than those relying on deeper soil water sources or groundwater (Wu et al. 2019). Many dryland plants have developed dimorphic root systems with both shallow and deep roots, which allow them to use water from different soil depths depending on temporal fluctuations in water availability (Bauerle et al. 2008; Prieto et al. 2012; Barbeta et al. 2015). Water from precipitation present in the topsoil after rainfall pulses may be used preferentially during the main growth period, as it favours microbial processes and nutrient uptake by roots (Querejeta et al. 2021). During drought, roots could potentially access deeper soil layers, where water availability is more stable (Ryel et al. 2008, 2010; Fan 2015; Rempe and Dietrich 2018).

Plant water use is not only controlled by root functioning, but also by shoot ecophysiological traits (Tiemuerbieke et al. 2018). Xerophyte communities in drylands host multiple functional types, such as different growth forms, and specific traits including leaf and/or

stem succulence, leaf reduction or different photosynthetic pathways (e.g., C_3 and C_4) (Rudov et al. 2020; Wertin et al. 2015). It has been proposed that C_4 plants have a higher water-use efficiency than C_3 plants (Morgan et al. 2011; Way et al. 2014; Taylor et al. 2014; Helliker and Ehleringer 2002). This assumption is based on the peculiar CO_2 concentrating mechanism present in C_4 plants, that allows avoidance of photorespiration even under reduced stomatal conductance and consequently reduces water demand (Ghannoum 2011).

Gypsum is an evaporitic mineral frequently present in soils within arid and semiarid landscapes. Endemic-rich gypsum ecosystems are frequently considered as agricultural badlands, and thus actively used as dumping places or affected by other negative anthropogenic activities like mining or urban expansion. The survival of plants in these ecosystems is affected by aridity and harsh edaphic conditions. Gypsum soils have low water retention (Herrero and Porta, 2000), which makes water a critical limiting factor for plants growing on them. However, gypsum holds two water molecules in its crystalline structure ($CaSO_4 \cdot 2H_2O$) and can be used as a water source by plants during dry periods (Palacio et al. 2014, 2017; de la Puente et al. 2021). Some plant species have adapted to cope with the physical and chemical restrictions of gypsum soils, behaving as edaphic endemics (gypsophiles) restricted to gypsum soils. In addition, there are gypsum tolerant species (gypsovags) that are not specialized to grow on gypsum soils, but are able to cope with their adverse properties (i.e. low water retention and adverse chemical effects of calcium and sulfate) (Meyer 1986; Cera et al. 2021). In a recent study of a plant community occurring on gypsum in the Iberian Peninsula, it was shown that crystallization water was mainly used by shallow rooted species, indicating a critical role of rooting depth to access deep, more stable water sources (de la Puente et al. 2021).

Gypsum deserts in the Irano-Turanian floristic region are highly diverse ecosystems, rich in local endemics. The dominant species in these ecosystems are extremely adapted subshrubs of different phylogenetic origins, gypsum affinity, photosynthetic pathways, shoot functional traits, etc., which have been proposed as driving factors for nutrient or water use in other arid ecosystems (Palacio et al. 2022; de la Puente et al, 2021; Moreno-Gutierrez et al 2012). These ecosystems are, however, highly understudied both floristically and ecologically. Practically nothing is known about the species occurring within these communities and the importance of their functional traits (such as succulence, photosynthetic pathways, gypsum affinity, root structure, etc.) on their ecohydrological mechanisms of adaptation and their community assemblage. Several of these taxa

(e.g., *Anabasis spp.*) are nevertheless of high scientific interest due to their occurrence in the most extreme parts of Iranian gypsum, clay, marl and gravel deserts. In these edaphically and climatically most extreme areas they often comprise the only existing sparse vegetation cover and thus, insights into their ecohydrological mechanisms are of particular interest.

The aim of this study was to define the water isotopic composition of xylem sap and the potential water sources used by five dominant woody shrub species coexisting in the arid Aladaghlar hills of NW Iran and to determine whether the species exhibit segregation of their ecohydrological niches according to different water sources used in spring and summer. We further sought to ascertain whether species, rooting architecture, gypsum affinity and photosynthetic pathway were determining factors for differences in water use among species and for their ability to use gypsum crystallization water. We hypothesized that (1) rooting architecture will be a determinant factor for water use, with species having a deep taproot using deep soil water throughout the year, while species with a dimorphic root system may use water from shallower soil level in spring to deeper level in summer. We further hypothesized that (2) shallow rooted species, independent of gypsum affinity, will use shallow water in spring, but mainly gypsum crystallization water during the dry season. Finally, regarding the different photosynthetic pathways of species, we hypothesized that (3) C_4 species with higher leaf-level water use efficiency and root systems, relative to C_3 species, should be less dependent on hydrological fluctuations, and thus, continue to rely on the scarce free soil water remaining in upper soil layers during summer.

MATERIALS AND METHODS

Study area and species

We conducted field sampling in the Aladaghlar hill area of NW Iran, on the border of the Zanjan and E Azerbaijan provinces (Figure 1, 2A and 2C) (Azizi et al. 2018). The area falls within the Upper Red Formation, which is composed of alternating layers of marl, conglomerate, sandstone and intercalated evaporite layers of crystalline gypsum (Figure 1, 2A and 2C) (Ghorbani 2019). It is located in the Zanjan basin, a remnant of the Tethys Sea of the Early Miocene, which has been shaped by subsequent sedimentation of marine and continental sediments (Rahimpour-Bonab et al. 2007; Alizadeh 2017). The gypsum content of hill slopes varies from 4% to 84% (Table S1, calculated with the methodology proposed by Porta,1998). The climate of the region is Irano-Turanian xeric continental (Djamli et al. 2011, 2012) with severe drought during summer. Climate data recorded over 55 years at the Zanjan meteorological station, ca. 115 km E of the study area, indicate that total annual precipitation is 313 mm and mean annual temperature is 11.5°C (minimum = −7.5°C, maximum = 31.9°C) (Figure 1).

The harsh climate of the Aladaghlar hill area and its edaphic and topographic peculiarities result in a sparse vegetation, dominated mainly by xerophytic hemicryptophytes and chamaephytes. The area is highly diverse with several endemic species, but is also understudied with scarce ecological information available in the scientific literature. We selected five dominant woody perennial plant species, *Anabasis eugeniae, Anabasis calcarea, Caroxylon gemmascens, Oreosalsola montana* (Amaranthaceae/Chenopodiaceae) and *Atraphaxis suaedifolia* (Polygonaceae) belonging to the order Carophyllales with different affinities to gypsum soils (Figure 2) and different photosynthetic pathways (Table 1) (Akhani et al. 2016; Rudov et al. 2020; Doostmohammadi et al. 2020). All dominant plant species of this area are succulent and retain large quantities of water in their leaves and/or photosynthetic shoots (Figure 2).

Plant, soil and water sampling

We conducted field sampling for isotope analyses in spring (April and May) 2018 and during the subsequent summer drought in August after a long rainless period. At each sampling date, we harvested the main stems (including the root crown) of five individuals per species. We selected vigorous, medium-sized individuals located at least 5 m apart. We harvested between 6:30 and 10:00 a.m., when we expected maximum transpiration rates and low evaporative demand to

prevent stem dehydration (Grammatikopoulos et al. 1995; Martín-Gomez et al. 2015). We removed the bark and phloem with a knife to avoid contamination with isotopically enriched phloem water and organic compounds present in living cells and/or in the bark (Ehleringer and Dawson 1992). We also collected two soil samples beneath each plant at two depths (10 and 20 cm), excluding roots. In spring, an extra water sample was collected from a temporary creek formed in the valley bottom by a strong rainstorm (14.04.2018) which recharged the groundwater aquifer. This water sample value was used as a representation of the deeper soil water source. All water, stem and soil samples were placed in airtight sealed tubes and immediately frozen in a portable freezer (Allison and Hughes 1983).

We observed the root system of at least four individuals per species up to a depth of 40 cm. Additionally, we observed the root system of *Anabasis eugeniae* and *A. calcarea* on eroded steep slopes, where their root systems were exposed due to soil movement. We compared our data with previously published data on root morphology of the investigated species (Persson and Baitulin, 1996; Wang et al. 2017; Fet and Atamuradov 1994; Smirnova et al. 1976).

Water extraction

We extracted xylem and soil water using a modified cryogenic vacuum distillation (Ehleringer and Dawson 1992) as in Palacio et al. (2014). Xylem water was extracted at CEBAS-CSIC (Murcia, Spain) using cryogenic vacuum distillation at 100 ºC and 10 millitorr vacuum pressure for 2 h (Querejeta et al. 2021). Soil samples were extracted at the laboratory of the Instituto Pirenaico de Ecología (IPE-CSIC, Zaragoza, Spain). Sample tubes were placed in a heated silicone oil bath, and connected with Ultra-Torr unions (Swagelok Company, Solon, OH, USA) to a vacuum system (ca. 10^{-2} mbar) including U-shaped water traps in series that were cooled with liquid nitrogen. After an extraction time of 90 min (West 2006; Meissner 2014), captured water was transferred into screw-capped 2 ml vials and stored at 4 ºC until isotope analysis. Soil samples were distilled in two steps: first at 35 ºC and then at 130 ºC to separate free and gypsum crystallization water while ensuring an almost complete dehydration of gypsum (Freyer and Voigt 2003; Palacio et al. 2014). When necessary, between the first and second distillation of soil samples, we kept sample tubes in a desiccator with silica gel to avoid any re-hydration with ambient moisture, which could contaminate the water extracted in the second step.

Stable isotope analyses

The use of water stable isotopes (^{2}H and ^{18}O) as natural tracers to understand water uptake by plants is extensive in plant ecology (Dawson et al. 2002). A vertical gradient in the isotopic composition of soil water is commonly found in arid ecosystems, where the upper soil areas are enriched in the heavier isotopes because of more pronounced evaporative isotopic fractionation (Allison and Hughes 1983; Orlowski et al. 2013).

We determined oxygen and hydrogen isotope composition ($\delta^{18}O$, $\delta^{2}H$) using Isotope Ratio Mass Spectrometers (IRMS) at the Center for Stable Isotope Biogeochemistry (CSIB) of the University of California at Berkeley (USA). $\delta^{2}H$ was determined by the dual inlet method, using a hot chromium reactor unit (H/Device™, Thermo Fisher Scientific, Waltham, MA, USA), coupled to a Thermo Delta Plus XL mass spectrometer (Thermo Fisher Scientific), using multiple standards in every run, and correcting for drifts using two internal standards with different isotope ratios. $\delta^{18}O$ was determined by the equilibration method, using a Thermo Gas Bench II interface, coupled to a Thermo Delta Plus XL mass spectrometer (Bremen, Germany). Briefly, ca. 20 µL of water were pipetted into 10 mL glass vials (Exetainer®, Labco Ltd., UK) and sealed. The vials were purged with 0.2 % CO_2 in helium and equilibrated at room temperature for 48 hours. The $\delta^{18}O$ value of CO_2 was then determined by IRMS. Long-term external precision is ± 0.60 ‰ and ± 0.12 ‰ for $\delta^{2}H$ and $\delta^{18}O$, respectively.

Statistical analyses

We ran all statistical analyses in R 4.0.0 (R Core Team, 2020). We used *ggplot2* (Wickham 2016) to visualize xylem sap isotopic composition and soil water at different sampling dates, the $\delta^{2}H$-$\delta^{18}O$ plot space and contributions of the water sources to plants. To account for seasonal changes (April-August) in isotopic composition in the water xylem sap of the five species and in the water sources (free and crystallization water), we used GLMs separately for each species and water source type (including season as a fixed factor) with the *lm* function of the *nlme* package (v3.1-152; Pinheiro et al., 2021). Differences among study species (fixed factor) and sampling dates (fixed factor) in xylem water isotopic composition ($\delta^{18}O$, $\delta^{2}H$) were evaluated using the *lmer* function, which uses the Maximum Likelihood estimation. Similarly, the effects of season, rooting architecture, gypsum affinity, photosynthetic pathway and their interaction with the season of sampling on the xylem isotopic composition of the species were also checked with the *lmer*

function. In this case, rooting architecture, gypsum affinity, photosynthetic pathway and season were considered as fixed factors, whereas species and plant family nested within species were included as random terms to account for potential phylogenetic effects. When the effect of the factor was significant on the xylem sap isotopic composition, groups were detected using a post-hoc Tukey test. Over- and under-dispersion of model residuals were checked using the *simulateResiduals* function in *DHARMa* package v.0.3.1 (Hartig 2021).

The relative contribution of different water sources to the xylem sap of plants was estimated using Bayesian Mixing Models for stable isotope data, using the package *MixSIAR* (Stock et al. 2018). The package, originally developed for dietary studies, estimates the relative contribution of alternative preys (sources) to a given consumer (mixture), considering the multidimensional space formed by two or more biotracers (e.g. stable isotope composition) in the sources and the consumers. We used the isotopic values (δ^2H and $\delta^{18}O$) of xylem water (consumer) in each individual sample (i.e. every single replicate of all the species) as inputs for the model. The mean and standard deviation (per species) for each alternative soil source (free and crystallization water, at 10 cm and 20 cm soil depth collected beneath each individual plant), and the water value of the creek, representing deep soil water, were considered as inputs for the sources. For the creek water collected in spring, only one replicate sample was available, so we had the same value for all plant species and replicates. As a conservative approximation of the standard deviation for this source, we choose the mean of the standard deviation of the other two free water sources. Alternatively, we also tested the models using the analytical error, as the only measured uncertainty for this source (0.4 for δ^2H and 0.1 for $\delta^{18}O$; Supplementary Materials, Methods S2). These two alternative models did not show noticeable differences in the trends of water sources used by study species.

To simplify the interpretation of the Bayesian Mixing Model, the output was combined *a posteriori* through the addition of the estimated contributions of each source into three simplified sources. First, crystal water (i.e., gypsum crystallization water from the soil) that was calculated by adding the contributions to the xylem of plants from crystallization water at the two depths initially considered (10 and 20 cm). Second, free water (i.e., free water in the shallow soil), was calculated by adding the contribution to the xylem from the free water at 10 and 20 cm. Third, deep soil free water (i.e., free water in the deep soil) was kept unmodified.

RESULTS

Water sources and species xylem sap isotopic composition

The oxygen isotopic composition of gypsum crystallization water was stable in both seasons, but in August it was less enriched in ^{2}H. Free soil water changed its $\delta^{2}H$ and $\delta^{18}O$ composition among sampled months (Figure 3, Table S3). We found differences in $\delta^{2}H$ and $\delta^{18}O$ of xylem sap between spring and summer for *Anabasis calcarea* and *A. eugeniae*, but only in $\delta^{2}H$ for *Atraphaxis suaedifolia* (Figure 3, Table S3).

Species, season and their interaction were all significant factors explaining differences in xylem sap isotopic composition ($\delta^{2}H$, $\delta^{18}O$), except for seasonal variation in $\delta^{18}O$ (Table 2). Contrastingly, neither plant rooting architecture, gypsum affinity nor photosynthetic pathway had a significant effect on the isotopic composition of the xylem sap of plants (Table 2). However, we observed a marginally significant interaction between species gypsum affinity and sampling month for $\delta^{18}O$ xylem sap variation (Table 2,) though a post-Hoc Tukey test did not separate clear groups.

Contribution of different water sources to the xylem water of plants

The analysis of the isotopic values ($\delta^{2}H$ and $\delta^{18}O$) of xylem sap and water sources (Figure 4) showed that crystallization water isotope values were the most distant from those of xylem sap in both April and August. The deep soil water source was less exposed to evaporation and therefore showed less evaporatively enriched isotopic values than shallower free soil water (Figure 4). Species could be separated according to their xylem sap isotopic composition with *Aanabasis calcarea* (DT), *Aatraphaxis suaedifolia* (T) *and Caroxylon gemmascens* (S) exhibiting very similar compositions in April, close to that of shallow free water at 10 cm depth. In contrast, *Anabasis eugeniae* (DT) and, especially, *Oreosalsola montana* (T), had xylem sap isotopic values that were less enriched and similar to those of deeper free water. These relationships changed in August, with *C. gemmascens (S)* and *A. eugeniae* (DT) having a relatively similar isotopic composition in their xylem sap, close to that of shallow free water, whereas the three other species, and especially *A. calcarea* (DT) and *O. montana (T)*, had compositions close to that of deep free water (Figure 4).

Bayesian Mixing Model estimation of the most likely sources of water used by the species revealed some notable differences between them (Figure 5, Table 3, Table S4). *Oreosalsola montana* used deep soil water as its main water source in both seasons, whereas the other species, and especially

A. eugeniae and *C. gemmascens*, used free water available from the upper layers of the soil profile as their main water source in spring. In summer, *A. eugeniae* and *C. gemmascens* were least dependent on deep soil water, whereas the three other species were heavily dependent on this water source (Figure 5, Table 3, Table S4). The model confirmed that crystallization water from gypsum was a less important water source for each of the species (Figure 5), though in April it contributed 33% and 25% to the water used by *A. suaedifolia* and *C. gemmascens*, respectively (Figure 5, Table 3, Table S4).

DISCUSSION

Our results revealed the different water sources used by five dominant subshrub species in the poorly studied gypsum rich Aladaghlar hills in NW IRAN, and contribute to an understanding of how such differences might be important to niche segregation and the coexistence of these species under the conditions of extreme aridity and harsh soil conditions they are subject to.

According to our results, the species showed differences in their vertical ecohydrological niches, resulting in different water uptake patterns. Ecohydrological niche segregation has been observed in other desert regions of the world, such as in the Gurbantunggut Desert of China (Wu et al. 2013; Timuerbieke et al. 2018), the Glen Canyon in Utah, North America (Ehleringer et al. 1991) and the Namib Desert in Africa (Schachtschneider, 2010). In these regions, the divergent rooting depths of coexisting species were shown to play an important role in water use segregation, thereby avoiding or minimizing interspecific competition for scarce water during drought (Dawson and Pate 1996). In the present study we hypothesised that rooting depth might also be a significant factor affecting water source uptake of species and their ecohydrological niche segregation, and this prediction was fulfilled for three of the five species studied. *Oreosalsola montana*, whose root system consists of a deep taproot, was found to dependent on deep soil water in both spring (April) and summer (August). *Caroxylon gemmascens*, with a shallow root system, used high quantities of superficial water in both of these seasons. On the other hand, *A. calcarea*, with a dimorphic root system described also in other *Anabasis* species (Persson and Baitulin 1996), shifted from using preferentially shallow water in spring to mainly deep soil water during summer drought. Such a shift to the use of deeper water sources during summer is in accordance with previous findings from studies in other arid ecosystems. Such shifts have been suggested to serve as an strategy to maximize nutrient uptake during the wet spring season, while securing access to deeper and more reliable and abundant water sources during the dry summer when evaporative demand is high (Querejeta et al. 2021).

Contrary to our hypothesis, however, *Anabasis eugeniae*, which also has a dimorphic root system, used preferentially water from the shallow topsoil layer in both spring and summer, potentially indicating a primary anchoring function of its taproot. Interestingly, *A. suaedifolia*, which has only a long taproot (Persson and Baitulin 1996; Wang et al. 2017), was nonetheless also able to use shallow topsoil water in spring. This period coincides with generative shoot formation and

flowering in *Atraphaxis* spp. and thus may require additional nutrient uptake from the nutrient rich soil surface (Kostina and Yurtseva 2021). These results are intriguing, since dimorphic roots have not been recorded for *A. suedifolia* in the field. More observations on the root system of this species should be made to ascertain its rooting system in detail.

Our hypothesis about the potential use of crystallization water held in gypsum by shallow rooted species was not supported by our results. We found that among the potential water sources available to plants, the isotopic composition of gypsum crystallization water was the most distant and dissimilar from the isotopic composition of plant xylem water in all the species (Figure 4), and Bayesian Mixing Models analysis showed crystallization water as the least likely water source used by plants. Although it is not possible to completely rule out crystallization water as a minor water source for some of the species studied, the low probability of its use, compared to what has been reported for other ecosystems (e.g. de la Puente et al 2022), suggests that it is not a very significant water source in this ecosystem. Recent studies have shown that coexisting plants use different water sources depending on their rooting depth and position along gypsum hills (Palacio et al. 2017; de la Puente et al. 2021). Shallow-rooted plants growing on soils with high gypsum content showed a widespread use of gypsum crystalline water during summer, while deep-rooted plants tended to use free soil water from deep soil layers (de la Puente et al. 2021). Previous studies reporting substantial use of gypsum crystallization water were conducted in gypsum hills where gypsum content in the soil was homogeneous and accounted for more than 60% (Palacio et al. 2017; de la Puente et al. 2021). However, soils beneath the plants in our studied ecosystem varied from ca. 84% gypsum content to 4% on the same slope (Table S1), suggesting that the highly spatially variable gypsum content of the soil may be a limitation to its use. It is, hence, probable that the presence of gypsum in the rhizosphere of many or all of our study plants was minimal. Alternatively, the availability of other potential free soil water sources was sufficient to supply plants with water even during the dry summer.

In relation to the isotopic composition of the deep water source considered in our study, we propose that, in future studies, more rigorous sampling is employed with more detailed characterization of soil water isotopic composition at different depths of the soil profile. We considered a generic deep soil water pool (using creek water as a proxy for groundwater), without specifying an exact depth, to refer to water that is less evaporated and shows no evidence of evaporative isotopic enrichment in the heavier isotopes in this seasonally dry area (Allison and

Hughes 1983; but see Sánchez-Martín et al, 2021; Ding et al. 2021).Therefore, our results of water use by the plant species should be taken as an approximation of the proportions of water uptaken from different depths in the different seasons; and not as an exact quantification of the water sources uptaken by the plant species.

The traditional assumption of absence of isotopic fractionation during water uptake has been questioned in some studies (Ellsworth and Williams 2007; Barbeta et al. 2020), which found that δ^2H of xylem water can be more depleted than the considered water source. However, in our study we observed that xylem sap δ^2H values were not more negative than those of the water sources available to plants as observed in those cited previous works. This result suggested that the δ^2H of xylem water accurately reflected the δ^2H of the soil water sources in our study, thereby facilitating the data analysis with Bayesian Mixing models using both water isotopes.

Our results further showed that water niche segregation strategies are not directly related to the photosynthetic pathway in the five desert species analysed. For example, *A. calcarea* (C_4) shifted to deep soil water sources during the period of summer drought, similarly to the C_3 species *A. suaedifolia*. On the other hand, we could explain the use of shallower and scarce free water by *A. eugeniae* and *C. gemmascens* by their photosynthetic pathway, both species are highly drought tolerant C_4 plants, so their photosynthetic pathway adapted to warm and dry climatic conditions may contribute to a higher water-use efficiency, decreasing their reliance on deep water. Nevertheless, we also observed that the relative of *A. eugeniae*, *A. calcarea*, also C_4, used a big proportion of deep water in summer. Similar patterns have been described in plants from other desert ecosystems. For example, ecohydrological niche segregation has been observed between the closely related C_4 desert shrubs *Haloxylon ammodendron* and *H. persicum* (Dai et al. 2015, Wu et al. 2019). Although these two *Haloxylon* species share common habitats (sand deserts) and have comparable extensive root systems, *H. ammodendron* switches from shallow water use to groundwater use during the hot dry season, while *H. persicum* continues to depend on free topsoil water during the entire dry season (Dai et al. 2015, Wu et al. 2019. Furthermore, deep-rooting C_3 desert shrubs, such as *Tamarix* species, have been shown to rely on deep soil water as the main water source during the whole year, similarly to *O. montana*, *w*hile shallow-rooting species, such as *Nitraria tangutorum* (C_3), *Reaumuria songarica* (C_3) and *Calligonum leucocladum* (C_4), continue to rely largely on the scarce free soil water pool even during dry periods (Xu and Li 2006;

Xu et al. 2007; Wu et al. 2014; Tiemuerbieke et al. 2018), similar to our findings for *C. gemmascens* and *A. eugeniae*.

The reason for the differences in water source use between the two *Anabasis* species and the most important functional traits related to them still need further attention, but studies on the dimorphic root systems of related *Anabasis* species point to their ability to rely on several complementary water sources. In contrast to C_4 monocots, C_4 eudicots typical for the Irano-Turanian deserts, have a higher diversity of functional traits and ecophysiological adaptations to their environment. Thus, differences in ecohydrological strategies of phylogenetically close taxa may, at least partly, be related to the variety of adaptations and functional traits they present (Akhani 2006; Cayssials and Rodriguez 2013; Rudov et al. 2020). In our case, the two *Anabasis* species differ in their shoot morphology, *A. eugeniae* being a leaf-succulent subshrub, while *A. calcarea* is a caudex forming stem-succulent. The genus *Anabasis* is known to include some of the most extremophilic desert species, growing in exceptionally dry habitats and forming species-poor vegetation types (Lauterbach et al. 2019). It presents a variety of ecotypes, functional traits and growth forms, that include annuals, and perennial leaf and stem succulents, which may affect ecohydrological strategies of each species (Rudov et al. 2020).

CONCLUSIONS

The five species studied showed distinct ecohydrological niche segregation, using different proportions of free soil water acquired from different depths. Although growing on a gypsum rich substrate in certain patches of soil, these species did not rely on gypsum crystallization water as a main water source during the summer drought. Neither gypsum affinity nor photosynthetic pathway had any significant effect on the water sources used by the different coexisting species. These xerophytic species revealed three basic strategies of water utilization. First, deep soil water was the main source throughout the growing season by *O. montana*. Second, a drastic shift from topsoil water in April to deep soil water in August by *A. calcarea* and *A. suaedifolia.* Third, moderate variation in use of more topsoil water in April to more deep water in August by *A. eugeniae* and *C. gemmascens*. Rooting architecture was an explanatory factor for the water sources used for *O. montana*, *A. calcarea* and *C. gemmascens*.

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TABLES AND FIGURES

Table 1. Plant species included in the study with indication of their average plant size, affinity for gypsum soils and taxonomic family. Growth forms: SSS = stem succulent subshrub; LSS = leaf succulent subshrub. Root systems: DT = long taprrot with the presence of a dimorphic root system; T = long taproot; Shallow rootsystem (less than 1 m depth). Gypsum affinity: GV = gypsovag; GP = gypsophile. References: 1) Persson & Baitulin, 1996; 2) Wang *et al*. 2017; 3) Fet and Atamuradov 1994; 4) Rudov − unpublished data; 5) Smirnova *et al*. 1976.

Id.	Species	Growth form & plant size	Root system	Gypsum affinity	Photo-syntetic type	Family
An.ca	*Anabasis calcarea* (Charif & Aellen) Bokhari & Wendelbo	SSS 20-40 cm	DT (1)	GV	**C_4**	Amaranthaceae
An.eu	Anabasis eugeniae Iljin	LSS 20 cm	DT (1)	GP	**C_4**	Amaranthaceae
At.su	*Atraphaxis suaedifolia* Jaub &Spach	LSS 40 cm	T (1, 2)	GP	**C_3**	Polygonaceae
Ca.ge	*Caroxylon gemmascens (Pall.) Tzvelev*	LSS 20-40 cm	S (3, 4)	GV	**C_4**	Amaranthaceae
Or.mo	*Oreosalsola montana* (Litv) Akhani.	LSS 50 cm	T (5)	GV	**C_3**	Amaranthaceae

Table 2. ANOVA (Type II Wald F tests with Kenward-Roger df) of linear mixed models analysing differences in the isotopic composition of the xylem sap among species and seasons, and among four fixed factors of the species and their interaction with the season. The three factors are:

The observed rooting depth of the species (Three levels: DT, S, T see Table 1)

The affinity for gypsum soils (gypsophily) with two levels: gypsovag and gypsophile, season and their interaction on the isotopic composition (δ18O, δ2H)

The photosynthetic pathway of the species (two levels: c3 and c4)

The phylogeny (four levels: recent, medium-recent, medium-ancient and ancient)

The plant maximum height (Three levels: small, medium, big)

Species, season and their interaction were included as fixed factors ($p<0.05$) in the first model and for the rest of the models, "Family" and "Family" nested with "Species" were included as random factors. *F-ratio*s and *p-value*s are shown. Significant effects are highlighted in bold type.

	δ^2H (‰)		$\delta^{18}O$ (‰)	
	F-ratio	*p-value*	*F-ratio*	*p-value*
Species	6.19	**<0.001**	7.29	**<0.001**
Season	9.84	**0.004**	0.23	0.637
Species:Season	10.71	**<0.001**	7.44	**<0.001**
Gypsophily	0.06	0.814	0.16	0.724
Gypsophily : Season	1.41	0.244	4.40	**0.044**
Photosyntetic pathway	0.19	0.701	0.21	0.678
Photosyntetic pathway: Season	0.91	0.348	1.15	0.291
Rooting depth	0.59	0.665	0.99	0.550
Rooting depth:Season	1.01	0.377	0.61	0.547

Table 3. Bayesian Mixing Model result shown as the percentage of estimated contribution of the three potential water sources, combined from the initial five potential sources, in the xylem sap of the subshrub species**.**

Season	Species	Crystallization water (%)	Shallow free water (%)	Deep free water (%)
SPRING	*Oreosalsola montana*	11.66	29.69	58.65
	Caroxylon gemmascens	25.69	45.21	29.11
	Atraphaxis suaedifolia	33.05	39.46	27.49
	Anabasis eugeniae	10.86	53.44	35.69
	Anabasis calcarea	24.22	42.2	33.57
SUMMER	*Oreosalsola montana*	9.3	17.92	72.78
	Caroxylon gemmascens	21.73	31.51	46.76
	Atraphaxis suaedifolia	6.49	25.45	68.05
	Anabasis eugeniae	23.82	37.69	38.49
	Anabasis calcarea	8.94	16.14	74.93

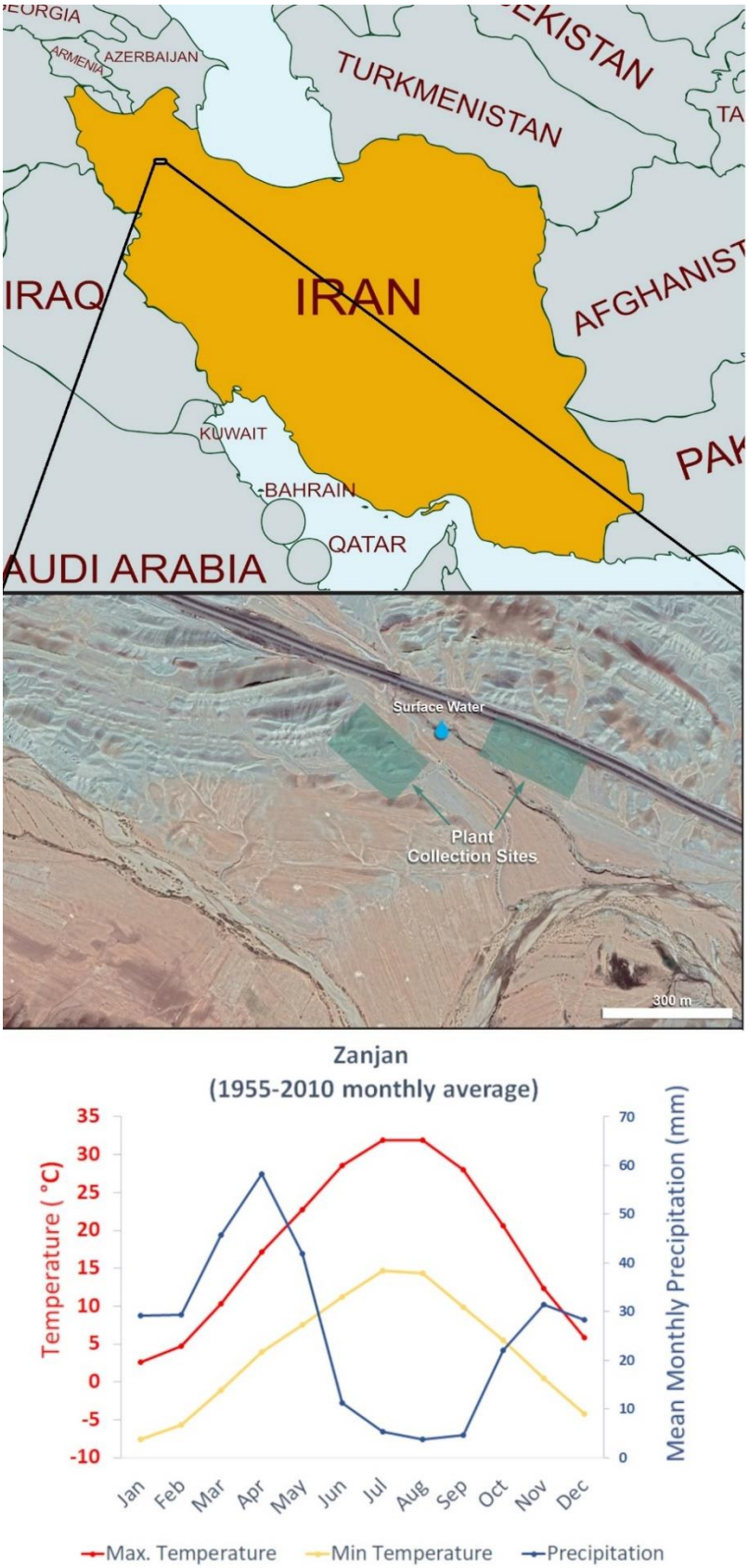

Figure 1. Location of the study area in NW Iran and Sampling sites. Blue droplets denote the location of water samples and the transparent green rectangles denote the areas were plant samples were collected. The lower left panel is the climatogram based on long term climate data collected at major meteorological stations in the region.

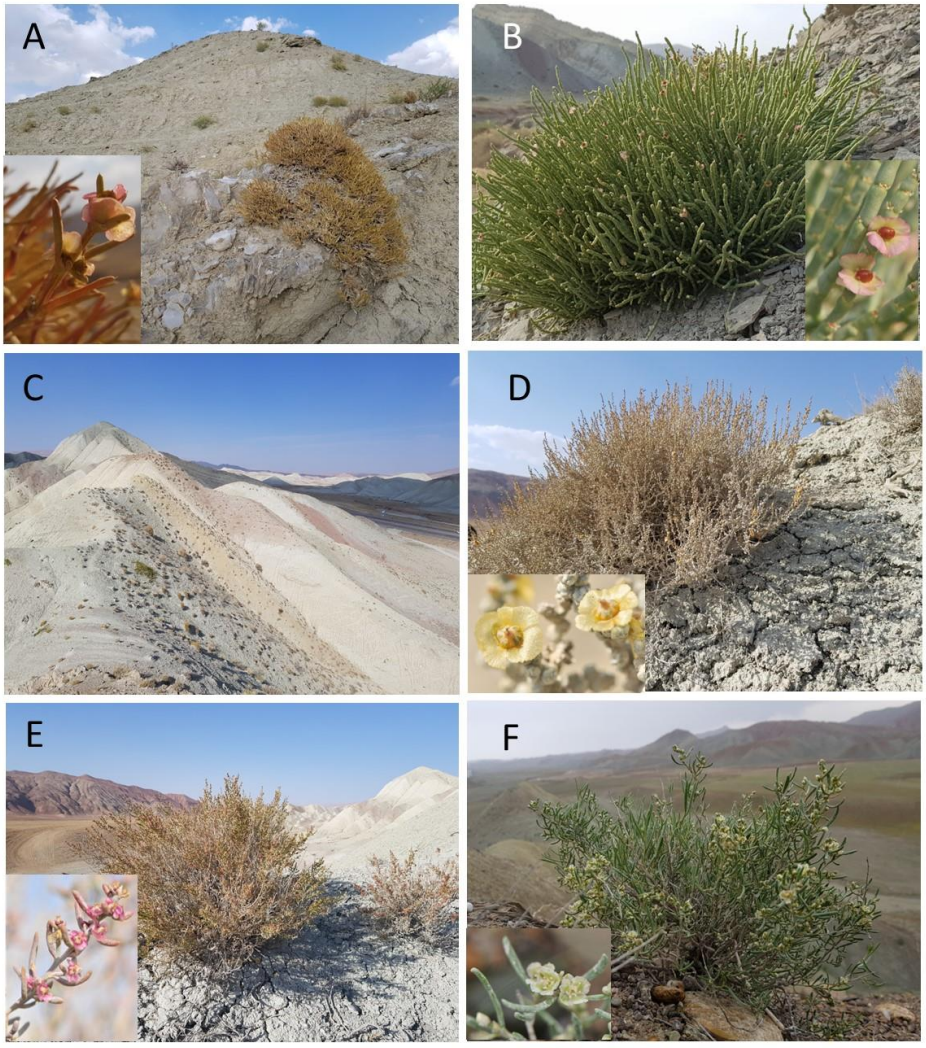

Figure 2. Habitat and plant species studied for ecohydrological niche segregation in a gypsym-calcareous habitat in NW Iran. A. Gypsum-calcareous hills with showing *Anabasis eugeniae* in foreground and A. calcarea in background, inset shows close-up of a fruiting branch of *A. eugeniae* on a gypsum outcrop; B. *Anabasis calcarea*; C. Marl hill with *Caroxylon gemmascens*, *Oreosalsola montana* and *Atraphaxis suaedifolia*; D. Habit and close up of a fruiting branch of Caroxylon gemmascens; E. Habit and close up of a fruiting branch of Oreosalsola montana; F. Habit and flowering branch of *Atraphaxis suaedifolia*.

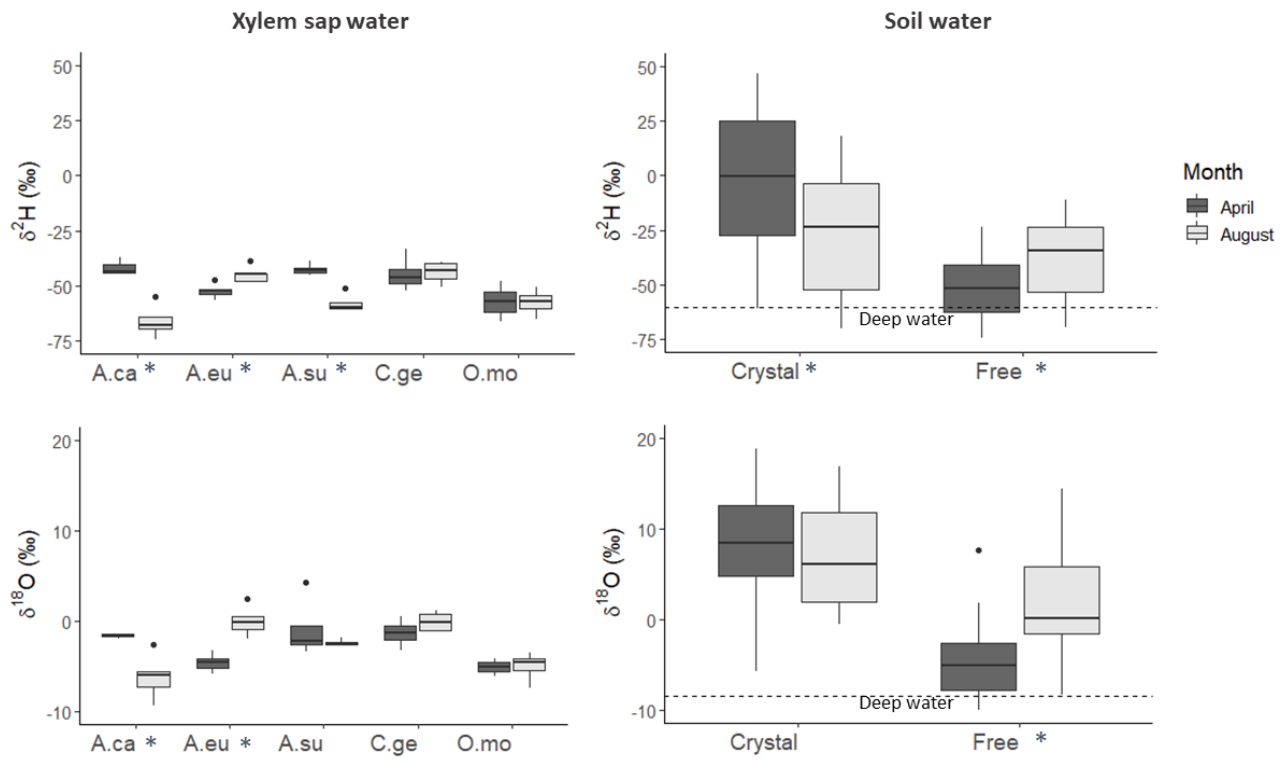

Figure 3. $\delta^{2}H$ and $\delta^{18}O$ values of the xylem sap of the plant species and soil in both months (April and August). Labels for plant species correspond to *Anabasis calcarea* (Aca), *Anabasis eugenie* (Aeu*), Atraphaxis suaedifolia* (Asu), *Caroxylon gemmascens* (Cage) *and Oreosalsola montana* (Omo). The asterisk (*) in the labels indicate significate changes of the stable isotopes in the xylem sap of the species and in the free and crystallization water among seasons indicated in the linear mixing model (nlme package)

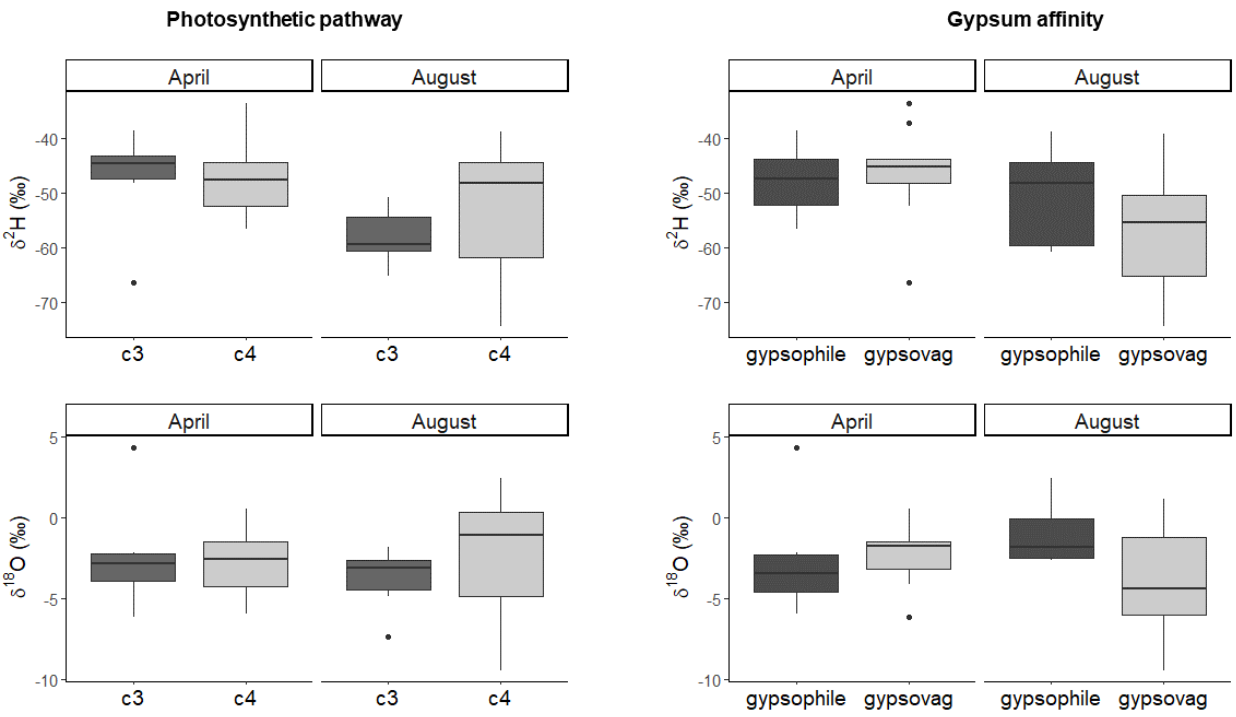

Figure 4. Xylem sap isotopic composition of the species grouped by their gypsum affinity and photosynthetic pathway for both seasons. (These grouping factors did not show any significant influence on the xylem sap isotopic composition of the subshrub species, see Table 2)

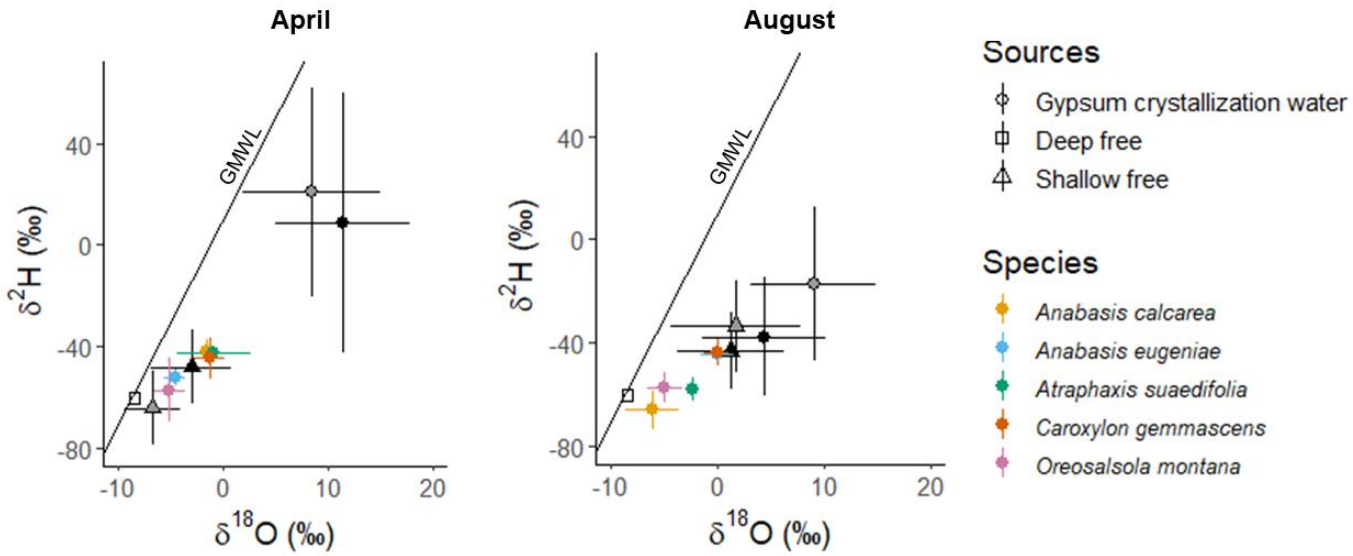

Figure 5. Plots showing the δ^2H (‰) and $\delta^{18}O$ (‰) composition of the different sources of water and water xylem sap of the five different species and the global meteoric water line (GMWL) in spring and summer. Values are means ± plus SD. The colour in the sources symbols are for the depth of the source: black= 10cm depth, grey=20 cm depth.

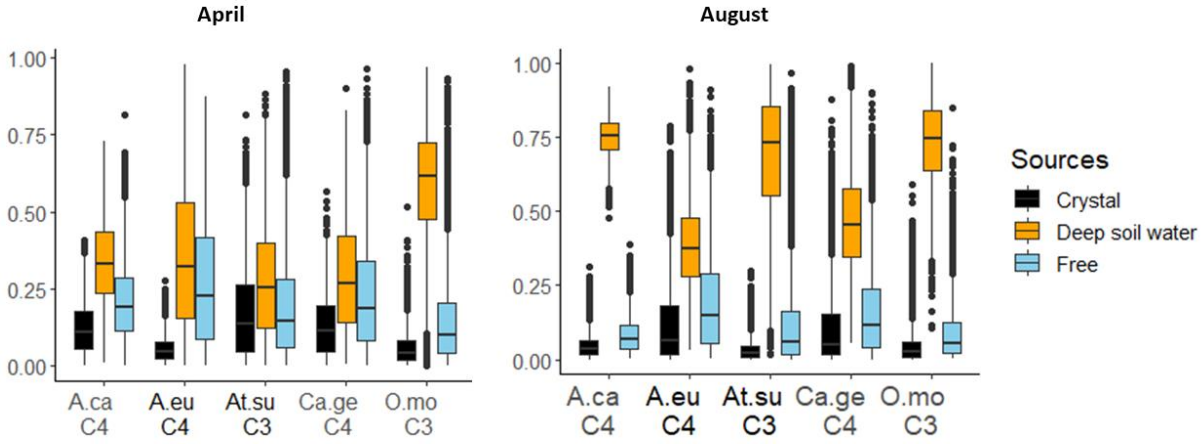

Figure 6. Results from Bayesian stable isotope mixing models showing the estimated contribution of different water sources to the xylem water of the five plant species from community studied in April and in August. The sources named "Crystal" and "Free" included gypsum crystallization water and free water, respectively, sampled at two different depths: 10 and 20 cm underneath the plants; "Deep soil water" represented deeper water.

Chapter 3

Helianthemum squamatum flowers.

Picture by I. Soriano, downloaded from Herbario Jaca (IPE-CSIC)

Margins designed by Virginia de la Iglesia and Laura de la Puente

Chapter 3

Integrated above and below-ground responses of the gypsum specialist *Helianthemum squamatum* (L.) Dum. Cours. to drought*

*To be submitted shortly

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ABSTRACT

Gypsum endemics (i.e. gypsophiles) have adapted to live in gypsum-rich soils where nutrient unbalance and drought can be extreme. Despite their relevance as plants adapted to extreme conditions, a complete analysis of the physiological responses of gypsophiles to drought is still lacking. *Helianthemum squamatum* (L.) Dum. Cours. is a conspicuous Iberian gypsophile that has been reported to use gypsum crystallization water during the driest period, but the mechanisms behind this process are unknown. To characterize gypsophile responses to drought and unravel the mechanisms underlying gypsum crystalline water use, *H. squamatum* plants were grown in pots with natural gypsum soil and gypsum soil with deuterium-labelled crystalline water. After three years, a drought experiment was carried out and whole-plant responses were investigated. Unexpectedly, the labelling treatment affected soil physicochemical characteristics and reduced microbial biomass and organic matter content, decreasing plant aerial biomass. *H. squamatum* plants did not use gypsum crystallization water during simulated drought neither in the labelled soil, nor in the natural one. Drought reduced plant transpiration, stomatal conductance, water use, photosynthetic rate and the foliar concentration of most nutrients except P and N, which were higher in the drought stressed plants. We detected increased root exudation of choline, an osmoprotector, by drought stressed plants. The drought treatment also affected the structure of microbial communities but did not reduce the relative abundance of functional microbial groups, highly adapted to the natural drought pulses. Our results highlight an integrated water-saving strategy of *H. squamatum* plants in the short-term, where responses at the leaf level are combined with belowground processes, like altered root exudation. Our findings also underline the remarkable resistance to drought of microbial communities present in gypsum soils.

INTRODUCTION

Gypsum soils are widespread in arid and semi-arid regions around the world (FAO, 1990; Verheye and Boyadgiev, 1997). Gypsum regions constitute unique and singular landscapes in Western Europe (Blanca, 1993; European Community 1992). These atypical soils host numerous edaphic endemics, whose mechanisms to cope with the harsh physical and chemical properties of the soil, and the remarkable droughtness of these ecosystems have intrigued scientists for decades (Meyer *et al*, 1992; Escudero *et al*, 1999). Plant growth in gypsum soils is particularly constrained by high Ca and S concentrations (Escudero *et al*, 2015), low porosity, low fertility and low water availability (Guerrero Campo *et al*, 1999; Moore *et al*, 2014). Amongst the mechanisms displayed by gypsum edaphic endemics to cope with these limitations are: the foliar accumulation of Ca, S and Mg (Palacio *et al*, 2022; Sánchez-Martín *et al*, 2021; Palacio *et al*, 2007), a great depth of water acquisition (Palacio *et al*. 2017; Sánchez-Martín *et al*, 2021) or the interaction with below-ground microbiota (Palacio *et al*, 2012; Cera *et al* 2021b). Water is recognised as the most limiting factor for plant life in these habitats due to the aridity in addition to the low water retention of gypsum soils (Herrero and Porta, 2000), which increases plant vulnerability to the increasingly drier conditions derived from anthropogenic effects (Mendoza-Fernandez *et al*, 2014; Collins *et al*, 2013). Previous studies evaluated the long-term responses to warming and rainfall reduction of gypsum plant species (León-Sánchez *et al*, 2019; León-Sanchez *et al*, 2017), showing their potential vulnerability to forecasted climate change. However, information on the whole-plant immediate response to experimental drought (crucial to understand their physiological adaptation to drought stress) and the effects of drought on the soil microbiota, is still lacking.

Helianthemum squamatum (L.) Pers is one of the most conspicuous and frequent perennial gypsum endemisms (i.e. gypsophile) of the Iberian Penisula and North Africa (Rivas Goday *et al*, 1957). Its flowering period extends until the end of July (Aragon *et al*, 2007) corresponding with summer drought, and showing an optimum physiological status when there is almost no water in the soil (Aragón *et al*, 2008). According to previous studies, *H. squamatum* shows no stomatal regulation of photosynthesis, with a decrease of assimilation rate but increased transpiration and stomatal conductance under drought (León-Sánchez *et al*, 2017; Querejeta *et al*, 2021). This profligate use of water could suggest a shift in the water sources used during drought by this species. Plants may shift to deeper sources of water, more available during drought, although this may have

consequences for plant nutrient status (Querejeta *et al*, 2021). Foraging deeper soil layers in arid conditions implies lower access to nutrients, reducing the average foliar N, P, Fe, K and Cu concentrations (León-Sánchez *et al*, 2020), and decreasing above-ground biomass of plants (Peñuelas *et al*. 2018; Wright & Westoby, 2002). Exploring *H. squamatum* physiological performance in humidity controlled pots along with above-ground responses could help identify its mechanisms to face drought and cope with the restrictive conditions of gypsum soils.

Another strategy of *H. squamatum* to survive drought is related to the uptake of gypsum crystallization water in the dry season (Palacio *et al*, 2014; de la Puente *et al*., 2021). Gypsum mineral ($CaSO_4\ 2H_2O$) contains two water molecules in its crystalline structure, accounting for up to 20.8% of its weight (Bock, 1961). This crystallization water is prone to be used by plants growing on gypsum when free water in the soil is scarce (Palacio *et al* 2014, 2017; de la Puente, 2021), supporting the water-expender activity of *H. squamatum* without a need to rely on deeper water sources. The source of water used by plants can be traced by comparing the stable isotope composition (δ^2H and $\delta^{18}O$) of water in the xylem sap of plants with that of the different soil water pools (Meisner *et al*. 2014; Penna *et al*, 2018). For gypsum plant communities, δ^2H and $\delta^{18}O$ under natural abundance have provided strong evidence for the use of crystallization water by plants, including *H. squamatum* (Palacio *et al* 2014; Palacio *et al*, 2017; de la Puente, 2019). However, to date no direct evidence has shown the use of this source of water by plants. The use of isotopically-labelled gypsum could provide unequivocal evidence of the uptake of this water pool by plants. Deuterium-labelled water can be traced not only in the xylem water of plants to identify the water sources used (Dawson *et al*, 2002), but also in the bulk organic plant material (e.g. leaves, roots) or in recent plant metabolites (Schleucher, 2020). However, this labelling approach has not been tested in gypsum-endemic plants. Despite the existing evidence of the use of crystallization gypsum water by plants, the biotic and abiotic factors needed to activate this process, and the mechanisms behind it, are still unknown. Only recently, some studies have reported the induction of a phase transformation from gypsum ($CaSO_4\ 2H_2O$) to anhydrite ($CaSO_4$) caused by the release of organic acids by microorganisms living in gypsum rocks in the Atacama Desert (Huang *et al*, 2020a; 2020b). Hence, the ability of plants to exudate organic compounds that could alter gypsum hydration or the occurrence of plant-microbial interactions leading to altered soil conditions may be crucial to cope with water stress in gypsum soils.

Many works have focused on the effect of drought as a shaping factor for root microbial communities (Hartman & Tringe, 2019). Previous studies indicate that plant-microbial interactions can lead to improved drought resistance for plants (Naylor *et al*, 2017; Fiztpatrick *et al*, 2018), although such effects remain unexplored in gypsum soils. *H. squamatum*, as many other dryland species, shows decreased diversity and relative abundance of its ectomycorrhizal communities with drought stress (León-Sanchez *et al*, 2018). However, no previous studies have evaluated the responses to drought of non-mycorrhizal soil fungal and bacterial communities linked to this model gypsum specialist species. In this context, plant root exudation is a vital component of plant drought responses (Karlowsky *et al*, 2018), as it constitutes a direct communication pathway with microbial life in the soil, contributing to plant performance under certain abiotic stresses (Bai *et al*, 2022; Pineda *et al*, 2013). The connection between rhizosphere chemical and microbial responses to drought stress has only been explored recently (Sasse al., 2018; Williams & de Vries, 2019). Root exudation has been shown to influence rhizosphere colonization, promoting arbuscular mycorrhizal fungi (Quiroga *et al*, 2017; Huang *et al*, 2017), or specific bacteria by the exudation of organic acids (Henry al., 2007; Kumar *et al*, 2016). The potential interplay between the microbiota and root exudates in response to drought in species living in drylands remains poorly explored.

The aim of this work was to perform an integrated analysis of the responses to experimental drought of the gypsophile *H. squamatum* cultivated on gypsum soil with labelled crystallization water and natural gypsum soils. We characterized processes in relation to water use, plant aerial status (physiology, biomass, water content and foliar nutrient composition) and the effects belowground (plant-soil microbial interactions and root exudation). We hypothesized that (1) the main water source used by this species during drought will be the crystallization water of gypsum. Moreover, if it was used any time in the plant life, we expected to detect the deuterium labelling not only in the xylem sap, but also in the transpired water or in the bulk organic matter of its tissues, such as leaves or roots. In addition, we hypothesized that, (2) stomatal conductance and transpiration would be maintained under drought stress, due to the reported non stomatal regulation of *H. squamtum* (León Sánchez *et al*, 2017) and the potential use of crystallization water, but photosynthetic rate may be affected. As a consequence, (3) plant aerial biomass will decrease with drought, as well as leaf elemental concentrations. Finally, we postulated that (4) drought will affect the soil microbiota and plant-soil interactions, leading to a reduction in the microbial biomass and

an increase in the stress of the microbial communities in the soil, and modifying the concentration of certain molecules exuded by plant roots.

MATERIAL AND METHODS

Experimental design

The study species was *Helianthemum squamatum* (L) Pers (Cistaceae), which grows only on gypsum soils and is considered a diagnostic species of Iberian gypsum vegetation (Braun-Blanquet & J. Bolòs, 1957). This species is a small (10-30 cm), evergreen, woody sub-shrub (Mota *et al*, 2011), mainly distributed in the eastern half of the Iberian Peninsula, with other localities in Northern Algeria (López- Gonzalez, 1993). Its root system is shallow with most of fine roots occurring in the first 25 cm of the soil (Guerrero-Campo *et al*. 2006). Seeds were collected from several individuals from natural populations near Villamayor, Zaragoza. They were sorted in the laboratory and stored in paper envelopes at room conditions until their germination in the pots. Seeds were scarified with sand paper and kept in distilled water for 24 hours before sowing. Pot surface was covered with coconut fiber to prevent rapid soil dehydration by evaporation.

In May 2019, we started a common garden experiment at the Instituto Pirenaico de Ecología- CSIC in Jaca, Huesca, Spain. Twenty pots of 15 x 15 x 20cm (3.6 L) were germinated with *Helianthemum squamatum* seeds to obtain one seedling per pot. Ten plants were grown on each of two different types of soil: natural gypsum soil and gypsum soil with deuterium-labelled crystallization water. Natural gypsum soil was collected from gypsum outcrops in the Middle Ebro Bassin (Villamayor del Gállego, Zaragoza, NE Spain, 41º41´44.5N 0º44´26.7W). The dominant substrate in this area is gypsum with a few inserted outcrops of marls and clays (Quirantes, 1978). The soil in the experiment is representative of natural gypsum soils where gypsum endemics frequently grow. Soil was kept in polypropylene raffia bags in the warehouse at room conditions until its use in the pots. Labelled gypsum soil consisted of an artificial soil resulting from heating natural gypsum soil at 100 ºC for three days to promote dehydration and loss of gypsum crystallization water and then, re-hydrating it with deuterated water obtained by adding 1 ml of deuterium oxide (99.9 % atom D, CAS 7789-20-0, Sigma-Aldrich, Germany) to 5 litres of water. The recrystallized gypsum soil was then broken into small pieces using a hammer and subsequently ground to pass a 2 mm sieve. The resulting gypsum labelled soil had lower organic matter content than the natural gypsum soil, was much sandier and had slightly different electrical conductivity (see Table 1). Its physicochemical properties considerably changed the growing

conditions of plants, which constitutes an important methodological limitation to consider when interpreting the results.

Plants were regularly watered with tap water to keep the soil moist, they were located in the open air until temperature dropped below 0 ºC, when they were moved inside a greenhouse to prevent root freezing. From the 28th Jun 2022, a progressive drought treatment designed to reduce water availability as a function of water use was applied. Plants were watered every second day and they were weighted each morning and just before watering to calculate the amount of water lost by evapotranspiration. Five plants from each type of soil (natural and labelled) were watered with the same amount of water they had lost (control), whereas the other five were watered with 25% of the water lost (drought treatment). To avoid the death of some plants caused by a sharp rise in temperatures, on 10th July we exceptionally watered all plants to field capacity, including the drought treatment replicates. Water content in the pots was also registered every fifteen minutes by EC-5 humidity sensors placed inside three pots per treatment and type of soil (12 sensors). The weight of pots was also used to monitor daily water use by plants.

Plant harvest was performed on the 22nd July 2022, 24 days after the beginning of drought treatments and when plants were three-year-old. From each pot, different portions of shoot, roots and soil were collected and stored for subsequent analyses (detailed below). Specifically, the different fractions collected were: bulk and rhizosphere soil, fine roots, including tips and aboveground plant biomass (main stems, branches and leaves).

Soil physicochemical characterization

Bulk soil samples were collected by considering pot soil that was not in direct contact with the fine roots. One aliquot of each sample was used for water isotope analyses and a second aliquot was immediately frozen at -20ºC and then freeze-dried for microbial analyses (see below). The remaining bulk soil was air dried during three months at room temperature and then sieved through a 2 mm sieve prior to physical and chemical analyses. The hydration of labelled gypsum was likely to differ from natural gypsum, leading to an underestimation of gypsum content when applying standard gravimetric procedures based on gypsum dehydration (e.g. Artieda *et al*., 2006). To overcome this limitation, we analysed the total sulphur (S) content in the soil (Casby-Norton *et al*, 2016) using an elemental analyser (LECO CNS928) and estimated the amount of gypsum content

in the soil by considering the molecular weight of gypsum and assuming that all S in the soil was in the form of gypsum. Soil texture was determined with a particle laser analyser (Mastersizer 2000 Hydro G, Malvern, UK). Soil pH and electrical conductivity were 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. Available Olsen-Phosphorus (P Olsen) was determined following standard methods (Anderson and Ingram, 1989). To analyse organic matter, a subsample of bulk soil was finely ground using a ball mill (Retsch MM200, Restch GmbH, Haan, Germany) and analysed following the Anderson and Ingram (1989) methodology. All these analyses took place at the Instituto Pirenaico de Ecología-CSIC.

Identification of water sources used by plants

We collected the main stems (including the upper coarse root portion) of study plants. The bark and phloem were scrapped off the stems with a knife to avoid the evaporative surface of the stem and contamination with organic compounds. Right after harvest, stems and a subsample of bulk soil were placed in individual airtight sealed tubes (Duran GL18) and immediately frozen (-20 ºC) until distillation.

Xylem and soil water was extracted by cryogenic vacuum distillation (Ehleringer and Dawson, 1992), adapted as described in Palacio *et al*. (2014) at the Instituto Pirenaico de Ecología (IPE-CSIC, Jaca, Spain). Sample tubes were placed in a heated silicone oil bath, and connected with Ultra-Torr unions (Swagelok Company, Solon, OH, USA) to a vacuum system (approx. 10–2 mbar) including U-shaped water traps in series that were cooled with liquid nitrogen. Four lines were installed. After an extraction time of 90 min for plant and soil samples (West *et al*, 2006; Meisner *et al*, 2014), captured water was transferred into screw capped 2 mL vials, and stored at 4 °C until isotope analysis. Xylem water was distilled at 130 °C for 90 min, whereas gypsum soils were distilled in two steps: first at 35 °C, and then at 130 °C to separate free and crystallization gypsum water and ensure complete dehydration of gypsum for 120 min each (Freyer and Voigt, 2003; Palacio *et al*., 2014). Between the first and second distillation, sample tubes were kept in a desiccator with silica gel to avoid any re-hydration with ambient moisture, which could contaminate the next extraction water. Distilled samples were completely dried in the oven for 24

h at 60 °C. The samples were weighed before and after each distillation and after oven-drying to measure water content and confirm complete distillation.

Oxygen and hydrogen isotope composition ($\delta^{18}O$ and $\delta^{2}H$) were determined by cavity ring-down spectroscopy (CRDS) at the scientific services of the Instituto Pirenaico de Ecología (CSIC), using a Picarro L2130-i with vaporizer A0211. The estimated precision was 0.10 % for $\delta\ ^{18}O$ and 0.40 % for $\delta\ ^{2}H$. Deuterium excess was calculated according to Dansgaard (1964), as the divergence from the Global Meteoric Water Line as:

$$Dex = \delta\ ^{2}H - 8 \times \delta\ ^{18}O$$

We did not consider the eventual role of organic contaminants because in labelling experiments their effect is negligible. For the $\delta^{2}H$ and ^{18}O analyses of transpired water, we used a cryogenic vapour trapping system (see Supplementary Figure 1 B, C), as described elsewhere (Ferrio *et al*. 2009). Three plants per treatment and type of soil were wrapped with a plastic bag and connected to suction pumps (i.e. reversed aquarium pumps), in line with a glass U-tube immersed in a mixture of ethanol and dry ice (at ca. -70 ºC). For each plant, air was pumped at *ca.* 1 L min^{-1} for about 2 h. After thawing, the collected water was immediately transferred into sealed 2 mL vials, and stored at 4 °C until isotope analyses.

Differences in $\delta^{2}H$ in bulk leaf tissue among treatments and type of soils were analysed at the Serveis Cientifics i Tècnics of the Universitat de Barcelona trough pyrolysis TC/EA (Thermo Fisher Scientific) coupled with a mass spectrometer IRMS with isotopic ratios (DELTA PLUS XP).

Presence of deuterium in root metabolites was analysed at Estación Experimental Aula Dei-CSIC (EEAD-CSIC), through Liquid Chromatography- High Resolution Mass Spectometer (LC-HRMS), and based on untargeted metabolomics following the protocol indicated in Pezzatti *et al*. (2020).

Plant aerial status: physiological parameters, water content, biomass and foliar nutrient concentrations

Gas exchange and photosynthesis were measured using a portable gas photosynthesis system CIRAS-3 (PP-Systems, Amesbury, MA, USA), fitted with an automatic universal leaf cuvette (PLC3-U, PP-Systems) and a chlorophyll fluorescence module (CFM, PP-Systems). All measurements were conducted under standard environmental conditions: cuvette CO_2 concentration of 400 μmol mol^{-1}, cuvette temperature of 25 °C, reference humidity set to 100% of ambient conditions, and saturating PPFD of 1200 μmol m^{-2} s^{-1}. The following gas exchange variables were determined: net CO_2 assimilation rate (A_n - μmol CO_2 m^{-2} s^{-1}), transpiration rate (E - mmol H2O m^{-2} s^{-1}) and stomatal conductance (g_s - mol H_2O m^{-2} s^{-1}). To estimate foliar area included in the cuvette, we took a picture of the leaves on a measurement scale, and subsequently calculated leaf area with Image J 1.5r (National Institutes of Health, USA) (see Supplementary Figure 1) approximating it to that of an ellipse (see Supplementary Figure 1 D). These measurements were used to calculate gas-exchange parameters on a per leaf area basis and to estimate mean leaf area from each individual (see below). As the first day of measures was an exceptionally cloudy and cold day, leading to overall limitations of photosynthesis, we kept for analysis only the measures from the second round, on the 21st of July, i.e. just before plant harvest.

On the harvest day (22th July), ten leaves were separated from each plant and rinsed with tap water to remove soil or dust, then weighed in a precision scale (42 g / 0.00001 g, MS105DU, Mettler Toledo, Columbus-OH, USA) and dried at 50 ºC. Once dried, they were weighed again to calculate leaf water content. The rest of plant leaves and shoot biomass were separated, dried on the stove at 50 ºC and weighed. Dried leaves were subsequently finely ground using a ball mill (Retsch MM200, Restch GmbH, Haan, Germany) to analyse nutrient content. N and C concentrations were analysed with an elemental analyzer (TruSpec CN, LECO, St. Joseph-MI, USA) in the Insituto Pirenaico de Ecología-CSIC. The elemental composition of Al, As, Ca, Cd, Co, Cr, Cu, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Ti, V, Zn was measured by extracting samples with HNO_3-H_2O_2 (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). ICP elemental analyses were performed by EEZ-CSIC Analytical Services. To approximate mean leaf area from each individual we used the same data as for IRGA measures (see above).

Belowground effects: soil microbial community analyses

The characterization of the main functional groups within soil microbial communities was made using Phospholipid Fatty Acid (PLFA) analyses of soil microbia carried out at IRNASA-CSIC Salamanca, Spain. Aliquots of 2 g of lyophilized bulk soil were used for lipid extraction. Lipids were extracted with a one-phase chloroform–methanol-phosphate buffer solvent. Phospholipids were separated from non-polar lipids and converted to fatty acid methyl esters before analysis, following the methodology described by Buyer and Sasser (2012). The resulting fatty acid methyl esters (FAMEs) were separated by gas chromatography using an Agilent 7890A GC System (Agilent Technologies, Wilmington, DE, USA) equipped with a 25-m Ultra 2 (5%-phenyl)-methylpolysiloxane column (J&W Scientifc, Folsom, CA, USA) and a flame ionization detector. The identification and quantification of FAMES was carried out using the PLFAD1 method of Sherlock software version 6.3 from MIDI, Inc (Newark, DE, USA). The internal standard 19:0 phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) was used for the quantification of the FAMEs. Total microbial biomass was estimated by summing the contents of all individual PLFAs and reported as nanomoles of PLFAs per gram of soil.

Specific PLFAs were used as biomarkers to quantify the biomass of broad taxonomic microbial groups, according to their characteristic fatty acids: eukaryote, Gram negative and Gram positive bacteria (hereafter G- and G + , respectively), saprophytic fungi and arbuscular mycorrhizal fungi (AMF) (Frostegard and Baath, 1996). Saturated to unsaturated fatty acids (hereafter Sat/Unsat) and cyclopropyl fatty acids to their monoenoic precursors (cy17:0 + cy19:0) / (16:1ω7 + 18:1ω7; hereafter G- cy/pre) ratios were calculated as proxies of physiological or nutritional stress in the bacterial communities (Frostegård *et al*. 2011; Willers *et al*. 2015).

Belowground responses: root exudates collection and analysis

Root exudates were collected following the methodology in Teodoro *et al* (2019) with small modifications for the fine rooted species included in this study. A small portion of fine root tips was rapidly collected, weighed up to about 60 mg, and shaken in 0.5 mL of 0.01% miliQ water-dissolved formic acid to avoid microbial consumption of the organic compounds (Abrahao *et al*, 2014; Teodoro *et al*, 2019). After ten minutes, the root tips were removed, the samples were filtered with a 0.22 µm cellulose acetate filter and frozen at -80ºC until analysis.

To identify the released carboxylates, we used an ultra-high-performance liquid chromatography (UHPLC) system coupled to a quadrupole-time-of-flight (maXis Impact HR Q-TOF-MS, Bruker Daltonik GmbH, Bremen, Germany) orthogonal accelerated Q-TOF mass spectrometer, equipped with an electrospray ionization source (ESI). UHPLC analyses were made at the Metabolomics Platform of CEBAS-CSIC (Centro de Edafología y Biología Aplicada del Segura). Organic acids are the main compounds known to be released by roots and to have direct or indirect effects on the acquisition of mineral nutrients required for plant growth (Dakora & Phillips, 2013). Consequently, we focused on the following organic acids: citric, isocitric, malic, oxalic, succinic, lactic, maleic, tartaric, malonic and fumaric acid. In addition, sugar alcohols or polyols, such as myo inositol, galactinol, xylitol, sorbitol-mannitol and the osmolyte choline were detected just by using spectral features (exact mass, isotopic distribution, elution order and fragmentation profile) with external and internal databases (using the generated molecular formula) and information provided in the literature about plant metabolome (Garcia *et al*., 2016). Analytical results of organic acids are show as µg/mL (ppm) but the rest of compounds (sugar alcohols and choline) are shown as a ratio of areas under the peaks (AUP), calculated with respect to the highest value detected, for comparative purposes only, without quantifying its concentration, and assuming that matrix composition variation is low (Warwick *et al*., 2005).

Calculations and statistical analyses

All statistical and graphical analyses were carried out using R version 4.0.0 (R Core Team, 2020). Graphs were designed with ggplot2 package 3.3.1 (Wickham, 2016). The effects of treatments on the deuterium excess of xylem and soil water were assessed by linear models using lm function (Chambers, 1992) in the *stats* package, which is part of R (R Core Team, 2020). The model included "drought treatment" (drought /control) and soil "labelling treatment" (labelled/natural) as fixed factors, and the interaction between them. The relative contribution of potential water sources to xylem sap was estimated using Bayesian Mixing Models for stable isotopic data with *MixSIAR* (Stock *et al*, 2018). This procedure estimates the proportion of source contributions to a mixture, using as "consumers" the isotopic values (δ^2H and $\delta^{18}O$) of xylem water in each individual. These results were compared with the visualization of both isotopes composition of free and crystallization water, xylem sap and the Global Meteoric Water Line to avoid misleading interpretations of Mixing Models.

Differences among treatments ("drought treatment", "labelling treatment" and its interaction) in the stomatal conductance, transpiration rate, water use and assimilation rate, as well as in the concentration of each exudate detected and the total aboveground biomass of the plant, were also evaluated with the lm and ANOVA functions from *stats* package. Residuals were visually checked using the *DHARMa* package (Hartig, 2020).

We visualized relationships among elemental concentrations and treatments using a Redundancy Analysis with RDA function of the *vegan* package version 2.5-7 in R (Oksanen *et al*. 2020). Then, we plotted the Principal Component Analysis (PCA) using the first and second Principal Components as the X and Y axis. We used elements with concentrations above the detection limit of the ICP-OES spectrometer and N and C concentration data. All elemental data were transformed to Center Log-Ratio coordinates (Aitchison., 1982) using compositions (van den Boogaart *et al*. 2022) to maintain relationships between elements regardless of the concentration, which allows studying joint patterns among elements (Prater *et al*. 2019). Differences in nutrient composition between treatments were assessed using Permutational Multivariate Analysis of Variance (PERMANOVA) based on distance (Adonis function on *vegan* package version 2.5-7 in R) with "drought treatment" (drought / control) and "labelling treatment" as fixed factors and using the Euclidean as distance from Center Log-ratio coordinates. For univariate approaches, we analysed the effect of treatments and their interaction (independent variables) with individual ANOVAs run on the 18 elements analysed separately. As above, residuals were visually checked using the *DHARMa* package (Hartig. 2020).

Relative abundance percentages of individual fatty acids were arcsine-transformed for normality before statistical analyses. A PCA was performed to represent and analyse the differences in soil microbial community composition (PLFAs) between experimental treatments. This PCA was conducted with CANOCO v 5.15 (Braak, & Smilauer., 2002). To test for the differences in PLFA composition of soil microbia between treatments and the interaction between them, PERMANOVA was used. Data on relative abundances of microbial groups (eukaryote, Gram negative bacteria, Gram positive bacteria, actynobacteria, saprophytic fungi and mycorrhizal fungi) and stress indexes were analysed, in the same way than the leaf elements, by ANOVA with treatments and their interaction as independent variables. Holm–Sidak's multiple comparisons test was used for further comparison when a significant difference was found in ANOVA.

RESULTS

Water sources used by plants

The isotopic composition of gypsum crystallization water of pot soil varied markedly with the labelling treatment (Table 2). Crystallization water of labelled gypsum soil was highly ^{2}H-enriched reflecting the stability of the labelling treatment (Supplementary Table 1, Supplementary Figure 2 and 3). Compared with free water, crystallization water from natural soils was always more $\delta^{2}H$ and $\delta^{18}O$ enriched (Figure 1, Supplementary Table1, Supplementary Figure 2 and 3). Free water $\delta^{2}H$, $\delta^{18}O$ and Deuterium-excess varied between control and drought treatments but, as expected, not with labelling treatments. However, there was a significant interaction between labelling and drought treatments on the $\delta^{18}O$ composition and Deuterium excess of free water (Table 2), showing more evaporated values under drought conditions for the labelled soil than in the natural soil (Figure 1, Supplementary Table 1, Supplementary Figure 2 and 3). On the other hand, we found significant effects of both treatments and their interaction on the isotopic composition and Deuterium-excess of xylem sap water: D-excess of the control treatment plants was always higher than those of the drought treatment, but this difference was much stronger in the labelled soil than in the natural one. However, the isotopic composition of transpiration water did not change significantly with any of the treatments or their interaction (Table 2, Supplementary Figure 2), indicating no use of labelled gypsum crystallization water during drought. Similarly, we did not detect the use of labelled crystallization water during plant life: $\delta^{2}H$ values of bulk leaf biomass were not significantly different between the labelled treatment: -91.0 ±2.7 (mean± SD); and the natural treatment; -96.5±5.0 (mean± SD).

The estimation of the most likely sources of water used by plants by Bayesian Models indicated that plants grown in the labelled soil did not use crystallization water, but free water was the main source used, showing a high contribution (97 % and 98% for the labelled drought and labelled control plants, respectively). Conversely, plants grown in natural soil showed a certain contribution of gypsum crystallization water in their xylem sap water according to Bayesian models. The model estimated that gypsum crystallization water was the main water source (61.7%) under drought, but a secondary water source (27.8%) in control pots (Supplementary Figure 3). However, contrasting with the model outcomes, the $\delta^{2}H$-$\delta^{18}O$ biplot of xylem water and the different sources (Figure 1) showed that xylem sap water of both labelled and natural pots fell on the same evaporation line of

soil free water. Plants in the drought treatment had more evaporated values (i.e. higher δ^2H and $\delta^{18}O$) than in the control, with the most evaporated values found in the labelled-drought treatment (i.e. L8, L9, L6). These results indicate no use of crystallization water by plants in this experiment.

Drought effects on plants

Drought caused a significant reduction of stomatal conductance, transpiration and assimilation rates and also of whole-plant water use in *H. squamatum* (Figure 2). The interaction between drought and labelling treatments had also a slight effect on the amount of water used by plants, being lower under drought for the labelled pots than for the pots with natural soil (Figure 2, Supplementary Table 2).

Plants cultivated in the labelled soil had significantly lower aerial biomass but drought treatment did not modify this trait. Leaf and root water content did not change with any of the treatments or their interaction (Supplementary Table 3). On the other hand, both treatments had an independent effect on the elemental composition of plant leaves (Table 3, Figure 3). Plants subjected to drought showed a different foliar elemental composition tan control plants (*p-value* = 0.001; *F-ratio* = 2.64), with significantly higher concentrations of N, P, Zn and C, and lower concentrations of Mg, Mn, Ca and Cu, S, Sr, Li and Ti (see Figure 3). Moreover, plants subjected to the labelling treatment showed significantly higher concentrations of Na, Ca, Al, S, Sr, Fe, Ti and Li, but lower C, K, Mn in their leaves (Table 3, Figure 3).

Drought effects on plant-soil interactions: microbial communities and plant root exudation

PERMANOVA showed significant effects of both treatments on the phospholipid fatty acids (PLFA) composition in the soil, indicating a considerable modification of the structure of microbial populations with drought and labelling (Table 4, Figure 4). Univariate analyses showed that the labelling treatment reduced drastically the total microbial biomass, as well as the relative abundance of Eukaryota, AM Fungi, Saprophitic Fungi and the Fungi/ Bacteria ratio. Conversely, effects of drought on these parameters were not significant. The labelling treatment showed significantly higher indices of microbial stress (indicated by the indexes “G- cy/pre” and “Sat/Unsat”; Table 4, Figure 4).

We did not find differences in the concentration of organic acids and alcohols exuded by plants between the labelling and natural treatment. However, in the drought treatment we detected a remarkably higher exudation of the amine choline by the roots of plants subjected to drought as compared to control plants (Figure 5, Supplementary Table 4).

DISCUSSION

Labelling gypsum crystallization water strongly modified soil characteristics

The labelling treatment applied in this experiment in order to trace gypsum water crystallization along the soil-plant system resulted in a gypsum soil with different physicochemical properties, respect to the original natural soil (Table1). This fact forced us to consider the labelling of the soil as an additional treatment that caused sharp responses by plants. The effect of the soil labelling was reflected in most of the parameters analysed in this work; from the temperature of gypsum dehydration when extracting it to calculate gypsum content in the soil (Material and Methods: Soil physicochemical characterization), to changes in the microbial biomass (Table 5, Figure 5), and including plant photosynthetic rate, plant aerial biomass or leaf elemental concentrations. Labelled soil had a sandier texture and lower organic matter content, and consequently, dried faster, and presented a significantly reduced microbial biomass and the microbiota colonizing it was subjected to higher stress (Tejada *et al*, 2006; Ros *et al*, 2003). Therefore, plants growing on the labelled soil showed the effects of increased water stress and lower soil fertility, rendering less productivity (i.e. lower aerial biomass). The leaf elemental composition of plants also changed as a result of soil labelling; plants growing on the labelled soil showed higher contents of certain metals (e.g. Na, Al, Fe and Li), but lower K compared to the natural soil. The lower K values in the plants growing on labelled soil could be the result of lower uptake, as the lack of microbial activity in this poorer soil might have limited K availability for plants (Meena *et al*, 2014).

Free water in the soil was the only water source used by plants, even during drought

Contrary to our hypothesis, our pot-grown *H. squamatum* plants did not use gypsum crystallization water, even when subjected to drought. These experimental results do not agree with previous field studies that evidenced the presence of gypsum crystallization water in the xylem sap of this species and other shallow rooted species at the driest time of the year (Palacio *et al*, 2014; de la Puente *et al*, 2021). Unlike our study, these works showed isotopic values of the *H. squamatum* xylem water much better aligned with crystallization water than with free water evaporation line during summer.

According to our results, the isotopic composition of the xylem sap of plants growing in the labelled soil differed from that of plants growing on natural soil. However, the differences could not be explained by the different water sources used by plants, but by the faster evaporation of free

soil water in the labelled soil exposed to drought (owing to the different physicochemical features of the labelled soil), so plants growing in this soil had more evaporated water in the xylem. The study of the water sources in this experiment let us make a call for caution in the interpretation of Bayesian Mixing Models, which should always be supported by the biplot showing the δ^2H-$\delta^{18}O$ data of the potential water sources and the xylem of plants (Figure1). Bayesian Mixing Models estimated a big proportion of gypsum crystalline water use in the natural drought treatment, however, values were comparatively much lower in the labelled drought treatment (Supplementary Figure 4). Without a proper interpretation of the biplots, this fact could have been interpreted as evidence that gypsum water in the labelling treatment was more difficult to uptake by plants (as it was more difficult to extract by heating), and let us confirm the use of gypsum crystallization water during drought in natural soils. However, when looking at Figure 1, we observed that all xylem water isotopic values (including those of the natural drought treatment) were aligned with the free water evaporation line, but not with the gypsum crystallization water line, ruling out the possibility of crystallization water use by the plants included in our experiment.

The lack of crystallization water use could be due to the artificial environment where the plants grew (i.e. watering and pot conditions), compared to the natural environment in the field, where previous studies were performed. There are several environmental conditions that were modified in our experimental approach and could have affected the development and performance of plants, and hence their water up-take mechanisms. Although care was taken to mimic natural conditions as much as possible, our experimental set up might have potentially altered water and nutrient availability, root foraging ability, soil temperature and microbial communities composition and performance (Lynch *et al*, 2012). More research is needed to identify experimental approaches that increase reproducibility of natural conditions and enable tracing the path of gypsum crystallization water in the plant-soil system.

Drought treatment decreased transpiration, stomatal conductance, photosynthesis and leaf elemental composition

This experiment showed, for the first time, short-time responses of *H. squamatum* to drought. Contrary to our hypothesis about the water spender strategy of the species based on previous long-term studies (Querejeta *et al* 2021, León-Sánchez, 2018), we observed a coordinated water-saving response in the short-term. This different behaviour could be linked to the inability to acquire

gypsum crystallization water that would otherwise have allowed the plant to maintain transpiration and stomatal conductance.

The closure of stomata and associated decrease in transpiration rate recorded, might have decreased transpiration-driven mass flow of soil nutrients to roots (Matimati *et al*, 2014). Accordingly, we observed a reduction of some leaf macronutrients (S, Ca, Mg) and micronutrients (Mn and Cu) with drought. These results agree with other manipulative studies on this species (Prieto & Querejeta, 2019). However, contrary to previous results of lower N leaf content with drought (León-Sánchez *et al*, 2017, Querejeta *et al*, 2021), we could see an increase of this macronutrient in the leaves of plants from the drought treatment. This observation could be the result of N recycling from leaves shed at the onset of a partial defoliation of stressed plants. The higher concentration of Ca and Mg in control plants leaves could be due to the higher transpiration rate of these plants. These elements are not easily remobilized from shed leaves, but their uptake from soil largely depends on the transpiration stream (White & Broadley, 2003)

Drought did not decrease soil microbial biomass but changed root exudation

The process of labelling involved heating and dehydration of the soil and, thus, extreme conditions for microbial life (Meisner *et al*, 2015; Fierer *et al*, 2003) that negatively affected a significant portion of the native microbiota. Moreover, the labelling process also altered the physico-chemical properties of the soil, mostly by decreasing the organic matter content and modifying its texture, being both soil characteristics reported as strong drivers of microbial soil communities (Chau *et al*, 2011; Grandy *et al*, 2009). Consequently, labelled gypsum soils showed increased relative abundance of Actinobacteria, a phylum renowned for its resistance to extreme conditions (Naylor & Coleman, 2018).

Contrastingly, drought did not alter the abundance of the different functional microbial groups or the stress indexes calculated from PLFA profiles. Native microbial communities of the gypsum natural soil might be highly adapted to this abiotic stress. This result agrees with previous findings reporting a high resistance of soil microorganisms like fungi to drought (Yuste *et al*, 2011; Barnard *et al*, 2013). However, our results contrast with previous studies on bacteria, which showed an increased sensitivity to drought (Griffin, 1985) and tended to become enriched in the phylum Actinobacteria (Naylor & Coleman, 2018), as observed for labelled gypsum soil in this study.

We detected a significant increase in the exudation of choline at the rhizosphere of drought stressed plants. This compound has an osmoprotective role which mitigates drought stress by the stabilization of biological structures (Sakamoto & Murata, 2001). This molecule is synthesized by plants (Zia *et al*. 2020), induced by certain bacteria in the soil (Zhang *et al*, 2011) or by rhizospheric microbes (Aslam *et al*, 2022; Mathesius. 2019), but our data do not enable clarifying the origin of this molecule in this study.

CONCLUSIONS

We performed an integrated study of the short-term responses of the gypsum specialist *Helianthemum squamatum* to experimental drought, identifying a water-saving strategy that contrasts with former findings. Contrary to previous field-work evidences, we could not demonstrate the use of gypsum crystallization water under experimental conditions by this species, probably due to the marked differences between environmental conditions in our experimental set up and those in the wild. We highlight the need to combine Bayesian Mixing Models with the observation of the xylem isotopic data in the δ^2H-$\delta^{18}O$ plot to infer potential water sources used by plants. In this study, the constraint of *H. squamatum* plants to use only the scarce free water content in the pot soil, likely forced them to reduce stomatal conductance and transpiration rates, showing a previously undescribed water-saving strategy in the short-term. Linked to these physiological responses, foliar nutrient concentrations decreased in drought-stressed plants, except for P and N, which could have been reabsorbed from senescing leaves of stressed plants. In addition, *H. squamatum* roots increased choline exudation, an important osmo-protective molecule, to face drought. Such exudation could, at least partly, be supported by the gypsum soil microbiota, which showed a strong resistance to arid conditions. Altogether, these results point at an integrated, conservative strategy of this gypsum specialist plant, at least in the short-term. Our study revealed interesting reactions of a gypsum specialist species and its microbiome under severe drought, prone to serve as a model species for future forecasted drought intensification.

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TABLES AND FIGURES

Table 1. Mean and standard deviation of principal soil physicochemical properties grouped by treatments. EC, electric conductivity; P, available P Olsen; OM, organic matter content**.**

	pH	EC	%gypsum	SAND	SILT	CLAY	P	OM
Labelled-Control	7.6 ±0.2	4.5±2.3	60.1±1.9	74.6±2.1	17.8±1.4	7.6±0.8	0.1±0.1	1.6±0.2
Labelled-Drought	7.4±0.4	4.4±1.1	60.8±0.8	75.2±2.1	6.4±1.6	6.4±0.7	0.0±0.0	1.5±0.1
Natural-Control	7.5±0.3	3.4±1.1	54.7±0.6	40.0±3.6	15.4±2.4	15.4±1.7	0.0±0.0	2.3±0.1
Natural-Drought	7.7±0.1	3.4±0.9	54.0±1.8	43.2±2.4	12.3±1.8	12.3±0.7	0.0±0.0	2.2±0.2

Table 2. Results from linear models on the effects of treatments in the isotopic composition of xylem sap of the plants, transpiration water and water sources in the soil (free and crystallization). *F-ratio*s and *p-value*s are provided. Significant effects (at α=0.05) are highlighted in bold.

XYLEM SAP WATER						
	δ²H		***δ¹⁸O***		***D-excess***	
	F	*p*	*F*	*p*	*F*	*p*
Drought	38.33	**<0.001**	49.30	**<0.001**	47.61	**<0.001**
Labelling	4.87	**0.045**	10.67	**0.006**	12.82	**0.003**
Drought:Labelling	18.34	**<0.001**	21.58	**<0.001**	19.92	**<0.001**
SOIL FREE WATER						
	δ²H		***δ¹⁸O***		***D-excess***	
	F	*p*	*F*	*p*	*F*	*p*
Drought	4.08	**0.062**	25.59	**<0.001**	37.45	**<0.001**
Labelling	2.80	0.115	1.46	0.246	0.17	0.687
Drought:Labelling	2.94	0.107	4.94	**0.042**	3.84	**0.069**
SOIL CRYSTALLIZATION WATER						
	δ²H		***δ¹⁸O***		***D-excess***	
	F	*p*	*F*	*p*	*F*	*p*
Drought	1.89	0.190	4.24	**0.057**	8.79	**0.010**
Labelling	5240.35	**<0.001**	94.51	**<0.001**	8747.21	**<0.001**
Drought:Labelling	1.38	0.258	7.64	**0.014**	0.11	0.743
TRANSPIRATION WATER						
	δ²H		***δ¹⁸O***		***D-excess***	
	F	*p*	*F*	*p*	*F*	*p*
Drought	0.49	0.49	2.39	0.145	4.48	0.053
Labelling	0.04	0.841	0.12	0.739	0.06	0.809
Drought:Labelling	1.98	0.18	0.51	0.486	0.01	0.941

Table 3 Multivariate test (PERMANOVA) testing the effect of drought, soil labelling and their interaction on the elemental composition of plant leaves. *F-ratio*s and *P-value*s are shown. Bold type indicates significant effects at $\alpha = 0.05$. Results of univariate linear models (ANOVA) testing the effect of the treatments and their interaction on the foliar concentration of each element analyzed separately are also shown.

PERMANOVA		
	F-ratio	*p-value*
Drought	2.64	**0.001**
Labelling	2.20	**0.004**
Drought*Labelling	0.74	0.811

ANOVA						
	Labelling		Drought		Drought*Labelling	
	F-ratio	*p-value*	*F-ratio*	*p-value*	*F-ratio*	*p-value*
Al	12.78	**0.003**	2.87	0.111	1.04	0.325
Ca	9.97	**0.006**	28.97	**<0.001**	2.64	0.125
Cu	1.94	0.184	5.46	**0.034**	1.45	0.246
Fe	5.99	**0.027**	0.64	0.438	1.93	0.185
K	9.15	**0.008**	1.11	0.308	0.46	0.508
Mg	0.64	0.433	7.38	**0.015**	0.16	0.696
Mn	19.36	**<0.001**	19.81	**<0.001**	1.13	0.304
Na	4.96	**0.041**	0.02	0.792	0.366	0.554
P	0.061	0.807	13.84	**0.002**	0.12	0.738
S	6.77	**0.020**	12.29	**0.003**	0.74	0.405
Zn	1.24	0.283	0.75	0.399	0.03	0.855
Sr	24.31	**<0.001**	1.09	**<0.001**	1.11	0.309
N	0.84	0.375	6.01	**0.028**	0.02	0.891
C	11.84	**0.004**	36.41	**<0.001**	6.10	**0.027**
Li	20.4	**<0.001**	12.13	**0.003**	1.10	0.310
Cr	2.31	0.149	0.13	0.716	1.64	0.220

Ti	6.28	**0.024**	5.17	**0.038**	0.98	0.336

Table 4. Multivariate test (PERMANOVA) testing the effect of the treatments on the molar percentage of all phospholipid fatty acids analysed in the pot soil and ANOVA results comparing the relative abundance of different microbial groups and of two stress indexes among treatments and their interaction. *F-ratio*s and *p-value*s are shown. Significant effects (at α=0.05) are highlighted in bold.

PERMANOVA		
	F-ratio	*p-value*
Drought	4.00	**0.014**
Labelling	8.64	**<0.001**
Drought*Labelling	1.83	0.134

ANOVA						
	Labelling		**Drought**		**Labelling* Drought**	
	F ratio	***p-value***	***F ratio***	***p-value***	***F ratio***	***p-value***
Microbial biomass	11.79	**0.004**	0.01	0.958	3.04	0.102
%Eukariota	14.80	**<0.001**	1.83	0.196	0.12	0.728
%Gram Negative	0.93	0.349	8.90	**0.009**	1.56	0.231
%Gram Positive	1.59	0.226	0.18	0.677	0.07	0.800
%Actinobacteria	0.36	0.555	6.85	**0.019**	0.86	0.369
%Fungi	8.19	**0.012**	1.16	0.301	0.58	0.460
%AM Fungi	29.54	**<0.001**	0.83	0.378	0.70	0.416
G- cy/pre	26.77	**<0.001**	1.92	0.186	0.09	0.771
Sat/Unsat	19.83	**<0.001**	1.56	0.230	0.01	0.911

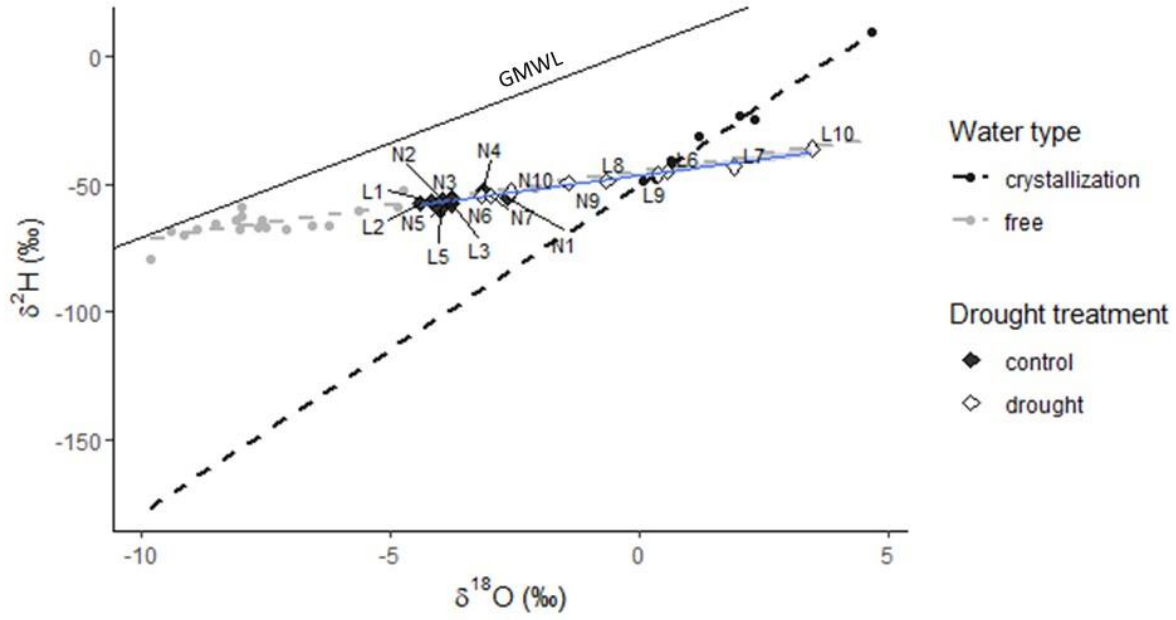

Figure 1. Isotopic composition (δ2H and δ18O) of each potted plant and the water sources in the soil. Diamonds indicate xylem water composition of plants growing either on labelled ("L") or natural soil ("N"). Dots stand for crystallization (black) and free soil (grey) water isotopic composition in the soil. Regression lines are represented to compare the slopes: black and grey dashed lines for crystallization and free soil, respectively; Solid black line: global meteoric water line (GMWL); solid blue line: xylem water of all potted plants.

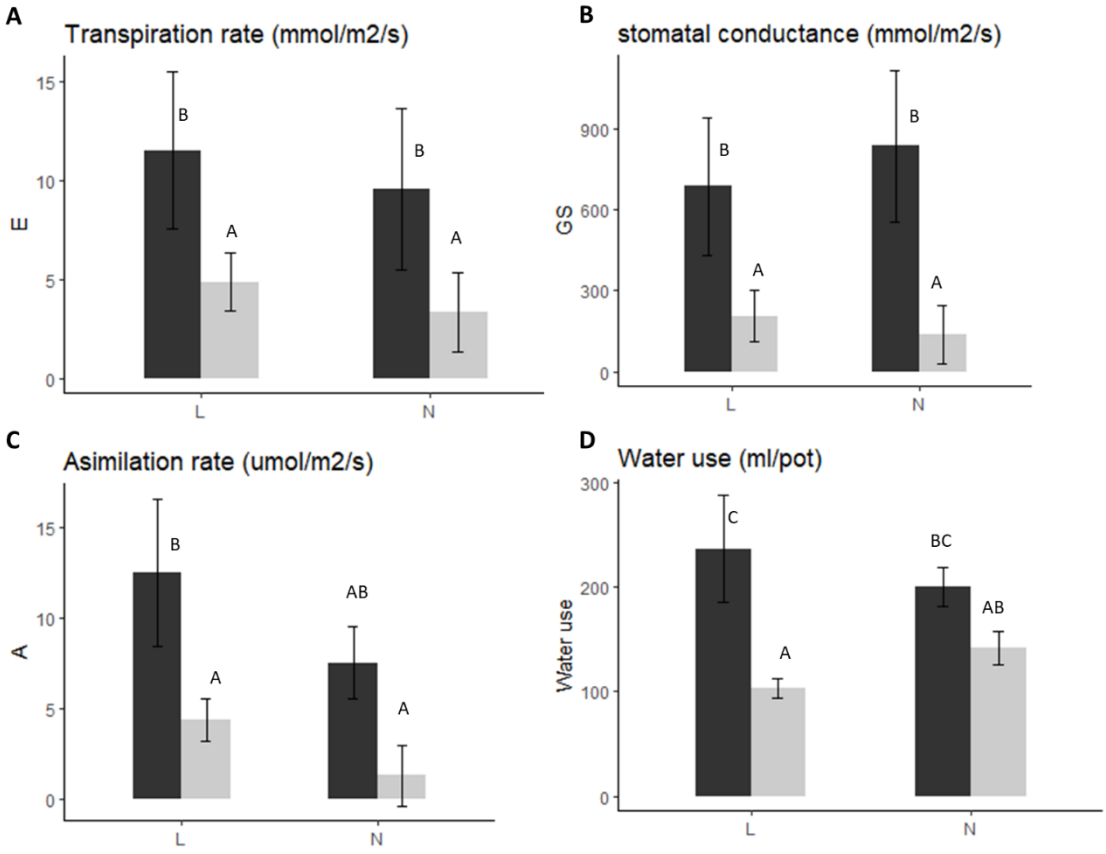

Figure 2. Comparison of tomatal conductance, transpiration rate, assimilation rate and water use in *H. squamatum* plants subjected to different treatments. L stands for labelled and N for natural treatment. Black bars are for the control and grey bars for the drought treatment. Different letters indicate significant differences across all treatments after multiple comparisons Tukey tests ($\alpha = 0.05$).

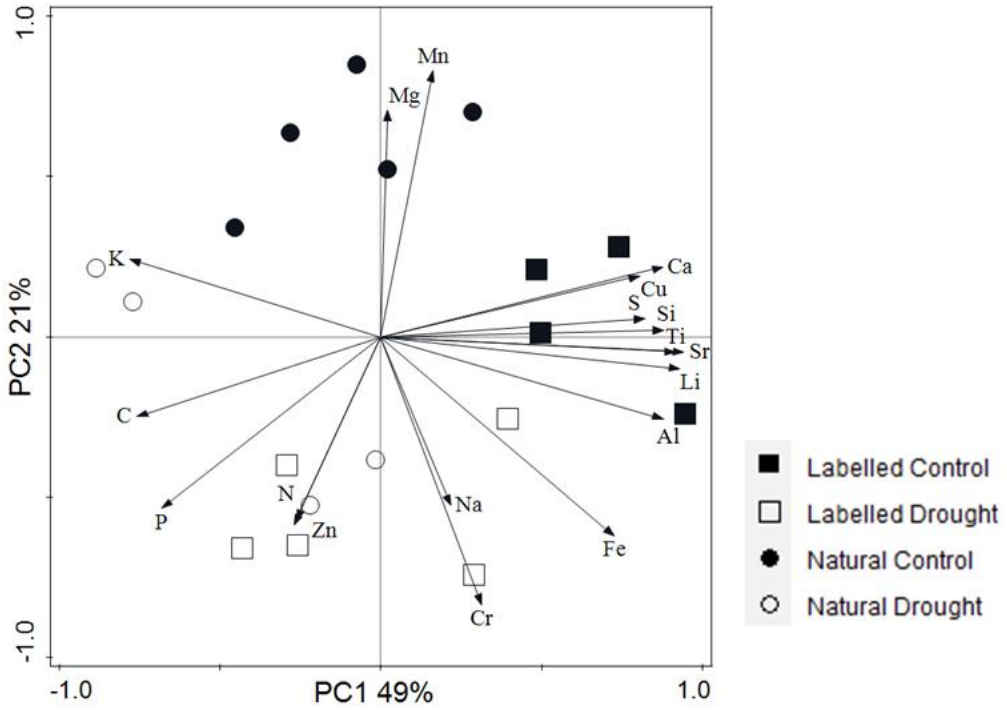

Figure 3. Distance plot of principal components analysis (PCA) from leaf elemental concentration data. Arrows indicate the loadings of each element (Al, As, Ca, Cd, Co, Cr, Cu, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Ti, V, Zn). Symbols stand for the scores of individual plants under control (squares) and drought (circles) conditions, separated by labelling (white symbols) and natural treatments (black symbols)

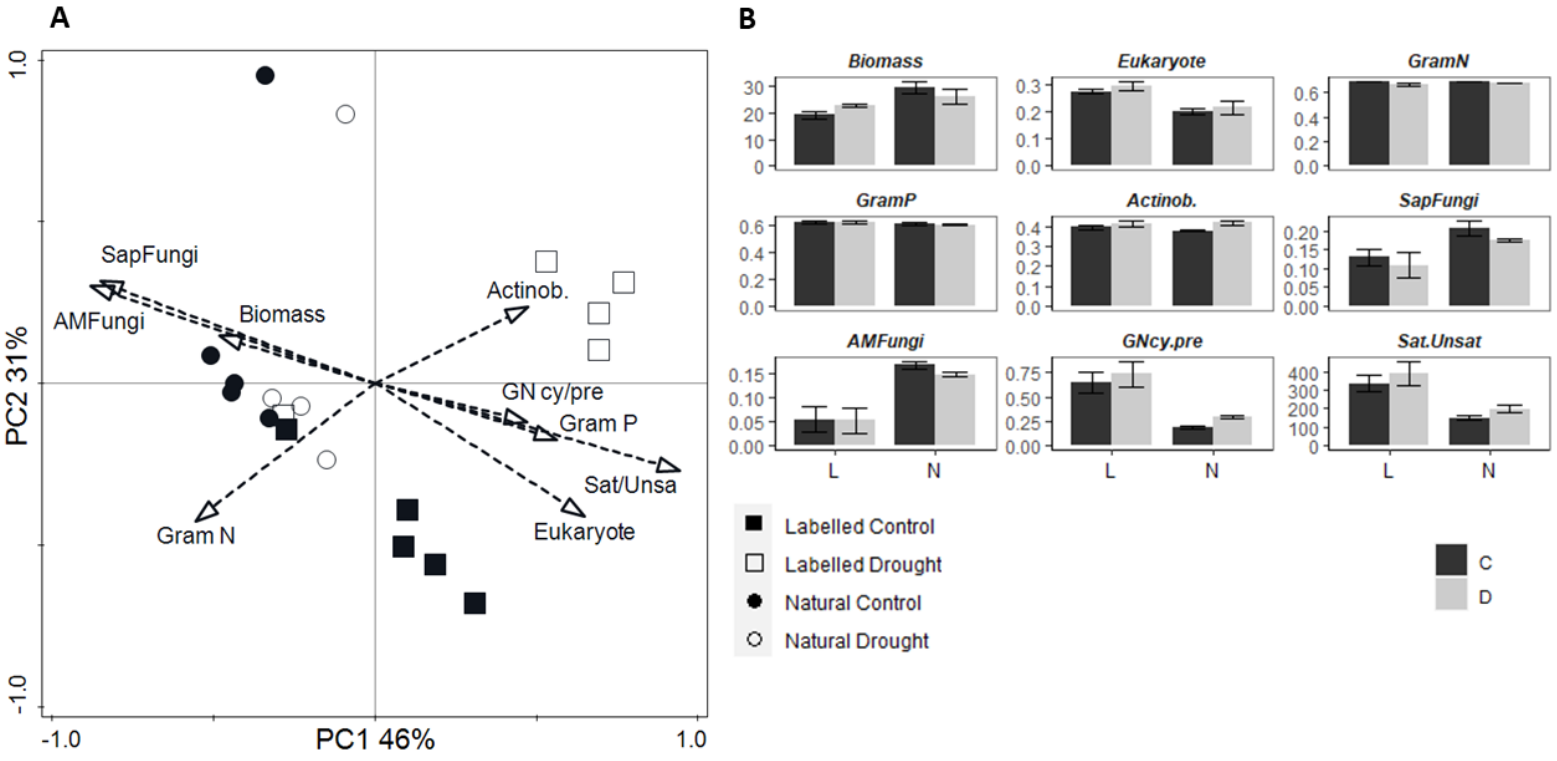

Figure 4. A. Biplot showing the results of PCA on the matrix of relative abundance of all PLFAs detected in soil samples. Arrows represent variables passively projected into the PCA diagram but not included in the calculation (accessory variables): relative abundance of eukaryote, Gram negative bacteria (Gram N), Gram positive bacteria (Gram P), Actinobacteria (Actinob.), saprophytic fungi (SapFungi), mycorrizal fungi (AMFungi), saturated to unsaturated fatty acids ratio (Sat/Unsat), cyclopropyl fatty acids to monoenoic precursors ratio (GN cy/pre) and total microbial biomass (Biomass). Values on the axes indicate percentages of total variation explained by each axis. Symbols stand for the scores of individual soils under labelled (squares) and natural (circles) conditions separated by drought (white symbols) and control treatments (black symbols). B. Mean and standard error of microbial parameters grouped by labelling treatment. L: Labelled. N: - Natural. Black bars are for the control treatment (C) and grey bars are for the drought treatment (D).

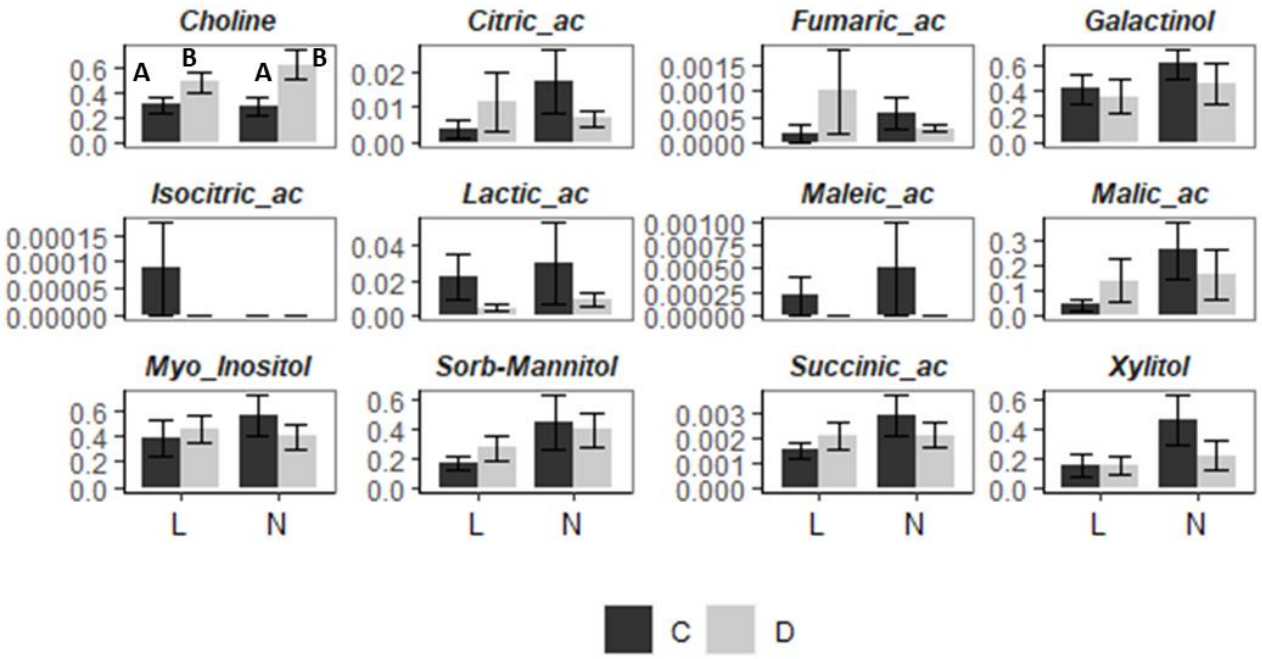

Figure 5. Mean and standard error of root exudate concentration grouped by labelling treatment. -L: Labelled N: Natural. Black bars are for the control treatment (C) and grey bars are for drought treatment (D). Choline was the only exudate which changed with the drought treatment (F=9.28; *p-value*=0.008). No effects of the labelling treatment were observed in the concentration of these compounds in the rhizosphere.

Chapter 4

Ononis tridentata flowers.

Picture by Gabriel Montserrat, downloaded from Herbario Jaca (IPE-CSIC)

Margins designed by Visrginia de la Iglesia and Laura de la Puente

Chapter 4

Soil microorganisms and root exudation mediate rhizosphere acidification of the gypsum specialist *Ononis tridentata* Devesa & G. López*

Article under review

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ABSTRACT

Background and Aims: Plants living on gypsum are adapted to uptake nutrients in extremely poor alkaline soils. Under such extreme conditions, processes affecting the chemical conditions of the rhizosphere may be crucial for plant survival and growth. Rhizosphere acidification in plants living on gypsum soils has never been reported before and the effect of root exudation and microbial fungi on the rhizosphere pH remains undescribed.

Methods: In this study we cultivated seeds of the gypsum specialist *Ononis tridentata* in rhizoboxes with natural gypsum soil and with fungi-sterile gypsum soil, and monitored changes in the rhizosphere pH with planar optodes coupled to a calibrated image recording system. Soil microbial life and root exudation were characterised.

Results: The acidification was steep in both treatments, more intense in the root tip. The higher presence of fungi led to lower pH values in the natural soil treatment. In the fungi-sterile treatment, however, rhizosphere acidification was more extensive across the root surface. Several organic acids and alcohols were exuded by plant roots, with a significantly higher concentration of some compounds in fungi-sterile roots, potentially due to the reduced fungal activity. However, the exudation of lactic acid, a compound related to rhizosphere bacteria, was higher in plants grown in the natural treatment.

Conclusion: Root exudation seemed to be a fundamental process to acidify the rhizosphere in gypsum soil, and fungal microbiota participated in the process without showing a dependency for plant growth. The direct visualization of pH changes at the rhizosphere helped to describe an important mechanism of plant life on gypsum.

Keywords: gypsum soils, rhizosphere acidification, pH, soil microbiota, *Ononis tridentata*.

INTRODUCTION

Soils with high contents of gypsum are present in arid and semiarid areas worldwide (Herrero & Porta, 2000). Its life-limiting properties such as water scarcity and nutrient imbalance have important consequences for plant nutrition and survival (Escudero et al, 2015; FAO, 1990). Gypsum chemical composition ($CaSO_4$. $2H_2O$) results in an alkaline soil solution with exceptionally high ionic concentrations of calcium and sulphate, oversaturating the cation-exchange complex and leading to remarkably low nutrient availability (Meyer et al, 1992). Thus, plants growing in gypsum soils have to cope with extremely high contents of Ca, S and Mg ions and extremely low concentrations of available P, N and K (Moore et al, 2014). However, some plant species have adapted to the harsh condition of gypsum soils, which host a diverse flora rich in edaphic endemics, frequently named gypsophiles (Mota et al, 2003).

It is known that the gypsophile *Ononis tridentata* tends to accumulate S and Ca in the leaves as a strategy to uptake nutrients from gypsum soil while avoiding the toxicity derived from the high contents of these elements in the soil (Cera et al, 2021a). Root nutrient uptake is coupled with the uptake or release of protons, and therefore is commonly associated with root-induced changes in rhizosphere pH (Neumann& Römheld, 2012). The spatial extent of these pH changes into the rhizosphere strongly depends on the buffering capacity of the soil. Rhizosphere pH changes affect nutrient solubilisation in soils and nutrient uptake, so that, generally, cation uptake decreases with declining pH whereas anion uptake is inhibited when pH increases (Neumann & Romheld, 2012). Soil nutrients are not equally available for plants across the pH spectrum, and the optimal availability for most of them has been reported to be in a slightly acidic pH (Lauchli & Grattan, 2012). Therefore, rhizosphere acidification is necessary for nutrient solubilisation and uptake from this alkaline gypsum soil, however, it has never been observed *in situ*.

The formation of mycorrhizal symbiosis is considered to be one of the most successful and widespread strategies to maximize the access of plant roots to available P, playing an important role in nutrient uptake efficiency (Smith and Read, 2008). Several studies have shown that plants living on gypsum soils rely on mycorrhizal fungi to acquire the scarce P available in the soil (Palacio et al, 2012; Cera et al, 2021b). Mycorrhiza is also known for alleviating different plant stresses (Smith et al, 2010) and so, this fungi-root symbiosis has been suggested as an important factor for plant edaphic adaptation in stressful habitats (Schechter and Bruns, 2008). Rhizosphere

pH has been speculated to be decreased by the activity of mycorrhiza, as an indirect effect of mychorrizal facilitation of the P availability in the root (Rigou et al, 1995) or by external measures of the pH in agar with different ectomycorrhizal cultivation (Arvieu et al, 2003), but there is no direct evidence of a quantitative change in pH in the rhizosphere due to mycorrhizal infection. Several studies have assessed the diversity of AM fungi in gypsum plant communities (Alguacil et al, 2009; Menendez-Serra et al, 2018). Other works evaluated the differences in AM colonization between gypsum-tolerant and gypsum-exclusive plant species (Palacio et al, 2012), as well as the variability of colonization among seasons (Cera et al, 2021b). However, comparative studies aimed to elucidate the role of fungi in the interaction between plants and gypsum soils are lacking.

Besides AM fungi, previous studies indicate that gypsophiles may use alternative mechanisms (such as root exudation) to achieve an efficient nutrient uptake, whereas gypsum-tolerant plant species are more dependent on mycorrhizal fungi (Palacio et al, 2011; Cera et al., 2021b). The production of root exudates to mobilize unavailable nutrients is a well-known mechanism to enhance nutrient acquisition in nutrient-limited soils (Marschner, 1995; Lambers et al, 2008). However, there are no studies analysing root exudation in gypsophiles. Major fractions of Low-Molecular-Weight (LMW) compounds are permanently lost from root cells by diffusion. They include sugars, organic acid anions, amino acids and various phenolics that may contribute to nutrient cycling as C and N sources for rhizosphere microorganisms (Jones et al., 2005). In addition, LMW compounds such as organic acids have also been proposed to be released as a controlled excretion by roots in response to different stresses, such as a shortage of oxygen (Xia and Roberts, 1994), Al-toxicity in acid soils (Kochian et al., 2004) or P deficiency, to mobilize P in the soil (Neumann and Römheld, 2000). Thus, in a soil where fungi are scarce, root exudation should be the key agent in charge of nutrients uptake and mobilization by soil acidification (Yan et al., 2002). However, research on pH changes and root exudation under different scenarios of microorganism presence are needed to better understand the role of root activity in the adaptation of plants to nutrient-poor soils like gypsum. Such studies cannot take place directly in the field. However, rhizoboxes coupled with optode technology to track dynamic and spatial pH changes offer a unique opportunity to gain new knowledge on the effect of fungi on rhizosphere soil pH.

The aim of this study was to analyse the plant-soil interaction of a gypsophile plant cultivated in natural and a fungi-sterile gypsum soil with a special focus on processes at the rhizosphere level. We intended to determine the importance of soil fungi and the role of root exudation in the acidification of the rhizosphere of *Ononis tridentata* seedlings growing in alkaline gypsum soils with contrasting fungal presence. We hypothesized (1) that the presence of fungi in the soil will promote rhizosphere acidification, reaching lower pH in natural than in fungi-sterile soils. We also hypothesized (2) that root exudation will be promoted in the fungi-sterile soil treatment, as a response to a higher nutrient stress.

MATERIALS & METHODS

Experimental conditions

Ononis tridentata is a perennial gypsophile shrub widespread in gypsum soils across Spain and reaching Northern Africa (Mota *et al*., 2011). Seeds from *O.tridentata* were collected from mixed adult individuals from a natural population growing in Villamayor (41°41'48.3"N 0°44'34.7"W, Zaragoza Province, Aragón, Spain). The soil for the experiment was collected in the same area from the top 0.5-1m of the soil, removing the top litter and biological crust layer, and then, homogenized and cultivated in the Pyrenean Institute of Ecology (IPE-CSCIC), Jaca (Huesca province, Aragón, Spain).

To understand the influence of fungi on the rhizosphere pH and the root exudation profile of *O. tridentata*, plants were grown in either natural soil or in fungi-sterile soil. Sterile soil was obtained after gamma irradiation (25 kGy) with a ^{60}Co source (Aragogamma, Granollers, Spain), and afterwards the sterilized soil was inoculated with a bacterial filtrate from the natural soil in order to add the bacterial community present in it. This bacterial inoculum was obtained by stirring natural soil in sterile distilled water in a proportion of 1/10 (weight/volume) for 5 minutes, and then passing the suspension sequentially through filter paper and a 9 µm ø filter to eliminate soil particles and fungal spores and propagules (Doubková et. al, 2011; Chaudhary *et al*, 2020), but not bacteria. Each fungi-sterile pot was inoculated with 50 mL of this bacterial inoculum.

We cultivated *O. tridentata* plants in rhizoboxes (i.e. plastic containers of 220 x 170 x 15 mm with a transparent plexiglass window that enabled root observation, see Figure 1A) and filled them with 561 ml of either natural soil or fungi-free soil. Five rhizoboxes per soil treatment were used.

Before cultivation, seeds were carefully scarified using a sandpaper, rehydrated with distilled water for 24 hours, and germinated in petri dishes humidified with distilled water. Afterwards, three germinated seeds of *O. tridentata* were planted in each rhizobox. Altogether there were 15 plants sown per treatment (natural and sterilized soil), although only one seedling per rhizobox was finally included in the soil pH measurements (the most vigorous one), amounting five replicates per treatment. During cultivation, the transparent windows of the rhizoboxes were covered with dark acetate sheets to prevent light from reaching the roots. Rhizoboxes were kept at a ~ 45º angle to ensure root growth towards the window (see Figure1B) and had several holes in

its bottom to drain excess water. Plants were watered with distilled water every 12 hour for 1 minute, maintaining soil humidity at field capacity. However, due the low water retention of gypsum soils (Herrero and Porta, 2000), and to guarantee the constant humidity of the VisiSens sensor foils (see pH visualization), rhizoboxes were placed in a plastic tray that retained the excess irrigation water, which could be absorbed to keep the field capacity in the soil. Plants were grown in a room under natural non-direct sunlight and supplementary 12h artificial illumination (full spectrum LED light, 380nm-800nm). They were harvested after 22 days of cultivation.

pH visualization

Changes in rhizosphere pH were monitored by obtaining pictures of optodes placed at the root tip of selected seedlings and the resulting images were edited with the VisiSens TD® software (PreSens GmbH). VisiSens is a 2D pH mapping device with PC data acquisition and evaluation. Six days after cultivation, VisiSens sensor foils of 2 cm^2 were installed between the transparent plexiglas of the rhizobox and the soil, covering the root tip in the healthier seedling of each rhizobox (see Figure 1C). After that, images were taken daily at 1 p.m. during a period of 14 days, to ensure the roots have grown and passed behind the sensor foil. The pictures were taken in a small dark chamber where the only light source came from the VisiSens TD camera. The camera was re-fixed for each image taken, placed in contact with the plexiglass, as near as possible of the sensor foil and focusing it whole (see graphic scheme in Figure 1D and Figure 1E). Images were edited by IDL evaluation pH software (VisiSens^{TM} Analytical 2 Software) using previous calibration data of the sensor foils. Maximum, minimum and average pH were noted for each rhizobox, during the monitoring period (Figure 1F).

Plant harvest and analysis of root exudates

After 14 days of pH measures, the transparent methacrylate (Plexiglas) of the rhizobox was carefully removed to access the roots. The aboveground plant part was separated from the root part. The roots were cleaned with a brush to remove rhizosphere soil. All the root tips of the rhizoboxes were fresh-weighted in a precision scale (42 g / 0.00001 g, MS105DU, Mettler Toledo, Columbus-OH, USA) and, as quickly as possible, placed in a small vial with 1 mL of a 0.01% formic acid solution (obtained by adding 10 µL of 98% purity Formic acid to 100 mL Mili-Q water, the resulting solution had a pH of 3.06). Fine roots were then shaken for 10 minutes at

medium speed in an automated agitator (Rotabit, JPselecta). Exudates were then filtered through a 0.22 µm of 13 mm ø cellulose acetate filter (CA Syringe Filter 1mL, Filter-Lab. REF: JS1) and kept at -80 ºC. The rest of the root was weighted fresh and dried in an oven at 50 ºC for a minimum of three days, then weighted in a precision scale (42 g / 0.00001 g, MS105DU, Mettler Toledo, Columbus-OH, USA) to obtain the dry weight of roots.

Exudates were analysed at the metabolomics service/lab (CEBAS-CSIC) with a ultra-high-performance liquid chromatography system with a triple quadrupole mass spectrometer (UPLC-QToF-MS). Organic acids are the most common exudates, so organic standards were used and results are provided as µg/mL (ppm). Contrastingly, alcohols were analysed without standards, as the study focused on organic acids. Consequently, results for alcohol measurements are reported as a ratio of concentration, comparing each data with the highest value detected and for comparative purposes among treatments only.

PLFAs analyses

To characterize soil microbial communities and to detect shifts in the main functional groups of microbes, we used phospholipid fatty acid (PLFA) profiling. Aliquots of 2 g of lyophilized bulk soils were used for lipid extraction. Lipids were extracted with a one-phase chloroform–methanol-phosphate buffer solvent. Phospholipids were separated from non-polar lipids and converted to fatty acid methyl esters (FAMEs) before analysis, following the methodology described by Buyer and Sasser (2012). The resulting FAMEs were separated by gas chromatography using an Agilent 7890A GC System (Agilent Technologies, Wilmington, DE, USA) equipped with a 25-m Ultra 2 (5%-phenyl)-methylpolysiloxane column (J&W Scientific, Folsom, CA, USA) and a flame-ionization detector. The identification and quantification of FAMES was carried out using the PLFAD1 method of Sherlock software version 6.3 from MIDI, Inc (Newark, DE, USA). The internal standard 19:0 phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) was used for the quantification of the FAMEs. Microbial biomass was estimated by summing the contents of all individual PLFAs and reported as nanomoles of PLFAs per gram of soil.

Specific PLFAs were used as biomarkers to quantify the biomass of broad taxonomic microbial groups, according to their characteristic fatty acids: eukaryote, Gram negative and Gram positive bacteria (hereafter G- and G +, respectively), saprophytic fungi and arbuscular mycorrhizal fungi (AMF) (Frostegard and Baath, 1996). The ratio between cyclopropyl fatty acids and their

monoenoic precursors [(cy17:0 + cy19:0) / (16:1ω7 + 18:1ω7); hereafter GN cy/pre] were calculated as proxies for physiological or nutritional stress in the bacterial communities (Frostegård *et al*. 2011; Willers *et al*. 2015).

Statistical analyses

The plotted pH data were filtered to select only the replicates where the root passage was clearly observed through the optode in PreSens VisiSens software.

All statistical analyses but the PCA for PLFAs (see below) were carried out using *R* version 4.0.0 (R Core Team, 2020). Graphs were created with ggplot2 package 3.3.1 (Wickham, 2016).

Statistical differences in pH among treatments were calculated with Kruskal Wallis test (Hollander & Wolfe, 1973) as data were not parametric and Shapiro Test indicated they were not normally distributed. The same procedure was used to analyse differences in the quantified exudates detected, creating an individual data set for each compound.

Relative abundance percentages of individual fatty acids were arcsine-transformed for normality before statistical analyses. A PCA was performed to represent and analyse the differences in soil microbial community composition (PLFAs) between experimental treatments. Then, we selected the parameters of the relative abundance of Saprophytic and Arbuscular Fungi, Gram Positive, Gram Negative and Actinobacteria; and the ratios Gram+/Gram- and G- cy/pre to project on the PCA plot to a facilitate visualization of the differences on the microbial composition on the soil of the different treatments. PCA analysis was run with CANOCO software (Braak & Smilauer, 2002). The comparison between fungi-sterile soils and natural soils for the selected parameters related to fungi and bacterial composition or to microbiota stress were individually analysed by a one-way ANOVA test (Welch, 1951) or a Kruskal Wallis test (Hollander & Wolfe, 1973) when data were not parametric. Normality of the data was double checked by Shapiro Test (Royston, 1995) and by checking the residuals of the *lm* model (Chambers, 1992) with the DHARMa package (Hartig, 2020).

RESULTS

Effects of experimental conditions on soil microbial communities

We observed a clear segregation of the treatments according to the presence and relative abundance of PLFAs in soil (Figure 2), which indicates the existence of distinct microbial communities. Although we detected fungal presence in both treatments, the relative abundance of saprophytic fungi and AM fungi was, however, significantly lower in the "fungi-sterile" treatment ($p = 0.016$ and $p = 0.012$, respectively; Table 1). Therefore, our methodology worked in terms of reducing the presence of fungi, so for simplicity we will keep referring to natural and fungi-sterile treatments although we acknowledge that there was some fungal presence in the fungi-sterile treatment. Moreover, there was a higher relative abundance of Gram-negative bacteria ($p < 0.001$) and, consequently, a lower ratio Gram+/Gram- in the fungi-sterile soils ($p < 0.001$). On the other hand, the microbiota living in the fungi-sterile soil showed higher ratio of GN cy/pre ($p= 0.047$), indicating that it was suffering higher nutritional stress than that in the natural soil (White *et al*. 1996; McKinley *et al*. 2005)

Rhizosphere pH acidification

Ononis tridentata seedlings developed a single apical radicle, whose position and morphology coincided with the images revealed by the VisiSens software. We observed an acidification in the rhizosphere for both treatments, with a pH decline of 2.4 units on average in natural replicates and 2.1 units in fungi-sterile replicates (Supplementary Table 1), calculated from the values of the mean of the pH maximum and pH minimum within the replicates. Figure 3 shows a darker blue area corresponding with the root tip, and how the intensity of this coloured signal descended as the root passed by the sensor over the days. The lowest minimum pH was reached in the rhizosphere of the seedlings growing in the natural soil, significantly lower than the minimum pH reached in the rhizosphere of seedlings growing in the fungi-sterile soil ($p = 0.014$; Figure 4). However, the mean pH in the rhizosphere was lower in the fungi-sterile soil ($p < 0.001$, Figure 4), indicating a greater acidification surface across the roots of plants grown with decreased fungal presence. The picture of the acidified rhizosphere was more intense and defined in the roots of the natural treatment, contrasting with the less intense but more widespread acidification in the fungi sterile rhizosphere.

Differences in exudation patterns

The UPLC analysis detected several organic acids (citric, malic, succinic, lactic, malonic and fumaric) as root exudates in both treatments, accounting for significantly higher quantities of malonic acid in the fungi-sterile treatment ($p = 0.036$). The isocitric acid was detected only in the fungi-sterile treatment, contrary to the natural treatment, whose seedlings did not exudate this compound. Conversely, lactic acid was significantly higher in the exudation profile of the seedlings growing in the natural treatment ($p = 0.016$). Some polyols were also detected in both treatments (myo-Inositol, galactinol, xylitol and sorbitol-mannitol), but only one of them presented significant differences in its abundance proportion: sorbitol-mannitol was significantly higher in the fungi-sterile treatment ($p = 0.021$; Table 2, Figure 5). In general, as we expected, we found a greater proportion of root exudation in the fungi-sterile soil replicates, except for lactic acid, an organic acid related to microbial activity (Juturu & Wu, 2015).

DISCUSSION

Effects of experimental conditions on soil microbial communities

The lower G+/G- ratio found in the gamma irradiated soil might be a consequence of some G- bacteria being r-strategists that, after the inoculation of the sterilized soil, may have surpassed in growth rate Gram+ bacteria under the elevated labile organic C conditions induced by radiation (McNamara et al., 2003; Zhang et al., 2016). Moreover, gamma-irradiation of soil is known to increase the pool of available inorganic nitrogen (Lensi et al., 1991; McNamara et al., 2003), which can stimulate the processes of nitrification and denitrification, both of them mostly conducted by Gram - bacteria (Hayatsu et al., 2008). Although increased relative abundance of Gram- bacteria after soil gamma-irradiation has been also reported in other researches (Yim et al., 2015; Zhang et al., 2016), it is far from being a general fact (Ogwu et al., 2019).

Plants from natural gypsum soil reached lower rhizosphere pH than those from fungi-sterile soil but the root acidification area was smaller

The key findings in our research were that (1) natural gypsum soils reached lower pH in the rhizosphere, showing a steeper rhizosphere gradient of pH but (2) fungi-sterile soils acidified a greater area in the soil, showing a larger extent of the rhizosphere. There is no previous literature about pH changes in such alkaline soils, especially in wild plant species. To the best of our knowledge, this is the first time an acidification of more than 2 pH units is shown *in situ* in a rhizosphere of a species not used for cropping purposes. The maximum pH observed in the soil (pH~9.4, see Figure 2 and Supplementary Figure S1) could be due to the natural intrusions of calcium carbonate in the gypsum soil, which increases significantly the soil pH and reduces the Ca^{2+} activity (FAO, 1990). Given this high soil pH, plants might have needed to acidify the soil to favour nutrient solubilisation and uptake. *O. tridentata* seedlings growing in natural soil seemed to be helped by its microbiota, which promoted a greater soil acidification by the release of H^{+} protons to the rhizosphere (Zhu et al, 2023). In this way, a steeper rhizosphere acidification could be indicating a greater P and N availability in the soil (Ma et al, 2021; Cakmak & Marschner, 1990). Rhizosphere acidification may be also indicating the uptake of calcium and magnesium cations, which is balanced by a net release of protons (Neumann & Röhmheld, 2012). These cations can be found in excess in the gypsum soil (Cashby-Horton et al, 2015), and are extensively accumulated in *O. tridentata* leaves (Cera et al., 2021a).

The fact that in plants grown in fungi-sterile the rhizosphere soil was broader could be due to the different pH buffering capacity of the soil. Altered microbial composition could reduce the presence of secondary minerals that contribute to the pH buffering capacity (Bilyera et al, 2022). In addition, rhizosphere acidification under P-limiting conditions has often been associated with the release of organic acids, which were detected in both treatments. However, the rapid microbial decomposition of root exudates (Jones et al., 2005) could be affecting the natural replicates, but not the fungi-sterile rhizosphere, where exudates could be increasing the space and time residence in the soil (Boudot, 1992). Another possibility to explain this extended acidification could be due to an increased growth of the root of nutrient-deprived plants cultivated in the fungi sterile treatment to promote soil foraging (de la Fuente et al., 2020) or the extension of its bacterial microbiota due to the lack of fungi that also contributes to the acidification of the soil.

The release of root exudates differed between soil treatments

Until now, most studies investigating root exudation have focused on agricultural plants (Henry et al., 2008) but see Williams et al. (2020 and 2022). However, the exudation profiles in different ecosystems may significantly differ and play important roles in shaping the microbial community (Baudoin et al, 2003; Ma et al, 2021). It is difficult to find direct evidence of the influence of a certain root exudate on soil microbial communities, because of the complexity of the rhizosphere environment and the difficulty in studying belowground processes (Biedrzycki & Bais, 2009). Our work revealed the differences in the release of exudates at the rhizosphere of *O. tridentata* in plants interacting with contrasting microbial communities. These results have important implications for the understanding of the plant-soil interactions mediating plant nutrition and survival in alkaline nutrient poor soils like gypsum.

The main contribution of this work about root exudation was that plants growing with a reduced fungal population in the soil, exuded, in general, greater quantities of several compounds, thus seeming to compensate for the lack of the fungal interaction on making nutrient available. Fungi-sterile replicates exuded higher quantities of different organic acids and a polyol, likely as a consequence of their nutrient deficiency due to a reduced fungal and bacterial activity in the soil. In low nutrient environments, root exudation could be employed as symbiotic signals to soil microbes involved in nutrients procurement (Dakora & Phillips, 2002). Moreover, our study showed that this species of plant adapted to such high pH soils is able to reduce the pH very

significantly and, thus, facilitate nutrition processes through the exudation of different organic acids. Isocitric acid and malonic acid were found in greater quantities in the fungi-sterile rhizosphere. These compounds are in the class of aliphatic acids, whose function as root exudates includes growth regulation (Oliveros-Bastidas et al, 2009) and have been reported as major compounds influencing the dissolution of minerals (Sokolova, 2019), thus contributing to *O. tridentata* nutrition.

We detected additional compounds in fungi-sterile replicates: the sugar alcohols sorbitol-mannitol. These compounds are also involved in the improvement of soil nutrient environment and soil nutrient cycling and, as such, they are beginning to be used as fertilisers in agriculture (Yu et al, 2014; Ichimura et al, 2016). Additionally, they have an important role in shaping and increasing different rhizosphere microbial communities (Yu et al, 2023).

Conversely, the exudation of lactic acid was significantly higher in *O. tridentata* replicates growing on natural soils, with an unaltered microbial composition. This compound has been reported to be a product of several bacteria associated to roots and is known by its beneficial effects on crops (Minervini et al, 2015). Significant increases in the abundance and diversity of soil bacteria communities have been reported after the artificial addition of lactic acid (Hengjing et al, 2011). In addition, this compound has also been reported to be exuded by plants in the rhizosphere to detoxify its high concentration in cells under oxygen deprivation caused by high soil moisture(Badri & Vivanco, 2009 and cites therein). Other studies showed a biocontrol function of lactic acid in tomato plants (Wang et al, 2019), or a detoxification function in response to copper or cadmium toxicity (Lyubenova et al, 2013; Chiang et al, 2006 respectively).

Combining planar optodes with root exudation and a treatment of partial soil sterilization in a gypsum soil helped understand *Ononis tridentata* rhizosphere behaviour. However, the high soil humidity requirements of the pH sensor restricted our observations of root activity to well-watered conditions. This situation in gypsum ecosystems would take place in the most favourable moment of the year, when gypsum plants tend to grow and maximize their demand for N and P (Cera et al., 2021b).

It is also important to mention a potential consequence of the root exudation shown by plants living on gypsum, related to the use of gypsum crystallization water. Gypsum ($CaSO_4 \cdot 2H_2O$) contains two water molecules in its crystalline structure, and several authors (Palacio et al., 2014; de la

Puente et al., 2021; Huang et al., 2020) have reported the use of this water as the main water source by plants or other microorganisms growing on gypsum during drought. The mechanism to obtain this water proposed by Huang et al. (2020) for cyanobacteria consists on gypsum dissolution by the acidification caused by the exudation of organic acids. However, the mechanisms displayed by plants to acquire gypsum crystallization water remain unknown. Our results show the ability to exude different organic acids by plants living on gypsum, which may affect the release of gypsum crystallization water. Similarly, the release of low molecular weight alcohols may also alter the thermodynamic conditions of calcium sulphate phases, modifying its hydration (Van Driessche et al, 2017; Tritschler et al, 2015). Although adult *O. tridentata* plants have been reported to rely mainly on deep water and not gypsum crystalline water (Palacio et al, 2014; de la Puente et al, 2021), the changes in rhizosphere pH observed in this study correspond to seedlings, whose access to gypsum crystalline water may be crucial for their survival at early stages. Preliminary results on root acidification similar to those observed in *O. tridentata* on other gypsum endemics plants with shallow root systems previously reported to rely on gypsum crystalline water, like *Helianthemum squamatum* or *Helianthemum syriacum* (Palacio et al, 2014; de la Puente et al, 2021) (see preliminary data in Supplementary Material Fig.S2), further support the potential role of root exudation in gypsum plants as a mechanism to obtain gypsum crystalline water.

CONCLUSIONS

For the first time, we visualized *in situ* rhizosphere acidification in wild plants growing in alkaline gypsum soils. According to our results, rhizosphere acidification seems to be a necessary process to grow on gypsum. We conclude that soil fungi participates in the rhizosphere acidification, as we observed a steeper pH decline in natural-soil replicates. However, the roots of *O. tridentata* also contributed directly to this acidification, through the exudation of different organic aliphatic acids and sugar-alcohols. This indicates that *O. tridentata* is not fully dependent on microbiota for nutrient acquisition, although it is facilitated by their presence, and has ways to compensate for the missing symbionts. We further conclude that root exudation is a determining factor for plant adaptation to atypical gypsum soils that could mediate plant nutrition and access to gypsum crystalline water.

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TABLES AND FIGURES

Table 1. Significance of the differences (p<0.05) between fungi-sterile and natural soils in different phospholipid fatty acids (PLFA) related to fungal and bacterial composition or to microbiota stress, based on one-way ANOVA or Kruskal Wallis test when data were not normally distributed. Significant *p-value*s are in bold.

	F	Kruskal-Wallis chi-squared	*p-value*
% Total Fungi	8.60		**0.033**
% Saprophyte Fungi		5.77	**0.016**
% AM Fungi	12.14		**0.012**
Fungi/Bacteria	9.26		**0.026**
Total Bacteria	5.38		**0.049**
Gram+/Gram-	60.17		**<0.001**
GN cy/pre	6.46		**0.047**
Gram -	46.64		**<0.001**
Gram +	20.68		**0.003**
Actinobacteria	100.39		**<0.001**

Table 2. Significance of the differences ($p<0.05$) between fungi-sterile and natural soils in root exudates, based on one-way ANOVA or Kruskal Wallis test when data were not normally distributed. Significant *p-value*s are in bold.

	F	Kruskal-Wallis chi square	*p-value*
Citric		0.54	0.459
Isocitric		**5.54**	**0.019**
Malic		0.10	0.754
Succinic	0.23		0.645
Lactic		**5.77**	**0.016**
Malonic	**7.45**		**0.036**
fumaric		1.33	0.249
Myo_Inositol		0.27	0.602
Galactinol	4.18		0.093
Xilitol	4.26		0.074
Sorbitol-Manitol	8.25		**0.021**
Choline	3.91		0.093

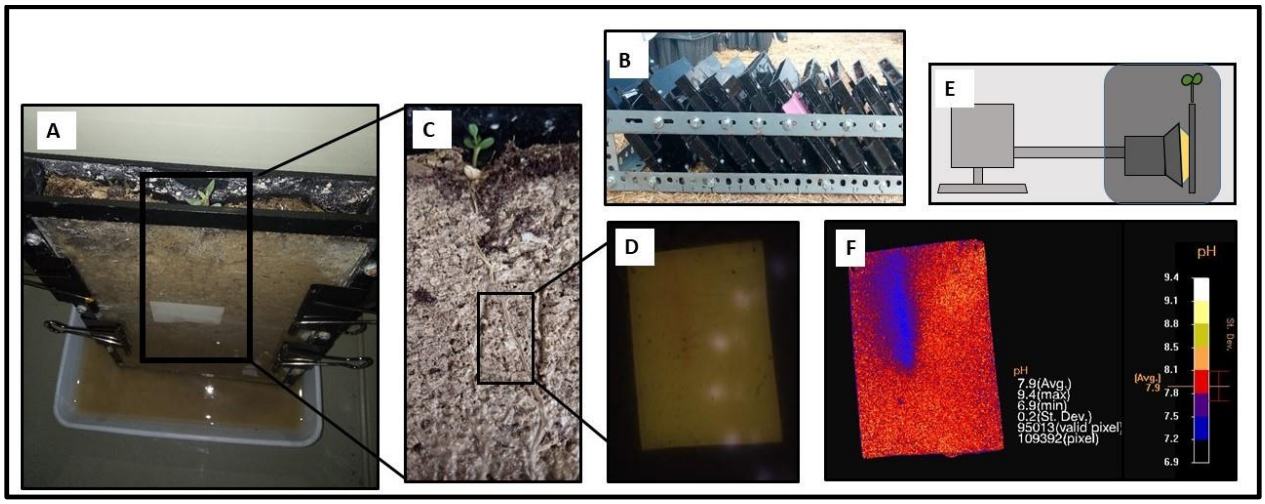

Figure 1. Methods. **A**. Picture of one of the rhizoboxes where seedlings were grown. **B**. rhizoboxes positioned *ca*. 45º to force the roots to be visible through the transparent plexiglass. **C**. Picture of the area where the sensor foil was installed, taken after the pH measures. **D**. Image acquired with the VisiSens PreSens camera before its edition with the calibrated pH. **E**. Graphic scheme of the procedure to take pictures. The software in the computer was connected to the camera, which was placed as close as possible to the sensor in contact with the transparent window. Pictures were taken with the camera light source. **F**. Example of the final image given by the software, indicating the maximum, minimum and mean pH of all the pixels corresponding to the sensor.

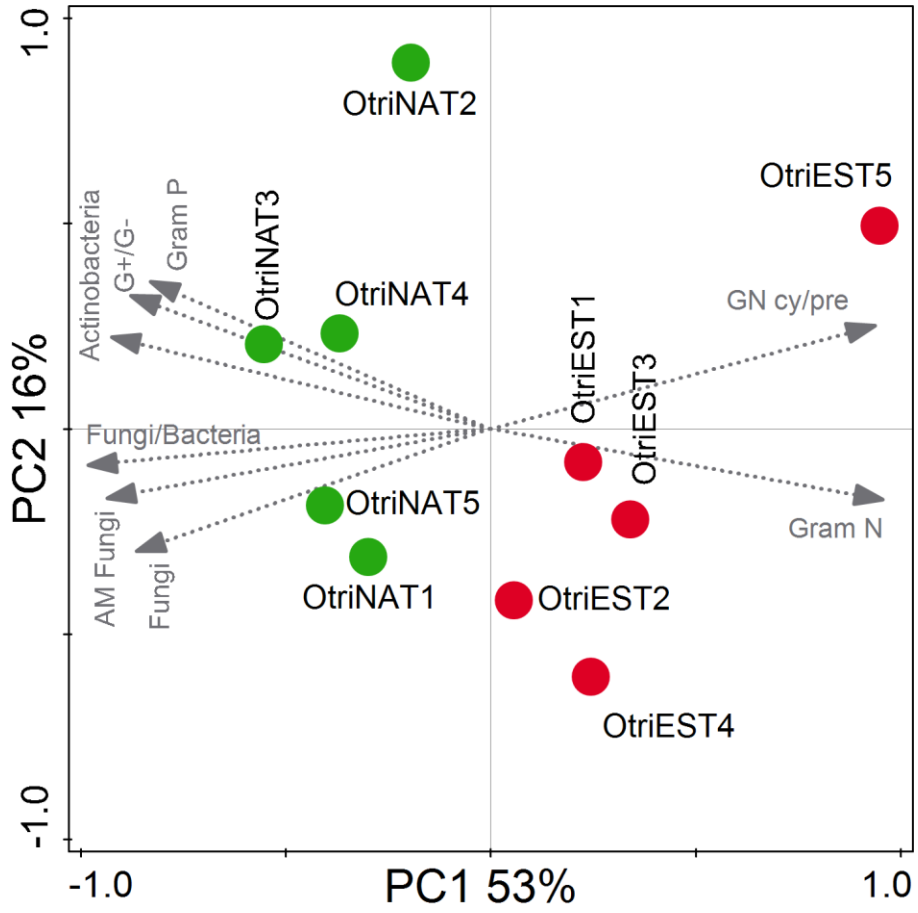

Figure 2. Biplot of the results of a PCA performed on the matrix of relative abundance of all PLFAs detected in soil samples. Arrows represent variables passively projected into the PCA diagram but not included in the calculation (accessory variables): relative abundance of Gram negative bacteria (Gram N), Gram positive bacteria (Gram P), Actinobacteria (Actinobacteria), saprophytic fungi (Fungi), mycorrizal fungi (AM Fungi), cyclopropyl fatty acids to monoenoic precursors ratio (GN cy/pre) and the ratio of the relative abundance of Gram Positive and Gram Negative Bacteria. Values on the axes indicate percentages of total variation explained by each axis. Green points stand for the scores of the five replicates of natural soil treatments and red points for the five replicates of fungi-sterile treatment.

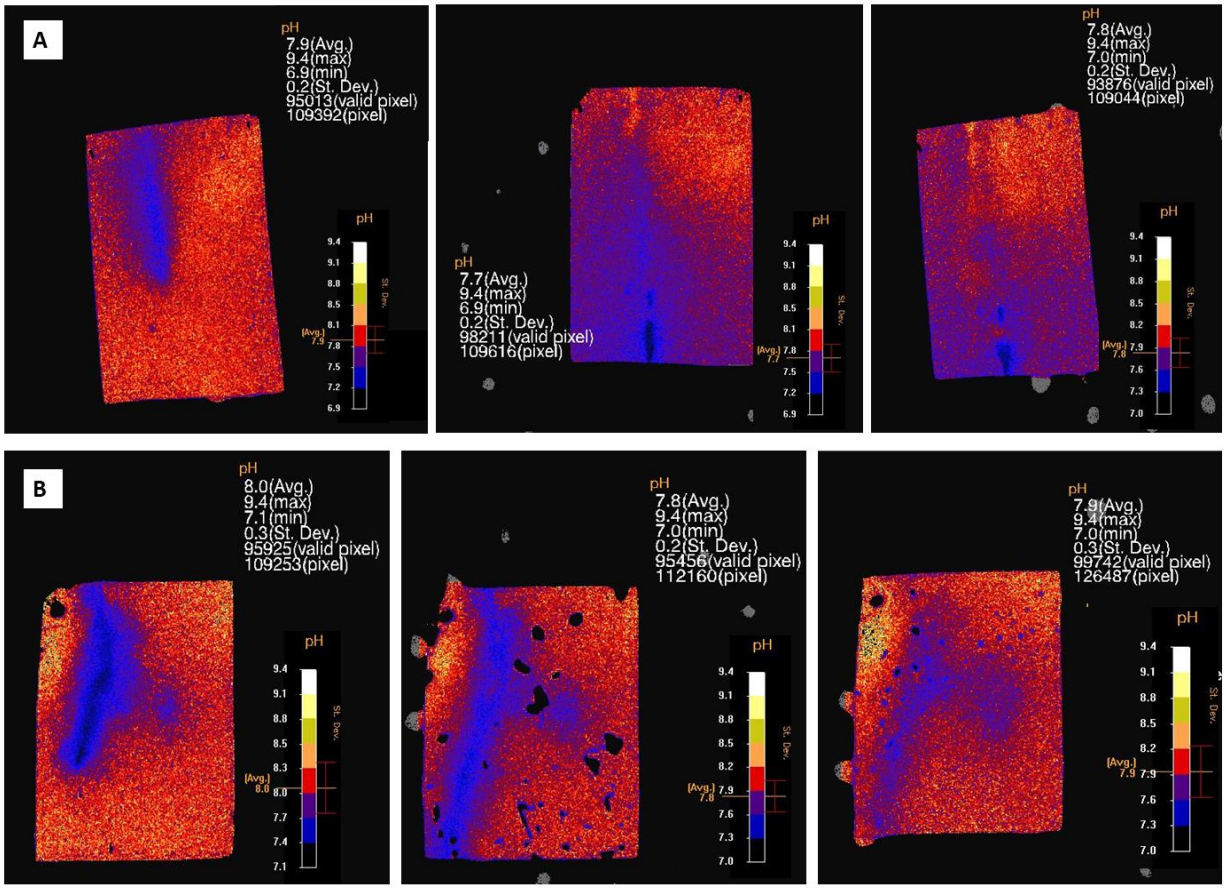

Figure 3. IDL evaluation images of growing roots taken with VisiSens PreSens. Sequential images show the movement of the acidification front due to the root tip passing through the sensor. **A**. Sequential images of one replicate of *Ononis Tridentata* ("rep1 natural") seedling root growing in natural gypsum soil in different days (day 1, 4 and 9). **B.** Sequential images of one replicate of *Ononis Tridentata* ("rep 2 sterile") seedling root growing in the fungi-sterile gypsum soil in different days (day 1, 3 and 9).

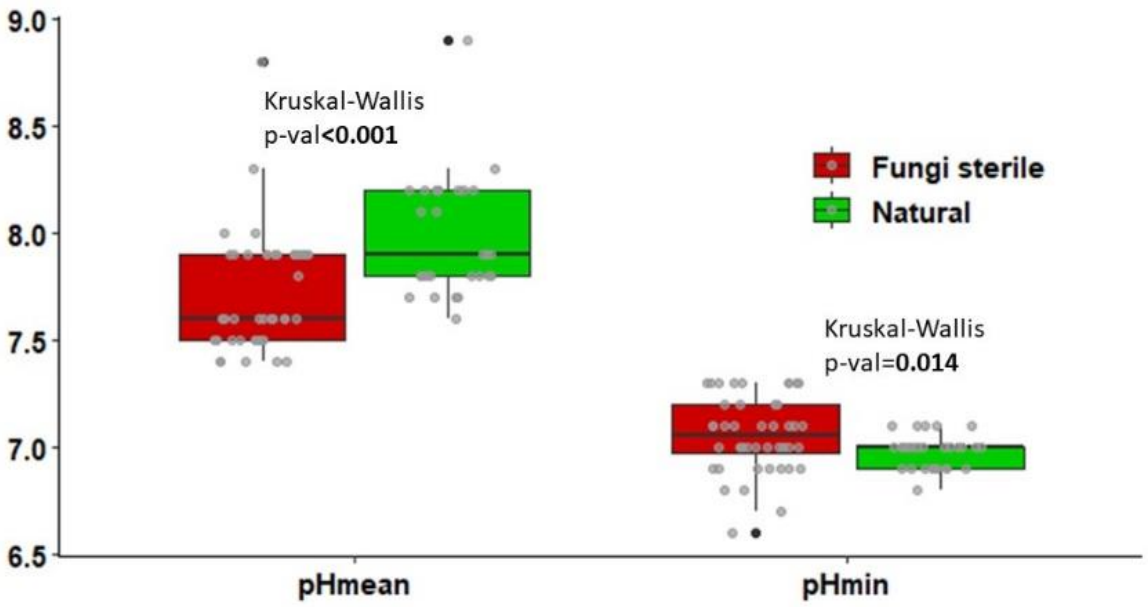

Figure 4. Boxplot of minimum and mean pH observed in the sensor foils placed in the roots of the growing seedlings of *Ononis tridentata*, compared per soil treatment: green boxes for the natural treatment, red boxed for the fungi-sterile treatment. *P-value*s of Kruskal Wallis tests show the significance of the differences between treatments.

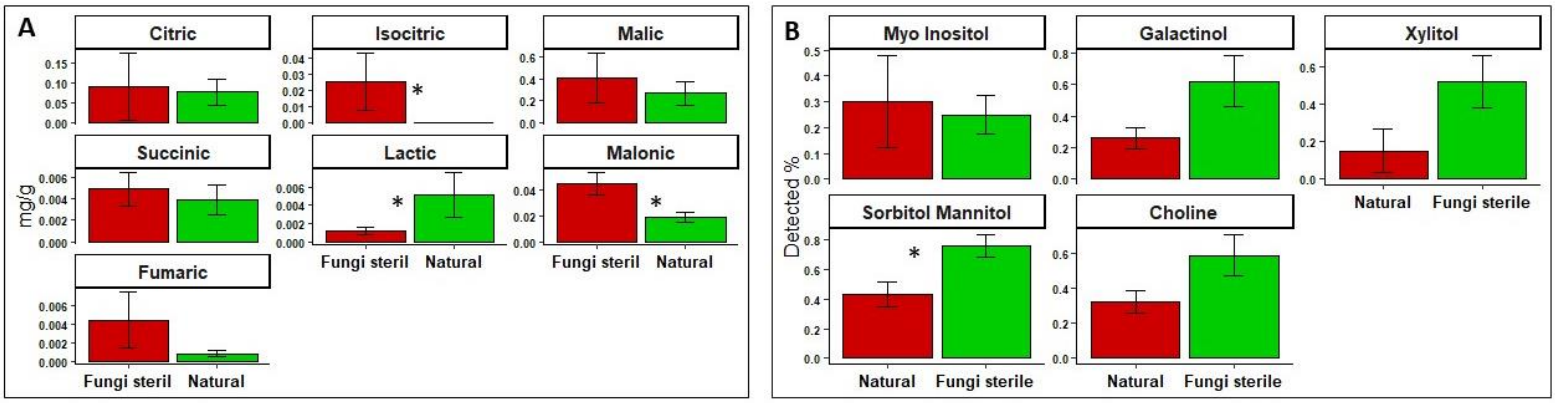

Figure 5. Bar plots showing the mean and SD of the exudates analysed in Ononis tridentata roots growing in natural gypsum soil (green bars) and in fungi-sterile gypsum soil (red bars). Organic acids were calculated as mg of the compound exuded by g of root (A), whereas the other exudates that belong to the polyols family or amine (such as choline) were calculated as ratio of detection, but not as exact concentration (B). The asterisk (*) indicate significant differences in each exudate between treatments (see also Table 2)

General Discussion

This thesis brought new knowledge about the ecology and physiology of plants living on gypsum soils. We contributed with novel information on the use of water in different gypsum plant communities, including plants growing in remote areas of Iran, and characterized the integrated ecophysiological response to drought by a gypsum specialist species, at the whole-plant level. Additionally, we showed, for the first time, evidence of the relevance of soil fungi on important rhizosphere processes that mediate plant life on gypsum.

The inherent aridity of gypsum ecosystems, and thus, the variability and seasonal water scarcity, is an important factor that shapes these diverse communities and conditions the water partitioning between neighbour species growing on gypsum soils. The results presented in this Thesis showed a clear eco-hydrological niche segregation, most pronounced during the drought season, in both gypsum ecosystems studied. Contrary to our hypothesis, the factors underlying species segregation in hydrological niches were not related to plant gypsum affinity (Chapter 1, 2) nor their photosynthetic pathway (Chapter 2). Contrastingly, rooting depth (in Chapter 1) seemed to be a key determinant factor explaining differences in the main water sources used by plants during seasonal shifts in water availability.

The most relevant contribution of Chapter 1 was the identification of gypsum crystallization water as the main water source used by almost all shallow-rooted species from a gypsum plant community in NE Spain during summer. This is a remarkable contribution that rules out the possibility of gypsum crystallization water being only available to gypsophile species. Deep-rooted species used deep water stored in the soil in summer; however, all the species in spring used the water available in the shallow soil. These findings have important implications for gypsum plant community structuring and maintenance, as evidenced by the hydrological niche segregation of plants. Chapter 2 did not show the use of crystallization water by any of the plant species analysed, but evidenced differences on the depth of the water used by species coexisting in remote drylands of Iran. This Chapter revealed three main strategies of water sources use among the studied plants: a permanent use of deep water throughout the year, a change from shallow soil water in spring to deeper soil water in summer and a principal use of the shallow soil water in both

seasons. Although limited in its methodological approach for logistic reasons, the study in Chapter 2 provides novel information on an understudied ecosystem, with a heterogeneous soil chemical composition including gypsum, and shows new information on the biology of five xerophytic shrubs that dominate the vegetation in the poorly studied Aladaghlar hill area (Iran-Turkey).

After verifying the widespread use of gypsum crystallization water during drought by shallow-rooted plants in gypsum plant communities (Chapter 1), we aimed to experimentally demonstrate its use and analyse whole-plant physiological adaptations to survive drought stress on gypsum. To that end, in Chapter 3 we performed an integrated analysis of the short-term responses to experimental drought of *H. squamatum* plants grown in natural gypsum soil and labelled gypsum soil. Unfortunately, our approach did not allow verifying the use of gypsum crystalline water by plants experimentally (see further comments below). Contrary to former findings, we observed a water-saving strategy in *H. squamatum*, reducing stomatal conductance and transpiration rate, and exuding choline by roots, an important osmo-protector molecule to face drought. This new strategy observed in our potted *H. squamatum* plants was associated to the sole use of the scarce free water contained in the pot soil, as no use of gypsum crystallization water was detected in the experiment. Contrary to the plant responses, soil microbiota composition in the pots did not vary or display signs of stress during drought, showing a good adaptation to soil desiccation.

After evaluating the above and belowground responses of gypsophiles under drought, we deepened our analysis into belowground processes in gypsum plants, specifically, into processes at the rhizosphere of the gypsophile *O. tridentata*. In Chapter 4 we showed how root growth in alkaline gypsum soils led to a remarkable acidification of the rhizosphere. This acidification was strongly affected by soil fungi. As we hypothesized, fungal presence in the soil led to a steeper acidification of the rhizosphere compared to plants grown in fungi-sterile soil. Our study also demonstrated the ability of gypsum plants to exude different organic acids and sugar alcohols (Chapter 3, 4), and showed how soil fungi can affect root exudation, with potential effects on soil pH, plant nutrition, gypsum crystalline water use and plant-fungal interactions.

In what follows, we explain each of the main findings of these PhD Thesis in more detail.

Eco-hydrological niche segregation revealed different seasonal water uptake strategies among species coexisting in gypsum soils

Previous studies comparing the isotopic composition of xylem water, groundwater and soil water, showed that the uptake of certain water sources by roots depends on different factors such as tree size (Dawson, 1996) or vegetation rooting patterns (Rossatto *et al*., 2014; White *et al*., 1985; Ehleringer & Dawson, 1992). This Thesis confirmed the predicted hydrological niche segregation in the two habitats studied, contributing to answer the question about how general this process is in natural plant communities (Silvertown *et al*., 2015). The observed pattern is in accordance with previous works done in arid or semiarid ecosystems: the partitioning of water sources depends on the variation of water distribution along horizontal and vertical gradients in the soil, and over time (Terradas *et al*., 2009). To identify water distribution patterns, we carefully characterised all potential sources of water available for plants considering variation in space, i.e. soil depths and its spatial replicates, and time, i.e. the wet and dry season (White & Smith, 2015; Sprenger *et al*., 2016). Thus, unlike classical approaches to niche segregation in coexisting plants, which considered temporal fluctuations of limited relevance (Araya *et al*., 2010), we measured water sources for plants in different seasons, as severe seasonal fluctuations in water an nutrients availability occur in arid and semiarid ecosystems (Austin *et al*., 2004).

The plant community studied in the gypsum hill in NE Spain (Chapter 1) showed that all species were mainly using the shallower soil water in the wet season, whereas there was a shift of the water sources used during water scarcity that could be explained according to the species rooting depth. Gypsum crystallization water was the main water source for most shallow-rooted species, whereas soil water from 50-100 m depth was the main water source for deep-rooted species. These results showed, for deep-rooted plants, that root water uptake is governed by the availability of water, and is also conditioned by plant rooting depth. We showed that most of the shallow-rooted plants use gypsum crystallization water during drought. The pattern observed in deep-rooted species is in accordance with Ryel *et al*. (2010), who concluded that dryland plants rapidly develop roots to use the soil moisture in the shallow soil, in order to maximize nutrient capture during the growth period, but exploit the deeper vadose zone to maintain transpiration and survive drought. Thus, not only water availability, but also nutrients, could be behind the importance of the rooting depth on the water uptake patterns observed in plants. All the plants in our study relied in the fertile topsoil

("growth pool") (Ryel *et al*., 2010) during the wet season. However, during the dry season, plants shifted to the utilization of water from deeper, wetter, but less fertile subsoil layers ("maintenance pool") (Barbeta *et al*., 2015) or to the utilization of crystallization water, a water source still to explore in terms of nutrients. We should emphasize that the main water source in summer for deep-rooted species was the water from 50-100 cm depth (i.e "rock moisture", Rempe and Dietrich, 2018), contrary to other studies, which identified groundwater as the main water source enabling the maintenance of activity during drought for deep-rooted species (Palacio *et al.*, 2017; Koirala *et al.*, 2016; Salvucci & Entekhabi, 1995; Fan *et al.*, 2017). Rock moisture allows taking advantage of the oxygenated conditions compared to groundwater, and seems to be a crucial source in arid ecosystems (Dwivedi *et al.*, 2019; Oshun *et al.*, 2019; Rempe and Dietrich, 2018; Hahm *et al.*, 2020). We suggest that the use of gypsum crystallization water needs to be considered as an alternative water source, at least to maintain transpiration during drought, even though the impact of its use on nutrient uptake is still unknown. In the Aladaghlar hill plant community studied (Chapter 2), several species were also able to change their water uptake pattern during the drought period. These results provide further evidence of nutrient availability being the main factor underlying root water uptake depth in the wet period but soil water availability being the main factor determining water uptake depth during the dry period, in agreement with previous works (Querejeta *et al*., 2021; Rose *et al*., 2003; Dai *et al*., 2015). The possibility to shift water sources from shallower to deeper soil water depending on soil moisture is due to the presence of dimorphic root systems in certain dryland plants (Dawson & Pate, 1996; Rempe & Dietrich, 2018). Contrastingly, some of the studied species, like *Oreosalsola montana* and *Caroxylon gemmascens* (Chapter 2) showed a constant main use of groundwater or superficial water, respectively, in spring and summer. These last strategies showed a dependency of water uptake on the rooting depth, independent of water or nutrient availability.

By unveiling the water uptake pattern of the five species in Aladaghlar hill (Chapter 2), we contributed to the description of the potential rooting depth and functioning of these poorly studied plant species. In addition, our study of water sources could serve as an indirect approximation to predict the impacts of global warming in the plant communities studied. Previous studies such as Berg *et al*., (2017) conclude that the reduction of soil moisture will affect, above all, the soil surface and not the deeper soil layers. Thus, whenever possible, the vegetation will shift to the use of deeper soil layers to keep transpiration. However, as evidenced by our results, not all the species

will have the same plasticity to change water sources; and the species which may be able to change, may be affected by a reduction of nutrient availability (Querejeta *et al*., 2021; Peñuelas *et al*., 2018; Luo *et al*., 2018). Further, plants will potentially decrease nutrient uptake and reduce water use efficiency, aboveground biomass growth and drought survival (Querejeta *et al*., 2021). Considering these arguments, we could expect the less vulnerable species to global warming from the Aladaghlar hill (Chapter 2) would be *O. montana,* which used groundwater throughout the year. Moreover, in the gypsum plant community located in NE Spain (Chapter 1), the observed water up-lift by species with dimorphic root systems could help other species in the community to survive the increasing drought forecasted. Nevertheless, several studies show the increasing vulnerability of deep-rooted species in drylands, particularly in areas with overexplotation of deep-water reservoirs such as Doñana National Park (South West Spain) and in the Pampa del Tamarugal Basin (Northern Chile) (Serrano & Serrano, 1996; Chávez *et al*., 2016).

The understanding of eco-hydrological niche segregation and its relation to water and nutrient availability in the soil is needed to promote the conservation of vegetation in habitats that face changing hydrology caused by human water use and climate change. Such information is needed to improve water use efficiency in vegetation management actions, which is one of the key issues for the sustainability of agricultural production, rural development and environmental protection (Pálfai, 2000; Somlyódy, 2000; Sutor & Gombos, 2006).

Gypsum crystallization water as the main water source during drought was observed only in the field

Our results of gypsum crystallization water use by plants during drought, shown in Chapter 1, are in accordance with previous studies. Palacio *et al* (2014) found evidence of the presence of gypsum crystallization water in the xylem sap of the gypsophile *H. squamatum*, and a transect study in Palacio *et al* (2017) revealed that the species using gypsum crystallization water were preferentially distributed on the slope of the gypsum hill, where the water table was far from the plants roots. The study done in Chapter 1 added new plant species to better characterize the community, and focused on the species distributed on the top of the hill, where groundwater was as far as possible. We confirmed the use gypsum crystallization water as the main water source in summer by almost all the shallow rooted plants analysed, contributing with the explanation of one of the plant strategies to survive drought in gypsum ecosystems. From these results, we strongly

recommend to incorporate this potential water source in water balance studies dealing with ecosystems developed on gypsum soils, which reach a big surface in all continents (Eswaran and Gong, 1991).

Nevertheless, Chapter 2 showed contrasting results with regard to the use of gypsum crystallization water by plants; although we could appreciate a minimum contribution of gypsum crystallization water to the xylem sap of several of the studied species, none of them relied on this water as the main source, contrary to the findings from the study in NE Spain. This result could presumably be due to the heterogeneity of the soil at the study site, with an irregular gypsum content that ranged from 4 to 84% in a few centimetres, and gypsum being scattered in different soil horizons along the same slope. Thus, plant species living on these soils could have developed other strategies to survive drought different to rely on the potential scarce gypsum crystallization water contained in the soil.

Contrary to our hypothesis of the potential higher use of gypsum crystallization water by gypsophiles, in both case studies (NE Spain and SW Iran) we found that affinity for gypsum was not a significant factor to explain this water use. Therefore, the ability to use gypsum crystallization water does not seem to contribute to the specialization of plants to this atypical soil. The use of gypsum crystallization water does not differ between gypsophiles and gypsovags, but seemed to be a common strategy shared by most shallow-rooted species in pure gypsum soils, such as those in NE Spain.

Our physiological approach in Chapter 3 to prove the use of gypsum crystallization water experimentally did not arrive to a direct observation of the process. First, the labelling treatment changed important physical and chemical properties of the soil, resulting in a decrease in the soil microbial biomass, sandier soil texture which dried faster and whose gypsum thermodynamics were also altered (see Chapter 3). Thus, plants grown in the deuterium-labelled gypsum soil had lower biomass and were more stressed by the drought treatment, showing reduced photosynthetic rate and generally reduced leaf nutrient concentration. In addition, and contrary to our hypothesis, plants subjected to the labelling and drought treatment, did not show the use of gypsum crystallization water, i.e. the labelling was just detected in the crystalline water of the soil, but not in the xylem or the bulk biomass of plants. It could be argued that the changed soil characteristics

could be affecting the process of gypsum crystallization water use by plants. However, plants growing on natural gypsum soil subjected to the drought treatment in pots did not show the use of crystalline water as a strategy to survive experimental drought either. We can conclude that the short-term drought experiment developed in the potted *H. squamatum* plants did not favour the strategy of using gypsum crystallization water to survive, and plants were forced to decrease the use of free soil water by physiological adjustments. The lack of crystallization water use in our pot trial could be due to the artificial environment where plants grew compared to natural environments in the field (e.g. Chapter 1). Although care was taken to mimic natural conditions as much as possible, our experimental set up might have potentially altered water and nutrient availability, root foraging ability, soil temperature and microbial communities composition and performance (Lynch *et al*, 2012).

Despite these limitations, in Chapter 3 and Chapter 4 we evidenced the ability of two gypsum specialists to exude different organic acids and sugar alcohols, which could potentially affect the release of gypsum crystallization water (Van Driessche *et al*., 2017; Tritschler *et al*., 2015). Similarly, Huang *et al* (2020) showed gypsum dissolution by organic acid exudation was the mechanism behind gypsum crystallization water use by Actinobacteria growing in gypsum rocks from the Atacama Desert.

Above and belowground physiological performance of two cultivated gypsophiles showed new specific strategies to cope with gypsum soil limitations

The physiological strategy to face drought of *H. squamatum* previously reported in works such as Querejeta *et al*., (2021) or León-Sánchez *et al*., (2018) involved a water-spender strategy with limited stomatal regulation. However, in our study, we showed for the first time, a short-term response of this species to drought that included a coordinated drought avoidance strategy decreasing stomatal conductance and transpiration; and releasing choline as root exudate. This molecule is an osmoprotector, reflecting a plant active response to avoid drought stress (Sakamoto & Murumata, 2021). The decrease in water use was associated with reduced nutrient uptake, leaf senescence and nutrient recycling, reflected in the generalised lower leaf nutrient content in the individuals subjected to drought, except for N and P content that could be recycled from the senescent leaves. These plant responses have been previously reported as indicators of drought stress in other works (Suriyagoda *et al*., 2014; Hussain *et al*., 2018; Munné-Bosch & Alegre, 2004).

Several differences in our methodological approach can explain the contrasting results obtained in relation to previous studies. Factors such as drought intensity, frequency, duration and different soil variables have a substantial impact on the overall effect and duration of drought-related symptoms in plants (Ali *et al*., 2022). Accordingly, our approach was developed in plants cultivated in pots from the seeds for 3-years and then subjected to a 24 day-long drought treatment, without any associated temperature change. Contrastingly, León-Sánchez *et al*. (2018) and Querejeta *et al*. (2021) did a manipulative field experiment assessing the effects of warming and rain reduction where plants were subjected to treatments for 4 and 6 years, respectively. Our short-term drought treatment did not alter the relative abundance of soil fungi with respect to the natural treatment, whereas León-Sánchez *et al*. (2018) reported a significant reduction of the mycorrhizal fungi with the associated nitrogen and phosphorus leaf reduction. These elements were not shown to decrease in our short-term drought experiment, as *H. squamatum* was potentially recycling them from the senescent leaves or the unaltered microbiota was still favouring their absorption, even if the transpiration flow was reduced. This new reported response reflects the species plasticity to act as a water-spender or water-saver according to the type of drought it is facing. Additionally, the water-saver strategy observed could be a consequence of the inability to use gypsum crystallization water in potted plants, which could have also restricted transpiration flow.

Contrasting with the rapid response recorded in plant physiology, no changes were observed in the abundance or stress indicators of the soil microbiota according to PLFAs analyses, likely due to its tight adaptation to changes in soil moisture. This is an important remark for plant life on gypsum, as the resistant microbiota could play a key role on the acquisition of nutrients and water and the reduction of plant stress in the longer term (Marasco *et al*., 2012; Zhang *et al*., 2021). In fact, this PhD Thesis provided evidence of a strong impact of the soil microbiota, specifically soil fungi, on the rhizosphere chemical environment and the release of exudates by plants, both processes related to resource uptake by plants. In addition, non- reported results from the pot Trial showed that *H. squamatum* did not survive to become adult when cultivated on fungi-sterile soils (all individuals cultivated from seed on fungi-sterile soil died before reaching 2-years of life, N = 20). This could be an evidence of the strong dependence of *H. squamatum* on soil fungi to complete its life cycle. Indeed, this species is known to form symbiotic associations both with arbuscular and ectomycorrhizal fungi (Palacio *et al*., 2012). These results agree with numerous studies highlighting the crucial role of soil fungi for plant nutrition and growth (Bridge& Spooner, 2001;

Behie, & Bidochka, 2014; Pennisi, 2004), which seem to be fundamental in nutrient poor soils like gypsum (Liang *et al*., 2022; Van Der Hejiden *et al*., 2008)

In Chapter 4 we showed that *O. tridentata* seedlings growing in natural soil showed a steeper acidification of the rhizosphere than individuals growing in fungi-sterile soil. Gypsum soils show neutral to alkaline pHs, sometimes reaching remarkably high pH of 9 due to calcareous intrusions (Poch *et al*., 2018). Under these circumstances, rhizosphere acidification may be crucial for plant grow. The release of protons (H^+) to the rhizosphere is promoted by soil fungi (Zhu *et al*., 2023), which increases nutrient availability for plants (Ma *et al*., 2021).

Another belowground response recorded in the gypsum specialists studied includes the release of different root exudates to the soil. Different organic acids such as Citric, Isocitric, Lactic, Fumaric, Maleic, Malic and Succinic; and different sugar alcohols such as Galactinol, Myo-Inositol, Sorbitol, Mannitol and Xylitol were released by the roots of studied species. These compounds could be acting as signals promoting symbiotic or mutualistic microorganisms related to nutrient and/or water acquisition (Dakora & Philips, 2022; Yu *et al*., 2023). Indeed, as reported in Chapter 4, they were increasingly released by fungi-deprived individuals. Additionally, root exudation could have a direct role in nutrient acquisition allowing *O. tridentata* seedlings to grow in soils with reduced fungal presence and to acidify the rhizosphere, even if the acidification does not reach the same degree of replicates growing in natural gypsum soil. Our results showed that *O. tridentata* is not fully dependent on the microbiota for nutrient acquisition, although it may facilitate the process. We also showed the ability of both gypsophiles (*H. squamatum* and *O. tridentata*) to exude organic acids and sugar alcohols that could be mediating plant nutrition or the access to gypsum crystallization water. The study included in Chapter 4 constitutes a pioneering work on the physiological mechanisms underlying rhizosphere acidification in a wild plant species, contrasting with most previous studies that focused on cultivated plants.

Limitations of the study and future lines of research

Limitations and future work on data collection in the field

Chapter 1 corroborated the presence of gypsum crystallization water in the xylem of the majorityof shallow rooted plants from a gypsum hill community of NE Spain in summer. Nevertheless, there

could be local particularities involving plant species or environmental conditions that vary from this to other gypsum ecosystems (Mota el al, 2011). Thus, to ascertain if the use of gypsum crystalline water is a common strategy of shallow-rooted plants growing in gypsum plant communities during drought, future research should perform similar studies in other gypsum ecosystems around the world.

Chapter 1 and 2 revealed eco-hydrological niche segregation, highly marked in the dry season, which evidences the ability of plant communities to face aridity. However, finding a clear pattern of water partitioning according to different plant traits was difficult in Chapter 2. This was due to the very different traits of the plant species studied concerning morphology, tissue organization or rooting depth, which could not be fully characterised by our model (for example considering trait interactions) due to insufficient species replication. To overcome these issues, future analyses of plant water use should be performed including below and aboveground parameters and additional data concerning plant total size to better understand the water use in under-studied dryland communities, like the one we analysed in the Alaghladar hills in Iran. Additionally, we recommend future studies on the use of gypsum crystallization water to focus on homogeneous gypsum landscapes, where gypsum mineral is clearly conditioning plant life.

In Chapter 1, we could observe an indirect evidence of hydraulic lift because there were several plant species with shallow rooting system using deep water. In addition, several deep-rooted species (*Gypsophila struthium*, *Rosmarinus officinalis* and *Thymelaea tinctoria*) showed similarities in the δ^2H between the shallow soil beneath them and their xylem composition. However, we had no spatial data to account for the position of shallow-rooted and deep-rooted species potentially involved in this process, so neighbouring species data are required to confirm the occurrence of hydraulic lift in our studied ecosystem (Filella and Peñuelas, 2003). This future line of research could help, additionally, to explain the use of gypsum crystallization water as a generalised strategy of shallow-rooted species and its potential impact on the water use of accompanying species.

Limitations of experimental procedures

The novel methodology applied in Chapter 3 to unveil the mechanisms displayed by plants to use gypsum crystallization water led to severe limitations. Labelling of gypsum crystallization water

resulted in a gypsum soil with different physicochemical properties that substantially modified *H. squamatum* environment, as compared to the natural soil replicates. This was considered a methodological limitation that hampered addressing the objective of the study. Soil labelling increased the temperature of gypsum dehydration, affecting calculations on soil gypsum content based on thermo-gravimetric procedures. In addition, the labelling procedure, which implied previous soil dehydration, resulted in a soil with sandier texture and lower organic matter that dried faster (Ros *et al*., 2003). Because of all these changes, labelled soil modified the soil microbial biomass, plant photosynthetic rate, aerial biomass and leaf elemental concentration. Plants growing on the labelled soil showed the effects of increased water stress and lower soil fertility. New methodologies should be applied in the future to trace gypsum crystallization water use by plants experimentally without such remarkable alterations.

On the other hand, in Chapter 4 we showed for the first time *in situ* rhizosphere acidification of a gypsum specialist growing in gypsum soil, a process facilitated by soil fungi and root exudation of organic acids and sugar alcohols. However, it would be very interesting to compare these processes in different gypsum plant species, including gypsum specialist and other generalist species. We initially designed the experiment to compare this species with a closely-related gypsovag species (*Ononis fruticosa*), as well as two shallow-rooted species, *Helianthemum squamatum* (gypsophile) and *Helianthemum syriacum* (gypsovag). The difficulties to keep a sufficient number of replicates alive in other species in addition to the roots redirection that avoided passing thought the sensor, restricted our results to the observation of the processes in the rhizosphere of *Ononis tridentata* Unfortunately, seedling survival and development in this artificial micro-environment was limited for the other species, whose interesting preliminary results about rhizosphere acidification, however, are shown in the Supplementary Material. An improved methodology is needed in future research on the belowground physiological adaptations of gypsophiles and gypsovags, which will undoubtedly contribute to unveil the factors underlying the ecological adaptations of these species.

Lessons learned from unsuccessful experiments

The study shown in Chapter 3, concerning *H. squamatum* cultivation in pots subjected to a labelling treatment of the gypsum crystallization water and to a short-term drought treatment, considered initially an additional treatment including soil fungi sterilisation, identical to the

treatment applied in Chapter 4, which was not finally included in the experiment results. This idea was applied with the aim to test if soil fungi were involved in the rooting uptake of gypsum crystallization water during drought. However, the treatment was not successful for our experiment of three-year duration because none of the *H. squamatum* seedlings cultivated in fungi-sterile pots from the seed survived. Thus, the answer to our question about the role of fungi in the uptake of gypsum crystallization water remains unanswered. New methods are needed in future research addressing the use of gypsum crystalline water by plants, focusing on the study of adult plants but maintaining the environmental conditions as similar as possible to the ones in the field. Nevertheless, these negative results have provided very interesting information about the serious consequences of the elimination of the soil fungi for *H. squamatum*. Future research should corroborate the strong dependency on soil fungi of this species.

Another attempt at the experimental observation of the process of gypsum crystallization water involved X-ray diffractometry to quantify different phases of calcium sulphate (namely gypsum, basanite and anhydrite). If gypsum dehydration was taking place in the rhizosphere during drought, we expected to observe anhydrite deposits by chemical X-ray diffractometry as found by Huang *et al*., (2020). To this end, during my PhD Thesis we performed a proof of concept involving the cultivation of *H. squamatum* in mini-rhizoboxes, as described in the General Methodology. The aim of that proof was to use X-ray diffractometry to observe the formation of anhydrite deposits in the rhizosphere. In our experiment, *H. squamatum* seedlings were grown in natural gypsum soil contained in mini-rhizoboxes and were subjected to a drought treatment with the aim to force the use of gypsum crystallization water, as observed in the field. However, the seedlings of barely twenty days of life, did not endure the stressful conditions and died before the analysis. The control (well-watered) replicates that survived, gave us preliminary results (data not shown) where only gypsum was found next to the roots surface of the seedlings. No signs of anhydrite were found under well-watered conditions.

Future directions to unveil the mechanisms of gypsum crystallization water use

We found contrasting results regarding crystalline gypsum water use by plants. It could be suggested that the detection of gypsum crystallization water is an artefact caused by the recrystallization of gypsum inside the xylem of the plants due to drying. Recent studies have shown that gypsophile plants tend to accumulate dissolved gypsum in their tissues at oversaturation, likely

inside cell vacuoles. The disruption of cell osmotic conditions by drying leads to gypsum crystallization (Cera *et al*. submitted), which would be subsequently extracted in the cryogenic distillation line. This process would be similar to the reported precipitation of gypsum in pipes or in laboratories simulations with temperatures from 20ºC to 45ºC (Hoang *et al*., 2007; Liu & Nancollas, 1970; Kontrec *et al*., 2002; Witkamp *et al*., 1990). However, this process will not explain the detection of big amounts of gypsum crystallization water in non-specialist gypsum plant species by de la Puente et., (2021) and Palacio *et al*., (2017), which are known to block S uptake at fine root level and avoid oversaturation of calcium sulphate in the stem and leaves, hence avoiding calcium and sulphate accumulation (Cera *et al*., 2022).

Future research should try to answer the question of: which were the factors that, present in the field and absent in the potted experiment, determine the use of the gypsum crystallization water by plants? We, definitely, encourage to continue this line of research. To get closer to this explanation, future research could address the biological similitudes of shallow-rooted plant species, or focus on neighbouring plant facilitation, where some species could be benefiting from others to obtain gypsum crystallization water, similar to restauration studies showing the improvement of micro-environmental conditions by some shrubs (Foronda-Vázquez *et al*., 2019). Our findings also point out at the release of root exudates like organic acids and sugar alcohols as potential mechanisms underlying gypsum crystalline water use by plants. Another potential mechanism could engage surface gypsum thermodynamics, as a passive dehydration where plants do not actively participate, but they benefit from the released water. All these hypotheses open up new avenues of exciting future research.

Main Conclusions/ Conclusiones Principales

- Hydrological niche segregation occurs during the drought season in a gypsum plant community in NE Spain, where the water source used by each species depends on the rooting depth of the species, but not on its gypsum affinity.
- Gypsum crystallization water was detected as the main water source for almost all the shallow rooted species living on the top of the hill, confirming its important role for life in gypsum drylands.
- Deep soil water (50 cm-100 cm depth) is the main water source for deep-rooted species, constituting, together with gypsum crystallization water, an important water source for the formation of diverse plant communities adapted to water scarcity.
- The dominant xerophytic shrubs in the Aladaghlar hill area (NW Iran) show highly marked hydrological niche segregation in the drought season. These species partition the use of shallower and deeper soil water among coexisting species without relying on gypsum crystallization water to survive drought.
- In NW Iran, the water sources used in each season (wet and dry) do not depend on the photosynthetic pathway or gypsum affinity of plants. Rooting architecture, however, was a good proxy for the water sources used by three of the five studied species.
- The experimental set up of the potted *H. squamatum* plants did not allow the demonstration of the use of gypsum crystallization water during a short-term drought treatment. Additionally, the constraint to use only the scarce free water available in the pot during drought may promote a water-saving strategy, not described before for this species.
- *H. squamatum* is able to exude different organic acids and sugar alcohols. In addition, it can increase choline exudation during drought, as an osmotic protection under drought stress.
- The gypsum soil microbiota is adapted to the strong drought-pulses of gypsum ecosystems, reflected on the stable PLFAs composition of the soil under the short-term drought applied.

The relative abundance of different soil microbial groups was not affected and the PLFA indexes analysed did not reflect microbial stress.

- Rhizosphere acidification is needed to grow in poor-nutrient alkaline gypsum soils and it is more intense in the root tip of the growing root.
- Soil fungi mediate rhizosphere soil pH decreases, as the fungi-sterile rhizospere of *O. tridentata* did not reach pH as lower as the natural replicates.
- Like *H. squamatum*, *O. tridentata*, also exudes organic aliphatic acids and sugar-alcohols which contribute to pH acidification in rhizosphere soil.
- The absence of fungi in the soil modified root exudation profiles of *O. tridentata*, which increased exudation of certain compounds related to fungi-attraction, likely as a means to help with nutrient acquisition in fungi-sterile soils.

- Hay segregación de nichos hidroecológicos durante la estación seca en la comunidad de plantas de suelos de yeso estudiada en el NE de España, donde la fuente de agua utilizada por las especies depende de su profundidad de raíz, y no de su afinidad al yeso.
- El agua de cristalización del yeso es la principal fuente de agua usada en verano por casi todas las especies de raíz somera que habitan las zonas altas de las colinas de yeso (más alejadas del nivel freático), lo que confirma su importante papel para la vida en los ecosistemas de suelos de yeso.
- El agua profunda del suelo (50-100 cm de profundidad) es la principal fuente de agua para las especies de raíz profunda, constituyendo, junto con el agua de cristalización del yeso, una importante fuente de agua para la formación de diversas comunidades de plantas adaptadas a la escasez de agua.
- Los arbustos xerófitos dominantes que coexisten en las colinas de Aladaghlar (NO Irán) presentan segregación de nichos hidroecológicos, más marcada en la estación seca. Estas especies se reparten el agua más superficial y más profunda libre en el suelo, sin depender del agua de cristalización para sobrevivir a la sequía.
- En el NO Irán, las fuentes de agua utilizadas en las distintas estaciones (húmeda y seca) no dependen de la vía fotosintética (C3/C4) o la afinidad al yeso de las plantas. La arquitectura del sistema radicular, sin embargo, es un factor explicativo de las fuentes de agua usadas por tres de las cinco especies estudiadas.
- El montaje experimental de las plantas de *H. squamatum* cultivadas en macetas no permitió demostrar el uso de agua de cristalización del yeso durante el tratamiento de sequía aplicado a corto plazo. La restricción a utilizar el escaso agua libre disponible en el suelo de la maceta, puede promover una estrategia de ahorro de agua que no se había descrito todavía para esta especie.
- *H. squamatum* tiene la capacidad de exudar distintos ácidos orgánicos y alcoholes de bajo peso molecular. Además, puede incrementar la exudación de colina, como protección osmótica durante el estrés por sequía.
- La microbiota del suelo de yeso está adaptada a los pulsos de sequía que habitualmente sufren los ecosistemas de yeso, ya que la abundancia relativa de los distintos grupos de microorganismos y los índices de estrés observados en la composición de PLFAs no varió en el tratamiento de sequía aplicado a las macetas.

- La acidificación de la rizosfera es necesaria para que las plantas puedan crecer en el suelo de yeso, que es alcalino y pobre en nutrientes. La acidificación es más intensa en la punta de la raíz en crecimiento.
- Los hongos del suelo median esta disminución del pH, ya que la rizosfera de las plántulas que crecieron en el suelo con menos presencia de hongos no alcanzó pH tan bajos como las réplicas que crecieron en suelo natural.
- *O. tridentata* también exuda ácidos orgánicos alifáticos y alcoholes de bajo peso molecular, que contribuyen con la acidificación de la rizosfera en el suelo.
- La disminución de la presencia de hongos en el suelo modificó el perfil de exudación de *O. tridentata*, que aumentó para ciertos compuestos relacionados con la atracción de hongos y, probablemente, con la adquisición de nutrientes.

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SUPPORTING INFORMATION

CHAPTER 1. Disentangling water sources in a gypsum plant community. Gypsum crystallization water is a key source for shallow-rooted plants

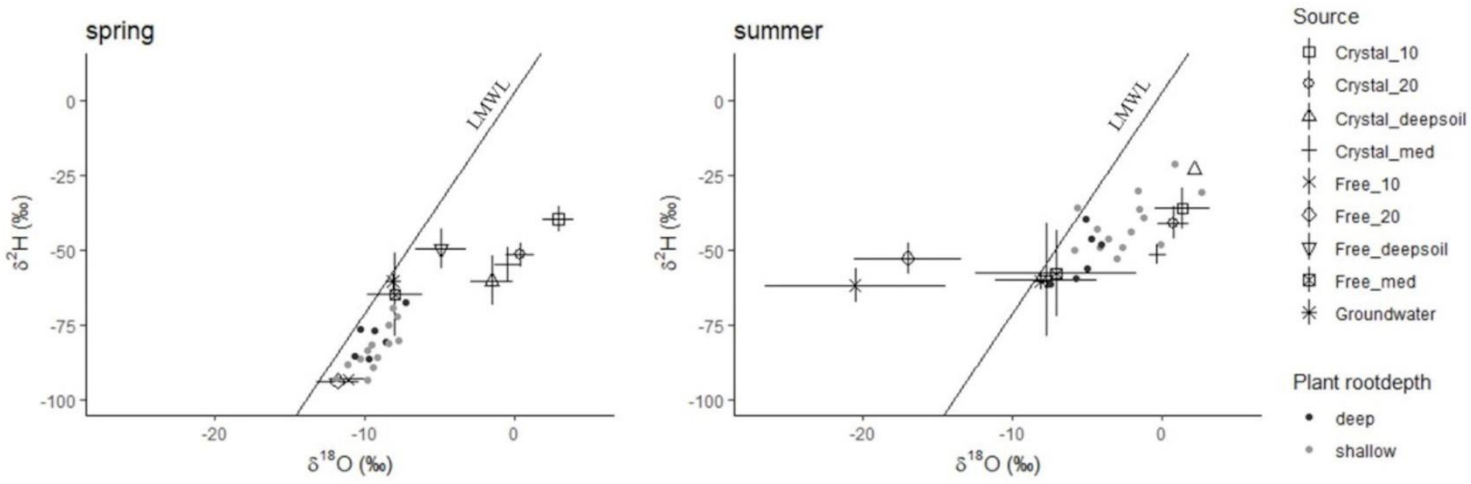


Fig. S1. δ^2H and $\delta^{18}O$ composition of the xylem sap of the plant species and eight different water sources. Water sources include gypsum crystallization water ("Crystal") and free water ("Free") in the soil at different depths. 10 and 20 cm deep soil was collected under the plants and deeper soil was collected from the profiles. "med" represents the mean composition of the water extracted from the soil at 30 and 40 cm deep, and "deepsoil" represents a mean composition of the soil from 50 to 100 cm deep. Grey points are for shallow rooted plants and black points for deep rooted plants. Groundwater is also represented. LMWL: local meteoric water line

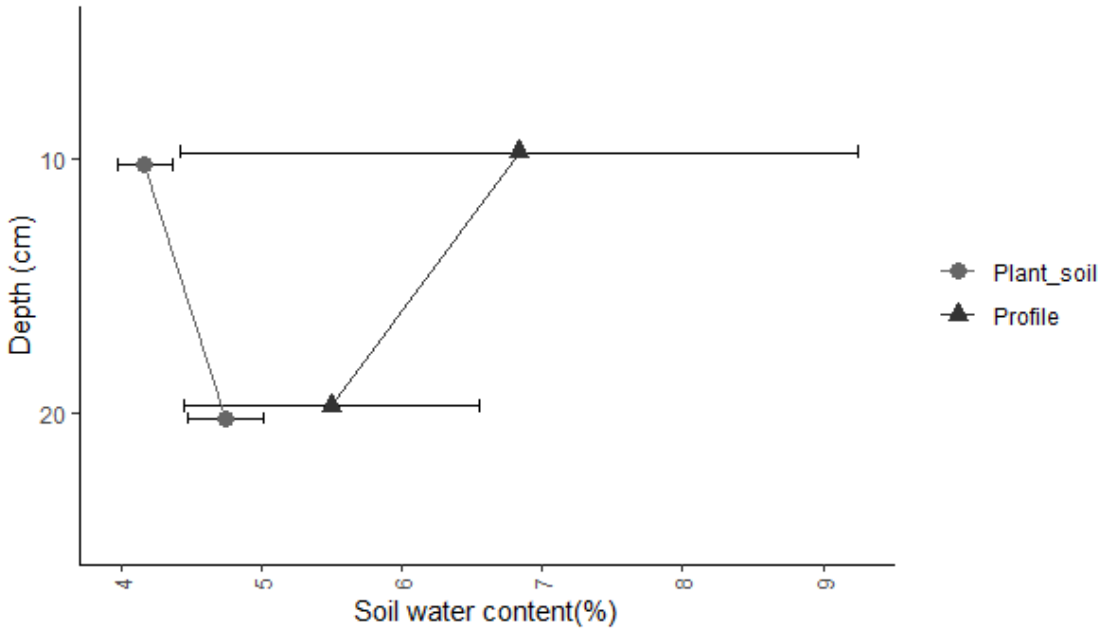


Figure S2. Summer water content in the first 10 and 20 cm of the soil. Grey points show values for the water underneath the plants and black triangles the values in the profiles (bare soil).

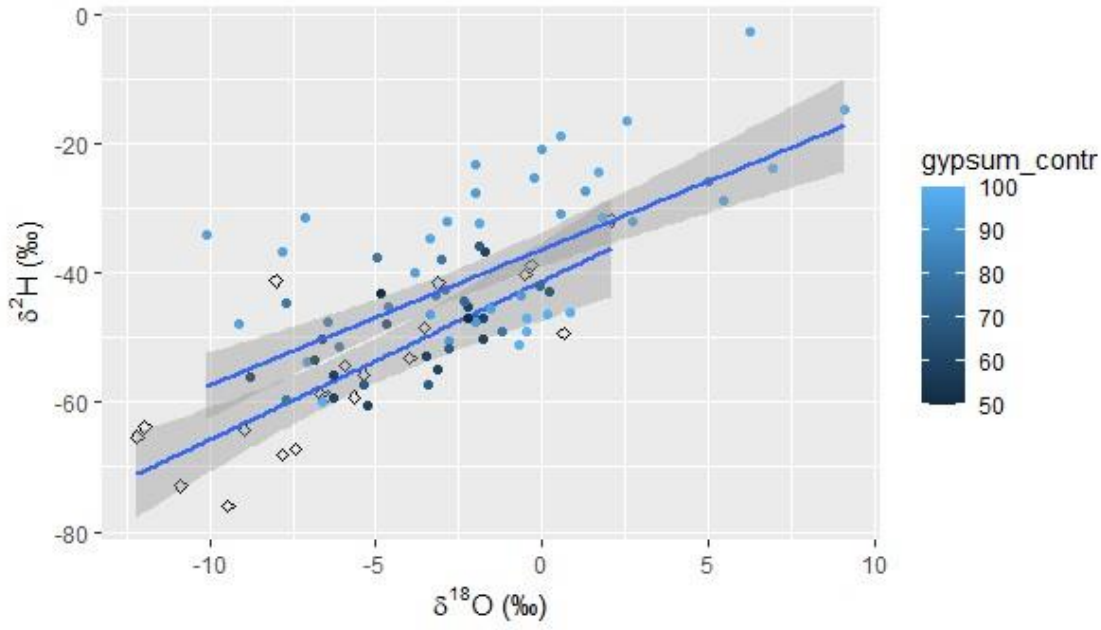


Figure S3 Isotopic composition of the free water in the bare soil up to 50 cm deep, obtained from the soil profiles (empty diamonds) and isotopic composition of the xylem of shallow rooted plants in summer (filled circles). The colour scale of the points represents gypsum water contribution in the species xylem sap according to the MixSIAR model. Lighter blue for larger contribution and darker blue for smaller contribution. The regression lines show the different slopes of each group of isotopic data (xylem sap of the plants; and soil water).

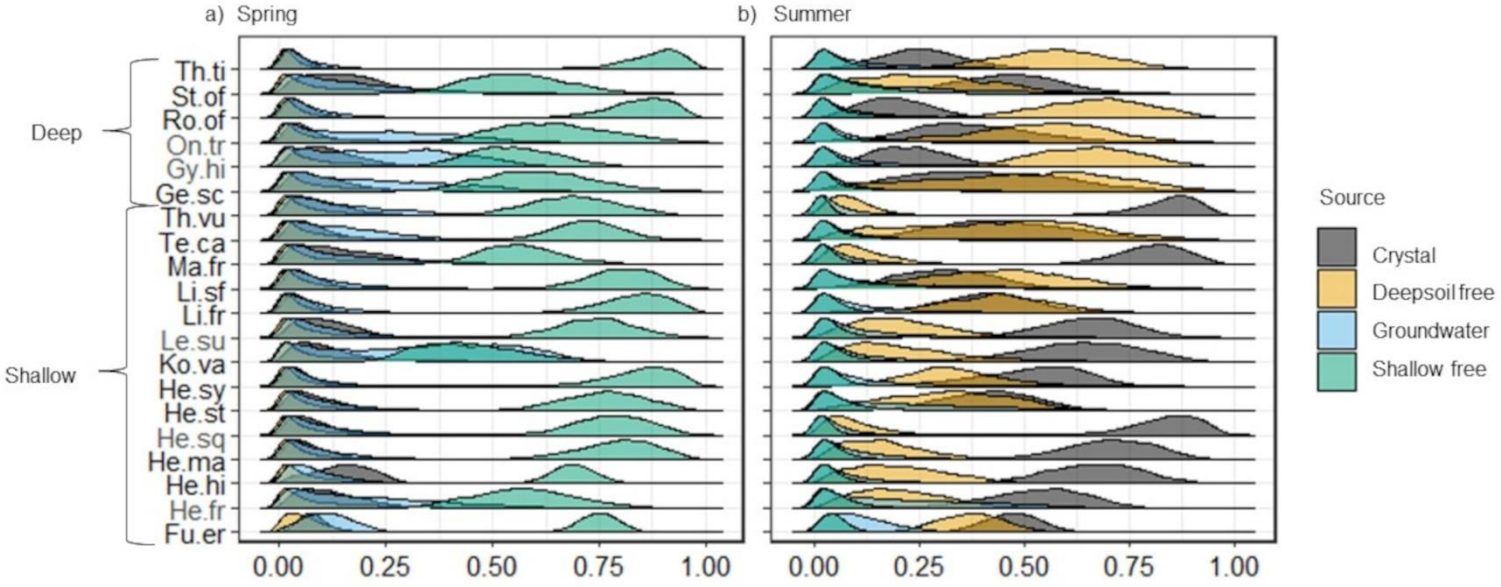


Figure S4. Results from Bayesian stable isotope mixing models showing the estimated contribution of shallow free water (10 – 20 cm), deep free water (50 – 100 cm), groundwater and gypsum crystallization water (all depths combined) to the xylem water of 20 dominant species coexisting in a gypsum hill in NE Spain. The first six species were deep-rooted, and the following 14 were shallow-rooted. Gypsovag species names are typed in black, those of gypsophyles are in grey.

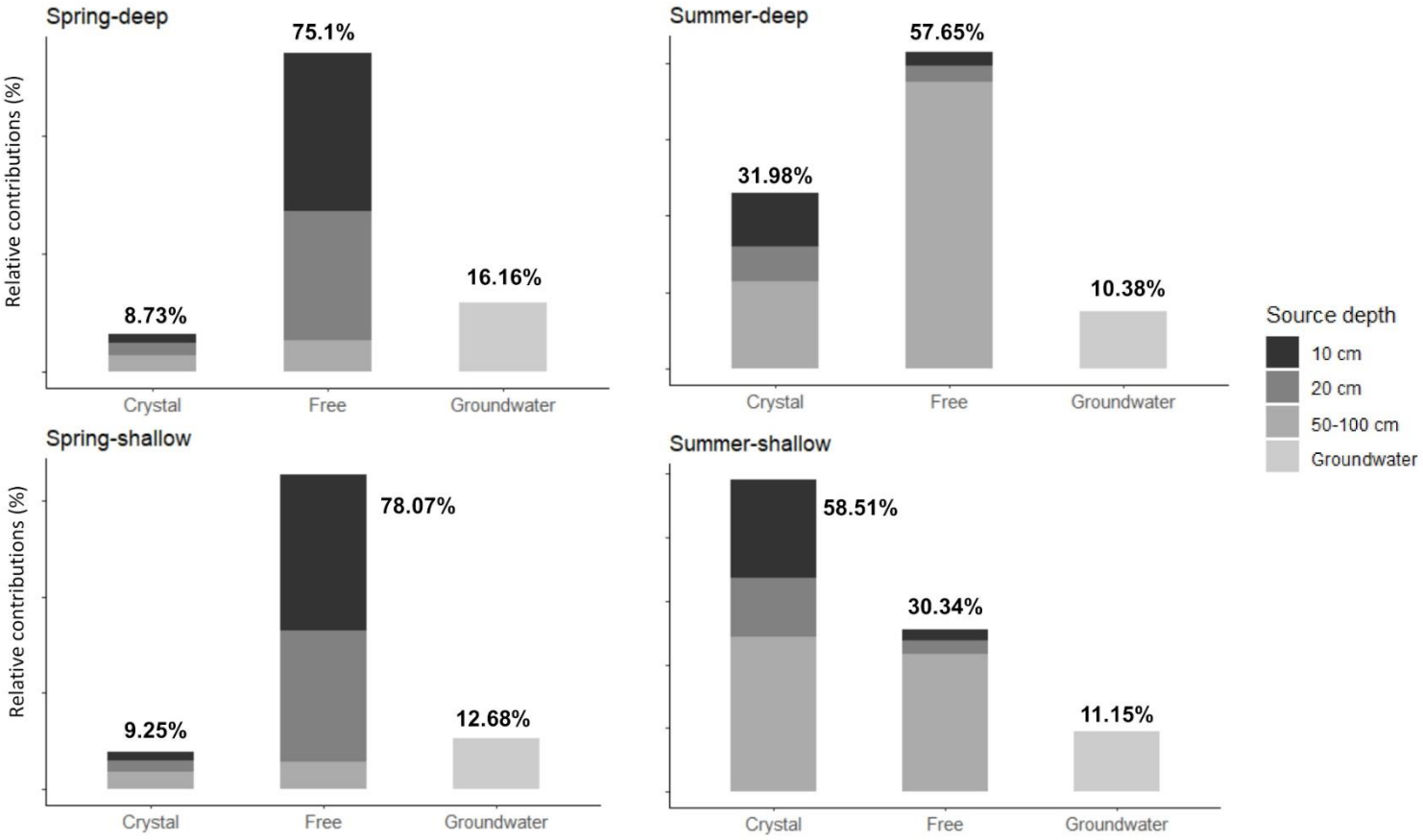


Figure S5. Results from Bayesian stable isotope mixing models showing the contribution of seven different water pools to the xylem water of plants, analysed separately for deep- and shallow-rooted species in each season. Percentages show the total contribution of gypsum crystallization water (crystal), free soil water and groundwater to the xylem of the shallow and deep rooted species, in spring and summer.

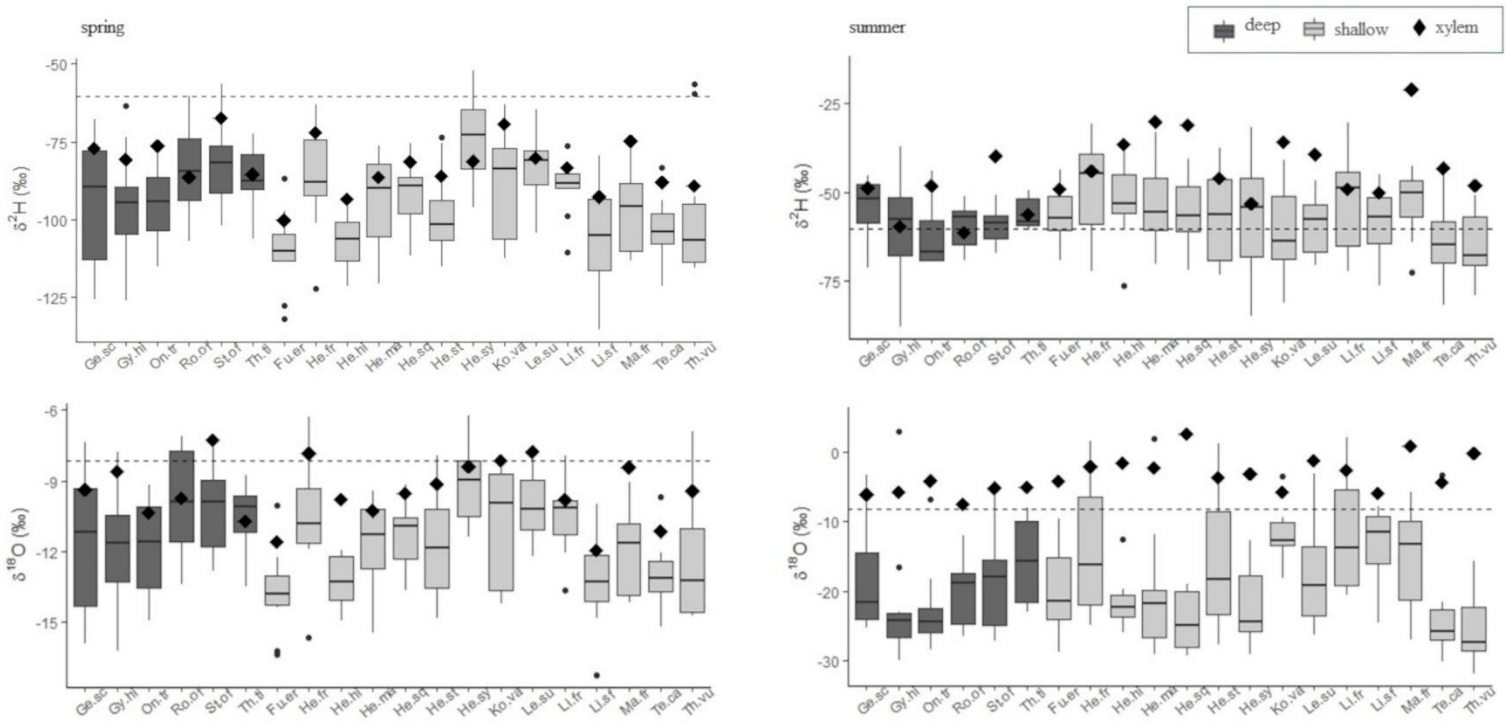


Figure S6. Spring and summer isotopic values of δ^2H $\delta^{18}O$ of free water in the soil underneath plants (boxes) and mean values for the xylem water of each species (diamonds). Boxes indicate the mean per species for the two soil depths analysed: 0-10 and 10-20 cm (N = 10 replicates), plus the upper and lower quartiles, and whiskers show maximum and minimum values. Darker boxes denote deep-rooted species; light grey boxes are shallow-rooted plants. Dashed line indicates groundwater values.

Table S1. Water isotopic values of composite monthly samples of precipitation in Zaragoza in the hydrological year 2017-2018. As a reference, long-term means (1981-2010 for precipitation; 2000-2016 for isotopes) are shown between brackets. Isotope values were contributed by the REVIP (Red de Vigilancia de Isótopos en Precipitación), managed by CEDEX (Centro de Estudios de Técnicas Aplicadas del Centro de Estudios y Experimentación de Obras Públicas), in collaboration with AEMET (Agencia Estatal de Meteorología). Meteorological data were provided by AEMET OpenData (https://opendata.aemet.es/). It should be noted that precipitation recorded in August occurred after sampling.

Date	**Precipitation (mm)**	**$\delta^{2}H$ (‰)**	**$\delta^{18}O$ (‰)**
10/2017	4 (36)	-21.40 (-42.56)	-2.29 (-6.21)
11/2017	1 (30)	-5.84 (-61.15)	2.49 (-8.89)
12/2017	8 (21)	-46.96 (-51.55)	-6.19 (-7.49)
01/2018	48 (21)	-93.22 (-60.15)	-12.68 (-8.32)
02/2018	31 (22)	-69.82 (-61.41)	-10.47 (-8.46)
03/2018	38 (19)	-57.36 (-43.16)	-7.44 (-6.40)
04/2018	109 (39)	-111.33 (-39.93)	-14.81 (-5.85)
05/2018	82 (44)	-34.79 (-31.47)	-4.97 (-4.70)
06/2018	11 (26)	-25.98 (-31.27)	-3.09 (-4.42)
07/2018	47 (17)	-29.34 (-24.60)	-4.39 (-3.60)
08/2018	66 (17)	-25.63 (-22.68)	-4.37 (-3.56)
09/2018	18 (30)	-20.53 (-30.62)	-3.61 (-4.71)

Table S2. Statistics of GLMM analyzing the effects of the root depth, affinity for gypsum soils (gypsophily), season and their interaction on the isotopic composition ($\delta^{18}O$, $\delta^{2}H$, D-excess) of the xylem water of plants. Species, family and species nested within family were included as random terms. *F-ratio*s and *p-value*s are shown. Bold type indicates significant effects at $\alpha < 0.05$.

Factor	$\delta^{2}H$ (‰)		$\delta^{18}O$ (‰)		D excess (‰)	
	F	*p*	*F*	*p*	*F*	*p*
Gypsophily	0.65	0.434	2.81	0.113	3.06	0.100
Rootdepth	0.10	0.333	3.86	0.070	6.88	**0.021**
Season	606.82	**<0.001**	305.50	**<0.001**	48.27	**<0.001**
Gypsophily:rootdepth	0.55	0.476	1.25	0.285	0.77	0.398
Gypsophilly:season	1.62	0.205	0.03	0.873	1.33	0.251
Rootdepth:season	23.95	**<0.001**	17.69	**<0.001**	6.03	**0.015**
Gypsophily:rootdepth:season	0.09	0.759	0.08	0.774	0.50	0.480

Table S3. Results of GLMMs analyzing the effects of root depth, affinity for gypsum soils (gypsophily) and their interaction on the isotopic composition ($\delta^{18}O$, $\delta^{2}H$, D-excess) of the xylem water of plants in spring and summer. Species, family and species nested within family were included as random terms. *F-ratio*s and *p-value*s are shown. Bold type indicates significant effects at $\alpha < 0.05$.

		$\delta^{2}H$(‰)		$\delta^{18}O$(‰)		D-excess (‰)	
		F	*p*	*F*	*p*	*F*	*p*
Spring	Gypsophily	1.22	0.287	1.62	0.222	0.82	0.377
	Rootdepth	0.25	0.628	0.00	0.981	2.47	0.138
	Gypsophily x rootdepth	1.00	0.341	0.97	0.349	0.20	0.666
Summer	Gypsophily	0.09	0.768	1.48	0.244	1.81	0.200
	Rootdepth	4.61	**0.049**	9.22	**0.010**	5.73	**0.032**
	Gypsophily x rootdepth	0.25	0.626	0.71	0.419	0.49	0.499

Table S4. Results of GLMMs analyzing the effects of root depth in the isotopic composition of the soil water underneath the plants. Different analyses were run for each isotope and season (two levels: spring and summer). Models included taxonomic family and species nested within family. *F* ratios and *p* values are shown. Bold type indicates significant effects at $\alpha < 0.05$

Season	**Factor**	**Isotope**	***F***	***p-value***
Spring	Gypsophily	δ^2H	0.51	0.487
		$\delta^{18}O$	0.38	0.547
	Roothdepth	δ^2H	0.87	0.365
		$\delta^{18}O$	0.88	0.364
	interaction	δ^2H	3.52	0.086
		$\delta^{18}O$	3.59	0.084
Summer	Gypsophily	δ^2H	0.13	0.727
		$\delta^{18}O$	2.08	0.149
	Rootdepth	δ^2H	0.00	0.949
		$\delta^{18}O$	0.47	0.501
	interaction	δ^2H	2.79	0.121
		$\delta^{18}O$	1.30	0.275

CHAPTER 2. Ecohydrological niche segregation among desert shrubs in a gypsum-calcareous formation (NW Iran)

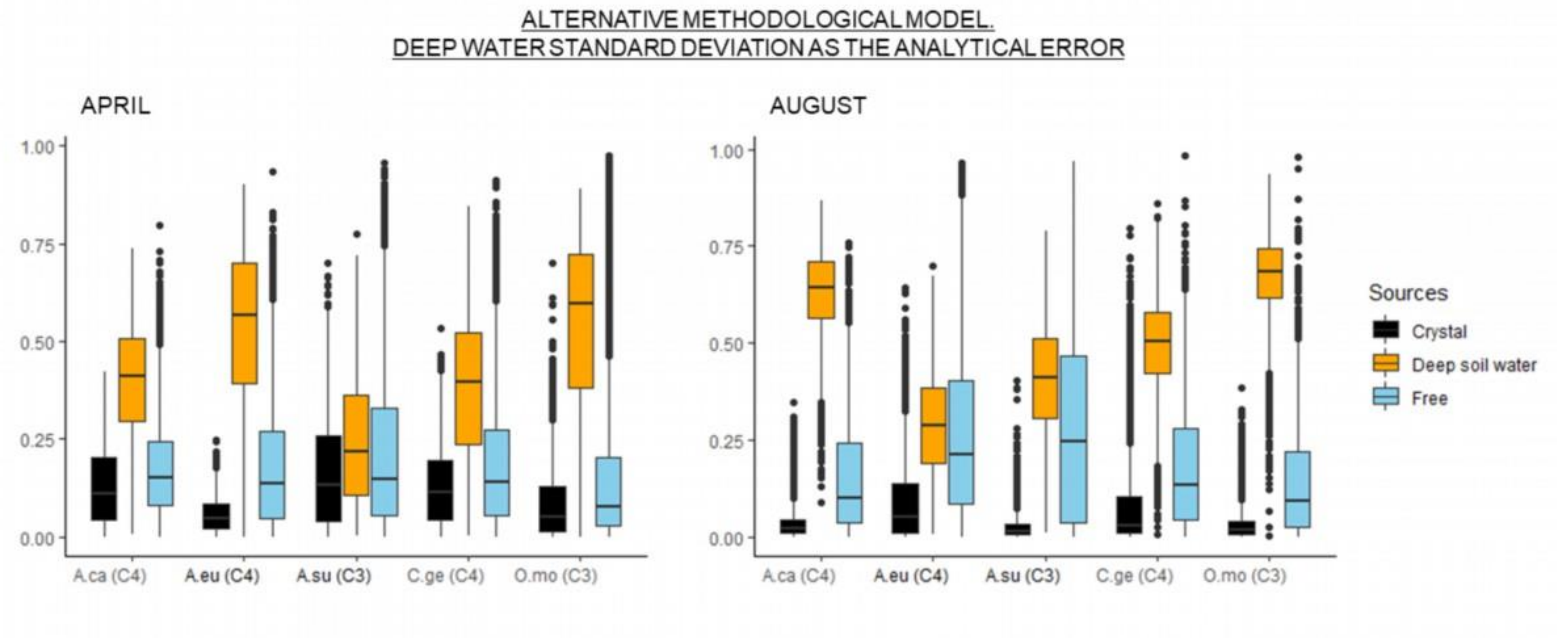
Methods S1. Comparison of the isotopic composition of the xylem sap of *Anabasis* species in April and May.

Results for the analysis of the differences in the composition of the xylem sap between April and May in *Anabasis* species (species as a random factor, using *lmer* function)

As indicated in the table above differences among spring samplings were not significant and, consequently, we used April samples for all species as representative of spring conditions.

Factor	Isotope	*p*	*F*
Month	δ^2H	0.617	0.26
	$\delta^{18}O$	0.425	0.67

Methods S2. Bayesian Mixing Model result for the alternative standard deviation of the "deep water source" considered as the analytical error (0.1 for ^{18}O and 0.4 for ^{2}H)



Methods S2. April and August estimated contribution of each combined source to the water xylem sap of the species. Deep water source standard deviation considered by the Bayesian Mixing Models as the analytical error.

Supplementary Table 1. Gypsum content of different plots sampled in the Upper Red Formation (NW Iran). Plots number 7, 8 and 9 correspond to the studied hill where plants sampling was performed**.** The oven-drying method was used to measure the percentage of gypsum (Porta, 1998). In this method, the weight of each empty crucible was measured, then approximately 2 grams of the homogenized soil samples were transferred into the crucibles, and the crucibles with the soil were weighed again. Gypsum mineral was added to one of the crucibles as a control sample. The samples were placed in an oven at a temperature of 50 degrees Celsius for at least four hours. After this period, the samples were weighed again. In the next step, the crucibles were placed in an oven at a temperature of 105 degrees Celsius for at least four hours, and after this time, they were weighed again.

Plot number	Gypsum %	Plot number	Gypsum %
1	13.71	20	39.22
2	91.09	21	81.95
3	86.70	22	94.12
4	15.09	23	93.40
5	58.82	24	91.00
6	11.03	25	71.29
7	**7.92**	**26**	**91.43**
8	**15.24**	**27**	**93.66**
9	**25.00**	**28**	**69.90**
10	23.76	29	83.02
11	3.96	30	79.21
12	74.51	31	88.46
13	74.51	32	91.87
14	11.37	33	86.27
15	37.58	34	71.03
16	11.76	35	15.76
17	3.76	36	67.33
18	11.37	37	34.78
19	15.53	38	43.35

Supplementary Table 2. Mean values of the species xylem sap isotopic composition for the different seasons sampled.

Species	**Month**	**Mean δ^2H**	**sd SD δ^2H**	**Mean $\delta^{18}O$**	**Sd SD $\delta^{18}O$**
Anabasis_ calcarea	April	-41.83	4.11	-1.64	0.25
	May	-52.38	7.76	0.63	6.91
	August	-66.26	7.32	-6.18	2.50
Anabasis_ eugeniae	April	-52.44	3.32	-4.64	1.01
	May	-47.86	5.54	-4.00	1.61
	August	-44.82	3.83	-0.02	1.64
Atraphaxis_ suaedifolia	April	-42.73	2.87	-0.89	3.52
	August	-58.13	4.51	-2.40	0.40
Caroxylon_ gemmascens	April	-44.8	8.10	-1.35	1.56
	August	-43.88	5.23	-0.10	1.18
Oreosalsola_ montana	April	-57.3	12.87	-5.14	1.46
	August	-57.65	6.05	-5.02	1.66

Supplementary Table 3. ANOVA results for linear models showing significant changes in the isotopic composition of the water xylem of species and type of water source between the sampled months, analysed separately. *F-ratios* and *p-values* are shown, significant differences in bold type (~Figure3).

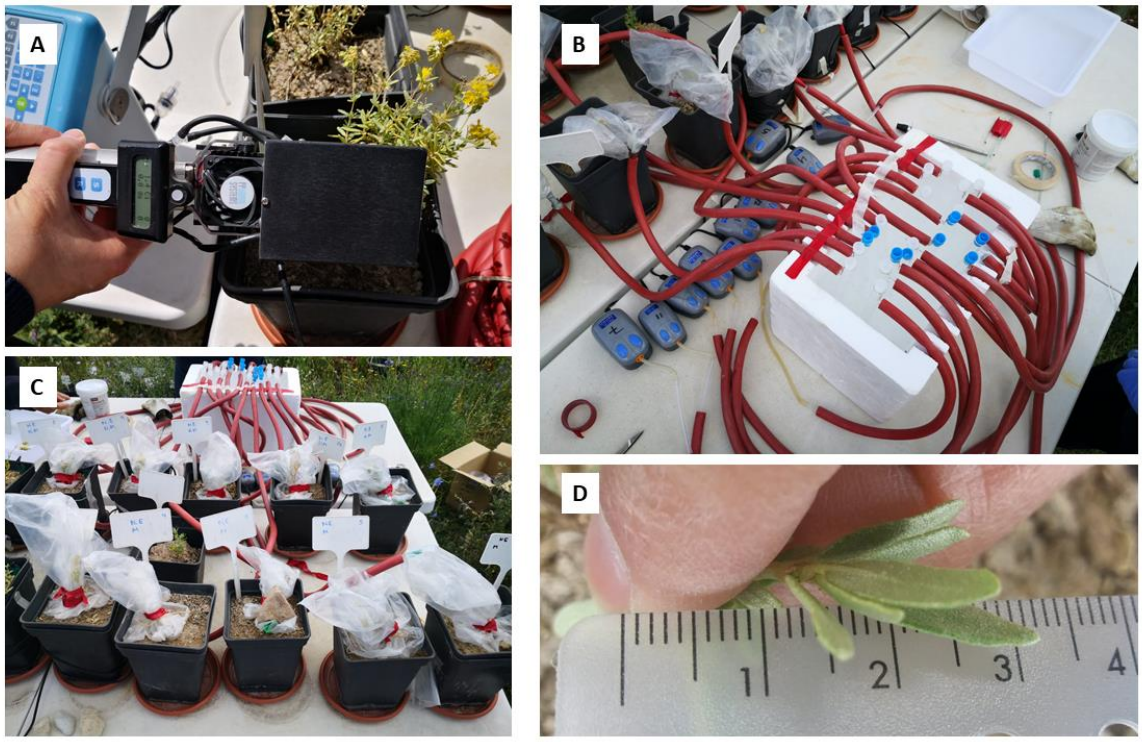
	δ^2H (‰)		$\delta^{18}O$ (‰)	
Species	*F-ratio*	*p-value*	*F-ratio*	*p-value*
Anabasis calcarea	**27.049**	**<0.001**	**9.19**	**0.023**
Anabasis eugeniae	**11.29**	**<0.001**	**28.64**	**<0.001**
Atraphaxis suaedifolia	**33.18**	**<0.001**	0.7302	0.426
Caroxylon gemmascens	0.04	0.854	1.65	0.247
Oreosalsola montana	0.00	0.964	0.01	0.934
Water sources				
Free	**20.77**	**<0.001**	**33.16**	**<0.001**
Crystallization	**22.91**	**<0.001**	3.39	0.070

Supplementary Table 4. Summarized output statistics ("credible intervals") of Bayesian Mixing Models showing the mean and standard deviation (SD); the median (percentile 50%), and percentiles 2,5% and 97.5%, which can be interpreted as the confidence interval (95%) for each source and species in both sampling moths.

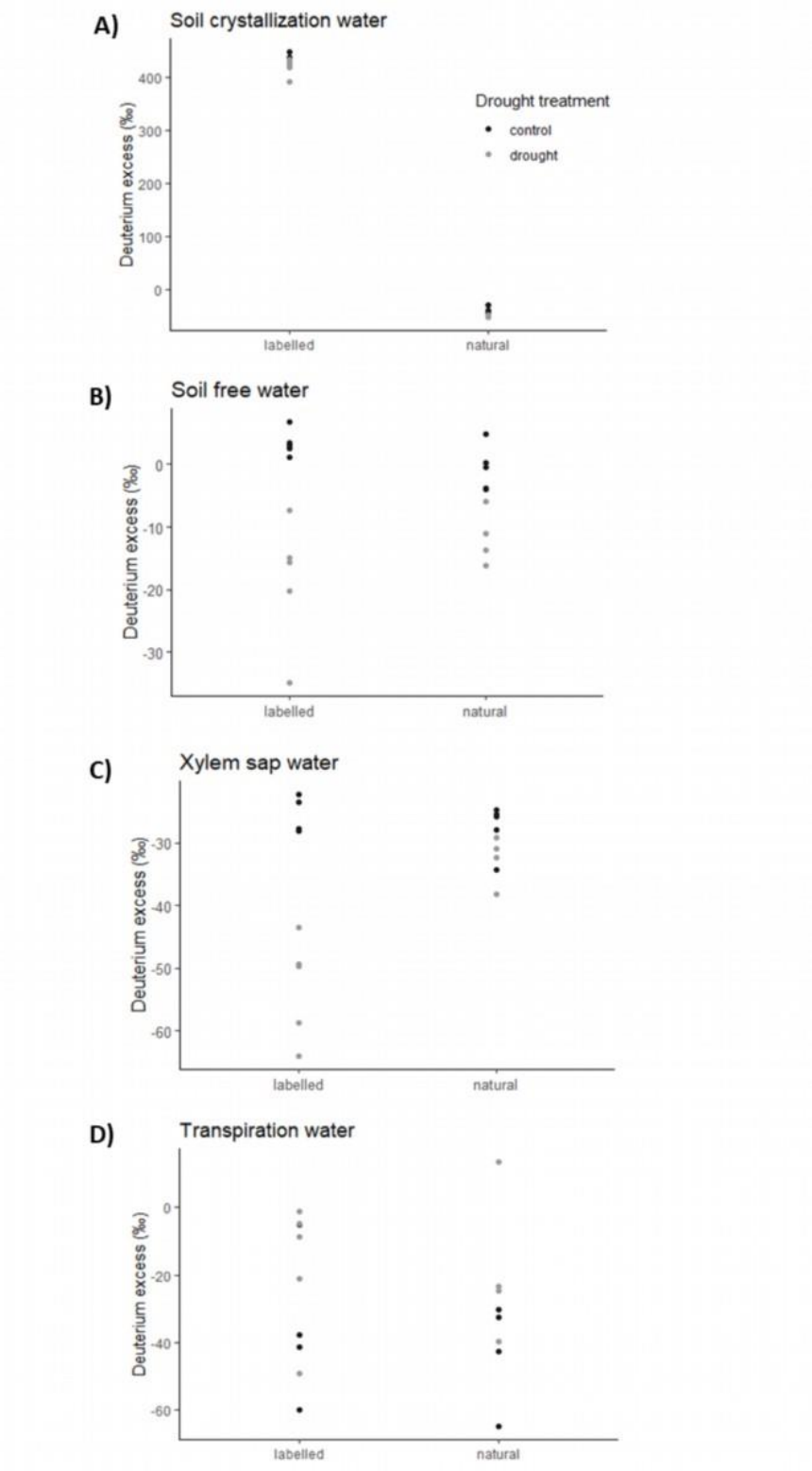
APRIL						
Source	**Species**	**Mean**	**SD**	**2.5%**	**50%**	**97.5%**
Crystal_10	*Anabasis_calcarea*	0.168	0.070	0.030	0.168	0.303
	Anabasis_eugeniae	0.064	0.040	0.008	0.058	0.156
	Atraphaxis_suaedifolia	0.216	0.134	0.013	0.211	0.492
	Caroxylon_gemmascens	0.177	0.097	0.013	0.174	0.371
	Oreosalsola_montana	0.076	0.058	0.006	0.062	0.217
Crystal_20	*Anabasis_calcarea*	0.074	0.054	0.004	0.063	0.197
	Anabasis_eugeniae	0.046	0.040	0.001	0.036	0.145
	Atraphaxis_suaedifolia	0.114	0.117	0.002	0.069	0.401
	Caroxylon_gemmascens	0.083	0.073	0.002	0.062	0.263
	Oreosalsola_montana	0.041	0.042	0.001	0.027	0.155
Free_10	*Anabasis_calcarea*	0.186	0.117	0.016	0.169	0.456
	Anabasis_eugeniae	0.183	0.146	0.006	0.148	0.519
	Atraphaxis_suaedifolia	0.227	0.209	0.006	0.159	0.758
	Caroxylon_gemmascens	0.212	0.178	0.007	0.166	0.662
	Oreosalsola_montana	0.142	0.155	0.004	0.090	0.594
Free_20	*Anabasis_calcarea*	0.244	0.132	0.027	0.233	0.526
	Anabasis_eugeniae	0.359	0.216	0.012	0.366	0.734
	Atraphaxis_suaedifolia	0.174	0.138	0.010	0.140	0.502
	Caroxylon_gemmascens	0.241	0.167	0.011	0.212	0.589
	Oreosalsola_montana	0.161	0.150	0.007	0.117	0.592
Deep soil	*Anabasis_calcarea*	0.329	0.136	0.091	0.320	0.614
	Anabasis_eugeniae	0.349	0.233	0.026	0.308	0.830
	Atraphaxis_suaedifolia	0.269	0.173	0.022	0.245	0.639
	Caroxylon_gemmascens	0.287	0.179	0.030	0.266	0.665
	Oreosalsola_montana	0.579	0.194	0.096	0.606	0.879
AUGUST						

Source	Species	Mean	SD	2.5%	50%	97.5%
Crystal_10	Anabasis_calcarea	0.056	0.04	0.003	0.046	0.162
	Anabasis_eugeniae	0.152	0.147	0.002	0.101	0.515
	Atraphaxis_suaedifolia	0.040	0.041	0.001	0.026	0.150
	Caroxylon_gemmascens	0.151	0.159	0.002	0.093	0.565
	Oreosalsola_montana	0.062	0.075	0.001	0.035	0.274
Crystal_20	Anabasis_calcarea	0.033	0.028	0.001	0.026	0.104
	Anabasis_eugeniae	0.087	0.101	0.001	0.047	0.360
	Atraphaxis_suaedifolia	0.026	0.031	0.000	0.015	0.113
	Caroxylon_gemmascens	0.068	0.084	0.001	0.0350	0.307
	Oreosalsola_montana	0.029	0.034	0.000	0.017	0.126
Free_10	Anabasis_calcarea	0.084	0.062	0.003	0.071	0.235
	Anabasis_eugeniae	0.189	0.176	0.003	0.135	0.616
	Atraphaxis_suaedifolia	0.123	0.160	0.001	0.056	0.607
	Caroxylon_gemmascens	0.152	0.144	0.002	0.107	0.506
	Oreosalsola_montana	0.105	0.118	0.001	0.062	0.438
Free_20	Anabasis_calcarea	0.076	0.051	0.006	0.067	0.197
	Anabasis_eugeniae	0.186	0.149	0.004	0.153	0.529
	Atraphaxis_suaedifolia	0.128	0.17	0.002	0.056	0.686
	Caroxylon_gemmascens	0.161	0.138	0.004	0.129	0.494
	Oreosalsola_montana	0.073	0.074	0.001	0.048	0.275
Deep soil	Anabasis_calcarea	0.750	0.064	0.617	0.754	0.868
	Anabasis_eugeniae	0.386	0.147	0.126	0.372	0.709
	Atraphaxis_suaedifolia	0.683	0.216	0.164	0.740	0.962
	Caroxylon_gemmascens	0.468	0.169	0.182	0.452	0.853
	Oreosalsola_montana	0.730	0.146	0.412	0.749	0.952

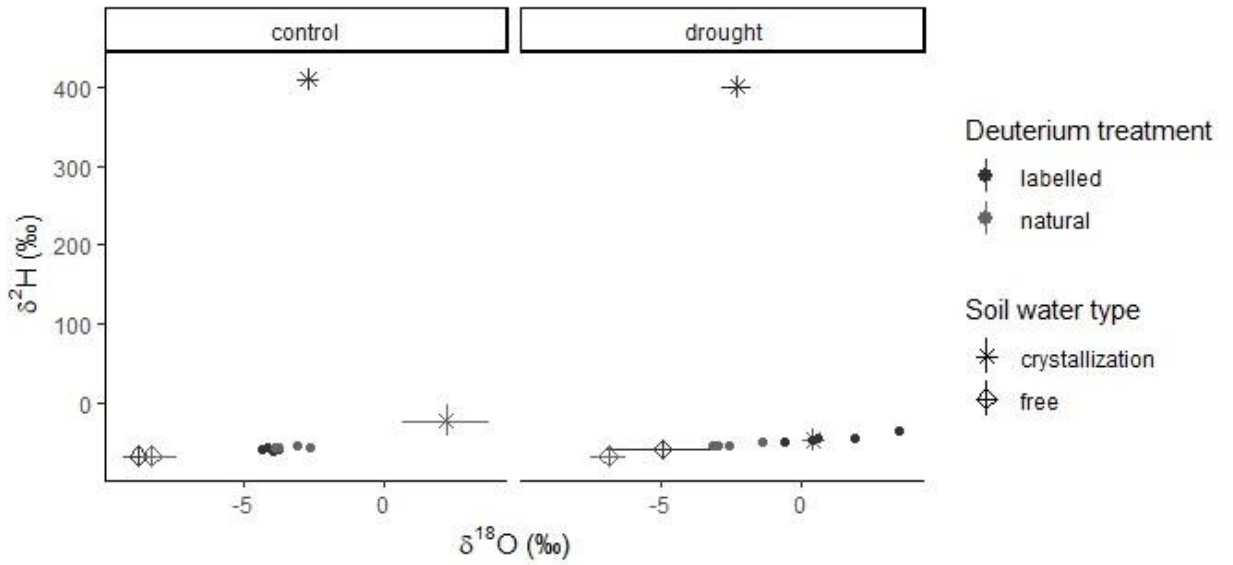
CHAPTER 3. Integrated above and below-ground responses of the gypsum specialist *Helianthemum squamatum* (L.) Dum. Cours. to drought



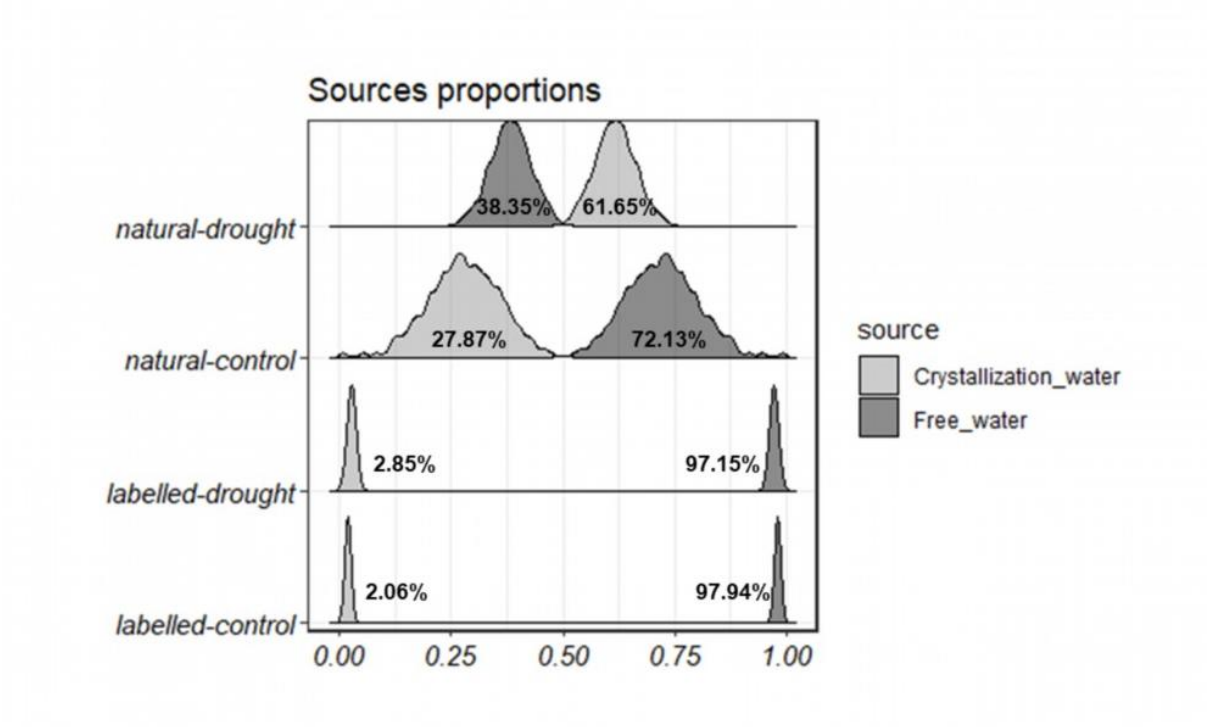
Supplementary Figure 1. Pictures of the methodology for transpiration water caption and physiological parameters measurement. **A**. Portable gas exchange and photosynthesis measuring system (CIRAS 3, PP Systems Internationa, Inc, Amesbury, Madison, USA). **B**. Cryogenic vapour trapping system for transpired water. **C**. Plants covered with plastic bags to trap transpired water. **D**. Portion of plant leaves of one replicate used for the analysis of the physiological parameters



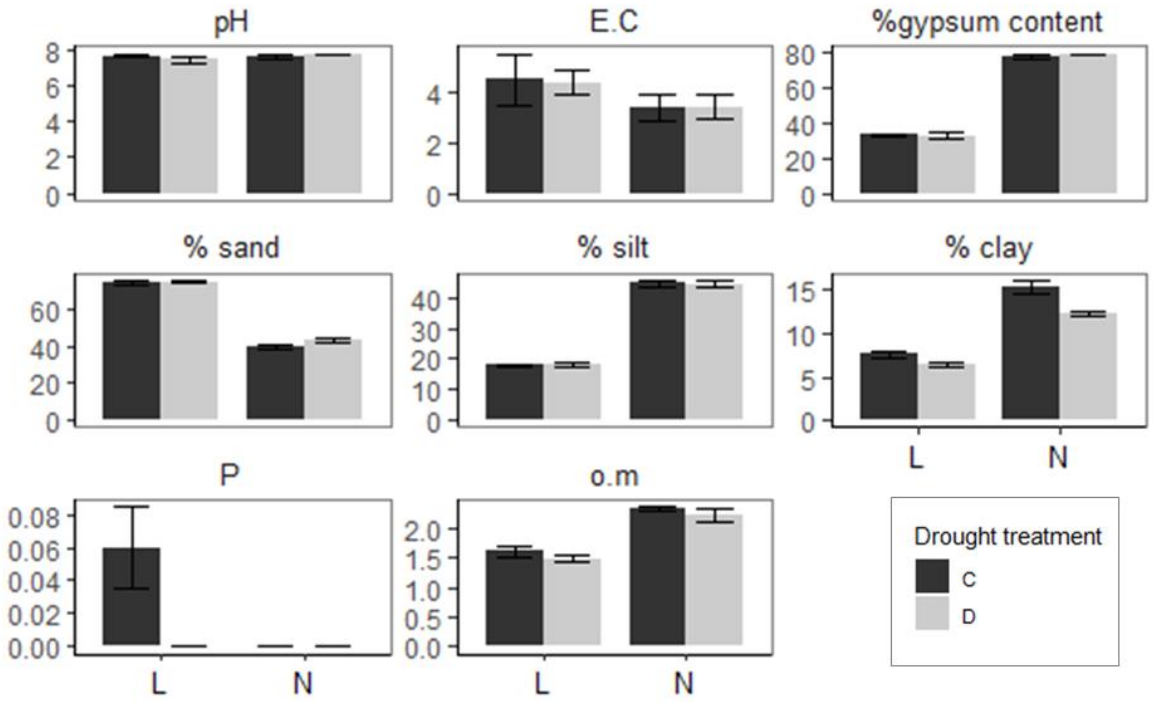
Supplementary Figure 2. Deuterium excess composition of: A) crystallization soil water, B) free soil water, C) xylem sap water and D) transpiration water



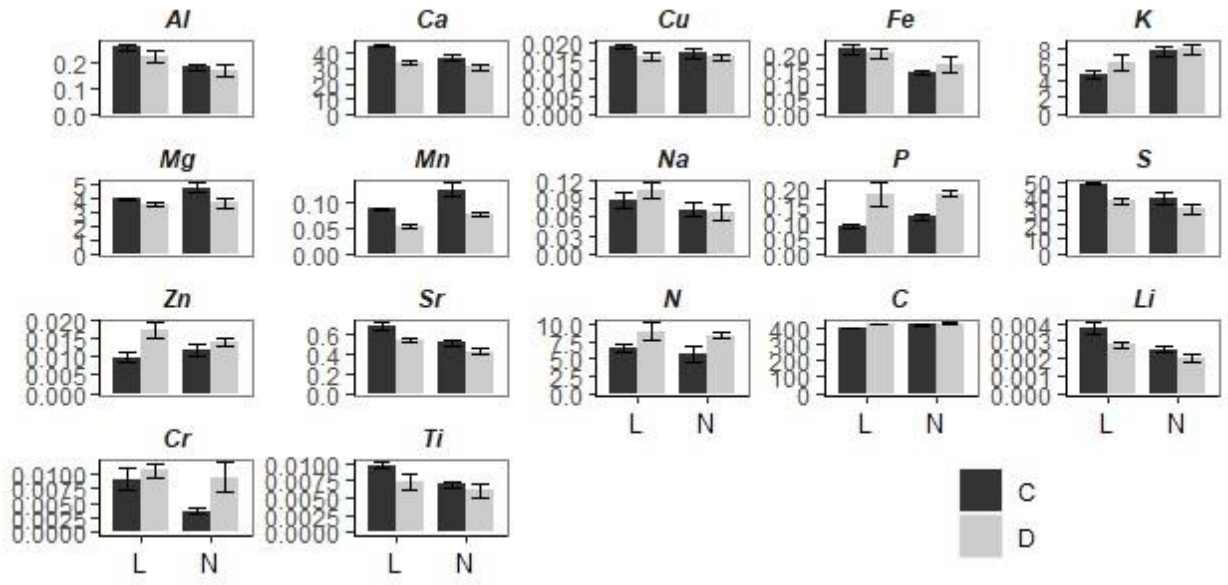
Supplementary Figure 3. δ^2H and $\delta^{18}O$ composition of the xylem sap of the plant species and the two potential water sources. Grey symbols indicate plants or soil corresponding to the natural soil treatment, while black points belong to the labelled gypsum treatment.



Supplementary Figure 4. Results of Bayesian Isotopes Mixing Models representing the contribution of the potential sources (crystallization and free water) in the xylem sap water of *Helianthemum squamatum* grouped by treatments (drought and labelling).



Supplementary Figure 5 Means and standard errors of principal physicochemical properties of the soil (pH; E.C: electric conductivity; % gypsum content, % sand, % silt and % clay, phosphorus: P and organic matter, o.m.) grouped by treatments. L: Labelled, N: Natural. Black bars are for control treatment (C), and grey bars for drought treatment (D).



Supplementary Figure 6. Mean and standard error of leaf elemental concentration grouped by labelling treatment. L: Labelled, N: - Natural. Black bars are for the control treatment (C) and grey bars are for the drought treatment (D).

Supplementary Table 1. Average isotopic composition of the two soil water sources analyzed in each type of soil (labelling treatment and natural soil) and drought treatment.

Labelling treatment	**Drought Treatment**	**Water source**	***δ^2H***	***$\delta^{18}O$***	***D-excess***	***n***
Labelled	Drought	Crystallization water	400.62±12.73	-2.30±0.52	419.02±15.97	5
		Free water	-58.16±6.42	-4.94±1.95	-18.61±10.19	5
	Control	Crystallization water	409.96 ±10.02	-2.73±0.30	431.84±11.58	5
		Free water	-66.81±2.80	-8.77±0.55	3.35±2.11	5
Natural	Drought	Crystallization water	-46.07±3.59	0.40±0.25	-49.30±2.25	4
		Free water	-66.69±0.90	-6.87±0.63	-11.72±4.37	4
	Control	Crystallization water	-22.45±19.48	2.17±1.55	-39.83±7.37	5
		Free water	-66.95±7.47	-8.29±0.88	-0.06±3.60	5

Supplementary Table 2. Results of the ANOVA of linear models analyzing the effect of the treatments and their interaction on four physiological parameters: stomatal conductance, transpiration rate, assimilation rate and water use. Bold numbers indicate significant effects ($p < 0.05$).

	Drought treatment		**Labelling treatment**		**Drought treatment*Labelling treatment**	
	F	*p*	*F*	*p*	*F*	*p*
Stomatal conductance (GS)	14.10	**0.002**	0.93	0.351	0.44	0.513
Transpiration rate (E)	10.33	**0.006**	0.20	0.662	0.21	0.651
Assimilation rate (A)	2.01	0.177	2.85	0.112	0.73	0.405
Water use	59.01	**<0.001**	0.62	0.444	5.94	**0.028**

	Drought treatment		**Labelling treatment**		**Drought treatment*Labelling treatment**	
	F	*p*	*F*	*p*	*F*	*p*
Stomatal conductance (GS)	28.75	**<0.001**	0.01	0.718	1.03	0.334
Transpiration rate (E)	15.50	**0.003**	1.09	0.322	0.02	0.897
Assimilation rate (A)	27.80	**<0.001**	8.24	**0.017**	0.44	0.521
Water use	38.71	**<0.001**	0.00	0.959	5.21	**0.045**

Supplementary Table 3. Mean and standard deviation (sd) of the water content of leaves and roots (calculated as Fresh mass/Dry mass (FM/DM)), aerial biomass (g) and leaf area (cm^2), indicated for each drought and labelling treatment. Capital letters indicate significant differences between treatments (i.e. all four). Linear model ANOVA comparing growth traits in the different treatments. *F-ratio*s and *P-value*s are shown. Bold type indicates significant effects at $\alpha = 0.05$

	FM/DM LEAVES			**FM/DM ROOTS**			**AERIAL BIOMASS**			**MEAN LEAF AREA**			
	Mean	sd		Mean	sd		Mean	sd		Mean	sd		N
Labelled-control	3.452	0.512	A	1.644	0.268	A	1.917	1.496	A	0.358	0.089	A	6
Labelled-drought	3.178	0.283	A	1.582	0.125	A	2.504	1.243	A	0.137	0.050	B	7
Natural-control	3.053	0.354	A	1.603	0.093	A	4.724	1.798	B	0.230	0.059	A	5
Natural-drought	2.882	0.133	A	1.361	0.417	A	3.495	0.456	B	0.202	0.095	B	4

Supplementary Table 4. Linear model ANOVA comparing growth traits in the different treatments. *F-ratio*s and *P-value*s are shown. Bold type indicates significant effects at $\alpha = 0.05$

	Drought		**Labelling**		**Drought *Labelling**	
	F	*p*	*F*	*p*	*F*	*p*
Aerial Biomass	0.07	0.798	10.66	**0.004**	2.27	0.147
FW/DCW leaves	2.26	0.150	4.39	**0.051**	0.11	0.743
FW/DCW roots	1.68	0.215	1.08	0.315	0.63	0.440
Mean leaf area	22.71	**<0.001**	0.07	0.793	3.25	0.092

Supplementary Table 5. PERMANOVA analyzing the effect of the drought treatment, labelling treatment and their interaction on the components exuded by roots and linear model ANOVA testing the effect of the different treatments on the root exudation detected in each compound analyzed separately. *F-ratio*s and *P-value*s are shown. Bold type indicates significant effects at α = 0.05

PERMANOVA		
	F-ratio	*p-value*
Drought treatment	1.26	0.281
Labelling treatment	1.64	0.171
Drought*Labelling	0.69	0.555

ANOVA						
	Drought treatment		**Labelling treatment**		**Drought *Labelling**	
	F-ratio	*p-value*	*F-ratio*	*p-value*	*F-ratio*	*p-value*
Citric acid	0.03	0.867	0.56	0.466	1.82	0.197
Isocitric acid	0.99	0.335	0.89	0.361	0.88	0.362
Malic acid	0.00	0.949	2.08	0.170	1.30	0.272
Succinic acid	0.00	0.949	2.08	0.170	1.30	0.272
Lactic acid	1.77	0.203	0.26	0.616	0.00	0.948
Maleic acid	1.44	0.248	0.36	0.556	0.25	0.622
Fumaric acid	0.39	0.541	0.10	0.757	1.39	0.255
Myo-Iositol	0.10	0.753	0.25	0.625	0.75	0.398
Galactinol	0.70	0.416	1.44	0.249	0.16	0.693
Xylitol	0.958	0.346	3.16	0.095 .	1.16	0.299
Sorbitol-Mannitol	0.08	0.777	2.91	0.109	0.43	0.523
Choline	9.28	**0.008**	0.32	0.577	0.81	0.382

CHAPTER 4. Soil microorganisms and root exudation mediate rhizosphere acidification of the gypsum specialist Ononis tridentata Devesa & G. López

Methodological challenges

For the first time, planar optodes were combined with root exudation and a treatment of partial soil sterilization in a gypsum soil to visualize root activity in this particular alkaline soil. As addressed in the Discussion, the high soil humidity requirements of the pH sensor restricted our observations of root activity to well-watered conditions. This situation in gypsum ecosystems will not always represent the natural conditions, but it would take place in the most favourable moment of the year, when gypsum plants tend to grow and maximize their demand for N and P (Cera et al., 2021b). Unfortunately, this technique cannot be used to monitor plant-soil interactions under drought when, for example, acidification to retrieve gypsum crystalline water would be most needed.

The physical properties of gypsum soil and its high solubility in water led to soil movements in the rhizobox visible surface, including the area in contact with the sensor foil, sometimes creating air bubbles or intrusions of soil particles that altered the signal of the sensor. In addition to the changing conditions of the soil, the rapid and variable speed of root growth in the different individuals limited the number of pictures that could be evaluated for each replicate. After measuring fourteen consecutive days, five replicates of both treatments (i.e. theoretically 5 x 14 = 70 pictures per treatment), only 29 and 44 pictures (from natural and fungi-sterile treatments, respectively) showing clear root activity could be included in the analyses.

On the other hand, the sterilization treatment and posterior bacteria inoculation changed the microbial communities in the soil and, as shown in Figure 2, the relative abundance of Fungi was reduced, but did not completely disappear. It has been reported that fungi are more sensitive to gamma-irradiation than bacteria, and typically, a gamma-irradiation of 10 kGy will eliminate fungi and Actinobacteria, while the majority of soil bacteria are eliminated by 20 kGy (McNamara et al., 2003). In our case, the presence of fungi might be due to an insufficient dose of irradiation or, alternatively, to the non-sterile conditions in which the experiment was conducted and the fact that the rhizoboxes containing gamma-irradiated soil were placed next to those containing natural soil. Hence, fungal spores or propagules might had reached the rhizoboxes containing gamma-

irradiated soil and thrived there until the end of the experiment. Despite these complications, we could compare root behaviour in natural gypsum soil and in gypsum soil with an altered microbial life and decreased fungal presence, observing similar trends in all treatment replicates.

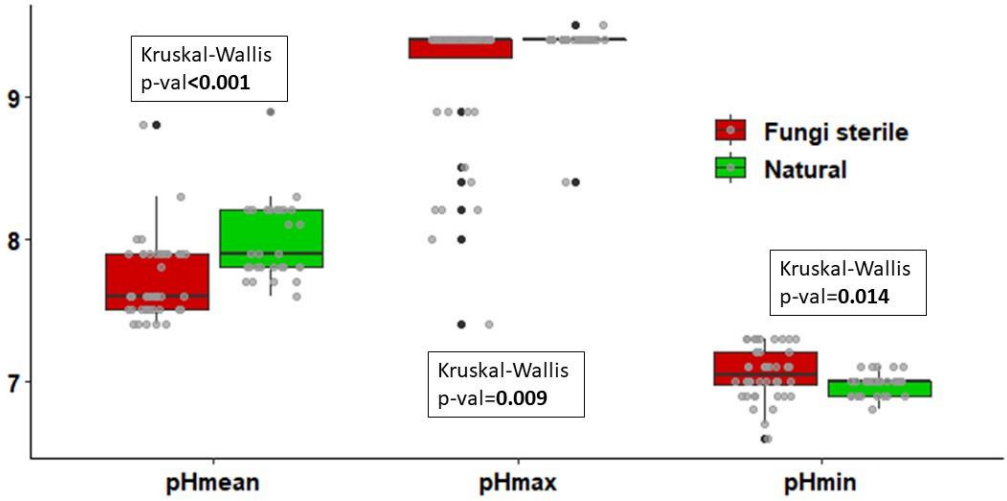


Figure Supplementary 1. Maximum, minimum and mean pH observed in the sensor foils placed in the roots of the growing seedlings of Ononis tridentata, compared per soil treatment: green boxes for the natural treatment, red boxed for the fungi-sterile treatment. *P-value*s of Kruskal Wallis tests show the significance of the differences between treatments. The variability of the maximum pH could be due to the calcium carbonate with variable presence in each piece of soil observed.

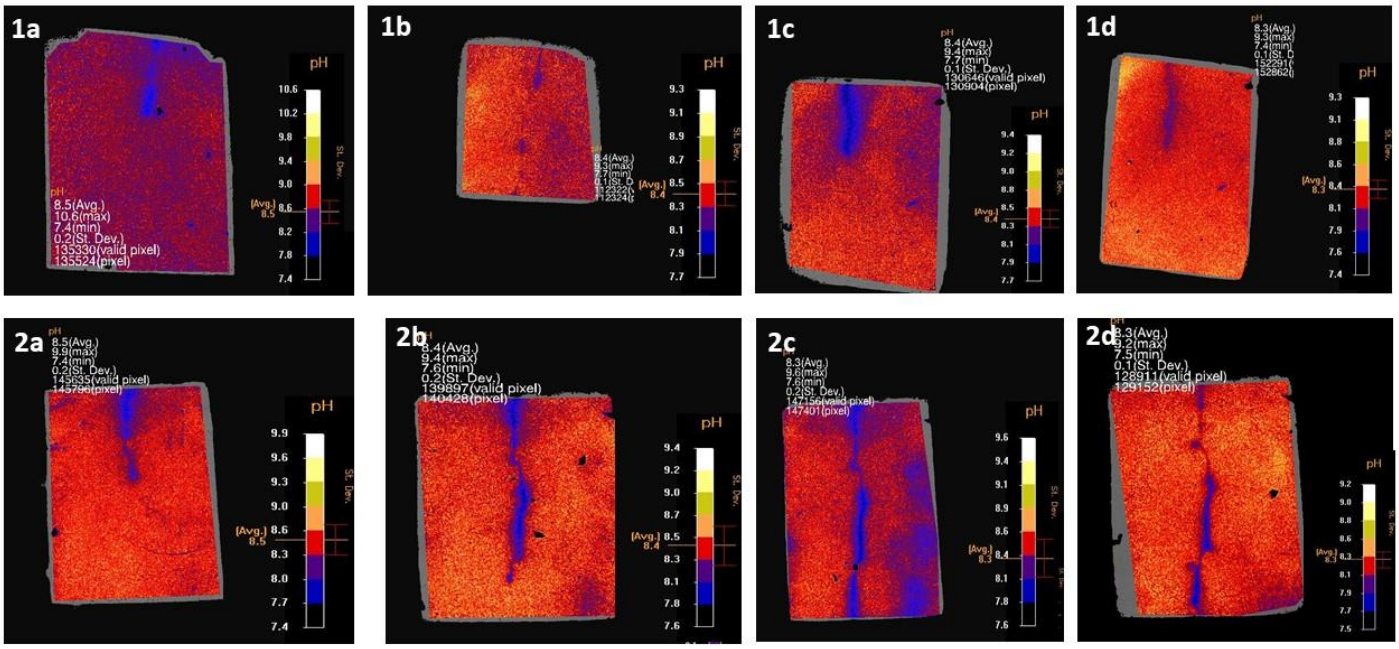


Figure Supplementary 2. IDL evaluation images of a preliminary experiment done with the methodology described, but with other species living on gypsum. 1) Helianthemum squamatum: fungi-sterile treatment first day for 1a and ninth day for 1b; 1c and 1d represent H. squamatum natural treatment first day and fourth day, respectively. 2 a, b, c, d) Helianthemum syriacum fungi-sterile treatment days 1st, 4th, 6th and 11th respectively.

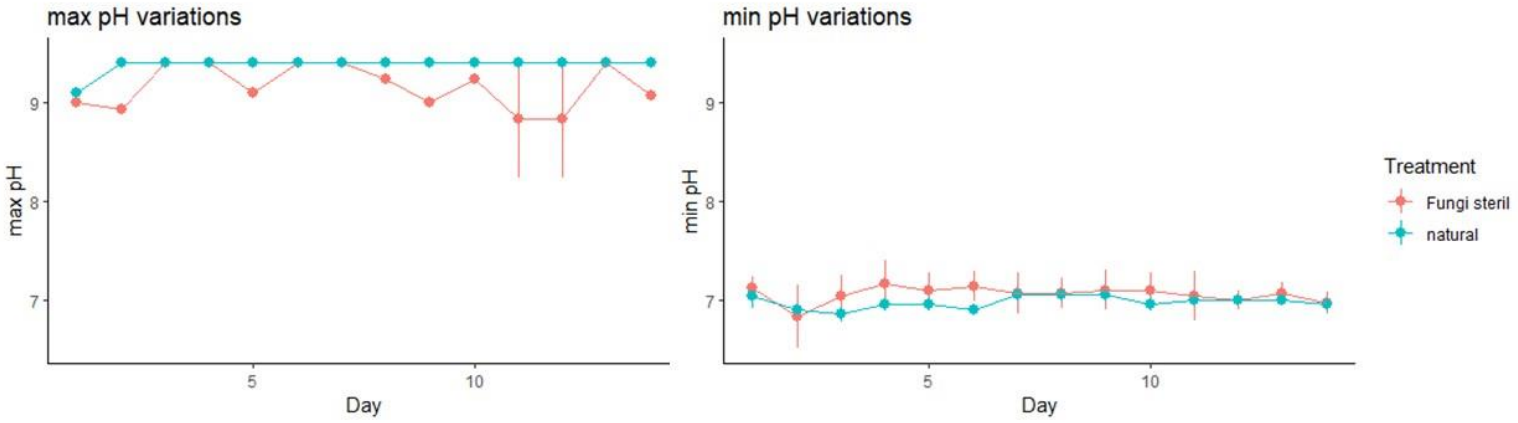


Figure Supplementary 3. pH min and pH max variations by experimental days. These data were not treated in the main analysis due to the different developmental status of each individual, which made these data not comparable.

Table S1. Mean and SD values observed for mean, maximum and minimum pH observed in the different replicates during the experiment measures. Treatment differences for pHmean, pH min and pH max are included in Figure 3. Acidification was also significantly different among treatments (Kruskal- Wallis, p-val=<0.001)

	pHmean		**pHmin**		**pHmax**		**acidification**		**N**
	mean	SD	mean	SD	mean	SD	mean	SD	
Natural	7.98	0.28	6.98	0.08	9.37	0.19	2.39	0.26	29
Fungi sterile	7.72	0.28	7.05	0.17	9.15	0.49	2.09	0.48	44

Table S2. Measured values for each replicate x day combination for mean pH, maximum pH and minimum pH observed with the optodes for the filtered pictures with a clear appreciation of the root passing through the sensor.

Treatment	rep	day	pHmean	pHmax	pHmin	Acidification
NAT	1	1	7.6	8.4	7.1	1.3
NAT	1	2	7.7	9.4	6.9	2.5
NAT	1	3	7.7	9.4	6.8	2.6
NAT	1	4	7.7	9.4	6.9	2.5
NAT	1	5	7.8	9.4	6.9	2.5
NAT	1	6	7.7	9.4	6.9	2.5
NAT	1	7	7.8	9.4	7.1	2.3
NAT	1	8	7.8	9.4	7	2.4
NAT	1	9	7.8	9.4	7	2.4
NAT	1	10	7.8	9.4	6.9	2.5
NAT	1	11	7.8	9.4	7	2.4
NAT	1	12	7.8	9.4	7	2.4
NAT	1	13	7.8	9.4	7	2.4
NAT	1	14	7.8	9.4	6.9	2.5
NAT	3	1	8.9	9.5	6.9	2.6
NAT	5	1	7.9	9.4	7.1	2.3
NAT	5	2	7.9	9.4	6.9	2.5
NAT	5	3	7.9	9.4	6.9	2.5
NAT	5	4	8.1	9.4	7	2.4
NAT	5	5	8.1	9.4	7	2.4
NAT	5	6	8.2	9.4	6.9	2.5
NAT	5	7	8.2	9.4	7	2.4
NAT	5	8	8.3	9.4	7.1	2.3
NAT	5	9	8.2	9.4	7.1	2.3
NAT	5	10	8.2	9.4	7	2.4
NAT	5	11	8.2	9.4	7	2.4
NAT	5	12	8.2	9.4	7	2.4
NAT	5	13	8.2	9.4	7	2.4
NAT	5	14	8.2	9.4	7	2.4
FUNGI ST	1	1	7.9	9.4	7.1	2.3
FUNGI ST	2	1	8	9.4	7.1	2.3
FUNGI ST	2	2	7.9	9.4	7.2	2.2
FUNGI ST	2	3	7.8	9.4	7.2	2.2
FUNGI ST	2	4	7.9	9.4	7.3	2.1
FUNGI ST	2	5	7.9	9.4	7.3	2.1
FUNGI ST	2	6	7.9	9.4	7.3	2.1
FUNGI ST	2	7	7.9	9.4	7.3	2.1

FUNGI ST	2	8	7.9	9.4	7.2	2.2
FUNGI ST	2	9	7.9	9.4	7.3	2.1
FUNGI ST	2	10	7.9	9.4	7.3	2.1
FUNGI ST	2	11	7.9	9.4	7.3	2.1
FUNGI ST	2	12	7.9	9.4	7.1	2.3
FUNGI ST	2	13	7.9	9.4	7.2	2.2
FUNGI ST	2	14	7.9	9.4	7.1	2.3
FUNGI ST	3	1	7.6	9.4	7	2.4
FUNGI ST	3	2	7.6	9.4	6.7	2.7
FUNGI ST	3	3	7.5	9.4	6.8	2.6
FUNGI ST	3	4	7.5	9.4	6.9	2.5
FUNGI ST	3	5	7.6	8.5	7	1.5
FUNGI ST	3	6	7.6	9.4	7.1	2.3
FUNGI ST	3	7	7.6	9.4	6.9	2.5
FUNGI ST	3	8	7.6	8.9	6.9	2
FUNGI ST	3	9	7.6	9.4	6.9	2.5
FUNGI ST	3	10	7.6	9.4	7	2.4
FUNGI ST	3	11	7.5	8.9	6.8	2.1
FUNGI ST	3	12	7.5	8.9	7	1.9
FUNGI ST	3	13	7.5	9.4	7	2.4
FUNGI ST	3	14	7.5	9.4	6.9	2.5
FUNGI ST	4	1	8.8	7.4	7.3	0.1
FUNGI ST	5	1	8.3	9.4	7.1	2.3
FUNGI ST	5	2	7.5	8	6.6	1.4
FUNGI ST	5	3	8	9.4	7.1	2.3
FUNGI ST	5	4	7.9	9.4	7.3	2.1
FUNGI ST	5	5	7.6	9.4	7	2.4
FUNGI ST	5	6	7.6	9.4	7	2.4
FUNGI ST	5	7	7.5	9.4	7	2.4
FUNGI ST	5	8	7.5	9.4	7.1	2.3
FUNGI ST	5	9	7.4	8.2	7.1	1.1
FUNGI ST	5	10	7.5	8.9	7	1.9
FUNGI ST	5	11	7.4	8.2	7	1.2
FUNGI ST	5	12	7.4	8.2	6.9	1.3
FUNGI ST	5	13	7.4	9.4	7	2.4
FUNGI ST	5	14	7.4	8.4	6.9	1.5

Eco-physiological mechanisms of gypsum plants to survive drought

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