



# Seasonal variation in AMF colonisation, soil and plant nutrient content in gypsum specialist and generalist species growing in P-poor soils

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## Abstract

**Aims** Gypsum soils are P-limited atypical soils that harbour a rich endemic flora. These singular soils are usually found in drylands, where plant activity and soil nutrient availability are seasonal. No previous studies have analysed the seasonality of P nutrition and its interaction with the arbuscular mycorrhiza fungi (AMF) colonisation in gypsum plants. Our aim

was to evaluate the seasonal changes in plant nutrient status, AMF colonisation and rhizospheric soil nutrient availability in gypsum specialist and generalist species.

**Methods** We evaluated seasonal variation in the proportion of root length colonised by AMF structures (hyphae, vesicles and arbuscules), plant nutrient status (leaf C, N and P and fine root C and N) and rhizospheric soil content (P, organic matter, nitrate and ammonium) of three gypsum specialists and two generalists throughout a year.

**Results** All species showed arbuscules within roots, including species of *Caryophyllaceae* and *Brassicaceae*. Root colonisation by arbuscules (AC) was higher in spring than in other seasons, when plants showed high leaf P-requirements. Higher AC was decoupled from inorganic N and P availability in

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rhizospheric soil, and foliar nutrient content. Generalists showed higher AC than specialists, but only in spring.

**Conclusions** Seasonality was found in AMF colonisation, rhizospheric soil content and plant nutrient status. The mutualism between plants and AMF was highest in spring, when P-requirements are higher for plants, especially in generalists. However, AMF decoupled from plant demands in autumn, when nutrient availability increases in rhizospheric soil.

**Keywords** Mediterranean · Semiarid and arid environments · Functional ecology · Gypsophiles · Gypsosvags · Leaf elemental concentration

## Introduction

Nitrogen (N) and phosphorus (P) are the most common limiting nutrients in a wide variety of terrestrial ecosystems (Vitousek et al. 2010). Nutrient availability underlies the nutritional strategy of plants (Chapin 1980). In the case of nutrient-poor environments as drylands, plants have frequently evolved a retention strategy *versus* a rapid growth strategy, affecting acquisition, use, storage and resorption of nutrients (Aerts and Chapin 1999). These nutritional strategies are reflected in plant nutrient concentration (Grime et al. 1997), which summarises the functioning of plants in relation to their environment (Peñuelas et al. 2019).

Plant nutrient concentrations vary throughout the year due to shifts in nutrient availability and plant activity imposed by climate seasonality (Chapin 1980). Plant phenology of perennial species in Mediterranean drylands is characterized by predominant shoot growth in spring, root growth mainly in autumn and flowering in spring and early summer (Orshan 1989; Palacio and Montserrat-Martí 2007). Shoot growth requires high N and P in leaves (Palacio et al. 2014), while flowering demands high P (Milla et al. 2005). However, nutrient availability in drylands strongly depends on soil moisture (Querejeta et al. 2021). The availability of inorganic P is high in late summer (Magid and Nielsen 1992), and inorganic N is high in autumn in soils from Mediterranean drylands (Delgado-Baquerizo et al. 2011). Consequently, peak plant demands for N and P may be decoupled from soil availability in Mediterranean drylands.

Unfortunately, seasonal studies linking nutrient acquisition strategies, plant nutrient status and soil nutrient availability in Mediterranean drylands are scarce (but see Palacio et al. 2014).

Plant strategies for nutrient acquisition in soils vary depending on the structural and functional features of roots, and the association of roots with microorganisms (Richardson et al. 2009). The association of roots with microorganisms has been broadly explored as a strategy to enhance N and P acquisition in nutrient poor-environments (Aerts and Chapin 1999). Plants may be associated with symbionts to improve N uptake, as N-fixing bacteria or ectomycorrhizal fungi (Chalot and Brun 1998; Miller and Cramer 2005), and with arbuscular mycorrhiza fungi (AMF) to improve N and P acquisition (Vance et al. 2003). AMF symbiosis generally improves plant growth in P-limited soils (Johnson 2010), providing plants with access to low-mobility P inorganic forms, such as phosphates (Hawkesford et al. 2012), although there is variability in the benefit provided by different AMF species with some fungi even behaving as cheaters (Kiers and Denison 2008). Root colonisation by AMF is seasonal, as it relates to plant activity (Jakobsen et al. 2003) and soil nutrient availability (Hoeksema et al. 2010). However, few studies have demonstrated a relationship between seasonal AMF colonisation and soil P concentration in natural populations of wild plants (i.e. Mullen and Schmidt 1993). Consequently, shifts in AMF colonisation may be determined by the interaction of soil nutrient availability and plant demands, which ultimately define C supply by plants to the fungi.

The analysis of AMF structures within roots allows us to understand fungal activity in relation to plant activity (Jakobsen et al. 2003). Arbuscules appear when nutrient plant requirements and nutrient exchanges rates between fungi and plants are high, whereas at other times they may be absent (Allen 1983; Mullen and Schmidt 1993). Contrastingly, vesicles are storage structures, which appear in periods without high nutrient plant acquisition (Abbott et al. 1984). Seasonal shifts in AMF colonisation within roots have been described in drylands (Roldán and Albaladejo 1993; Varela-Cervero et al. 2016; Fakhech et al. 2019), and have been related to plant activity (López-Sánchez and Honrubia 1992). Previous studies found high AMF colonisation in spring (Roldán and Albaladejo 1993) generally when plants

sprouted or flowered, and slightly high in autumn (López-Sánchez and Honrubia 1992). Most of the studies on AMF seasonality on drylands only provided hyphal colonisation (hereafter, HC). However, seasonal studies on arbuscular colonisation (hereafter, AC) and vesicular colonisation (hereafter, VC) are required to improve knowledge on AMF activity in nutrient acquisition (Jakobsen et al. 2003).

Nutrient limitation increases in soils with minimal content of clay and organic matter, such as gypseous soils (Casby-Horton et al. 2015). Gypseous are special soils with high gypsum (calcium sulphate dehydrate) content (Herrero and Porta 2000), which frequently occur in drylands around the world (Verheye and Boyadgiev 1997). The high gypsum content of gypseous soils modifies the physical and chemical properties of soils (Herrero et al. 2009). For example, the high solubility of gypsum produces high  $\text{Ca}^{2+}$  activity in the soil solution (Casby-Horton et al. 2015), leading to a decrease in macronutrient availability and plant acquisition, particularly P (Stout et al. 1951). These features of gypseous soils severely limit plant life (FAO 1990). Despite these limitations, gypsum environments host a unique flora, identified as an international conservation priority (Escudero et al. 2015; Ochoterena et al. 2020).

Gypsum plants are adapted to a harsh substrate (Moore et al. 2014), where there is a strong seasonality in water and nutrient availability (Delgado-Baquerizo et al. 2011; Palacio et al. 2017). There are two types of gypsum plants according to their gypsum affinity (Meyer 1986): specialist species (also referred as gypsophiles), and generalist species (gypsovags). Gypsum specialist species are considered edaphic endemics with specific features related to gypseous soils (Duvigneaud and Denaeyer-De Smet 1968). Gypsum specialist species differ from generalist species in their foliar S, Ca and Mg concentrations (Palacio et al. 2007; Merlo et al. 2019), but not in their leaf P and N (Muller et al. 2017; Sánchez-Martín et al. 2021). In addition, plants growing on gypseous soils show low foliar P concentrations (Cera et al. 2021). Previous studies analysed the differences in AMF colonisation between gypsum specialist and generalist species. They found higher AMF colonisation and higher phylogenetic diversity of AMF in roots of gypsum generalist vs. specialist species (Palacio et al. 2012; Torrecillas et al. 2014). However, these studies

were usually performed in spring, and no previous studies have evaluated the seasonality in AMF colonisation in gypsum plants, or the possible links with soil nutrient availability and plant activity and nutrient demands which are seasonal in these ecosystems (Palacio and Montserrat-Martí 2005; Delgado-Baquerizo et al. 2011).

The aim of this study was to evaluate the seasonal changes in plant nutrient status, AMF colonisation and rhizospheric soil nutrient availability and their interaction in five studied plant species, which included both gypsum specialists and generalists. Root colonisation by AMF (accounting for hyphae, vesicles and arbuscules separately), concentration of C, N and P in leaves and of C, N in fine roots and  $\text{P}_{\text{Olsen}}$ , organic matter content, and concentration of nitrate and ammonium in the rhizospheric soil were analysed four times throughout a year. We hypothesised that: 1) All species will display AMF structures (hyphae, vesicles and arbuscules) indicative of AMF colonisation/symbiosis throughout the year, because gypseous soils are remarkably P-improvised; 2) The degree of AMF colonisation will vary seasonally, according to previous studies in semi-arid environments (Varela-Cervero et al. 2016); 3) The seasonality of AMF colonisation will follow plant nutrient content and rhizospheric soil nutrient concentration (especially P), displaying the highest HC and AC in autumn and spring, when nutrient plant concentration will be high, and the highest VC in summer, when both plants and fungi have to cope with the harshest environmental conditions, 4) Generalist gypsum species will show higher HC and AC than specialist gypsum species according to previous studies (Palacio et al. 2012).

## Materials and methodology

### Study site

This study was conducted at one locality in the Middle Ebro Basin (Villamayor, Zaragoza, NE Spain, 41°42'39.2"N 0°44'22.8"W; 295 m a.s.l.), within a sampling area of approximately 3000 m<sup>2</sup>. The main lithology is an extensive area of massive gypsum deposits and gypseous soils with high contents of gypsum (Palacio et al. 2012) with a few thin inserted outcrops of marls and clays (Quirantes 1978; Table 1). The locality has a semi-arid Mediterranean

climate, with an annual average rainfall of 322 mm and a mean annual temperature of 15.5 °C (data from the nearest weather station at Zaragoza 41°37'15''N, 0°56'6''W, between 1981–2010). Vegetation was composed predominantly of shrubs, forbs and grasses, like, *Gypsophila struthium subsp. hispanica* (Willk.) G. López, *Helianthemum squamatum* Pers., *Helianthemum syriacum* (Jacq.) Dum. Cours., *Herniaria fruticosa* L., *Lepidium subulatum* L., *Rosmarinus officinalis* L., *Thymus vulgaris* L., *Plantago albicans* L., *Brachypodium retusum* (Pers.) P. Beauv., *Stipellula parviflora* (Desf.) Röser & Hamasha.

### Sampling design

Five plant species were selected for analysis. All of them were sub-shrubs, which are prevalent growth forms in

gypsum outcrops (Parsons 1976; Martínez-Hernández et al. 2011). They included two Cistaceae: a specialist (*Helianthemum squamatum* Pers.) and its congener generalist (*Helianthemum syriacum* (Jacq.) Dum. Cours.); two Brassicaceae: a specialist (*Lepidium subulatum* L.) and a con-familial generalist (*Matthiola fruticulosa* (L.) Maire); and a Caryophyllaceae specialist (*Gypsophila struthium* Loeffl.).

Five specimens of each species were collected in the same locality at four different times: late autumn (28<sup>th</sup> November 2017), spring (26<sup>th</sup> April 2018), summer (21<sup>st</sup> August 2018) and late autumn (13<sup>th</sup> December 2018). We chose isolated individuals located at least five meters apart from each other. Selected individuals were healthy adult plants with their foliage exposed to full sunlight. We selected spring as the main period of growth, summer as the period of arrested shoot growth (Palacio and

**Table 1** Generalised linear models of mycorrhizal colonisation with gypsum affinity and season as fixed factors, and family and species nested within family as random factors

	Hyphal colonisation <sup>a</sup>		Arbuscular colonisation <sup>b</sup>		Vesicular colonisation <sup>a</sup>	
	Chisq	Pr(> Chisq)	Chisq	Pr(> Chisq)	Chisq	Pr(> Chisq)
Gypsum affinity	0.077	0.779	0.619	0.432	0.006	0.937
Season	10.423	0.015	284.962	<0.001	72.755	<0.001
Gypsum affinity x Season	2.588	0.460	104.096	<0.001	1.630	0.653

<sup>a</sup> Models were fitted to a Gaussian distribution

<sup>b</sup> Models were fitted to a Binomial distribution and weighted by total counts per individuals

**Table 2** Generalised linear models of plant organ concentrations of C, N and P with gypsum affinity and season as fixed factors and family and species nested within family as random factors

	Fine root N <sup>b</sup>		Fine root C <sup>b</sup>	
	Chisq	Pr(> Chisq)	Chisq	Pr(> Chisq)
Gypsum affinity	0.040	0.841	2.012	0.156
Season	18.497	0.004	6.410	0.093
Gypsum affinity x Season	14.242	0.003	1.660	0.646
	Leaf N <sup>a</sup>		Leaf C <sup>b</sup>	
	Chisq	Pr(> Chisq)	Chisq	Pr(> Chisq)
Gypsum affinity	0.919	0.338	2.589	0.108
Season	62.377	<0.001	9.382	0.025
Gypsum affinity x Season	6.958	0.073	2.710	0.439
	Leaf P <sup>a</sup>		Leaf N:P <sup>b</sup>	
	Chisq	Pr(> Chisq)	Chisq	Pr(> Chisq)
Gypsum affinity	0.730	0.393	0.947	0.331
Season	60.377	<0.001	8.049	0.045
Gypsum affinity x Season	9.036	0.029	1.378	0.711

<sup>a</sup> Models were fitted to a Gaussian distribution

<sup>b</sup> Models were fitted to a Negative Binomial distribution

Montserrat-Martí, 2005; Table 2), and autumn as the period with high soil nutrient availability (Delgado-Baquerizo et al. 2011). The autumn harvest in 2017 followed a dry summer (with 79.9 mm of rainfall) and a dry autumn (with only 14.3 mm precipitation; Fig. 1). Contrastingly, the autumn harvest in 2018 followed a wet summer (128.6 mm) and autumn (93.1 mm). We collected complete specimens, with rhizospheric soil attached, placed them individually in polyethylene bags and transported them to the laboratory, where plant tissues were separated from the soil and processed.

### Soil analyses

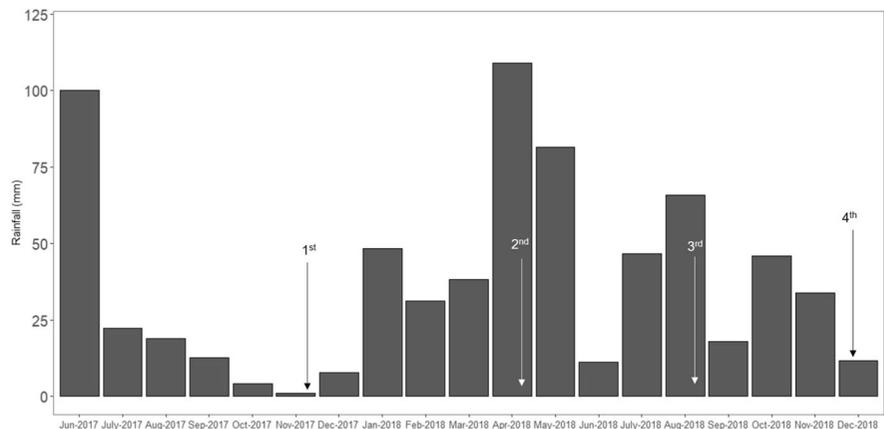
Physical and chemical soil properties were analysed from the five replicates per species collected on every sampling date ( $N=100$ ). Rhizospheric soil, here considered as soil adhered to the root system, was gently separated from the fine roots using dissection forceps, and subsequently divided into two subsamples: one to be sieved at 2 mm and air dried for 2 months at room temperature prior to physical and chemical analyses, and another one to be stored at 4°C prior to extraction with KCl for nitrate and ammonium analyses. Gravimetric soil water content was measured in all soil samples before drying and storage, weighing before and after drying in the oven at 40 °C during five days, this temperature was selected to avoid gypsum de-hydration, which would alter soil water content estimates (Herrero et al. 2009). Dried soils were used to measure the following variables: gypsum content, measured according to Artieda et al. (2006); carbonate content, measured by Bernard calcimetry (Muller and Gatsner 1971); soil texture, determined with a particle laser

analyser (Mastersizer 2000 Hydro G, Malvern, UK); soil pH and conductivity, measured with a pH/conductivity meter (Orion StarA215, Thermo Scientific, Waltham-MA, USA) by diluting samples with distilled water to 1:2.5 (w/v) and 1:5 (w/v), respectively; and available Olsen-P following standard methods (Anderson and Ingram 1989). A subsample of each dried and sieved soil was finely ground using a ball mill (Retsch MM200, Restch GmbH, Haan, Germany) and subsequently used to analyse organic matter following standard methods (Anderson and Ingram 1989). For nitrate and ammonium analyses, 10 g of fresh soil were extracted with 50 mL KCl (1 M). Extracts were shaken and filtered through a filter (7–9 µm pore, 0.160 mm thickness). Ammonium concentration in the extracts was estimated by colorimetry (salicylate method, Kempers and Zweers, 1986). Nitrate concentration was analysed according to Kaneko et al. (2010) as the difference between absorbance between 260 and 220 nm.

### Plant analyses

Leaves and a subsample of fine roots were collected at each harvest, washed and dried to a constant weight at 50 °C for 5 days and subsequently finely ground using a ball mill (Retsch MM200, Restch GmbH, Haan, Germany) to measure P, N and C concentrations. P concentration was determined by vanado-molybdate colorimetry (Becker 1961). N and C concentrations were measured with an elemental analyzer (TruSpec CN, LECO, St. Joseph-MI, USA). N, C analyses were performed by EEZ-CSIC Analytical Services, and P analyses by IPE-CSIC Analytical Services.

**Fig. 1** Diagram of rainfall near the sampling location with indication of sampling times throughout the period of study



## Mycorrhizal colonisation

A subsample of fine roots was separated from each plant, washed in distilled water to remove soil and stored in 50% ethanol at 4 °C. Mycorrhizal colonisation was analysed by cutting the roots into approx. 1 cm fragments and rinsing them in distilled water. Dead and old fine roots were removed under a stereo microscope. Root samples were cleared in 10% KOH for 20 min at 120 °C (5 min longer for some species with very dark roots) as in Brundrett et al. (1996) and stained with trypan blue in lactoglycerol as in Phillips and Hayman (1970). Later, the roots were mounted on glass slides with Hoyer's medium (Cunningham 1972) for examination under the microscope. The proportion of root length containing arbuscules, vesicles and hyphae (i.e. arbuscular (AC), vesicle (VC) and hyphal colonisation (HC)) was calculated under an optical microscope following the magnified intersections method (McGonigle et al. 1990). The average number of total intersections observed ranged between 332 and 405 per individual plant per species and season, in order to obtain high statistical power, especially in the analysis of arbuscular colonisation (Palacio et al. 2012).

## Statistical analyses

All statistical analyses and graphics were performed using R version 4.0.2. The effect of season and gypsum affinity on mycorrhizal colonisation, plant nutrient concentrations and rhizospheric soil characteristics was evaluated using generalised linear mixed models (GLMMs) with season and gypsum affinity as fixed factors and family and species nested within family as random factors. We also analysed the effect of season within each species on mycorrhizal colonisation, plant nutrient concentrations and rhizospheric soil characteristics using generalised linear models (GLMs) with season as a fixed factor. Shapiro–Wilk and Bartlett's K-squared tests were performed to check for normality and homoscedasticity of residuals. Models were run with the *glm* or *glmer* functions (Bates et al. 2007). When residuals were normally distributed, models were fitted to a Gaussian distribution. While when not normally distributed, models were fitted to a: Gamma distribution if data were continuous, had a constant coefficient of variation and variances increased with means (McCullagh

and Nelder 1989); Binomial distribution if dealing with mycorrhizal colonisation (Alvarez-Santiago et al. 1996); and Negative Binomial distribution if data were proportions (McCullagh and Nelder 1989). Dispersion of residuals for data without normal distribution was checked using *simulateResiduals* function in DHARMA package version 0.3.1 (Hartig 2017). If residuals were dispersed, we ran analyses with a Quasibinomial distribution or Binomial distribution weighted by total of intercepts for mycorrhizal colonisation data (Hartig 2017), and with *glm-TMB* (Magnusson et al. 2019) for other variables. When differences were statistically significant, multiple comparisons among levels of fixed factors were assessed with the *glht* function in multcomp package version 1.4–13 in R (Hothorn et al. 2009).

To analyse the relationships among soil features, we performed a Principal Component Analysis (PCA) with the rhizospheric soil features measured underneath each plant using the *rda* function in the vegan package version 2.4–6 (Oksanen et al. 2007).

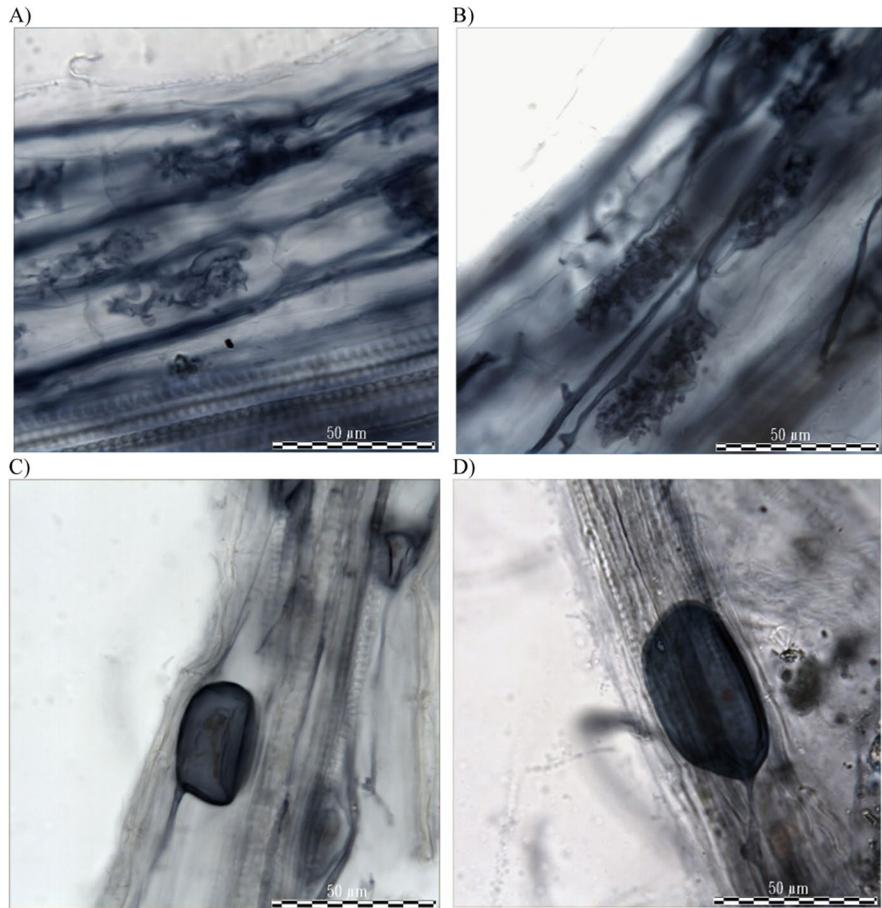
## Results

### AMF colonisation

All species displayed arbuscular mycorrhizal fungi in their fine roots, showing typical structures of arbuscular mycorrhizas (hyphae, vesicles and arbuscules) in all samples throughout the year studied (Fig. 2). The main differences in AMF colonisation were between different families, while individuals from the same species showed similar colonisation (data not shown). A significant effect of sampling time (season) was found for hyphal, arbuscular and vesicular colonisation (Table 2, Fig. 3). Gypsum affinity was not a significant factor affecting AMF colonisation, although we found a significant interaction between gypsum affinity and season for arbuscular colonisation (Table 2, Fig. 3). The highest HC was observed in spring and the lowest in autumn 2018, while the highest VC was in summer. The highest AC was in spring, when gypsum generalists also showed higher AC than specialist species, and the lowest AC was in summer (Fig. 3).

As for the differences in AMF colonisation between seasons for each plant species (Fig. 4), *L. subulatum* did not show seasonality in any AMF

**Fig. 2** Arbuscules in *Gypsophila struthium* (*Caryophyllaceae*) (A) and *Lepidium subulatum* (*Brassicaceae*) (B). Vesicules in *Helianthemum syriacum* (*Cistaceae*) (C) and in *Gypsophila struthium* (D). All structures were recorded in autumn 2017



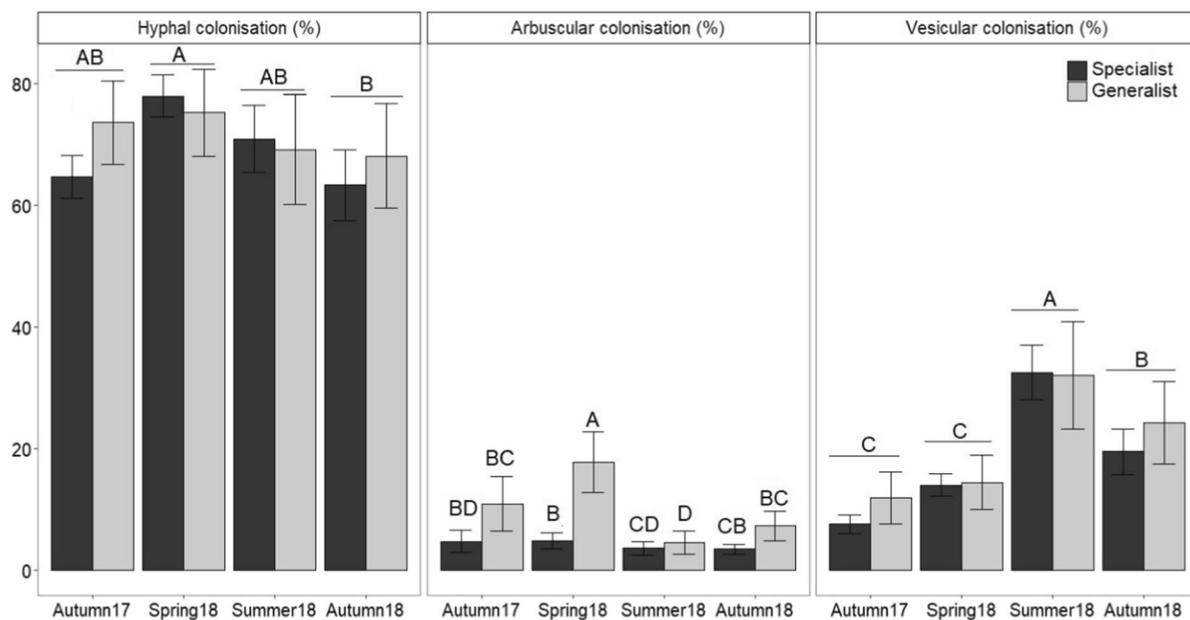
structures, whereas the rest of species showed seasonality in some of the structures (Supplementary Data, Tables A2 and A3). Significant differences in HC were found for *G. struthium*, showing higher values in spring. VC varied significantly among seasons in *G. struthium* and both *Helianthemum* species. Seasonal shifts in AC were significant in *M. fruticulosa* and *H.squamatum*. However, while the trend was to show an increase in AC in spring, *H.squamatum* showed also a peak in AC in autumn 2017.

#### Plant nutrient content

Leaf C, N, P, N:P ratio and fine root N concentration showed significant seasonal variability (Table 3). Gypsum affinity was not a significant factor affecting plant nutrient content, although a significant interaction between gypsum affinity and season was found for leaf P and fine root N (Table 3). Overall, the highest leaf C and N concentrations were found in

autumn, and the lowest in summer (Table 4, Fig. 4). Similarly, the highest P was observed in autumn and the lowest in summer, but specialist species showed higher leaf P than generalist species in autumn 2017 and spring (Table 4). The highest fine root N concentrations were observed in autumn and the lowest in summer (Table 4). Generalist species showed higher fine root N than specialist species in both autumns, and lower values in spring and summer. The highest leaf N:P ratio was in spring, and the lowest in autumn 2017 (Table 4).

When we analysed each species separately (Fig. 4, see GLMs and means with SE for each species in Supplementary Data, Tables A4 and A5), leaf N, C and P concentrations varied seasonally in all species ( $P < 0.05$ ). Species showed similar patterns of seasonal variation for leaf C, N and P, except for *H. syriacum*, with highest leaf N and leaf P in spring, and *Helianthemum* species, with the highest leaf C in summer and the lowest in autumn 2017. In



**Fig. 3** Arbuscular mycorrhizal colonisation in plants with different affinity to gypseous soils between autumn 2017 and autumn 2018. Data are means  $\pm$  SE. Different letters indicate

significant differences among gypsum affinity and seasons after multiple comparisons (Tukey test)

the case of fine roots, *M. fruticulosa* and *L. subulatum* showed significant seasonal differences for fine root C ( $P < 0.05$ ), with the lowest C concentration in spring and the highest in autumn. *M. fruticulosa* also displayed seasonality for fine root N ( $P < 0.05$ ), following general trends. Season was a significant effect for leaf N:P ratio only in *Helianthemum* species ( $P < 0.05$ ).

#### Rhizospheric soil chemical characteristics

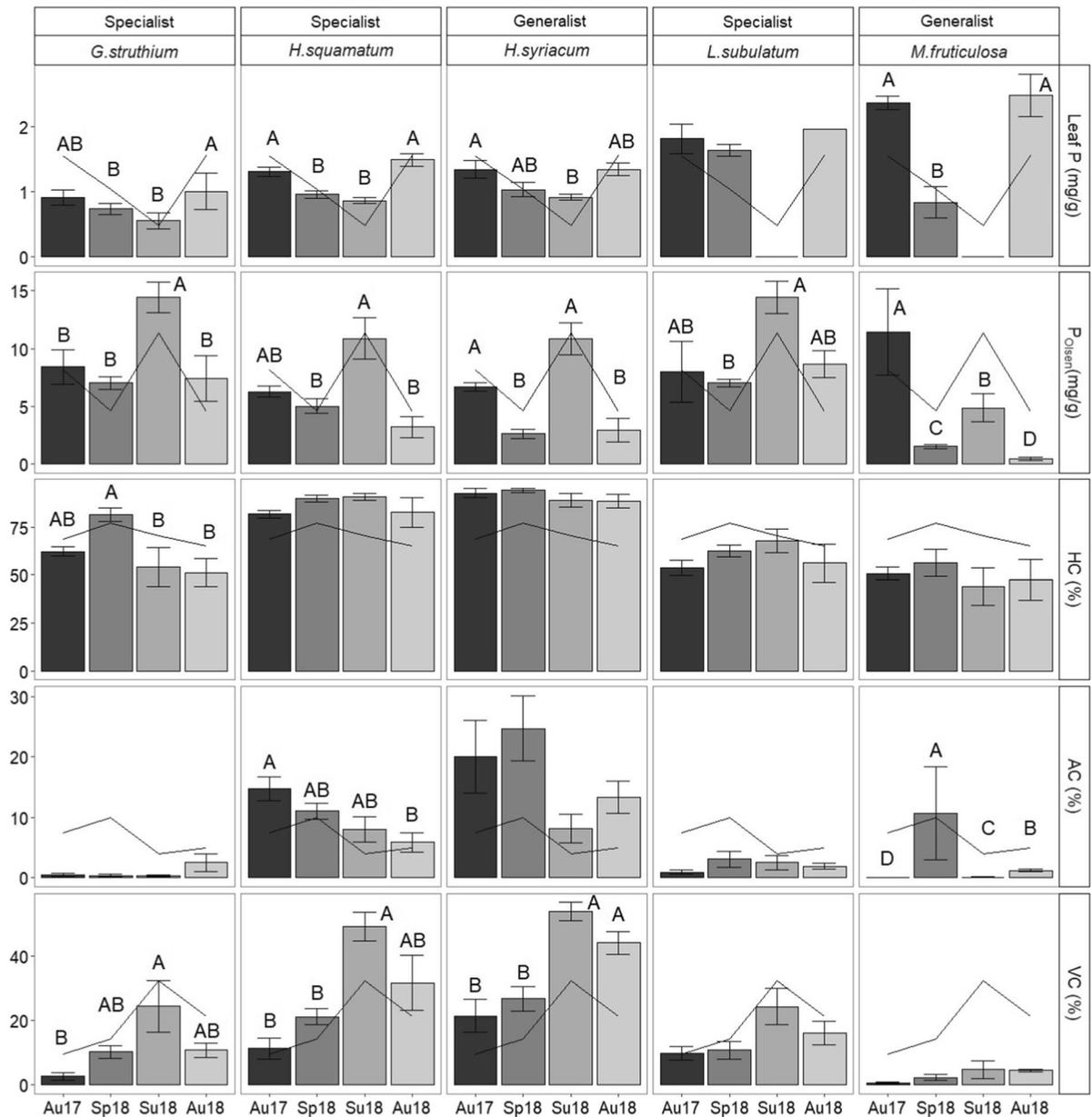
Season was a significant factor affecting all variables measured in the rhizospheric soil, except for organic matter ( $P = 0.312$ ). Gypsum affinity had only a marginally significant effect ( $P = 0.072$ ) for ammonium concentration (Table 4). In general, the highest soil water content was found in spring and autumn 2018, and the lowest in autumn 2017 and summer (Table 5). The highest soil nitrate and ammonium concentrations were in autumn 2018, whereas the lowest nitrate was in autumn 2017 and summer (Table 5). We recorded the highest  $P_{\text{Olsen}}$  in summer, while other seasons displayed similar concentrations (Table 5, Fig. 4). However, the rhizospheric soil collected underneath generalist species showed higher P

content than that of specialist species in all seasons, except in autumn 2018 (Tables 4 and 5).

Rhizospheric soil underneath each species showed different ranges in gypsum content, conductivity, carbonate content and pH (Supplementary Data, Tables A6 and A7). Season was also a significant factor affecting the water content of the rhizospheric soil of all species ( $P < 0.05$ , see Supplementary Data, Tables A6 and A7). All species had different  $P_{\text{Olsen}}$  in their rhizosphere in different sampling dates following the general trend (Fig. 3), except for *M. fruticulosa*, with highest  $P_{\text{Olsen}}$  content in autumn 2017, since they were collected in very low gypsum content (Supplementary Data, Tables A6 and A7). The only species displaying significant seasonal changes for ammonium and nitrate were *G. struthium* and *M. fruticulosa*, respectively (Fig. 4).

#### Discussion

According to our first hypothesis, all gypsum species studied displayed AMF in their fine roots, showing typical structures of arbuscular mycorrhizae (hyphae, vesicles and arbuscules) in all



**Fig. 4** Differences in leaf P, soil P<sub>Olsen</sub> and mycorrhizal colonisation in different sampling dates for each study species. Bars are means with standard errors. Different letters indicate significant differences among seasons within each species (see

GLM in Supplementary Tables) after multiple comparisons (Tukey test). Lines are means for all species. Soil P<sub>Olsen</sub> values of *M. fruticulosa* were divided by 10. HC: Hyphal colonisation. AC: Arbuscular colonisation. VC: Vesicular colonisation

samples throughout the year. In support to our second hypothesis, AMF colonisation was seasonal, since the highest VC was in summer and the highest AC was in spring. Contrary to our third hypothesis, the highest AMF root colonisation did not concur with the highest foliar or lowest rhizospheric soil P

content, but with the time of maximum P demand for plant growth (i.e. the time when leaf N:P ratios were lowest). Finally, in partial support of our last hypothesis, gypsum generalist species showed higher AMF colonisation than specialist species, although only for AC in spring.

**Table 3** Means and standard errors of plant organ concentrations. Different letters indicate significant differences among gypsum affinity and seasons after multiple comparisons (Tukey test) on generalised models with family and species nested within family as random factors

	Autumn 2017		Spring 2018		Summer 2018		Autumn 2018	
	Specialist	Generalist	Specialist	Generalist	Specialist	Generalist	Specialist	Generalist
Fine root C (mg g <sup>-1</sup> ) <sup>b</sup>	466.77 ± 5.53	466.44 ± 3.59	442.50 ± 6.61	454.56 ± 7.22	456.58 ± 6.56	460.50 ± 7.14	451.54 ± 7.76	463.13 ± 3.32
Fine root N (mg g <sup>-1</sup> ) <sup>b</sup>	11.20 ± 1.37 AC	12.81 ± 2.86 A	11.12 ± 1.28 AC	8.79 ± 0.85 BC	10.89 ± 1.62 AC	8.67 ± 1.05 C	13.61 ± 1.30 AB	15.97 ± 3.77 A
Leaf C (mg g <sup>-1</sup> ) <sup>b</sup>	377.20 ± 11.29	409.73 ± 4.06	385.07 ± 13.21	406.70 ± 9.41	362.60 ± 22.76	439.80 ± 1.02	399.38 ± 10.75	419.40 ± 5.78
Leaf N (mg g <sup>-1</sup> ) <sup>a</sup>	24.65 ± 3.46 A	27.22 ± 3.92 A	22.02 ± 2.11 B	19.21 ± 1.20 B	12.05 ± 0.60 C	13.53 ± 0.76 C	28.64 ± 3.24 A	30.66 ± 4.23 A
Leaf N:P ratio <sup>a</sup>	18.61 ± 1.94	14.72 ± 1.05	20.81 ± 1.77	19.23 ± 1.37	19.52 ± 2.74	14.93 ± 0.86	16.55 ± 1.15	16.09 ± 1.40
Leaf P (mg g <sup>-1</sup> ) <sup>a</sup>	1.33 ± 0.13 A	1.80 ± 0.18 AC	1.11 ± 0.11 B	1.04 ± 0.08 BC	0.71 ± 0.08 BC	0.91 ± 0.05 BC	1.44 ± 0.10 A	1.84 ± 0.24 A

<sup>a</sup> Models were fitted to a Gaussian distribution

<sup>b</sup> Models were fitted to a Negative Binomial distribution

Gypsum species showed seasonal differences in AMF colonisation

All five gypsum plant species analysed displayed AMF, with the formation of arbuscules throughout the year. They included *Brassicaceae* and *Caryophyllaceae* species, which are usually cited as non-mycorrhizal families (Brundrett 2009). Colonisation by arbuscules had already been found in *L. subulatum* and *G. struthium* on gypsum (Palacio et al. 2012) and in other taxa of *Lepidium*, *Matthiola* and *Gypsophila* from other environments (Hempel et al. 2013), which calls for caution when assuming the inability of *Brassicaceae* and *Caryophyllaceae* to interact with AMF. Studied species of *Cistaceae* showed the highest hyphal colonisation and *Brassicaceae* showed the lowest, independently of their affinity to gypsum soils. Apart from AMF, we observed Hartig nets typical of ectomycorrhiza fungi in both *Helianthemum* species, although we did not quantify their root colonisation. Gypsum plants in our study also had colonisation of dark septate endophytes, such as those described by Porras-Alfaro et al. (2014) in plants growing on gypseous soils of the Chihuahuan Desert.

Previous studies had reported AMF colonisation in plants from gypseous soils (Alguacil et al. 2009; Palacio et al. 2012; Torrecillas et al. 2014; Hernández y Hernández et al. 2020, but seasonality was neglected and most of these studies were conducted only in spring, when plants show high growth activity (Alguacil et al. 2009). Our results confirm that arbuscular mycorrhizal colonisation in gypsum species varies seasonally, similar to previous studies in other drylands (Roldán and Albaladejo 1993; Varela-Cervero et al. 2016; Fakhech et al. 2019). Most of these previous studies measured the highest hyphal colonisation in spring, but did not account for vesicular or arbuscular colonisation. Our results for arbuscular colonisation agree with those for hyphal colonisation of previous studies. However, these results are not fully comparable, since arbuscules and hyphae differ in functionality. Arbuscules are the unique structures involved directly in nutrient transfer to the plant (Allen 1983; Mullen and Schmidt 1993), whereas hyphae are the vegetative structures of fungi (Brundrett 2009), and vesicles are storage structures (Jakobsen et al. 2003). We observed seasonality in arbuscular (AC) and vesicular colonisation (VC), but not in hyphal colonisation (HC). AC was

**Table 4** Generalised linear model of rhizospheric soil features with gypsum affinity and season as fixed factors and species as a random factor

	Ammonium <sup>a</sup>		Nitrate <sup>b</sup>		Organic matter <sup>a</sup>		P <sub>Olsen</sub> <sup>b</sup>		Water content <sup>b</sup>	
	Chisq	Pr(> Chisq)	Chisq	Pr(> Chisq)	Chisq	Pr(> Chisq)	Chisq	Pr(> Chisq)	Chisq	Pr(> Chisq)
Gypsum affinity	3.23	0.072	0.01	0.913	0.32	0.571	0.95	0.331	1.11	0.293
Season	14.60	0.002	20.57	<0.001	3.57	0.312	97.92	<0.001	148.15	<0.001
Gypsum affinity x Season	5.25	0.154	4.38	0.223	6.04	0.110	54.33	<0.001	2.03	0.565

<sup>a</sup> Models were fitted to a Gaussian distribution

<sup>b</sup> Models were fitted to a Negative Binomial distribution

high in spring, when the highest AM fungal activity is expected in the Mediterranean climate (Alguacil et al. 2009), and low in summer, when plants showed reduced growth activity in our study system (Palacio and Montserrat-Martí 2005). In addition, VC was high in summer, since vesicles appear at later stages of fungal colonisation (Jakobsen et al. 2003) and during arbuscule senescence (Brundrett 2009). AM fungi are not the unique root-associated fungi with seasonal colonisation (Mandyam and Jumpponen 2008), and consequently we also found seasonal colonisation of dark septate endophytes (DSE) between autumn 2017 and spring (data not shown). While the beneficial role of arbuscules formed by AM fungi on plant nutrition is well-established (Johnson 2010), the structures of DSE (hyphae and microsclerotia) cannot be interpreted as interfaces for nutrient exchange between fungi and their hosts (Newsham 2011).

Both gypsum specialist and generalist species showed increased root colonisation by arbuscules during high P-requirements in spring

All plants analysed showed the highest foliar P and N concentrations in autumn, after the peak of P<sub>Olsen</sub> rhizospheric soil concentration in summer, and concurring with maximum nitrate and ammonium concentrations in the soil. Such increased nutrient foliar concentrations were decoupled from arbuscular colonisation, since we observed low root colonisation by arbuscules in summer and autumn. We expected a high arbuscular colonisation when plants demanded P, either autumn or spring, since gypsum are very P-impooverished soils (FAO 1990). For example, gypseous soils led to lower plant growth and lesser P accumulation on leaves than other similar calcareous soils (Cera et al. 2021). Hernández y Hernández et al.

(2020) also found a negative correlation between AMF root colonisation, dissolved organic nutrients in soil and microbial N and P in gypseous soils from the Chihuahua Desert. These results may indicate that, despite the low N and P concentration in gypseous soils, gypsum plants use other acquisition strategies, different to AMF, to uptake P and N, especially when nutrient availability in the soil is high (for example in autumn with high water content). Symbiosis with AM fungi may benefit plants when P demand by the plant exceeds the capacity of the root system to uptake nutrients independently of AMF (Fitter 1991).

In the seasonal environment analysed, most studied species arrested growth in summer and some species, like *Lepidium subulatum* and *Matthiola fruticulosa* are summer deciduous. Gypsum plants restart their growth at the end of summer (Palacio and Montserrat-Martí 2005), probably remobilising nutrients from storage organs (Milla et al. 2005; Palacio et al. 2014) and absorbing nutrients with acquisition strategies not only related to AMF symbiosis, but to phosphatase and organic acid exudation, or enhanced expression of P<sub>i</sub> transporters (Vance et al. 2003; Lambers et al. 2018). All study species but *G. struthium* have shallow roots (Guerrero-Campo et al. 2006), without specialised root architecture to enhance P-mining (Palacio et al. 2012). However, the main root growth in these plants is in autumn (Palacio and Montserrat-Martí 2007), which can favour nutrient uptake. For example, *Lepidium subulatum* shows an opportunistic growth to exploit sporadic N pulses in autumn (Palacio et al. 2014), probably with rapid root proliferation to enhance nutrient acquisition in seasonal environments (Jackson and Caldwell 1989; Palacio and Montserrat-Martí 2007). A decrease in AMF colonisation may occur when P supply by roots is high and plants limit the symbiosis with fungi to

**Table 5** Means and standard errors of rhizospheric soil concentrations. Different letters indicate significant differences among gypsum affinity and seasons after multiple comparisons (Tukey test) on generalised models with species as a random factor

	Autumn 2017		Spring 2018		Summer 2018		Autumn 2018	
	Specialist	Generalist	Specialist	Generalist	Specialist	Generalist	Specialist	Generalist
Ammonium (mg kg <sup>-1</sup> ) <sup>a</sup>	11.07 ± 0.93 B	11.88 ± 0.91 B	9.37 ± 0.84 B	12.45 ± 0.34 B	10.56 ± 0.80 B	12.12 ± 0.48 B	13.65 ± 0.65 A	13.20 ± 0.37 A
Nitrate (mg kg <sup>-1</sup> ) <sup>b</sup>	34.40 ± 5.66 B	35.50 ± 6.02 B	59.69 ± 12.72 AB	40.59 ± 7.10 AB	38.86 ± 6.33 B	40.60 ± 8.43 B	59.74 ± 7.34 A	92.77 ± 18.58 A
Organic matter (mg g <sup>-1</sup> ) <sup>a</sup>	11.35 ± 1.16	11.26 ± 1.40	12.14 ± 1.06	12.92 ± 2.05	10.40 ± 1.19	13.21 ± 2.44	8.05 ± 1.12	13.60 ± 2.53
P <sub>Olsen</sub> (mg g <sup>-1</sup> ) <sup>b</sup>	7.60 ± 0.98 BCD	64.45 ± 23.10 AB	6.33 ± 0.37 CD	8.10 ± 2.19 DE	13.25 ± 0.93 AE	26.36 ± 7.53 AC	5.71 ± 1.03 CD	3.60 ± 0.80 DE
Water content (%) <sup>b</sup>	1.07 ± 0.13 B	1.42 ± 0.17 B	6.04 ± 0.82 A	6.19 ± 1.07 A	1.19 ± 0.20 B	1.27 ± 0.22 B	7.13 ± 1.56 A	10.86 ± 1.27 A

<sup>a</sup> Models were fitted to a Gaussian distribution<sup>b</sup> Models were fitted to a Negative Binomial distribution

reduce associated carbon costs (Lambers et al. 2008). Accordingly, we found that N content in fine roots was high in autumn, indicating high fine root activity (Roumet et al. 2016). In addition, during the wet autumn (2018), species showed higher vesicular colonisation than in autumn 2017 (dry), probably because AMF may not be providing nutrients to the host plants, but keeping them to support growth or storage (Johnson 1993; Koyama et al. 2017). In addition, the inter-annual climate variability observed in these two autumns indicates the importance of studying AMF colonization over different seasons and years.

In spring, the studied species showed high leaf N:P ratio, which indicates high P requirements in leaves and P limitation to primary productivity (Güsewell 2004). At this time of the year, most study species showed a peak in shoot growth rate (Palacio and Montserrat-Martí 2005), flowering in spring and early summer (data not shown), with increased demand for P (Milla et al. 2005). However, such increased demand concurred with decreased P-inorganic (measured as P<sub>Olsen</sub>) availability in rhizospheric soils. It is, hence, not surprising that the highest arbuscular colonisation (AC) was recorded in spring, when plants can benefit from AMF getting extra P than that available to their roots alone.

The gypsum generalist species studied displayed higher arbuscular colonisation than specialist species, although only in spring. This result was similar to another previous study on gypsum outcrops (Palacio et al. 2012), indicating that spring is the most discriminating season to analyse responses in AMF between gypsum generalist and specialist species. According to Palacio et al. (2012) and Torrecillas et al. (2014), specialist species seem to be more specialised to gypsum soils, and to its P cycle and seasonal availability, likely displaying other mechanisms of nutrient acquisition, because they displayed reduced AMF-symbiosis. On the other hand, the dependence of generalist species on AMF symbiosis would indicate a stress-tolerant strategy to cope with the limiting conditions in gypsum environments (Palacio et al. 2012).

## Conclusions

Studied gypsum species showed seasonal AMF colonisation, decoupled from seasonal shifts in foliar N and P content and from shifts in N and P rhizospheric

soil availability. Arbuscular colonisation was higher in spring, when P demand by the plant may exceed the capacity of the root system to uptake sufficient nutrients due to low soil availability. These trends were particularly marked in studied generalist species. Our results exemplify the need to study seasonal changes in plant-AMF-soil interactions to gain insight into P-acquisition strategies in plants growing in nutrient-limited environments.

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