Tesis Doctoral
Angela Illuminati

The importance of belowground processes in drylands:
from an individual to a whole plant community perspective

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Resumen abreviado

Antecedentes

Un enfoque basado en los rasgos y la identificación de 'trade-offs' funcionales en plantas son herramientas clave en ecología vegetal. En las últimas décadas, los rasgos de la raíz han recibido mayor atención con el objetivo de integrarlos con rasgos de hoja y tallo y lograr así una perspectiva completa a nivel de individuo de las estrategias funcionales de la planta. La discrepancia entre los datos disponibles de la parte aérea y subterránea sigue siendo muy grande, lo que dificulta el desarrollo de un marco general y una teoría que describa el funcionamiento de las plantas y las fuentes de variación fenotípica. En este contexto, en ambientes áridos, donde el agua y los nutrientes del suelo representan las principales limitaciones para la supervivencia y el desarrollo de las plantas, las estrategias de las plantas relacionadas con el uso del agua y los nutrientes son de especial interés, ya que están directamente relacionadas con el desempeño de los individuos y la dinámica de la comunidad. Hay evidencia creciente de que la escala espacial del estudio también afecta de manera dramática los patrones observados de coordinación entre rasgos y 'trade-offs' funcionales, ya que los procesos que filtran los rasgos que caracerizan las comunidades dependen íntimamente de la escala espacial. Además, la historia evolutiva ejerce un fuerte efecto sobre la caracterización funcional de la comunidad vegetal, por lo que exige su consideración cuando se investigan 'trade-offs' funcionales. Recientemente, se han llevado puesto en marcha metodologías para caracterizar la distribución de especies en el suelo, gracias a técnicas como el 'DNA metabarcoding', que proporcionan las herramientas necesarias para investigar lo que durante mucho tiempo se ha conocido como la parte oculta de las comunidades vegetales. Dada la importancia de las interacciones planta-planta y planta-suelo (tanto factores bióticos como abióticos) en ambientes áridos, la evaluación de los patrones de diversidad funcional y taxonómica en el suelo sin duda arrojará luz sobre los mecánismos claves que determinan el ensamblaje de comunidades y la coexistencia de especies.

Objetivos

En este trabajo, centramos la atención en el componente subterráneo de las comunidades de plantas, integrando los rasgos de las raíces en el análisis de las estrategias de uso del agua y nutrientes. Utilizamos una evaluación de los patrones de diversidad funcional y taxonómica en el suelo, a escala espacial muy fina, considerando su vínculo tanto con el compartimento aéreo, como con los factores bióticos y abióticos del suelo.

Objetivos principales:

Capítulo 1 Evaluar la presencia de segregación de nicho entre especies coexistentes mediante el uso de isótopos estables del agua. Caracterizar la integración entre la estrategia de uso de agua y la estrategia de uso de nutrientes a nivel de hoja teniendo en cuenta el efecto potencial de la historia evolutiva. Determinar la asociación entre la estrategia de uso de agua y la estrategia de uso de nutrientes a nivel de hoja y el 'performance' de las especies en condiciones naturales.

Capítulo 2 Comparar los 'trade-offs' funcionales a nivel individual observados a escala local en la comunidad vegetal con los 'trade-offs' funcionales globales descritos.

Evaluar la asociación potencial de la estrategia de uso de nutrientes a nivel de raíces con la estrategia de uso de agua y el 'performance' de las especies en la comunidad.

Capítulo 3 Caracterizar y comparar patrones de riqueza y distribución espacial de especies entre los compartimentos aéreos y subterráneos, teniendo en cuenta las posibles variaciones observadas a diferentes escalas espaciales.

Evaluar el efecto de la heterogeneidad del suelo en los patrones de diversidad observados tanto en el compartimento aéreo como en el subterráneo.

Capítulo 4 Analizar la diversidad funcional de las plantas en el suelo y evaluar la presencia de patrones funcionales no aleatorios en el ensamblaje de la comunidad.

Determinar el efecto de la heterogeneidad del suelo y de la comunidad microbiana en los patrones funcionales subterráneos.

Metodología

Capítulo 1 Muestreamos en condiciones naturales 24 especies perennes coexistentes en un matorral Mediterráneo semiárido. Medimos los rasgos funcionales de las hojas relacionados con el uso de agua y nutrientes y recolectamos tallos basales y/o cuellos de raíces para extraer el agua del tallo y estimar las proporciones de las diferentes fuentes de agua del suelo utilizadas por cada especie a través del uso de técnicas isotópicas.

Capítulo 2 Llevamos a cabo un experimento de jardín común con rizotrones con 23 especies coexistentes en el mismo matorral mediterráneo. Medimos 10 rasgos funcionales de raíces relacionados con la estrategia de uso de nutrientes o el tamaño de la planta, y rasgos funcionales de hojas relacionados con el uso de nutrientes, en individuos cultivados en condiciones óptimas de agua y nutrientes.

Capítulo 3 Evaluamos la diversidad aérea y subterránea de la comunidad a escala muy fina. Utilizamos un enfoque espacialmente explícito, estableciendo una parcela de 64 m^2 donde todos los individuos del compartimento aéreo fueron mapeados, y se tomaron 94 muestras de suelo

dispuestas en una cuadrícula regular en el suelo. Se utilizaron técnicas de 'DNA metabarcoding' desarrolladas previamente en nuestro grupo para identificar las especies presentes en las muestras de raíces.

Capítulo 4 Integramos la información sobre rasgos funcionales recolectada en los capítulos 1 y 2 con los datos de diversidad radical obtenidos en el capítulo 3.

Resultados

*Capítulo 1*Observamos una fuerte segregación de nicho de agua entre especies durante el pico fe nológico de la comunidad. Además, detectamos un claro 'trade-off' funcional entre la profundidad de absorción de agua y los rasgos foliares relacionados con el espectro económico de la hoja, con una absorción de agua más superficial relacionada con estrategias más adquisitivas. También encontramos que una estrategia de uso de carbono y nutrientes más conservadora, así como una estrategia de uso de agua a nivel de hoja ahorradora, se correlacionaron positivamente con el 'performance' de las especies en la comunidad.

Capítulo 2 Encontramos 'trade-offs' funcionales a nivel de individuo, congruentes en parte con los 'trade-offs' globales, aunque con algunas discrepancias. Nuestros resultados evidenciaron la fuerte relación entre una estrategia de ahorro de agua a nivel de hoja y una mayor densidad de tejido radical. También observamos una correlación positiva entre una estrategia coordinada de uso de agua a nivel de hojas y una alta densidad de tejido radicular y el 'performance' de las especies en la comunidad.

Capítulo 3 Detectamos una alta diversidad de especies en el suelo a escala espacial muy fina y una fuerte discrepancia con la diversidad de especies en el compartimento aéreo. La máxima similaridad entre los compartimentos aéreos y subterráneos se encontró a escalas muy diferentes. También encontramos que los factores del suelo que favorecen la riqueza aérea y subterránea de especies solo coincidían parcialmente, y solamente cuando se consideraba la capa de suelo menos profunda, mientras que diferían notablemente a mayor profundidad.

Capítulo 4 Observamos patrones no aleatorios que indican una fuerte diversificación funcional a escala muy fina, particularmente en el caso del diámetro de la raíz, rasgo relacionado con el 'gradiente de colaboración' del espacio económico de la raíz. Nuestros resultados también señalaron el efecto significativo y positivo de la riqueza de hongos en el suelo en la alta diversidad funcional de raíces observada.

Conclusiones

Encontramos alta diversidad funcional en la comunidad estudiada, un matorral mediterráneo muy rico en especies, con una coordinación patente entre diferentes aspectos asociados a la estrategia de uso de agua y nutrientes. En concreto, una estrategia de uso adquisitivo de carbono y nutrientes a nivel de hoja se asoció con un mayor uso de fuentes de agua poco profundas, provenientes de capas superficiales del suelo ricas en nutrientes, mientras que una estrategia más conservadora de uso de carbono y nutrientes a nivel de hoja se relacionó con el uso de fuentes de agua más profundas. En cambio, una mayor densidad de tejido radical se asoció fuertemente con un uso ahorrador de agua a nivel de hoja, una coordinación funcional que puede estar asociada a un mayor soporte mecánico proporcionado por una mayor densidad de tejido. Como es esperable en ambientes áridos, una estrategia más conservadora de uso de carbono y nutrientes a nivel de hoja y una estrategia de uso de agua más ahorradora, especialmente cuando se coordinó con una mayor densidad de tejido radical, se relacionaron positivamente con el 'performance' de las especies en condiciones naturales.

La diversidad taxonómica en el compartimento aéreo y subterráneo difirieron fuertemente y estuvieron determinadas por procesos determinísticos que actúan mayoritariamente a diferentes escalas espaciales. Los patrones funcionales no aleatorios observados en el suelo resultaron fuertemente regulados por procesos determinísticos, en su mayoría atribuibles a la competencia por los nutrientes pero también a procesos de facilitación. La riqueza de hongos en el suelo afectó positivamente los patrones observados, apoyando la idea de que los 'feedbacks' entre plantas y suelo pueden jugar un papel clave en la coexistencia de especies a escalas espaciales locales.

En este trabajo desentrañamos varios aspectos clave relacionados con el funcionamiento de las plantas y la dinámica de la comunidad a escala local en ambientes mediterráneos. Una de las principales novedades de este trabajo fue detectar que, si bien las estrategias de nutrientes a nivel de hojas y raíces estaban completamente desacopladas, ambas estaban fuertemente asociadas a diferentes aspectos clave de la estrategia de uso de agua de la planta. Además, mostramos la alta diversidad taxonómica y funcional de la comunidad en el suelo a escala espacial muy fina, y aportamos evidencia de que los procesos determinísticos, especialmente las interacciones bióticas, tanto planta-planta como planta-microbiota, están íntimamente involucrados en la caracterización funcional del compartimento subterráneo de comunidad.

Summary

Background

A plant trait-based approach and the identification of functional trade-offs are key tools in plant ecology to link plant form and function. In the last decades, increasing attention has been given to root traits to integrate them with leaf and stem traits and achieve a whole-individual perspective of plant functional strategies. The gap between aboveground and belowground data is still very high, hampering the development of a general framework and theory describing plant functioning and sources of phenotypic variation. In this context, in drylands, where soil water and nutrients represent the main constraints for plant survival and development, plant strategies related to both water and nutrient use are of special interest as they are directly connected to plant performance and community dynamics. A growing body of evidence points out that the spatial scale of the study strongly affects the observed patterns of trait coordination and functional trade-offs, because the processes that filter the traits shaping plant communities are spatial-scale dependent. Furthermore, evolutionary history exerts a strong effect on the functional characterization of the plant community, thus demanding its consideration when searching for plant functional trade-offs. Recently, increasing efforts have been carried out to characterize species distribution belowground, as a result of new straightforward techniques, such as DNA metabarcoding, which provide the necessary tools to investigate what for long has been coined the hidden part of plant communities. Given the importance of plant-plant and plant-soil (including both biotic and abiotic factors) interactions in arid environments, the assessment of both taxonomical and functional diversity patterns belowground will likely shed some light on the mechanistic aspects of community assembly and species coexistence.

Objectives

In this work, we focused the attention to the belowground plant community component, by integrating root traits in the analysis of plant water and nutrient use strategies in a whole-individual perspective. We used a fine spatial scale assessment of taxonomical and functional diversity patterns belowground, considering its link with both the aboveground compartment and with soil abiotic and biotic factors.

Main aims:

Chapter 1 To assess the presence of water niche segregation between coexisting species through the use of water stable isotopes. To characterize the integration between plant water-use strategy and the leaf-level nutrient-use strategy accounting for the potential effects of evolutionary history. To determine the potential association between the water use strategy and the leaf-level nutrient-use strategy with species performance in the plant community.

Chapter 2 To compare whole-individual functional trade-offs observed at a local plant community scale with the described global functional trade-offs. To assess the potential association of the root-level nutrient use strategy with the plant water use strategy and species performance in the plant community.

Chapter 3 To characterize and compare patterns of species richness and species spatial distribution between aboveground and belowground compartments, accounting for the potential variations across spatial scales. To evaluate the effect of soil heterogeneity in both aboveground and belowground patterns of species diversity.

Chapter 4 To analyse plant belowground functional diversity and assess the presence of non-random functional patterns on community assembly. To determine the effect of soil heterogeneity and microbial community in the belowground patterns.

Methods

Chapter 1 We sampled 24 perennial coexisting species growing in natural conditions in a rich semiarid Mediterranean shrubland. We measured leaf functional traits related to water- and nutrient-use and collected basal stems and/or root necks to extract stem water and estimate proportions of different soil water sources used by each species through isotopic techniques.

Chapter 2 We carried out a common garden, rhizotron experiment with 23 coexisting species from the same Mediterranean shrubland. We measured 10 root functional traits related with either the nutrient-use strategy or the plant size, and leaf nutrient-use functional traits in individuals grown in optimal water and nutrient conditions.

Chapter 3 We assessed the diversity of the aboveground and belowground plant community at a very fine scale. We used a spatially explicit approach, establishing a 64m^2 plot where all individuals were mapped aboveground, and where a regular grid of 94 cores was sampled belowground. DNA metabarcoding techniques previously developed in our group were used to identify species in roots samples.

Chapter 4 We integrated the functional trait variation assessed in chapters 1 and 2 with the belowground species diversity assessment from chapter 3.

Results

Chapter 1 We observed a strong water niche segregation between species during the phenological peak of our community and detected an important functional trade-off between water uptake depth and leaf traits related with the leaf economic spectrum, with a shallower water uptake related with more acquisitive strategies. Furthermore, we observed that a more conservative carbon and nutrient use strategy, as well as a saver leaf-level water use strategy, was positively correlated with species performance in natural conditions.

Chapter 2 We observed functional trade-offs at the whole-plant level, which were partly congruent with global trade-offs, yet with some discrepancies. Our results evidenced the strong relationship between a saver leaf-level water use strategy and a higher root tissue density. We also observed a positive correlation between a coordinated saver leaf-level water use strategy and high root tissue density and species performance in the field.

Chapter 3 We detected high species diversity belowground at the neighbourhood scale of a few cm and a strong discrepancy with aboveground species diversity. The maximum similarity between aboveground and belowground compartments was encountered very different scales. We also found that soil factors driving species richness aboveground and belowground only partly matched when considering the shallower soil layer, while remarkably differed for larger depths.

Chapter 4 We observed important non-random patterns indicating a strong functional diversification of traits at a very fine scale, particularly for root diameter, related with the collaboration gradient of the root economic space. Our findings also pointed out the significant and positive effect of soil fungi richness on the high root functional diversity observed.

Conclusions

We found high functional variability in the study plant community, a rich Mediterranean shrubland, with a patent coordination between different aspects associated with the water and nutrient use strategy. Indeed, an acquisitive leaf-level carbon and nutrient use strategy was associated with a greater use of shallow water sources from nutrient-rich topsoil layers, while a more conservative leaf-level carbon and nutrient use strategy was linked to uptake of deeper water sources. Higher root tissue density was instead strongly associated with a saver leaf-level water use, a functional coordination that may be associated to a higher mechanical support provided by a higher root tissue density. As may be expected in drylands, a more conservative leaf-level carbon and nutrient use strategy and a saver water use strategy, especially when coordinated with a higher root tissue density, were positively related with species performance.

Aboveground and belowground plant species diversities strongly differed and were driven by fine-scale deterministic processes mostly acting at different spatial scales. Deterministic processes, mostly attributable to competition for nutrients but also facilitation, regulated non-random functional patterns observed belowground. Soil fungi richness positively affected the observed patterns supporting the idea that plant soil feedbacks may exert a key role for species coexistence at local spatial scales.

In this work we elucidated several key aspects related to plant functioning and local scale community dynamics characterizing Mediterranean environments. One of the main novelties of this work was to find that while leaf- and root-level nutrient strategies were completely decoupled, they were strongly associated to different key aspects of the plant water use strategy. In addition, we showed the high belowground both taxonomical and functional diversity at a very fine spatial scale, and provided evidence that deterministic processes, especially biotic, both plant-plant and plant-microbiota, interactions, are especially involved in the characterization of the belowground plant community.

General introduction

Background

The hidden part of plant communities: from a taxonomical to a functional perspective

Terrestrial ecosystems can be divided in aboveground and belowground communities, which interact through both positive and negative feedbacks (Wardle *et al.*, 2004). Ecologists are becoming increasingly aware of the role of aboveground–belowground relationships as drivers of the structure and functioning of ecosystems, as well as in the regulation of their response to global change across a hierarchy of temporal and spatial scales (Bardgett *et al.*, 2005; Bardgett, 2018). For this reason, during the past few decades, research efforts have been directed to explore belowground communities and their functional significance for plant communities (Bardgett *et al.*, 2005). Indeed, plants exert a fundamental role as they connect, directly or indirectly, aboveground and belowground components of terrestrial ecosystems (Van Der Putten, 2012). The belowground component of plant communities is commonly referred to as the *hidden* compartment, as most knowledge to date is related to the aboveground counterpart.

For a long time, the lack of straightforward sampling techniques strongly limited the exploration of belowground communities (e.g. Rewald *et al.*, 2012). Recent advances in molecular techniques such as DNA metabarcoding, which allows the simultaneous identification of multiple taxa through next generation sequencing, has considerably shifted this scenario (Hiiesalu *et al.*, 2012; Deiner *et al.*, 2017; Cabal *et al.*, 2021). This powerful molecular tool has opened new venues to explore the hidden compartment of plant communities by identifying all the species present in root mixtures, even allowing in some cases the estimation of species-specific root biomass (e.g. Matesanz *et al.*, 2019). Plant ecologists have indeed observed a portion of diversity, i.e. 'dark diversity', which remains undetected when sampling is limited to the aboveground compartment (e.g. Pärtel *et al.*, 2011; Carrasco-Puga *et al.*, 2021), and found contrasting spatial patterns of species distribution above and belowground (e.g. Wildová, 2004; Price *et al.*, 2012). The assessment of whether discrepancies exist in species diversity and spatial distribution patterns between aboveground and belowground compartments thus represents a necessary first step to investigate mechanistic processes determining plant community structure. However, only the integration of this information with a functional trait-based approach, which links form and function, may provide an understanding of the mechanisms driving ecosystems' structure and functioning (McGill *et al.*, 2006; Violle *et al.*, 2007; Escudero & Valladares, 2016). Finally, a the estimation of functional diversity is also of primary interest for conservation purposes, given its significant association with ecosystem services (e.g. Cadotte *et al.*, 2011). Therefore, in parallel with a growing attention for

belowground taxonomic diversity, the last decades have seen a booming interest on root phenotyping and investigation of the links of root traits with multiple plant and ecosystem functions (e.g. Klimešová *et al.*, 2018).

Whole-Plant Ecological Strategies: integrating aboveground and belowground parts

Trait-based plant ecology searches for leading dimensions of species variation that can describe functional strategies from the observation of general phenotypic patterns (Westoby *et al.*, 2002; McGill *et al.*, 2006). In other words, plant ecological strategy schemes classify plants according to meaningful axes of plant adaptation and specialization (Grime, 1979; Westoby, 1998; Díaz *et al.*, 2004). Each of these axes represents a trade-off between different plant functions and biological realization -morphological, biochemical, physiological- (Diaz & Cabido, 1997) that limit possible investments of resources to different parts of cells, tissues and organs (Freschet *et al.*, 2010). Sets of plant functional traits are widely recognized as powerful proxies for these plant functional trade-offs (Freschet *et al.*, 2010). Among the most relevant pioneering works in the field are the Raunkiaer' life forms classification and the Grime's CSR model (Raunkiaer, 1934; Grime, 1979). Two decades later, Westoby (1998) proposed the important leaf–height–seed (LHS) plant ecology strategy, defining three main axes of species variation related with specific leaf area, plant size and seed mass. Yet only the work by Wright *et al.* (2004) first, followed by other notable frameworks (Chave *et al.*, 2009; Díaz *et al.*, 2016; Saatkamp *et al.*, 2019), described a spectrum of plant form and function at a global scale. The leaf economic spectrum (Wright *et al.*, 2004), identified by one single axis representing a gradient from conservative to acquisitive nutrient-use strategies is characterized by a functional trade-off of chemical and morphological leaf-traits. The plant economic spectrum (Díaz *et al.*, 2016) adopted the first integrative whole-plant perspective, and defined a new fundamental axis of species variation related with both plant size and seed mass, additional to the axis related with the leaf-level nutrient-use. It is worth to note that the root component was still neglected. Only very recently, the definition of a root economic space (Bergmann *et al.*, 2020), defining two main axes of species variation, a nutrient-use conservation gradient and a collaboration gradient directly associated with the occurrence of mycorrhizal symbiotic associations, has brought some light on functional strategies related with fine-root traits trade-offs. Thus far, the most integrative whole-plant effort has been carried out by latter works (Carmona *et al.*, 2021; Weigelt *et al.*, 2021), which assessed for a coordination between aboveground and belowground functional trade-offs. Even though a fundamental step forward to an integrative plant perspective at global scale has been taken, we are still far from reaching a unifying whole-plant framework. Indeed, while Carmona *et al.* (2021) observed no association between the aboveground

and belowground main axes in a global data set, Weigelt *et al.* (2021) identified coordination between aboveground and belowground dimensions. Notably, still much effort is needed to fill the large gap between aboveground and belowground information (279.845 versus 6.214 species recorded, data respectively from Kattge *et al.* (2020) and Guerrero-Ramírez *et al.* (2021)). In addition, if we consider the actual number of species of which at least one key trait has been recorded both aboveground and belowground, the number of species decreases much further (2510 species, from Weigelt *et al.* (2021), and even more dramatically if we select the species with a full set of key traits (301 species, from Carmona *et al.* (2021). In last instance, because of functional traits are known to mirror not only plant functions but also adaptive and plastic individual responses to biotic and abiotic factors (Albert *et al.*, 2010), the general patterns described at global scale may differ importantly at local scales (i.e. realized assemblages) (e.g. Benavides *et al.*, 2021; Matesanz *et al.*, 2021). Indeed, we still have little knowledge about functional trait variation across spatial scales, especially of root traits (McCormack et al. 2017). Some authors (e.g. Messier *et al.*, 2017) have even suggested that, at local spatial scales, to constrain species functional variation in a few axes may be not possible, while trait networks would be more appropriate to describe the multiple high correlations existing among functional traits. Moreover, to understand how functional traits trade-offs vary across species and biomes, it is necessary to consider the effect of phylogenetic relatedness (e.g. Westoby *et al.*, 1995; Münkemüller *et al.*, 2012; Ma *et al.*, 2018), because the observed functional traits trade-offs may be mirroring a correlated evolutionary divergence (e.g. Prinzing *et al.*, 2001; Westoby *et al.*, 2002), instead of actual ongoing selection processes (e.g. Harvey & Rambaut, 2000; Westoby *et al.*, 2002).

Plant community assembly and species coexistence

Since the early works on the mechanisms explaining coexistence in plant communities (Clements, 1916; Gleason, 1926), an intense debate has ensued, and two main contrasting theories have emerged: one recognizes the importance of deterministic processes (Diamond, 1975) and the other of stochastic processes (Hubbell, 2001) as the main driving force of community assembly and thus species coexistence. Recent perspectives integrated deterministic versus stochastic processes as complementary mechanisms, rather than exclusive ones, as part of the same complex interactive process (e.g. Gravel *et al.*, 2006), where the relative importance of each factor can vary across scales and environments (e.g. Valladares *et al.*, 2015; Escudero & Valladares, 2016). The contemporary coexistence theory based on Chesson's ideas (Chesson, 2000) assumes that local plant communities mirror cumulative effects of these processes and outline the relevance of both stabilizing niche differences and fitness differences for species coexistence (HilleRisLambers *et al.*, 2012). Stabilizing

niche differences are those that cause species to more strongly limit themselves than others through, for example, resource partitioning, host-specific natural enemies, or storage effects, while fitness differences reflect differences between species that predict the outcome of competition in the absence of stabilizing niche differences (HilleRisLambers *et al.*, 2012). Evidently, competition is widely recognized as a dominant force influencing community structure as a major driver of species' exclusions, and is experienced with neighbourhood plants at very fine scales (Lotka, 1926; Lekberg *et al.*, 2018). At similar fine spatial scales, within a maximum of a few multiples of the size of the adult focal individual's canopy (Mack & Bever, 2014), plants are simultaneously involved in complex interactions with soil biota, shaped as plant-soil feedbacks, which have been shown to have an important contribution to promote species coexistence when strong resource competition would otherwise lead to exclusion (e.g. Bever, 2003; Lekberg *et al.*, 2018). Within the same plant community, if we increase the lens of the spatial scale, i.e. from one meter to ten meters, the main processes affecting plant community structure may change abruptly, with environmental filtering gaining a major role than plant to plant interactions (e.g. Siefert, 2012). Thus, a growing body of evidence suggests that every single process affecting plant community structure and functioning is strongly dependent on the spatial scale (Escudero & Valladares, 2016).

The importance of belowground processes in arid and semi-arid environments

Arid and semi-arid ecosystems cover approximately 50% of the land surface of the earth (Bailey, 1996). They are characterized by mean annual potential evapotranspiration (PET) that exceeds mean annual precipitation (MAP) (Schenk & Jackson, 2002). Despite the harsh limiting conditions, they account for 30-50% of the net primary productivity of terrestrial ecosystems (Field *et al.*, 1998; Ryel *et al.*, 2008). While in fertile environments the main factor driving plant to plant competition is light, in nutrient-poor arid and semi-arid environments competition is mostly belowground for water and nutrients (Casper & Jackson, 1997; Aerts, 1999), and, as such, it is assumed to be an important mechanism determining species coexistence (Silverton et al. 2015). For resource-mediated competition to occur belowground, a plant must have a negative effect on the availability of a belowground key resource to which another plant shows a positive response in growth, survival, or reproduction (Casper & Jackson, 1997). A typical plant morphological response to the high belowground competition characterizing arid and semi-arid environments is observed in the higher plant root biomass allocation compared with other environments (Schenk & Jackson, 2002). Moreover, in most arid and semi-arid ecosystems, vegetation is organized into two-phase mosaics with high-cover vegetation patches interspersed in a matrix of low or null plant cover (Noy Meir, 1981; Fuentes *et al.*, 1984; Couteron & Kokou, 1997; Cipriotti & Aguiar, 2015) and belowground

competition has been suggested as a factor determining this patterns (Phillips & MacMahon, 1981; Martens *et al.*, 1997). An emerging body of evidence indicates that a net balance of positive and negative interactions among plants, i.e. facilitation and competition, controls the vegetation patch dynamics observed in arid ecosystems (Holzapfel & Mahall, 1999; Olff *et al.*, 1999; Arroyo *et al.*, 2015; Cipriotti & Aguiar, 2015). As well as negative (competitive) interactions, roots may have positive effects (facilitation) on other roots, by increasing the availability of certain soil resources (e.g. Holzapfel & Mahall, 1999; Hauggaard-Nielsen & Jensen, 2005; Schenk, 2006).

The complex interaction network formed by roots and the soil biota

Soil biota represents one of the largest reservoirs of biodiversity on Earth, comprising an enormous number, i.e. 1 gram of soil may contain 5-10 thousands, of microorganisms and larger organisms, such as nematodes, arthropods, earthworms, ants, and moles (Torsvik *et al.*, 1990; De Deyn *et al.*, 2003; Wardle *et al.*, 2004). Early research (e.g. Anderson *et al.*, 1983) conducted in the early 1980s showed the importance of soil trophic interactions for ecosystem processes, paving the way for the growing number of studies carried out during the last decades to understand the key role of soil biota in a multitude of processes such as nutrient and carbon cycling, plant community dynamics, and eco-evolutionary responses to global change (Bardgett & Van Der Putten, 2014). Recently, some authors (Teste *et al.*, 2017; Inderjit *et al.*, 2021) have also identified plant-soil feedbacks as possible key drivers of species coexistence at local spatial scales. However, understanding the mechanistic processes underlying the role of soil biota on ecosystem structure and functioning is not a straightforward task. Because each component of soil biota has a different effect on plant communities, the resulting outcome depends on the sum of both negative and positive feedbacks. Parasites, pathogens, and root herbivores in the rhizosphere produce a negative feedback on plant growth, while mutualistic symbionts such as mycorrhizal fungi determine a positive feedback on plant productivity, by enhancing access to limiting nutrients (e.g. Bever *et al.*, 1997; Smith & Read, 2010). Moreover, the variety of soil biotic interactions operate both directly and indirectly, for example by changing soil nutrient availability, or plants interactions with plant-feeding organisms and symbionts belowground, and also with multitrophic communities aboveground (Wardle *et al.*, 2004; Bardgett & Van Der Putten, 2014). At this regard, although host-specific plant-microbes associations have for long captured most attention, some authors (Semchenko *et al.*, 2022) have recently recognized the potential relevance of generalist microbial pathogens, mutualists and decomposers, to generate differential effects on plant communities. Worth to note that the same group of soil organisms can exert a positive or a negative effect according to the context. For example, arbuscular mycorrhizal fungi affect positively or negatively plant diversity, which in turn

has been associated with net primary productivity and thus temporal stability, depending on which species, subordinate or dominant, form profitable relationships with them (Urcelay & Díaz, 2003; Yang *et al.*, 2018). Besides, the magnitude of the effect of arbuscular mycorrhizal on net primary productivity has been observed to be much higher in low diversity systems, possibly for its effects on plant species functional redundancy (Klironomos *et al.*, 2000). Despite the complexity of the plant-soil interactions processes, the numerous gaps remaining in our understanding of the link between soil biota and plant communities are also highly related to the fact that most of the studies (e.g. Van Der Heijden *et al.*, 1998; Wagg *et al.*, 2011; Yang *et al.*, 2014; Pellkofer *et al.*, 2016) have been carried out considering exclusively the aboveground compartment of plant communities. Recently, attention has raised on plant traits as a possible key to unveil mechanistic processes behind the wide range of soil biota effects on plant communities (e.g. Baxendale *et al.*, 2014). Interestingly, a trait-based study (Teste *et al.*, 2017), recently carried out in a species-rich Mediterranean shrubland, has evidenced that nutrient-acquisition strategies at the root level, i.e. more specifically the presence and type of symbiotic associations (arbuscular mycorrhizas, ectomycorrhizas, nitrogen-fixing bacteria, no symbiosis), strongly affected plant responses to the soil biota.

Objectives

The present thesis project was born in the context of the key questions introduced in the previous sections. We argue that even though much progress has been made in the last decades regarding the assessment, characterization and integration of the belowground component with that aboveground, there is still a large gap at both the individual and plant community level. Thus, the main aim of this study was to integrate these two components to better understand plant functioning and the factors driving plant community structure in a very rich semi-arid Mediterranean shrubland, considering both a whole-individual and a community perspective. In this context, the specific aims of the thesis were the following:

- To assess the presence of water niche segregation among coexisting species, describe plant water-use strategies and their link with leaf-level nutrient use and species performance in natural conditions (Chapter 1).
- To consider a root trait-based approach to assess whole-plant functional trade-offs at a local spatial scale (neighbourhood) and the potential relationship between root-level nutrient use strategies with plant water-use and species performance in the plant community (Chapter 2).
- To consider the potential effect of evolutionary history on plant phenotypic integration of traits related to water and nutrient use strategies (Chapter 1 and 2)
- To describe species diversity patterns aboveground and belowground, analyse discrepancies between the two components, and assess the effect of soil heterogeneity on the observed patterns (Chapter 3).
- To assess belowground functional diversity patterns, determine the potential influence of deterministic processes on the patterns observed, and evaluate the potential effects of both abiotic (soil heterogeneity) and biotic interacting (fungi and bacteria) factors (Chapter 4).

Methods

The choice of the study system

Mediterranean ecosystems present a variety of ombroclimates, but most of them occur in arid and semi-arid conditions, with summers characterized by high temperatures and low precipitation (Rana & Katerji, 2000; Deitch *et al.*, 2017). Although they occupy only 2% of the emerged surface, Mediterranean ecosystems have been recognized as the third major biodiversity hotspot, with 22.500 plant species, and an exceptionally high number of endemic species, around 11.700 (Cowling *et al.*, 1996; Médail & Quezél, 1999; Myers *et al.*, 2000; Mittermeier, 2004). As such, they represent a priority target for conservation efforts, especially in consideration of the multiple global change drivers co-occurring in these regions (Lavorel *et al.*, 1998; Matesanz & Valladares, 2014). Therefore, a Mediterranean shrubland was an ideal and appropriate plant system of study according to the main objectives of this project. The selected plant community was located in the south of Madrid province (40°17'17.5" N 3°12'19.4" W, 760 m asl). The plant community is dominated by small-sized shrubs and grasses, and it occurs in calcareous soils with a variable content of gypsum which creates a patchy environment with many species (Fig. 1 and 2).

Fig. 1 Picture of the semiarid Mediterranean shrubland selected for the study. *(Photo credit: Angela Illuminati)*

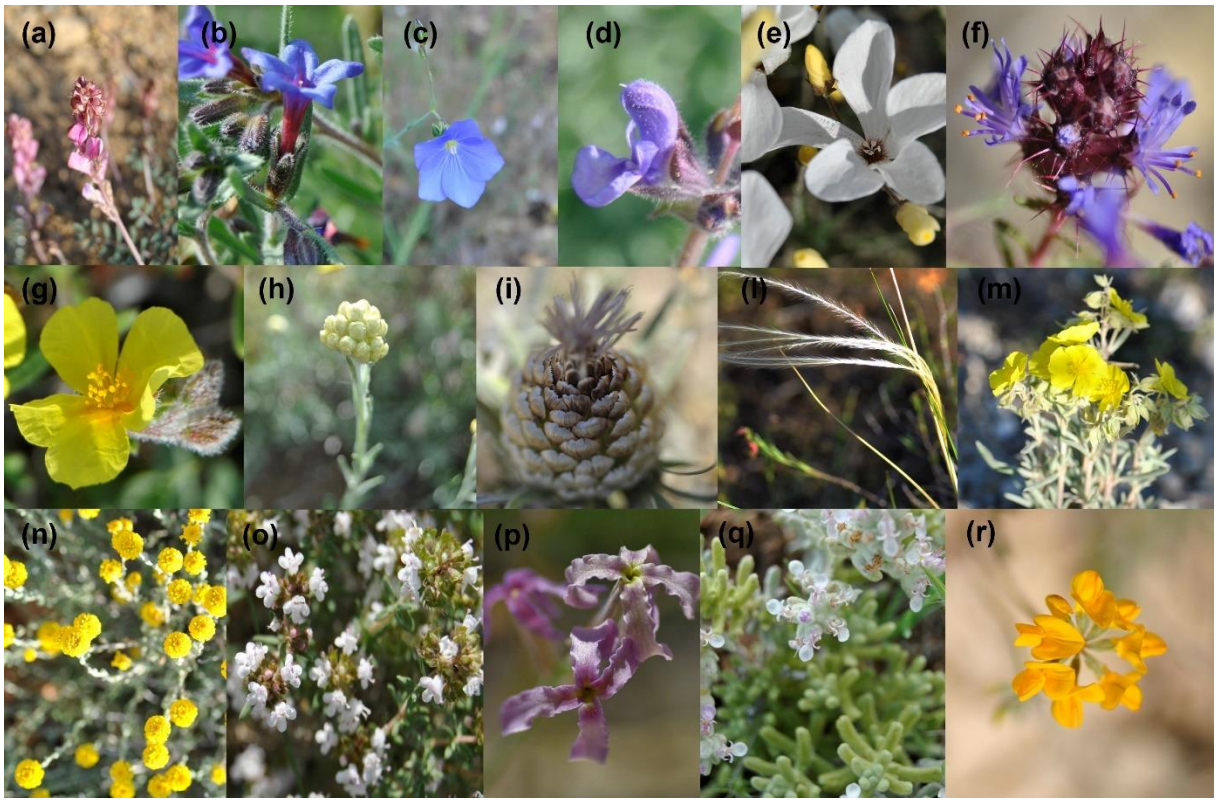

Fig. 2 Some of the species (dominant and non) characterizing the plant community. **(a)** *Astragalus incanus*, **(b)** *Lithodora fruticosa*, **(c)** *Linum narbonense*, **(d)** *Salvia lavandulifolia*, **(e)** *Linum suffruticosum*, **(f)** *Coris monspeliensis*, **(g)** *Helianthemum hirtum*, **(h)** *Helichrysum serotinum*, **(i)** *Leuzea* conifera, **(l)** *Stipa pennata*, **(m)** *Helianthemum syriacum*, **(n)** *Santolina chamaecyparissus*, **(o)** *Thymus vulgaris*, **(p)** *Matthiola fruticulosa*, **(q)** *Teucrium capitatum*, **(r)** *Hippocrepis comosa. (Photos credit: Angela Illuminati)*

Sampling and experimental design

We used different methodologies and experimental approaches to address the general objectives:

Chapter 1. We established three 30 x 30 m plots with a similar slope (≈ 10%), orientation (north-west) and species composition. Distance between plots was ≈0.5 km. We sampled three individuals per species (one per plot) of a total of 24 species and 72 individuals. From each individual, we collected one segment of the basal stem and several well-developed leaves to measure both stem water isotopic composition, specific leaf area (SLA) and leaf traits related with water use ($\delta^{13}C$ and $\delta^{18}O$). We also sampled three 1m-deep soil profiles by using a hand auger of 4 cm of diameter.

Chapter 2. We carried out a common garden rhizotron experiment in which we grew a total of 162 individuals of 23 species in homogeneous conditions at the CULTIVE facilities at URJC (https://urjc-cultive.webnode.es; Madrid), specifically 12 species in 2018 and 11 species in the

2019. Seedlings were cultivated individually in tubes (4.5 cm of radius and 60 cm of height) placed in the soil. We measured 10 root traits and key leaf traits related with nutrient-use and plant size.

Chapter 3. We sampled the plant community at a very fine spatial scale by establishing a 64 m^2 plot where all the aboveground perennial individuals were mapped (at their rooting point) with centimetric resolution. In the same plot, we also sampled the belowground plant community, by collecting 94 soil cores (5 cm of diameter, 30 cm of depth), and 84 soil cores 10cm-deep, which were located adjacent to the root cores.

Chapter 4. We integrated data collected from the work described in Chapters 1, 2 and 3. Specifically, we selected 16 species which were detected in the belowground community (presence-absence data in root cores, Chapter 3) and estimated functional diversity by using traits related with both the water- and nutrient-use strategy (Chapters 1 and 2).

List of manuscripts

Chapter 1 Illuminati A, Querejeta JI, Pías B, Escudero A, Matesanz S 2022. Coordination between water uptake depth and the leaf economic spectrum in a Mediterranean shrubland. Published in *Journal of Ecology*.

Chapter 2 Illuminati A, Matesanz S, de la Cruz M, Pías B, Sánchez AM, Ramos-Muñoz M, López-Angulo J, S. Pescador D, Escudero A. Integrating root traits to identify local-scale trade-offs and water use strategies in a Mediterranean shrubland. *In preparation*.

Chapter 3 Illuminati A, López-Angulo J, de la Cruz M, Chacón-Labella J, S. Pescador D, Pías B, Sánchez AM, Escudero A, Matesanz S 2021. Larger aboveground neighbourhood scales maximise similarity but do not eliminate discrepancies with belowground plant diversity in a Mediterranean shrubland. Published in *Plant and Soil* 460: 497–509.

Chapter 4 Illuminati A, Matesanz S, López-Angulo J, de la Cruz M, Escudero A. Plant-plant interactions and soil microbiome feedbacks determine belowground segregation patterns of root nutrient-use strategies. *In preparation*.

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References

Aerts R. **1999**. Interspecific competition in natural plant communities: Mechanisms, trade-offs and plant-soil feedbacks. *Journal of Experimental Botany* **50**: 29–37.

Albert CH, Thuiller W, Yoccoz NG, Douzet R, Aubert S, Lavorel S. **2010**. A multi-trait approach reveals the structure and the relative importance of intra- vs. interspecific variability in plant traits. *Functional Ecology* **24**: 1192–1201.

Anderson JM, Ineson P, Huish SA. **1983**. Nitrogen and cation mobilization by soil fauna feeding on leaf litter and soil organic matter from deciduous woodlands. *Soil Biology and Biochemistry* **15**: 463–467.

Arroyo AI, Pueyo Y, Saiz H, Alados CL. **2015**. Plant-plant interactions as a mechanism structuring plant diversity in a Mediterranean semi-arid ecosystem. *Ecology and Evolution* **5**: 5305–5317.

Bailey RG. **1996**. *Ecosystem geography*. Springer New York, NY, USA.

Bardgett RD. **2018**. Linking Aboveground–Belowground Ecology: A Short Historical Perspective. In: Aboveground–belowground community ecology. Springer, Cham, 1–17.

Bardgett RD, Bowman WD, Kaufmann R, Schmidt SK. **2005**. *A temporal approach to linking aboveground and belowground ecology*. Elsevier Current Trends.

Bardgett RD, Van Der Putten WH. **2014**. Belowground biodiversity and ecosystem functioning. *Nature* **515**: 505–511.

Baxendale C, Orwin KH, Poly F, Pommier T, Bardgett RD. **2014**. Are plant–soil feedback responses explained by plant traits? *New Phytologist* **204**: 408–423.

Benavides R, Carvalho B, Matesanz S, Bastias CC, Cavers S, Escudero A, Fonti P, Martínez-Sancho E, Valladares F. **2021**. Phenotypes of Pinus sylvestris are more coordinated under local harsher conditions across Europe. *Journal of Ecology* **109**: 2580–2596.

Bergmann J, Weigelt A, Van Der Plas F, Laughlin DC, Kuyper TW, Guerrero-Ramirez N, Valverde-Barrantes OJ, Bruelheide H, Fresche GT, Iversen CM, *et al.* 2020. The fungal collaboration gradient dominates the root economics space in plants. *Science Advances* **6**: eaba3756.

Bever JD. **2003**. Soil community feedback and the coexistence of competitors: conceptual frameworks and empirical tests. *New Phytologist* **157**: 465–473.

Bever JD, Westover KM, Antonovics J. **1997**. Incorporating the Soil Community into Plant Population Dynamics: The Utility of the Feedback Approach. *Journal of Ecology* **85**: 561–

573.

Cabal C, De Deurwaerder HPT, Matesanz S. **2021**. Field methods to study the spatial root density distribution of individual plants. *Plant and Soil*: 1–19.

Cadotte MW, Carscadden K, Mirotchnick N. **2011**. Beyond species: functional diversity and the maintenance of ecological processes and services. *Journal of Applied Ecology* **48**: 1079–1087.

Carmona CP, Bueno CG, Toussaint A, Träger S, Díaz S, Moora M, Munson AD, Pärtel M, Zobel M, Tamme R. **2021**. Fine-root traits in the global spectrum of plant form and function. *Nature* **597**: 683–687.

Carrasco-Puga G, Díaz FP, Soto DC, Hernández-Castro C, Contreras-López O, Maldonado A, Latorre C, Gutiérrez RA. **2021**. Revealing hidden plant diversity in arid environments. *Ecography* **44**: 98–111.

Casper BB, Jackson RB. **1997**. PLANT COMPETITION UNDERGROUND. *Annual Review of Ecology and Systematics* **28**: 545–570.

Chave J, Coomes D, Jansen S, Lewis SL, Swenson NG, Zanne AE. **2009**. Towards a worldwide wood economics spectrum. *Ecology Letters* **12**: 351–366.

Chesson P. **2000**. Mechanisms of Maintenance of Species Diversity. *Annual Review of Ecology and Systematics* **31**: 343–366.

Cipriotti PA, Aguiar MR. **2015**. Is the balance between competition and facilitation a driver of the patch dynamics in arid vegetation mosaics? *Oikos* **124**: 139–149.

Clements FE. **1916**. The development and structure of biotic communities. *Journal of Ecology* **5**: 12–21.

Couteron P, Kokou K. **1997**. Woody vegetation spatial patterns in a semi-arid savanna of Burkina Faso, West Africa. *Plant Ecology 1997 132:2* **132**: 211–227.

Cowling RM, Rundel PW, Lamont BB, Arroyo MK, Arianoutsou M. **1996**. Plant diversity in mediterranean-climate regions. *Trends in Ecology & Evolution* **11**: 362–366.

Deiner K, Bik HM, Mächler E, Seymour M, Lacoursière-Roussel A, Altermatt F, Creer S, Bista I, Lodge DM, de Vere N, *et al.* 2017. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology* **26**: 5872–5895.

Deitch MJ, Sapundjieff MJ, Feirer ST. **2017**. Characterizing Precipitation Variability and Trends in the World's Mediterranean-Climate Areas. *Water* **9**: 259.

De Deyn GB, Raaijmakers CE, Zoomer HR, Berg MP, De Ruiter PC, Verhoef HA, Bezemer TM, Van der Putten WH. **2003**. Soil invertebrate fauna enhances grassland

succession and diversity. *Nature* **422**: 711–713.

Diamond JM. **1975**. The island dilemma: lessons of modern biogeographic studies for the design of natural reserves. *Biological Conservation* **7**: 129–146.

Diaz S, Cabido M. **1997**. Plant functional types and ecosystem function in relation to global change. *Journal of Vegetation Science* **8**: 463–474.

Díaz S, Hodgson JG, Thompson K, Cabido M, Cornelissen JHC, Jalili A, Montserrat-Martí G, Grime JP, Zarrinkamar F, Asri Y, *et al.* 2004. The plant traits that drive ecosystems: Evidence from three continents. *Journal of Vegetation Science* **15**: 295–304.

Díaz S, Kattge J, Cornelissen JHC, Wright IJ, Lavorel S, Dray S, Reu B, Kleyer M, Wirth C, Colin Prentice I, *et al.* 2016. The global spectrum of plant form and function. *Nature* **529**: 167–171.

Escudero A, Valladares F. **2016**. Trait-based plant ecology: moving towards a unifying species coexistence theory. *Oecologia* **180**: 919–922.

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P. **1998**. Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science* **281**: 237–240.

Freschet GT, Cornelissen JHC, van Logtestijn RSP, Aerts R. **2010**. Evidence of the 'plant economics spectrum' in a subarctic flora. *Journal of Ecology* **98**: 362–373.

Fuentes ER, Otaiza RD, Alliende MC, Hoffmann A, Poiani A. **1984**. Shrub clumps of the Chilean matorral vegetation: structure and possible maintenance mechanisms. *Oecologia* **62**: 405–411.

Gleason HA. **1926**. The Individualistic Concept of the Plant Association. *Bulletin of the Torrey Botanical Club* **53**: 7–26.

Gravel D, Canham CD, Beaudet M, Messier C. **2006**. Reconciling niche and neutrality: the continuum hypothesis. *Ecology Letters* **9**: 399–409.

Grime JP. **1979**. Primary strategies in plants. *Transactions of the Botanical Society of Edinburgh* **43**: 151–160.

Guerrero-Ramírez NR, Mommer L, Freschet GT, Iversen CM, McCormack ML, Kattge J, Poorter H, van der Plas F, Bergmann J, Kuyper TW, *et al.* 2021. Global root traits (GRooT) database. *Global Ecology and Biogeography* **30**: 25–37.

Harvey PH, Rambaut A. **2000**. Comparative analyses for adaptive radiations. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **355**: 1599–1605.

Hauggaard-Nielsen H, Jensen ES. **2005**. Facilitative root interactions in intercrops. In: Root physiology: From gene to function. Springer, Dordrecht, 237–250.

Van Der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R,

Boller T, Wiemken A, Sanders IR. **1998**. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* **396**: 69–72.

Hiiesalu I, Öpik M, Metsis M, Lilje L, Davison J, Vasar M, Moora M, Zobel M, Wilson SD, Pärtel M. **2012**. Plant species richness belowground: Higher richness and new patterns revealed by next-generation sequencing. *Molecular Ecology* **21**: 2004–2016.

HilleRisLambers J, Adler PB, Harpole WS, Levine JM, Mayfield MM. **2012**. Rethinking Community Assembly through the Lens of Coexistence Theory. *Annual Review of Ecology, Evolution, and Systematics* **43**: 227–248.

Holzapfel C, Mahall BE. **1999**. BIDIRECTIONAL FACILITATION AND INTERFERENCE BETWEEN SHRUBS AND ANNUALS IN THE MOJAVE DESERT. *Ecology* **80**: 1747–1761.

Hubbell SP. **2001**. *The Unified Neutral Theory of Biodiversity and Biogeography (MPB-32)*. Princeton University Press.

Inderjit, Callaway RM, Meron E. **2021**. Belowground feedbacks as drivers of spatial self-organization and community assembly. *Physics of Life Reviews* **38**: 1–24.

Kattge J, Bönisch G, Díaz S, Lavorel S, Prentice IC, Leadley P, Tautenhahn S, Werner GDA, Aakala T, Abedi M, *et al.* 2020. TRY plant trait database – enhanced coverage and open access. *Global Change Biology* **26**: 119–188.

Klimešová J, Martínková J, Ottaviani G. **2018**. Belowground plant functional ecology: Towards an integrated perspective. *Functional Ecology* **32**: 2115–2126.

Klironomos JN, McCune J, Hart M, Neville J. **2000**. The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. *Ecology Letters* **3**: 137–141.

Lavorel S, Canadell J, Rambal S, Terradas J. **1998**. Mediterranean terrestrial ecosystems: research priorities on global change effects. *Global Ecology & Biogeography Letters* **7**: 157–166.

Lekberg Y, Bever JD, Bunn RA, Callaway RM, Hart MM, Kivlin SN, Klironomos J, Larkin BG, Maron JL, Reinhart KO, *et al.* 2018. Relative importance of competition and plant–soil feedback, their synergy, context dependency and implications for coexistence. *Ecology Letters* **21**: 1268–1281.

Lotka AJ. **1926**. Element of physical biology. *Science Progress in the Twentieth Century* **21**: 341–343.

Ma Z, Guo D, Xu X, Lu M, Bardgett RD, Eissenstat DM, McCormack ML, Hedin LO. **2018**. Evolutionary history resolves global organization of root functional traits. *Nature* **555**:

94–97.

Mack KML, Bever JD. **2014**. Coexistence and relative abundance in plant communities are determined by feedbacks when the scale of feedback and dispersal is local. *Journal of Ecology* **102**: 1195–1201.

Martens SN, Breshears DD, Meyer CW, Barnes FJ. **1997**. Scales of aboveground and below-ground competition in a semi-arid woodland detected from spatial pattern. *Journal of Vegetation Science* **8**: 655–664.

Matesanz S, Blanco-Sánchez M, Ramos-Muñoz M, de la Cruz M, Benavides R, Escudero A. **2021**. Phenotypic integration does not constrain phenotypic plasticity: differential plasticity of traits is associated to their integration across environments. *New Phytologist* **231**: 2359–2370.

Matesanz S, Pescador DSDS, Pías B, Sánchez AMAM, Chacón-Labella J, Illuminati A, de la Cruz M, López-Angulo J, Marí-Mena N, Vizcaíno A, *et al.* 2019. Estimating belowground plant abundance with DNA metabarcoding. *Molecular Ecology Resources* **19**: 1265–1277.

Matesanz S, Valladares F. **2014**. Ecological and evolutionary responses of Mediterranean plants to global change. *Environmental and Experimental Botany* **103**: 53–67.

McGill BJ, Enquist BJ, Weiher E, Westoby M. **2006**. Rebuilding community ecology from functional traits. *Trends in Ecology and Evolution* **21**: 178–185.

Médail F, Quezél P. **1999**. Biodiversity Hotspots in the Mediterranean Basin : Setting Global Conservation Priorities. *Conservation Biology* **13**: 1510–1513.

Messier J, Lechowicz MJ, McGill BJ, Violle C, Enquist BJ. **2017**. Interspecific integration of trait dimensions at local scales: the plant phenotype as an integrated network. *Journal of Ecology* **105**: 1775–1790.

Mittermeier R. **2004**. *Hotspots revisited*. Cemex.

Münkemüller T, Lavergne S, Bzeznik B, Dray S, Jombart T, Schiffers K, Thuiller W. **2012**. How to measure and test phylogenetic signal. *Methods in Ecology and Evolution* **3**: 743–756.

Myers N, Mittermeler RA, Mittermeler CG, Da Fonseca GAB, Kent J. **2000**. Biodiversity hotspots for conservation priorities. *Nature* **403**: 853–858.

Noy Meir I. **1981**. Spatial effects in modelling of arid ecosystems. In: Arid-land ecosystems:ecosystems: structure, functioning and management. Vol 2. Cambridge Univ. Press, 411–432.

Olff H, Vera FWM, Bokdam J, Bakker ES, Gleichman JM, De Maeyer K, Smit R. **1999**.

Shifting Mosaics in Grazed Woodlands Driven by the Alternation of Plant Facilitation and Competition. *Plant Biology* **1**: 127–137.

Pärtel M, Szava-Kovats R, Zobel M. **2011**. Dark diversity: shedding light on absent species. *Trends in Ecology & Evolution* **26**: 124–128.

Pellkofer S, Van Der Heijden MGA, Schmid B, Wagg C. **2016**. Soil Communities Promote Temporal Stability and Species Asynchrony in Experimental Grassland Communities. *PLOS ONE* **11**: e0148015.

Phillips DL, MacMahon JA. **1981**. Competition and Spacing Patterns in Desert Shrubs. *The Journal of Ecology* **69**: 97–115.

Price JN, Hiiesalu I, Gerhold P, Pärtel M. **2012**. Small-scale grassland assembly patterns differ above and below the soil surface. *Ecology* **93**: 1290–1296.

Prinzing A, Durka W, Klotz S, Brandl R. **2001**. The niche of higher plants: evidence for phylogenetic conservatism. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **268**: 2383–2389.

Van Der Putten WH. **2012**. Climate Change, Aboveground-Belowground Interactions, and Species' Range Shifts. *Annual Review of Ecology, Evolution, and Systematics* **43**: 365–383.

Rana G, Katerji N. **2000**. Measurement and estimation of actual evapotranspiration in the field under Mediterranean climate: a review. *European Journal of Agronomy* **13**: 125–153.

Raunkiaer C. **1934**. *The life forms of plants and statistical plant geography; being the collected papers of C. Raunkiaer*. Oxford: Clarendon Press.

Rewald B, Meinen C, Trockenbrodt M, Ephrath JE, Rachmilevitch S. **2012**. Root taxa identification in plant mixtures - current techniques and future challenges. *Plant and Soil* **359**: 165–182.

Ryel RJ, Ivans CY, Peek MS, Leffler AJ. **2008**. Functional Differences in Soil Water Pools: a New Perspective on Plant Water Use in Water-Limited Ecosystems. In: Progress in Botany. Springer, Berlin, Heidelberg, 397–422.

Saatkamp A, Cochrane A, Commander L, Guja LK, Jimenez-Alfaro B, Larson J, Nicotra A, Poschlod P, Silveira FAO, Cross AT, *et al.* 2019. A research agenda for seed-trait functional ecology. *New Phytologist* **221**: 1764–1775.

Schenk HJ. **2006**. Root competition: Beyond resource depletion. *Journal of Ecology* **94**: 725–739.

Schenk HJ, Jackson RB. **2002**. Rooting depths, lateral root spreads and below-ground/above-ground allometries of plants in water-limited ecosystems. *Journal of Ecology* **90**: 480–494.

Semchenko M, Barry KE, de Vries FT, Mommer L, Moora M, Maciá-Vicente JG. **2022**. Deciphering the role of specialist and generalist plant–microbial interactions as drivers of plant–soil feedback. *New Phytologist* **234**: 1929–1944.

Siefert A. **2012**. Incorporating intraspecific variation in tests of trait-based community assembly. *Oecologia* **170**: 767–775.

Smith SE, Read DJ. **2010**. *Mycorrhizal Symbiosis*. ACADEMIC PRESS.

Teste FP, Kardol P, Turner BL, Wardle DA, Zemunik G, Renton M, Laliberté E. **2017**. Plant-soil feedback and the maintenance of diversity in Mediterranean-climate shrublands. *Science* **355**: 173–176.

Torsvik V, Salte K, Sorheim R, Goksoyr J. **1990**. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. *Applied and Environmental Microbiology* **56**: 776–781.

Urcelay C, Díaz S. **2003**. The mycorrhizal dependence of subordinates determines the effect of arbuscular mycorrhizal fungi on plant diversity. *Ecology Letters* **6**: 388–391.

Valladares F, Bastias CC, Godoy O, Granda E, Escudero A. **2015**. Species coexistence in a changing world. *Frontiers in Plant Science* **6**: 866.

Violle C, Navas ML, Vile D, Kazakou E, Fortunel C, Hummel I, Garnier E. **2007**. Let the concept of trait be functional! *Oikos* **116**: 882–892.

Wagg C, Jansa J, Stadler M, Schmid B, Van Der Heijden MGA. **2011**. Mycorrhizal fungal identity and diversity relaxes plant-plant competition. *Ecology* **92**: 1303–1313.

Wardle DA, Bardgett RD, Klironomos JN, Setälä H, Van Der Putten WH, Wall DH. **2004**. Ecological linkages between aboveground and belowground biota. *Science* **304**: 1629–1633.

Weigelt A, Mommer L, Andraczek K, Iversen CM, Bergmann J, Bruelheide H, Fan Y, Freschet GT, Guerrero-Ramírez NR, Kattge J, *et al.* 2021. An integrated framework of plant form and function: the belowground perspective. *New Phytologist*.

Westoby M. **1998**. A leaf-height-seed (LHS) plant ecology strategy scheme. *Plant and Soil* **199**: 213–227.

Westoby M, Falster DS, Moles AT, Vesk PA, Wright IJ. **2002**. Plant Ecological Strategies: Some Leading Dimensions of Variation Between Species. *Annual Review of Ecology and Systematics* **33**: 125–159.

Westoby M, Leishman MR, Lord JM. **1995**. On Misinterpreting the `Phylogenetic Correction'. *The Journal of Ecology* **83**: 531–534.

Wildová R. **2004**. Below-ground spatial pattern of rhizomes in a grassland community and its

relevance to above-ground spatial pattern. *Plant Ecology* **174**: 321–338.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornellssen JHC, Diemer M, *et al.* 2004. The worldwide leaf economics spectrum. *Nature* **428**: 821–827.

Yang G, Liu N, Lu W, Wang S, Kan H, Zhang Y, Xu L, Chen Y. **2014**. The interaction between arbuscular mycorrhizal fungi and soil phosphorus availability influences plant community productivity and ecosystem stability. *Journal of Ecology* **102**: 1072–1082.

Yang G, Wagg C, Veresoglou SD, Hempel S, Rillig MC. **2018**. How Soil Biota Drive Ecosystem Stability. *Trends in Plant Science* **23**: 1057–1067.

Chapters

Chapter 1

Coordination between water uptake depth and the leaf economic spectrum in a Mediterranean shrubland

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Summary

Water is the most limiting resource for plant survival and growth in arid environments, but the diversity of water-use strategies among coexisting species in dryland communities is not well understood. There is also growing interest in assessing whether a whole-plant coordination exists between traits related to water-use and the leaf economic spectrum (LES). We used water stable isotopes ($\delta^{2}H$, $\delta^{18}O$) to quantify water uptake proportions from different soil depths by 24 species in a Mediterranean shrubland. Leaf traits associated with water use efficiency, stomatal regulation ($\delta^{13}C$, $\delta^{18}O$) and the LES (SLA, N, P, K concentrations) were also measured. We assessed potential trade-offs between the above-mentioned leaf traits, water uptake depth and their relationship with species abundance. We found distinct ecohydrological niche segregation among coexisting species. Bayesian models showed that our shrubland species used a median of 37% of shallow soil water (0-30 cm) and 63% of deep water (30-100 cm). Still, water source proportions varied considerably among species, as shallow soil water-use ranged from a minimum of 6.4% to a maximum of 68%. Interspecific variability in foliar carbon investment (SLA) and nutrient concentrations was remarkably high, indicating diverse nutrient-use strategies along the LES. Leaf $\delta^{18}O$, $\boldsymbol{\delta}^{13}C$ and $\boldsymbol{\delta}^{15}N$ values also differed widely among species, revealing differences in stomatal regulation, water use efficiency and nitrogen acquisition mechanisms. After accounting for evolutionary history effects, water uptake depth was coordinated with the LES: species using shallower soil water from fertile topsoil layers exhibited a more acquisitive carbon- and nutrient-use strategy, whereas water uptake from deeper but less fertile soil layers was linked to a more conservative nutrient-use strategy. Leaf-level water-use traits significantly influenced species abundance, as water-savers with tight stomatal regulation and high water use efficiency were dominant. Greater utilization of water stored in nutrient-rich topsoil layers favoured a more acquisitive nutrient-use strategy, whereas a deeper water uptake pattern appeared to constrain access to nutrients. Our findings thus suggest a largely inescapable trade-off and coordination between soil water uptake depth and carbon- and nutrient-use strategies in low-fertility drylands.

Introduction

One of the main questions in plant ecology is understanding whole-plant phenotypic integration driving plant form and function (Freschet et al. 2010). As water is the most limiting resource for plant survival and growth in arid environments and is thus the main driver of competition between coexisting plant species (e.g. Kulmatiski *et al.*, 2010; Kulmatiski & Beard, 2013), increasing attention is paid to traits related to plant water-use strategy, especially in drylands such as Mediterranean shrublands. These ecosystems harbour diverse plant communities highly susceptible to species extinctions or composition shifts under climate change and aridification (West *et al.*, 2012; León-Sánchez *et al.*, 2020). Plant water-use strategy is defined by traits related to leaf-level stomatal regulation, water use efficiency and root water acquisition (Moreno-Gutiérrez *et al.*, 2012). In dryland ecosystems, leaf $\delta^{13}C$ and leaf $\delta^{18}O$ can serve as time-integrated proxies of leaf-level intrinsic water use efficiency (WUEi) and stomatal conductance (g_s), respectively (e.g. Farquhar *et al.*, 1989; Barbour, 2007; Moreno-Gutiérrez *et al.*, 2012). Prieto *et al.* (2018) suggested that these leaf isotopic traits could be an overlooked component of the leaf economic spectrum (LES, thereafter; Wright *et al.*, 2004) in water-limited plant communities, based on the tight coupling and coordination observed between LES traits and water-use isotopic traits across Mediterranean shrubland species.

Water uptake depth in the soil profile is another key aspect of plant water-use strategy related to water acquisition (Schulze *et al.*, 1996; Williams, D. G., & Ehleringer, 2000). Thus far, contrasting patterns have been observed regarding the relationship between water uptake depth and the leaf-level water-use strategy. Some works (e.g. Moreno-Gutiérrez et al., 2012; Brum et al., 2017; Jiang et al., 2020a; Ding et al., 2021) have found that species with a profligate water-use strategy (water-spenders), i.e. with high g_s and low WUEi, generally use water stored in shallower soil layers, while coexisting species with a water- saver strategy (water-savers), i.e. with low g_s and high WUEi, tend to use comparatively deeper soil water sources. In contrast, other studies (e.g. del Castillo et al., 2016; Beyer et al., 2018; Rodríguez-Robles et al., 2020) have found variable or even opposite patterns that are highly dependent on the species and the sampling season. In order to obtain an integrative perspective of the whole-plant water-use strategy and niche segregation among species in drylands, it is necessary to further investigate the relationship between soil water uptake depth and aboveground leaf traits related to stomatal regulation and water use efficiency (e.g. Moreno-Gutiérrez *et al.*, 2012; Sánchez-Martín *et al.*, 2021).Especially in drylands, ecohydrological niche segregation, i.e. the use of water from different soil depths or the spatial partitioning of species along soil moisture gradients in realised assemblages (Araya *et al.*, 2011; Silvertown *et al.*, 2015) among coexisting plant species is expected. Silvertown et al. (2015) reported that ecohydrological

niche segregation is commonly found in multiple habitats. Yet, only a few studies to date (e.g. Moreno-Gutiérrez et al. 2012; Palacio et al. 2017) have explored ecohydrological niche separation in drylands at the plant community level, i.e. considering many species coexisting at fine spatial scales. In this context, maximum rooting depth has been often considered a proxy of the water source used by plants (Bucci *et al.*, 2009; Zhou *et al.*, 2020). However, rooting depth is difficult to measure in field conditions, and often fails to robustly predict water sources actively accessed by plants (e.g. Ehleringer & Dawson, 1992; Holdo, 2013). As a powerful alternative, the isotopic composition of stem water can provide an estimate of the relative proportion of water sources used by plants, especially when steep vertical gradients of oxygen and hydrogen stable isotopes ratios ($\delta^{18}O/\delta^{16}O$ and $\delta^{2}H/\delta H$, respectively) of water along soil depth are formed due to evaporative isotopic fractionation (Allison *et al.*, 1983; Dawson *et al.*, 2002). As a result, plants using water stored in shallow soil layers tend to present stem water that is more enriched in the heavier isotopes (^{18}O and ^{2}H) because of intense evaporative isotopic fractionation of their main water source, compared with plants using deeper and less evaporated water sources(Moreno-Gutiérrez *et al.*, 2012; Querejeta *et al.*, 2021; Ding *et al.*, 2021).

If species coexisting in diverse dryland communities segregate their water niches by adopting different whole-plant water-use strategies, an additional remaining question is whether such differentiation affects species performance, i.e. abundance, at the community level. Contrasting, but equally successful, strategies to cope with water stress possibly coexist in drylands (Jacobsen *et al.*, 2008; Moreno-Gutiérrez *et al.*, 2012; West *et al.*, 2012). Therefore, contrasting water-use strategies could lead to niche complementarity to minimise competition and favour coexistence (McDowell et al. 2008; West et al. 2012). However, ecophysiological constraints determining inevitable trade-offs among plant traits could limit the number of feasible trait combinations and water-use strategies (Moreno-Gutiérrez *et al.*, 2012; Wang *et al.*, 2021). Several studies have highlighted the advantage of tapping deep water sources in drought-prone ecosystems (West *et al.*, 2012; Rempe & Dietrich, 2018; McCormick *et al.*, 2021), which also concurs with the greater biomass allocation to roots typical of these environments in comparison with more mesic vegetation types (Schenk & Jackson, 2002). It is also known that the most dominant and abundant species in a plant community tend to exhibit trait values closer to the 'optimal' strategy in any given ecosystem (Jiang *et al.*, 2020b). However, our current understanding of the relationship between depth of water uptake in the soil profile, leaf-level water- and nutrient-use strategy, and species abundances in plant communities at local scales is still poor.

In this study, we aimed to gain insight into the potential role played by plant water-use strategy in the assembly of plant communities in drylands. We measured the soil water isotopic profile with

depth along with stem water isotopic composition (δ^2H and $\delta^{18}O$) to identify the water sources used by the different coexisting species during the phenological peak of the community (late spring). We also measured leaf $\delta^{18}O$ and $\delta^{13}C$ as proxies of time-integrated g_s and WUEi, respectively, as well as specific leaf area (SLA) and leaf nutrients (N, P, K) known to be key functional traits in the LES. We first assessed the presence of water niche partitioning between 24 species coexisting in a Mediterranean shrubland. Then, we assessed the potential coordination of water uptake depth with leaf traits related to stomatal regulation ($\delta^{13}C$, $\delta^{18}O$) and carbon- and nutrient-use strategy (i.e. LES traits). As evolutionary history can importantly affect the level of integration between plant traits (e.g. Zhou et al. 2018; Long and Medeiros 2021), we accounted for the phylogenetic relatedness among species in our analyses (Caruso *et al.*, 2020). Based on plant functional diversity theory (Reich, 2014) and the findings of recent studies conducted in other water-limited ecosystems (Prieto *et al.*, 2018; Ding *et al.*, 2021), we expected a tight coordination between water uptake depth, leaf-level water-use strategy and LES traits across species. In last instance, we aimed to assess if any particular plant water- and nutrient-use strategy was advantageous and linked to greater species abundance in this dryland community.

Materials and Methods

Study area, plant and soil sampling

We sampled a species-rich Mediterranean shrubland located in the south of Madrid (Spain) (40°17'17.5" N 3°12'19.4" W, 760 m a.s.l.) characterised by a semiarid continental climate, with cold winters and intense summer droughts, a mean annual temperature of 12.8°C and a mean annual precipitation of 452 mm. The area presents low fertility calcareous soils with a variable content of gypsum, which creates a fine-scale patchy environment. The perennial plant community is very diverse and dominated mainly by dwarf shrubs and scrubs, hemicrytophytes and grasses, being the most abundant species *Bupleurum fruticescens* L., *Thymus vulgaris* L., *Linum suffruticosum* L., *Helianthemum cinereum* Pers., and *Stipa pennata* L.

We established three 30 x 30 m plots with similar slope (≈ 10%), orientation (north-west) and species composition, located 0.5 km away from each other. We sampled three individuals per species (one per plot) for a total of 24 species and 72 individuals. These species encompass more than the 90% of the plant community in terms of cover and abundance. All samples were collected on the same day in the first week of June. The sampling date was carefully chosen to: 1) match the flowering peak of the community; 2) collect samples at the onset of the summer drought period (Spring-Summer transition period), and 3) ensure a preceding 2-week period with no rainfall. The

latter was necessary to obtain a steep isotopic soil water gradient, which is formed in response to soil water evaporation and isotopic fractionation during rainless periods (Allison et al., 1983). We collected one segment of the basal stem from each individual to measure stem water isotopic composition, in order to avoid any potential bias due to the proximity of leaves or photosynthetic stems where transpiration takes place (Schwinning 2008). In a few cases in which the aerial part of the plant was completely herbaceous (e.g., *Stipa pennata*), we sampled the root crown instead. We immediately stored these plant samples in 5 ml glass vials, capped and wrapped with parafilm, before placing them in a cooler. We also sampled well-developed and healthy leaves of the same individuals for specific leaf area (SLA) measurement and elemental and isotopic analyses (see details below).

On the same date, we sampled three soil profiles down to 1 metre of depth by using a hand auger of 4 cm of diameter. Soil cores were sampled from the centre of each selected plot. Soil cores were separated into 10 different portions, corresponding to different soil depth intervals (0–5, 5–10, 10-15, 10–20, 20–30, 30–40, 40–50, 50–65, 65–80, 80–100 cm, see Fig. S1), which were stored in separate sealable bags and maintained in a cooler. Within the same day we transported all soil and plant samples in coolers to the lab, where they were kept at -20 C°, except for the leaf material which was stored at 4 C° and processed the day after field sampling. No permissions were required to carry out the fieldwork.

Trait measurement and isotopic analyses

We extracted all water contained in plant stems and soil samples using a cryogenic vacuum extraction line (2 h extraction time at 100°C and 10 millitorr vacuum pressure; Ehleringer *et al.*, 2000). Stem and soil water contents were calculated based on sample weights before and after the extraction. Extracted water samples were shipped to the Center for Stable Isotope Biogeochemistry of the University of California (Berkeley, USA) for isotopic analyses. Specifically, we determined oxygen and hydrogen stable isotopes ratios in both stem and soil water by isotopic ratio mass spectrometry (IRMS), through a Thermo Gas Bench II and a hot chromium reactor unit (H/Device™), interfaced to a Thermo Delta V Plus mass spectrometer. Hydrogen (δ^2H) and oxygen ($\delta^{18}O$) isotopic composition are expressed in ‰ notation relative to the standard V-SMOW (Vienna standard Mean Ocean Water), according to the equation:

δ^2H ($\delta^{18}O$) = 1000 * ($R_{sample}/R_{standard}$) - 1

where R_{sample} and $R_{standard}$ represent the ratio of the heavy to light isotope ($^2H/H$ or $^{18}O/^{16}O$) of the sample and the standard, respectively (Dawson *et al.*, 2002). As it is considered a good indicator of

evaporative isotopic fractionation processes (Craig & Gordon, 1965; Gat, 1996), we estimated water deuterium excess (Dansgaard, 1964) as:

d-excess = $\delta^2H - 8 * \delta^{18}O$

The Local Meteoric Water Line (LMWL) representing the isotopic composition of precipitation for Central Spain was obtained from Díaz-Teijeiro et al. (2009):

$\delta^2H = 12.40 + 8.49 * \delta^{18}O$

We selected five mature leaves of each sampled individual to calculate SLA. First, we scanned the leaves and measured leaf area (LA). Thereafter, we dried them at 60 Cº for 48 hours to assess the dry weight (DW). SLA (cm^2/g) was calculated as = LA/DW. For the leaf isotopic analyses, we ground the dried leaves into a fine powder using a ball mill, and samples were weighted and encapsulated in tin ($\boldsymbol{\delta}^{13}C/\boldsymbol{\delta}^{15}N$, 4 mg) or silver capsules ($\boldsymbol{\delta}^{18}O$, 0.3 mg). Leaf samples were shipped to the Centre for Stable Isotope Biogeochemistry of the University of California (Berkeley, USA) for analysis. Measurements of leaf $\boldsymbol{\delta}^{13}C$ and $\boldsymbol{\delta}^{15}N$, and leaf C and N concentrations (%), were carried out by continuous flow (CF) dual isotope analysis using a CHNOS Elemental Analyzer interfaced to an IsoPrime100 mass spectrometer. Long-term external precision for C and N isotope determinations is ± 0.10‰ and ± 0.20‰, respectively. Leaf $\boldsymbol{\delta}^{18}O$ was measured in CF using an Elemental PYRO Cube interfaced to a Thermo Delta V mass spectrometer. Long term external precision for IAEA-V-9 (cotton cellulose) is ± 0.20‰. Isotopic composition values are expressed in delta notation (**‰**) relative to the international standard (V-SMOW for $\delta^{18}O$, V-PDB for ^{13}C**)**. Leaf P and K concentrations (%) were measured by inductively coupled plasma optical emission spectrometry (ICP- OES, Thermo Elemental Iris Intrepid II XDL, Franklin, MA, USA) at CEBAS-CSIC.

Phylogenetic tree construction

We sequenced the 24 species using the barcoding locus rbcL. First, we collected leaves from three individuals of each species in the same study site. Then, we air dried and stored 20 mg of leaf material in silica gel. DNA extraction was carried out using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). The rbcL barcode was amplified in a 25 μL reaction with 2 μL of DNA and 23 μL mix reaction composed by 2.5 μL of Taq buffer with 2 mM MgCl2, 1 μL of dNTP Mix (0.4 mM), 1.25 μL of reverse and forward primer and 1.25 U Taq DNA Polymerase (Biotools, Madrid, Spain). PCR amplification was performed on a S1000 Thermal Cycler (Bio Rad, Hercules, CA, USA). Primers and PCR conditions are provided in Table S1. Amplified PCR products were purified using the ExoSap purification kit® (USB Corporation, Cleveland, OH, USA), and sequenced by MACROGEN (Seoul, Korea and Amsterdam, Netherlands). Consensus sequences

were assembled using Sequencher 4.1.4 software (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned with MAFFT online v. 7, then checked manually with Mesquite version 2.6. Sequences were used to build a phylogenetic tree by maximum likelihood methods using the R package 'Phangorn' (Schliep, 2011), using the GTR + G + I model and 100 bootstrap replicates (Violle *et al.*, 2011).

Statistical analysis

To estimate the proportion of different water sources used by each species, we used a Bayesian mixed modelling approach (*run_model* function, *MixSIAR* package, Stock et al. 2018). Given the growing uncertainties related to the widespread δ^2H depletion observed in plant stem water relative to soil water sources (Barbeta *et al.*, 2020, 2022; de la Casa *et al.*, 2021; Chen *et al.*, 2021), we first assessed if plant water values presented a significant δ^2H depletion compared with the soil isotopic values (Fig. 1 and Fig. S2 for individual values). As the two regression lines corresponding to plant and soil water values showed different slopes, we carried out the Bayesian model using only the $\delta^{18}O$ data. Researchers have traditionally reduced the number of sources through aggregation in order to improve model inference (e.g. Ben-David *et al.*, 1997; Stock *et al.*, 2018). Thus, we selected three soil intervals as main water sources: shallow (5 to 30 cm), intermediate (30 to 50 cm) and deep (50 to 100 cm). However, since two water sources (30-50 and 50-100 cm) resulted to be highly correlated (R = 0.84, Fig. S3), we carried out an "*a posteriori*" aggregation, i.e. after running the mixing model, of the two sources, combining them into a single water source pool (*combine_sources* function, *MixSIAR* package), as suggested by Stock *et al.* (2018). We calculated the mean and standard errors for the $\delta^{18}O$ values of soil water at each soil depth interval from the three plots. The calculated means (±SD) were included as water source data input in the model, while the raw stem $\delta^{18}O$ values of the 72 individuals were considered as the 'consumers' data input. We run the model setting manually the number of iterations, burn-in and thinning to 500000, 200000 and 300000, respectively. Model diagnosis was performed via the Gelman–Rubin and Geweke tests (Stock & Semmens, 2013). Therefore, we finally obtained proportions of water sources used by each study species from two different soil depth intervals (shallow 0-30 and deep, 30-100 cm).

We assessed whether plant traits related to water-use strategy, i.e. stem water $\delta^{18}O$, leaf $\delta^{18}O$ and leaf $\delta^{13}C$, were coordinated with leaf N and SLA by carrying out a Principal Components Analyses (*prcomp* function, *stats* package) considering mean species values. SLA and leaf N concentration were selected because they are the main representative traits of the LES. We selected the stem water $\delta^{18}O$ for the PCA because it is considered a more reliable proxy of water source utilization compared with stem water δ^2H (Barbeta *et al.*, 2020, 2022; Chen *et al.*, 2021). When necessary,

variables were log-transformed to adjust for normality, and all were scaled before running the analysis. We carried out the PCA (phylPCA hereafter) including the phylogenetic distance among species to account for the evolutionary history of our study species (method = BM, i.e. Brownian Motion; *phyl.pca* function, *phytools* package). We also run the same PCA without including phylogeny to assess for potential discrepancies between the two outputs.

Finally, in order to assess if the plant water- and/or nutrient-use strategy affected species abundances, we tested the existence of correlation (Kendall's correlations; *cor.test* function, *stats* package) between the species scores in the first two axes of both PCAs, with and without phylogeny, and the species abundance in the plant community. Species abundance data were collected in a previous study where we fully mapped the same plant community (for more details see Illuminati et al. 2021). Specifically, we considered both the cover (%) and the number of individuals as indicators of the species dominance and abundance at the community level (Table S2).

Results

Vertical soil water isotopic gradient and ecohydrological niche segregation among species

Soil water content ranged from a minimum of 3.66% (SD = ± 0.70) in the topsoil layer (0-5 cm), to a maximum of 11.38% (SD = ± 4.67) in the deepest soil layer (80 to 100 cm; Fig. 2). We found a steep gradient in the isotopic composition of water along the soil profile, with major differences between sampled layers in the first 0-30 cm of soil depth (Fig. 2a, b). Both soil water δ^2H and $\delta^{18}O$ values became more negative with depth along the soil column, whereas d-excess values were most negative near the surface and increased with depth (Fig. 2c). While standard deviations of water δ^2H partially overlapped among soil layers, standard deviations of water $\delta^{18}O$ were smaller and most of the soil layers presented a clearly distinct isotopic composition (Fig. 2a, b). The observed patterns indicate strong evaporative isotopic fractionation of soil water stored in the upper layers, which progressively decreased with depth. The slope of the linear model relating the isotopic composition of soil water across soil samples was flatter than that of the LMWL, which further indicates strong evaporative enrichment of water stored in upper soil layers (Fig. 1).

The 24 coexisting species displayed large and clinal differences in stem water isotopic composition (δ^2H, $\delta^{18}O$, d-excess) and water content (Fig. S4). The Bayesian model calculations (Fig. 3) showed that the 24 coexisting species used a median of 37% of shallow soil water (0-30 cm) and 63% of deep soil water (30-100 cm). Still, water source proportions varied considerably between species, with several species, i.e. *Leuzea conifera, Thymus vulgaris, Stipa pennata, Koeleria vallesiana, Coris*

monspeliensis, Sideritis incana, relying more on water stored in the shallow topsoil layer (0-30 cm) than in deeper layers. Indeed, shallow soil water use by plants ranged widely from a minimum of 6.4% for *Linum narbonense*, to a maximum of 68% for *Leuzea conifera*.

Species variation in leaf nutrient and isotopic traits

The 24 coexisting species showed a wide range of variation in functional leaf traits related to water- and nutrient-use strategies. Interspecific variability in foliar carbon investment (SLA) and leaf N, P and K concentrations (Fig. 4 and S5) was remarkably high. Species mean SLA values ranged from 50.61 to 133.74 cm^2/g; while species mean leaf N, P and K concentrations ranged from 1.16 to 4.54%, 0.02 to 0.06% and 0.14 to 0.97%, respectively. Species mean leaf $\delta^{18}O$ values ranged from 28.22 to 40.15 ‰, leaf $\boldsymbol{\delta}^{13}C$ ranged from -30.16 to -26.48 ‰, and leaf $\boldsymbol{\delta}^{15}N$ ranged from -4.67 to 0.67 ‰, suggesting large differences in stomatal regulation, water use efficiency and nitrogen acquisition mechanisms among coexisting species (Fig. 4, Fig. S6).

Coordination between LES, water-use traits and species abundance

The first axis of the phylPCA explained 43% of the total variance in key plant traits, while PC2 and PC3 explained an additional 24% and 18%, respectively (Fig. 5, Table S3). The first axis largely reflected species variation along the LES, with species showing higher SLA and leaf N values representing a more acquisitive carbon- and nutrient-use strategy in one side, and species with lower SLA and leaf N representing a more conservative strategy at the opposite side (Table S3). Reinforcing this interpretation of the first phylPCA axis as a LES gradient, we observed significant (or marginally significant) correlations of this axis with both leaf P ($R = -0.31$, $p = 0.04$) and leaf K concentrations ($R = -0.27$, $p = 0.07$), which are known to be related to the LES. Interestingly, stem water $\delta^{18}O$ also loaded heavily on the first PCA axis ($R = 0.59$) (Table S3), indicating greater utilization of deep soil water in species with more conservative nutrient-use strategies along the LES.

The second phylPCA axis was largely related to leaf-level water-use traits, with heavy loadings of both leaf $\delta^{13}C$ and $\delta^{18}O$ (Table S3). Species exhibiting higher leaf $\delta^{13}C$ and $\delta^{18}O$ values representing a water-saver strategy with tighter stomatal regulation of transpiration and higher intrinsic water use efficiency plotted on one side of the PC2 axis. Species with lower leaf $\delta^{13}C$ and $\delta^{18}O$ values representing a more water-spender strategy with high stomatal conductance and low intrinsic water use efficiency plotted on the opposite side of the second phylPCA axis. Results of the PCA carried out without including phylogeny were rather similar to the phylPCA, although an important

discrepancy emerged in the coordination of stem water $\delta^{18}O$ with the second PCA axis, instead of with the first PCA axis (Fig. S7, Table S4).

Species abundances, i.e. both the cover and the number of individuals were significantly correlated to the species scores along the second phylPCA axis (R = 0.30 and 0.37; p = 0.04 and 0.01, respectively; Fig. 6), indicating dominance by species with water-saver strategies. We did not find any correlation of species abundances with their scores along the first phylPCA axis. Interestingly, different results were found when considering the PCA carried out without phylogeny. The only marginally significant correlation observed in this case was that between the first axis and the species cover (R = 0.27; p = 0.06), suggesting that the plant community was dominated by species with a conservative LES strategy.

Discussion

Ecohydrological niche segregation and variation in leaf functional traits

Coexisting perennials in this semiarid shrubland showed a marked vertical soil water partitioning and ecohydrological niche segregation during the phenological peak. The pattern observed of heavy deep water use by many species is similar to that found in other drylands where deep root systems are widespread (Schenk & Jackson, 2002). Nevertheless, several species such as *Leuzea conifera*, *Thymus vulgaris*, *Coris monspeliensis* and the grasses *Stipa pennata* and *Koeleria vallesiana* relied more heavily on shallow soil water sources (0-30 cm) than on deeper soil water pools (30-100 cm). (Fig. 3).

Silvertown et al. (2015) found that ecohydrological niche segregation is widespread and occurs in a broad range of vegetation types, from drylands to tropical forests, although most of this evidence relied on a relatively small number of species per vegetation type. Our results showed that a clear complementary pattern in the exploitation of the most limiting resource (i.e. soil water) exists when a diverse array of coexisting species is considered. Indeed, an evaluation of all the species coexisting in a plant community is necessary to assess if ecohydrological niche segregation explains fine-scale species coexistence and assembly (Silvertown 2004). To our knowledge, few previous works (e.g., Palacio et al. 2017; Sohel et al. 2021) have reported such well-structured and distinct water niche segregation among multiple coexisting species within the same plant community at fine spatial scales.

The large variability in leaf morphological (SLA) and nutrient (N, P and K) traits (Fig. 4 and S5) encountered among the 24 coexisting species indicated a high functional diversity regarding carbon- and nutrient-use strategy along the LES (Wright et al. 2004). This was further evidenced

by the large interspecific variation in leaf $\delta^{15}N$ values (Fig. S6), which largely reflects different nitrogen acquisition mechanisms related to the presence, or lack thereof, of different root-microbe symbiotic associations (Craine *et al.*, 2009). The highest leaf $\delta^{15}N$ values were found in non-mycorrhizal or facultative-mycorrhizal species, whereas leaf $\delta^{15}N$ values around zero were found in N_2 fixing species (*Fabaceae*). Obligate mycorrhizal species (arbuscular and/or ectomycorrhizal) showed negative leaf $\delta^{15}N$ values, with ectomycorrhizal species in particular showing the most negative values of all (Fig. S6) (Brundrett, 2009; Craine *et al.*, 2009).

Alongside the wide variation in LES traits related to carbon- and nutrient-use strategy, the species presented large variability in leaf isotopic traits ($\delta^{13}C$ and $\delta^{18}O$) linked to stomatal regulation and leaf-level water use efficiency. This indicated the coexistence of species with sharply contrasting water-use strategies within the same plant community, ranging from water-saver to water-spender species (Moreno-Gutiérrez *et al.*, 2012; Prieto *et al.*, 2018).

Linking water-use strategy, the leaf economic spectrum and species' abundances

Despite the remarkably large interspecific variability along the LES, most species plotted on the conservative side of the carbon- and nutrient-use strategy gradient in the multidimensional trait space (see phylPCA in Fig. 5). Dominance of conservative carbon- and nutrient-use strategies has been previously predicted and reported in several nutrient-poor dryland plant communities (Chapin et al., 1993; Wright et al., 2004; Reich, 2014). Interestingly, stem water $\delta^{18}O$ also loaded substantially on (and covaried with) the LES axis, indicating that species on the resource conservative side of the LES primarily used water stored in deeper soil layers with more temporally stable moisture content. Conversely, species with more acquisitive nutrient-use strategies along the LES used a much greater proportion of water stored in shallower soil layers that are richer in nutrients but are exposed to sharper fluctuations in moisture content (Fig.1 and 5). It is worth to note that species using isotopically enriched water stored in the shallower soil layers during spring may not necessarily be characterized by a shallow root system. Indeed, several species may have dimorphic root systems which can capture water from different soil depths (e.g. Dawson & Pate, 1996; Filella & Peñuelas, 2003), according to the fluctuating soil moisture conditions in upper soil layers which are subjected to sharp seasonal variations in arid and semi-arid environments (e.g. Schwinning & Ehleringer, 2001), but also depending on other important factors such as plant phenology (Reynolds et al., 2004; Ryel et al., 2008).

Greater use of water stored in nutrient-rich topsoil layers may favour a more acquisitive nutrient-use strategy along the LES, particularly after rainfall pulses that enhance nutrient mineralization, solubilisation and uptake by roots in the fertile topsoil (Schwinning & Sala, 2004; Huxman et al.,

2004). Our findings are in agreement with the two-pools hypothesis postulated by Ryel et al. (2008), according to which the shallow soil water pool, also named "growth" pool (rich in nutrients), would be preferentially exploited during the main resource acquisition and growth period, i.e. in the wet season. The shallow water pool would therefore be the milieu where high inter-specific competition dynamics would take place (Ryel et al., 2008; Schenk, 2008), and thus species characterized by acquisitive nutrient-use strategies may gain an advantage over more conservative species. The deep water pool, also termed the "maintenance" water pool, would instead represent a water source used by those perennial species which do not senesce but maintain physiological activity, even if low, during drought periods (Ryel et al., 2008; Schenk, 2008). In agreement with this hypothesis, some recent studies (e.g. Kulmatiski et al., 2017, 2020) comparing trees and grasses have indeed shown that nitrogen and water uptake may in part be spatially decoupled in trees, with a preference for shallow nutrient-rich layers for nitrogen uptake.

Conversely, the species relying primarily on water stored in deeper and nutrient-poor soil layers may have more limited access to essential nutrients like nitrogen, which may be necessarily coupled with a more conservative nutrient-use strategy along the LES (Fig. 5). Moreover, root access to deeper and more stable soil water pools may favour longer leaf lifespans in conservative species by buffering them against severe drought stress during extended rainless spells in drylands, which would be in agreement with the LES theory and predictions (Wright et al. 2004).

Our findings suggest a probably inevitable trade-off and coordination between soil water uptake depth and LES traits in Mediterranean semiarid shrubland communities, which may also occur in other nutrient-poor dryland ecosystems (Fig. 7). Previous pioneer works, such as Walter' two-layers hypothesis describing hydrological segregation between coexisting trees and grasses in dry savannas, and later extended to other arid and semi-arid environments, may have implicitly suggested this potential trade-off (Walter, 1939; Ryel et al., 2008; Ward et al., 2013). Even though water source partitioning was generally interpreted as the result of different rooting depths and water-use strategies between woody vegetation and grasses, the hydrological niche segregation observed in these studies may have also implied different nutrient-use strategies. Indeed, nutrient-use strategies have been shown to vary strongly between different life forms, especially between woody and non-woody species (e.g. Díaz et al., 2016).

In addition, coexisting species appeared scattered along a second orthogonal gradient related to leaf-level water-use strategies (Fig. 5). The second axis of the phylPCA explained 24% of the variance and showed strong loadings of both leaf $\delta^{13}C$ and $\delta^{18}O$, indicating that it was primarily related to stomatal behaviour (Barbour et al., 2000). According to previous studies conducted in Mediterranean ecosystems (e.g. Moreno-Gutiérrez et al., 2012; Prieto et al., 2018), this second axis

segregated species with tight stomatal regulation of transpiration and high water use efficiency (water-savers) from species with loose stomatal regulation, low water use efficiency and more profligate water use (water-spenders). In sub-humid Mediterranean shrublands located in southern France, Prieto et al. (2018) found a tight coordination between plant traits related to the LES (SLA and leaf N) and traits related to leaf-level water-use strategy (leaf $\delta^{13}C$ and leaf $\delta^{18}O$) converging along a single main PCA axis. Our results partly agree with this pattern, as leaf $\delta^{13}C$ also showed substantial loading on the first axis of the phylPCA (Table S3), indicating that species on the conservative side of the LES generally show higher WUEi than their neighbour nutrient-acquisitive species. However, the heavy loadings of both leaf $\delta^{13}C$ and leaf $\delta^{18}O$ along a distinct second dimension (phylPCA axis 2) that was orthogonal to the LES (phylPCA axis 1) in the multi-trait space suggested a separation between the leaf-level water use continuum and the LES continuum in our semiarid shrubland.

Interestingly, we found an unexpected discrepancy between the PCA results accounting (or not) for species phylogeny and relatedness (Fig. 5 and Fig. S7). When evolutionary history was not considered in the PCA, stem water $\delta^{18}O$ was tightly coordinated with leaf $\delta^{13}C$ and leaf $\delta^{18}O$. This agrees with previous studies (e.g. Moreno-Gutiérrez et al. 2012; Ding et al. 2021), in which plant species with a water-saver strategy exploited deeper soil water sources, whereas species with a more profligate water-use strategy preferentially used shallower soil water sources. This finding highlights how phylogeny strongly affects the observed coordination between plant traits. We suggest that the apparent coordination of leaf $\delta^{13}C$ and $\delta^{18}O$ with stem water $\delta^{18}O$ along the same PCA axis may be due to common evolutionary history that is probably connected with the existence of strong ecological filters, rather than to the existence of a true universal trade-off between these functional traits. Similarly, a recent study (Zhou *et al.*, 2021) showed how variation in traits related to water-use strategy was greatly influenced by phylogeny and that certain traits were more strongly variable in certain clades compared to others. It is thus critically important to consider the potential role played by evolutionary history when exploring the coordination and trade-offs among multiple functional traits.

Finally, we observed that the species in this Mediterranean shrubland were rather scattered and evenly distributed along the leaf-level water-use gradient (phylPCA axis 2, Fig. 5). Whereas most of the species in the plant community clearly showed a conservative carbon- and nutrient-use strategy along the LES (low SLA, low N content) and strong reliance on deep soil water sources, there were nearly as many water-spender as water-saver species along the phylPCA second axis. Species dominance (cover %), but not abundance (number of individuals), was negatively associated, even if only marginally, to the first axis of the PCA carried out without phylogeny,

indicating a link between a nutrient-conservative strategy and species performance (Fig.S8). However, when we constrained this PCA by adding phylogenetic relatedness, we found that the first axis, mostly related with the nutrient-use strategy, became unrelated to species dominance. Instead, species dominance and abundance were significantly correlated with the second phylPCA, which was related to the water-use strategy. These differences support the notion that evolutionary history significantly affects community assembly processes at ecological time scale Our findings may also suggest that the coexistence of species with sharply contrasting water-use strategies may represent a key driver of plant community assembly in drylands where water is the most limiting resource. Given the dominance of conservative carbon- and nutrient-use strategies along the LES in nutrient-poor drylands, species also presenting a more conservative water-use strategy at leaf-level would be expected to further improve their whole-plant resource-use efficiency (Reich, 2014), and therefore their dominance and abundance within the plant community.

The remarkably wide diversity of plant functional traits and resource-use strategies encountered in this species-rich semiarid plant community suggests that an efficient and exhaustive acquisition and exploitation of the limiting soil resources available across the whole soil profile may reduce competition and may be ruling the assembly of this community (e.g. Peñuelas et al. 2011; Escudero & Valladares 2016). Vertical soil water niche segregation and complementarity among coexisting species would be expected to enhance the primary productivity and drought resistance and resilience of water-limited Mediterranean shrublands. Moreover, the presence of a few species with acquisitive LES strategy and/or profligate water use pattern may enhance soil resource capture and utilization during the short water and nutrient pulses after rainfall, thereby helping maximise overall plant community productivity in semiarid shrublands largely dominated by water-saver species. In particular, water-spender species with acquisitive nutrient-use strategy may be capable of achieving more efficient exploitation of water (and dissolved nutrients) stored in topsoil, which is a relatively ephemeral resource pool that is rapidly lost to unproductive direct soil evaporation in dryland ecosystems.

In conclusion, we provide evidence of strong vertical ecohydrological niche segregation among coexisting species in a diverse Mediterranean shrubland. Water source partitioning among neighbours likely enhances complementarity and decreases competition for water, the most critical limiting resource in drylands. Water uptake depth was coordinated with LES traits across species, with nutrient-conservative species relying more heavily on deep soil water sources, and nutrient-acquisitive species using a greater proportion of shallow water from the fertile topsoil layer, although the latter could be either shallow-rooted species or species with dimorphic root systems that may also be capable of using deep water sources during the dry summer season. To our

knowledge, this is the first study reporting a strong trade-off and coordination between soil water uptake depth and the leaf economics spectrum in nutrient-poor dryland plant communities. Moreover, leaf-level water use pattern represented a second distinct functional dimension in multi-trait space, and we encountered a significant link between tighter stomatal regulation, higher water use efficiency and greater species cover and abundance in semiarid shrublands. Finally, this study highlights the need to account for the evolutionary history and phylogenetic relationships among coexisting species when assessing functional traits trade-offs defining whole-plant resource-use strategies.

Figures

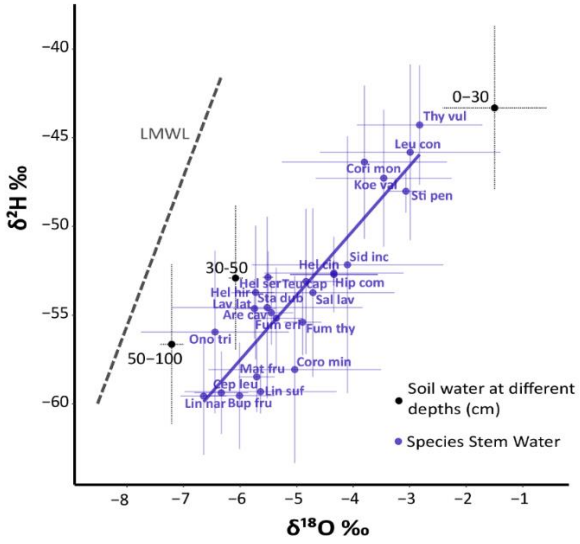

Fig. 1 Regression line of plant stem water isotopic composition (δ^2H and $\delta^{18}O$) and the Local Meteoric Water Line (LMWL). The points represent mean values (+/-SD) of 24 coexisting plant species and soil water isotopic composition at the three different depth intervals used in the mixed Bayesian model.

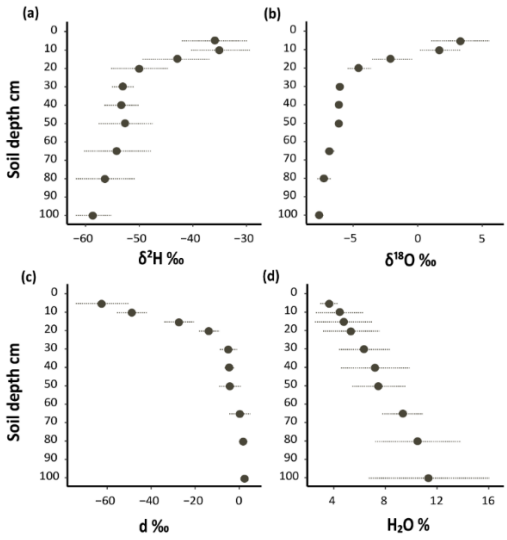

Fig. 2 Steep gradients with depth (from 5 to 100 cm) of soil water δ^2H ‰ **(a)**, $\delta^{18}O$ ‰ **(b)**, d-excess ‰ (i.e. deuterium excess), **(c)** and soil water content % **(d)**. Mean (+/-SD) are represented and calculated as the mean of three replicate soil samples (one soil sample from each of the three different plots).

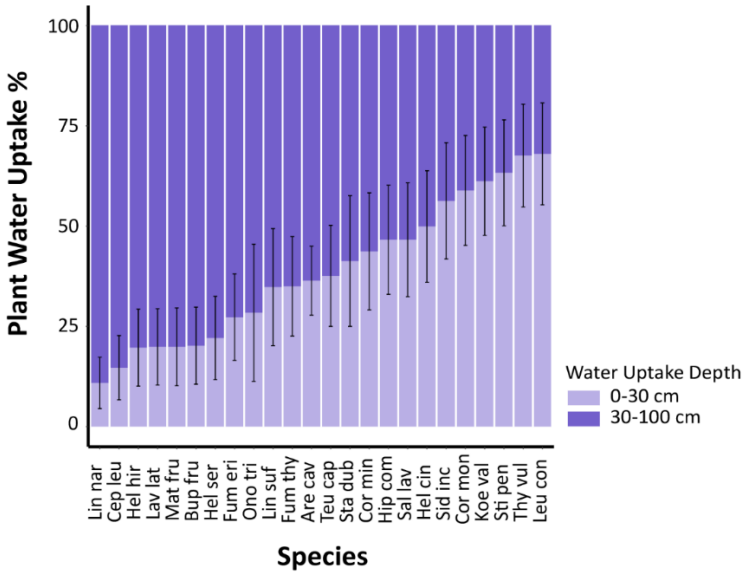

Fig. 3 Proportions (%) of two different water sources (0-30 and 30-100 cm of depth), and their standard deviations, estimated by the mixed Bayesian model, captured by 24 species coexisting in the same plant community corresponding to the moment of vegetative peak.

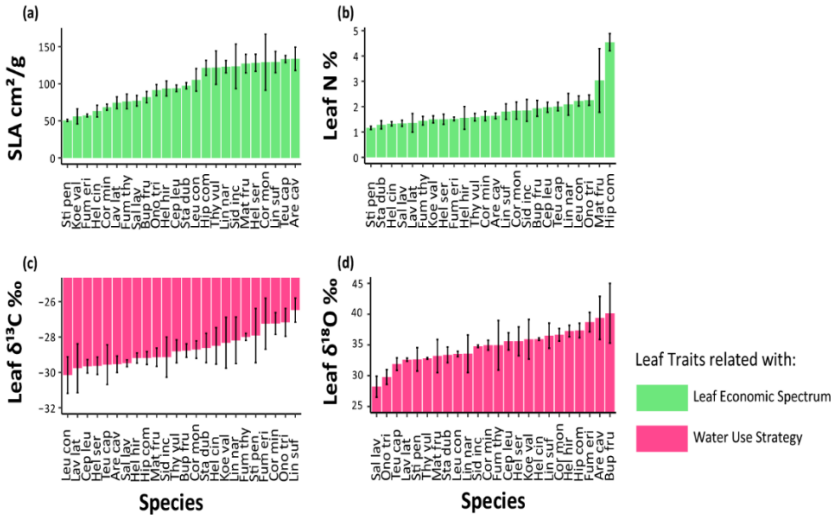

Fig. 4 Variation of SLA (cm^2/g **(a)**, leaf N (%) **(b)** and leaf isotopic traits ($\delta^{13}C$ and $\delta^{18}O$, **(c)** and **(d)** respectively) across the 24 coexisting species.

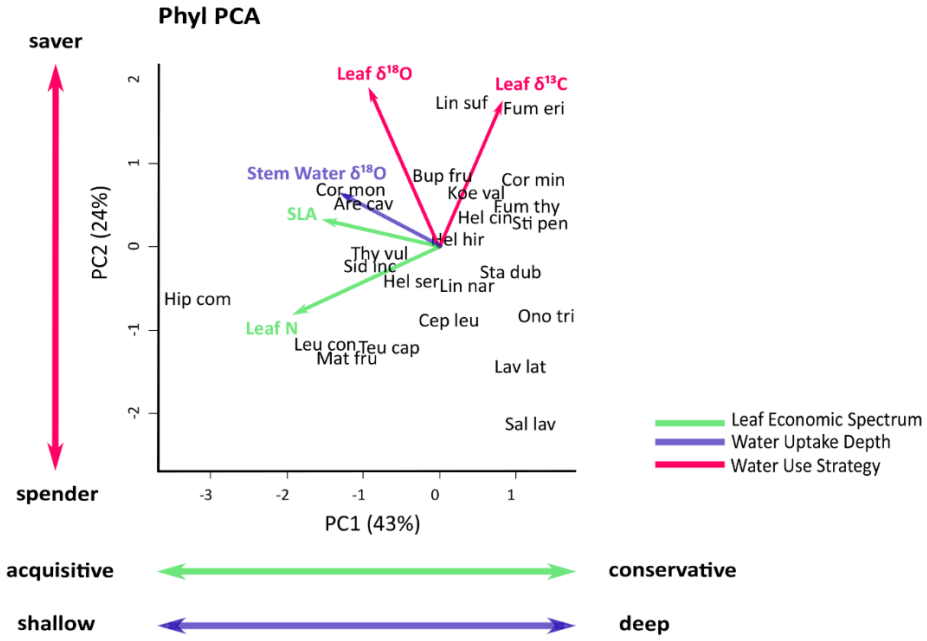

Fig. 5 Biplot of the two first axes of the phylPCA, where phylogenetic distances between species were considered and the species mean values were used as the input variables. The first phylPCA axis represents species variation both along the leaf economic spectrum and in water uptake depths. The second phylPCA axis depicts species variation along a leaf-level water use strategy gradient ranging from water-saver to water-spender. Blue arrow indicates water uptake depth (shallow/deep); red arrow indicates leaf-level water-use strategy and stomatal regulation (water-spender/water-saver); green arrow indicates carbon- and nutrient-use strategy along the leaf economic spectrum (conservative/acquisitive).

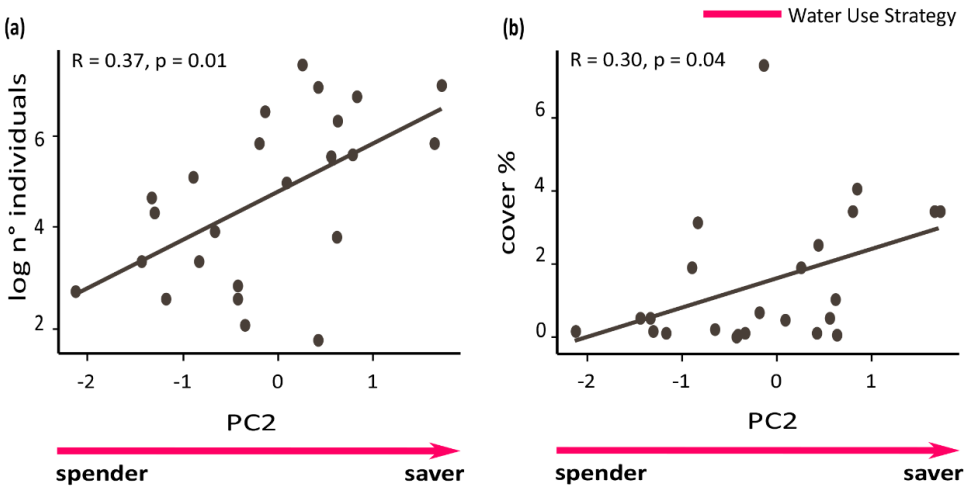

Fig. 6 Kendall's correlation tests carried out between the PC2 axis of the phylPCA, representing the continuum from a water-spender to a water- saver strategy, and the species abundances in the plant community (number of individuals **(a)** and the cover % **(b)**).

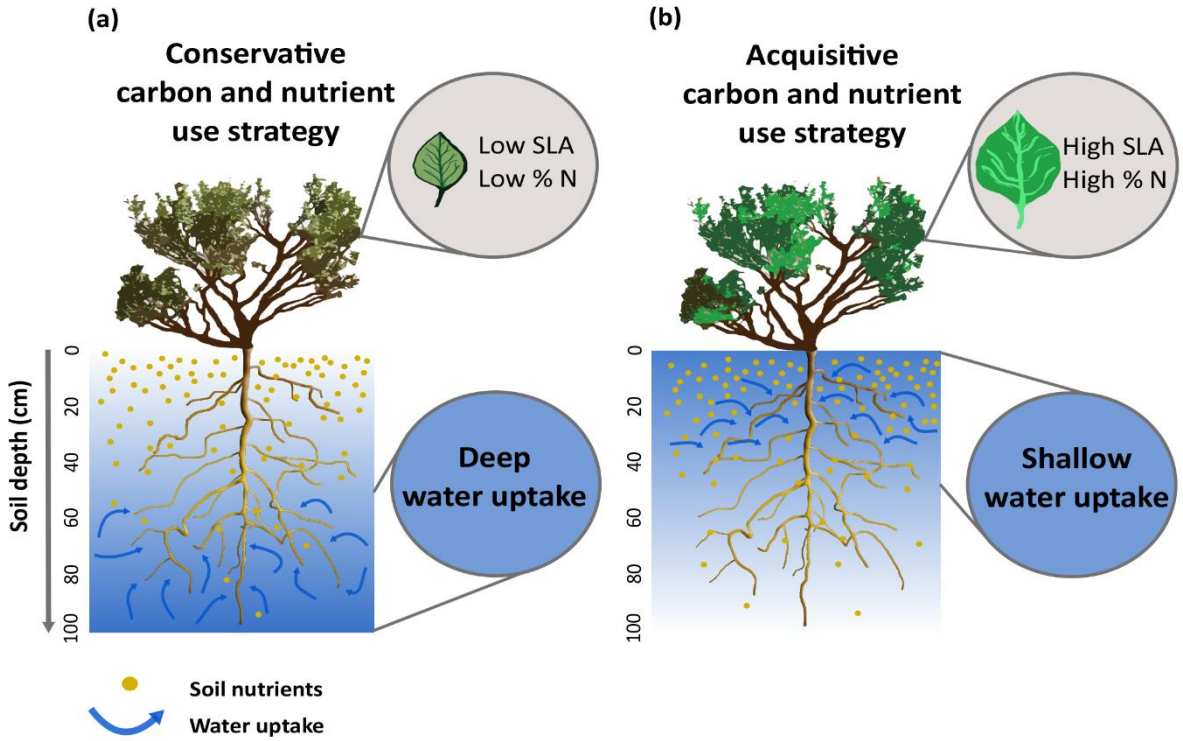

Fig. 7 Conceptual model of the proposed trade-off between leaf-level carbon- and nutrient-use strategy (Leaf Economic Spectrum) and water uptake depth in drought-prone environments. Panel **(a)** shows the coupling between a more conservative carbon and nutrient strategy at the leaf level (low SLA and leaf nitrogen content) and a deeper water uptake pattern. Deeper soil layers are poorer in nutrients but reliably store more water during prolonged rainless periods. Panel **(b)** represents the coupling between a more acquisitive carbon- and nutrient-use strategy (high SLA and leaf nitrogen content) and a shallower water uptake pattern, which enhances acquisition of dissolved nutrients from the fertile topsoil layer after rainfall pulses. Dryland soils typically exhibit a steep vertical gradient in nutrient distribution, as nutrient availability to roots is highest in topsoil layers but steeply declines with depth (Jobbágy & Jackson, 2001; Ryel *et al.*, 2008, 2010; Querejeta *et al.,* 2021)

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References

Allison GB, Barnes CJ, Hughes MW. 1983. The distribution of deuterium and 18O in dry soils 2. Experimental. *Journal of Hydrology* **64**: 377–397.

Araya YN, Silvertown J, Gowing DJ, McConway KJ, Peter Linder H, Midgley G. 2011. A fundamental, eco-hydrological basis for niche segregation in plant communities. *New Phytologist* **189**: 253–258.

Barbeta A, Burlett R, Martín-Gómez P, Fréjaville B, Devert N, Wingate L, Domec JC, Ogée J. 2022. Evidence for distinct isotopic compositions of sap and tissue water in tree stems: consequences for plant water source identification. *New Phytologist* **233**: 1121–1132.

Barbeta A, Gimeno TE, Clavé L, Fréjaville B, Jones SP, Delvigne C, Wingate L, Ogée J. 2020. An explanation for the isotopic offset between soil and stem water in a temperate tree species. *New Phytologist* **227**: 766–779.

Barbour MM. 2007. Stable oxygen isotope composition of plant tissue: A review. *Functional Plant Biology* **34**: 83–94.

Barbour MM, Fischer RA, Sayre KD, Farquhar GD. 2000. Oxygen isotope ratio of leaf and grain material correlates with stomatal conductance and grain yield in irrigated wheat. *Australian Journal of Plant Physiology* **27**: 625–637.

Ben-David M, Flynn RW, Schell DM. 1997. Annual and seasonal changes in diets of martens: evidence from stable isotope analysis. *Oecologia* **111**: 280–291.

Beyer M, Hamutoko JT, Wanke H, Gaj M, Koeniger P. 2018. Examination of deep root water uptake using anomalies of soil water stable isotopes, depth-controlled isotopic labeling and mixing models. *Journal of Hydrology* **566**: 122–136.

Brum M, Teodoro GS, Abrahão A, Oliveira RS. 2017. Coordination of rooting depth and leaf hydraulic traits defines drought-related strategies in the campos rupestres, a tropical montane biodiversity hotspot. *Plant and Soil* **420**: 467–480.

Brundrett MC. 2009. Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant and Soil* **320**: 37–77.

Bucci SJ, Scholz FG, Goldstein G, Meinzer FC, Arce ME. 2009. Soil water availability and rooting depth as determinants of hydraulic architecture of Patagonian woody species. *Oecologia* **160**: 631–641.

Caruso CM, Mason CM, Medeiros JS. 2020. The Evolution of Functional Traits in Plants: Is the Giant Still Sleeping? *International Journal of Plant Sciences* **181**: 1–8.

del Castillo J, Comas C, Voltas J, Ferrio JP. **2016**. Dynamics of competition over water in a mixed oak-pine Mediterranean forest: Spatio-temporal and physiological components. *Forest Ecology and Management* **382**: 214–224.

Chen Y, Helliker BR, Tang X, Li F, Zhou Y, Song X. **2021**. Stem water cryogenic extraction biases estimation in deuterium isotope composition of plant source water. *Proceedings of the National Academy of Sciences of the United States of America* **117**: 33345–33350.

Craig H, Gordon LI. **1965**. *Deuterium and oxygen 18 variations in the ocean and the marine atmosphere.*

Craine JM, Elmore AJ, Aidar MPM, Bustamante M, Dawson TE, Hobbie EA, Kahmen A, Mack MC, McLauchlan KK, Michelsen A, *et al.* 2009. Global patterns of foliar nitrogen isotopes and their relationships with climate, mycorrhizal fungi, foliar nutrient concentrations, and nitrogen availability. *New Phytologist* **183**: 980–992.

Dansgaard W. **1964**. Stable isotopes in precipitation. *Tellus* **16**: 436–468.

Dawson TE, Mambelli S, Plamboeck AH, Templer PH, Tu KP. **2002**. Stable Isotopes in Plant Ecology. *Annual Review of Ecology and Systematics* **33**: 507–559.

Dawson TE, Pate JS. **1996**. Seasonal water uptake and movement in root systems of Australian phraeatophytic plants of dimorphic root morphology: a stable isotope investigation. *Oecologia 1996 107:1* **107**: 13–20.

Díaz-Teijeiro F, Rodríguez-Arévalo J, Castaño S. **2009**. La Red Española de Vigilancia de Isótopos en 561 la Precipitación (REVIP): distribución isotópica espacial y aportación al conocimiento del ciclo hidrológico. *Ingeniería Civil* **155**: 87–97.

Díaz S, Kattge J, Cornelissen JHC, Wright IJ, Lavorel S, Dray S, Reu B, Kleyer M, Wirth C, Colin Prentice I, *et al.* 2016. The global spectrum of plant form and function. *Nature* **529**: 167–171.

Ding Y, Nie Y, Chen H, Wang K, Querejeta JI, Kelin W, Querejeta JI. **2021**. Water uptake depth is coordinated with leaf water potential, water-use efficiency and drought vulnerability in karst vegetation. *New Phytologist* **229**: 1339–1353.

Ehleringer JR, Dawson TE. **1992**. Water uptake by plants: perspectives from stable isotope composition. *Plant, Cell & Environment* **15**: 1073–1082.

Ehleringer JR, Roden J, Dawson TE. **2000**. Assessing Ecosystem-Level Water Relations Through Stable Isotope Ratio Analyses. In: Methods in Ecosystem Science. Springer, New York, NY, 181–198.

Esteban GJ, Robert BJ. **2001**. The distribution of soil nutrients with depth: Global patterns and the imprint of plants. *Biogeochemistry* **53**: 51–77.

Farquhar GD, Hubick KT, Condon AG, Richards RA. **1989**. Carbon isotope fractionation

and plant water-use efficiency. In: Stables Isotopes in Ecological Research. Springer, New York, NY, 21–40.

Filella I, Peñuelas J. **2003**. Partitioning of water and nitrogen in co-occurring Mediterranean woody shrub species of different evolutionary history. *Oecologia* **137**: 51–61.

Freschet GT, Cornelissen JHC, van Logtestijn RSP, Aerts R. **2010**. Evidence of the 'plant economics spectrum' in a subarctic flora. *Journal of Ecology* **98**: 362–373.

Gat JR. **1996**. Oxygen and hydrogen isotopes in the hydrologic cycle. *Annual Review of Earth and Planetary Sciences* **24**: 225–262.

Holdo RM. **2013**. Revisiting the Two-Layer Hypothesis: Coexistence of Alternative Functional Rooting Strategies in Savannas. *PLoS ONE* **8**: 69625.

Huxman TE, Snyder KA, Tissue D, Leffler AJ, Ogle K, Pockman WT, Sandquist DR, Potts DL, Schwinning S. **2004**. Precipitation pulses and carbon fluxes in semiarid and arid ecosystems. *Oecologia* **141**: 254–268.

Illuminati A, López-Angulo J, de la Cruz M, Chacón-Labella J, S. Pescador D, Pías B, Sánchez AM, Escudero A, Matesanz S. **2021**. Larger aboveground neighbourhood scales maximise similarity but do not eliminate discrepancies with belowground plant diversity in a Mediterranean shrubland. *Plant and Soil* **460**: 497–509.

Jacobsen AL, Pratt RB, Davis SD, Ewers FW. **2008**. Comparative community physiology: nonconvergence in water relations among three semi-arid shrub communities. *New Phytologist* **180**: 100–113.

Jiang P, Meinzer FC, Wang H, Kou L, Dai X, Fu X. **2020a**. Belowground determinants and ecological implications of shrub species' degree of isohydry in subtropical pine plantations. *New Phytologist* **226**: 1656–1666.

Jiang P, Wang H, Meinzer FC, Kou L, Dai X, Fu X. **2020b**. Linking reliance on deep soil water to resource economy strategies and abundance among coexisting understorey shrub species in subtropical pine plantations. *New Phytologist* **225**: 222–233.

Jobbágy EG, Jackson RB. **2001**. The distribution of soil nutrients with depth: Global patterns and the imprint of plants. *Biogeochemistry* **53**: 51–77.

Kulmatiski A, Adler PB, Stark JM, Tredennick AT. **2017**. Water and nitrogen uptake are better associated with resource availability than root biomass. *Ecosphere* **8**.

Kulmatiski A, Beard KH. **2013**. Root niche partitioning among grasses, saplings, and trees measured using a tracer technique. *Oecologia* **171**: 25–37.

Kulmatiski A, Beard KH, Holdrege MC, February EC. **2020**. Small differences in root distributions allow resource niche partitioning. *Ecology and Evolution* **10**: 9776–9787.

Kulmatiski A, Beard KH, Verweij RJT, February EC. **2010**. A depth-controlled tracer technique measures vertical, horizontal and temporal patterns of water use by trees and grasses in a subtropical savanna. *New Phytologist* **188**: 199–209.

de la Casa J, Barbeta A, Rodríguez-Uña A, Wingate L, Ogée J, Gimeno TE. **2021**. Revealing a significant isotopic offset between plant water and its sources using a global meta-analysis. *Hydrology and Earth System Sciences Discussions*: 1–31.

León-Sánchez L, Nicolás E, Prieto I, Nortes P, Maestre FT, Querejeta JI. **2020**. Altered leaf elemental composition with climate change is linked to reductions in photosynthesis, growth and survival in a semi-arid shrubland. *Journal of Ecology* **108**: 47–60.

Long RW, Medeiros JS. **2021**. Water in, water out: root form influences leaf function. *New Phytologist* **229**: 1186–1188.

McCormick EL, Dralle DN, Hahm WJ, Tune AK, Schmidt LM, Chadwick KD, Rempe DM. **2021**. Widespread woody plant use of water stored in bedrock. *Nature* **597**: 225–229.

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI. **2012**. Isotopes reveal contrasting water use strategies among coexisting plant species in a mediterranean ecosystem. *New Phytologist* **196**: 489–496.

Palacio S, Montserrat-Martí G, Ferrio JP. **2017**. Water use segregation among plants with contrasting root depth and distribution along gypsum hills (R Michalet, Ed.). *Journal of Vegetation Science* **28**: 1107–1117.

Peñuelas J, Terradas J, Lloret F. **2011**. Solving the conundrum of plant species coexistence: Water in space and time matters most. *New Phytologist* **189**: 5–8.

Prieto I, Querejeta JI, Segrestin J, Volaire F, Roumet C. **2018**. Leaf carbon and oxygen isotopes are coordinated with the leaf economics spectrum in Mediterranean rangeland species. *Functional Ecology* **32**: 612–625.

Querejeta JI, Ren W, Prieto I. **2021**. Vertical decoupling of soil nutrients and water under climate warming reduces plant cumulative nutrient uptake, water use efficiency and productivity. *New Phytologist* **230**: 1378–1393.

Reich PB. **2014**. The world-wide 'fast-slow' plant economics spectrum: A traits manifesto. *Journal of Ecology* **102**: 275–301.

Rempe DM, Dietrich WE. **2018**. Direct observations of rock moisture, a hidden component of the hydrologic cycle. *Proceedings of the National Academy of Sciences of the United States of America* **115**: 2664–2669.

Reynolds JF, Kemp PR, Ogle K, Fernández RJ. **2004**. Modifying the 'pulse-reserve' paradigm for deserts of North America: Precipitation pulses, soil water, and plant responses. *Oecologia* **141**:

194–210.

Rodríguez-Robles U, Arredondo JT, Huber-Sannwald E, Yépez EA, Ramos-Leal JA. **2020**. Coupled plant traits adapted to wetting/drying cycles of substrates co-define niche multidimensionality. *Plant, Cell & Environment* **43**: 2394–2408.

Ryel RJ, Ivans CY, Peek MS, Leffler AJ. **2008**. Functional Differences in Soil Water Pools: a New Perspective on Plant Water Use in Water-Limited Ecosystems. In: Progress in Botany. Springer, Berlin, Heidelberg, 397–422.

Ryel RJ, Leffler AJ, Ivans C, Peek MS, Caldwell MM. **2010**. Functional Differences in Water-Use Patterns of Contrasting Life Forms in Great Basin Steppelands. *Vadose Zone Journal* **9**: 548–560.

Sánchez-Martín R, Querejeta JI, Voltas J, Ferrio JP, Prieto I, Verdú M, Montesinos-Navarro A. **2021**. Plant's gypsum affinity shapes responses to specific edaphic constraints without limiting responses to other general constraints. *Plant and Soil*.

Schenk HJ. **2008**. The Shallowest Possible Water Extraction Profile: A Null Model for Global Root Distributions. *Vadose Zone Journal* **7**: 1119–1124.

Schenk HJ, Jackson RB. **2002**. Rooting depths, lateral root spreads and below-ground/above-ground allometries of plants in water-limited ecosystems. *Journal of Ecology* **90**: 480–494.

Schliep KP. **2011**. phangorn: Phylogenetic analysis in R. *Bioinformatics* **27**: 592–593.

Schulze E-D, Mooney HA, Sala OE, Jobbagy E, Buchmann N, Bauer G, Canadell J, Jackson RB, Loreti J, Oesterheld M, *et al.* 1996. Rooting depth, water availability, and vegetation cover along an aridity gradient in Patagonia. *Oecologia* **108**: 503–511.

Schwinning S, Ehleringer JR. **2001**. Water use trade-offs and optimal adaptations to pulse-driven arid ecosystems. *Journal of Ecology* **89**: 464–480.

Schwinning S, Sala OE. **2004**. Hierarchy of responses to resource pulses in arid and semi-arid ecosystems. *Oecologia* **141**: 211–220.

Silvertown J. **2004**. Plant coexistence and the niche. *Trends in Ecology and Evolution* **19**: 605–611.

Silvertown J, Araya Y, Gowing D. **2015**. Hydrological niches in terrestrial plant communities: a review (W Cornwell, Ed.). *Journal of Ecology* **103**: 93–108.

Sohel MSI, Grau AV, McDonnell JJ, Herbohn J. **2021**. Tropical forest water source patterns revealed by stable isotopes: A preliminary analysis of 46 neighboring species. *Forest Ecology and Management* **494**: 119355.

Stock BC, Jackson AL, Ward EJ, Parnell AC, Phillips DL, Semmens BX. **2018**. Analyzing mixing systems using a new generation of Bayesian tracer mixing models. *PeerJ* **2018**.

Stock B, Semmens B. **2013**. MixSIAR GUI user manual: version 1.0. Available from

https://github.com/brianstock/MixSIAR. : 1–42.

Violle C, Nemergut DR, Pu Z, Jiang L. 2011. Phylogenetic limiting similarity and competitive exclusion. *Ecology Letters* **14**: 782–787.

Walter H. 1939. Grasland, Savanne und Busch der arideren Teile Afrikas in ihrer ökologischen Bedingtheit. *Jahrbücher für Wissenschaftliche Botanik* **87**: 750–860.

Wang J, Wen X, Lyu S, Guo Q. 2021. Transition in multi-dimensional leaf traits and their controls on water use strategies of co-occurring species along a soil limiting-resource gradient. *Ecological Indicators* **128**: 107838.

Ward D, Wiegand K, Getzin S. 2013. Walter's two-layer hypothesis revisited: Back to the roots! *Oecologia* **172**: 617–630.

West AG, Dawson TE, February EC, Midgley GF, Bond WJ, Aston TL. 2012. Diverse functional responses to drought in a Mediterranean-type shrubland in South Africa. *New Phytologist* **195**: 396–407.

Williams, D. G., & Ehleringer JR. 2000. Intra- and interspecific variation for summer precipitation use in pinyon-juniper woodlands. *Ecological Monographs* **70**: 517–537.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornellssen JHC, Diemer M, *et al.* 2004. The worldwide leaf economics spectrum. *Nature* **428**: 821–827.

Zhou M, Bai W, Li Q, Guo Y, Zhang W. 2021. Root anatomical traits determined leaf-level physiology and responses to precipitation change of herbaceous species in a temperate steppe. *New Phytologist* **229**: 1481–1491.

Zhou M, Bai W, Zhang Y, Zhang W-H. 2018. Multi-dimensional patterns of variation in root traits among coexisting herbaceous species in temperate steppes (L Mommer, Ed.). *Journal of Ecology* **106**: 2320–2331.

Zhou Y, Wigley BJ, Case MF, Coetsee C, Staver AC. 2020. Rooting depth as a key woody functional trait in savannas. *New Phytologist* **227**: 1350–1361.

Supporting information

Methods S1 Results of PCA carried out without phylogeny

The first two axes of the PCA, carried out without including phylogeny, explained 57 % of variance (PC1 = 33 %; PC2 = 24 %; PC3 = 18 %) (Table S4 and Fig. S7). The two first axes of the PCA roughly represented species variation along the LES and water use strategy, similarly to phylPCA. However, stem water $\delta^{18}O$ was coordinated (R = -0.56) with the water use strategy axis in this PCA, while it was better coordinated (R = 0.59) with the LES axis in the phylPCA. Stem water $\delta^{18}O$ was highly coordinated also with the third axis of both PCAs, as well as the leaf $\delta^{18}O$.

Table S1. Primers used and PCR conditions applied for the phylogenetic tree construction.

Barcoding locus	Primer	Sequence (5´ à 3´)	PCR conditions	Ref.
rbcLa	SI_F	ATGTCACCACAAACAGAGACTAAAGC	95ºC 3min; [34 cycles: 94ºC 30s; 55ºC 30s; 72ºC 1min]; 72ºC 10min	Kress et al. (2005)
	SI_R	GTAAAATCAAGTCCACCRCG	95ºC 3min; [34 cycles: 94ºC 30s; 55ºC 30s; 72ºC 1min]; 72ºC 10min	Kress et al. (2009)

Table S2. Species abundances (total number of individuals and cover %) in the aboveground plant community of study (data from Illuminati et al. 2021).

Species	Family	Individuals	Cover %
Arenaria cavanillesiana	*Caryophyllaceae*	260	0.51
Bupleurum fruticescens	*Apiaceae*	899	4.08
Cephalaria leucantha	*Caprifoliaceae*	169	1.91
Coris monspeliensis	*Primulaceae*	49	0.03
Coronilla minima	*Fabaceae*	270	3.44
Fumana ericoides	*Cistaceae*	339	3.43
Fumana thymifolia	*Cistaceae*	6	0.11
Helianthemum cinereum	*Cistaceae*	1095	2.49
Helianthemum hirtum	*Cistaceae*	154	0.48
Helichrysum serotinum	*Asteraceae*	14	0.02
Hippocrepis comosa	*Fabaceae*	55	0.18
Koeleria vallesiana	*Poaceae*	545	1.00
Lavandula latifolia	*Lamiaceae*	30	0.52
Leuzea conifera	*Asteraceae*	14	0.07
Linum narbonense	*Linaceae*	18	0.01
Linum suffruticosum	*Linaceae*	1154	3.42
Matthiola fruticulosa	*Brassicaceae*	81	0.12
Ononis tridentata	*Fabaceae*	30	3.11
Salvia lavandulifolia	*Lamiaceae*	16	0.15
Sideritis incana	*Lamiaceae*	343	0.65
Stahelina dubia	*Asteraceae*	8	0.11
Stipa pennata	*Poaceae*	1756	1.92
Teucrium capitatum	*Lamiaceae*	112	0.49
Thymus vulgaris	*Lamiaceae*	654	7.47

Table S3. Loadings of the input variables in the phylogenetic PCA axes with mean species values

Input variables	PC1 (43%)	PC2 (24%)	PC3 (18%)
SLA	-0.77	0.12	0.12
Leaf N	-0.87	-0.28	0.15
Leaf δ13C	0.49	0.75	-0.19
Leaf δ18O	-0.44	0.74	0.42
Stem water δ18O	-0.58	0.21	-0.77

Table S4. Loadings of the input variables in the non-phylogenetic PCA axes with species mean values

Input variables	PC1 (33%)	PC2 (24%)	PC3 (18%)
SLA	-0.66	-0.005	-0.13
Leaf N	-0.59	-0.13	0.06
Leaf δC13	0.43	-0.51	-0.07
Leaf δ18O	-0.12	-0.56	0.76
Stem water δ18O	0.11	0.64	0.62

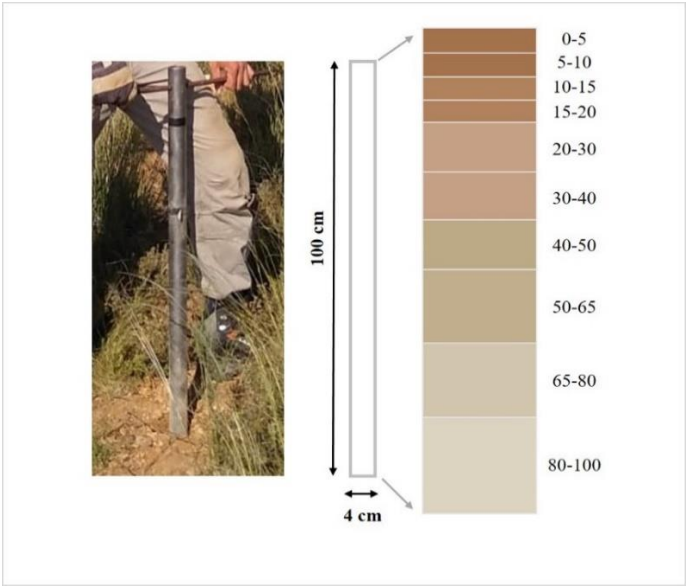

Fig. S1 Picture of the soil core used in the field to dig the soil up to 1 meter of depth (left) and representation of the different 10 soil layers sampled (right).

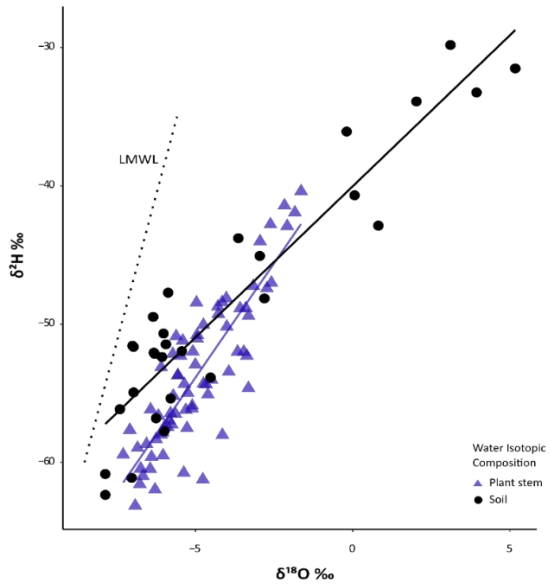

Fig. S2 Isoplot showing both water $\delta^{18}O$ and δD of plant individuals and soil samples. Regression lines corresponding to plant and soil samples and the Local Meteoric Water Line (LMWL) are also represented.

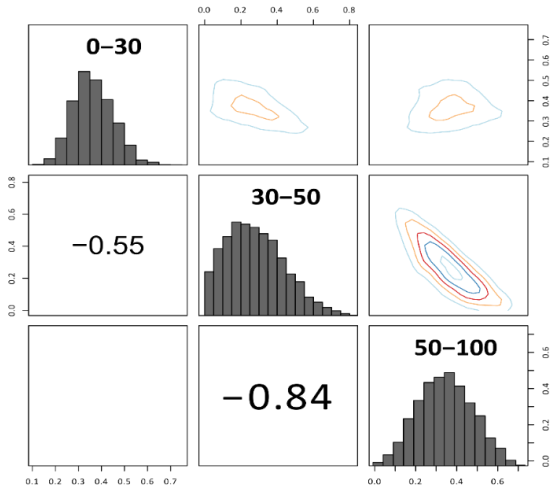

Fig. S3 Diagonal cells show probability of distribution of each water source (0-30, 30-50 and 50-100 cm of depth), calculated in the MixSIAR model. Correlation coefficients between water sources pairs are represented in the lower cells, while contours of the probability of distribution for combined pairs of water sources are shown in the upper cells. Given the high correlation (R = -0.84) between the 30-50 and 50-100 cm water sources, they were combined in one unique source for the final estimation.

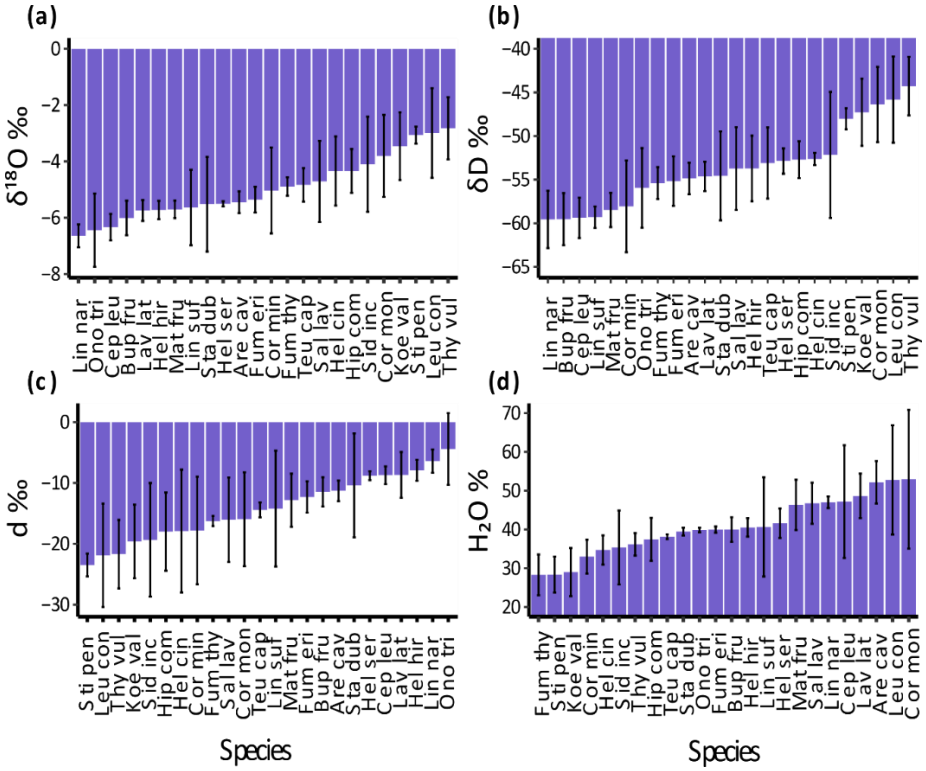

Fig. S4 Panels **(a)-(c)** show stem water isotopic composition and its variation between the 24 coexisting species. Specifically, they represent $\delta^{18}O$ ‰ **(a)**, δD ‰ **(b)** and the d ‰, deuterium excess, **(c)**, while panel **(d)** reports the water content (%).

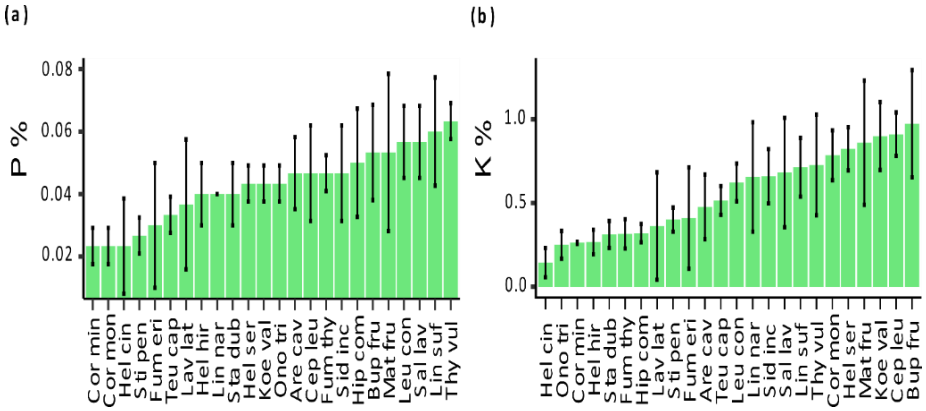

Fig. S5 Panels **(a)** and **(b)** represent variation of leaf P (%) and K (%), respectively, in the 24 species coexisting in the plant community.

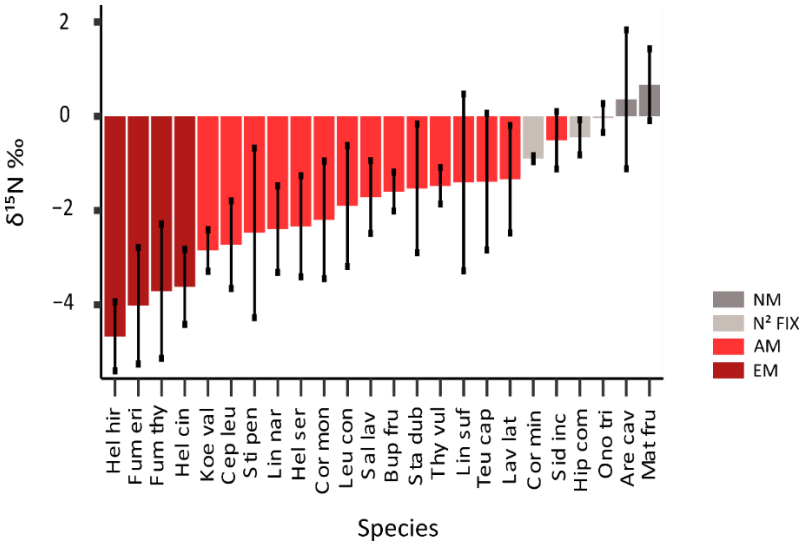

Fig. S6 Variation of the $\delta^{15}N$ content (‰) across the 24 coexisting species. Species are also grouped according to four different categories: no mycorrhizal (NM), N^2 fixing (N^2 FIX), arbuscular mycorrhizal (AM), ectomycorrhizal (EM), according to the family (see Brundrett 2009).

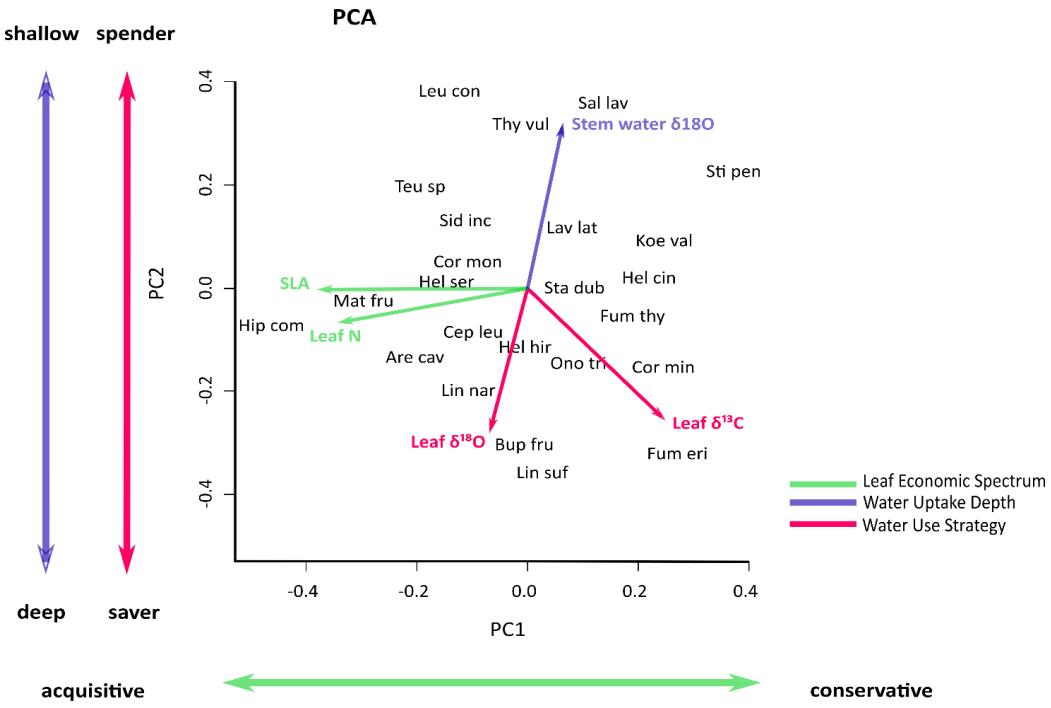

Fig. S7 PCA carried out with species mean values without considering phylogenetic relatedness among species.

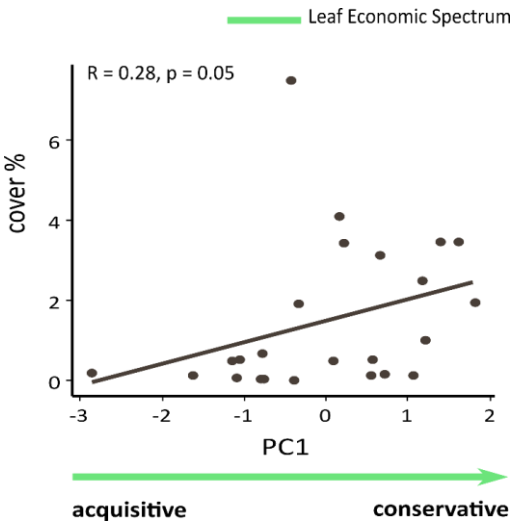

Fig.S8 Kendal's correlation test carried out between the PC axis of the PCA without phylogeny and interpreted as the leaf economic spectrum and the species dominance in the plant community (cover %).

References

Brundrett MC (2009) Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil 2009 3201 320:37–77. https://doi.org/10.1007/S11104-008-9877-9

Illuminati A, López-Angulo J, de la Cruz M, et al (2021) Larger aboveground neighbourhood scales maximise similarity but do not eliminate discrepancies with belowground plant diversity in a Mediterranean shrubland. Plant Soil. https://doi.org/10.1007/s11104-020-04796-7

Kress WJ, Erickson DL, Jones FA, et al (2009) Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. Proc Natl Acad Sci U S A 106:18621–18626. https://doi.org/10.1073/pnas.0909820106

Kress WJ, Wurdack KJ, Zimmer EA, et al (2005) Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci U S A 102:8369–8374. https://doi.org/10.1073/pnas.0503123102

Chapter 2

Integrating root traits to identify local-scale trade-offs and water use strategies in a Mediterranean shrubland

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Key words: *Functional trade-offs, root traits, plant water strategy, leaf-level water use, root economic space, Mediterranean shrublands, water isotopes, water use efficiency, plant performance*

Summary

In the last decades, increasing efforts have focused on integrating root traits to achieve a whole-individual perspective of plant form and function. In arid environments, root traits, together with leaf and stem traits related to water use, play a key role given the limited availability of water and nutrients, which exacerbate belowground competition processes. However, a unifying framework is still missing to identify both whole-plant functional trade-offs and water use strategies. Using a common garden rhizotron experiment, we measured root traits of 23 species coexisting in a Mediterranean shrubland, and integrated these data with traits related with water use measured in natural conditions. We found three leading dimensions of functional variation at the whole-plant level, related to different key aspects of plant functioning, in partial agreement with global models. Root traits characterized one independent gradient, but were also strongly associated with a second axis of variation mostly related to plant size. We also detected a strong trade-off between root tissue density and the leaf-level water use strategy. Higher root tissue density was coordinated with a saver leaf-level water use, which in turn correlated with higher species performance in the plant community. Our results highlight the link between the root-level nutrient use strategy and leaf-level water use, and provide evidence of the effect of functional coordination on plant performance on natural conditions.

Introduction

A major goal of plant functional ecology is to establish the links between plant form and function, as a necessary step to understand the mechanisms driving ecosystems' structure and functioning (e.g. McGill *et al.*, 2006; Violle *et al.*, 2007; Freschet *et al.*, 2010). Functional trade-offs along meaningful axes of variation allow to identify ecological strategies in which species are easily delimited (e.g. Westoby, 1998; Díaz *et al.*, 2004).

Functional trait variation among species is affected by a variety of biotic and abiotic processes (e.g. Albert *et al.*, 2010), which in turn vary across scales. Therefore, some authors (e.g. Messier *et al.*, 2017b,a) pointed out that functional trade-offs, even the widely recognized leaf economic spectrum (LES, Wright *et al.*, 2004), may not hold at small spatial scales, and suggested that the identification of clear functional trade-offs within plant communities may be limited by the multiple interdependence between functional traits (see also Escudero & Valladares, 2016; McCormack *et al.*, 2017). Another key aspect in this context is the inevitable effect of evolutionary history in the assessment of functional trade-offs across species, which may constrain variation among species and blur the functional patterns observed (e.g. Westoby *et al.*, 1995; Cavender-Bares *et al.*, 2009; Kraft *et al.*, 2015; Ma *et al.*, 2018).

These yet-unresolved questions are even more remarkable when considering root traits (McCormack *et al.*, 2017). Recently, a new framework defined as the root economic space (RES, sensu Bergmann *et al.*, 2020), characterized by two orthogonal components, has been proposed at a global scale. The first component, defined as the root 'conservation' gradient, is characterized by high root tissue density and low nitrogen content on one side, and by low root tissue density and high root nitrogen content in the other (Bergmann *et al.*, 2020). The second component, the so-called 'collaboration' gradient, describes species variation from a 'do it yourself' strategy associated with high specific root length and low root diameter to an 'outsourcing' strategy favouring mycorrhizal associations and characterized by low specific root length and high root diameter (Bergmann *et al.*, 2020).

In an attempt to build a global scale theoretical background at whole-plant level, a few recent studies (Carmona *et al.*, 2021; Weigelt *et al.*, 2021) have included root functional traits into plant global models, but a general unifying framework is still missing. Indeed, despite the increasing efforts, lack of information on root traits is still remarkably high compared with its aboveground counterpart (Iversen *et al.*, 2017; Laliberté, 2017). While the presence of a root functional dimension connecting the maximum root depth with plant height is emerging (Weigelt *et al.*, 2021), contrasting results have been found regarding the coordination between the root 'conservation' gradient and

the leaf economic spectrum (Carmona *et al.*, 2021; Weigelt *et al.*, 2021). Such growing focus on root traits is endorsed by the growing awareness about the importance of belowground processes, and thus of roots, as the missing piece for the understanding of whole-plant functioning, plant community assembly processes and responses to a rapidly changing environment (e.g. Bardgett *et al.*, 2014; Laughlin, 2014; Inderjit *et al.*, 2021). A root functional approach is especially needed in stressful environments such as Mediterranean ecosystems, where belowground biomass is larger and competition for soil resources is critical for survival and coexistence (Casper & Jackson, 1997; Aerts, 1999; Silvertown *et al.*, 2015).

Since water represents the most limiting factor for plant growth in drylands, several studies (e.g. Moreno-Gutiérrez *et al.*, 2012; Ding *et al.*, 2021) have also focused on traits related to plant water use strategy. In this context, a key trait is root water uptake depth (Schulze *et al.*, 1996; Williams, D. G., & Ehleringer, 2000) which can be robustly estimated by measuring stem water $\delta^{18}O$ (Dawson *et al.*, 2002; Barbeta *et al.*, 2020). It is also well-known that water use strategy is associated to leaf-level water use efficiency (WUE) and stomatal conductance (g_s), of which leaf $\delta^{13}C$ and leaf $\delta^{18}O$, respectively, have been showed to be good time-integrated proxies (e.g. Farquhar *et al.*, 1989; Barbour, 2007; Moreno-Gutiérrez *et al.*, 2012). A few studies (e.g. Domec *et al.*, 2009; Fort *et al.*, 2017; Zhou *et al.*, 2021) explored the relationships existing between root traits and plant water use strategy. However, we still lack robust information on how root anatomy and morphology may influence water uptake and leaf-level water use (Long & Medeiros, 2021).

The environmental constraints that characterize drylands are predicted to promote a conservative resource-use strategy (e.g. Matesanz & Valladares, 2014; Carvajal *et al.*, 2019). However, some authors (Weltzin & Tissue, 2003; Carvajal *et al.*, 2019) have pointed out that an acquisitive, i.e. fast, resource-use strategy may be advantageous during short periods of high water availability, which may have important implications for species coexistence under a niche partitioning perspective (Chase & Leibold, 2003; Silvertown, 2004). This would lead to the existence of different resource-use strategies in the same plant community.

Here, we assessed the whole-plant functional structure of 23 species coexisting in a Mediterranean shrubland, with a special focus on root functional aspects. We carried out a common garden rhizotron experiment, growing plants under similar, optimum conditions and removing the effects of plant-plant interactions. We measured 11 root functional traits, mostly related with the nutrient-use strategy as well as several key aboveground traits related with the leaf economic spectrum and plant size. Our first aim was to assess the presence of main dimensions of root functional variation at the plant community scale, and their coordination with the aboveground counterparts, accounting for potential effects related with the evolutionary history of the species. Second, we

assessed the relationship between root traits linked with the RES and traits associated with the plant water-use strategy. And finally, we evaluated if any particular water-use and root nutrient-use strategy may affect species performance in the plant community.

Methods

Description of the plant community and experimental design

We measured root functional traits of 23 species of a Mediterranean shrubland characterised by calcareous soils, with a variable content of gypsum, of the southern half of Madrid region (40°17'17.5" N 3°12'19.4" W, 760 m a.s.l., Spain). These plants coexist and are dominant in these shrublands (Chacón-Labella *et al.*, 2016; Illuminati *et al.*, 2021). Climate is dry Mediterranean, with mean annual temperature of 12.8 °C, mean annual precipitation of 452 mm, sharp summer drought and cold winters. The plant community has high species diversity and is dominated mostly by dwarf shrubs, hemicryptophytes and grasses. The most abundant species are *Bupleurum fruticescens* L., *Helianthemum cinereum* Pers., *Linum suffruticosum* L., *Stipa pennata* L. and *Thymus vulgaris* L.

We grew a total of 162 individuals of the selected species (see Table S1) in a common garden at the CULTIVE facilities at URJC (https://urjc-cultive.webnode.es; Madrid) from late autumn until spring, specifically 12 species in the 2018-2019 and 11 species in the 2019-2020 periods. We collected at least 500 seeds for each species during two summers (2017, 2018), from several randomly-distributed individuals in the same site. We sowed a minimum of 20 to 50 randomly selected seeds of each species according to their germination rates (unpublished data). Each seed was put in a single seedbed with humid substrate in chambers with photoperiods of 16 hours at 20°C alternated with dark periods of 8 hours at 15°C. After germination, seedlings were grown for a minimum of ten days before being transplanted individually into polycarbonate tubes (4.5 cm of radius and 60 cm of height) previously filled with a mixed substrate and placed in the soil outdoors. We mixed 50 % fine and 50 % gross river sand to optimize root washing and extraction, mixed with fertilizer (4 g/L) and an additional upper layer of peat (2 cm thick), to guarantee optimal nutrient availability. The tubes were organized in 16 blocks (80 x 80 cm), to which seedlings of each species (5-8 samples per species) were assigned randomly. Ten seedlings for each species were instead collected and dried at 60°C for 48 hours to estimate initial dry mass.

Plant harvest

After a growing period of around five months (minimum of four and maximum of six) plants were harvested. We selected five healthy and mature leaves for the next measurement of specific leaf area (SLA). We measured plant height (H) and the two main aerial orthogonal diameters (D_1, D_2) which were then used to estimate the aerial mean diameter, AD, as $D_1+D_2/2$. We then extracted the tube from the soil, opened it vertically in two halves and removed the plant root carefully, to preserve it intact while eliminating all the substrate. After washing off the substrate, maximum root length (MRL) and the root neck diameter (RN), by means of two orthogonal diameter measures, were measured. The cleared substrate was washed twice, and all the water filtered through a double 1-mm mesh filter, to collect the few potential root fragments broken during washing. The entire root was cut at the neck level, placed in a plastic zipped bag and frozen at -20 °C.

Functional traits measurement

We scanned five leaves per individual to estimate the leaf area (LA). Then, we dried them at 60 °C for 72 hours, to estimate their dry mass (DM) and calculate specific leaf area (cm^2/g) as SLA = LA/DM. We dried and weighed the remaining aerial plant tissues to quantify the total aerial dry mass (AM). We then quantified N and P contents (LN and LP, respectively) in leaf dry tissues through Kjeldahl digestion as described in (Radojevic & Bashkin, 1999).

We measured 10 root functional traits (Table 1) directly related either to the plant-size or the nutrient-use strategy. Here, we considered as root traits related to the nutrient-use strategy both traits directly associated to the nutrient uptake and use, and indirectly, referred to as exploitative traits, since they improve the exploitation of a limited volume of soil (Freschet *et al.*, 2021). Some of the selected traits (specific root length, root diameter, root tissue density) related to the nutrient-use strategy represent key traits of the root economic space (RES sensu Bergmann *et al.*, 2020). Most root traits were measured in fine roots (diameter <1 mm). Such cut-off diameter has been recommended over the widely used <2 mm diameter, especially when different woody species are involved (Freschet & Roumet, 2017). We scanned all fine roots which corresponded to the entire root system in several species (See Table S2). To avoid estimation errors in the scanned images, as suggested by Delory *et al.* (2017), we cut the roots in fragments to minimize root overlapping, and thus diameter overestimation. The images were taken at 600 bpi with an Epson Expression 10000XL scanner. Then, we oven-dried the scanned roots at 60°C for 72 hours and weighed them to determine the fine root mass (RM, hereafter).

We carried out the image analysis using WinRHIZO 2009a,b,c and estimated the following traits: mean root diameter (RD), total root area (RA), very fine (<0.02 mm) root % (VFR) and total root length (TRL). We also calculated the specific root length (SRL) as TRL/RM, and the specific root area, SRA, as RA/RM. To calculate the root tissue density (RTD), we first measured the mean root

volume (RV), by estimating the volume occupied by the cylinder defined by height (corresponding to the TRL) and diameter (corresponding to the RD). Then, we estimated RTD as RV/RM. Finally, we weighed the thick component of the root system to determine the total root mass (TRM) and the total plant dry mass (PM = TRM+AM) Finally, we estimated the root mass fraction (RMF) by applying the formula = TRM/PM *100. We also assessed the relative growth rate (RGR) as AM-AM_i/N, where AM_i was the initial aerial mass measured as the mean dry mass of each species at the moment of transplant in the tubes, and N was the number of days between transplant and plant harvest. Finally, isotopic traits (leaf $\delta^{13}C$, leaf $\delta^{18}O$ and stem water $\delta^{18}O$) data related to the plant water-use strategy for the study species, as well as the species mean height, mean aerial diameter, abundance (number of individuals) and dominance (cover %) were obtained from previous studies performed in natural conditions (Illuminati *et al.*, 2021, 2022).

Phylogenetic tree construction

Phylogenetic tree construction has been described with detail in Illuminati *et al.* (2022). Briefly, we sampled leaves in three individuals per species in the same plant community and used the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) to carry out the DNA extraction by considering the rbcL barcode. PCR amplification was performed on a S1000 Thermal Cycler (Bio Rad, Hercules, CA, USA) and PCR products were sequenced by MACROGEN (Seoul, Korea and Amsterdam, Netherlands). From the obtained sequences the phylogenetic tree was built by applying maximum likelihood methods (*Phangorn* package, Schliep, 2011), using the GTR + G + I model and 100 bootstrap replicates (Violle *et al.*, 2011).

Statistical analysis

Root functional traits and a whole-plant phenotypic integration

First, we carried out Spearman's pairwise correlation tests (*rcorr* function, *Hmisc* package) between all functional traits measured in the common garden experiment and considering individual values. To check for discrepancies related to the year when each species was grown, we considered all functional traits and carried out a PERMANOVA, Permutational Multivariate Analysis of Variance (*adonis* function, *vegan* package), using Euclidean distance and checked for the assumption of homogeneity of groups dispersions (*betadisper* function, *vegan* package).

To assess the presence of leading dimensions of root functional variation and their coordination with their aboveground counterparts, we carried out a Principal Component Analysis (PCA) with all functional traits measure in the common garden experiment. Trait values were log- (alternatively, logit for percentage variables) transformed when necessary, and scaled for standardization before

being used as input variables in the analyses. When root traits were highly correlated (>0.80%) with other root traits, they were discarded from the PCA analysis. The same selection was carried out for aerial traits. We carried out a PCA considering all individual trait values, but also three additional PCAs, two considering individual trait values of the species grown in each year separately, to check for potential discrepancies related to interannual differences, and another one with species' mean values to check for consistency in our results. We finally repeated the analysis by accounting for the effect of evolutionary history on the observed relationships among traits, by carrying out a phylogenetic PCA, including the phylogenetic relatedness information (phylogenetic tree) and using the species mean values as input variables (method = BM, *phyl.pca* function, *phytools* package). In addition, to assess if the plant size of individuals in the common garden was linked to field-grown adult size and/or to RGR, we carried out Spearman' correlation tests (*cor.test* function, *stats* package) between aboveground traits related with plant size (H, AD, AM) with both mean height and mean aerial diameter measured in the field, and the RGR measured in the same experiment.

Root economic space, plant water-use strategy and species performance in the plant community

We assessed the relationships between traits related to the RES and the plant water-use strategy by carrying out a PCA considering species mean values of six selected functional traits for the 21 species with complete data (both from the common garden and the field). We selected SRL, RD and RTD as critical root traits of the RES (*sensu* Bergmann *et al.*, 2020) and leaf $\delta^{13}C$, leaf $\delta^{18}O$, associated with the leaf-level water use, and stem water $\delta^{18}O$, related with the soil water uptake depth, as key traits associated with the plant water-use strategy. We again quantified how the evolutionary history affected relationships between the selected functional traits, by carrying out a phylogenetic PCA with the species mean values (log-transformed when necessary) of the selected traits (*phyl.pca* function, *phytools* package). To assess the potential relevance of either or both a particular root nutrient-use strategy and plant water-use strategy for species performance, we extracted the species scores along the two main axes of the PCA. We carried out a Spearman' correlation test between the extracted species scores and the species abundance and dominance in the plant community.

Results

Whole-plant functional trade-offs at the plant community scale

We found high interspecific trait variation across 23 coexisting species in all traits, showing the high functional diversity of the plant community (see Results S1, Table S3, Fig. S1, S2, S3). We found high and significant correlations between root and aerial traits (Fig. 1) related with plant size. AM was highly correlated with AD and RN ($r = 0.88$ and 0.66 respectively, $p<0.001$), and also with TRM and TRL ($r = 0.90$, 0.91; $p<0.001$), and to a lower extent with RN and MRL ($r= 0.66$, 0.54; $p<0.001$). H was less tightly correlated to other plant-size related traits such AM, TRM, RN and MRL ($r= 0.41$, 0.41, 0.44, 0.26; $p<0.001$, respectively). On the contrary, LN and SLA, leaf traits related to the LES, did not correlate significantly with any other plant trait. Similarly, root traits related with the nutrient-use strategy were not correlated with any plant-size related traits, except for RMF and SRA, which were correlated to the TRL ($r = -0.41$, 0.51, respectively; $p <0.001$), a plant-size related trait (Fig. 1). Root traits related to the nutrient-use strategy were all highly correlated between them, except for RTD which was highly correlated only with SRA ($r = -0.69$; $p <0.001$) (Fig. 1).

PERMANOVA results showed that a very small fraction of the variance (5%) was related to variation between years ($p < 0.001$). However, we found similar results in the two PCAs carried out with individuals and species mean values, as well as when considering both years combined and separately. Consequently, we present our results of the analyses performed on the combined dataset.

The relationship between traits were considerably altered when accounting for evolutionary history. While the first axis (32% of variance) of the PCA without including phylogeny was only associated with plant-size related traits, the second and fourth axes (19% and 9% of variance, respectively) were associated with root traits related to nutrient-use, also considered key traits in the RES (SRL, RD and RTD) (Table S4, S5). In the phylogenetic PCA the first axis resulted instead highly connected to both plant-size and nutrient-use root traits (Table S6, S7). Specifically, species characterized by high SRL, low RD, low RTD and low RMF also showed higher AM, TRM, MRL and RN (Fig. 2a). Though root traits related to nutrient-use were highly linked to the first axis of the phylogenetic PCA, still an important portion of variance (15%) associated to the third axis, represented species variation from high SRL, low RD and low RMF values at one side to low SRL, high RD and high RMF values at the other side (Fig. 2b). On the other hand, SLA and LN, leaf traits closely related with the LES, were mostly aligned along another axis independent from plant-size in both PCAs, except for H, which was partly associated to these traits in the phylogenetic

PCA, with higher SLA and LN linked to lower H (Fig. 2a, Table S7). This axis, related to leaf-level nutrient use, explained 15% and 22% of variance, respectively, in the PCA without and with phylogeny. We found significant negative correlations between aerial traits related with plant size measured in the experiment and in the field. In particular, AM and AD were negatively correlated with the mean aerial diameter in the field (r = -0.53, -0.47; p <0.01, < 0.05 respectively); and only marginally with the mean height in the field (r = -0.42, -0.38; p = 0.05, 0.07). On the contrary, both AM and AD were highly positively correlated, while H only marginally so with RGR (r = 0.97, 0.93, 0.37; p < 0.001, 0.08).

RES, water-use strategy and species performance

The phylogenetic PCA carried out with the species mean values of six selected traits related with the RES (SRL, RD, RTD) and the water-use strategy (leaf $\delta^{13}C$, leaf $\delta^{18}O$ and stem water $\delta^{18}O$) showed a gradient of species variation along the first axis, explaining 36% of the variance, from high SRL and low RD values to low SRL and high RD values, which corresponded to the so-called 'collaboration gradient', one of the main dimensions of the RES (Fig. 3, Table S8, S9). Two species, *Arenaria cavanillesiana* and *Matthiola fruticulosa*, not characterized by mycorrhizal associations, were positioned both in the left side of the gradient (species scores, S = -1.38, -2.04, respectively), corresponding to high SRL and low RD, and to a 'do it yourself' strategy. Ectomycorrhizal species presented medium values along the gradient (S = 0.38, -0.62, -0.37), except *Helianthemum hirtum* which was located in the right side of the gradient (S = 1.36). N-fixing species of the *Fabaceae* family, *Coronilla minima* and *Hippocrepis comosa*, were both located in the right side of the gradient (S = 0.99, 1.82, respectively). Arbuscular mycorrhizal species presented a higher variability along the gradient, from species such as *Thymus vulgaris* and *Koeleria vallesiana* (S = -2.43, -1.60, respectively) in one side of the gradient, to species such *Cephalaria leucantha* and *Stipa pennata* in the other side (Fig. 3).

The second axis of the phylogenetic PCA explained 26% of variance and was highly associated with leaf $\delta^{13}C$, leaf $\delta^{18}O$ and RTD, representing a gradient of species variation from high leaf $\delta^{13}C$ and leaf $\delta^{18}O$, related with a more saver leaf-level water-use strategy, and high RTD, associated to the 'conservation' gradient of the RES and a more conservative root-level nutrient-use strategy. On the other side, species had low leaf $\delta^{13}C$ and leaf $\delta^{18}O$, a more spender leaf-level water-use strategy, and log RTD which could be associated with a more acquisitive root-level nutrient-use strategy (Fig. 3). The stem water $\delta^{18}O$, proxy of the soil water uptake depth, was slightly coordinated with both the first and second axis (loadings L = -0.35, -0.32, respectively), showing only a weak link with these two gradients. Indeed, stem water $\delta^{18}O$ was more strongly associated with the third axis of the phylogenetic PCA (L = 0.60; and 19% of the TVE). The stem water $\delta^{18}O$ was

coordinated with the RTD (L= 0.55) along the third axis, while they presented opposite signs along the first and second axes (Table S9). The main PCA outputs were similar when not accounting for evolutionary history (Table S10).

Spearman's correlation tests showed that the first axis of the phylogenetic PCA was not significantly correlated with neither the species abundance nor dominance in the plant community. The second axis of the phylogenetic PCA, related to the leaf-level water-use and to the 'conservation gradient' of the RES, was highly correlated to both species abundances and dominance (r = 0.48, 0.58; p = 0.028, 0.006, respectively), showing that the most abundant and dominant species tended to present a more saver water-use strategy at the leaf-level coordinated with a more conservative nutrient-use strategy at the root-level (Fig. 4).

Discussion

Whole-plant functional trade-offs at a local scale

We identified three main gradients of functional variation at the local plant community scale, the first mostly associated with plant size-related traits, the second to the leaf economic spectrum (Wright *et al.*, 2004), and the third to root traits linked to the 'collaboration' gradient of the root economic space (RES *sensu* Bergmann *et al.*, 2020). In agreement with Weigelt *et al.* (2021), we observed that aerial and root traits related to plant size were highly correlated and coordinated along the same gradient represented by the first axis of our phylogenetic PCA (Fig.s 1, 2). However, while aerial mass (AM) and total root mass (TRM) were very tightly related, the link between height (H) and its root counterpart (maximum root length, MRL) was weaker, showing that aerial mass and diameter and the root neck were better proxies of both TRM and MRL. Key leaf traits related with the leaf economic spectrum, such as SLA and LN, formed a gradient of functional variation along the second axis of the phylogenetic PCA. This second dimension was independent from both the plant size gradient and the so-called 'collaboration gradient' of the root economic space, which was easily identifiable as a third axis mostly related to specific root length (SRL) and root diameter (RD) (Fig. 2). Although contrasting results have been found at local (i.e. community) spatial scales (e.g. Withington *et al.*, 2006; Holdaway *et al.*, 2011; Valverde-Barrantes *et al.*, 2015), our findings support the independence of the LES from the 'collaboration' gradient of the RES, in accordance with patterns and trade-offs identified at the global scale (Carmona *et al.*, 2021; Weigelt *et al.*, 2021).

Lack of a consistent pattern between the LES and the 'conservation gradient' of the RES, which is known to be especially related to both root tissue density (RTD), and root nitrogen content (Bergmann *et al.*, 2020) seems the norm both at global and local spatial scales (e.g. Craine *et al.*, 2001; Kramer-Walter *et al.*, 2016; Carmona *et al.*, 2021; Weigelt *et al.*, 2021). In this sense, we did not find strong links between RTD and any leaf trait related to the LES, as shown by the only weak loading of RTD along the second phylogenetic axis (Table S9). This is in agreement with other studies (e.g. Carmona *et al.*, 2021), suggesting that both RES dimensions were independent from the LES. However, we did not observe complete independence between the gradient related to plant size and root traits associated to the RES, in contrast with global-scale models (Weigelt *et al.*, 2021). Indeed, we observed a very high coordination of SRL with plant size traits and, to a lesser extent, of RD and RTD, which may be partly due to the higher correlated nature of traits at local spatial scale (Messier *et al.*, 2017a). However, our results, showing the high coordination between plant size related traits (AM, TRM, MRL) with traits related to the RES, may be alternatively explained by the fact that individual plant size was more representative of RGR, rather than the final size of adults observed in the field. Our findings, in accordance with other studies (e.g. Reich *et al.*, 1998; Hummel *et al.*, 2007; Fort *et al.*, 2017), indeed evidence that species with high SRL showed higher AM and higher RGR, which was also associated to lower RD and RTD values. We also observed that species with higher MRL, i.e. a very good proxy of maximum root depth, also presented lower RTD and higher RD values (see Fort *et al.*, 2017 for similar results). In addition, the negative correlation observed between plant size measured in our experiment and in the field may be linked to the fact that traits were measured in juveniles (≈6 months) grown at optimal water and nutrients conditions, which are expected to promote faster plant growth on more acquisitive species (Chapin *et al.*, 1993; Wright *et al.*, 2004).

Evolutionary history strongly affected patterns of trait coordination. For instance, the PCA carried out without phylogeny showed a patent independence between plant size related traits, aligned along the first axis, and root traits associated with the RES, which appeared coordinated along the second axis. This pattern was lost in the phylogenetic PCA where the historical relationships were taken into consideration. This discrepancy between the two approaches highlights the significant contribution of evolutionary history in the actual phenotype observed in our plant community, which was also blurring the not-negligible relationship existing between root traits related to the RES and plant size.

Link between root tissue density and the leaf-level water-use strategy

The phylogenetic PCA carried out with root traits related to the RES (SRL, RD, RTD) and the water use strategy (stem $\delta^{18}O$, leaf $\delta^{18}O$ and leaf $\delta^{13}C$) showed a first axis explaining a large fraction of the total variance. This axis could be interpreted as the 'collaboration' gradient of the RES (sensu Bergmann *et al.*, 2020), since it represented species variation from a 'do it yourself' strategy, with high SRL and low RD, to an 'outsourcing' strategy, with low SRL and high RD (Fig. 3). Accordingly, we found that the two non-mycorrhizal species, *Matthiola fruticulosa* and *Arenaria cavanillesiana*, were positioned in the left side of the gradient ,associated to a 'do it yourself strategy'. In addition, and in agreement with Bergmann *et al.* (2020), we observed that arbuscular mycorrhizal species showed a high diversity of strategies, while the ectomycorrhizal species showed less variability along the gradient (Fig. 3).

The second axis of the phylogenetic PCA showed an important coordination between root tissue density (RTD) and traits related to leaf-level water-use, both leaf $\delta^{18}O$ and leaf $\delta^{13}C$, proxies of the stomatal conductance and the time-integrated water use efficiency, respectively. RTD is highly linked to the 'conservation' gradient of the RES, where species with high RTD present longer life span and a slow resource return on investment, i.e. a conservative resource use strategy, while species with low RTD are characterized by short life spans but fast returns, i.e. an acquisitive resource use strategy (Bergmann *et al.*, 2020). Our results showed a coupling between a conservative resource use at the root-level with a tighter stomatal regulation and greater water use efficiency, corresponding to a saver leaf-level water use strategy. In parallel, species with an acquisitive resource use strategy, characterized by low RTD, also had lower leaf $\delta^{18}O$ and leaf $\delta^{13}C$ associated instead to a spender leaf-level water use strategy. Our findings concur with other studies (Fort *et al.*, 2017; Zhou *et al.*, 2021) suggesting that root morphology is importantly linked to plant water use strategy. In particular, we observed a strong coordination between a saver leaf water use strategy with a high RTD. The latter, which is highly related with the 'conservation' gradient of the RES, may provide the mechanical support needed to withstand dry conditions and maintain evapotranpiration processes (e.g. Hacke *et al.*, 2001), especially during summer droughts when soil water potentials may reach highly negative values (e.g. McDowell *et al.*, 2008). Therefore, we suggest that RTD may have a fundamental contribution to plant water use dynamics, especially in semiarid Mediterranean environments. It was also hypothetized (Fort *et al.*, 2017) that high RTD may be key trait for some species in Mediterranean environments capable to maintain their transpiration capacity for a long period albeit relying on shallow water sources.

The significant correlation observed between a saver leaf-level water use strategy and species dominance in the plant community (Illuminati *et al.*, 2022) was stronger when coordinated with higher RTD, further showing the important contribution of a conservative root nutrient use strategy in coordination with a saver leaf water use strategy (Fig. 4). Conversely, we did not find any coordination between leaf water use strategy and root traits related with the 'collaboration' gradient of the RES (Fig. 3). This result differs from previous findings which pointed out a clear relationship between them, e.g. RD and traits related with leaf water use (Fort *et al.*, 2017; Zhou *et al.*, 2021). Moreover, in agreement with other studies (Fort *et al.*, 2017), we did not find any clear relationship between water uptake depth and root traits related with the RES (Fig. 3, Table S9). Although root traits were measured in juveniles grown in controlled conditions, our results suggest a strong coordination between RTD and leaf-level plant water use strategy, which may result critical for species survival and success especially in Mediterranean environments.

Conclusions

In accordance with global models, our findings showed that whole-plant functional variation of 23 species coexisting in a semiarid Mediterranean shrubland was characterized by three main gradients of variation, clearly associated to plant size, the leaf-level nutrient use strategy and the collaboration gradient of the root economic space, respectively. However, the high coordination of key traits of the root economic space with the plant size axis also pointed out that plant size was strongly associated to the root-level nutrient use strategy, at least at the juvenile stage. We indeed found that species characterized by a more acquisitive root-level strategy were positively linked to higher plant sizes, both aboveground and belowground. We also detected an important functional trade-off between the leaf-level water use strategy and the root tissue density, with a tighter transpiration rate and higher time-integrated water use efficiency coordinated with a higher root tissue density. Such relationship could be attributable to a better mechanical support provided by a higher tissue density, which may prove crucial to endure the highly negative soil water potentials during summer drought. The better performance in the plant community observed in species characterized by a coordinated saver leaf-level water use strategy and a higher root tissue density further suggests the improved functionality provided by this coordination.

Table 1 List of root traits measured in 23 species coexisting in a Mediterranean shrubland, some related to the nutrient-use strategy and others to the plant size. The symbols plus (+) and minus (-) indicated a positive and a negative effect, respectively.

Root Trait	Acronym	Resource acquisition	Resource conservation	Exploitative capacity	References
Nutrient-use strategy					
Specific Root Length (m/g)	SRL	+	-	+	Ostonen *et al.* (2007); Freschet *et al.*, (2021)
Specific Root Area (cm2/g)	SRA	+	-	+	Pérez-Ramos *et al.* (2012); de la Riva *et al.* (2021)
Root Diameter (mm)	RD	-	+		Larson & Funk (2016); Ma *et al.* (2018)
Very Fine (0-0.02 mm) Roots (%)	VFR	+	-	+	Ryser (1996); Pagès & Picon-Cochard (2014); Roumet *et al.* (2016)
Root Mass Fraction (%)	RMF			+	Poorter *et al.* (2012); Freschet *et al.* (2015, 2021)
Roots Tissue Density (mg/cm3)	RTD	-	+		Ryser (1996); Eissenstat & Yanai (1997)
Plant size					
Max Root Length (cm)	MRL				Larigauderie & Richards (1994); Eissenstat *et al.* (2015); Freschet *et al.* (2021)
Tot Root Length (m)	TRL			+	
Root Neck Diameter (mm)	RN				
Total Root Mass (mg)	TRM				

Figures

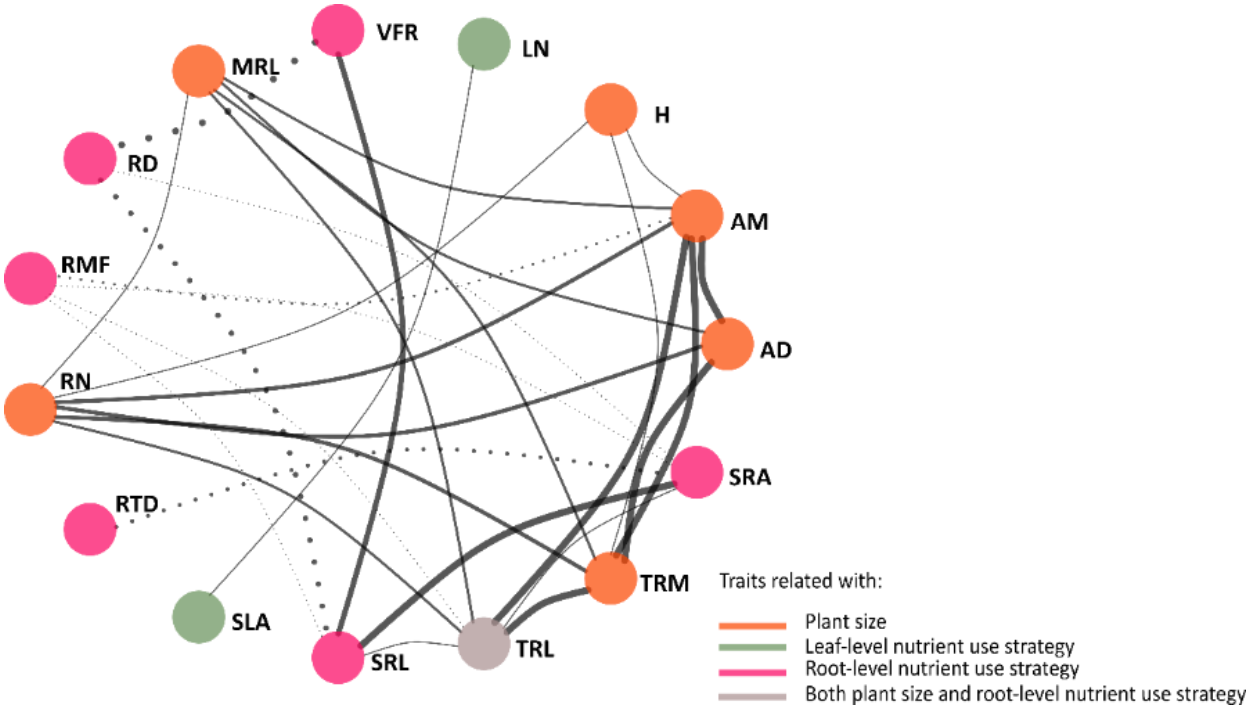

Fig. 1 Spearman' pairwise correlations between all traits considered. Only significant correlations with correlation values >0.40 are shown. The thickness of the links is proportional to the correlation coefficient. Dotted lines indicate negative correlations. Specific leaf area (SLA) and leaf nitrogen (LN) were the traits related with the leaf-level nutrient use strategy (LES). Height (H), aerial mass (AM), aerial diameter (AD), root neck (RN), maximum root length (MRL) and total root mass (TRM) were the aerial and root traits related with plant size. Total root length (TRL) was a trait related with both plant size and nutrient use strategy. Specific root length (SRL), specific root area (SRA), root diameter (RD), very fine roots (VFR), root mass fraction (RMF) and root tissue density (RTD) were the traits related with the root-level nutrient use strategy.

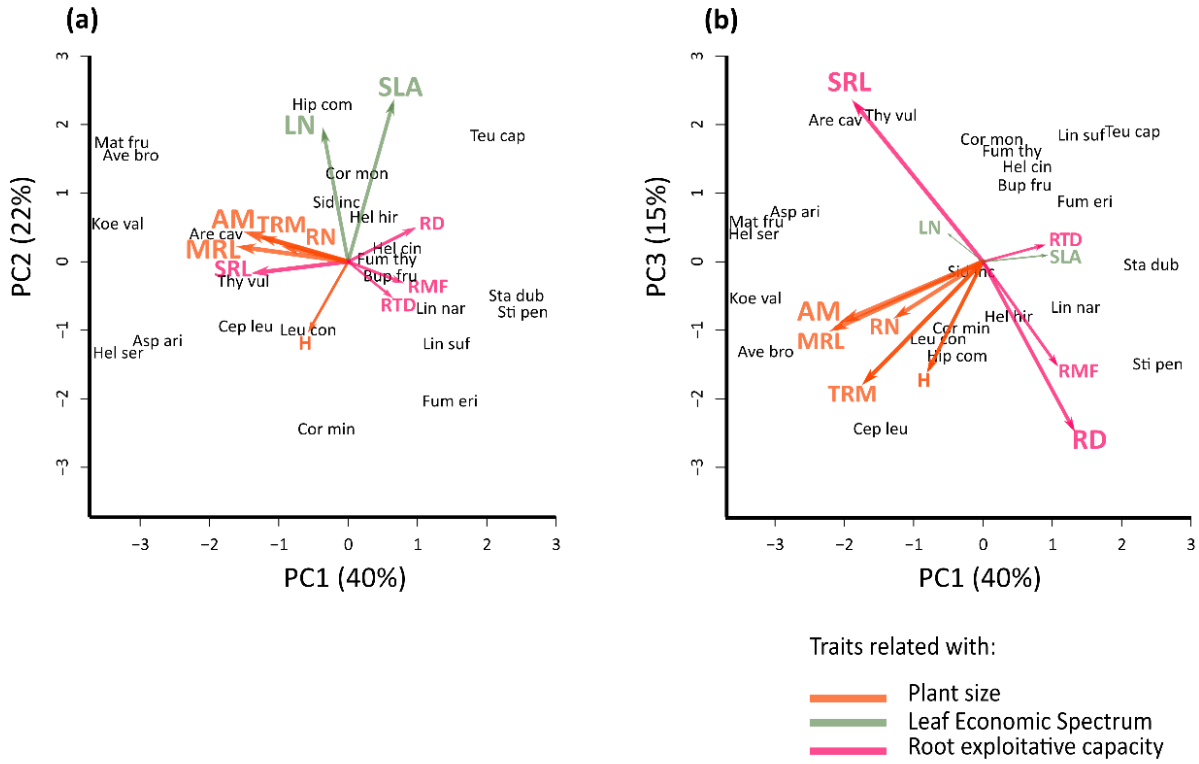

Fig. 2 Phylogenetic PCA carried out with species mean values of aerial and root traits. Panel **(a)** shows species variation along the first and second PCA axes, while **(b)** shows the first and third axes.

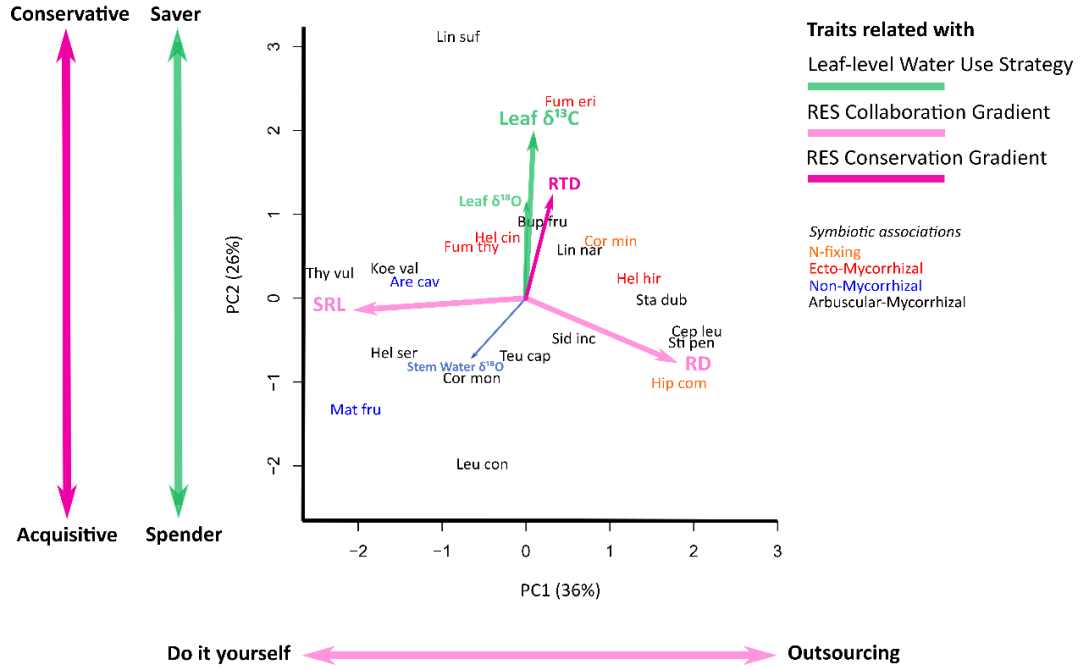

Fig. 3 Phylogenetic PCA carried out with species mean values of six selected traits, associated with the water-use strategy (leaf $\delta^{13}C$, leaf $\delta^{18}O$ and stem water $\delta^{18}O$) and root traits related with the RES (SRL, RD, RTD).

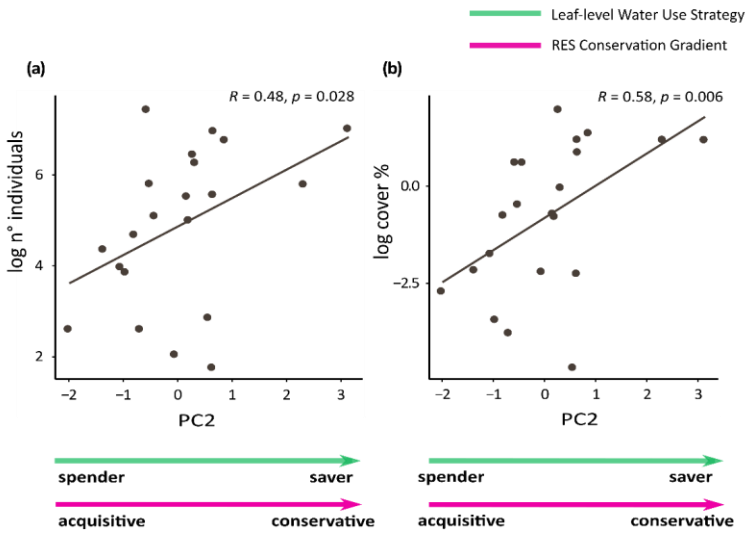

Fig. 4 Spearman's correlation tests carried out between the second axis (PC2) of the phylogenetic PCA and **(a)** the species abundance (log number of individuals) and **(b)** species dominance (log cover %).

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References

Aerts R. **1999**. Interspecific competition in natural plant communities: Mechanisms, trade-offs and plant-soil feedbacks. *Journal of Experimental Botany* **50**: 29–37.

Albert CH, Thuiller W, Yoccoz NG, Douzet R, Aubert S, Lavorel S. **2010**. A multi-trait approach reveals the structure and the relative importance of intra- vs. interspecific variability in plant traits. *Functional Ecology* **24**: 1192–1201.

Barbeta A, Gimeno TE, Clavé L, Fréjaville B, Jones SP, Delvigne C, Wingate L, Ogée J. **2020**. An explanation for the isotopic offset between soil and stem water in a temperate tree species. *New Phytologist* **227**: 766–779.

Barbour MM. **2007**. Stable oxygen isotope composition of plant tissue: A review. *Functional Plant Biology* **34**: 83–94.

Bardgett RD, Mommer L, De Vries FT. **2014**. Going underground: Root traits as drivers of ecosystem processes. *Trends in Ecology and Evolution* **29**: 692–699.

Bergmann J, Weigelt A, Van Der Plas F, Laughlin DC, Kuyper TW, Guerrero-Ramirez N, Valverde-Barrantes OJ, Bruelheide H, Fresche GT, Iversen CM, *et al.* 2020. The fungal collaboration gradient dominates the root economics space in plants. *Science Advances* **6**: eaba3756.

Carmona CP, Bueno CG, Toussaint A, Träger S, Díaz S, Moora M, Munson AD, Pärtel M, Zobel M, Tamme R. **2021**. Fine-root traits in the global spectrum of plant form and function. *Nature* **597**: 683–687.

Carvajal DE, Loayza AP, Rios RS, Delpiano CA, Squeo FA. **2019**. A hyper-arid environment shapes an inverse pattern of the fast–slow plant economics spectrum for above-, but not below-ground resource acquisition strategies. *Journal of Ecology* **107**: 1079–1092.

Casper BB, Jackson RB. **1997**. PLANT COMPETITION UNDERGROUND. *Annual Review of Ecology and Systematics* **28**: 545–570.

Cavender-Bares J, Kozak KH, Fine PVA, Kembel SW. **2009**. The merging of community ecology and phylogenetic biology. *Ecology Letters* **12**: 693–715.

Chacón-Labella J, de la Cruz M, Escudero A. **2016**. Beyond the classical nurse species effect: Diversity assembly in a Mediterranean semi-arid dwarf shrubland. *Journal of Vegetation Science* **27**: 80–88.

Chapin FS, Autumn K, Pugnaire F. **1993**. Evolution of Suites of Traits in Response to Environmental Stress. *The American Naturalist* **142**: S78–S92.

Chase JM, Leibold MA. **2003**. *Ecological niches: interspecific interactions*. Chicago University Press.

Craine JM, Froehle J, Tilman DG, Wedin DA, Chapin FS. **2001**. The relationships among root and leaf traits of 76 grassland species and relative abundance along fertility and disturbance gradients. *Oikos* **93**: 274–285.

Dawson TE, Mambelli S, Plamboeck AH, Templer PH, Tu KP. **2002**. Stable Isotopes in Plant Ecology. *Annual Review of Ecology and Systematics* **33**: 507–559.

Delory BM, Weidlich EWA, Meder L, Lütje A, van Duijnen R, Weidlich R, Temperton VM. **2017**. Accuracy and bias of methods used for root length measurements in functional root research. *Methods in Ecology and Evolution* **8**: 1594–1606.

Díaz S, Hodgson JG, Thompson K, Cabido M, Cornelissen JHC, Jalili A, Montserrat-Martí G, Grime JP, Zarrinkamar F, Asri Y, *et al.* 2004. The plant traits that drive ecosystems: Evidence from three continents. *Journal of Vegetation Science* **15**: 295–304.

Ding Y, Nie Y, Chen H, Wang K, Querejeta JI, Kelin W, Querejeta JI. **2021**. Water uptake depth is coordinated with leaf water potential, water-use efficiency and drought vulnerability in karst vegetation. *New Phytologist* **229**: 1339–1353.

Domec JC, Noormets A, King JS, Sun G, McNulty SG, Gavazzi MJ, Boggs JL, Treasure EA. **2009**. Decoupling the influence of leaf and root hydraulic conductances on stomatal conductance and its sensitivity to vapour pressure deficit as soil dries in a drained loblolly pine plantation. *Plant, Cell & Environment* **32**: 980–991.

Eissenstat DM, Kucharski JM, Zadworny M, Adams TS, Koide RT. **2015**. Linking root traits to nutrient foraging in arbuscular mycorrhizal trees in a temperate forest. *New Phytologist* **208**: 114–124.

Eissenstat DM, Yanai RD. **1997**. The Ecology of Root Lifespan. *Advances in Ecological Research* **27**: 1–60.

Escudero A, Valladares F. **2016**. Trait-based plant ecology: moving towards a unifying species coexistence theory. *Oecologia* **180**: 919–922.

Farquhar GD, Hubick KT, Condon AG, Richards RA. **1989**. Carbon isotope fractionation and plant water-use efficiency. In: Stables Isotopes in Ecological Research. Springer, New York,

NY, 21–40.

Fort F, Volaire F, Guilioni L, Barkaoui K, Navas ML, Roumet C. 2017. Root traits are related to plant water-use among rangeland Mediterranean species. *Functional Ecology* **31**: 1700–1709.

Freschet GT, Cornelissen JHC, van Logtestijn RSP, Aerts R. 2010. Evidence of the 'plant economics spectrum' in a subarctic flora. *Journal of Ecology* **98**: 362–373.

Freschet GT, Kichenin E, Wardle DA. 2015. Explaining within-community variation in plant biomass allocation: A balance between organ biomass and morphology above vs below ground? *Journal of Vegetation Science* **26**: 431–440.

Freschet GT, Roumet C. 2017. Sampling roots to capture plant and soil functions. *Functional Ecology* **31**: 1506–1518.

Freschet GT, Roumet C, Comas LH, Weemstra M, Bengough AG, Rewald B, Bardgett RD, Deyn GB De, Johnson D, Klimešová J, *et al.* 2021. Root traits as drivers of plant and ecosystem functioning: current understanding, pitfalls and future research needs. *New Phytologist* **232**: 1123–1158.

Hacke UG, Sperry JS, Pockman WT, Davis SD, McCulloh KA. 2001. Trends in wood density and structure are linked to prevention of xylem implosion by negative pressure. *Oecologia 2000 126:4* **126**: 457–461.

Holdaway RJ, Richardson SJ, Dickie IA, Peltzer DA, Coomes DA. 2011. Species- and community-level patterns in fine root traits along a 120 000-year soil chronosequence in temperate rain forest. *Journal of Ecology* **99**: 954–963.

Hummel I, Vile D, Violle C, Devaux J, Ricci B, Blanchard A, Garnier É, Roumet C. 2007. Relating root structure and anatomy to whole-plant functioning in 14 herbaceous Mediterranean species. *New Phytologist* **173**: 313–321.

Illuminati A, López-Angulo J, de la Cruz M, Chacón-Labella J, S. Pescador D, Pías B, Sánchez AM, Escudero A, Matesanz S. 2021. Larger aboveground neighbourhood scales maximise similarity but do not eliminate discrepancies with belowground plant diversity in a Mediterranean shrubland. *Plant and Soil* **460**: 497–509.

Illuminati A, Querejeta JI, Pías B, Escudero A, Matesanz S. 2022. Coordination between water uptake depth and the leaf economic spectrum in a Mediterranean shrubland. *Journal of*

Ecology.

Inderjit, Callaway RM, Meron E. **2021**. Belowground feedbacks as drivers of spatial self-organization and community assembly. *Physics of Life Reviews* **38**: 1–24.

Iversen CM, McCormack ML, Powell AS, Blackwood CB, Freschet GT, Kattge J, Roumet C, Stover DB, Soudzilovskaia NA, Valverde-Barrantes OJ, *et al.* 2017. A global Fine-Root Ecology Database to address below-ground challenges in plant ecology. *New Phytologist* **215**: 15–26.

Kraft NJB, Godoy O, Levine JM. **2015**. Plant functional traits and the multidimensional nature of species coexistence. *Proceedings of the National Academy of Sciences of the United States of America* **112**: 797–802.

Kramer-Walter KR, Bellingham PJ, Millar TR, Smissen RD, Richardson SJ, Laughlin DC. **2016**. Root traits are multidimensional: specific root length is independent from root tissue density and the plant economic spectrum. *Journal of Ecology* **104**: 1299–1310.

de la Riva EG, Prieto I, Marañón T, Pérez-Ramos IM, Olmo M, Villar R. **2021**. Root economics spectrum and construction costs in Mediterranean woody plants: The role of symbiotic associations and the environment. *Journal of Ecology* **109**: 1873–1885.

Laliberté E. **2017**. Below-ground frontiers in trait-based plant ecology. *New Phytologist* **213**: 1597–1603.

Larigauderie A, Richards JH. **1994**. Root proliferation characteristics of seven perennial arid-land grasses in nutrient-enriched microsites. *Oecologia 1994 99:1* **99**: 102–111.

Larson JE, Funk JL. **2016**. Seedling root responses to soil moisture and the identification of a belowground trait spectrum across three growth forms. *New Phytologist* **210**: 827–838.

Laughlin DC. **2014**. The intrinsic dimensionality of plant traits and its relevance to community assembly. *Journal of Ecology* **102**: 186–193.

Long RW, Medeiros JS. **2021**. Water in, water out: root form influences leaf function. *New Phytologist* **229**: 1186–1188.

Ma Z, Guo D, Xu X, Lu M, Bardgett RD, Eissenstat DM, McCormack ML, Hedin LO. **2018**. Evolutionary history resolves global organization of root functional traits. *Nature* **555**: 94–97.

Matesanz S, Valladares F. **2014**. Ecological and evolutionary responses of Mediterranean plants

to global change. *Environmental and Experimental Botany* **103**: 53–67.

McCormack ML, Guo D, Iversen CM, Chen W, Eissenstat DM, Fernandez CW, Li L, Ma C, Ma Z, Poorter H, *et al.* 2017. Building a better foundation: improving root-trait measurements to understand and model plant and ecosystem processes. *New Phytologist* **215**: 27–37.

McDowell N, Pockman WT, Allen CD, Breshears DD, Cobb N, Kolb T, Plaut J, Sperry J, West A, Williams DG, *et al.* 2008. Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? *New Phytologist* **178**: 719–739.

McGill BJ, Enquist BJ, Weiher E, Westoby M. 2006. Rebuilding community ecology from functional traits. *Trends in Ecology and Evolution* **21**: 178–185.

Messier J, Lechowicz MJ, McGill BJ, Violle C, Enquist BJ. 2017a. Interspecific integration of trait dimensions at local scales: the plant phenotype as an integrated network. *Journal of Ecology* **105**: 1775–1790.

Messier J, McGill BJ, Enquist BJ, Lechowicz MJ. 2017b. Trait variation and integration across scales: is the leaf economic spectrum present at local scales? *Ecography* **40**: 685–697.

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI. 2012. Isotopes reveal contrasting water use strategies among coexisting plant species in a mediterranean ecosystem. *New Phytologist* **196**: 489–496.

Ostonen I, Püttsepp Ü, Biel C, Alberton O, Bakker MR, Lõhmus K, Majdi H, Metcalfe D, Olsthoorn AFM, Pronk A, *et al.* 2007. Plant Biosystems Specific root length as an indicator of environmental change Specific root length as an indicator of environmental change.

Pagès L, Picon-Cochard C. 2014. Modelling the root system architecture of Poaceae. Can we simulate integrated traits from morphological parameters of growth and branching? *New Phytologist* **204**: 149–158.

Pérez-Ramos IM, Roumet C, Cruz P, Blanchard A, Autran P, Garnier E. 2012. Evidence for a 'plant community economics spectrum' driven by nutrient and water limitations in a Mediterranean rangeland of southern France (R Aerts, Ed.). *Journal of Ecology* **100**: 1315–1327.

Poorter H, Niklas KJ, Reich PB, Oleksyn J, Poot P, Mommer L. 2012. Biomass allocation to leaves, stems and roots: meta-analyses of interspecific variation and environmental control. *New Phytologist* **193**: 30–50.

Radojevic M, Bashkin V. **1999**. *Practical Environmental Analysis*. Royal Society of Chemistry.

Reich PB, Tjoelker MG, Walters MB, Vanderklein DW, Buschena C. **1998**. Close association of RGR, leaf and root morphology, seed mass and shade tolerance in seedlings of nine boreal tree species grown in high and low light. *Functional Ecology* **12**: 327–338.

Roumet C, Birouste M, Picon-Cochard C, Ghestem M, Osman N, Vrignon-Brenas S, Cao K fang, Stokes A. **2016**. Root structure–function relationships in 74 species: evidence of a root economics spectrum related to carbon economy. *New Phytologist* **210**: 815–826.

Ryser P. **1996**. The Importance of Tissue Density for Growth and Life Span of Leaves and Roots: A Comparison of Five Ecologically Contrasting Grasses. *Functional Ecology* **10**: 717.

Schliep KP. **2011**. phangorn: Phylogenetic analysis in R. *Bioinformatics* **27**: 592–593.

Schulze E-D, Mooney HA, Sala OE, Jobbagy E, Buchmann N, Bauer G, Canadell J, Jackson RB, Loreti J, Oesterheld M, *et al.* 1996. Rooting depth, water availability, and vegetation cover along an aridity gradient in Patagonia. *Oecologia* **108**: 503–511.

Silvertown J. **2004**. Plant coexistence and the niche. *Trends in Ecology and Evolution* **19**: 605–611.

Silvertown J, Araya Y, Gowing D. **2015**. Hydrological niches in terrestrial plant communities: a review (W Cornwell, Ed.). *Journal of Ecology* **103**: 93–108.

Valverde-Barrantes OJ, Smemo KA, Blackwood CB. **2015**. Fine root morphology is phylogenetically structured, but nitrogen is related to the plant economics spectrum in temperate trees. *Functional Ecology* **29**: 796–807.

Violle C, Navas ML, Vile D, Kazakou E, Fortunel C, Hummel I, Garnier E. **2007**. Let the concept of trait be functional! *Oikos* **116**: 882–892.

Violle C, Nemergut DR, Pu Z, Jiang L. **2011**. Phylogenetic limiting similarity and competitive exclusion. *Ecology Letters* **14**: 782–787.

Weigelt A, Mommer L, Andraczek K, Iversen CM, Bergmann J, Bruelheide H, Fan Y, Freschet GT, Guerrero-Ramírez NR, Kattge J, *et al.* 2021. An integrated framework of plant form and function: the belowground perspective. *New Phytologist*.

Weltzin JF, Tissue DT. **2003**. Resource pulses in arid environments - Patterns of rain, patterns of life. *New Phytologist* **157**: 171–173.

Westoby M. **1998**. A leaf-height-seed (LHS) plant ecology strategy scheme. *Plant and Soil* **199**:

213–227.

Westoby M, Leishman MR, Lord JM. **1995**. On Misinterpreting the `Phylogenetic Correction'. *The Journal of Ecology* **83**: 531–534.

Williams, D. G., & Ehleringer JR. **2000**. Intra- and interspecific variation for summer precipitation use in pinyon-juniper woodlands. *Ecological Monographs* **70**: 517–537.

Withington JM, Reich PB, Oleksyn J, Eissenstat DM. **2006**. COMPARISONS OF STRUCTURE AND LIFE SPAN IN ROOTS AND LEAVES AMONG TEMPERATE TREES. *Ecological Monographs* **76**: 381–397.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornellssen JHC, Diemer M, *et al.* 2004. The worldwide leaf economics spectrum. *Nature* **428**: 821–827.

Zhou M, Bai W, Li Q, Guo Y, Zhang WH. **2021**. Root anatomical traits determined leaf-level physiology and responses to precipitation change of herbaceous species in a temperate steppe. *New Phytologist* **229**: 1481–1491.

Supporting information

Results S1

Single-trait inter-specific functional variation

We found high interspecific trait variation across 23 coexisting species in all root traits, both in those related with nutrient-use such as SRL (species mean values range: 111.49-765.07 m/g), SRA (443.61-1645.81 cm^2/g), RD (0.15-0.43 mm), VFR (9-87%) and RTD (27.85-129.81 mg/cm^3), and those mostly related to the plant size, such as the RN (1.63-34.77 mm), TRM (45.03-1029.05 mg), TRL (6.43-399.30 m) and MRL (37.05-59.08 cm). High functional diversity was also observed in the measured leaf traits, including SLA (38.06-207.38 cm^2/g), LN (0.52-1.04%) and LP (0.04-0.16%) contents, which are related to the leaf nutrient-use strategy (LES) and in aerial traits related to plant size such as H (38.52-155.83 mm) and AM (43.45-2083.65 mg). Despite the very high interspecific differences, we also observed high intraspecific variability, especially in some species (Fig. S1, S2, S3).

Table S1 Species list considered in the study and their respective family, growth form and abundances (cover % and number of individuals) in the plant community.

Species	Family	Growth form	Cover %	N° individuals
Arenaria cavanillesiana	*Caryophyllaceae*	nano shrub	0.36	261
Asperula aristata	*Rubiaceae*	hemicryptophyte	0.03	67
Avenula bromoides	*Poaceae*	grass	0.07	169
Bupleurum fruticescens	*Apiaceae*	nano shrub	2.46	899
Cephalaria leucantha	*Caprifoliaceae*	hemicryptophyte	1.57	169
Coris monspeliensis	*Primulaceae*	nano shrub	0.03	49
Coronilla minima	*Fabaceae*	shrub	2.14	270
Fumana ericoides	*Cistaceae*	nano shrub	2.05	339
Fumana thymifolia	*Cistaceae*	nano shrub	0.10	6
Helianthemum cinereum	*Cistaceae*	nano shrub	2.04	1099
Helianthemum hirtum	*Cistaceae*	nano shrub	0.32	154
Helichrysum serotinum	*Asteraceae*	nano shrub	0.02	14
Hippocrepis commutata	*Fabaceae*	nano shrub	0.14	55
Koeleria vallesiana	*Poaceae*	grass	0.74	545
Leuzea conifera	*Asteraceae*	hemicryptophyte	0.05	14
Linum narbonense	*Linaceae*	hemicryptophyte	0.01	18
Linum suffruticosum	*Linaceae*	shrub	2.13	1155
Matthiola fruticulosa	*Brassicaceae*	nano shrub	0.11	81
Sideritis incana	*Lamiaceae*	shrub	0.51	344
Stahelina dubia	*Asteraceae*	hemicryptophyte	0.08	8
Stipa pennata	*Poaceae*	grass	1.11	1756
Teucrium capitatum	*Lamiaceae*	nano shrub	0.27	112
Thymus vulgaris	*Lamiaceae*	nano shrub	4.76	655

Table S2 Number of samples for each species, total fine root percentage (%) estimated as fine (<1mm) root dry mass (mg)/ total root dry mass (mg)*100. The percentage (%) of fine root which has been scanned for estimation of root traits such as SRL was also indicated.

Species	N° samples	Total Fine Root %	Fine Root % scanned
Arenaria cavanillesiana	7	78.47	100.00
Asperula aristata	5	94.19	100.00
Avenula bromoides	6	100.00	100.00
Bupleurum fruticescens	8	82.89	100.00
Cephalaria leucantha	8	69.46	100.00
Coris monspeliensis	8	93.19	100.00
Coronilla minima	5	58.17	100.00
Fumana ericoides	8	97.08	100.00
Fumana thymifolia	8	88.65	100.00
Helianthemum cinereum	6	95.82	100.00
Helianthemum hirtum	6	83.46	100.00
Helichrysum serotinum	5	73.05	99.85
Hippocrepis commutata	8	80.85	100.00
Koeleria vallesiana	3	100.00	100.00
Leuzea conifera	6	33.26	100.00
Linum narbonense	7	95.14	100.00
Linum suffruticosum	8	89.43	100.00
Matthiola fruticulosa	5	53.03	77.22
Sideritis incana	8	79.27	100.00
Stahelina dubia	7	86.83	100.00
Stipa pennata	8	99.61	100.00
Teucrium capitatum	8	82.81	100.00
Thymus vulgaris	7	82.99	99.92

Table S3 Mean species values of root traits measured in 23 species coexisting in a Mediterranean shrubland.

Species	Species code	RD (mm)	MRL (cm)	RMF (%)	SRA (cm^2/g)	SRL m/g	TRL (m)	TRM (mg)	VFR (%)	RN (mm)	RTD (mg/cm^3)
Arenaria cavanillesiana	Are cav	0.22	49.26	0.16	1127.11	522.58	43.97	102.73	0.66	1.99	53.949
Asperula aristata	Asp ari	0.19	52.78	0.27	1094.83	579.28	156.89	290.36	0.78	1.83	61.954
Avenula bromoides	Ave bro	0.23	55.57	0.32	685.88	294.73	302.42	1029.05	0.64	10.43	79.943
Bupleurum fruticescens	Bup fru	0.29	49.11	0.32	806.06	281.67	11.70	51.07	0.31	1.97	55.972
Cephalaria leucantha	Cep leu	0.36	55.01	0.29	471.40	132.35	56.62	622.44	0.15	5.02	75.907
Coris monspeliensis	Cor mon	0.31	42.03	0.21	1248.84	399.68	26.32	70.53	0.12	1.80	32.926
Coronilla minima	Cor min	0.35	53.26	0.32	705.19	212.21	11.45	101.02	0.27	2.26	55.259
Fumana ericoides	Fum eri	0.26	44.65	0.38	552.99	216.24	13.91	66.11	0.44	0.81	91.971
Fumana thymifolia	Fum thy	0.24	46.60	0.34	803.46	337.82	15.15	54.68	0.50	1.10	67.741
Helianthemum cinereum	Hel cin	0.23	50.07	0.38	612.90	266.14	32.58	133.64	0.48	1.06	90.442
Helianthemum hirtum	Hel hir	0.33	54.12	0.37	514.78	160.52	20.42	175.96	0.18	1.56	76.696
Helichrysum serotinum	Hel ser	0.22	59.08	0.26	1221.09	558.51	145.53	346.52	0.62	3.57	49.029
Hippocrepis commutata	Hip com	0.43	54.50	0.35	664.81	156.34	30.89	241.28	0.09	2.16	45.267
Koeleria vallesiana	Koe val	0.21	53.27	0.31	918.85	449.80	399.30	903.67	0.74	11.90	67.835
Leuzea conifera	Leu con	0.28	49.37	0.43	777.63	291.64	49.54	513.17	0.25	4.85	60.449
Linum narbonense	Lin nar	0.30	50.20	0.44	634.35	269.95	20.15	76.11	0.36	1.00	76.568
Linum suffruticosum	Lin suf	0.18	46.89	0.40	575.62	331.07	13.66	45.03	0.72	1.37	129.275
Matthiola fruticulosa	Mat fru	0.22	57.68	0.23	1645.81	765.07	119.76	383.84	0.62	4.06	27.850
Sideritis incana	Sid inc	0.30	48.75	0.27	563.20	187.92	33.34	225.58	0.25	2.40	76.021
Stahelina dubia	Sta dub	0.34	47.42	0.35	469.13	142.19	6.97	59.02	0.22	1.45	83.428
Stipa pennata	Sti pen	0.40	44.83	0.43	443.61	111.49	6.43	58.69	0.13	3.13	72.241
Teucrium capitatum	Teu cap	0.25	37.05	0.28	572.78	233.09	13.24	70.05	0.47	1.66	91.761
Thymus vulgaris	Thy vul	0.15	52.68	0.24	879.82	592.48	63.49	130.66	0.87	2.06	129.813

Table S4 Variance explained by the main axes of the PCA carried out with all traits measured in the common garden experiment and without phylogeny.

	PC1	PC2	PC3	PC4
SD	1.872	1.447	1.276	1.01707
Variance explained	0.3186	0.1903	0.148	0.09404
Cumulative variance	0.3186	0.5089	0.6569	0.75098

Table S5 Trait scores along the main axes of the PCA carried with all traits of the common garden experiment and without phylogeny.

	PC1	PC2	PC3	PC4
SRL	-0.20046	**0.55333**	0.24258	-0.16059
TRM	**-0.47204**	-0.16741	-0.00012	0.224946
RTD	0.253489	-0.03188	**0.385722**	**0.611002**
RN	**-0.40649**	-0.18864	-0.10729	0.251677
H	-0.2662	-0.15682	0.234469	0.144601
AM	**-0.50214**	0.062583	-0.02965	0.091366
RD	0.033603	**-0.52391**	**-0.4677**	-0.20303
MRL	-0.33435	-0.16941	0.174278	-0.02384
RMF	0.248916	**-0.37556**	0.141572	0.278733
SLA	0.080873	0.282088	**-0.43521**	**0.555052**
LN	-0.05551	0.277295	**-0.51618**	0.173562

Table S6 Variance explained by the main axes of the PCA carried out with all traits measured in the common garden experiment and accounting for phylogeny.

	PC1	PC2	PC3	PC4
SD	11.79363	8.822946	7.196755	5.173664
Variance explained	0.397752	0.22261	0.148112	0.076544
Cumulative variance	0.397752	0.620362	0.768474	0.845018

Table S7 Trait scores along the main axes of the PCA carried with all traits of the common garden experiment and without phylogeny.

	PC1	PC2	PC3	PC4
SRL	**-0.77189**	-0.07035	**0.588396**	0.031511
TRM	**-0.76595**	0.16419	**-0.48174**	0.32228
RTD	**0.477484**	-0.29321	0.080227	**0.562466**
RN	**-0.74572**	0.131622	-0.29237	0.339627
H	-0.37717	**-0.50334**	**-0.46195**	-0.17274
AM	**-0.90607**	0.196716	-0.23764	0.172811
RD	**0.591953**	0.22581	**-0.67664**	-0.34083
MRL	**-0.82151**	0.085106	-0.22974	-0.17871
RMF	**0.564038**	-0.16452	**-0.48591**	0.383636
SLA	0.322354	**0.863517**	0.020137	0.220198
LN	-0.21367	**0.854582**	0.105266	-0.25409

Table S8 Variance explained by the main axes of the PCA carried out with root traits especially related to the root economic space (RD, SRL, RTD), measured in the common garden experiment, and traits related to water use (Stem water $\delta^{18}O$, Leaf $\delta^{18}O$, Leaf $\delta^{13}C$), measured in the field, and accounting for phylogeny.

	PC1	PC2	PC3	PC4
SD	8.335882	7.049341	5.984577	5.060507
Variance explained	0.362437	0.259195	0.186809	0.133573
Cumulative variance	0.362437	0.621632	0.808441	0.942013

Table S9 Trait scores along the main axes of the PCA carried with carried out with root traits especially related to the root economic space (RD, SRL, RTD), and traits related to water use (Stem water $\delta^{18}O$, Leaf $\delta^{18}O$, Leaf $\delta^{13}C$), and accounting for phylogeny.

Traits	PC1	PC2	PC3	PC4	PC5
Leaf $\delta^{13}C$	0.048069	**0.876526**	0.287525	-0.12157	0.36319
Stem water $\delta^{18}O$	-0.34613	-0.32247	0.597624	0.635893	0.121133
RD	**0.920424**	-0.33149	-0.05903	0.091019	0.152328
Leaf ^{18}O	0.013319	**0.501209**	-0.64218	0.579144	-0.02821
SRL	**-0.9756**	-0.0568	-0.1581	-0.10464	0.048271
RTD	0.198372	**0.651292**	0.551852	0.075078	-0.47151

Table S10 Trait scores along the main axes of the PCA carried with carried out with root traits especially related to the root economic space (RD, SRL, RTD), and traits related to water use (Stem water $\delta^{18}O$, Leaf $\delta^{18}O$, Leaf $\delta^{13}C$), without phylogeny.

Traits	PC1	PC2	PC3	PC4
Leaf δ13C	0.05076264	**-0.4159072**	0.53679	-0.4732261
Stem water δ18O	-0.1206817	-0.3098449	-0.56636	-0.7027637
RD	**0.69651639**	0.1807282	-0.16125	-0.1451954
Leaf 18O	0.02622291	0.3913335	0.56829	-0.4045711
SRL	**-0.7049559**	0.2075936	-0.00007	-0.0685713
RTD	0.00857714	**-0.7086026**	0.20528	0.30449767

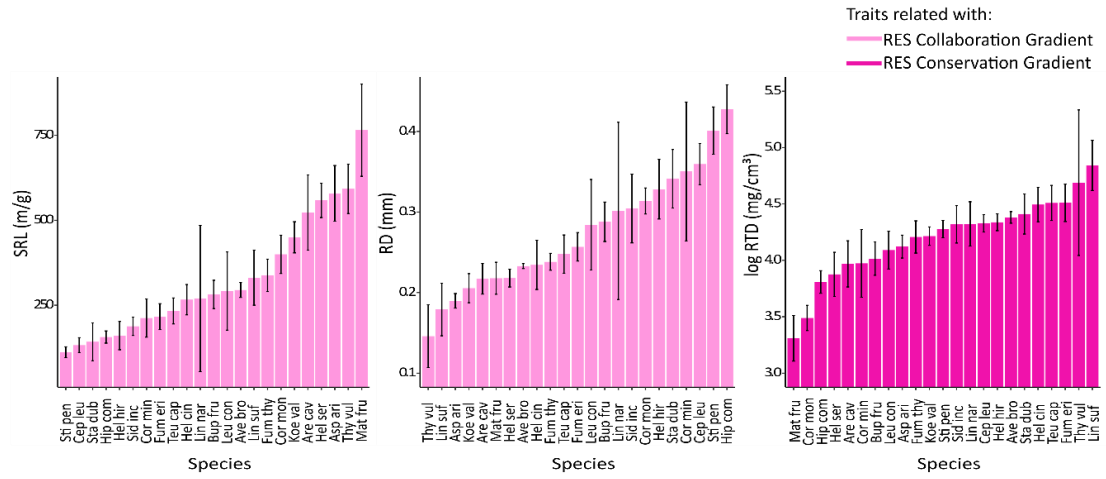

Fig. S1 Species mean (± SD) values of three key root traits (SRL, RD, RTD) related with the RES, showing inter-specific and intra-specific variation of each trait.

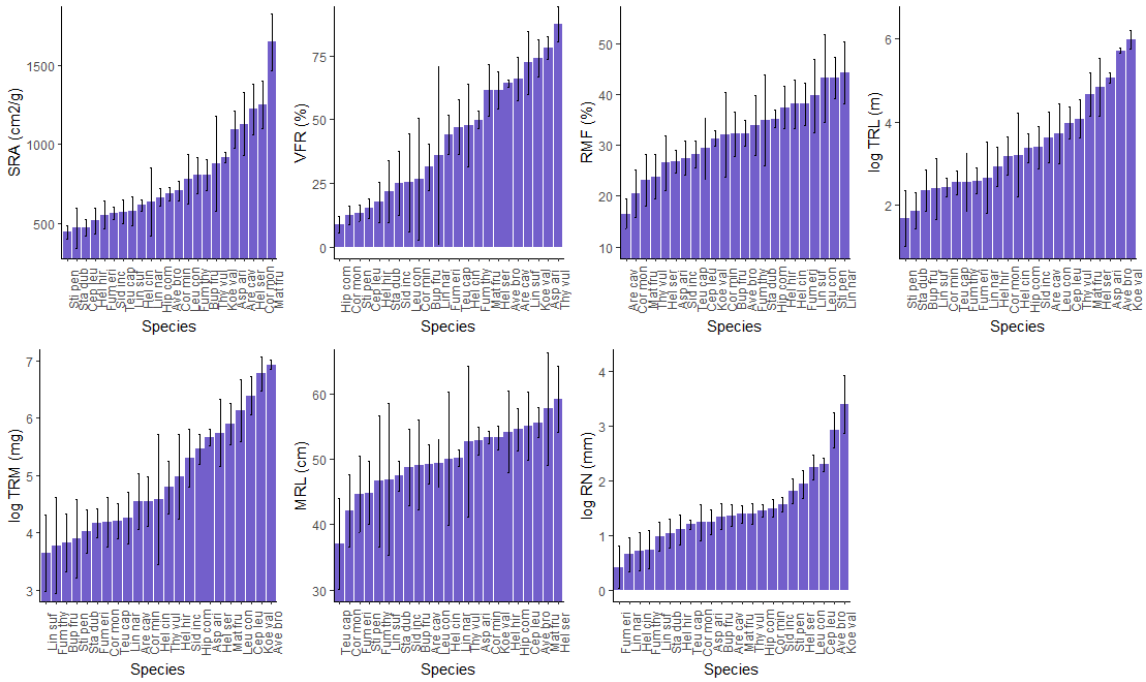

Fig. S2 Species mean (± SD) values of the other root traits considered in the study. Specific Root Area (SRA), Very Fine Roots (VFR), Root Mass Fraction (RMF), Total Root Length (TRL), Total Root Mass (TRM), Maximum Root Length (MRL), Root Neck (RN).

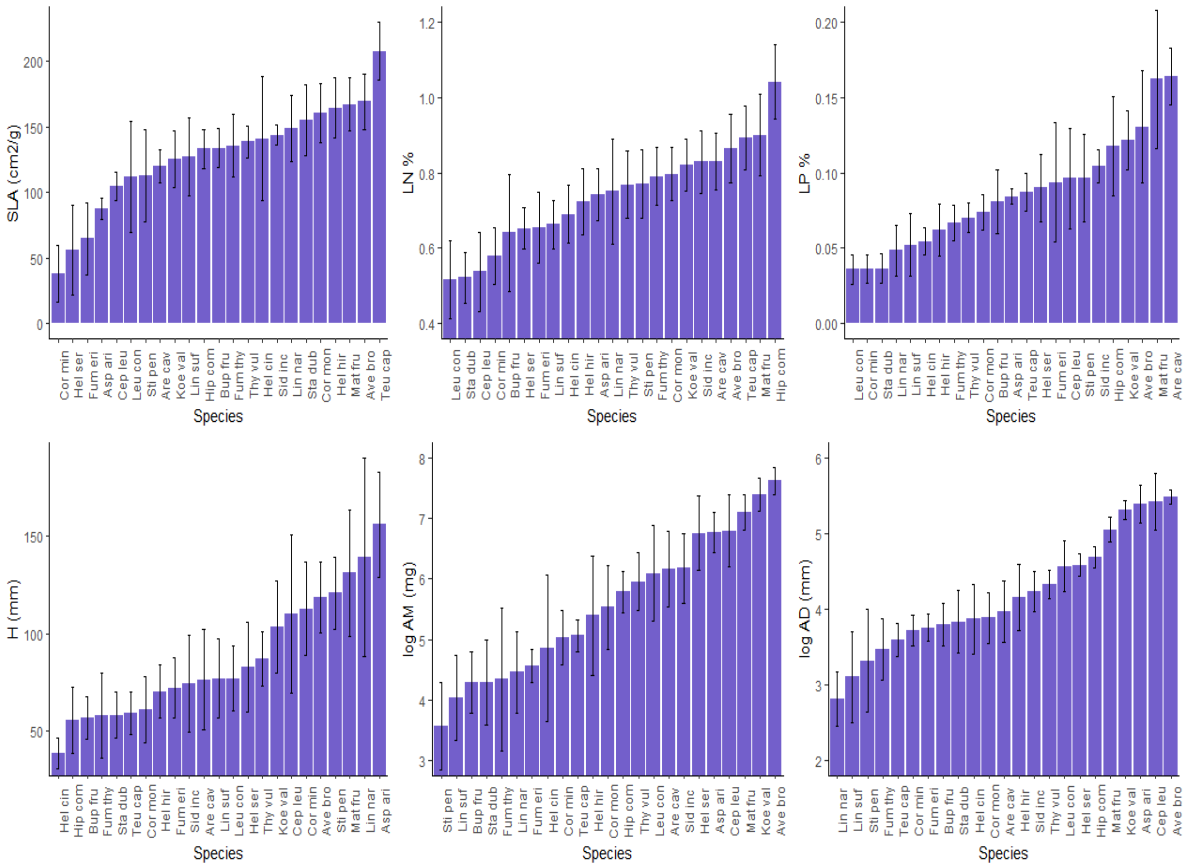

Fig. S3 Leaf traits related to the leaf economic spectrum and aerial traits related with the plant size. Specific Leaf Area (SLA), Leaf Nitrogen content (LN), Leaf Phosphorus content (LP); Height (H), Aerial Mass (AM), Aerial Diameter (AD).

Chapter 3

Larger aboveground neighbourhood scales maximise similarity but not eliminate discrepancies with belowground plant diversity in a Mediterranean shrubland

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Key words: *DNA metabarcoding; belowground plant diversity; community assembly; roots; shrubland; coexistence*

Summary

An unresolved question in plant ecology is whether diversity of the aboveground and belowground compartments of a plant community is similar at different neighbourhood scales. We investigated how the similarity between both compartments varies with the aboveground sampling grain and if significant discrepancies exist between aboveground and belowground plant diversity at the maximum similarity scale. We fully mapped the perennial plant community of a 64 m^2 plot in a Mediterranean shrubland and analysed the aboveground compartment by assessing diversity in 5 to 50 cm radii circles centred in soil cores. We sampled 2.5 cm radius root cores at two different depths and identified plant species by using DNA *metabarcoding* to characterise the belowground compartment. We quantified differences in species richness, composition and species' spatial distribution above- and belowground. The differences between aboveground and belowground were affected by the size of the aboveground sampling grain and were minimised when considering a circle of 20 cm radius in the aboveground. We found a significant dissimilarity in richness and composition between the two compartments, with larger differences when considering the deeper soil layer only. Our results showed that the spatial grain selected to sample a plant community aboveground and belowground is critical to characterise them in a comparable manner. Although their composition is related, species distribution patterns strongly differ, suggesting the simultaneous action of different assembly mechanisms. Our results call for caution when studying community assembly considering only the standing vegetation, since total plant diversity can be underappreciated.

Introduction

Roots are an important fraction of total ecosystem biomass in all vegetation types (Mokany et al. 2006). This is especially evident in stressful habitats such as water-limited environments, where plant root:shoot ratios are significantly higher than in more benign conditions (Schenk and Jackson 2002; Walter 1963). As early as in the 1960's, some authors tried to determine the relative weight of shoots and roots in plant communities (see *e.g.* Bray 1963; Davidson 1969). However, the lack of straightforward, feasible sampling techniques, strongly limited the integration of belowground information in the toolbox of plant community ecologists (Rewald et al. 2012). Recent advances in molecular techniques such as DNA metabarcoding, which allows the simultaneous identification of multiple taxa through next generation sequencing, are changing this scenario (Deiner et al. 2017; Hiiesalu et al. 2012). This powerful molecular tool has opened new venues to explore the hidden compartment of plant communities by identifying all the species present in root mixtures, and potentially, also their relative biomass partition (Matesanz et al. 2019). Incorporating a detailed characterization of the belowground compartment into the study of plant communities can help to unveil mechanisms controlling community assembly at fine spatial scales (Pärtel et al. 2012). In addition, it can be basic for the estimation of total plant diversity, which represents a priority in conservation ecology because of the known linkages between biodiversity and ecosystem functioning (Cardinale et al. 2012).

Only a few studies (*e.g.* Hiiesalu et al. 2012; Kesanakurti et al. 2011; Träger et al. 2019), have jointly assessed richness and composition of both the above- and belowground compartments of plant communities. A general pattern that emerged from these studies are the discrepancies in species richness, since the number of species is generally higher belowground than aboveground (*e.g.* Hiiesalu et al. 2012; Jones et al. 2011). This could be explained by several concomitant factors, including a higher prospective ability of roots in space and time, and a greater heterogeneity in the distribution of soil resources and conditions compared with those in the aboveground, which could in turn promote more opportunities for niche diversification (Pärtel et al. 2012). Although some studies (*e.g.* Kesanakurti et al. 2011; Li et al. 2017) observed similarity on the species distribution between the above- and belowground compartments (both in terms of presence-absence and abundance), they also reported a general asymmetry in species frequencies between both compartments and a sharp segregation of species with soil depth. Consequently, the arising paradigm is that species diversity and distribution observed aboveground are different from those belowground, thus limiting its value as a robust and integrative proxy of the total diversity structure in the plant community.

The species distribution asymmetry often observed in plant communities could be related to different processes that structure diversity in the two compartments. Some authors (*e.g.* Casper and Jackson 1997) suggested that plant to plant interactions may be more important and frequent belowground than aboveground. Price et al. (2012), however, suggested that such interactions (*e.g.* competition) are possibly more important in the aboveground, while abiotic factors, such as soil heterogeneity, would affect more directly species' patterns belowground. In this sense, it has been hypothesised that the mechanisms underlying patterns of richness and composition aboveground and belowground may act at different spatial scales (Pärtel et al. 2012). If true, this would suggest that the observed similarity (or dissimilarity) in species patterns between both compartments could vary along both horizontal and vertical spatial scales.

Previous studies exploring similarity between both compartments performed their comparisons at the same spatial scale (*i.e.* using the same sampling grain, Hiiesalu et al. 2012; Träger et al. 2019) or, alternatively, used different scales in the aboveground and belowground (Frank et al. 2010; Kesanakurti et al. 2011; Li et al. 2017), without any assessment of whether it was the most appropriate scale for comparison. Therefore, it cannot be excluded that the dissimilarities observed between the two compartments are simply a consequence of the sampling grain used. Furthermore, several studies (*e.g.* Hiiesalu et al. 2012; Träger et al. 2019) only sampled the most superficial soil layer (up to 10 cm), standing on the fact that the greater portion of root biomass is usually found in the most superficial part of the soil (Kesanakurti et al. 2011). However, sampling only the top layer, particularly on habitats characterised by deep root systems (see Schenk and Jackson 2002) could limit our understanding of how belowground and aboveground communities are structured. Accordingly, to assess whether a robust characterization of the entire plant community may be done using only the information of the aboveground compartment (or alternatively, the belowground), it is critical to first determine whether these communities differ. In this sense, firstly identifying the spatial scales and soil depths that maximise the similarity between the two compartments could be crucial.

In this study, we compared species richness and composition in the aboveground and belowground compartments of a rich Mediterranean shrubland, considering different aboveground sampling grains and soil depths. We conducted a spatially-explicit approach on a fully mapped Mediterranean dwarf shrubland in combination with DNA metabarcoding of the root fraction, to provide a high resolution of both aboveground and belowground compartments. Fully mapping the aboveground community allowed the subsequent application of different aboveground sampling grains to identify the scale at which the similarity with the belowground compartment is maximised. Because soil heterogeneity may differentially affect plant distribution both aboveground and belowground,

we also evaluated how soil composition affects diversity in both compartments. Specifically, we ask: (1) Do species richness, composition and distribution differ between the aboveground and belowground compartments of the plant community? (2) Which is the aboveground sampling grain maximising similarity between the aboveground and belowground compartments? And, finally (3), how does soil heterogeneity affect the two plant compartments?

Methods

Study area and sampling design

This study was conducted in a species-rich Mediterranean shrubland located in the south-easternmost part of Madrid province (Spain) (40°17'17.5" N 3°12'19.4" W, 760 m asl). The plant community is dominated by chamaephytes and hemicritophytes (mostly < 50 cm in maximum height), and it occurs in calcareous soils with a variable content of gypsum. This creates a patchy environment with many species, varying from gypsophiles, such as *Helianthemum squamatum* (L.) Dum. Cours, *Thymus lacaitae* Pau, *Centaurea hyssopifolia* Vahl, *Arenaria cavanillesiana* (Font Quer & Rivas Goday) Nieto Fel. and *Ononis tridentata* L., to gypsovags and calciphyllous plants (both on and off gypsum soils) as *Bupleurum fruticescens* L., *Thymus vulgaris* L., *Linum suffruticosum* L., *Helianthemum cinereum* Pers., *Stipa pennata* L., *Salvia lavandulifolia* Vahl and *Lithodora fruticosa* (L.) Griseb (Escudero et al. 2015).

In May 2016, we established an 8 × 8 m (64 m^2) plot (10% of slope) in a representative and well conserved area (Fig. 1), *i.e.* without recent evidences of human impact, and all the aboveground perennial individuals were mapped (at their centroid or rooting point) with centimetric resolution using a Leica Viva GS15 system (Leica, Wetzlar, Germany) (see *e.g.* Chacón-Labella et al. 2017). We also measured their major perpendicular diameters (length and width) and the maximum height of each plant (excluding the reproductive shoots). The crown of each individual was represented by a circle with diameter equal to the average of its major diameters. In addition, 64 sampling points were located on the nodes of a regular 1 × 1 m grid. In order to incorporate a finer spatial scale, 30 additional points were sampled in a similar 1 × 1 m grid offset 0.5 m from the first grid (see Fig. 1). To account for different aboveground neighbourhood scales, we sampled the aboveground community with circles centred in the location of each sampling point considering eight different sampling grains, *i.e.* with radii varying from 5 to 50 cm. For each point, we recorded all aboveground plants (thereafter converted to presence-absence data) whose crown was included within or intersected with the sampling circle (see Fig. 1). We sampled the belowground plant community in the same plot during the first two weeks of June 2016, just after the aboveground sampling was finished. We collected 94 soil cores, each in every sampling point (5 cm of diameter, 30 cm of

depth; root cores hereafter). Specifically, each root core was separated in two subsamples: the superficial fraction, between 0 and 10 cm, and the deeper fraction ranging from 10 to 30 cm, which is reported to include at least 50% of the total root biomass in most environments including Mediterranean shrublands (Schenk and Jackson 2002b), rendering a total of 188 root samples. In addition, to account for soil heterogeneity in the plot, in September 2016 we collected 84 soil cores reaching a depth of 10 cm, which were located adjacent to the root cores (see Fig. 1).

Root cores processing and soil properties analysis

Upon collection in the field, root samples were placed in a cooler, maintained at 4ºC and processed within 48 hours since collection, to avoid DNA degradation. We carefully washed all roots contained in each root sample, filtering them with a 1 mm mesh sieve. Then, we centrifuged roots at 3000 rpm for 30 seconds to remove excess water and weighed them to obtain fresh root biomass per root sample. Then, we thoroughly mixed the root fragments in each sample. From each sample, we took a subsample of 100 mg, snap-froze it with liquid nitrogen and stored it at -80ºC until DNA extraction.

Even though biochemical properties could be potentially altered on air-dried soil samples, Zornoza et al. (2009) showed that biochemical properties from Mediterranean semi-arid soils are stable in the medium-term in stored air-dried soil samples. Therefore, for practical reasons, the soil cores were air-dried for four weeks and stored for subsequent analysis. Then, they were sieved (2 mm mesh size) to determine both physical and chemical soil properties of the finest fraction. Texture was estimated following the Kettler et al. (2001) method. Electrical conductivity and pH were measured in deionised water, in a proportion of 1:2.5 and 1:5 (mass/volume), respectively, by using a conductivity meter GLP 31 and a pH meter GLP 21 (Crison, Barcelona, Spain). Soil organic C (SOC) was estimated by a wet oxidation procedure according to Yeomans and Bremner (1988). Total N and extractable P were estimated by Kjeldahl digestion (Anderson and Ingram 1993), while total K was determined applying Radojević and Bashkin (1999) methodology. Moreover, we quantified key soil enzymatic activities as an estimation of the current microbiome soil dynamics, which are relevant for soil quality assessment and functioning (Adetunji et al. 2017), by applying the techniques described by Eivazi and Tabatabai (1988) and Tabatabai and Bremner (1969), for the measurement of β-glucosidase activity and acid phosphatase, respectively.

Root identification through DNA metabarcoding

To identify all the plant species in each root sample, we used DNA metabarcoding using the *rbcL* gene as barcode (see Matesanz et al. 2019). We built a complete in-house reference library for the

identification of species in the study plant community, considering at least 95% of the perennial species rooting in the sampled plot. In addition, to account for either the occurrence of perennial organs belowground or roots from plants with their aerial part outside the plot, we also included other species which were not present in the plot but occurred in the surroundings. The final database contained the *rbcL* reference sequences of 45 species. A detailed description of the metabarcoding pipeline is provided in Methods S1 (Online Resource 1). Briefly, as a first step, DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, CA, USA) in the lab at Universidad Rey Juan Carlos, including negative controls for each extraction batch. Afterwards, DNA extractions were processed in the AllGenetics laboratories (AllGenetics & Biology SL, A Coruña, Spain). We amplified a fragment of the *rbcL* chloroplast gene using primers rbcLa-F (5' ATG TCA CCA CAA ACA GAG ACT AAA GC 3'; Levin et al. 2003) and rbcLa-R (5' GTA AA ATC AAG TCC ACC RCG 3'; Kress et al. 2009). A first PCR was performed to amplify the selected fragment of the *rbcL* chloroplast gene. A second PCR was required to attach the Illumina index sequences for multiplexing distinct libraries in the same sequencing pool. Four negative controls that contained no DNA were included to check for contamination during library preparation. The pool was sequenced in a run of the MiSeq PE300 (Illumina). Then, samples were demultiplexed, removing indexes and sequencing primers. Sequences were then dereplicated, clustered at a similarity threshold of 100% and sorted. The taxonomical assignment was performed by querying the clustered sequences against the in-house reference library in VSEARCH (usearch global option) with a 99% similarity threshold. The output was a table listing the number of sequences from each OTU found in each sample. We removed the OTUs with a number of sequences lower than 0.005% of the total number of sequences (Bokulich et al. 2013) to apply a quality filtering. Finally, we removed those OTUs that did not match any reference sequence in the database at a similarity of 99% and remained unidentified ('No hit'). These OTUs accounted for an average of 9.4% of the total reads before filtering. We corroborated by blasting them in GenBank that at least the 70% of them corresponded to bryophytes or *Thymus* sp. sequences of lower quality. Finally, we converted the OTUs abundance table into a species presence-absence table for subsequent analysis.

Statistical analysis

Similarities between the two plant community compartments were quantified as differences in terms of species richness and composition. In each sampling point (*i.e.* around each root core), we calculated the richness and composition dissimilarities (ΔR and *J* hereafter, respectively) between the aboveground and belowground compartments, for each horizontal (aboveground sampling grain) or vertical (soil depth) scale considered in the study. ΔR was calculated as the absolute

difference between aboveground and belowground richness (hereafter AR and BR, respectively), *i.e.* $\Delta R = |AR - BR|$. Species composition similarity between aboveground and belowground compartments was calculated the with the Jaccard dissimilarity index as:

$$J = (b + c) / (a + b + c),$$

where *a* is the number of species present in both compartments, *b,* the number of species present only aboveground and *c,* the number of species present only belowground. To assess for significant differences in Jaccard (J index) and richness differences (ΔR) between aboveground grains, for the three possible depths (0-10, 10-30 and 0-30 cm), we performed Tukey tests, when the variables had a normal distribution, and Dunn's tests when this was not feasible.

Once identifying the aboveground sampling grain with maximum similarity, we carried out the comparison between aboveground and belowground compartments considering this spatial scale. We performed a Mann-Whitney-Wilcoxon test to evaluate differences between aboveground richness and belowground richness estimated at different depths: i) 0-10 cm ($BR_{0\text{-}10}$), ii) 10-30 cm ($BR_{10\text{-}30}$) and iii) 0-30 cm ($BR_{0\text{-}30}$). We tested the correlation between aboveground and belowground richness, *i.e.* $BR_{0\text{-}10}$, $BR_{10\text{-}30}$ and $BR_{0\text{-}30}$, using Kendall rank correlation coefficients (Kendall 1976). Differences in species composition between the aboveground and belowground compartments were also evaluated at the three different depths, 0-10, 10-30 and 0-30 cm. For this, we carried out a PERMANOVA analysis (Anderson 2001). Moreover, we assessed the number of species shared between the two compartments (shared richness), those appearing only aboveground (additional aboveground richness), and, finally, the species found only belowground (additional belowground richness), considering all three different depths. For this estimation, we also considered the 5 cm radius grain and the 0-10 cm layer belowground, as it is the most commonly used in previous studies (*e.g.* Hiiesalu et al. 2012; Träger et al. 2019).

To assess the existence of a spatial concordance of individual species between the aboveground and belowground compartments, we implemented two complementary tests. First, for each species, we quantified its frequency in the 94 aboveground and belowground samples, for all the soil layers (0-10, 10-30 and 0-30 cm) and tested for differences in species' frequencies with Pearson's *Chi*-squared tests. Second, we explored spatial correspondence for each species between aboveground and belowground (again for the 0-10, 10-30 and 0-30 cm layers) with the McNemar's *Chi*-squared test (Agresti 1990). We controlled the false discovery rate for multiple testing using the approach of Benjamini and Hochberg (1995).

Finally, we analysed the effect of soil heterogeneity on aboveground and belowground richness. Since the effect of soil heterogeneity could change with the sampling scale, we again chose two different aboveground sampling grains: i) 5 cm, the most similar scale to that belowground, ii) and

20 cm radius circles, the scale where we observed the maximum similarity between the two compartments (see Results), and all different depths (0-10, 10-30 and 0-30 cm). After checking for collinearity, the soil variables considered were sand (%), C, N, K contents, glucosidase, phosphatase, conductivity and pH. First, we fitted Poisson GLMs, then we tested models residuals for spatial autocorrelation with Moran's tests and applied a simulation-based approach for other residual diagnostics (Hartig 2020). In the case of a significant spatial autocorrelation, we included a distance-weighted autocovariate into the model (F. Dormann et al. 2007). As in most cases (with the exception of AR at the 5 cm scale) we found a significant under-dispersion in the GLM, we fitted a VGLM following Hilbe (2014).

All statistical analyses were conducted in R (R Core Team 2020). Wilcoxon tests, correlation analysis, Pearson's and McNemar's *Chi*-squared tests, false discovery rate correction and GLMs were respectively performed with functions *wilcox.test*, *cor.test, chisq.test*, *mcnemar.test, p.adjust* and *glm* of the stats package (R Core Team 2020). Jaccard dissimilarity and PERMANOVA analysis were performed, respectively, with functions *vegdist* and *adonis* in the *vegan* package (Oksanen et al. 2019). Moran's tests and distance-weighted autocovariates were computed, respectively, with functions *moran.test and autocov_dist* in the *spdep* package (Bivand and Wong 2018). Residual diagnostics were computed with *simulateResiduals* and *testDispersion* functions in the DHARMa package (Hartig 2020). VGLMs were fitted with the *vglm* function in the *VGAM* package (Yee 2010).

Results

We mapped a total of 8551 perennial individuals aboveground, belonging to 45 species. In the belowground compartment, we retrieved a total of 1701120 sequence reads and assigned taxonomically 90.6% of them to species in our reference database. We identified a total of 30 taxa belowground, 26 at the species level and four at the genus level (*Thymus* sp., *Stipa* sp., *Teucrium* sp. and *Quercus* sp.), which were represented by two different species in the aboveground. The species that were mapped aboveground but not detected belowground had, in all cases, very low abundances, accounting together for 3.94% of the total number of individuals in the aboveground community (see Table S1, Online Resource 2).

Aboveground and belowground diversity across different spatial scales

The total number of species characterizing the aboveground compartment of the plant community ranged from 22 to 41, respectively, for the 5 and 50 cm sampling grains. Average aboveground and belowground richness varied consistently with the aboveground grain and depth (see Fig. S1, Online Resource 3). The average aboveground richness ranged from 1.61 ± 1.59 (mean ± SD) to

16.37 ± 3.49 species per sample, from the 5 to the 50 cm sampling grains, respectively. Average belowground richness was similar in the 0-10 (BR_{0-10}) and 10-30 cm (BR_{10-30}) layers, with 6.02 (± 1.82) and 6.05 (± 2.14) species per sample respectively, while BR in the complete 0-30 cm (BR_{0-30}) core was higher, with 7.90 (± 2.08) species per root core.

Richness and composition dissimilarities (ΔR and Jaccard index, *J*, respectively) between the above- and belowground (0-30 cm) compartments (see Fig. 2) changed across aboveground sampling grains but followed a similar pattern. They both were higher at the smaller and larger sampling grains (*i.e.* ΔR = 6.29 and *J* = 0.84 at 5 cm scale; ΔR = 8.53 and *J* = 0.64 at 50 cm scale) while reaching a minimum at 20 cm radius (ΔR = 2.15 and *J* = 0.53). When we separately accounted for the 0-10 and 10-30 belowground depths (see Fig. 2), results were similar, and the 20 cm radius had again the highest match. Even though there were not significant differences between the 15, 20 and 25 cm aboveground grains for any of the soil depths considered (see *P*-values of Tukey and Dunn's tests in Tables S3 and S4 in Online Resource 2), we selected the 20 cm radius grain for subsequent analyses, as it was the grain where the mean similarity was maximised. At the 20 cm radius aboveground and 10 cm of depth, 42.50% of species were found in both compartments, while 38.94% of them were found only in the aboveground and 18.55% in the belowground (Fig. 3). Similar results were obtained for the 10-30 cm layer and the complete 0-30 cm layer (Fig. 3), but species composition similarity (*i.e.* shared species) reached a maximum (47.04%) when considering the 0-30 cm layer, while it was minimised in the 10-30 (38.09%) cm layer. At the 5 cm grain, the shared species between aboveground and belowground (0-10 cm of depth) were only 18.35% (see Fig. 3), while most of the species were found only belowground, *i.e.* additional belowground richness, (75.54%).

Similarity between aboveground and belowground compartments

Aboveground richness at the sampling grain with the largest similarity (*i.e.* 20 cm, AR_{20}, hereafter) did not differ significantly from BR_{0-30} (Mann-Whitney-Wilcoxon's test $P = 0.21$). However, when comparing AR_{20} with both BR_{0-10} and BR_{10-30}, we found significant differences (Mann-Whitney-Wilcoxon's test $P < 0.0001$ in both cases). In parallel, Kendall tests (Fig. S2, Online Resource 3) showed that AR_{20} was significantly correlated with both BR_{0-10} and BR_{0-30} (BR_{0-10} $R = 0.32$, $P = 0.0008$; BR_{0-30} $R = 0.25$, $P = 0.007$), while BR_{10-30} was not correlated neither with AR_{20} ($P = 0.18$) nor BR_{0-10} ($P = 0.32$). In addition, results from PERMANOVA showed that species composition differed significantly between the aboveground (20 cm grain) and all three root depths ($F = 23.07$, 18.87 and 24.02, for 0-10, 10-30 and 0-30 respectively; $P < 0.001$ in all cases).

Species frequencies, *i.e.* the number of occurrences, were significantly different (Pearson's *Chi*-squared tests, $P < 0.0001$) between above- and belowground (0-10, 10-30 and 0-30 layers). However, these results were mostly driven by a few species (Fig. S3, Online Resource 3), such as *Lithodora fruticosa* and *Quercus* sp., which were significantly more frequent in the belowground, or *Koeleria vallesiana*, which instead, was more frequent in the aboveground. Indeed, most of the species had similar frequencies in both compartments (see Table S1, Online Resource 2). Spatial tests for each species (McNemar's *Chi*-squared tests) showed differences in the spatial distribution between aboveground and belowground for the 26.67% and 33.33% of species, respectively, considering the 0-30 cm layer and both the 0-10 and 10-30 cm layers (see Table S2, Online Resource 2). The species with a different distribution, regardless of the soil layer considered, were *Helianthemum cinereum*, *Arenaria cavanillesiana*, *Koeleria vallesiana*, *Sideritis incana* and *Quercus* sp.

Effects of soil heterogeneity on aboveground and belowground richness

We found only marginal effects of the soil heterogeneity on richness. Aboveground richness was significantly affected by the phosphatase activity at the two aboveground grains selected for the analyses (at 5 and 20 cm, $P = 0.047$ and $P = 0.017$, respectively) (Table 1). On the other hand, belowground richness was affected differently according to the soil layer considered. In the case of $BR_{0\text{-}30}$, the organic carbon content was the only significant and positive predictor. However, when we separately considered the two belowground layers (0-10 and 10-30), results were different: $BR_{0\text{-}10}$ was affected by carbon content and phosphatase, whereas $BR_{10\text{-}30}$ was significantly affected only by the potassium content.

Discussion

In semi-arid environments, such as Mediterranean shrublands characterised by higher biomass root allocation (Schenk and Jackson 2002) and sparse distribution of aboveground vegetation (Martens et al. 1997), we expected aboveground and belowground diversity patterns to be different. Indeed, our findings showed important diversity discrepancies, in terms of species richness, composition and spatial distribution, between the aboveground and belowground compartments. To understand how plant diversity is structured within aboveground and belowground fractions, and whether the aboveground robustly informs on the whole community diversity, pioneer studies (*e.g.* Hiiesalu et al. 2012) considered equivalent and unique scales of comparison, usually sampling units of 10 × 10 centimetres, both above- and belowground. Their results, specifically those related to the lack of congruence in the corresponding species-area curves (Hiiesalu et al. 2012), induced other authors (*e.g.* Pärtel et al. 2012) to suggest that different spatial scales of comparison should be considered.

Some studies adopted different aboveground sampling grains (*e.g.* Kesanakurti et al. 2011), but did not justify what scale was the most appropriate to compare both plant compartments. Given that the drivers of community structure are likely different above- and belowground, a previous and necessary step for an accurate description of a plant community should be identifying the scale at which the similarity between both components is maximised. Indeed, our study shows that the neighbourhood scale adopted to sample the aboveground community strongly affects the similarity between the aboveground and belowground compartments (Fig. 2), which represent different facets of the same plant community. Importantly, this result conditions the ability to robustly answer whether the aboveground can be a good surrogate of the whole plant community composition and structure.

In our community, the highest similarity in species richness and composition between aboveground and belowground compartments was registered with a 20 cm radius grain in the aboveground (and belowground root cores of 2.5 cm radius). It is also noteworthy that this scale was not affected by the different sampling depths. It is likely that the spatial scale at which the similarity reaches a maximum would vary with the plant community considered, as it may vary with the lateral spread of different species, which in turn depends on both their growth form and climatic conditions (*e.g.* Schenk and Jackson 2002). In our study case, this sampling grain roughly matched the maximum height of most individuals, which might be pointing to an allometric relationship between maximum plant height and the lateral root spread in these species. Interestingly, in a mesophytic grassland the best match between the aboveground and belowground richness was obtained when considering an aboveground cumulative sampling area three times larger than belowground (Hiiesalu et al. 2012; Pärtel et al. 2012). In other words, in another plant community, where dominating species are shorter than in our shrubland, maximum richness similarity was also encountered at a smaller aboveground scale. This hypothesis, however, needs further research and testing in other plant communities to be confirmed.

The comparison of the aboveground and belowground richness showed contrasting results according to sampling depth. We detected a high and positive correlation between richness of the two compartments when considering both the 0-10 and the 0-30 cm soil layers. This correlation was stronger in case of the 0-10 cm layer only, while it was not significant for the 10-30 cm layer. This result concurs with Li et al. (2017), who reported that aboveground richness was more correlated with richness in the most superficial soil layer (first 5 cm) than in the deepest one (≈10-15 cm of depth). When comparing average above- and belowground richness values, our results also varied with the soil layer. Above- and belowground richness were statistically similar only when the entire sampled soil profile (0-30 cm of depth) was considered, while a significant difference

emerged when considering the shallow or the deeper layer in the soil separately. These results suggest that, in environments dominated by a greater root allocation, we cannot reduce our consideration of the plant diversity only to the shallowest part of the soil (10 cm), as plant community dynamics invest even deeper layers and possibly vary consistently with soil depth. This idea is supported by the fact that, even though there was an obvious decrease in root biomass (Fig. S4, Online Resource 3), we did not observe any reduction in species richness with depth. Results of the composition similarity analysis did not differ considerably with the soil layer. Indeed, our results evidenced significant differences in species composition between aboveground and belowground compartments, regardless of the soil layer considered. However, the strength of dissimilarities changed according with the layer considered, due to the fact that the 0-10 cm and 10-30 cm layers had a consistent portion of unshared species (a mean value of 45.44%), showing a certain species turnover between the shallow and the deeper (10-30 cm) layers. Altogether, our results show that, even at the scale of comparison in which the maximum similarity is reached, the aboveground and belowground communities present significant dissimilarities in richness and composition.

This discrepancy may be due to different factors, including different species' frequencies and/or a different spatial distribution in the two compartments. Our results showed a generalised concordance in species frequencies between aboveground and belowground, with only a very few exceptions, such as, for instance, *Quercus* sp. This tree species was by far much more common in the belowground than in the aboveground compartment in our fully-mapped plot. It is worth noting that this tree is almost absent in the plot, with only a few seedlings, but it is relatively common in the vicinity, which informs on the strong ability of this species to spread its roots far beyond their canopies. The generalised symmetry in species frequencies observed for the majority of the species contrasts with other studies reporting clear asymmetries between the aboveground and belowground in grasslands (*e.g.* Kesanakurti et al. 2011; Hiiesalu et al. 2012). Interestingly, tests carried out to compare the distribution of individual species in the two compartments at the sampling point level evidenced that an important portion (ranging from 26.67% to 33.33%, according to the soil layer) of species presented a significantly different spatial distribution between the aboveground and belowground. In other words, our results indicate that although many of the species in our plant community have similar frequencies above- and belowground, several of them are differentially distributed in space in both compartments. This suggests that most of the composition dissimilarities observed are caused by species differentially prospecting the two compartments. This may be also the reason why we identified a significant amount (more than

50%), of unshared species per sampling point (circle/core) between the two compartments, for all the belowground layers.

A significant fraction of the diversity in each sampling point was only present in the belowground (*i.e.* additional belowground richness, see Fig. 3), regardless of the aboveground sampling grain considered. The detection of certain species only in the belowground is in agreement with previous studies (*e.g.* Hiiesalu et al. 2012; Träger et al. 2019), and reinforces the idea that the soil contains a very relevant fraction of the total diversity (*i.e.* hidden diversity; Pärtel 2014) that is systematically ignored when sampling is only conducted aboveground. The additional aboveground richness was also a relevant fraction of diversity (Fig. 3), suggesting that neither the aboveground nor the belowground community include all species present at small spatial scales. This could be related to the fact that not all the species can be easily detected in the belowground, contrarily to those in the aboveground, as molecular techniques still have some limitations (see *e.g.* Hiiesalu et al. 2012).

Richness variation in the two compartments showed contrasting responses to soil heterogeneity even at the fine scale of our study. This result differed from Kesanakurti et al. (2011) who observed that soil heterogeneity was able to structure species diversity only in the belowground, but not in the aboveground. In our case, although the number of significant predictors was low, phosphatase activity in the soil, a surrogate of microbial activity (Nannipieri et al. 2011), explained a small fraction of the aboveground richness, at both 5 and 20 cm radius scale, while the organic carbon content affected the root diversity at 0-30 cm of depth. In the case of the belowground richness at the shallow layer, 0-10 cm, the response was similar to the aboveground richness (*i.e.* both were positively affected by the phosphatase activity). Our findings disagree with those of Hiiesalu et al. (2012) who analysed richness variation at a local scale, very different from our very fine spatial scale, in a 2-ha diverse mesophytic grassland, and pointed out that both aboveground and belowground richness responded to the nitrogen content in the soil, but in different ways. Moreover, richness in the deepest layer, 10-30 cm, was positively and exclusively related to the level of potassium in the soil. The fact that richness was differently affected by soil heterogeneity with the layer considered suggests that the dynamics regulating belowground diversity patterns vary with depth. The results of our analysis, including the aboveground *vs.* belowground comparison as well as the richness variation with soil heterogeneity, shed light on another important issue, the huge complexity of belowground plant communities.

Our results challenge the current views of plant community assembly in Mediterranean arid and semi-arid shrubby vegetation. Indeed, the fact that we observed that species are strongly intermingled in very small spaces in the soil questions several hypotheses. First, species territoriality, *i.e.* defending soil spaces to avoid competitors to achieve resources, has been hypothesised to

represent a possible strategy to avoid competition for water and nutrients in the soil, mainly in environments where these are limited (Schenk et al. 1999). Species segregation along soil depth is also missing, contrary to what we could expect according to the niche differentiation theory, as we observed similar species frequencies in the two different soil layers (see Fig. S3 in Online Resource 3). However, we cannot rule out the possibility that, in our study, environment soil segregation with depth occurs at higher depths. The high belowground species richness detected also contrasts the idea that the sparse distribution of the aboveground vegetation in environments where light is abundant and soil resources (including water) are scarce responds to belowground competition (Cipriotti and Aguiar 2015; Deng et al. 2006; Martens et al. 1997). Contrary to this view, the coexistence of a high number of species in very small pockets in the soil (*i.e.* within 5 cm soil cores) suggests that belowground competition does not determine the plant diversity patterns observed in the aboveground, while competition for aboveground resources (*e.g.* light) could actually have a more important contribution. This idea could be further supported by studies (*e.g.* Price et al. 2012) showing that aboveground competition is an important driver of community assembly at small spatial scales, while abiotic processes could be more important in the belowground. Our results, however, do not completely support this hypothesis, since we found only a small effect of soil heterogeneity on species richness distribution both aboveground and belowground.

Conclusions

Identifying the spatial scale at which the similarity between aboveground and belowground compartments reaches a maximum is critical not only to understand if they differ or not, but also to properly assess the processes determining the diversity patterns of the plant community as a whole. In this work, we show the importance for sampling a larger aboveground scale than in the belowground to maximise the similarity in species richness and composition between these two compartments. However, our findings show that, even at the scale of maximum similarity, there are relevant discrepancies between above- and belowground richness (except when a complete profile of 0-30 cm of depth was considered) and composition (for any of the three soil layers). This result confirms that, although the above- and belowground compartments are clearly related, the processes operating in each compartment differ, limiting their reciprocity and their ability to characterise the plant community individually. This is further reinforced by the fact that soil heterogeneity exerts a different effect on richness patterns in the two compartments. We show that to identify the scales maximising the similarity between aboveground and belowground compartments is a necessary step to obtain a complete perspective of the diversity structure in a plant community. This is critical to infer the mechanisms controlling plant coexistence in natural communities, which represent one of the most important challenges of plant community ecology.

Table 1 Differential effects of soil heterogeneity on aboveground and belowground richness: Estimates (Est.) and *P*-values from GLMs and VGLMs for aboveground richness at 5 and 20 cm sampling grains (AR_5 and AR_{20}) and belowground richness at different depths ($BR_{0\text{-}30}$, $BR_{0\text{-}10}$, $BR_{10\text{-}30}$, 0-30 cm, 0-10 cm and 10-30 cm, respectively).

	AR_{20}		AR_5		$BR_{0\text{-}30}$		$BR_{0\text{-}10}$		$BR_{10\text{-}30}$	
	Est.	***P*-values**	**Est.**	***P*-values**	**Est.**	***P*-values**	**Est.**	***P*-values**	**Est.**	***P*-values**
(Intercept):1	-0.65	**0.003**	0.51	**0**	-0.76	**0.001**	-1.07	**0**	-0.32	0.078
(Intercept):2	2.39	**< 2e-16**	-		2.36	**< 2e-16**	2.19	**< 2e-16**	1.93	**<2e-16**
Sand	-0.06	0.06	-0.15	0.105	-0.05	0.125	-0.04	0.184	-0.07	0.06
C	-0.03	0.479	-0.07	0.548	-0.09	**0.019**	-0.11	**0.007**	-0.05	0.315
N	0.02	0.543	0	0.998	0.04	0.193	0	0.937	0	0.992
K	-0.01	0.872	-0.04	0.654	0.03	0.299	-0.06	0.09	0.09	**0.015**
Glucosidase	0.03	0.391	0.02	0.88	0.04	0.257	0.03	0.502	0.09	0.074
Phosphatase	0.09	**0.017**	0.23	**0.047**	0.06	0.144	0.13	**0.001**	-0.02	0.655
Conductivity	-0.06	0.194	0.1	0.328	-0.05	0.203	0.02	0.584	-0.05	0.298
pH	-0.04	0.362	0.04	0.741	0.01	0.797	0.05	0.225	-0.02	0.746
Autocovariate	0.05	**0**	0.06	0.069	**-**	**-**	**-**	**-**	**-**	**-**

Figures

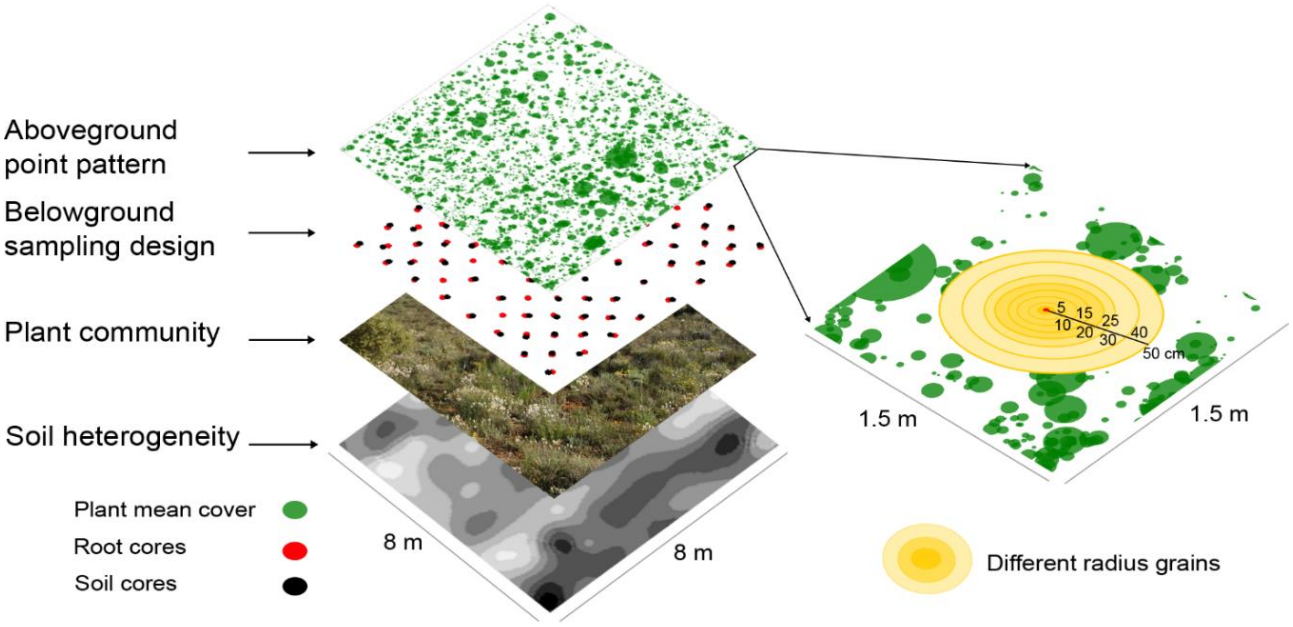

Fig. 1 Sampling design: From bottom to top, layers represent the soil heterogeneity, the sampled plant community (64 m^2), the grid of root and soil cores and the point pattern of the aboveground plant community (each point represents an individual, with size proportional to plant mean cover). In the right, a zoom of the aboveground point pattern is representing different radius circles, corresponding to different aboveground sampling grains, departing from the centre of a root core.

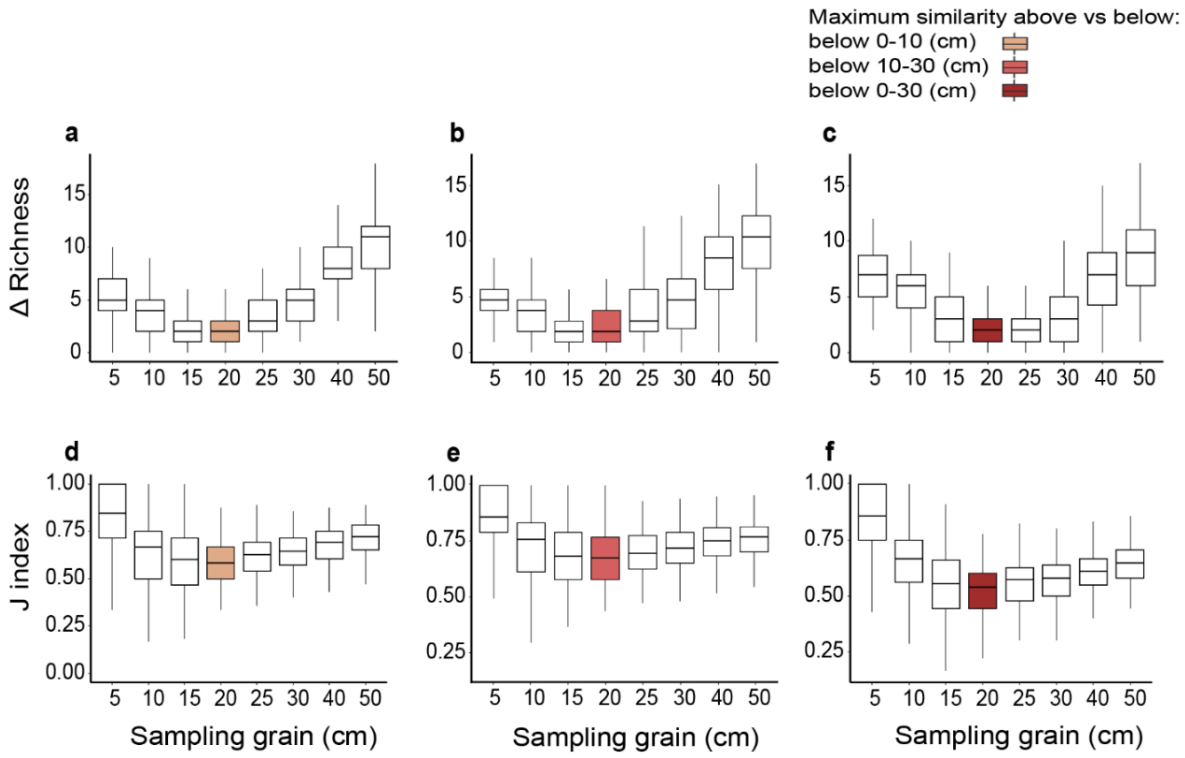

Fig. 2 Similarity between aboveground and belowground species richness and composition: a and **d** boxplots represent the median and the 1st and 3rd quartiles of both richness differences (ΔR) and Jaccard dissimilarity index (J), respectively, considering different aboveground sampling grains (circles with different radius size) aboveground vs the 0-10 cm depth layer belowground. **b** and **e** represent the same indices calculated with the belowground community at 10-30 cm, while **c** and **f** at 0-30 cm.

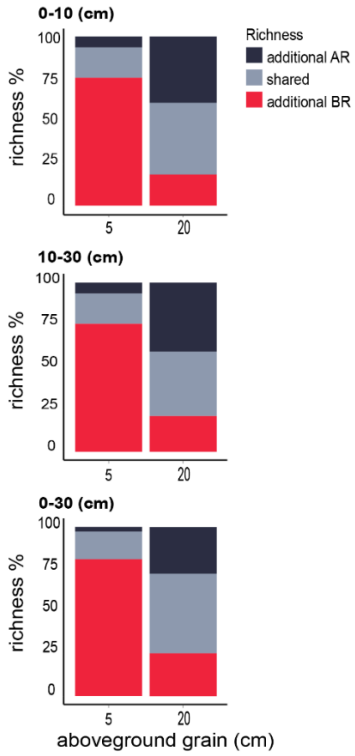

Fig. 3 Species composition similarities between aboveground and belowground: bar plot showing the species shared between the two compartments, the species found only aboveground (additional AR) and only belowground (additional BR), for the 5 and 20 cm sampling grains and the 0-10 , 10-30 and 0-30 cm of depth layers belowground.

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References

Adetunji AT, Lewu FB, Mulidzi R, Ncube B (2017) The biological activities of β-glucosidase, phosphatase and urease as soil quality indicators: A review. J. Soil Sci. Plant Nutr. 17:794–807

Agresti A (1990) Categorical Data Analysis. Wiley, New York.

Anderson JM, Ingram JSI (1993) Tropical Soil Biology and Fertility: A Handbook of Methods. J Ecol 78:547. https://doi.org/10.2307/2261129

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol 26:32–46. https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B 57:289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bivand RS, Wong DWS (2018) Comparing implementations of global and local indicators of spatial association. Test 27:716–748. https://doi.org/10.1007/s11749-018-0599-x

Bokulich NA, Subramanian S, Faith JJ, et al (2013) Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods 10:57–59. https://doi.org/10.1038/nmeth.2276

Bray JR (1963) Root Production and the Estimation of Net Productivity. Can. J. Bot. 41:65–72

Cardinale BJ, Duffy JE, Gonzalez A, et al (2012) Biodiversity loss and its impact on humanity. Nature 486:59–67

Casper BB, Jackson RB (1997) PLANT COMPETITION UNDERGROUND. Annu Rev Ecol Syst 28:545–570. https://doi.org/10.1146/annurev.ecolsys.28.1.545

Chacón-Labella J, de la Cruz M, Escudero A (2017) Evidence for a stochastic geometry of biodiversity: the effects of species abundance, richness and intraspecific clustering. J Ecol 105:382–390. https://doi.org/10.1111/1365-2745.12710

Cipriotti PA, Aguiar MR (2015) Is the balance between competition and facilitation a driver of the patch dynamics in arid vegetation mosaics? Oikos 124:139–149. https://doi.org/10.1111/oik.01758

Davidson RL (1969) Effect of Root/Leaf Temperature Differentials on Root/Shoot Ratios in Some Pasture Grasses and Clover. Ann Bot 33:561–569. https://doi.org/10.1093/oxfordjournals.aob.a084308

Deiner K, Bik HM, Mächler E, et al (2017) Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Mol Ecol 26:5872–5895. https://doi.org/10.1111/mec.14350

Deng JM, Wang GX, Morris EC, et al (2006) Plant mass-density relationship along a moisture

gradient in north-west China. J Ecol 94:953–958. https://doi.org/10.1111/j.1365-2745.2006.01141.x

Eivazi F, Tabatabai MA (1988) Glucosidases and galactosidases in soils. Soil Biol Biochem 20:601–606. https://doi.org/10.1016/0038-0717(88)90141-1

Escudero A, Palacio S, Maestre FT, Luzuriaga AL (2015) Plant life on gypsum: a review of its multiple facets. Biol Rev 90:1–18. https://doi.org/10.1111/brv.12092

F. Dormann C, M. McPherson J, B. Araújo M, et al (2007) Methods to account for spatial autocorrelation in the analysis of species distributional data: A review. Ecography (Cop.). 30:609–628

Frank DA, Pontes AW, Maine EM, et al (2010) Grassland root communities: species distributions and how they are linked to aboveground abundance. Ecology 91:3201–3209. https://doi.org/10.1890/09-1831.1

Hartig F (2020) DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package version 0.2.7. http://florianhartig.github.io/DHARMa/

Hiiesalu I, Öpik M, Metsis M, et al (2012) Plant species richness belowground: Higher richness and new patterns revealed by next-generation sequencing. Mol Ecol 21:2004–2016. https://doi.org/10.1111/j.1365-294X.2011.05390.x

Hilbe JM (2014) Modeling count data

Jones FA, Erickson DL, Bernal MA, et al (2011) The roots of diversity: Below ground species richness and rooting distributions in a tropical forest revealed by DNA barcodes and inverse modeling. PLoS One 6:. https://doi.org/10.1371/journal.pone.0024506

Kendall M (1976) Rank Auto Correlation Methods, 4th Edn., Griffin

Kesanakurti PR, Fazekas AJ, Burgess KS, et al (2011) Spatial patterns of plant diversity below-ground as revealed by DNA barcoding. Mol Ecol 20:1289–1302. https://doi.org/10.1111/j.1365-294X.2010.04989.x

Kettler TA, Doran JW, Gilbert TL (2001) to Accompany Soil-Quality Analyses. 852:849–852

Kress WJ, Erickson DL, Jones FA, et al (2009) Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. Proc Natl Acad Sci U S A 106:18621–18626. https://doi.org/10.1073/pnas.0909820106

Levin RA, Wagner WL, Hoch PC, et al (2003) Family-level relationships of Onagraceae based on chloroplast *rbc L* and *ndh F* data. Am J Bot 90:107–115. https://doi.org/10.3732/ajb.90.1.107

Li Z, Lamb EG, Piper CL, Siciliano SD (2017) Plant Belowground Diversity and Species Segregation by Depth in a Semi-Arid Grassland. Ecoscience 25:1–7. https://doi.org/10.1080/11956860.2017.1403242

Martens SN, Breshears DD, Meyer CW, Barnes FJ (1997) Scales of aboveground and below-ground competition in a semi-arid woodland detected from spatial pattern. J Veg Sci 8:655–664. https://doi.org/10.2307/3237370

Matesanz S, Pescador DS, Pías B, et al (2019) Estimating belowground plant abundance with DNA metabarcoding. Mol Ecol Resour 19:1265–1277. https://doi.org/10.1111/1755-0998.13049

Mokany K, Raison RJ, Prokushkin AS (2006) Critical analysis of root : shoot ratios in terrestrial biomes. Glob Chang Biol 12:84–96. https://doi.org/10.1111/j.1365-2486.2005.001043.x

Nannipieri P, Giagnoni L, Landi L, Renella G (2011) Phosphorus in Action. 26:215–243. https://doi.org/10.1007/978-3-642-15271-9

Oksanen J, Blanchet FG, Friendly M, et al (2019) Package "vegan" Title Community Ecology Package Version 2.5-6

Pärtel M (2014) Community ecology of absent species: Hidden and dark diversity. J Veg Sci 25:1154–1159. https://doi.org/10.1111/jvs.12169

Pärtel M, Hiiesalu I, Öpik M, Wilson SD (2012) Below-ground plant species richness: New insights from DNA-based methods. Funct Ecol 26:775–782. https://doi.org/10.1111/j.1365-2435.2012.02004.x

Price JN, Hiiesalu I, Gerhold P, Pärtel M (2012) Small-scale grassland assembly patterns differ above and below the soil surface. Ecology 93:1290–1296. https://doi.org/10.1890/11-1942.1

R Core Team (2020) R: The R Project for Statistical Computing. https://www.r-project.org/. Accessed 17 Apr 2020

Radojević M, Bashkin VN (1999) Water analysis. In: Radojevic M, Bashkin VN (eds) Practical Environmental Analysis. Royal Society of Chemistry, Cambridge, pp 138–273

Rewald B, Meinen C, Trockenbrodt M, et al (2012) Root taxa identification in plant mixtures - current techniques and future challenges. Plant Soil 359:165–182. https://doi.org/10.1007/s11104-012-1164-0

Schenk HJ, Callaway RM, Mahall BE (1999) Spatial Root Segregation: Are Plants Territorial? Adv Ecol Res 28:145–180. https://doi.org/10.1016/S0065-2504(08)60032-X

Schenk HJ, Jackson RB (2002a) Rooting depths, lateral root spreads and below-ground/above-ground allometries of plants in water-limited ecosystems. J Ecol 90:480–494. https://doi.org/10.1046/j.1365-2745.2002.00682.x

Schenk HJ, Jackson RB (2002b) The global biogeography of roots. Ecol Monogr 72:311–328. https://doi.org/10.1890/0012-9615(2002)072[0311:TGBOR]2.0.CO;2

Tabatabai MA, Bremner JM (1969) Use of p-nitrophenyl phosphate for assay of soil phosphatase

activity. Soil Biol Biochem 1:301–307. https://doi.org/10.1016/0038-0717(69)90012-1
Träger S, Öpik M, Vasar M, Wilson SD (2019) Belowground plant parts are crucial for comprehensively estimating total plant richness in herbaceous and woody habitats. Ecology 100:1–12. https://doi.org/10.1002/ecy.2575
Walter H (1963) The water relation of plants
Yee TW (2010) The VGAM package for categorical data analysis. J Stat Softw 32:1–34. https://doi.org/10.18637/jss.v032.i10
Yeomans JC, Bremner JM (1988) A rapid and precise method for routine determination of organic carbon in soil. Commun Soil Sci Plant Anal 19:1467–1476. https://doi.org/10.1080/00103628809368027
Zornoza R, Mataix-Solera J, Guerrero C, et al (2009) Storage Effects on Biochemical Properties of Air-Dried Soil Samples from Southeastern Spain. Arid L Res Manag 23:213–222. https://doi.org/10.1080/15324980903038727

Supporting information

Methods S1 Root identification through DNA metabarcoding

As a first step of the analysis, DNA extraction was carried out employing the DNeasy Plant Mini Kit (Qiagen, CA, USA) in the lab at Universidad Rey Juan Carlos, including negative controls for each extraction batch. Afterwards, DNA extractions were shipped to the AllGenetics laboratories (AllGenetics & Biology SL, A Coruña, Spain).

We amplified a fragment of the rbcL chloroplast gene (550 bp) using the primers rbcLa-F (5' ATG TCA CCA CAA ACA GAG ACT AAA GC 3'; Levin et al. 2003) and rbcLa-R (5' GTA AA ATC AAG TCC ACC RCG 3'; Kress et al. 2009). The Illumina sequencing primer sequences were attached at the 5' ends of primers. A first series of PCRs was performed to amplify the selected fragment of the rbcL chloroplast gene. It was carried out in a total volume of 25 μL, containing 2.5 μL of template DNA, 0.5 μM of the primers, 12.5 μL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water up to 25 μL. The reactions were run as follows: the mixture was incubated at 95 °C for 5 min, than it was subjected to 30 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, and finally to 72 °C for 10 minutes. A latter series of PCRs was required to attach the Illumina index sequences for multiplexing distinct libraries in the same sequencing pool. Thermocycling conditions were identical to first PCRs series but with only 5 cycles and an annealing temperature of 60°C. During library preparation, four negative controls, with no DNA, were included to the check for contamination. The obtained libraries were run on 2 % agarose gels, which were stained with GreenSafe (NZYTech), and then observed under UV light to verify their size. After that, they were purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek) and pooled in equimolar amounts. The pool was sequenced in a run of the MiSeq PE300 (Illumina). Then we proceeded with the demultiplexing step, which consists in removing indexes and sequencing primers. We carried out quality control on FASTQ files using the software FastQC and we filtered raw-reads in Geneious 11.1.2. PCR primers were eliminated and a region at the 3´ end

of each file was trimmed considering a minimum Phred score of 20. After, the R1 and R2 reads were concatenated (fuse.sh script, BBmap package, Bushnell 2014) and the sequences labelled (multiple split libraries.py) in Qiime (Caporaso et al., 2010). Labelling was crucial for the subsequent sample identification because sequences were combined later to perform downstream analysis. Next processing steps were carried out with the VSEARCH bioinformatics tool. Sequences were dereplicated, clustered at a similarity threshold of 100 %, and sorted. Furthermore, the bioinformatic pipeline included filters intended to reduce those artefacts, which normally generate during PCR and sequencing, and that can overestimate the number of OTUs. De novo chimera detection was implemented with the UCHIME algorithm (Edgar et al., 2011).

The taxonomical assignment was performed by querying the clustered sequences against the reference library in VSEARCH (usearch global option) with a 99% similarity threshold. As the query sequences mapped only to the 5´and 3´ends of the references sequences, their central region was previously removed, resulting in a final length of 517 bp. An OTU table resulted from the application of the script mesas-uc2clust.py. The new table listed the number of sequences from each OTU found in each sample. We removed the OTUs with a number of sequences lower than 0.005% of the total number of sequences (Bokulick et al., 2013) to apply a quality filtering. Moreover, the low abundance OTUs of each sample (0.1% threshold) were removed in order to contrast the phenomenon, which is normally referred to as mistagging, index jumping, tag jumping, etc. Indeed, a low percentage of the reads of a library can be misassigned to another library, during library preparation, sequencing and/or demultiplexing steps (Esling et al., 2015; Bartram et al., 2016; Guardiola et al., 2016). Finally, we removed those OTUs that did not match any reference sequence in the database at a similarity of 99% and remained unidentified ('No hit'). These OTUs accounted for an average of 9.4% of the total reads before filtering.

Table S1 Aboveground abundance in the entire 64 m² plot and aboveground (at 5 cm and 20 cm radius grains) and belowground (at three different depths) species frequencies, calculated as the sum of presence-absence values in the 94 circles/cores (i.e. values from 0 to 94).

Species	Aboveground abundance		Frequency									
	64 m2 plot		aboveground 20		aboveground 5		belowground 0-30		belowground 0-10		belowground 10-30	
	n° individuals	%	0-94	%	0-94	%	0-94	%	0-94	%	0-94	%
Stipa sp	1758	20.56	88	93.62	20	21.28	85	90.43	67	62.98	72	67.68
Linum suffruticosum	1154	13.5	69	73.4	18	19.15	63	67.02	49	46.06	48	45.12
Helianthemum cinereum	1095	12.81	76	80.85	11	11.7	56	59.57	46	43.24	38	35.72
Bupleurum fruticescens	899	10.51	55	58.51	16	17.02	55	58.51	46	43.24	48	45.12
Thymus sp	683	7.99	77	81.91	14	14.89	84	89.36	81	76.14	67	62.98
Koeleria vallesiana	545	6.37	57	60.64	8	8.51	10	10.64	6	5.64	5	4.7
Sideritis incana	343	4.01	44	46.81	5	5.32	25	26.6	19	17.86	16	15.04
Fumana ericoides	339	3.96	56	59.57	8	8.51	43	45.74	34	31.96	23	21.62
Coronilla minima	270	3.16	41	43.62	12	12.77	42	44.68	23	21.62	33	31.02
Arenaria cavanillesiana	260	3.04	26	27.66	6	6.38	13	13.83	8	7.52	7	6.58
Avenula bromoides	169	1.98	18	19.15	2	2.13	0	0	0	0	0	0
Cephalaria leucantha	169	1.98	21	22.34	3	3.19	30	31.91	24	22.56	25	23.5
Helianthemum hirtum	154	1.8	22	23.4	7	7.45	13	13.83	11	10.34	6	5.64
Teucrium sp	118	1.38	19	20.21	6	6.38	30	31.91	23	21.62	21	19.74
Matthiola fruticulosa	81	0.95	10	10.64	4	4.26	7	7.45	4	3.76	6	5.64
Thesium divaricatum	77	0.9	14	14.89	0	0	6	6.38	4	3.76	3	2.82
Asperula aristata	67	0.78	9	9.57	0	0	0	0	0	0	0	0
Hippocrepis commutata	55	0.64	8	8.51	0	0	10	10.64	5	4.7	7	6.58
Coris monspeliensis	49	0.57	6	6.38	1	1.06	0	0	0	0	0	0
Lavandula latifolia	30	0.35	6	6.38	3	3.19	16	17.02	11	10.34	12	11.28
Ononis tridentata	30	0.35	7	7.45	3	3.19	2	2.13	1	0.94	1	0.94
Quercus sp	25	0.29	4	4.26	1	1.06	90	95.74	74	69.56	82	77.08
Euphorbia nicaeensis	24	0.28	6	6.38	0	0	4	4.26	2	1.88	3	2.82
Thymelaea pubescens	19	0.22	6	6.38	2	2.13	7	7.45	3	2.82	6	5.64
Linum narbonense	18	0.21	2	2.13	0	0	2	2.13	1	0.94	2	1.88
Salvia lavandulifolia	16	0.19	1	1.06	0	0	5	5.32	3	2.82	3	2.82
Helychrisum serotinum	14	0.16	2	2.13	0	0	0	0	0	0	0	0
Leuzea conifera	14	0.16	2	2.13	0	0	0	0	0	0	0	0
Phlomis lychinitis	13	0.15	1	1.06	1	1.06	5	5.32	1	0.94	4	3.76
Eryngium campestre	12	0.14	7	7.45	1	1.06	5	5.32	0	0	5	4.7
Helianthemum syriacum	9	0.11	2	2.13	0	0	0	0	0	0	0	0
Santolina chamaecyparissus	9	0.11	2	2.13	0	0	1	1.06	1	0.94	1	0.94
Sanguisorba minor	8	0.09	2	2.13	0	0	0	0	0	0	0	0
Staehelina dubia	8	0.09	3	3.19	0	0	13	13.83	10	9.4	9	8.46
Fumana thymifolia	6	0.07	1	1.06	0	0	4	4.26	3	2.82	4	3.76
Astragalus incanus	4	0.05	0	0	0	0	0	0	0	0	0	0
Jurinea humilis	2	0.02	1	1.06	0	0	4	4.26	0	0	4	3.76
Lithodora fruticosa	2	0.02	1	1.06	0	0	13	13.83	6	5.64	8	7.52
Aristolochia paucinervis	1	0.01	0	0	0	0	0	0	0	0	0	0
Centaurea hyssopifolia	1	0.01	0	0	0	0	0	0	0	0	0	0
Sideritis hirsuta	1	0.01	1	1.06	0	0	0	0	0	0	0	0

Table S2 Adjusted P-values of McNemar Chi-squared test comparing spatial distribution of each species between aboveground and belowground. We considered 30 species, which are the species found both aboveground and belowground.

	Species	McNemar Chi-squared test adj. *P*-value Aboveground (20 cm) versus Belowground		
		0-30 cm	**0-10 cm**	**10-30 cm**
1	*Arenaria cavanillesiana*	**0.017445**	**0.000672**	**0.000414**
2	*Bupleurum fruticescens*	1	0.097711	0.21992
3	*Cephalaria leucantha*	0.242176	0.757856	0.633379
4	*Coronilla minima*	1	**0.005945**	0.343296
5	*Eryngium campestre*	0.778051	NA	0.715807
6	*Euphorbia nicaeensis*	0.825402	0.207845	0.592776
7	*Fumana ericoides*	0.144114	**0.001628**	**6.41E-05**
8	*Fumana thymifolia*	0.399899	0.614764	0.359909
9	*Helianthemum cinereum*	**0.002391**	**1.25E-05**	**2.35E-07**
10	*Helianthemum hirtum*	0.125238	**0.038929**	**0.000733**
11	*Hippocrepis commutata*	0.896483	0.614764	1
12	*Jurinea humilis*	0.512462	NA	0.512462
13	*Koeleria vallesiana*	**7.23E-10**	**9.11E-11**	**2.21E-11**
14	*Lavandula latifolia*	**0.033984**	0.207845	0.159707
15	*Linum narbonense*	NA	1	NA
16	*Linum suffruticosum*	0.402264	**0.000778**	**0.001807**
17	*Lithodora fruticosa*	**0.008678**	0.128867	0.119955
18	*Matthiola fruticulosa*	0.512462	0.088796	0.355526
19	*Ononis tridentata*	0.242176	0.088796	0.159707
20	*Phlomis lychinitis*	0.242176	1	0.359909
21	*Quercus* sp	**1.43E-18**	**4.54E-15**	**8.17E-17**
22	*Salvia lavandulifolia*	0.376439	0.719921	0.715807
23	*Santolina chamaecyparissus*	1	1	1
24	*Sideritis incana*	**0.002391**	**5.83E-05**	**2.65E-05**
25	*Staehelina dubia*	**0.033984**	0.091001	0.21992
26	*Stipa* sp	0.665662	**0.00017**	**0.004459**
27	*Teucrium* sp	0.131951	0.614764	0.907548
28	*Thesium divaricatum*	0.148308	**0.026249**	**0.028488**
29	*Thymelaea pubescens*	1	0.546874	1
30	*Thymus* sp	0.242176	0.614764	0.159707

Table S3 Adjusted *p*-values of Tukey and Nemenyi tests (i.e. only significant results are reported) carried out to compare results obtained considering different aboveground scales in the calculation of the Jaccard dissimilarity index (J index) and the richness differences (ΔR), i.e. the two measures considered to quantify dissimilarities between aboveground and belowground compartments (at three different depths).

Aboveground scale (cm)		Adj p-values J index						Adj P-values ΔR					
		Belowground depth (cm)						Belowground depth (cm)					
		0-10		10-30		0-30		0-10		10-30		0-30	
5	**vs. 10**	<0.0001	***	<0.0001	***	<0.0001	***	<0.01	*	0.01	*	0.01	*
	vs. 15	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***
	vs. 20	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***
	vs. 25	<0.0001	***	<0.0001	***	<0.0001	***	0.0001	***	<0.01	*	<0.0001	***
	vs. 30	<0.0001	***	<0.0001	***	<0.0001	***	1.00		1.00		<0.0001	***
	vs. 40	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	0.001	**	0.48	
	vs. 50	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	0.48	
10	**vs. 15**	0.36		0.18		0.0001	***	<0.01	*	0.02	·	<0.0001	***
	vs. 20	0.06		0.12		<0.0001	***	0.01	*	0.04	·	<0.0001	***
	vs. 25	0.89		0.77		<0.0001	***	1.00		1.00		<0.0001	***
	vs. 30	1.00		0.99		<0.0001	***	0.03	·	0.04	·	<0.0001	***
	vs. 40	0.57		1.00		0.31		<0.0001	***	<0.0001	***	0.42	
	vs. 50	0.03	·	0.71		0.99		<0.0001	***	<0.0001	***	<0.0001	***
15	**vs. 30**	0.51		0.71		1.00		<0.0001	***	<0.0001	***	1.00	
	vs. 40	0.001	**	0.03	·	0.28		<0.0001	***	<0.0001	***	<0.0001	***
	vs. 50	0.001	**	0.01	*	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***
20	**vs. 30**	0.11		0.58		0.43		<0.0001	***	<0.0001	***	0.20	
	vs. 40	0.001	**	0.01	*	<0.01	*	<0.0001	***	<0.0001	***	<0.0001	***
	vs. 50	0.001	**	0.001	**	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***
25	**vs. 30**	0.96		1.00		0.99		<0.01	*	0.02	·	0.19	
	vs. 40	0.03	·	0.31		0.10		<0.0001	***	<0.0001	***	<0.0001	***
	vs. 50	0.0001	***	0.03	·	0.001	**	<0.0001	***	<0.0001	***	<0.0001	***
30	**vs. 40**	0.42		0.77		0.53		<0.0001	***	<0.0001	***	<0.0001	***
	vs. 50	0.01	*	0.19		0.02	·	<0.0001	***	<0.0001	***	<0.0001	***

Table S4 Adjusted *p*-values of Tukey and Nemenyi tests comparing Jaccard dissimilarity index (J index) and richness differences (ΔR) between the 20 cm aboveground scale (i.e. the scale considered to maximise similarity between aboveground and belowground compartments) and the other aboveground scale

Aboveground scale (cm)		Adj p-values J index						Adj P-values ΔR					
		Belowground depth (cm)						Belowground depth (cm)					
		0-10		10-30		0-30		0-10		10-30		0-30	
20	**vs. 5**	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***
	vs. 10	0.06		0.12		<0.0001	***	0.01	*	0.04	·	<0.0001	***
	vs. 15	0.99		1.00		0.70		1.00		1.00		0.28	
	vs. 25	0.71		0.94		0.92		0.07		0.09		1.00	
	vs. 30	0.11		0.58		0.43		<0.0001	***	<0.0001	***	0.20	
	vs. 40	0.01	*	<0.01	*	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***
	vs. 50	0.001	**	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***	<0.0001	***

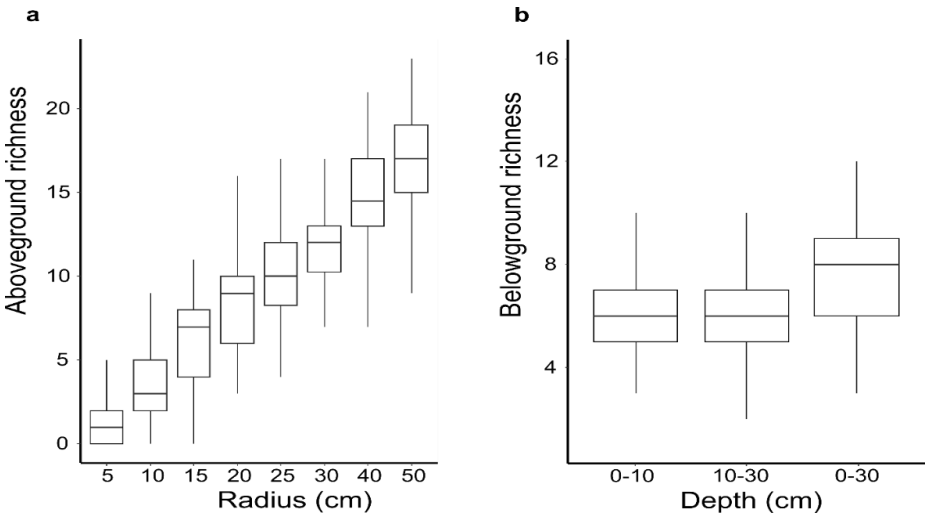

Fig. S1 Boxplots representing aboveground richness at different scales (**a**) and belowground richness at different depths (**b**).

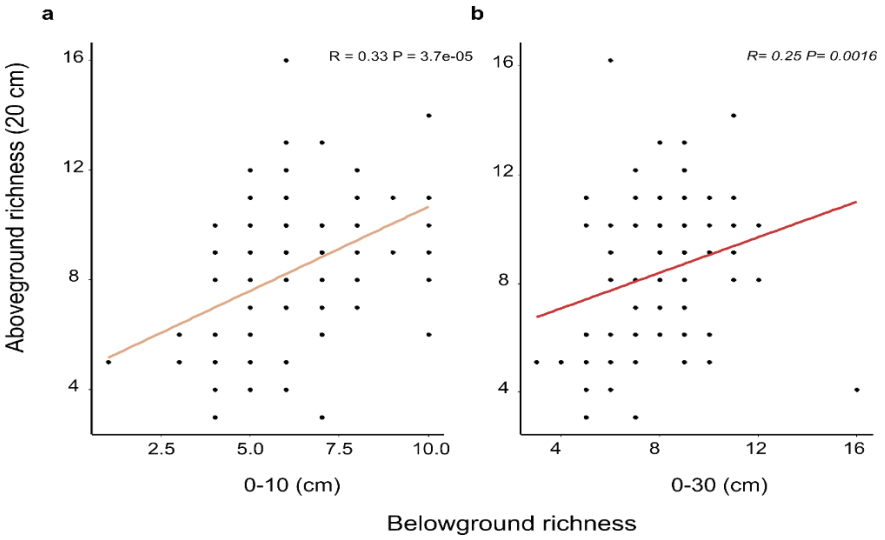

Fig. S2 Kendall correlation between aboveground richness at 20 cm of radius scale and belowground richness at different depths. **a**: belowground richness (0-10 cm); **b**: belowground richness (0-30 cm).

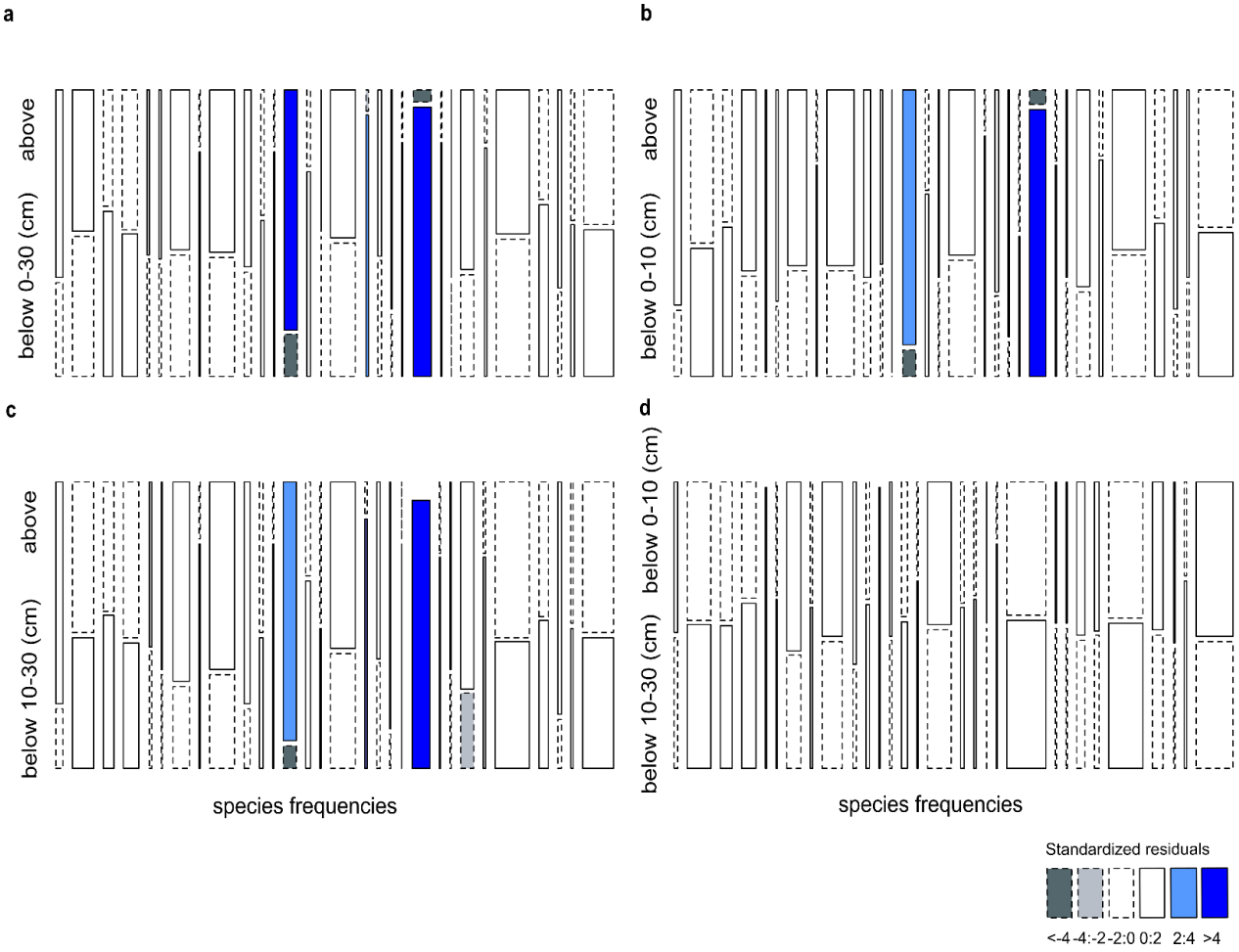

Fig. S3 Mosaic plots representing standardized residuals from the chi-squared test comparing species frequencies aboveground at 20 cm sampling grain vs belowground at different depths, 0-30, 0-10 and 10-30 cm (**a**, **b**, **c**, respectively), and belowground 0-10 vs belowground 10-30 (**d).** The size of the boxes is proportional to each species frequency. As shown by the Fig., most of the species frequencies are similar between compared layers, indeed only the coloured boxes correspond to significant differences from the chi-squared test.

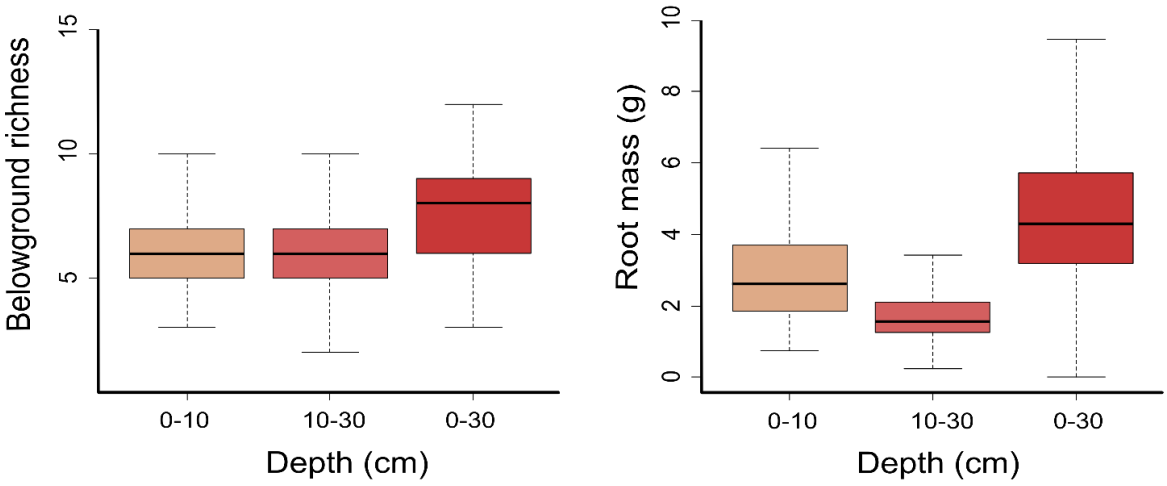

Fig. S4 Belowground richness and root biomass at three depths: 0-10, 10-30 cm and 0-30 cm.

Chapter 4

Plant-plant interactions and soil microbiome feedbacks determine high belowground segregation patterns of root nutrient-use strategies

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Summary

Competition is a key determinant of species coexistence at fine spatial scales. One mechanism to avoid competitive exclusion in realized communities is niche partitioning, which may be especially patent in the belowground compartment in stressful habitats, characterized by low water and nutrient availability. Despite the importance of belowground processes, only a few studies have assessed taxonomical diversity patterns belowground, and even fewer have considered a functional approach especially in the case of the root phenotypic variability. In the present study, we aimed to fill this gap by assessing belowground plant community assembly at the neighbourhood scale in a Mediterranean shrubland. Using a null model approach, we searched for evidence of non-random patterns of functional trait distribution in traits related to water and nutrient use, and mainly focusing on root traits. We detected clear non-random patterns showing high belowground functional segregation, especially in the root-level nutrient use strategies related to the presence and type of microbiome associations. The observed patterns can be attributable to strong plant-plant interactions, especially competition, but were also partly determined by the soil microbiota, with higher fungal richness associated to higher belowground functional diversity. Our results provide evidence of biotic deterministic processes, both plant-plant and plant-soil feedbacks, as key drivers shaping belowground plant community assembly in semiarid Mediterranean shrublands.

Introduction

Understanding plant coexistence represents one of the greatest challenges for plant community ecologists (Vellend, 2010). The last decades have seen a consensus on the interplay of both deterministic (Diamond, 1975) and stochastic (Hubbell, 2001) processes as integrative mechanisms driving the complexity of plant community assembly (e.g. Gravel *et al.*, 2006). However, a clear insight of the relative importance and effect of each force on plant community assembly across spatiotemporal scales and environments is lacking (e.g. Valladares *et al.*, 2015; Escudero & Valladares, 2016).

Among the so-called deterministic processes, plant to plant interactions (i.e. competition and facilitation) are thought to be one of the main drivers of plant community coexistence and structure at fine scales (Lotka, 1926; Lekberg *et al.*, 2018). In this sense, resource niche partitioning is a mechanism consisting of species functional diversification to minimize competition between neighbouring plants at very local scales, in turn promoting species coexistence (Chesson, 2000; HilleRisLambers *et al.*, 2012). Because of the expected link between niche partitioning and the intensity of competition, several studies (e.g. Siefert, 2012; Chacón-Labella *et al.*, 2016) have assessed the occurrence and strength of functional segregation to unveil the relative importance of competition in structuring plant communities at these fine neighbourhood scales.

In relatively productive environments, competitive interactions are strongly related to light availability in the canopy, which mostly involves the aboveground compartment of the community. However, in drylands and other stressful habitats, water and nutrients represent the most limiting factors driving belowground competition processes (Casper & Jackson, 1997; Aerts, 1999). In this context, assessing patterns of belowground trait functional variation may be crucial to unveil plant community assembly processes. However, our knowledge on belowground functional diversity still lags considerably behind that of aboveground functional diversity since, up to a decade ago, feasible techniques to identify species belowground were lacking (Rewald *et al.*, 2012). Accordingly, the information gap between root traits compared with leaves and stems functional traits is still very high (Laughlin & Laughlin, 2013; Laliberté, 2017). As such, the assessment of drivers of belowground functional diversity patterns is an increasing priority in plant community ecology.

While aboveground plant-plant competition is mainly driven by light, which is an unidirectional source, generally leading to asymmetric competition, soil water and nutrients are three-dimensional sources resulting in a more symmetric belowground plant-plant competition (e.g. Weiner, 1990; Connolly & Waine, 1996). However, some authors pointed out that in certain environmental conditions such as in nutrient patchy soils, plants may be subject to asymmetric competition (e.g.

Casper & Jackson, 1997; Schwinning & Weiner, 1998). In this context, contradictory results have been reported, as some authors observed symmetry (Cahill Jr & Casper, 2000; Von Wettberg & Weiner, 2003), while other studies (e.g. Fransen *et al.*, 2001; Rasmussen *et al.*, 2019) found evidence of asymmetry in belowground plant-plant competition. Therefore, fine-scale soil heterogeneity may exert a key role on belowground competition processes, which should be mimicked by the functional structure of the root space, and help to promote species coexistence, especially in plant communities characterized by high species diversity (Beck & Givnish, 2021).

The belowground component of plant communities is also involved in a complex network of interactions with the soil, with multiple positive and negative plant-soil feedbacks (Wardle *et al.*, 2004; Bardgett & Van Der Putten, 2014). A growing number of studies (e.g. Laliberté *et al.*, 2015; Laliberté, 2017; Yan *et al.*, 2022) have also suggested that the soil biota may exert an important role in shaping both plant species (i.e. taxonomic) and functional diversity as well as contribute to maintain plant diversity, especially in species-rich ecosystems. A plant trait-based approach, especially focused on root traits, could help to unveil mechanistic processes behind the wide range of soil biota effects on plant communities (e.g. Baxendale *et al.*, 2014; Teste *et al.*, 2017) especially in the case of plant communities where the light is not limiting. However, up to date, most of the studies exploring the link between plant community dynamics and soil biota have considered the aboveground plant community compartment only (e.g. Wagg *et al.*, 2011; Yang *et al.*, 2014). Therefore, still little knowledge is available on the mechanisms whereby plant-soil feedbacks may regulate plant community assembly and species coexistence especially in patchy and stressful plant communities.

In this study, we aimed to fill the gap of knowledge on the dynamics regulating belowground plant communities on species-rich semiarid Mediterranean shrublands. We used a spatial-explicit design at a very fine spatial scale, combined with a functional trait-based approach, considering traits related to water- and nutrient-use strategies, and particularly focusing on root traits. First, we explored functional diversity patterns characterizing the belowground plant community. Second, we assessed, through the use of null models (e.g. Gotelli & Mccabe, 2002), the potential importance of deterministic processes. According to the niche partitioning hypothesis, we expected non-random belowground functional diversity patterns. Finally, we explored the effects of soil heterogeneity and soil biota on dynamics of belowground plant community assembly.

Methods

Study area, sampling design and processing

This study was conducted in a species-rich Mediterranean shrubland located in the south of Madrid province (Spain) and characterized by calcareous soils with a variable content of gypsum. The plant community is dominated especially by dwarf shrubs, hemicryptophytes and grasses such as *Bupleurum fruticescens* L., *Thymus vulgaris* L., *Linum suffruticosum* L., *Helianthemum cinereum* Pers., and *Stipa pennata* L. (Illuminati *et al.*, 2021).

In June 2016, we established a 64 m^2 plot in which all the individuals in the aboveground fraction were mapped. After that, we collected 84 soil cores disposed in a regular grid, to identify belowground species composition. The soil cores had 5 cm diameter and 10 cm of depth. Upon collection, soil cores were placed in a cooler and then kept at 4ºC for a maximum of 48 hours to avoid DNA degradation. All samples were washed by using a 1 mm mesh sieve filter to isolate the roots which were centrifugated (3000 rpm, 30 seconds) and weighed, before being fragmented and mixed. From each sample, we took a subsample of 100 mg, snap-froze it with liquid nitrogen and stored it at -80ºC until DNA extraction.

In September 2016 we also collected 84 10 cm-deep additional soil cores adjacent to the other soil cores to characterize soil heterogeneity and microbial community (for more details on sampling design see Illuminati *et al.*, 2021). The soil cores were air-dried for four weeks and then stored until analysis.

Species plant identification through DNA metabarcoding

We used DNA metabarcoding techniques to identify species composition in each sample (for an accurate description see (Matesanz *et al.*, 2019; Illuminati *et al.*, 2021). Briefly, we built an in-house reference library including at least 95% of species present in the aboveground plant community (sampled plot and its surroundings), comprising the *rbcL* reference sequences of 45 species. DNA extractions were carried out using the DNeasy Plant Mini Kit (Qiagen, CA, USA) in the lab at Universidad Rey Juan Carlos. Afterwards, extracted DNA samples were processed in the AllGenetics laboratories (AllGenetics & Biology SL, A Coruña, Spain). We amplified a fragment of the *rbcL* chloroplast gene using primers rbcLa-F (Levin et al. 2003) and rbcLa-R (Kress et al. 2009). A first PCR was performed to amplify the *rbcL* chloroplast gene fragment. A second PCR was required to attach the Illumina index sequences for multiplexing distinct libraries in the same sequencing pool. The pool was sequenced in a run of the MiSeq PE300 (Illumina). Then, samples were demultiplexed, removing indexes and sequencing primers. Sequences were then dereplicated,

clustered at a similarity threshold of 100%. The taxonomical assignment was performed by querying the clustered sequences against the in-house reference library in VSEARCH (usearch global option) with a 99% similarity threshold. We removed both the OTUs (Operational Taxonomic Units) with a number of sequences lower than 0.005% of the total number of sequences (Bokulich *et al.*, 2013) to apply a quality filtering and those OTUs that did not match any reference sequence in the database at a similarity of 99% and remained unidentified ('No hit').

Assessment of soil heterogeneity and microbial community composition

A detail description of the analyses carried out to describe soil properties and identify the microbial community may be found in Illuminati *et al.* (2021) and López-Angulo *et al.* (2020) (2020). In brief, physical and chemical soil properties were measured on the finest soil fraction (diameter <2 mm). Soil texture was estimated according with Kettler et al. (2001). Electrical conductivity and pH were measured in deionised water, by using a conductivity meter GLP 31 and a pH meter GLP 21 (Crison, Barcelona, Spain), respectively. Soil organic carbon (SOC) was estimated by a wet oxidation procedure according to Yeomans and Bremner (1988). Total nitrogen (N) and phosphorus (P) contents were estimated by Kjeldahl digestion (Anderson & Ingram, 1993), while total potassium (K) content was determined applying Radojević and Bashkin (1999) methodology. We also estimated key soil enzymatic activities as surrogates of soil driven nutrient cycling, by the measurement of β-glucosidase activity and acid phosphatase, applying the techniques described by Eivazi and Tabatabai (1988) and Tabatabai and Bremner (1969), respectively.

We used DNA metabarcoding to identify fungi (total and arbuscular mycorrhizal) and bacteria taxa. First, DNA was extracted from 0.25 g of dry soil sampled in each soil core by using DNeasy PowerSoil isolation kit (Qiagen, CA, USA). The 16S rRNA gene sequences were amplified to identify bacteria taxa, while the ITS2 region and the 18S (SSU) rRNA gene sequences were amplified to detect all fungi taxa and the Glomeromycota fungi taxon (arbuscular mycorrhizal fungi), respectively. Sequencing was performed with the Illumina MiSeq PE300 v3 system at the Fundación Parque Científico de Madrid. Quality of raw reads was assessed with FASTQC software (Andrews, 2010) before carrying out paired-end assembly of the R1 and R2 reads with FLASH software (Magoč & Salzberg, 2011). Thereafter sequences were filtered considering a minimum of PHRED quality score of 20 and labelled in QIIME (Caporaso *et al.*, 2010). Sequences were dereplicated, clustered at a similarity threshold of 100% and sorted in VSEARCH (Rognes *et al.*, 2016).

The taxonomical assignment of bacteria taxa was performed by querying the clustered centroids against the SILVA reference database in Qiime (Quast *et al.*, 2013), with a 97% similarity threshold; while for taxonomical identification of fungi and the fungi taxon of Glomeromycota were instead

considered, respectively, the UNITE reference database (UNITE Community 2017) and the MaarjAM reference database (Öpik *et al.*, 2010), with a minimum similarity of 90% (Morgan & Egerton-Warburton, 2017). Quality filtering was applied to the OTUs tables by removing both OTUs occurring at a very low frequency in both the whole dataset (<0.005% threshold) and each sample (<0.1%). To account for the unequal number of sequences between samples, reads were rarefied to the minimum number of sequences in each sample considering reads for each group separately. To avoid further methodological biases, the OTUs table, representing the microbial community matrix, was transformed with Hellinger method (Legendre & Gallagher, 2001; López-Angulo *et al.*, 2020). The number of OTUs finally obtained in each sample for each group was used as a proxy of its richness in the sample.

Plant functional traits measurement

We measured functional traits for sixteen selected species among those which were detected in the soil cores and together accounted for 76% (SD = ±12%) of species mean richness found individual soil cores. A detailed description of the common garden experiment and trait measurement is reported in Chapter 2. Briefly, functional traits were measured in individuals (5-8 samples per species) cultivated in homogeneous outdoor conditions and grown individually in rhizotron tubes located in the soil for a period of five months. After the harvest, the entire root was washed carefully and filtered by a double 1-mm mesh filter, and frozen at -20 °C. Only the fine root component (<1 mm) was selected and scanned at 600 bpi with an Epson Expression 10000XL scanner. Thereafter, roots were dried at 60°C for 72 hours to measure the dry weight. We selected root traits importantly related with the nutrient-use strategy. Thus, we measured the specific root area (SRA), specific root length (SRL), root diameter (RD), very fine (<0.02 mm) roots percentage (VFR), root tissue density (RTD) and root mass fraction (RMF). Among them, the SRL and RD have been described as the traits most importantly associated with the so-called 'collaboration gradient' of the root economic space (RES, sensu Bergmann *et al.*, 2020) and the RTD as one of the main traits associated with the orthogonal 'conservation gradient' of the RES.

We scanned all the fine (<1mm) roots at 600 bpi using an Epson Expression 10000XL scanner. Then roots were we oven-dried at 60°C for 72 hours and weighed to determine the root mass. We carried out the image analysis by using WinRHIZO (2009a,b,c) to estimate the following traits: mean root diameter (RD), total root area, total root length, mean root volume and the percentage of the very fine (<0.02 mm) roots on total root length (VFR). The SRA was estimated by the formula root area / root mass, the SRL was instead calculated as root length / root mass and the RTD was estimated dividing the mean root volume by the root mass. The RMF was calculated as

the percentage of mass represented by roots (fine and coarse roots) on total plant mass (for details see Chapter 2). In addition, we considered the species scores along the PC axis (RES1-PC hereafter) which was interpreted as the 'collaboration' gradient of the RES in our plant community (see Fig. 3 Chapter 2), as an additional trait related with the root-level nutrient use strategy.

We also considered isotopic traits measured in the field associated with the plant water use strategy. Specifically, the stem water $\delta^{18}O$, as a proxy of water uptake depth, the leaf $\delta^{13}C$ and the leaf $\delta^{18}O$ as time integrated measures of water use efficiency and transpiration rate, respectively (see for more details Illuminati *et al.*, 2022). Moreover, we added the species scores along the PC axis (both cases with and without considering phylogenetic relatedness, respectively WU-phylPC and WU-PC hereafter) which can be easily interpreted as a species gradient of different water use strategies coordinated with the root tissue density (RTD) as an additional trait related with plant water use for further analyses (see Fig. 3 and Tables 9,10 in Chapter 2).

Finally, as additional key traits related with the leaf-level nutrient use strategy, we also considered the species scores along the PC axis associated with the leaf economic spectrum (both cases with and without considering phylogenetic relatedness, LES-phylPC and LES-PC) and the leaf $\delta^{15}N$, for its link to different nitrogen acquisition mechanisms related to root-microbe symbiotic associations (Craine *et al.*, 2009) (for more details see Illuminati *et al.*, 2022).

Statistical analysis

We estimated functional diversity in each soil core, by considering the functional traits (mean species values from Chapter 1 and 2) and the occurrence of the species in each soil core (site-by-species community data matrix (from Chapter 3). All the traits were log-transformed, when necessary, to approximate a normal distribution. To describe functional diversity within soil cores, we estimated the mean, standard deviation (SD), range, kurtosis and skewness, for each plant functional trait, as different measures of traits spacing encountered within each soil core, and the coefficient of variation (SD/mean) as an additional measure to compare trait variance across soil cores.

To assess if functional diversity patterns observed were driven by either deterministic or random processes, we used a null modelling approach, for each trait and each metric of functional variability, randomizing the site-by-species community data matrix (in our case, presence-absence of species in each core) (Hardy, 2008). We performed two different null models: a null model, which assumes that the total richness is determined by the carrying capacity of this tiny piece of soil, maintaining species richness of communities (cores) by swapping only columns (null model 1, hereafter), and another completely random null model permuting both rows and columns of the

data matrix (null model 2),) which assumes that the assembly process is stochastic. We generated 999 random community matrices for each null model. Each trait dispersion was estimated with the z-score, corresponding to the standardized effect size SES (Gotelli & Mccabe, 2002), as following: z-score = $T_{obs} – T_{exp} / \sigma_{exp}$, with T_{obs} representing the observed trait value, T_{exp} the expected trait value according to a random distribution (mean value estimated by 999 simulated random communities) and σ_{exp} indicating the standard deviation of the expectations (SD of trait values calculated from the 999 simulated communities). Z-scores differed significantly from a random distribution for a *p-value* <0.05, i.e. when the observed value had a percentile rank smaller or larger than 2.5th or 97.5th percentiles of the null distribution (see also López-Angulo *et al.*, 2021). Positive and negative values of the z-scores indicated an observed value greater or lower than the value expected under a random distribution

To assess if the deviations from a random distribution found in the observed values were related to the soil abiotic heterogeneity and/or the microbial community, we carried out linear models considering the z-scores of the traits that presented a high percentage (>15% of cores) of significant values in at least one of the two null models, as the response variable and explanatory soil variables. To reduce the total number of soil variables to be added to the model, we carried out a principal component analysis after log-transforming, when required, to approximate a normal distribution, and scaling all the soil variables. To maximize correlation between soil variables and PC axes, we carried out a PCA with varimax rotation (*principal* function, *psych* package), selecting four factors, which explained together 75% (of which respectively 29%, 25%, 24%, 23%) of the total variance, which were selected by the 'broken-stick' method (*screeplot* function, *vegan* package). The RC1 (Rotate Component 1) axis was mainly associated with soil sand, silt and clay contents, thus representing a soil texture gradient, the RC2 was related with glucosidase, phosphatase and C content, indicating a gradient of enzymatic activity and soil organic carbon; the RC3 represented a soil fertility gradient, mostly associated to soil N and P contents variability while the RC4 was related to soil salinity, i.e. pH and conductivity. Thus, we finally included in the lineal models the four RC axes representing diverse aspects of soil heterogeneity, as well as the richness of total fungi, arbuscular mycorrhizal fungi and total bacteria, estimated by the total number of OTUs found for each microbial group. Nine samples of the arbuscular mycorrhizal fungi group were removed from the analysis due to small amounts of sequences after all filtering steps, resulting in 75 samples. We carried out a type III ANOVA (*Anova* function, *car* package) and verified

assumptions of the linear models by checking for normality, independence, and homoscedasticity of residuals. All analyses were carried out in R v.4.2 (R Core Team, 2020).

Results

Belowground plant functional diversity

We observed high trait variability both within and across cores, as shown by the standard deviation (SD), range and coefficient of variation (CV) observed in each core, for all traits considered in the study. We report here (Fig. 1) the mean, SD and CV of selected traits that presented clear non-random patterns in the belowground community. In the case of root diameter (RD), CV values varied from 0.11 to 0.66 (scale 0 to 1), while the specific root length (SRL) showed the highest variability of CVs, with values ranging from 0.12 to 0.97. The root tissue density (RTD) and stem water $\delta^{18}O$ (SW $\delta^{18}O$) also presented less variability compared with RD and SRL, with CV values respectively ranging from 0.17 to 0.60 and from 0.05 to 0.41 (Fig. 1).

Non-random belowground functional patterns

Null models 1 and 2 provided similar results when considering trait mean values as response variables, although the percentage of significant z-scores was higher for null model 1 (Fig. 2). The highest number of significant z-scores was found for the mean values of WU-PC, i.e. the PC axis related with both plant water use and the root tissue density (RTD), the stem water $\delta^{18}O$ (SW $\delta^{18}O$) and the RTD (Fig. 2). The z-scores of WU-PC mean values were significant in 27.38% and 21.43% of cores based on null models 1 and 2, respectively. Similarly, z-scores of SW $\delta^{18}O$ mean values were significant in 22.62% and 20.24% of cores, and z-scores of RTD mean values were significant in 22.62% and 14.29% of cores, respectively for null models 1 and 2 (Fig. 2). All significant z-scores were higher for both null models carried out considering mean traits values, indicating that observed values were higher than expected by a random (null model 2), or partially random (null model 1), distribution (Fig. 3).

Results from null models carried out with the standard deviations (SD) of traits in each core showed a higher percentage of significant z-scores for several selected traits using null model 1, while results were not significant using null model 2 (Fig. 2). The significant z-scores observed from outputs of null model 1 were found for traits related with the collaboration gradient of the RES. In particular, root diameter (RD) showed the highest percentage (39.9%) of significant z-scores, followed by the percentage of very fine roots (VFR) with 23.81% of significant z-scores and the RES1-PC, i.e. the PC axis interpreted as the collaboration gradient of the RES in our plant community. Significant z-scores were all positive for RD and RES1-PC, and mostly positive in case of VFR. Null models

carried out with the range of trait values gave similar outputs to those carried out with SD trait values. Both null models carried out with skewness and kurtosis for all the selected traits detected significant results in a low number of cores.

Linking plant belowground functional diversity with biotic and abiotic soil heterogeneity

Linear models carried out with z-scores of SD trait values (RD, RES1-PC, VFR) showed that belowground microbial community had significant effects on the patterns observed. The z-scores of the root diameter (RD) were significantly related to soil fungi richness (p-value = 0.031) and, marginally to soil bacteria richness (p-value = 0.088) (Fig. 4 a, b). The linear model carried out with z-scores of RES1-PC showed similar results, with a small, yet significant portion of variance explained by both soil fungi and bacteria richness (p-value = 0.032, 0.083, respectively). On the contrary, the linear model carried out with z-scores of the percentage of very fine roots percentage (VFR) showed only a very marginal effect of the soil texture (p-value = 0.097). The linear models carried out considering the z-scores of WU-PC, SW $\delta^{18}O$, RTD did not find any significant relationship.

Discussion

We found high functional diversity between species coexisting at the neighbourhood scale of few centimetres (considering mean traits values of the species coexisting in each soil core), as showed by the high ranges and standard deviations observed for each selected trait (Fig. 1). The greater trait variability was observed in root traits related with the 'collaboration gradient' of the RES (Bergmann *et al.*, 2020), of which root diameter (RD) and specific root length (SRL) represent the most important traits (Fig. 1). However, functional diversity was heterogeneous at the fine spatial scale of the study, as showed by the high variability of the coefficients of variation estimated for each trait across soil cores, with some samples characterized by higher functional diversity than others (Fig. 1).

We found evidence that deterministic processes are critical structuring the belowground plant community at the neighbourhood scale of a few centimetres. Indeed, we detected functional diversity higher than expected by a random distribution in high fractions of local sampled points (soil cores). Moreover, we found evidence of clear functional segregation patterns especially related to the root-level nutrient-use strategy. We detected a very high percentage of significant positive z-scores from the null models carried out with the standard deviation of RD and very fine roots (VFR). Similar results were obtained for the RES1-PC, the PC axis which represented the

coordinated variation of RD, SRL and VFR, and interpreted as the 'collaboration gradient' of the RES (Fig. 2, Fig. S1) in our Mediterranean shrubland. This gradient defines root nutrient-use strategies ranging from a 'do it yourself' to 'outsourcing' strategies, characterized by either mycorrhizal or bacterial symbiotic associations (Bergmann *et al.*, 2020). Thus, our results pointed out that among the variety of possible deterministic processes, plant-plant competition possibly had a major role in structuring the belowground plant community. Indeed, in accordance with the niche partitioning hypothesis (e.g. Casper & Jackson, 1997), species characterized by different strategies related to nutrient uptake may more easily outcome competitive exclusion and coexist in the same location. However, we cannot exclude the potential contribution of other plant-plant interactions such as facilitation to the functional diversity patterns observed. Indeed, several studies have pointed out that facilitation can increase functional segregation and promote coexistence of functionally contrasted species (Gross *et al.*, 2009, 2013; Butterfield & Briggs, 2011).

Our findings also pointed out the key role of the microbial plant community as an important determinant driving belowground plant community assembly. Indeed, we detected that the higher variability of root nutrient-use strategies observed in some areas was significantly linked to both fungi and, marginally, bacteria richness (Fig. 4). Therefore, our results showed that plant-soil feedbacks related to the soil microbiota contribute to increase belowground plant functional diversity in Mediterranean environments. These results are in agreement with recent studies (Teste *et al.*, 2017; Yan *et al.*, 2022) suggesting plant soil feedbacks as a potential key driver of species coexistence at local spatial scales. Moreover, Teste *et al.* (2017) found that plant responses to the soil microbiota were highly dependent on the root-level nutrient strategies and, more specifically, on the type of symbiotic associations, in a species-rich Mediterranean environment, which further supports our findings linking higher root functional diversity related to the 'collaboration' gradient of the RES with the soil microbiota.

It is worth noting that we did not find any significant effect of the arbuscular mycorrhizal fungi richness on the functional root patterns observed. This result may be related to the fact that, even though arbuscular mycorrhizal fungi represent the most common symbiotic association, several species abundant in our plant community, such as *Helianthemum cinereum* and *Fumana ericoides*, are instead characterized by ectomycorrhizal associations (Craine *et al.*, 2009; Illuminati *et al.*, 2022). Moreover, some authors (Laliberté *et al.*, 2015) pointed out the potential relevance of soilborne fungi to promote species coexistence in species-rich shrublands, which may be an additional factor explaining why the total richness of fungi, and not only of arbuscular mycorrhizal fungi, detected in the soil affected significantly the functional characterization of our plant community. Finally, complementing a growing body of evidence, some authors (Semchenko *et al.*, 2022) have recently

suggested that generalist microbial pathogens, mutualists and decomposers may generate relevant differential effects on plant communities, which has been for long neglected.

Our study also suggests that, while biotic interactions, both plant-plant and plant-microbiota, represent important deterministic forces of belowground plant community assembly at the neighbourhood scale of a few centimetres, abiotic factors related to soil heterogeneity did not affect significantly belowground functional diversity patterns at the very fine spatial scale considered. However, it is possible that soil heterogeneity may exert a significant effect on belowground functional diversity patterns at larger local scales, i.e. metres rather than of centimetres. Indeed, some authors (e.g. Siefert, 2012) have provided evidence of niche differentiation at very fine scales (<1 meter) and of environmental filtering at higher local scales (1 -10 metres).

We also detected a non-random distribution of mean values for traits related with the plant water use and the root tissue density (RTD) (Fig. 2). In this case, the positive z-scores indicated that the mean trait values observed were higher (overdispersion) than expected by the simulated random distribution (Fig.3). In the case of stem water $\delta^{18}O$ (SW $\delta^{18}O$), higher mean values corresponded to a shallower water uptake. This result was in accordance with the fact that we analysed belowground community assembly at 0-10 cm of soil depth, where it is reasonable to expect a major abundance of species using shallower water sources. These findings suggested the possible species segregation along soil depth as a mechanism to reduce competition for water sources.

We also observed higher RTD mean values, which may be, at least in part, related to its relationship with leaf-level water use traits such as water use efficiency (Chapter 2). Indeed, we also observed that the gradient (PC-WU) of coordinated variation of water use efficiency, water uptake depth and the root tissue density presented a high percentage of significantly positive z-scores. This indicates that species characterizing the shallower layers of the belowground community had greater root tissue density, shallower water uptake depth and higher water use efficiency. These results were in agreement with previous hypotheses about the importance of the root tissue density in relation to plant water dynamics, especially in Mediterranean environments (e.g. Fort *et al.*, 2017, Chapter 2).

Some authors have previously recognized that different outcomes can be found according to the null model selected (e.g. Gotelli & Mccabe, 2002). In our case, results were not significant when considering a complete randomization model (the null model 2) for the standard deviation and the range, while we detected significant results when randomizing only species combinations, while keeping constant the species richness in each core (null model 1) (Fig. 2). It is worth noting that when the number of species is low, such in our core samples (mean species richness in each core = 6 ± 2, see Illuminati *et al.*, 2021), randomization on species richness may strongly determine the functional diversity observed under null models. To keep the species richness constant (null model

1) allowed to better compare the functional characterization observed with different species combinations, by removing the effect of species richness which is expected. It is possible to expect that, in case of lower trait variability, a complete randomization of species richness would affect the results to a lower extent. This would explain why in case of mean values of traits such as root tissue density and stem water oxygen, which presented a lower coefficient of variation compared with other traits (Fig. 1), we observed similar results from the two different models.

Conclusions

Our results highlight the importance of the root trait variation among coexisting species for explaining coexistence at fine spatial scales. We found evidence of the importance of deterministic processes, especially attributable to plant-plant interactions, as drivers of belowground plant community assembly at a very fine neighbourhood spatial scale in Mediterranean environments. Notably, we detected high functional segregation patterns especially related to different nutrient use strategies related to the 'collaboration' gradient of the root economic space. We also showed the relevant contribution of the soil microbiota, especially fungal richness, in promoting the functional segregation patterns detected. In last instance, we also observed that species encountered in the first cm of soil depth tended to use a greater portion of shallow water source, suggesting a possible spatial segregation along depth between species capturing different soil water sources.

Figures

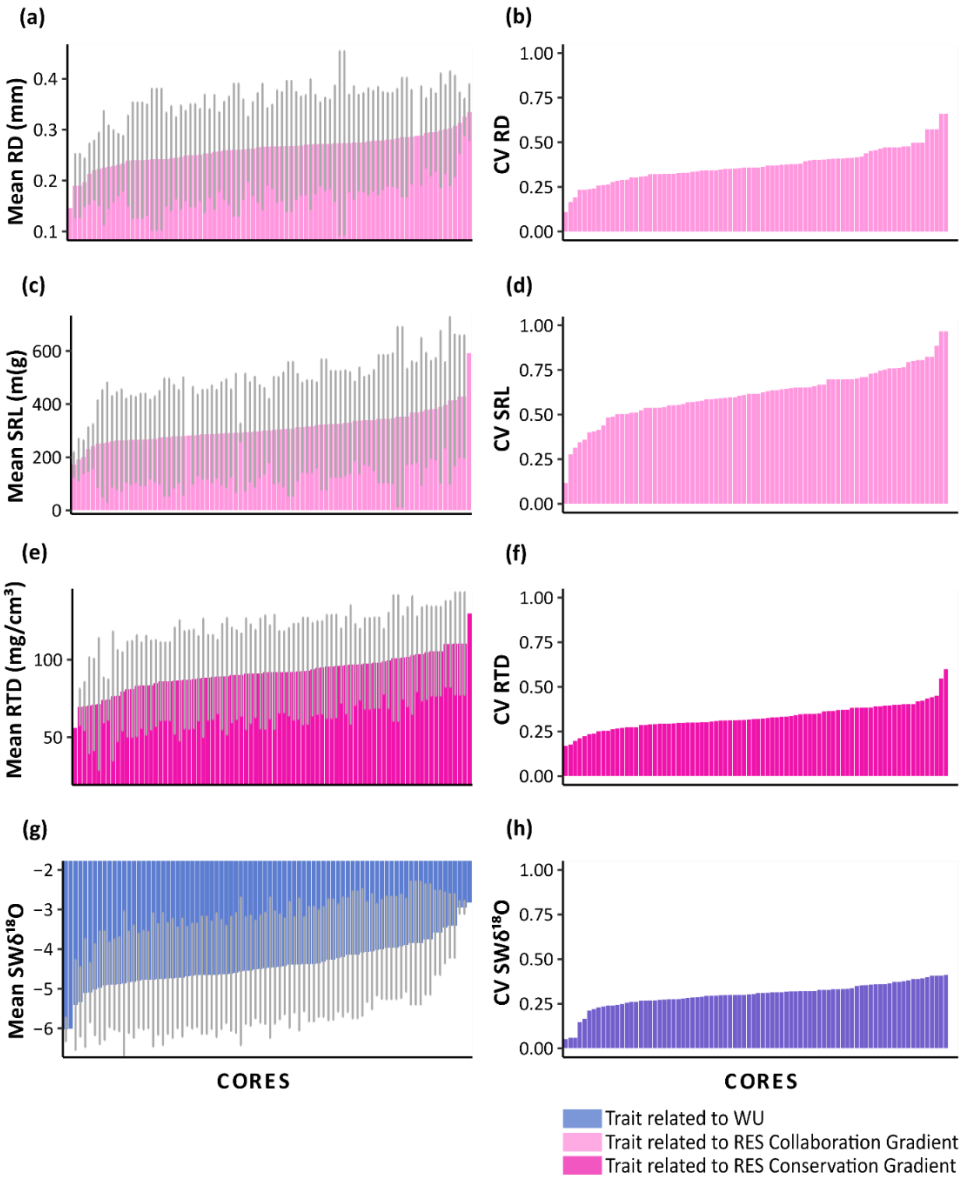

Fig. 1 Mean (± SD) and coefficient of variation (CV) observed in each core for the following traits: root diameter (RD, a, b); specific root length (SRL, c, d); root tissue density (RTD, c, d) and stem water $\delta^{18}O$ ($SW\delta^{18}O$, g, h).

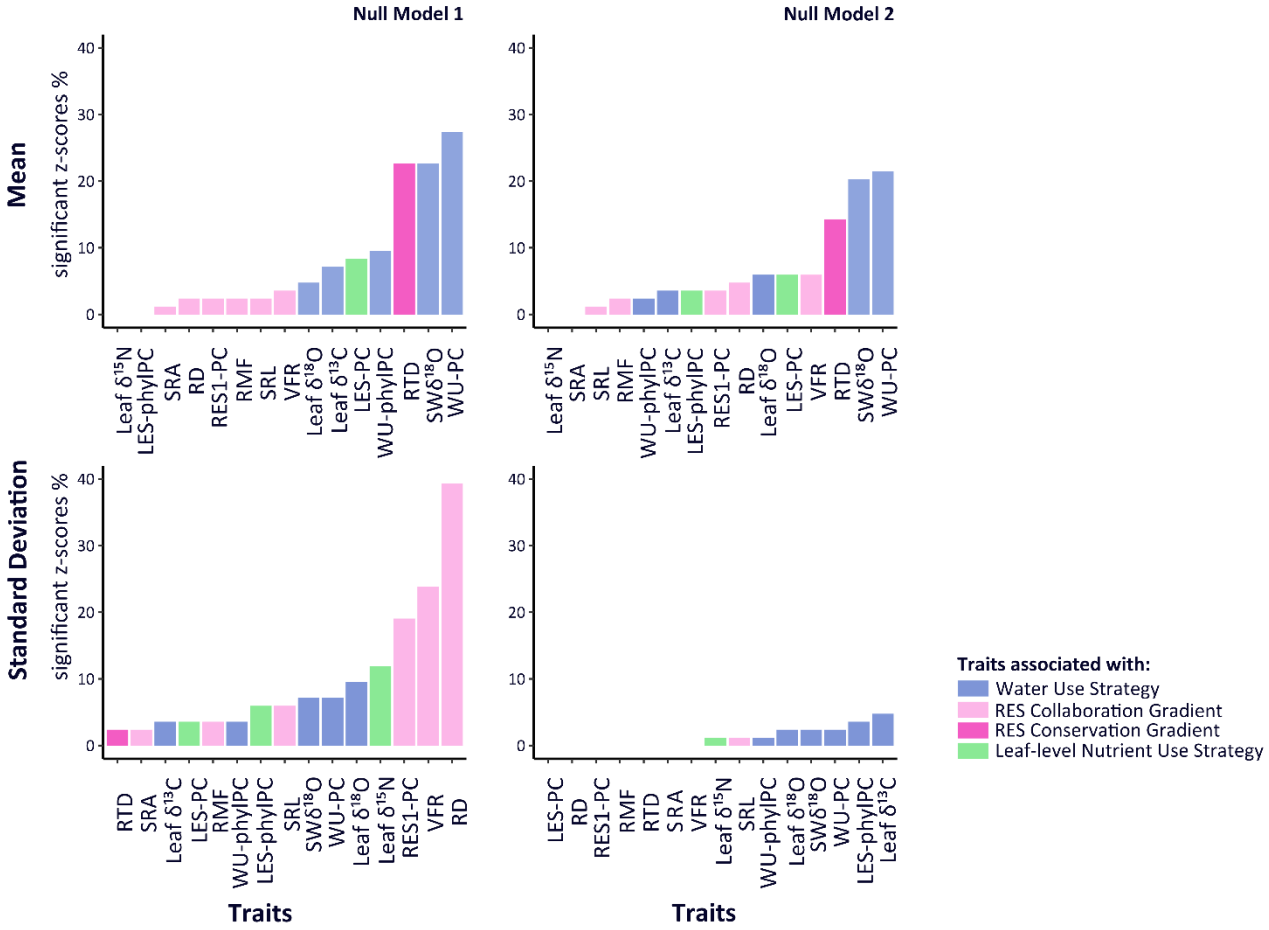

Fig. 2 Percentage (%) of cores with significant z-scores, i.e. indicating a non-random functional structure, calculated for each trait considering two different null models. Null model 1: species frequencies were kept constant. Null model 2: All random. Null models have been carried out considering both mean and standard deviations (SD) values of each trait per core. Mean and SD were calculated from species mean trait values of each species present in each core.

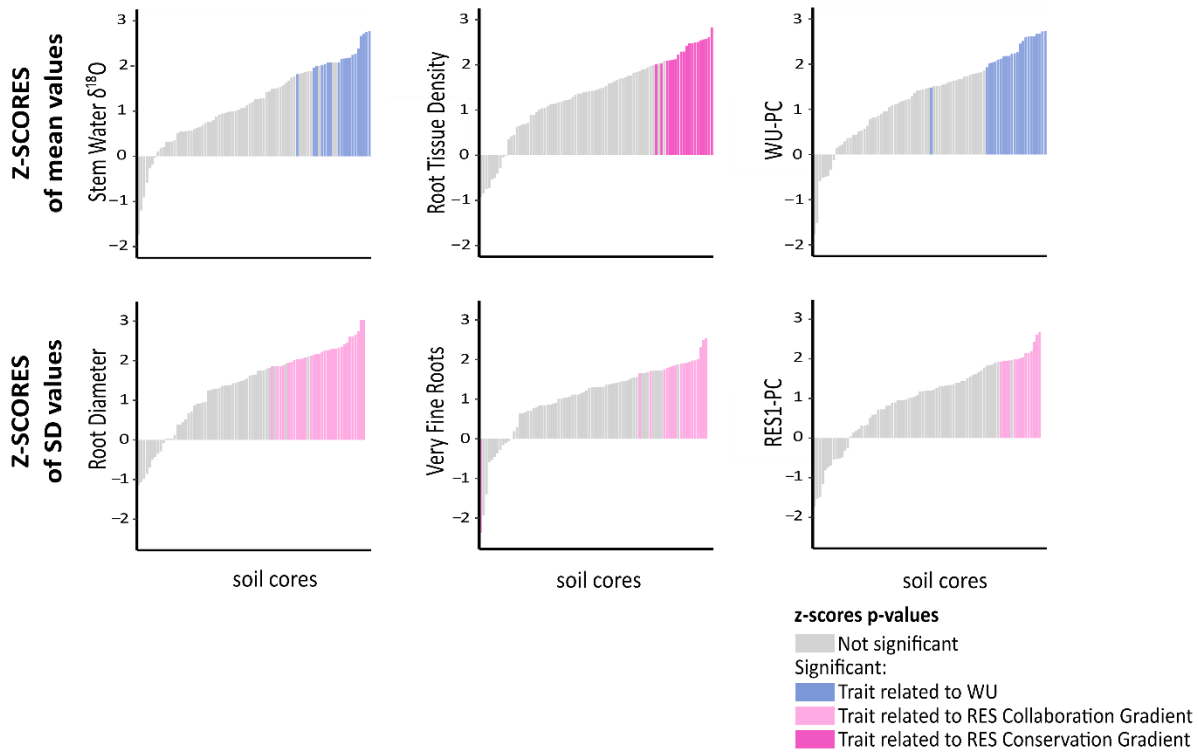

Fig. 3 Z-scores values from null model 1 reported for traits with a high percentage of significant *p*-values. The number of significant z-scores calculated on mean trait values per core were high for Stem Water $\delta^{18}O$ (SW $\delta^{18}O$), Root Tissue Density (RTD) and the WU-PC, i.e. species scores along the PC axis representing coordinated variation of SW $\delta^{18}O$, Leaf $\delta^{18}O$, Leaf $\delta^{13}C$, trait related with plant water use (WU), and RTD. Z-scores calculated on standard deviation (SD) values were high for the following traits: Root Diameter (RD), Very Fine Roots (VFR) and RES1-PC, species scores along the PC axis interpreted as representative of the Collaboration Gradient of the Root Economic Space (RES).

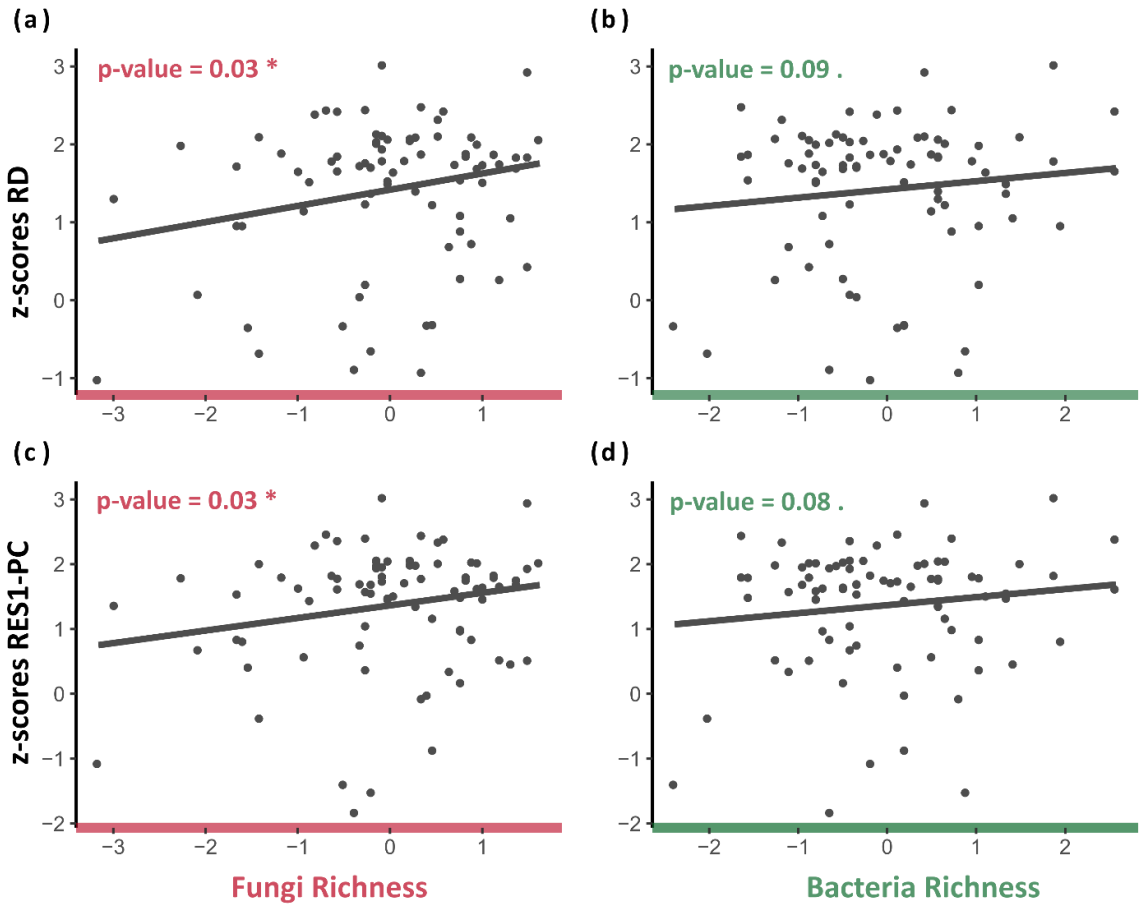

Fig. 4 Results of linear models carried out with z-scores (null model 1) of **(a)**, **(b)** Root Diameter (RD) and **(c)**, **(d)** the species scores along the PC axis interpreted as the collaboration gradient of the root economic space (RES1-PC), showing the significant and marginally significant effects of Fungi Richness (red) and Bacteria Richness (green), respectively, on both Root Diameter and species strategy along the collaboration gradient of the RES.

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References

Aerts R. **1999**. Interspecific competition in natural plant communities: Mechanisms, trade-offs and plant-soil feedbacks. *Journal of Experimental Botany* **50**: 29–37.

Anderson JM, Ingram JSI. **1993**. Tropical Soil Biology and Fertility: A Handbook of Methods. *The Journal of Ecology* **78**: 547.

Andrews S. **2010**. FastQCA quality controltoolfor high throughputsequence data.

Bardgett RD, Van Der Putten WH. **2014**. Belowground biodiversity and ecosystem functioning. *Nature* **515**: 505–511.

Baxendale C, Orwin KH, Poly F, Pommier T, Bardgett RD. **2014**. Are plant–soil feedback responses explained by plant traits? *New Phytologist* **204**: 408–423.

Beck JJ, Givnish TJ. **2021**. Fine-scale environmental heterogeneity and spatial niche partitioning among spring-flowering forest herbs. *American Journal of Botany* **108**: 63–73.

Bergmann J, Weigelt A, Van Der Plas F, Laughlin DC, Kuyper TW, Guerrero-Ramirez N, Valverde-Barrantes OJ, Bruelheide H, Fresche GT, Iversen CM, *et al.* 2020. The fungal collaboration gradient dominates the root economics space in plants. *Science Advances* **6**: eaba3756.

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG. **2013**. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods* **10**: 57–59.

Butterfield BJ, Briggs JM. **2011**. Regeneration niche differentiates functional strategies of desert woody plant species. *Oecologia* **165**: 477–487.

Cahill Jr JF, Casper BB. **2000**. Investigating the relationship between neighbor root biomass and belowground competition: field evidence for symmetric competition belowground. *Oikos* **90**: 311–320.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pẽa AG, Goodrich JK, Gordon JI, *et al.* 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**: 335–336.

Casper BB, Jackson RB. **1997**. PLANT COMPETITION UNDERGROUND. *Annual Review of Ecology and Systematics* **28**: 545–570.

Chacón-Labella J, de la Cruz M, Pescador DS, Escudero A. **2016**. Individual species affect plant traits structure in their surroundings: evidence of functional mechanisms of assembly. *Oecologia* **180**: 975–987.

Chesson P. **2000**. Mechanisms of Maintenance of Species Diversity. *Annual Review of Ecology and Systematics* **31**: 343–366.

Connolly J, Waine P. **1996**. Asymmetric competition between plant species. *Oecologia* **108**: 311–320.

Craine JM, Elmore AJ, Aidar MPM, Bustamante M, Dawson TE, Hobbie EA, Kahmen A, Mack MC, McLauchlan KK, Michelsen A, *et al.* 2009. Global patterns of foliar nitrogen isotopes and their relationships with climate, mycorrhizal fungi, foliar nutrient concentrations, and nitrogen availability. *New Phytologist* **183**: 980–992.

Diamond JM. **1975**. The island dilemma: lessons of modern biogeographic studies for the design of natural reserves. *Biological Conservation* **7**: 129–146.

Eivazi F, Tabatabai MA. **1988**. Glucosidases and galactosidases in soils. *Soil Biology and Biochemistry* **20**: 601–606.

Escudero A, Valladares F. **2016**. Trait-based plant ecology: moving towards a unifying species coexistence theory. *Oecologia* **180**: 919–922.

Fort F, Volaire F, Guilioni L, Barkaoui K, Navas ML, Roumet C. **2017**. Root traits are related to plant water-use among rangeland Mediterranean species. *Functional Ecology* **31**: 1700–1709.

Fransen B, De Kroon H, Berendse F. **2001**. SOIL NUTRIENT HETEROGENEITY ALTERS COMPETITION BETWEEN TWO PERENNIAL GRASS SPECIES. *Ecology* **82**: 2534–2546.

Gotelli NJ, Mccabe DJ. **2002**. SPECIES CO-OCCURRENCE: A META-ANALYSIS OF J. M. DIAMOND'S ASSEMBLY RULES MODEL. *Ecology* **83**: 2091–2096.

Gravel D, Canham CD, Beaudet M, Messier C. **2006**. Reconciling niche and neutrality: the continuum hypothesis. *Ecology Letters* **9**: 399–409.

Gross N, Börger L, Soriano-Morales SI, Le Bagousse-Pinguet Y, Quero JL, García-Gómez M, Valencia-Gómez E, Maestre FT. **2013**. Uncovering multiscale effects of aridity and biotic interactions on the functional structure of Mediterranean shrublands. *Journal of Ecology* **101**: 637–649.

Gross N, Kunstler G, Liancourt P, De Bello F, Suding KN, Lavorel S. **2009**. Linking individual response to biotic interactions with community structure: a trait-based framework. *Functional Ecology* **23**: 1167–1178.

Hardy OJ. **2008**. Testing the spatial phylogenetic structure of local communities: statistical performances of different null models and test statistics on a locally neutral community. *Journal of Ecology* **96**: 914–926.

HilleRisLambers J, Adler PB, Harpole WS, Levine JM, Mayfield MM. **2012**. Rethinking Community Assembly through the Lens of Coexistence Theory. *Annual Review of Ecology,*

Evolution, and Systematics **43**: 227–248.

Hubbell SP. **2001**. *The Unified Neutral Theory of Biodiversity and Biogeography (MPB-32)*. Princeton University Press.

Illuminati A, López-Angulo J, de la Cruz M, Chacón-Labella J, S. Pescador D, Pías B, Sánchez AM, Escudero A, Matesanz S. **2021**. Larger aboveground neighbourhood scales maximise similarity but do not eliminate discrepancies with belowground plant diversity in a Mediterranean shrubland. *Plant and Soil* **460**: 497–509.

Illuminati A, Querejeta JI, Pías B, Escudero A, Matesanz S. **2022**. Coordination between water uptake depth and the leaf economic spectrum in a Mediterranean shrubland. *Journal of Ecology*.

Kettler TA, Doran JW, Gilbert TL. **2001**. Simplified method for soil particle-size determination to accompany soil-quality analyses. **852**: 849–852.

Kress WJ, Erickson DL, Jones FA, Swenson NG, Perez R, Sanjur O, Bermingham E. **2009**. Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 18621–18626.

Laliberté E. **2017**. Below-ground frontiers in trait-based plant ecology. *New Phytologist* **213**: 1597–1603.

Laliberté E, Lambers H, Burgess TI, Wright SJ. **2015**. Phosphorus limitation, soil-borne pathogens and the coexistence of plant species in hyperdiverse forests and shrublands. *New Phytologist* **206**: 507–521.

Laughlin DC, Laughlin DE. **2013**. Advances in modeling trait-based plant community assembly. *Trends in Plant Science* **18**: 584–593.

Legendre P, Gallagher ED. **2001**. Ecologically meaningful transformations for ordination of species data. *Oecologia* **129**: 271–280.

Lekberg Y, Bever JD, Bunn RA, Callaway RM, Hart MM, Kivlin SN, Klironomos J, Larkin BG, Maron JL, Reinhart KO, *et al.* 2018. Relative importance of competition and plant–soil feedback, their synergy, context dependency and implications for coexistence. *Ecology Letters* **21**: 1268–1281.

Levin RA, Wagner WL, Hoch PC, Nepokroeff M, Pires JC, Zimmer EA, Sytsma KJ. **2003**. Family-level relationships of Onagraceae based on chloroplast *rbc L* and *ndh F* data. *American Journal of Botany* **90**: 107–115.

López-Angulo J, de la Cruz M, Chacón-Labella J, Illuminati A, Matesanz S, Pescador DS, Pías B, Sánchez AM, Escudero A. **2020**. The role of root community attributes in predicting

soil fungal and bacterial community patterns. *New Phytologist* **228**: 1070–1082.

López-Angulo J, de la Cruz M, Pescador DS, Sánchez AM, Escudero A. **2021**. A dimmer shade of pale: revealing the faint signature of local assembly processes on the structure of strongly filtered plant communities. *Ecography* **44**: 87–97.

Lotka AJ. **1926**. Element of physical biology. *Science Progress in the Twentieth Century* **21**: 341–343.

Magoč T, Salzberg SL. **2011**. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**: 2957–2963.

Matesanz S, Pescador DSDS, Pías B, Sánchez AMAM, Chacón-Labella J, Illuminati A, de la Cruz M, López-Angulo J, Marí-Mena N, Vizcaíno A, *et al.* 2019. Estimating belowground plant abundance with DNA metabarcoding. *Molecular Ecology Resources* **19**: 1265–1277.

Morgan BST, Egerton-Warburton LM. **2017**. Barcoded NS31/AML2 primers for sequencing of arbuscular mycorrhizal communities in environmental samples. *Applications in Plant Sciences* **5**: 1700017.

Öpik M, Vanatoa A, Vanatoa E, Moora M, Davison J, Kalwij JM, Reier Ü, Zobel M. **2010**. The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytologist* **188**: 223–241.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. **2013**. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**: D590–D596.

R Core Team. **2020**. R: The R Project for Statistical Computing.

Radojević M, Bashkin VN. **1999**. Water analysis. In: Radojevic M, Bashkin VN, eds. Practical Environmental Analysis. Cambridge: Royal Society of Chemistry, 138–273.

Rasmussen CR, Weisbach AN, Thorup-Kristensen K, Weiner J. **2019**. Size-asymmetric root competition in deep, nutrient-poor soil. *Journal of Plant Ecology* **12**: 78–88.

Rewald B, Meinen C, Trockenbrodt M, Ephrath JE, Rachmilevitch S. **2012**. Root taxa identification in plant mixtures - current techniques and future challenges. *Plant and Soil* **359**: 165–182.

Rognes T, Flouri T, Nichols B, Quince C, Mahé F. **2016**. VSEARCH: A versatile open source tool for metagenomics. *PeerJ* **4**: e2584.

Schwinning S, Weiner J. **1998**. Mechanisms determining the degree of size asymmetry in competition among plants. *Oecologia* **113**: 447–455.

Semchenko M, Barry KE, de Vries FT, Mommer L, Moora M, Maciá-Vicente JG. **2022**. Deciphering the role of specialist and generalist plant–microbial interactions as drivers of plant–soil feedback. *New Phytologist* **234**: 1929–1944.

Siefert A. **2012**. Incorporating intraspecific variation in tests of trait-based community assembly. *Oecologia* **170**: 767–775.

Tabatabai MA, Bremner JM. **1969**. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry* **1**: 301–307.

Teste FP, Kardol P, Turner BL, Wardle DA, Zemunik G, Renton M, Laliberté E. **2017**. Plant-soil feedback and the maintenance of diversity in Mediterranean-climate shrublands. *Science* **355**: 173–176.

Valladares F, Bastias CC, Godoy O, Granda E, Escudero A. **2015**. Species coexistence in a changing world. *Frontiers in Plant Science* **6**: 866.

Vellend M. **2010**. Conceptual Synthesis in Community Ecology. *The Quarterly Review of Biology* **85**: 183–206.

Wagg C, Jansa J, Stadler M, Schmid B, Van Der Heijden MGA. **2011**. Mycorrhizal fungal identity and diversity relaxes plant-plant competition. *Ecology* **92**: 1303–1313.

Wardle DA, Bardgett RD, Klironomos JN, Setälä H, Van Der Putten WH, Wall DH. **2004**. Ecological linkages between aboveground and belowground biota. *Science* **304**: 1629–1633.

Weiner J. **1990**. Asymmetric competition in plant populations. *Trends in Ecology & Evolution* **5**: 360–364.

Von Wettberg EJ, Weiner J. **2003**. Larger Triticum aestivum plants do not preempt nutrient-rich patches in a glasshouse experiment. *Plant Ecology* **169**: 85–92.

Yan X, Levine JM, Kandlikar GS. **2022**. A quantitative synthesis of soil microbial effects on plant species coexistence. *Proceedings of the National Academy of Sciences* **119**: e2122088119.

Yang G, Liu N, Lu W, Wang S, Kan H, Zhang Y, Xu L, Chen Y. **2014**. The interaction between arbuscular mycorrhizal fungi and soil phosphorus availability influences plant community productivity and ecosystem stability. *Journal of Ecology* **102**: 1072–1082.

Yeomans JC, Bremner JM. **1988**. A rapid and precise method for routine determination of organic carbon in soil. *Communications in Soil Science and Plant Analysis* **19**: 1467–1476.

General Discussion

General discussion

Whole-individual phenotypic integration of water and nutrient use strategies

Our findings constitute a step further to build a robust framework at the whole-individual level on phenotypic integration, coordination and functional trade-offs related to water and nutrient use functions in coexisting Mediterranean plants. This knowledge may enable a better understanding of plant functioning in drylands. Our results pointed out that different aspects of the plant water use strategy are tightly associated with specific functional traits related to nutrient use (Chapters 1 and 2). Indeed, the water uptake measured through stable isotopes was linked to the leaf-level nutrient use, while, in turn, water use efficiency was associated with the root tissue density, i.e. the conservation gradient of the root economic space (Bergmann *et al.*, 2020). Contrary to what we might have expected from previous studies carried out in Mediterranean environments (e.g. Moreno-Gutiérrez *et al.*, 2012; Prieto *et al.*, 2018), we did not observe a whole integration between plant water use strategy and leaf traits related with the nutrient use strategy, i.e. the leaf economic spectrum, LES (Wright *et al.*, 2004). We indeed found important phenotypic integration between traits related with the plant water strategy in species coexisting in the same plant community (Fig. S7, Chapter 1), but not with leaf traits related to the LES. However, it is worth noting that the phenotypic integration observed between traits related with the plant water use strategy was strongly affected by evolutionary history. Indeed, incorporating the phylogenetic relatedness in the analyses drastically altered the relationships observed among traits, suggesting that the phenotype did not mirror actual functional trade-offs.

We found high coordination between water uptake depth and the leaf economic spectrum. This result represents one of the main novelties of our work, because even though previous works (Walter, 1939; Ryel *et al.*, 2008; Ward *et al.*, 2013), such as the Walter' two-layer hypothesis, may have indirectly suggested a potential link between water uptake depth and the LES in arid environments, we provided the first evidence of the presence of a link between shallower water uptake and an acquisitive leaf-level nutrient use strategy. Interestingly, we also observed that leaf traits related to water use were especially associated with a key morphological root trait, the root tissue density. Indeed, higher water use efficiency and tighter transpiration rates showed a tight coordination with higher root tissue density (Fig. 3, Chapter 2). High values of root tissue density may provide the mechanical support necessary to withstand the very negative potentials typical of the soil layers during summer droughts (Hacke *et al.*, 2001). Some authors (Fort *et al.*, 2017) suggested that high root tissue density may be a key trait also to maintain transpiration capacity for a longer time, especially for those species capturing water in shallower layers, and thus are subjected

to the most extreme water potentials. In this context, our results did not show a clear relationship between water uptake depth and root tissue density. While several species with both high water use efficiency and root tissue density also tended to use shallower water sources, other species with similar high root density values were able to capture deeper water sources (Table S19, S10, Chapter 2).

Linking water and nutrient use strategies with species performance in natural conditions

The study species showed remarkable variability of nutrient use strategies, both at the leaf- and the root-level, showing the high functional diversification between the species coexisting in our Mediterranean plant community (Chapter 1 and 2). However, in accordance with the idea that environmental constraints tend to promote conservative resource strategies in Mediterranean environments (e.g. Matesanz & Valladares, 2014; Carvajal *et al.*, 2019), we observed that a conservative strategy was correlated with higher species performance in the plant community. Specifically, we found a significant relationship between species performance (i.e. dominance in the community) and a more conservative leaf-level nutrient-use strategy (Fig. S6, Chapter 1), and an even stronger link between species performance (both dominance and abundance) and a more conservative leaf-level water use strategy (Fig. S8, Chapter 1). Worth to note, however, that leaf-level nutrient use strategy was not coordinated with the leaf-level water use strategy (Fig. 5, Chapter 1). Indeed, even though we observed a positive effect of both a conservative leaf-level nutrient use and leaf-level water use on species performance, the most abundant species did not show a whole coordinated strategy in leaf-level water and nutrient use.

Furthermore, none of the two species gradients related to the root-level nutrient use had a significant correlation with our proxies of species performance, showing that different root-level nutrient use strategies can be equally successful in the plant community. This result is possibly related to the bi-dimensionality of the root economic space (Bergmann *et al.*, 2020), which also was detected at the local scale of our study (Fig. 3, Chapter 2). For example, a species characterized by high tissue density, i.e. more conservative nutrient use strategy, generally presents a lower nutrient acquisition capacity compared with species with a lower tissue density. However, through symbiotic associations, it may gain an acquisition capacity comparable with that of species characterized by a lower tissue density, i.e. more acquisitive nutrient use strategy, with no symbiotic associations. Although root morphology *per se* did not seem to affect species performance in the plant community, when coupled with leaf-level water use traits, it exerted an influence on species performance. In this sense, we observed that species with higher water use efficiency, tighter transpiration and higher root tissue density had better performance (in dominance terms) (Fig. 4,

Chapter 2. This result reinforced the idea that root tissue density may play a key role in relation with water evapotranspiration processes linking roots to leaves.

Conversely, water uptake depth during the phenological peak was not related to any surrogate of species performance, showing that species capturing both shallow- and deep-water sources may result equally successful in the plant community (Chapter 1). This is in agreement with the few studies (e.g. Jiang *et al.*, 2020) assessing the link between water uptake depth and species performance in the plant community, in which the most abundant species neither had very high nor very low reliance in deep water sources. However, Jiang *et al.* (2020), in a study carried out on understorey shrub species in subtropical coniferous plantations, observed that seasonal variation of reliance on deep water sources was lower in the most abundant species. It is known that several species characterizing Mediterranean environments may have a dimorphic root system, i.e. capturing water at different depths according to seasonal soil water availability fluctuations (e.g. Filella & Peñuelas, 2003). This would suggest that seasonal variation on the use of different water sources may affect species performance and thus plant community composition and dynamics in these ecosystems.

Spatial, water and nutrient niche segregation

We found a very strong discrepancy in patterns of diversity between the aboveground and belowground compartments of the plant community, together with higher richness at the neighbourhood scale of a few cm belowground (Fig. 3, Chapter 3). Although higher values of belowground richness have also been observed in other environments such as in species-rich temperate grasslands (e.g. Hiiesalu *et al.*, 2012), we detected a strongerdiscrepancy between aboveground and belowground richness at comparable spatial scales (average 1.61 vs 6.02 species) (Chapter 3). Our results contrast with the idea suggested by some authors (e.g. Martens *et al.*, 1997) that the typical patchy distribution of aboveground vegetation observed in drylands may be mirroring soil horizontal spatial segregation processes resulting from belowground competitive interactions. In our plant community, however, we observed that functional segregation represents a key factor driving species coexistence. We detected a clear pattern of water niche segregation at the plant community level with plants clearly organized as a function of water uptake depth. Water niche segregation is well-documented in the literature across multitude of environments (Silvertown *et al.*, 2015), our study considered a very high number (24) of species coexisting in the same environment, providing strong evidence of this phenomenon at the plant community level seldom shown before (e.g. Palacio *et al.*, 2017) (Fig. 3, Chapter 1). The clear presence of water

niche partitioning reflects the high competition triggered by the low availability of water sources in these environments.

We also detected the presence of clear non-random patterns in the first cm of soil depth of the mean value of stem water $\delta^{18}O$, i.e. calculated considering all the species present in a given soil core, showing that the mean water uptake depth was shallower than expected by a random distribution (Fig. 2, Chapter 4). This finding showed that species with shallower root distributions also tended to capture water at shallower depths. The observation of this clear non-random pattern belowground suggested that water niche segregation may affect the belowground community structure by promoting a soil vertical spatial segregation, i.e. along soil depth, among species using different water sources. These results were not obvious for the following reasons. First, though the maximum root depth has been commonly considered as a proxy of water uptake depth (Bucci *et al.*, 2009), as mentioned before, some species may be characterized by a dimorphic root system. Moreover, some authors (Kulmatiski *et al.*, 2017) observed a partial decoupling in some tree species between nutrient and water uptake, with shallower roots mostly related with nutrient uptake, and deeper roots to water uptake, suggesting that shallow roots may be more associated to nutrient, rather than to water, uptake. Previous studies (e.g. Chacón-Labella *et al.*, 2016) assessing aboveground taxonomical and functional spatial patterns at a fine spatial grain in the same plant community suggested the presence of niche complementarity as a fundamental driver of these species-rich Mediterranean shrublands. We found that a very high functional diversification in the root-level nutrient-use strategy was a mechanism driving belowground species coexistence at the very small scale of a few cm (Chapter 4). Notably, we found evidence of very strong non-random patterns showing a clear functional segregation related with root traits associated with the collaboration gradient of the root economic space (Bergmann *et al.*, 2020). These findings showed that deterministic processes related to fine-scale competition dynamics for nutrient sources may represent the main determinants of belowground plant community assembly.

Contrasting aboveground and belowground patterns and dynamics

The relevant difference we detected between aboveground and belowground compartments in both species richness and composition was not surprising (Chapter 3). Indeed, several works (e.g. Pärtel *et al.*, 2011; Träger *et al.*, 2019) pointed out that a unique component of diversity, which is commonly referred to as dark diversity, is present belowground but is not detected aboveground. As previously acknowledged by other authors (Hiiesalu *et al.*, 2012; Price *et al.*, 2012), these results suggest that the aboveground and belowground compartments of the plant community are regulated by very different processes which in turn determine divergent dynamics of plant

community assembly. Moreover, we observed that the maximum taxonomical similarity between the two compartments was achieved at very different spatial scales (20 *vs* 2.5 cm of radius, respectively, aboveground and belowground). This supported the hypothesis that the main drivers of the plant community not only differ, but also act at very different spatial scales aboveground and belowground (see also (Pärtel *et al.*, 2012) (Chapter 3). However, the two compartments of the plant community are inevitably linked, which means that the main processes regulating community assembly aboveground and belowground cannot be totally uncoupled. In this context, we detected a significant positive effect of enzymatic activity (soil phosphatase) in species richness both aboveground and belowground (0-10 cm of depth). In accordance with other works (e.g. Kesanakurti *et al.*, 2011), we also showed that drivers regulating species diversity (richness) may vary rapidly with soil depth: for example, enzymatic activity did not have a significant effect on species richness in deeper soil layers (Chapter 3). While in a previous study in a similar community (López-Angulo *et al.*, 2020), we showed that there was not a link between belowground plant richness and microbial richness, our findings (Chapter 4) highlighted that microbial diversity (particularly fungi richness) had a significant positive effect on belowground plant functional diversity. Our findings supported the emerging idea that plant-soil feedback may represent a key mechanisms driving plant community assembly at local scales (Teste *et al.*, 2017; Inderjit *et al.*, 2021). Moreover, our results also add support to the emerging body of evidence (e.g. Semchenko *et al.*, 2022) suggesting that the positive feedback on plant functional diversity is not linked to a specific group of fungi but to species richness of the whole fungal community, which includes a high variety of microorganisms, i.e. both host specific and generalist pathogens, mutualists and decomposers.

References

Bergmann J, Weigelt A, Van Der Plas F, Laughlin DC, Kuyper TW, Guerrero-Ramirez N, Valverde-Barrantes OJ, Bruelheide H, Fresche GT, Iversen CM, *et al.* 2020. The fungal collaboration gradient dominates the root economics space in plants. *Science Advances* **6**: eaba3756.

Bucci SJ, Scholz FG, Goldstein G, Meinzer FC, Arce ME. 2009. Soil water availability and rooting depth as determinants of hydraulic architecture of Patagonian woody species. *Oecologia* **160**: 631–641.

Carvajal DE, Loayza AP, Rios RS, Delpiano CA, Squeo FA. 2019. A hyper-arid environment shapes an inverse pattern of the fast–slow plant economics spectrum for above-, but not below-ground resource acquisition strategies. *Journal of Ecology* **107**: 1079–1092.

Chacón-Labella J, de la Cruz M, Escudero A. 2016. Beyond the classical nurse species effect: Diversity assembly in a Mediterranean semi-arid dwarf shrubland. *Journal of Vegetation Science* **27**: 80–88.

Filella I, Peñuelas J. 2003. Partitioning of water and nitrogen in co-occurring Mediterranean woody shrub species of different evolutionary history. *Oecologia* **137**: 51–61.

Fort F, Volaire F, Guilioni L, Barkaoui K, Navas ML, Roumet C. 2017. Root traits are related to plant water-use among rangeland Mediterranean species. *Functional Ecology* **31**: 1700–1709.

Hacke UG, Sperry JS, Pockman WT, Davis SD, McCulloh KA. 2001. Trends in wood density and structure are linked to prevention of xylem implosion by negative pressure. *Oecologia 2000 126:4* **126**: 457–461.

Hiiesalu I, Öpik M, Metsis M, Lilje L, Davison J, Vasar M, Moora M, Zobel M, Wilson SD, Pärtel M. 2012. Plant species richness belowground: Higher richness and new patterns revealed by next-generation sequencing. *Molecular Ecology* **21**: 2004–2016.

Inderjit, Callaway RM, Meron E. 2021. Belowground feedbacks as drivers of spatial self-organization and community assembly. *Physics of Life Reviews* **38**: 1–24.

Jiang P, Wang H, Meinzer FC, Kou L, Dai X, Fu X. 2020. Linking reliance on deep soil water to resource economy strategies and abundance among coexisting understorey shrub species in subtropical pine plantations. *New Phytologist* **225**: 222–233.

Kesanakurti PR, Fazekas AJ, Burgess KS, Percy DM, Newmaster SG, Graham SW, Barrett SCH, Hajibabaei M, Husband BC. **2011**. Spatial patterns of plant diversity below-ground as revealed by DNA barcoding. *Molecular Ecology* **20**: 1289–1302.

Kulmatiski A, Adler PB, Foley KM. **2020**. Hydrologic niches explain species coexistence and abundance in a shrub–steppe system. *Journal of Ecology* **108**: 998–1008.

Kulmatiski A, Adler PB, Stark JM, Tredennick AT. **2017**. Water and nitrogen uptake are better associated with resource availability than root biomass. *Ecosphere* **8**.

López-Angulo J, de la Cruz M, Chacón-Labella J, Illuminati A, Matesanz S, Pescador DS, Pías B, Sánchez AM, Escudero A. **2020**. The role of root community attributes in predicting soil fungal and bacterial community patterns. *New Phytologist* **228**: 1070–1082.

Martens SN, Breshears DD, Meyer CW, Barnes FJ. **1997**. Scales of aboveground and below-ground competition in a semi-arid woodland detected from spatial pattern. *Journal of Vegetation Science* **8**: 655–664.

Matesanz S, Valladares F. **2014**. Ecological and evolutionary responses of Mediterranean plants to global change. *Environmental and Experimental Botany* **103**: 53–67.

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI. **2012**. Isotopes reveal contrasting water use strategies among coexisting plant species in a mediterranean ecosystem. *New Phytologist* **196**: 489–496.

Palacio S, Montserrat-Martí G, Ferrio JP. **2017**. Water use segregation among plants with contrasting root depth and distribution along gypsum hills (R Michalet, Ed.). *Journal of Vegetation Science* **28**: 1107–1117.

Pärtel M, Hiiesalu I, Öpik M, Wilson SD. **2012**. Below-ground plant species richness: New insights from DNA-based methods (C Fox, Ed.). *Functional Ecology* **26**: 775–782.

Pärtel M, Szava-Kovats R, Zobel M. **2011**. Dark diversity: shedding light on absent species. *Trends in Ecology & Evolution* **26**: 124–128.

Price JN, Hiiesalu I, Gerhold P, Pärtel M. **2012**. Small-scale grassland assembly patterns differ above and below the soil surface. *Ecology* **93**: 1290–1296.

Prieto I, Querejeta JI, Segrestin J, Volaire F, Roumet C. **2018**. Leaf carbon and oxygen isotopes are coordinated with the leaf economics spectrum in Mediterranean rangeland species. *Functional Ecology* **32**: 612–625.

Ryel RJ, Ivans CY, Peek MS, Leffler AJ. **2008**. Functional Differences in Soil Water Pools: a New Perspective on Plant Water Use in Water-Limited Ecosystems. In: Progress in Botany. Springer, Berlin, Heidelberg, 397–422.

Semchenko M, Barry KE, de Vries FT, Mommer L, Moora M, Maciá-Vicente JG. **2022**. Deciphering the role of specialist and generalist plant–microbial interactions as drivers of plant–soil feedback. *New Phytologist* **234**: 1929–1944.

Silvertown J, Araya Y, Gowing D. **2015**. Hydrological niches in terrestrial plant communities: a review (W Cornwell, Ed.). *Journal of Ecology* **103**: 93–108.

Teste FP, Kardol P, Turner BL, Wardle DA, Zemunik G, Renton M, Laliberté E. **2017**. Plant-soil feedback and the maintenance of diversity in Mediterranean-climate shrublands. *Science* **355**: 173–176.

Träger S, Öpik M, Vasar M, Wilson SD. **2019**. Belowground plant parts are crucial for comprehensively estimating total plant richness in herbaceous and woody habitats. *Ecology* **100**.

Walter H. **1939**. Grasland, Savanne und Busch der arideren Teile Afrikas in ihrer ökologischen Bedingtheit. *Jahrbücher für Wissenschaftliche Botanik* **87**: 750–860.

Ward D, Wiegand K, Getzin S. **2013**. Walter's two-layer hypothesis revisited: Back to the roots! *Oecologia* **172**: 617–630.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornellssen JHC, Diemer M, *et al.* 2004. The worldwide leaf economics spectrum. *Nature* **428**: 821–827.

General conclusions

General conclusions

1. Leaf-level nutrient use strategies are notably coordinated with water uptake depth. An acquisitive leaf-level nutrient use strategy is associated with a greater use of shallow water sources from nutrient-rich topsoil layers, while more conservative leaf-level nutrient use strategies is linked to a deeper water sources uptake.
2. Root-level nutrient use strategies are highly coordinated with the leaf-level water use strategy.

   A conservative root-level nutrient use strategy is associated with a saver leaf-level water use strategy, and, conversely, a more acquisitive root-level nutrient use strategy is linked to a spender leaf-level water use strategy. A more conservative root nutrient use strategy may result essential to tolerate very negative soil water potentials under drought.
3. The functional trade-offs observed can be crucial in arid environments where the severity of environmental conditions highly constrains plant access to water and nutrients.
4. In line with the limited availability of water and nutrients in semiarid environments, a more conservative leaf-level nutrient use strategy and, to a larger extent, a saver leaf-level water use strategy coordinated with a more conservative root-level nutrient use strategy, positively affect species dominance in the plant community.
5. The high discrepancy observed between phenotypic integration and trade-offs detected when accounting for phylogeny highlights the important effect of evolutionary history.
6. Although aboveground and belowground species diversity (i.e. richness) are obviously linked, factors driving plant community patterns strongly differ and act at different spatial scales between compartments, determining a much higher belowground richness at very fine spatial scales.
7. A very high functional segregation is present belowground at very fine spatial scales, as showed by evident non-random functional patterns.
8. The belowground functional segregation processes determine a high diversity of root-level nutrient use strategies, especially related to the presence and/or type of symbiotic associations.
9. The high functional segregation detected belowground is mostly attributable to plant-plant competitive interactions, but also the positive effect exerted by the soil microbiota.