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Phylogenetic relatedness predicts plant–plant nitrogen transfer better than the duration of water scarcity periods

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Summary

- Intermittent water availability is a significant stress factor for plants, particularly in arid and semi-arid ecosystems. Plant nutrient demands often do not align with precipitation pulses that trigger nutrient mobilization and availability, but biotic interactions like plant facilitation (e.g. through nitrogen transfer among distant relatives) and mycorrhizal symbiosis may mitigate this asynchrony, enabling nutrient access despite temporal disparities.
- We conducted a field experiment with 324 plant individuals to test two hypotheses: (1) greater mycorrhizal fungi abundance increases the amount of ^{15}N transferred between plants, particularly under conditions of fluctuating water availability, and (2) the amount of ^{15}N transferred is affected by the phylogenetic relatedness between donor and receiver plants.
- We show that ^{15}N transfer is prevalent in the studied semi-arid communities, occurring between all species pairs in 68% of the trials. Interestingly, we observed an increase in ^{15}N transfer between distantly related species, and this phylogenetic pattern remained consistent across fungicide and water regime treatments, which did not affect ^{15}N transfer.
- Elucidating the drivers of N transfer between plants under different environmental conditions can improve our predictions on how plant communities will respond to future climate challenges, especially prolonged droughts in Mediterranean ecosystems.

Introduction

Intermittent water availability is a significant stress factor for plants, particularly in arid and semi-arid ecosystems where precipitation patterns are highly variable (Noy-Meir, 1973; Collins *et al.*, 2008; Gherardi & Sala, 2015). Arid environments experience long dry periods with small and sporadic rainfall events that trigger the mobilization of nutrients mediated by microbial activity (Huxman *et al.*, 2004), influencing their accessibility to plants. Additionally, natural systems can exhibit complex responses to water availability, with varying sensitivity depending on the timing and magnitude of rainfall events (Austin *et al.*, 2004; Schwinning & Sala, 2004; Post & Knapp, 2020). Considering the predominance of drier conditions in recent decades in the Mediterranean (Sousa *et al.*, 2011), it is relevant to understand how periods of limited water availability affect plant–plant interactions in natural communities and how biotic interactions may mitigate these impacts.

The majority of plant species form associations with arbuscular mycorrhizal fungi (AMF), which provide host plants with mineral nutrients and water in exchange for photosynthetic products (Smith & Read, 2008). The structure of AMF mycelium allows access to nutrients beyond the reach of plant roots (Smith *et al.*, 2000), enhancing nutrient uptake and promoting plant growth, especially in low-nutrient conditions (Meng *et al.*, 2015). Fungi are able to make use of nutrients at higher temperatures and lower water potentials than plants, making them significant drivers of processes in arid environments (Collins *et al.*, 2008). As a result, the mycorrhizal symbiosis can increase in plant desiccation resistance during dry periods and allow water and nutrient uptake during pulses of water availability (Allen, 2007). The high variability of water and nutrients in arid ecosystems is highly relevant as it conditions plant performance and consequently plant–plant interactions (Collins *et al.*, 2008; Gherardi & Sala, 2015). Despite the known relevance of plant–microbial interactions in arid and semiarid ecosystems, it is less clear to what extent they reduce variation in nutrient and water availability for plants. When pulses of resource availability are spaced out over time, plants may benefit from accumulating nutrients during these pulses to use or redistribute during unfavorable conditions, reducing reliance on future precipitation. In this context, the extent to which plant–microbial interactions might affect how plants access resources when rainfall is not synchronized with their nutritional needs deserves further investigation.

Additionally, plants can transfer resources among them. While the transfer of carbon among plants is under debate (Klein *et al.*, 2023), the transfer of nitrogen (N), water, and phosphorus is well documented (Newman & Eason, 1993; Egerton-Warburton *et al.*, 2007; Walder *et al.*, 2012; Ren *et al.*, 2013; Teste *et al.*, 2015; Fernandez *et al.*, 2020; Luo *et al.*, 2023). This transfer can occur directly via root exudates or through fungal networks

(Egerton-Warburton *et al.*, 2007; Li *et al.*, 2009; Meng *et al.*, 2015; Teste *et al.*, 2015; Montesinos-Navarro *et al.*, 2016, 2017; Montesinos-Navarro, 2023) that can mitigate the stress of neighboring plants during drought. On one hand, plants can release essential nutrients by root exudates, investing up to 20–40% of their photosynthetically fixed carbon (Whipps, 1990). Root exudates can contain ions, inorganic acids, and water but mainly consist of carbon-based compounds (Uren, 2000; Bais *et al.*, 2006). The latter can either be low-molecular-weight compounds, such as amino acids, organic acids, sugars, phenolics, and secondary metabolites, or high-molecular weight compounds like proteins (Badri & Vivanco, 2009), many of which are rich in N. On the other hand, N transfer between plants can also be mediated by mycorrhizal mycelium. The mycelia of mycorrhizal fungi can spread to the roots of nearby plants, forming common mycorrhizal networks that enable the transfer of resources both within and between different plant species (Newman, 1988; Simard & Durall, 2004; Selosse *et al.*, 2006). This resource transfer through mycorrhizal fungi can promote seedling growth and survival (Teste *et al.*, 2009; Booth & Hoeksema, 2010; Bingham & Simard, 2012). There are complex ways in which mycorrhizal fungi can affect the outcome of plant–plant interactions (Montesinos-Navarro *et al.*, 2018), and their impact can become more unpredictable with fluctuating water and nutrient availability.

Phylogenetic relationships between plants can influence both mycorrhizal-mediated facilitation and nutrient transfer between plants driven by N gradients. In terms of mycorrhizal facilitation, phylogenetically diverse plant neighborhoods can enhance the phylogenetic diversity of mycorrhizal fungi in the shared rhizosphere, considering that the different plant species involved in facilitative interactions can contribute to contrasted mycorrhizal communities (Montesinos-Navarro *et al.*, 2012). In turn, fungal richness and phylogenetic diversity can enhance plant biomass and promote plant facilitative interactions (van der Heijden *et al.*, 1998; Wagg *et al.*, 2011). As for the influence of plant phylogeny on nutrient transfer, two equally plausible scenarios could be proposed in this context. On one hand, as closely related plant species may share more fungi (Meng *et al.*, 2023), inter-plant N transfer through fungal connections may be enhanced between them. On the other hand, distantly related plant species may segregate or even complement their resource use, resulting in an increase of N availability in the soil that can affect N transfer. In addition, as N content is phylogenetically conserved, N gradients can be steeper between distantly related plant species, further encouraging nitrogen transfer (Montesinos-Navarro *et al.*, 2017). Plant associations with different types of mycorrhizal fungi are to some extent phylogenetically conserved, with some lineages of plants tending to associate with arbuscular, ecto-, ericoid, or orchid mycorrhiza, respectively (Meng *et al.*, 2023). As a result, the co-occurrence of phylogenetically diverse plant species with different mycorrhizal types can influence N availability, given that arbuscular- and ectomycorrhizal associated plants often interact differently with soil microorganisms responsible for soil N mobilization (Du *et al.*, 2024). These differences in N dynamics can shape nutrient transfer among plants, as it tends to occur following source-sink gradients (Bethlenfalvay *et al.*, 1991; Ren *et al.*, 2013; Kravchenko *et al.*, 2021) influenced by both N availability and plants' uptake. Since the foliar N content is phylogenetically conserved among plant species, ecosystems governed by legume–nursery facilitation have shown a higher N transfer between phylogenetically distant plants (Montesinos-Navarro *et al.*, 2017). However, in other ecosystems with lower legume abundance, other factors, such as species-specific temporal N demands driven by phenology, can also impact the N content of neighboring plants and affect N transfer between plants (Montesinos-Navarro, 2023).

In this study, we measured ^{15}N transfer between pairs of plants with varying degrees of phylogenetic relatedness and assessed the role of fungi in plant–plant N transfer under two contrasting regimes of water availability. Considering that fungi are able to connect plants' roots (e.g. Egerton-Warburton *et al.*, 2007) and access nutrients at lower water potentials than plants (Collins *et al.*, 2008), we hypothesize that greater mycorrhizal fungi abundance will significantly increase the amount of ^{15}N transferred between plants, especially under conditions of variable water availability. Additionally, considering that mechanisms have been proposed to promote nitrogen transfer in both closely (Meng *et al.*, 2023) and distantly (Montesinos-Navarro *et al.*, 2017) related plants, we hypothesize that the phylogenetic distance between plants will affect ^{15}N transfer. Understanding whether mycorrhizas affect plants' stress tolerance by influencing inter-plant N transfer during water and nutrient scarcity can improve our predictions on how plant communities may respond to potentially larger periods of drought in Mediterranean ecosystems.

Materials and Methods

Study site

The study was conducted in a set of three gypsum outcrops, located < 25 km apart within the Vinalopó Valley, Alicante (38°29′N, 0°44′W; elevation: 568 m) in southeastern Spain. In this semiarid region, the mean temperature is 16 ± 6°C, ranging from a minimum of −2.4°C in January to a maximum of 39.5°C in August, and the average annual rainfall is 365 mm, most of which falls during spring and autumn, with scarce and irregular rainy periods during the rest of the year (365.9 ± 97.7 mm) (Delalandre & Montesinos-Navarro, 2018; Montesinos-Navarro, 2023). These soils are characterized by a slightly alkaline pH (7.91 ± 0.06), very low phosphorus content (0.005 ± 0.0006%), low total nitrogen (0.06 ± 0.01%), and low total organic material (1.14 ± 0.11%). They also contain moderate carbonate content ($CaCO_3$ 9.59 ± 1.74) and high sulfate concentration (1646 ± 9.6 ppm). The vegetation is a xeric shrubland community dominated by small shrubs and chamaephytes forming vegetation patches that occupy 20% of the vegetation cover, surrounded by 80% of bare ground. Some of the common species in these environments belong to different families, such as Cistaceae (*Fumana ericoides* (Cav.) Gand. in Magnier, *Helianthemum squamatum* (L.) Dum. Cours., *Helianthemum syriacum* (Jacq.) Dum. Cours.), Poaceae (*Stipa*

parviflora Desf., *Brachipodium retusum* (Pers.) Beauv.), and Lamiaceae (*Thymus vulgaris* L., *Thymus moroderi* Pau ex Mart. Mart., and *Teucrium libanitis* Schreb.) (Delalandre & Montesinos-Navarro, 2018; Montesinos-Navarro, 2023). The vegetation is distributed in patches *c.* 28 cm in diameter (n = 69, maximum diameter = 28.3 ± 1.3 cm, mean ± SE), typically with no more than four plant species per patch (98% of the 69 patches) and generally one individual per species (97% of the cases) (Delalandre & Montesinos-Navarro, 2018). On average, the closest distance between two vegetation patches is relatively small (35.1 ± 2.9 cm).

Several features make this study site suitable for addressing the proposed hypotheses. First, the prevalence of water stress, caused by low and intermittent water availability, can enhance the benefits of interplant nutrient exchange. Second, the patchy vegetation structure allows identifying the proper scale for assessing plant–plant interactions and studying how these interactions are influenced by water stress. Finally, in similar gypsum systems in southeastern Spain, many co-occurring plant species share some AMF species, although there are also some specificities showing distinct fungal communities between edaphic specialists and generalists (Torrecillas *et al.*, 2014).

Species and unit selection

Four focal species of chamaephytes with a similar size were selected to ensure that the effect of the treatments on their biomass was comparable. The species belong to two families: Cistaceae (*H. squamatum* and *H. syriacum*) and Lamiaceae (*T. moroderi* and *T. libanitis*). Individual plants of these four species were selected in a paired design (hereafter experimental unit).

Each experimental unit included a donor plant, randomly selected to be labeled with ^{15}N, and two conspecific individuals of a given focal species, one growing associated with the donor plant (associated) and the other outside their vegetation patch (solitary), no more than 1.5 m apart. The solitary plant served as a spatial control, where no ^{15}N transfer was expected (Supporting Information Fig. S1). In order to avoid confounding effects from variation in size, the units were selected so that the two paired conspecifics (growing associated vs solitary) had similar sizes. The height and diameter of the two conspecifics in each experimental unit were measured at the beginning of the study, and this comparison was verified in advance (r = 0.46; P < 0.0001; df = 157).

A total of 162 experimental units were sampled, 40 of which were of *H. squamatum*, 40 of *H. syriacum*, 41 of *T. moroderi*, and 41 of *T. libanitis*. To complete the design balance, we searched for units across three nearby gypsum outcrops of *c.* 1 ha each, all of which were relatively homogeneous and flat. Two of the outcrops had units of all the focal species, one with 22–28 units per species and the other with 3–17 units per species. The last outcrop had 9–14 units of three out of the four focal species.

Field experiments

Two field experiments were conducted to (1) assess the effectiveness of the fungicide and the duration of its effect (pilot experiment) and (2) test the effect of the fungicide and the water regime on N transfer (main experiment).

Pilot experiment In February 2022, we started two pilot experiments to test fungicide effectiveness. We applied the same fungicide treatments used in the main experiment to other plants and then measured the reduction in AMF root colonization.

We used the fungicide Iprodione (Rovral 50 P.H., Philadelphia, PA, USA), which has been shown to effectively eradicate fungi, especially AMF, without impacting soil insects and bacteria (Gange *et al.*, 1990; Ganade & Brown, 1997; Hernández-Dorrego & Mestre-Parés, 2010; see also https://www.lebanonturf.com/education-center/biological-plant-treatments/fungicide-effects-on-mycorrhizae). The fungicide was applied by technicians with phytosanitary products handling licenses, diluted in 2.0 g l^{-1} of water using *c.* 1.5 l per vegetation patch (25 cm^2) in each application. The fungicide was applied using inverted containers placed in the soil following Sortibran *et al.* (2019).

In one set of plants, we sampled fine roots of 40 individuals (five individuals per species) before (t_0) and 40 d after (t_1) three applications of the fungicide (conducted 1, 14, and 34 d after the first root collection). In order to isolate the effect of the fungicide from the water required to dissolve it, on the same dates, another set of 96 plants received three treatments: (1) fungicide (dissolved in water); (2) only water; and (3) no treatment (randomly assigning eight individuals for each of the four species to each of the three treatments, 8 × 4 × 3 = 96). In this case, individuals across the three treatments were sampled once, 67 d after the first application (Fig. 1). Out of the 136 root samples collected, in seven of them it was not possible to extract enough root tissue to estimate colonization; thus, the final sample size for the pilot experiment, including the two sets of plants, was 129 plants.

Fine roots were sampled from each individual cutting, 20 fragments, 2 cm in length per plant, stored in 50% ethanol until they were processed in the laboratory. Roots were cleared in a 10% aqueous solution of KOH (w/v) for 10 min under pressure at 120°C, washed with 10% H_2O_2 and acidified with 1% HCl (v/v) and stained with 0.05% trypan blue (w/v) in lactoglycerol. The percentage of root colonization was estimated following Phillips & Hayman's (1970) method.

Main experiment A factorial design was used, combining the two treatments (Table 1): a reduction of mycorrhizal fungi and contrasting water supply regimes. We assigned 10–11 experimental units per focal species in each combination of the 2 × 2 factorial design (i.e. two fungicide treatments and two water regimes). For the fungicide treatments, half of the units had natural AMF colonization (80 control units), and the other half had fungicide application to reduce AMF colonization (82 units with fungicide; 162 units in total). For the water regimes, half of the units (83) received 1 l of water weekly (multi-pulse), while the other half (79) received the same total amount in a single application (mono-pulse). The treatments were applied simultaneously to all conspecifics (within 2–3 d). The fungicide treatment was applied on the 23 February, 8 March, and 29 March 2022. After

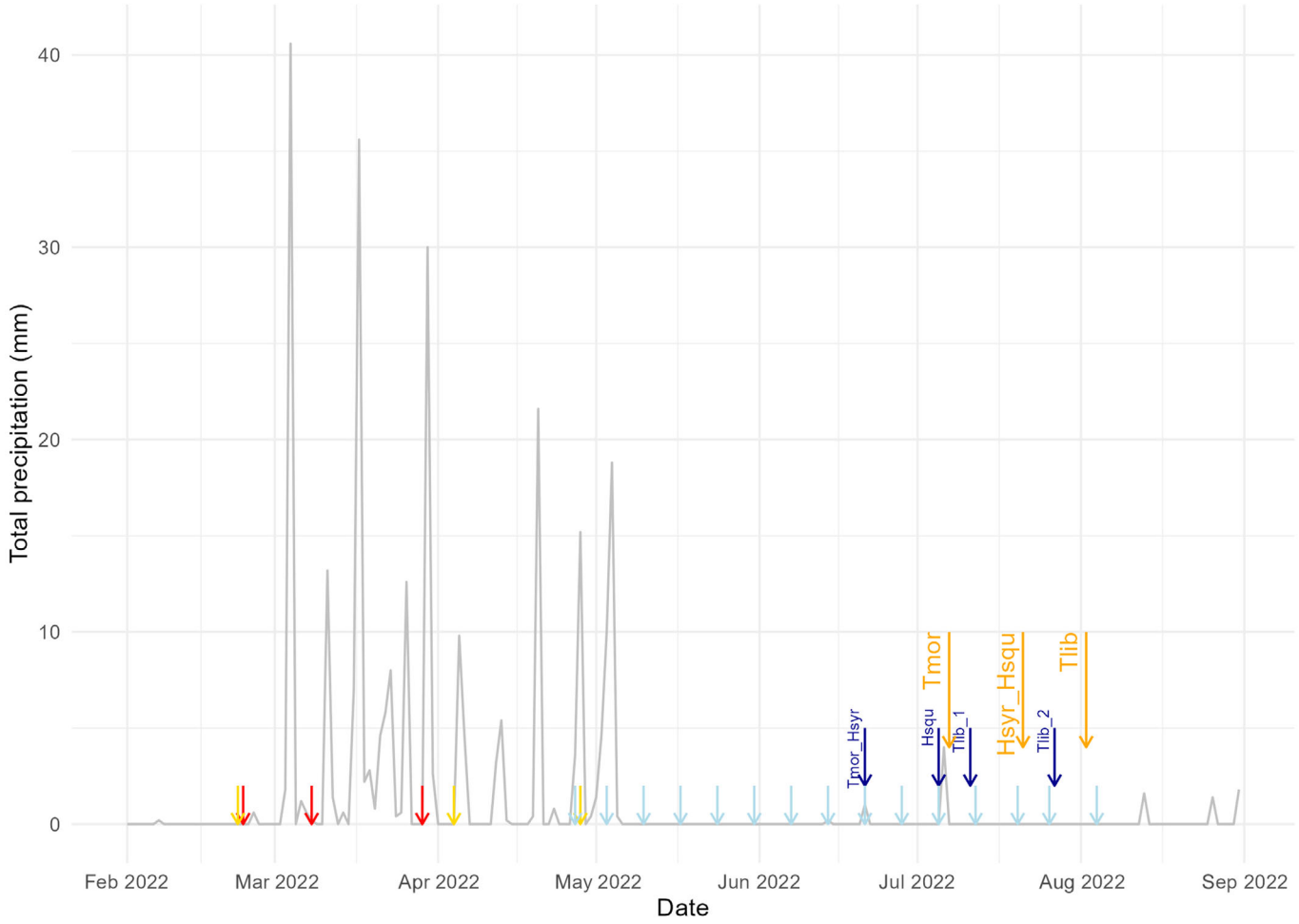

Fig. 1 Natural precipitation and treatments applications. Representation of the natural precipitation (total millimeters of precipitation in grey) during the year of the study (February to August 2022) and the dates of application of all the treatments. Blue arrows indicate the application of water treatments (dark for mono-pulse and light for multi-pulse), red arrows indicate the application of fungicide, yellow arrows indicate dates when roots were collected in some plants of the pilot experiment to quantify the percentage of mycorrhization, and large orange arrows indicate the application of ^{15}N to donor plants of the species that were flowering at that time. The codes indicate which focal species were treated in each application, depending on their phenology: *Thymus moroderi* (Tmor), *Helianthemum syriacum* (Hsyr), *Helianthemum squamatum* (Hsqu), and *Teucrium libanitis* (Tlib).

a month, in late April, the multi-pulse water applications started (Fig. 1). It is relevant to emphasize that extending fungicide applications beyond that point was not feasible since it requires dissolving the fungicide in water, which would have interfered with the mono-pulse water application used in half of the experimental units.

Fungicide treatment. In those experimental units that received fungicide (fungi (−) = reduced fungi abundance), the application and concentration of the fungicide were done using the same procedure described in the pilot experiment (Fig. 1). The rest of the experimental units received the same amount of water with no fungicide (fungi (+) = natural fungi abundance), simulating the addition of the water needed to dissolve the fungicide in the other treatment.

Water treatment. All experimental units received the same amount of water but with different supply regimes: in the multi-pulse supply, the soil experienced shorter periods of desiccation between water applications (Table 1), and in the mono-pulse supply, the soil had longer intervals of desiccation. In order to decide the total amount of water supplied, we based it on the precipitation records provided by the online database of AVAMET (https://www.avamet.org/mxo-mxo.php) for the study site. The difference between the average precipitation from the last 50 yr and the average rainfall of the wettest year on record was used in order to simulate a realistic supply of water. In the multi-pulse water supply regime, plants received 1 l of water weekly throughout their annual growing season until their peak of flowering. In the mono-pulse water supply regime, the units received the same total amount in a single application, trying to overlap with natural precipitation, if it occurred. In this case, the water application was at different times across species, attending to each species' phenology to ensure that every species received it at the same phenological stage, at its peak of flowering (Fig. 1). For *T. libanitis*, which flowers in the beginning of August,

Table 1 Factorial design to assess the combined effects of water and fungi treatment on plant ^{15}N transfer.

	Multi-pulse water supply regime (83 units)	Mono-pulse water supply regime (79 units)
High soil fungal abundance (80 units)	41 experimental units (10–11 per 4 species) × 2 individuals/experimental unit = 82 samples	39 experimental units (10–11 per 4 species) × 2 individuals/experimental unit = 78 samples
Low soil fungal abundance (82 units)	42 experimental units (10–11 per 4 species) × 2 individuals/experimental unit = 84 samples	40 experimental units (10–11 per 4 species) × 2 individuals/experimental unit = 80 samples

High soil fungal abundance treatment represents control treatments where mycorrhizal fungi abundance was manipulated, while low soil fungal abundance treatment has received fungicide application. The mono-pulse water supply regime is more dispersed over time (i.e. longer time lapses between application/pulses) in contrast to the multi-pulse water supply regime, which occurs more consistently over time (i.e. fewer gaps between pulses, more consistent soil moisture). The numbers show the number of replicates (i.e. individuals sampled) per treatment and the number of experimental unit ('units') in which they were located. Each experimental unit included a donor plant, randomly selected to be labeled with ^{15}N, and two conspecific individuals of a given focal species, one growing associated to the donor plant and the other outside their vegetation patch, no more than 1.5 m apart. The four focal species studied are *Thymus moroderi*, *Helianthemum syriacum*, *Helianthemum squamatum*, and *Teucrium libanitis*.

considerably late compared to the rest of the species, the mono-pulse water supply regime was divided into two pulses, to make the potential effects of the water supply more comparable to the rest of the species (Fig. 1). The water was applied using inverted containers placed in the soil to ensure a slow delivery, avoiding water loss through runoff.

^{15}N transfer

^{15}N transfer between plants was quantified in each unit using a ^{15}N-enriched tracer applied to the donor plant (i.e. a plant directly labeled with ^{15}N-enriched urea) and recovered in two conspecifics of a focal species, one associated with the donor in the same vegetation patch (i.e. potential receiver) and another growing solitary within 1 m (i.e. paired control).

In the laboratory, we prepared a solution of 2 g of urea enriched in ^{15}N ($^{13}CH_4{}^{15}N_2O$, 99 atom% ^{13}C, 98 atom% ^{15}N; Cambridge Isotope Laboratories; Sercon Ltd, Cheshire, UK) and 1 ml of surfactant, dissolved in 1 l of distilled water.

After the fungicide and water treatment, in July to August 2022 depending on each species phenology (Fig. 1), we quantified ^{15}N transfer from the donor plant to the potential receiver and its paired control in each experimental unit. Leaves of donor and focal plants were sampled before and 9 d after the tracer application to the donor.

To ensure a systemic absorption of the tracer in the donor tissues, 1 ml of the tracer solution was applied to each plant by placing a leafy branch in a 1.5 ml vial (Sarstedt, Nümbrecht, Germany) containing the solution, secured to the stem and sealed with playdough to prevent evaporation and spillage. The branch was immersed until the solution was absorbed and then was cut to remove the vials without spillage (Montesinos-Navarro, 2023; adapted from Putz *et al.*, 2011; Montesinos-Navarro *et al.*, 2016, 2017). The amount of N in the tracer applied to the donor is three orders of magnitude smaller than typical plant N content, making it unlikely to impact the donor plant's overall N content. The systemic application of the tracer and the short period between measurements (9 d) ensured that the tracer entered the system only through the live tissue of the donor plant, as litter production and root decomposition are minimal during the growing season.

Each foliar sample was dried at 50°C for 3 d. Afterwards, 3 mg of leaf material per plant was weighed and encapsulated into tin capsules (8 × 5 mm; Elementar Americas, New York, NY, USA) for N isotope analysis ($\delta^{15}N$) and total N concentration measurements. The $\delta^{15}N$ analyses were conducted at the University of California, Davis Stable Isotope Facility (SIF), using a PDZ Europa ANCAGSL elemental analyzer interfaced to a PDZ Europa 20–20 continuous flow isotope ratio mass spectrometer (Sercon Ltd). We employ conventional delta (δ) notation to express the isotope ratios of foliar samples, denoted in parts per thousand (‰), in relation to international standards, specifically atmospheric N (N_2). The analytical error for $\delta^{15}N$ measurements is within ±0.3‰.

All $\delta^{15}N$ values were log transformed, and to not alter their signs, multiplied by their original sign. Then, we estimated the amount of ^{15}N transferred from the donor to the receiver plant as the difference in the transformed values of $\delta^{15}N$ (after − before) in the potential receiver. This increase was corrected for that observed in its paired control by subtracting the two increases (i.e. solitary plant (control) − associated (potential receiver)). We only considered ^{15}N transfer when the $\delta^{15}N$ increase in the receiver plant exceeded that of its paired control, which was used as a proxy for the natural variation during this time. Otherwise, ^{15}N transfer was assumed to be zero.

Statistical analyses

First, we describe the patterns of ^{15}N natural abundance observed in the study system. We tested whether the value of $\delta^{15}N$ before the application of the tracer differs among the species using a linear model with $\delta^{15}N$ values as a dependent variable and species as a fixed effect.

Second, using the plants of the pilot experiment, we tested for the effectiveness of fungicide treatment by comparing the percentage of AMF root colonization (1) before and after 40 and 67 d from the first fungicide application, and (2) across three application types after 67 d: no treatment at all, application of water only, and application of fungicide dissolved in water. Two linear mixed models were used with the percentage of AMF colonization as the dependent variable and (1) fungicide application time or (2) application type as a fixed factor, including species as a

random factor and weighted by the number of intersections used in each root sample to assess the percentage of AMF colonization.

Third, we assessed whether the tracer application resulted in an enrichment in donor and potential receiver plants. For donor plants, we used a *t*-test to assess whether the increment in $\delta^{15}N$ after − before the application of the tracer was significantly larger than zero. For the rest of the plants, we conducted two analyses: (1) We tested whether applying the tracer altered the correlation between $\delta^{15}N$ levels in the potential receivers and their paired spatial controls. We expected $\delta^{15}N$ values to be correlated before tracer application due to spatial distribution among nearby conspecifics. However, after tracer application, this pattern might blur due to increased ^{15}N transfer to plants associated with donor plants compared to solitary controls, and (2) we tested whether, overall, the amount of ^{15}N transferred to associated plants was significantly different from that observed in their paired-control plant, by using a paired *t*-test on their respective differences in $\delta^{15}N$ after-before the application of the tracer to the donor.

Finally, we analyzed the patterns of ^{15}N transfer by focusing only on the experimental units where transfer was detected, assuming zero transfer in cases where none was observed. Specifically, we tested whether the phylogenetic relatedness between donor and receiver plants (categorized as across species within genus (i.e. *Helianthemum* spp.), across genera within family, and across families (i.e. Cistaceae, Lamiaceae)) explains the amount of ^{15}N transferred and whether fungicide and water supply regimes influence this pattern. To do so, we fitted a generalized linear mixed model with the amount of ^{15}N transferred as the dependent variable and the ordered category of phylogenetic relatedness, the fungicide treatment, the water regime, and their interaction as fixed factors. In the model we included the species of the receiver as a random factor, and controlled for the leaf N gradient between donor and receiver plants, and the $\delta^{15}N$ in the donor after labeling. As phylogenetic relatedness was considered as ordered, both linear and quadratic fits were tested. We used a generalized linear model with a Tweedie distribution that allows modeling the zero-inflation and a Gaussian distribution of non-zero values simultaneously. We used the CAR (Fox & Weisberg, 2019) and GLMMTMB (Brooks *et al.*, 2017) packages in the R v.4.3.1 (R Core Team, 2023) for fitting general linear models and generalized linear mixed models, respectively, after checking that all the assumptions of normality and homoscedasticity were fulfilled using the DHARMA package (Hartig, 2022). The significance of differences between groups was assessed computing the estimated marginal means and the 95% low and upper confidence interval for the specified factors or factor combinations in each linear model, using EMMEANS package (Lenth, 2024). Figures were drawn with GGPLOT2 (Wickham *et al.*, 2016) using a color-blind palette. All datasets (Datasets S1–S4) and the code to run the analyses (Notes S1) are available in the Supporting Information.

Results

The natural abundance of $\delta^{15}N$ in plants varied among species, with the two Cistaceae (*H. squamatum* and *H. syriacum*) showing lower values than the Lamiaceae (*T. libanitis* and *T. moroderi*) (Fig. 2).

The pilot experiment confirmed the effectiveness of the fungicide treatment, showing that overall, the fungicide reduced the root AMF colonization from 43.6 ± 2.03% to 17.14 ± 0.65% after 40 d, and 23.44 ± 0.56% after 67 d ($\chi^2 = 1857$; df = 2; *P*-value < 0.01). Additionally, the root AMF colonization was higher when plants received no treatment (28.42 ± 1.12%) or only water (32.21 ± 0.55%) than when they received fungicide (dissolved in water) (22.89 ± 0.53%) ($\chi^2 = 332$; df = 2; *P*-value < 0.01) (Fig. 3).

To quantify ^{15}N transfer between plants, we first ensured that all donor plants exhibited positive increments in $\delta^{15}N$ after tracer application (mean increments ± SE = 1187 ± 357; range (0.14–35 965); $n = 153$; $t = 3.32$; $P < 0.001$).

Second, we verified that solitary plants can be used as a spatial control that, while accounting for microenvironmental variation within the sampling unit, do not show signs of enrichment after the application of the tracer. The $\delta^{15}N$ (logged) values in the receiver plants before the application of the tracer exhibited a significant correlation with those of nearby solitary plants ($r = 0.58$; $P < 0.001$; df = 160; Fig. 4). This implies that plants in close proximity spatially tend to share similar $\delta^{15}N$ values. However, the introduction of the tracer disrupted this spatial correlation ($r = 0.07$; $P = 0.37$; df = 152; Fig. 4).

Third, we found that, overall, the relative increment in the receiver plants was significantly different from zero (mean relative increment and 95% confidence interval = 0.45 (0.22, 0.68); $t = 3.91$; df = 153; $P = 0.0001$), indicating that associated plants were, on average, significantly enriched after the application of the treatment. We detected $\delta^{15}N$ transfer between plants in 68% of the units (87% for *H. squamatum*, 72% for *H. syriacum*, 43% for *T. libanitis*, and 70% for *T. moroderi*).

Finally, we tested whether ^{15}N transfer was explained by the phylogenetic relatedness between the donor and the receiver, the fungicide treatment, and the water regime, accounting for the leaf N gradient between both plants and the $\delta^{15}N$ in the donor plant after labeling. Only the phylogenetic relatedness showed a significant, positive, and linear effect on ^{15}N transfer (Table 2). This trend of increasing transference between distantly related plants was consistent across fungicide treatment and water regime that showed no effects on ^{15}N transfer (Fig. 5). The $\delta^{15}N$ enrichment in the donor plant and the leaf N gradient between the donor and receiver plants did not significantly explain the ^{15}N transfer between them (Table 2), likely due to the small gradients of N generated in the species studied, which ranged from −14.5 to 6.8 mg g^{-1}.

Discussion

Using a ^{15}N labeling experiment, we significantly enriched ^{15}N donors after establishing a rigorous spatial and temporal control that allows us to account for natural ^{15}N fluctuations. We detected plant–plant transfer in 68% of the studied pairs.

We found the highest percentage of receiver individuals with evidence of ^{15}N transfer in species from the Cistaceae family

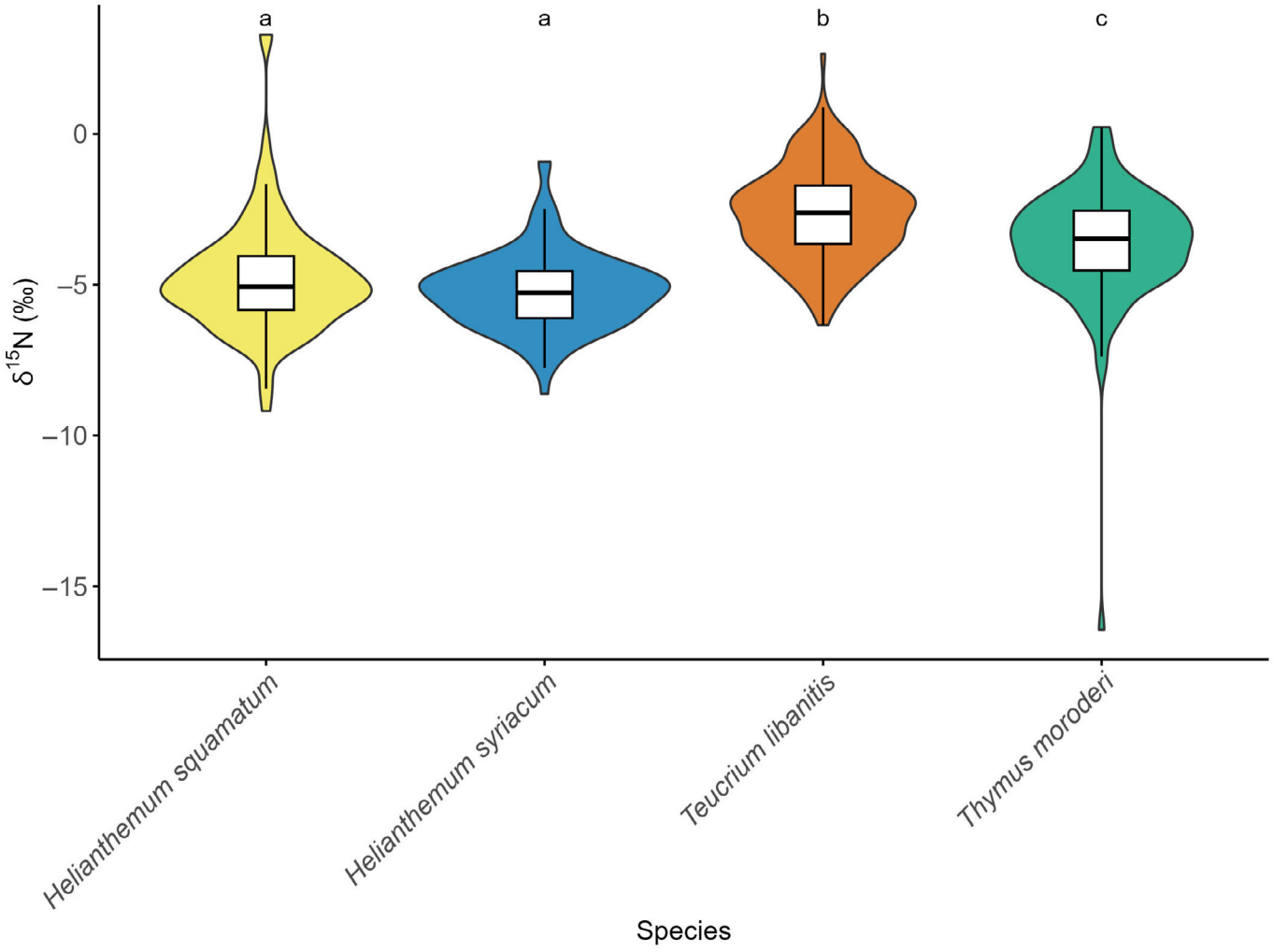

Fig. 2 Differences in $\delta^{15}N$ natural abundance across species. Violin plot showing the natural abundance of $\delta^{15}N$ in the four study species before the application of the tracer. In the boxplots, the box represents the interquartile range (IQR), covering from the 25th to the 75th percentile, with the line inside marking the median. The whiskers extend to the smallest and largest values within 1.5 times the IQR. Different letters are indicative of significant differences in the abundances of $\delta^{15}N$ between species ($P < 0.05$; Tukey tests).

(*H. squamatum* (87%) and *H. syriacum* (72%)), while species from the Lamiaceae family showed lower percentages (*T. moroderi* (70%) and *T. libanitis* (43%)). The amount of ^{15}N transfer between species of different families was higher than between species of the same family or congeneric species, and this is true for any water regime and fungicide treatment. The potential recovery of the mycorrhizal symbiosis since the last fungicide application to the measurement of ^{15}N transfer may have contributed to the observed lack of effect of the fungicide treatment on ^{15}N transfer. However, the impact of the fungicide might not have been limited to immediate effects, and a reduction in AMF levels maintained over time could have influenced N uptake in the previous months, affecting plant N content and gradients, and consequently, plant-to-plant N transfer.

Neighboring plants have been previously shown to have similar values of $\delta^{15}N$ natural abundance (Rascher *et al.*, 2012; Hellmann *et al.*, 2016). This could be due to a spatial distribution of fungal abundance or soil texture, which makes it more feasible for plants to take up N through mycorrhizas rather than directly through their roots. We show that there is ^{15}N transfer between plants living in the same patch, but this transfer is significantly reduced for isolated plants growing a meter apart. This spatial heterogeneity may contribute to the typical patchiness of plant communities structured by facilitation, where islands of fertility are interspersed with barren land (Aguiar & Sala, 1999; Rietkerk & Van de Koppel, 2008). Regarding species variation in $\delta^{15}N$ natural abundance, usually interpreted in terms of species dependence on fungi to obtain N (Dawson *et al.*, 2002), our results indicate that species from the Cistaceae family exhibit lower values than those from the Lamiaceae family. Other studies have also reported lower values of foliar $\delta^{15}N$ in Cistaceae compared to other Mediterranean small shrub species (Saura-Mas & Lloret, 2010), which could be related to the dual association of species belonging to this family with both arbuscular and ectendomycorrhizal symbiosis, despite being taxonomically distant from other species known to form ectomycorrhizae (Malloch &

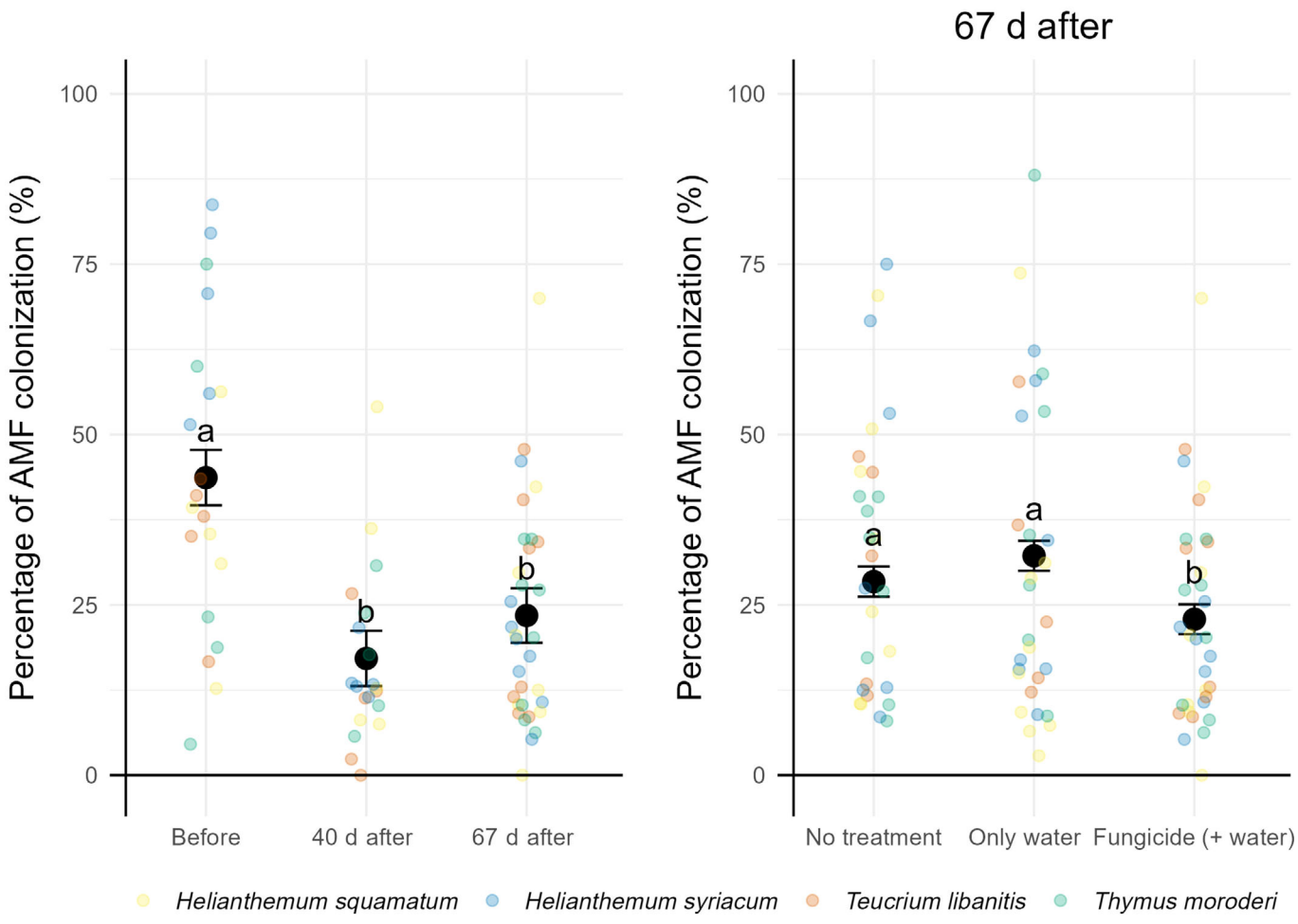

Fig. 3 Effect of the fungicide application in the pilot experiment. Both panels represent the arbuscular mycorrhizal fungi (AMF) colonization percentage in the plant roots from the pilot experiment. Left panel: AMF colonization at three root collection times, before any fungicide application, 40 d, and 67 d after three applications of fungicide. Right panel: AMF colonization at the last sampling time in plants that received no treatment, only water, or fungicide dissolved in water (Fungicide (+ water)). The black points and bars represent the estimated marginal means ± 95% low and upper confidence intervals, while letters denote significant differences in AMF colonization percentages among treatments, based on non-overlap in their 95% confidence intervals. Colors indicate individuals from different species.

Thorn, 1985). The high prevalence of N transfer between all the pairs of species studied here demonstrates that this may be an important process in this community, which aligns with findings from other ecosystems. Similar patterns have been observed in Mexican deserts, where *Mimosa luisana* transferred ^{15}N to 13 out of the 14 spatially associated species (Montesinos-Navarro *et al.*, 2017), and also in ecosystems with nutrient-impoverished soils, such as coastal sand dunes (Laliberté *et al.*, 2012), where nutrients like P and N can be co-limiting (Jalonen *et al.*, 2009; Teste *et al.*, 2015). Other studies have reported that, in managed agroecosystems, the percentage of N in receiving plants, derived from donor plants, can range from 20% to 50%, primarily involving one-way N transfer from legumes to non-legumes (He *et al.*, 2003). However, the impact of ^{15}N transfer on plant fitness in non-managed ecosystems may be even more significant than in managed agroecosystems, suggesting that this process can play an important role, beyond competition, in structuring plant communities and preserving biodiversity.

The phylogenetic distance between plant species has been previously proposed as a predictor of successful nutrient transfer. Specifically, this pattern in which ^{15}N transfer increases with phylogenetic distance between plants has been observed in Mexican desert communities, although limited to interactions between a legume and another species (Montesinos-Navarro *et al.*, 2017). In that context, greater phylogenetic distance was associated with a more pronounced N gradient, ultimately enhancing ^{15}N transfer. In our study, however, we did not observe an effect of the N gradient between the donor and receiver plant on ^{15}N transfer between them. This discrepancy is likely due to the relatively small N gradient between our study species, which is threefold lower than that reported in the Mexican community. Nevertheless, other mechanisms may operate between distant relatives that can also affect N transfer between them. The enhancement of ^{15}N transfer between distantly related species can be directly mediated by mycorrhizal networks or indirectly through plant exudates. In the first case, this may occur because species from

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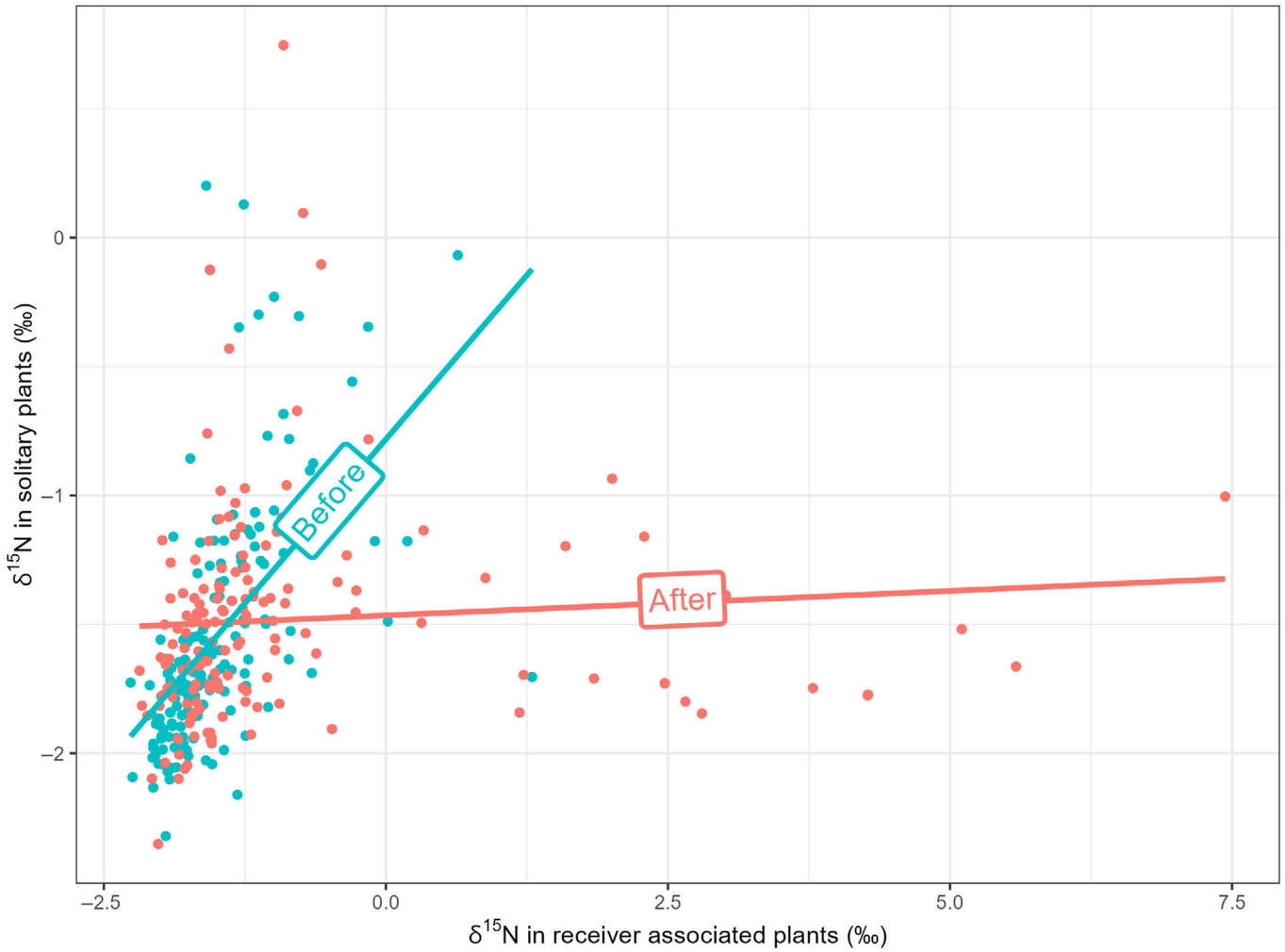

Fig. 4 Evidence for ^{15}N transfer in plants associated to donor plants. The values of $\delta^{15}N$ in spatially close plants growing solitarily and in association with other plants (including a donor) is significantly correlated before the application of the ^{15}N tracer to each donor (blue line 'Before'), but the correlation vanishes afterward (red line 'After'). All $\delta^{15}N$ values were log transformed, and to not alter their signs, multiplied by their original sign. The species considered are *Helianthemum squamatum, Helianthemum syriacum, Thymus moroderi,* and *Teucrium libanitis*.

Table 2 Generalized linear model (GLM) testing the effects of phylogenetic relatedness between the donor and the receiver plant, the nitrogen gradient between both plants, the $\delta^{15}N$ in the donor after labeling, the fungicide treatment, and the water regime on the ^{15}N transfer between plants.

	Estimate	SE	*Z*-value	*P*-value
Intercept	−0.84	0.31	−2.7	< 0.001
Phylogenetic relatedness (linear)	1.07	0.38	2.8	< 0.001
Phylogenetic relatedness (quadratic)	0.22	0.41	0.54	0.59
Fungicide (yes)	−0.13	0.32	−0.39	0.70
Water regime (multi-pulse)	−0.34	0.33	−1.04	0.30
Fungicide × Water regime	0.42	0.45	0.93	0.35
Nitrogen gradient	0.07	0.39	0.18	0.85
Donor δ^{15} N after the labeling	6×10^{-6}	2×10^{-5}	0.22	0.82

Phylogenetic relatedness was coded as an ordered factor grouping donor and receiver plants into three groups from shorter to distant phylogenetic distances: (1) different species within the same genus, (2) different genera within the same family, and (3) different families. Linear ('linear') and quadratic ('quadratic') relationships between phylogenetic relatedness and ^{15}N transfer were tested. The reference level of the fungicide treatment was the absence of fungicide (i.e. the Estimate refers to the increase when the fungicide was present ('yes')), while the reference level for the water regime was the mono-pulse regime (i.e. the Estimate refers to the increase when the regime was 'multi-pulse'). *Z*-value is the Wald statistic used in the GLM to tests the hypothesis that the estimate is zero, and *P*-value indicate the significance of factors in the GLM, with $P < 0.05$ considered significant.

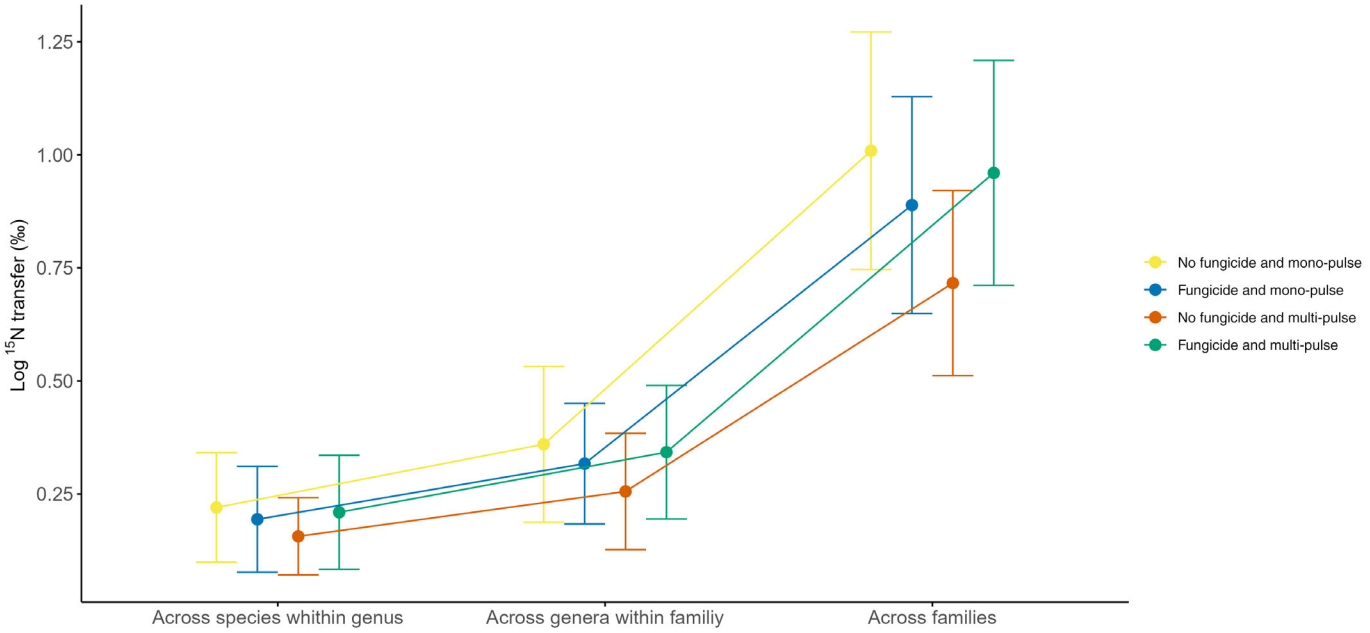

Fig. 5 Amount of transfer of ^{15}N between plants with different phylogenetic distance. Each line shows the effects of the phylogenetic relatedness between the donor and the receiver plant in the transfer of ^{15}N between plants across the fungicide and water regime treatments. The N transfer was measured as the increment (log-transformed) in the $\delta^{15}N$ contained in the receiver plant after the application of the N tracer to the donor plant. The points and bars represent the estimated marginal means ± 95% lower and upper confidence interval. The species considered are *Helianthemum squamatum, Helianthemum syriacum, Thymus moroderi*, and *Teucrium libanitis*.

different families provide functionally complementary fungi to the shared soil (Montesinos-Navarro *et al.*, 2012). However, this phylogenetic pattern is context-dependent, with some studies finding a significant phylogenetic signal in the plant-AMF interaction while others do not (Montesinos-Navarro *et al.*, 2012; Reinhart & Anacker, 2014; Veresoglou & Rillig, 2014). In particular, in gypsum outcrops like the ones studied here, there is some evidence that aligns with this argument. Specifically, it has been reported that species belonging to the Lamiaceae family, such as *T. libanitis*, and Cistaceae, such as *H. squamatum*, share the most common mycorrhizal fungi present in the system, although they also harbor other mycorrhizal fungal taxa in their roots that are found exclusively in *T. libanitis* and *H. squamatum*, respectively (Alguacil *et al.*, 2009), potentially resulting in certain complementarity among them.

Another phylogenetic mechanism, which may or may not involve fungi, is that increased transfer between different plant families could arise from a phylogenetic signal in the resource use timing (e.g. phenology, seasonal growth activity) or the temporal dynamics of root exudate production. This could result in greater temporal complementarity, where receiver species may align their N uptake with the timing of exudate production by the donor species from different plant families. This hypothesis is plausible given that although the exudation of primary metabolites (e.g. amino acids) is diffusion-driven, the efflux of sugars, organic acids, and amino acids can be mediated by specific transporters and efflux channels that are genetically regulated (Badri *et al.*, 2008), potentially resulting in phylogenetic patterns in root exudation. In addition, there is some evidence of phylogenetic conservatism in the exudation of some particular metabolites, although there is no evidence of phylogenetic conservatism of the metabolome composition of exudates (Rathore *et al.*, 2023). In our study, these phylogenetic effects, whereby distantly related plants transfer more ^{15}N to each other, prevailed across fungicide treatments and different water treatments. These results may indicate that distantly related plants are capable of buffering the stress conditions simulated by our treatments by recovering the functionality of fungal communities during the experiment. The ability of distantly related plants to restore soil properties has been previously reported, showing that plant phylogenetic diversity enhances soil microbial biomass, activity, and respiration in gypsum soils (Navarro-Cano *et al.*, 2014).

This work supports the idea that ^{15}N transfer between plants is a prevalent process that can occur among all studied plant species. However, this transfer occurred most frequently toward Cistaceae, which, given their low $\delta^{15}N$ natural abundance (Fig. 2), likely rely heavily on mycorrhizal fungi, suggesting that certain species might be more sensitive to the loss of these interactions than others. Furthermore, the phylogenetic diversity of communities could act as a 'buffer' to prevent the loss of these interactions by maintaining ecosystem functions despite abiotic stress conditions. Considering the projected increase in dry periods in future climate change scenarios, understanding the drivers underlying N transfer between plants under different environmental conditions can help us more accurately predict plant community responses to future climate challenges.

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Competing interests

None declared.

Author contributions

AM-N, SC, CD and MV planned and designed the research. SC and CD performed experiments. AM-N and MV analyzed data and wrote the manuscript.

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Data availability

Datasets S1–S4, Figure S1 and Notes S1 are available in the Supporting Information.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Dataset to conduct analyses related to nitrogen transfer.

Dataset S2 Dataset to conduct analyses related to natural abundance of ^{15}N.

Dataset S3 Dataset to conduct analyses related to the pilot experiment.

Dataset S4 Dataset to conduct plots showing natural precipitation and the times for each treatment application.

Fig. S1 Schematic representation of the experimental design.

Notes S1 Script file containing the R code to run all the analyses and draw the figures in the manuscript.