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Corrigendum

Plant isotopic composition provides insight into mechanisms underlying growth stimulation by AM fungi in a semiarid environment

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The sixth sentence of the abstract contained an error, introduced by the author during the revision process. The correct sentence is:

Shoot $\delta^{18}O$ (a proxy measure of stomatal conductance) was less enriched in AMF-inoculated compared to control *Pistacia* seedlings, indicating enhanced cumulative transpiration in the former.

The third sentence of the sixth paragraph in the Introduction also contained an error. The correct sentence is:

We hypothesised that improved plant water status and enhanced transpiration are key mechanisms involved in plant growth stimulation by native AMF in semiarid calcareous ecosystems, and predicted that shoot $\delta^{18}O$ would be less enriched in shrubs pre-inoculated with native AMF than in control ones.

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Plant isotopic composition provides insight into mechanisms underlying growth stimulation by AM fungi in a semiarid environment

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Abstract. We hypothesised that improved plant water status and enhanced transpiration are key mechanisms involved in plant growth stimulation by native arbuscular mycorrhizal fungi (AMF) in semiarid calcareous soils. Seedlings of the dryland shrubs *Pistacia lentiscus* L. and *Retama sphaerocarpa* L. were pre-inoculated with a mixture of eight native *Glomus* spp. fungi, or left un-inoculated, before transplanting into a degraded site in south-eastern Spain. Pre-inoculated *Pistacia* and *Retama* shrubs grew faster after transplanting, despite spontaneous colonisation of control plants by local AMF. Pre-inoculation enhanced shoot water content and shoot $\delta^{15}N$ in both shrub species. Increased potassium uptake and improved water relations were key mechanisms behind growth stimulation by native AMF in *Pistacia*. Shoot $\delta^{18}O$ (a proxy measure of stomatal conductance) was significantly less negative in AMF-inoculated than in control *Pistacia* seedlings, indicating enhanced cumulative transpiration in the former. In contrast, shoot $\delta^{18}O$ was unaffected by AMF inoculation in *Retama*, a leafless leguminous shrub with photosynthetic stems. Growth stimulation by native AMF in *Retama* was attributed to increased phosphorus uptake, enhanced atmospheric nitrogen fixation and a largely nutrient-mediated improvement of plant water status. Shoot $\delta^{13}C$ was not significantly influenced by AMF inoculation in either shrub species, thus suggesting roughly parallel upshifts in photosynthetic and transpiration rates which did not affect plant water use efficiency.

Additional keywords: *Pistacia lentiscus*, *Retama sphaerocarpa*, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$.

Introduction

Arbuscular mycorrhizal fungi (AMF) can greatly facilitate plant performance in harsh environments by enhancing nutrient acquisition and by increasing resistance to a wide range of abiotic stresses (Allen 1991). However, taxonomically impoverished AMF communities in severely degraded soils may lack effective mutualistic fungal partners capable of ensuring successful re-establishment of native plants (Requena *et al*. 2001; Azcón-Aguilar *et al*. 2003; Ferrol *et al*. 2004). Restoration sites are optimal study locations to study microbial symbioses because they often contain little AMF inoculum (e.g. Allen and Allen 1980; Carpenter and Allen 1988). Non-mycorrhizal plants are rare and anomalous in nature, so instead of comparing mycorrhizal *v.* non-mycorrhizal plants under controlled glasshouse conditions, it may be more informative to examine in the field the ecophysiology of plants at varying levels of AM colonisation, or using different indigenous inocula (Augé 2001). Manipulative field experiments involving different combinations of AMF and plant taxa could shed much light on diverse ecophysiological aspects of the arbuscular mycorrhizal symbiosis.

AMF strains adapted to local climatic and soil conditions are generally better able to survive and improve plant growth than non-native AMF (Requena *et al*. 2001; Ferrol *et al*. 2004). This might be especially true for semiarid ecosystems, as several glasshouse studies have demonstrated that drought-adapted AMF can acquire nutrients in dry soil and alleviate plant drought stress significantly better than non-native AMF (Tobar *et al*. 1994; Ruiz-Lozano and Azcón 1995; Ruiz-Lozano *et al*. 1995; Marulanda *et al*. 2003). However, the degree of compatibility between naturally coexisting plant and AMF taxa is known to vary widely (e.g. Van der Heijden *et al*. 1998). Helgason *et al*. (2002) showed that AMF can altogether fail to form mycorrhizal association with some of their naturally co-occurring AM plant species. Klironomos (2003) demonstrated that plant growth responses to inoculation with naturally co-occurring AMF can range from highly parasitic to highly mutualistic. Besides, plant growth responses to AMF are temporally variable and age dependent, so it may be beneficial for plants to be colonised by different AMF taxa in different seasons or at different developmental stages (Van der Heijden *et al*. 2006). Pre-inoculating transplants with a mixture of AMF taxa isolated from nearby relic patches of native vegetation could ensure good AMF adaptation to local conditions in vegetation restoration projects (Requena *et al*. 2001; Ferrol *et al*. 2004), and might at the same time increase the chance of attaining both optimal

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plant-AMF matches and functional complementarity among fungal strains (Van der Heijden *et al*. 1999; Koide 2000).

Analyses of $\delta^{13}C$ signatures of plant material can help evaluate the physiological response of host plants to AMF colonisation (Handley *et al*. 1999; Querejeta *et al*. 2003, 2006). Plant $\delta^{13}C$ is positively related to intrinsic water use efficiency (WUE), defined as the ratio between photosynthetic rate and stomatal conductance (Farquhar *et al*. 1989). Plant performance in dry environments is often positively associated with WUE (Ehleringer *et al*. 1992; Tsialtas *et al*. 2001). Enhanced nutrient status in mycorrhizal plants can differentially stimulate photosynthesis over stomatal conductance, thus, increasing WUE (Allen *et al*. 1981; Querejeta *et al*. 2003). Further, AMF colonisation creates a 'sink' demand for photosynthates that can lead to an extra 4–26% drain of carbon from the host plant (Miller *et al*. 2002). This increased demand for carbohydrates can directly stimulate a plant's rate of carbon assimilation independently of nutrient effects (Miller *et al*. 2002).

$\delta^{18}O$ in plant biomass provides a time-integrated measure of stomatal conductance and transpiration rate during the growing season (Barbour and Farquhar 2000; Cernusak *et al*. 2003; Jaggi *et al*. 2003; Barbour 2007). At a given air temperature and humidity, plant $\delta^{18}O$ decreases with increasing stomatal conductance and transpiration rate. Therefore, $\delta^{18}O$ complements the use of $\delta^{13}C$ by providing information about stomatal conductance independently of the effects of photosynthetic rate on $\delta^{13}C$ (Scheidegger *et al*. 2000). Querejeta *et al*. (2006) first showed that plant $\delta^{18}O$ analysis can help evaluate the effects of AMF colonisation on the water relations of shrubs growing under semiarid conditions.

Plant $\delta^{15}N$ cannot be reliably used as a tracer of nitrogen source in the field (Evans 2001). However, it does provide a synthesis of the $\delta^{15}N$ of the nitrogen source and isotopic fractionation events that take place during N uptake by mycorrhizal symbionts and during assimilation and translocation of N within the plant. Furthermore, measuring $\delta^{15}N$ in plant material can help assess whether AMF colonisation affects the relative weights of atmospheric nitrogen fixation and soil nitrogen uptake in legume species (Evans 2001). Legumes obtaining most of their nitrogen through atmospheric N_2 fixation usually show $\delta^{15}N$ values around 0‰ (Shearer *et al*. 1983).

In this paper we examine the internal relationships among mycorrhizal, nutrient, isotopic and growth variables in seedlings of two shrub species (*Pistacia lentiscus* and *Retama sphaerocarpa*), with the aim of determining the specific physiological mechanisms involved in host plant growth enhancement by native AMF under semiarid conditions. Plant growth in Mediterranean semiarid calcareous ecosystems is primarily limited by soil water availability (Jonasson *et al*. 1997). We hypothesised that improved plant water status and enhanced transpiration are key mechanisms involved in plant growth stimulation by native AMF in semiarid calcareous ecosystems, and predicted that shoot $\delta^{18}O$ would be significantly less negative in shrubs pre-inoculated with native AMF than in control ones. We further predicted that pre-inoculation with native AMF would enhance plant WUE through differential stimulation of carbon assimilation capacity over stomatal conductance, thus leading to enriched shoot $\delta^{13}C$.

Materials and methods

Study site and target plant species

The study was conducted in the foothills of the El Picarcho range in the province of Murcia, south-eastern Spain (400 m above sea level). The climate in the area is semi-arid Mediterranean with a pronounced rainless season from May to September. A description of the climate, topography, soil and vegetation characteristics in the experimental area was provided in a previous report (Querejeta *et al*. 2006). Two climax plants from semiarid shrublands in south-eastern Spain, namely *Pistacia lentiscus* L. (Anacardiaceae) and *Retama sphaerocarpa* (L.) Boissier (Fabaceae), were selected as target species. *Retama* is a leguminous shrub which develops symbiotic associations with both nitrogen-fixing rhizobial bacteria (*Rhizobium* sp.) and AMF, and the evergreen sclerophyllous shrub *P. lentiscus* is a non-fixer AM species. In *Retama*, leaves are small and have a very brief lifespan (3–4 weeks per year), so carbon is fixed entirely by photosynthetic stems during most of the year (Haase *et al*. 2000).

Mycorrhizal treatments

The mycorrhizal inoculum used in the study was a mixture of native AMF ecotypes isolated from a nearby patch of climax vegetation where the target shrubs grow naturally. The mixture of native fungi included *Glomus geosporum* (Nico. and Gerd.) Walker (EEZ 31), *Glomus albidum* Walker and Rhodes (EEZ 39), *Glomus microaggregatum* Koske, Genma & Olexia (EEZ 40), *Glomus constrictum* Trappe (EEZ 42), *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe (EEZ 43), *Glomus coronatum* Giovannetti (EEZ 44), *Glomus intraradices* Schenk & Smith (EEZ 45) and an unidentified *Glomus* sp. (EEZ 46). The acronym EEZ refers to the AMF collection of the Estación Experimental Zaidín-CSIC in Granada (Spain).

Mycorrhizal inoculum was bulked in an open pot culture of *Sorghum* sp. and consisted of a mixture of rhizospheric soil, spores, mycelia and infected root fragments. Once germinated, shrub seedlings were transplanted into the growing substrate of peat and cocopeat (1 : 1, v : v, Paisajes del Sur, Granada) mixed at a rate of 5% (v : v) with the corresponding AMF inoculum. Non-mycorrhizal treatments received the same amount of autoclaved inoculum together with a filtrate (<20 µm) of the inoculum. Inoculated and non-inoculated seedlings were grown for 8 months under nursery conditions without fertiliser addition.

Experimental design and layout

The experiment was arranged in a randomised block design, with two factors (shrub species and AMF inoculation) and five replicate blocks. The first factor was shrub species (*P. lentiscus* or *R. sphaerocarpa*), and the second factor was inoculation with either the mixture of eight native *Glomus* species, or no inoculation (control). In early January, an area of 1200 m^2 was mechanically prepared by soil ripping. Four rows (1 m wide, 3 m apart) were established in each block, and pre-inoculated or control seedlings of *P. lentiscus* or *R. sphaerocarpa* were randomly assigned to them (in order to prevent 'contamination' of control seedlings by the AMF inoculum). Seedlings within the same row were planted in individual holes 1 m apart from each

other. Fifteen seedlings of each shrub species × mycorrhizal treatment combination were planted per replication block (to a total of 300 plants). The field experiment was conducted under strictly natural conditions, without any watering or fertiliser treatments. Total rainfall during the 12 month duration of the field experiment was ~240 mm.

Sampling and laboratory procedures

Five seedlings of each shrub species × mycorrhizal treatment combination were randomly harvested in the nursery just before field transplanting. Twelve months after outplanting, one seedling per treatment combination was randomly selected and harvested from each replicate block (five seedlings per treatment). During seedling harvesting in the field, damage to roots was minimised by carefully excavating a soil rhizosphere volume of 40 × 40 × 40 cm. Shoot fresh weights were measured in the laboratory within 2 h of seedling harvesting in order to estimate shoot water contents. Dry (65°C, 24 h) mass of shoots was measured afterwards. Shoot water content was calculated as [(fresh weight − dry weight)/fresh weight × 100]. The mean biomass of seedlings harvested in the greenhouse before transplanting was used as the value for initial mass in calculations of relative growth rate (RGR) at time of final harvest (Hoffmann and Poorter 2002), according to the formula [(ln final dry mass) − (ln initial dry mass)/time].

Percent AM colonisation was evaluated as described by Querejeta *et al*. (2006). Three sub-samples from the upper, middle and lower root system of each seedling were sampled, and the percentage of root length colonised by AMF was calculated by the gridline intersect method (Giovannetti and Mosse 1980) after staining with trypan blue.

Only new shoots and leaves produced after field transplanting were used for nutrient and isotopic determinations. Oven-dried plant tissues were finely ground before chemical analysis. After digestion of the leaf samples in nitric-perchloric acid (5 : 3) for 6 h at 210°C, foliar P was measured by colorimetry, foliar N was determined by the Kjeldahl method, and foliar K was estimated by flame photometry as described by Caravaca *et al*. (2003).

Shoot $\delta^{13}C$ and $\delta^{15}N$ were measured at the Stable Isotope Facility of the University of California-Davis on a continuous flow CF-IRMS isotope ratio mass spectrometer (Europa Scientific Hydra 20/20, Chelshire, UK), interfaced with a CN sample converter. The standard for $\delta^{13}C$ was Pee Dee Belemnite, and for $\delta^{15}N$, atmospheric N_2. Shoot $\delta^{18}O$ was measured at the Stable Isotope Facility of the Paul Scherrer Institute (Villigen, Switzerland). Samples were pyrolysed to CO for oxygen isotope analysis (Jaggi *et al*. 2003) with an elemental analyzer (EA-1108, Carlo Erba, Italy) connected to a continuous flow mass spectrometer (DELTA-S Finnigan MAT, Bremen, Germany). The glassy carbon in the pyrolysis tube was replaced every 200 samples to minimise memory effects and every sample was measured twice. The isotope signature is expressed in $\delta^{18}O$, relative to the internationally accepted standard (Vienna Standard Mean Oceanic Water).

Statistical procedures

The effects of plant species and AMF inoculation on the measured variables were evaluated by 2-way analysis of variance. Within shrub species, differences between inoculated and non-inoculated seedlings were assessed by Student's *t*-tests. Correlation coefficients among measured variables were calculated with Pearson's correlation analyses for each shrub species. Statistical procedures were conducted using the software package SPSS 13.0 (SPSS Inc., Chicago, IL).

Results

AMF-inoculated seedlings of *Pistacia* and *Retama* generally showed higher foliar nutrient concentrations than their non inoculated counterparts before field transplanting (Table 1). In addition, mycorrhizal *Retama* seedlings had higher shoot water content than control ones, but plant water status was unaffected by AMF inoculation in *Pistacia*. Despite differences in nutrient status, mycorrhizal and control seedlings did not differ in aboveground shoot biomass at time of field transplanting in either shrub species.

Twelve months after field transplanting, the survival rate of *Pistacia* (80–85%) was unaffected by AM inoculation, but pre-inoculated *Retama* seedlings showed significantly greater survivorship (100%) than control ones (80%; $P < 0.05$). Lower seedling survival rate in the control than in the pre-inoculated treatment may have introduced some small bias in the results obtained with *Retama*, because drought-stress mortality differentially removed the smaller plants within the control treatment (and, therefore, differences between treatments may have been underestimated). Pre-inoculation with the mixture of native *Glomus* fungi affected the AMF colonisation level, nutrient and water status, growth rate and stable isotope composition of seedlings at time of harvest (Table 2). However,

Table 1. Percentage AM colonisation of roots, shoot dry biomass, shoot nutrient concentrations, and shoot water content of 8-month-old nursery grown *Pistacia* and *Retama* seedlings

Means ± s.e. are shown ($n = 5$). Within plant species, significant differences between AMF inoculated and control plants are indicated: *$P < 0.05$

	Pistacia lentiscus		*Retama sphaerocarpa*	
	Control	Mix of native AMF	Control	Mix of native AMF
AM root colonisation (%)	0	86 ± 1*	0	42 ± 4*
Shoot dry biomass (g)	0.29 ± 0.04	0.30 ± 0.09	0.64 ± 0.19	0.48 ± 0.07
Nitrogen (mg g^{-1})	8.9 ± 0.3	13.6 ± 0.6*	12.4 ± 0.9	13.8 ± 0.2
Phosphorus (mg g^{-1})	1.56 ± 0.12	2.22 ± 0.15*	0.64 ± 0.03	1.14 ± 0.05*
Potassium (mg g^{-1})	7 ± 0.4	8.9 ± 0.3*	8.7 ± 0.3	12.6 ± 0.2*
Shoot water content (%)	58.6 ± 1	58.8 ± 0.9	61.4 ± 1.2	64.5 ± 0.8*

Table 2. Percentage AM colonisation of roots, aboveground dry biomass, foliar nutrient concentrations, carbon, oxygen and nitrogen isotope composition, shoot water content and relative growth rate of *Pistacia* and *Retama* seedlings 1 year after transplanting

Means ± s.e. are shown ($n = 5$). Within plant species, significant differences between AMF-inoculated and control plants are indicated: *$P < 0.05$. Results of a 2-way analysis of variance testing the effects of plant species and mycorrhizal inoculation on the measured variables are also shown (P-values presented)

	Pistacia lentiscus		*Retama sphaerocarpa*	
	Control	Mix of native AMF	Control	Mix of native AMF
AM root colonisation (%)	43 ± 16	67 ± 3	8 ± 1	73 ± 5*
Shoot dry biomass (g)	1.0 ± 0.1	4.5 ± 0.6*	3.3 ± 0.5	4.5 ± 0.3
Nitrogen (mg g^{-1})	8.3 ± 0.7	8.4 ± 0.7	16.1 ± 0.6	20.4 ± 0.9*
Phosphorus (mg g^{-1})	0.56 ± 0.02	0.63 ± 0.03	0.29 ± 0.01	0.54 ± 0.07*
Potassium (mg g^{-1})	3.7 ± 0.3	6.2 ± 0.3*	4.8 ± 0.1	6.1 ± 0.2
$\delta^{13}C$ (‰)	−29.9 ± 0.2	−29.1 ± 0.4	−31.6 ± 0.2	−31.5 ± 0.3
$\delta^{18}O$ (‰)	30.2 ± 1.0	26.9 ± 0.3*	35.8 ± 0.6	35.4 ± 0.5
$\delta^{15}N$ (‰)	2.2 ± 0.2	3.3 ± 0.4*	−0.2 ± 0.3	0.8 ± 0.4*
Shoot water content (%)	49.3 ± 0.7	51.7 ± 0.8*	47.8 ± 0.6	52.4 ± 0.5*
Relative growth rate (mg g^{-1} d^{-1})	1.4 ± 0.1	3.2 ± 0.1*	1.9 ± 0.2	2.6 ± 0.1*

	AM colonisation	N	P	K	Water content	$\delta^{13}C$	$\delta^{18}O$	$\delta^{15}N$	Relative growth rate
Shrub species	0.147	0.000	0.001	0.600	0.526	0.000	0.000	0.000	0.893
AMF	0.000	0.010	0.003	0.000	0.000	0.129	0.010	0.060	0.000
Shrub × AMF	0.048	0.011	0.079	0.007	0.121	0.268	0.039	0.813	0.003

the factorial analysis of variance detected significant interactions between AMF inoculation and shrub species for most of the variables measured, indicating that the response to inoculation was plant species-specific (Table 2).

Pre-inoculated *Pistacia* seedlings showed higher shoot K concentration, shoot water content, shoot $\delta^{15}N$, RGR, and aboveground biomass than non-inoculated ones (Table 2). Inoculated *Pistacia* seedlings also exhibited lower shoot $\delta^{18}O$ values than the controls. Shoot N and P concentrations were not significantly affected by AMF inoculation in *Pistacia*, although pre-inoculated seedlings showed higher total N and P contents because they were larger. Shoot $\delta^{13}C$ did not differ significantly between control and pre-inoculated plants. We noted that 43% of root length in non-inoculated *Pistacia* seedlings was mycorrhizal at harvest, indicating rather extensive colonisation by local AMF after transplanting (significant shrub species × AMF interaction, Table 2). Despite of this result, the divergence in growth rate between control and pre-inoculated seedlings was much larger in *Pistacia* than in *Retama* (highly significant shrub species × AMF interaction for RGR in Table 2).

Pearson's correlation analysis showed that RGR in *Pistacia* was positively correlated with shoot K concentration and shoot water content, and negatively correlated with shoot $\delta^{18}O$ (Table 3). Shoot $\delta^{18}O$ was strongly negatively correlated with shoot K concentration, and $\delta^{13}C$ correlated positively with P concentration. Shoot $\delta^{15}N$ correlated positively with shoot water content in *Pistacia*, and was also marginally positively associated with AM colonisation, shoot K concentration and relative growth rate ($P = 0.066$, $P = 0.078$ and $P = 0.076$, respectively).

Pre-inoculated *Retama* seedlings exhibited significantly higher AM colonisation, shoot N and P, shoot water content, shoot $\delta^{15}N$ and relative growth rate than their non-inoculated controls at time of harvest (Table 2). Shoot biomass and shoot K concentration were also marginally higher in pre-inoculated *Retama* seedlings than in the controls ($P = 0.078$ and $P = 0.058$, respectively). Shoot $\delta^{13}C$ and $\delta^{18}O$ were not significantly affected by AMF inoculation in *Retama*.

Pearson's correlation analysis showed that *Retama* growth rate was significantly positively correlated with percent AM colonisation, shoot N concentration and shoot water content (Table 4), and marginally positively associated with shoot P concentration ($P = 0.088$). In contrast with results obtained with *Pistacia*, shoot $\delta^{13}C$ and $\delta^{18}O$ showed no significant correlation with any of the other measured variables in *Retama*. Shoot $\delta^{15}N$ was positively correlated with percentage AM colonisation, and was also marginally positively associated with shoot K concentration ($P = 0.059$) and shoot water content ($P = 0.084$).

Discussion

Percent AM colonisation remained lower in control than in pre-inoculated shrubs throughout the duration the field experiment, despite spontaneous infection of non-inoculated seedlings by local AMF (Table 2). As previously reported by Azcón-Aguilar *et al*. (2003), the taxonomic diversity of the AMF community as well as the abundance of fungal propagules are rather low in the experimental area. Sparse density and patchy spatial distribution of AMF spores and mycelial matrices often compromise colonisation of transplants in degraded soils (Ferrol *et al*. 2004). However, the extent of AM colonisation of non-inoculated seedlings was on average 5-fold higher in *Pistacia* than in *Retama*, indicating a remarkably high degree of host specificity by the local AMF community in this degraded semiarid site (Van der Heijden *et al*. 1998; Helgason *et al*. 2002; Husband *et al*. 2002).

Table 3. Pearson's correlation coefficients among measured variables calculated for control and AMF-inoculated *Pistacia lentiscus* shrubs
$n = 10$ (five control + five pre-inoculated plants) at time of final harvest. RGR, relative growth rate

	% AM	N	P	K	$\delta^{13}C$	$\delta^{18}O$	$\delta^{15}N$	Water content	RGR
% AM	1	−0.245 ($P = 0.495$)	0.152 ($P = 0.674$)	0.486 ($P = 0.155$)	0.389 ($P = 0.267$)	−0.347 ($P = 0.326$)	0.602 ($P = 0.066$)	0.584 ($P = 0.076$)	0.448 ($P = 0.194$)
N		1	−0.061 ($P = 0.866$)	0.032 ($P = 0.930$)	0.009 ($P = 0.979$)	−0.068 ($P = 0.851$)	0.214 ($P = 0.553$)	−0.213 ($P = 0.555$)	−0.009 ($P = 0.981$)
P			1	0.55 ($P = 0.094$)	0.647 ($P = 0.043$)	−0.403 ($P = 0.248$)	0.169 ($P = 0.641$)	0.406 ($P = 0.245$)	0.488 ($P = 0.153$)
K				1	0.484 ($P = 0.156$)	−0.825 ($P = 0.003$)	0.582 ($P = 0.078$)	0.634 ($P = 0.049$)	0.969 ($P < 0.000$)
$\delta^{13}C$					1	−0.504 ($P = 0.137$)	0.200 ($P = 0.581$)	0.541 ($P = 0.107$)	0.524 ($P = 0.120$)
$\delta^{18}O$						1	−0.270 ($P = 0.451$)	−0.495 ($P = 0.146$)	−0.808 ($P = 0.005$)
$\delta^{15}N$							1	0.744 ($P = 0.014$)	0.585 ($P = 0.076$)
Water content								1	0.667 ($P = 0.035$)
RGR									1

Table 4. Pearson's correlation coefficients among measured variables calculated for control and AMF-inoculated *Retama sphaerocarpa* shrubs
$n = 10$ (five control + five pre-inoculated plants) at time of final harvest. RGR, relative growth rate

	% AM	N	P	K	$\delta^{13}C$	$\delta^{18}O$	$\delta^{15}N$	Water content	RGR
% AM	1	0.762 ($P = 0.010$)	0.727 ($P = 0.017$)	0.582 ($P = 0.078$)	0.259 ($P = 0.470$)	−0.147 ($P = 0.685$)	0.668 ($P = 0.035$)	0.857 ($P = 0.002$)	0.777 ($P = 0.008$)
N		1	0.888 ($P = 0.001$)	0.419 ($P = 0.228$)	0.198 ($P = 0.583$)	−0.467 ($P = 0.173$)	0.470 ($P = 0.170$)	0.900 ($P = 0.000$)	0.665 ($P = 0.036$)
P			1	0.182 ($P = 0.615$)	0.341 ($P = 0.335$)	−0.356 ($P = 0.312$)	0.401 ($P = 0.251$)	0.775 ($P = 0.009$)	0.566 ($P = 0.088$)
K				1	−0.273 ($P = 0.446$)	−0.274 ($P = 0.444$)	0.615 ($P = 0.059$)	0.721 ($P = 0.019$)	0.532 ($P = 0.114$)
$\delta^{13}C$					1	−0.383 ($P = 0.275$)	0.013 ($P = 0.972$)	0.017 ($P = 0.962$)	0.413 ($P = 0.235$)
$\delta^{18}O$						1	−0.247 ($P = 0.492$)	−0.336 ($P = 0.343$)	−0.437 ($P = 0.206$)
$\delta^{15}N$							1	0.572 ($P = 0.084$)	0.398 ($P = 0.255$)
Water content								1	0.757 ($P = 0.011$)
RGR									1

Although control and pre-inoculated *Pistacia* seedlings had similar biomass at transplanting, initial differences in nutrient status between treatments may have contributed to faster early growth of pre-inoculated shrubs in the field. Despite substantial colonisation of control *Pistacia* shrubs by local AMF (43%), differences in total N, P, and K content between pre-inoculated and control seedlings increased, rather than decreased, after field transplanting. Total N, P and K contents were, 1.6, 1.5 and 1.3 times greater, respectively, in pre-inoculated than in control *Pistacia* seedlings at transplanting; at harvest, these differences had increased to 4.9, 5.6 and 8.8 times greater, respectively, in pre-inoculated than in control seedlings. Large inter-treatment differences in seedling biomass at harvest indicate that the local AMF community was far less effective than the inoculum obtained from relic patches of climax vegetation for enhancing *Pistacia* growth. Sub-optimal match between *Pistacia* and the local AMF community (Klironomos 2003) or a possible time lag required for the local AMF to colonise the transplants could have both played a role in the comparatively poor performance of control *Pistacia* seedlings in the field.

Unexpectedly, relative growth rate in *Pistacia* was not significantly correlated with either percent AM colonisation of roots, shoot phosphorus or nitrogen concentrations (Table 3). Although shoot N and P concentrations were rather low in both inoculated and control plants (Jonasson *et al*. 1997; Milla *et al*. 2006), values were roughly similar to those recorded in *Pistacia* seedlings that were fertilised with compost during a parallel study conducted in the same experimental area (8.66 and 0.52 mg g^{-1}, respectively; Caravaca *et al*. 2003). Further, RGR in *Pistacia* was strongly positively correlated with shoot K concentration (Table 3). Pre-inoculation with native AMF enhanced shoot K concentration to a greater extent in *Pistacia* than in *Retama* (highly significant shrub species × AMF interaction in Table 2). Plant growth in degraded semiarid calcareous soils is frequently co-limited by multiple nutrient elements (Azcón and Barea 1997). Inoculation with some AMF (mostly in the genus *Glomus*) has been reported to enhance shoot K concentration along with P concentration (Clark and Zeto 2000), often concurrently with improved growth of host plants under drought conditions (Augé 2001; Kaya *et al*. 2003). Azcón and Barea (1997) reported that *Lavandula spicata* shrubs inoculated with *Glomus mosseae* had significantly higher shoot K concentration than their non-inoculated controls when cultivated in degraded semiarid calcareous soils. Potassium is known to play a critical role in the opening and closing of stomata in plant leaves (Humble and Raschke 1971). Stomatal number and aperture size may also be affected by K nutrition (Cooper *et al*. 1967). Potassium deficiency can severely impair plant-water relations (Jordan-Meille and Pellerin 2004), and can lead to stomatal as well as biochemical limitations to photosynthetic activity (Bednarz *et al*. 1998).

The superior ability of the native *Glomus* inoculum (compared with local AMF) to enhance the K status of *Pistacia* appeared to be key in the better performance of pre-inoculated seedlings. Shoot water content in *Pistacia* was uncorrelated with N or P concentration, but correlated positively with K concentration, suggesting an at least partially K-mediated amelioration of drought stress in pre-inoculated seedlings. Shoot $\delta^{18}O$ was strongly negatively correlated with K concentration, which further supports a K-mediated improvement of water relations in pre-inoculated *Pistacia*. Since the $\delta^{18}O$ of plant biomass decreases in response to increased stomatal conductance (e.g. Barbour 2007), we interpret the large inter-treatment difference in $\delta^{18}O$ as an indication that growing season cumulative transpiration was much higher in pre-inoculated seedlings than in control plants colonised by local AMF. AMF species differ widely in their ability to enhance transpiration and to improve the water status of host plants under water-limiting conditions (Allen and Boosalis 1982; Stahl and Smith 1984; Ruiz-Lozano and Azcón 1995; Ruiz-Lozano *et al*. 1995; Marulanda *et al*. 2003; Querejeta *et al*. 2006). Differences in seedling size and rooting depth may have also contributed to the large divergence in $\delta^{18}O$ between control and pre-inoculated plants, as the latter might have had access to less evaporated source water stored in deeper soil (Querejeta *et al*. 2006).

It is crucial to note that, under mesic glasshouse conditions, higher shoot concentrations of N, P and K in AMF-inoculated *Pistacia* seedlings than in control ones did not lead to any significant difference in shoot water content (Table 1). In contrast, *Pistacia* shrubs pre-inoculated with native AMF had significantly higher shoot water content than control shrubs when grown under semiarid field conditions, even though differences in both percent AM colonisation and shoot nutrient concentrations were much smaller at this stage (Table 2).

Shoot $\delta^{13}C$ did not differ significantly between control and pre-inoculated *Pistacia* shrubs, and was uncorrelated with either RGR or shoot $\delta^{18}O$. The only variable that significantly correlated with $\delta^{13}C$ was shoot phosphorus concentration, indicating that improved P status tended to enhance the water use efficiency of *Pistacia* seedlings in this P-poor soil. Improved P nutrition can lead to differential enhancement of photosynthetic rate over stomatal conductance in mycorrhizal plants, thus, increasing WUE (Querejeta *et al*. 2003). According to the conceptual model developed by Scheiddeger *et al*. (2000), the lower $\delta^{18}O$ but similar $\delta^{13}C$ of pre-inoculated compared with control *Pistacia* seedlings indicates that native AMF enhanced both transpiration and photosynthetic rates, but without greatly affecting WUE (Querejeta *et al*. 2003, 2006). The significantly greater difference in shoot $\delta^{18}O$ than in shoot $\delta^{13}C$ between the two treatments suggests that faster growth rate in pre-inoculated *Pistacia* was more the result of reduced stomatal limitation to photosynthesis than of enhanced biochemical capacity to assimilate carbon. Overall, the results obtained with *Pistacia* highlight the critical role that some (but not all) drought-adapted native AMF can play at improving the water relations of co-occurring host plants in drought-prone environments.

Higher shoot $\delta^{15}N$ in pre-inoculated compared with control *Pistacia* seedlings might reflect the greater mean total uptake of soil nitrogen in the former (which was on average 33.1 mg in pre-inoculated plants *v.* 5.1 mg in control ones). The local topsoil (0–20 cm) was enriched in the heavier isotope of nitrogen ($\delta^{15}N_{soil} = 4.6 \pm 0.3‰$, $n = 5$), so greater total uptake of soil N by pre-inoculated seedlings than by control ones likely led to higher $\delta^{15}N$ in the former. Shoot $\delta^{15}N$ in *Pistacia* was positively associated with AM colonisation, shoot water content and shoot K concentration (Table 3). This correlation pattern was very similar to that found for *Retama* (Table 4). The similar

correlation patterns found in a N-fixer and a non-fixer shrub species support the view that high percent AM colonisation may be *per se* a major contributor to the oft-reported $\delta^{15}N$ enrichment in mycorrhizal plants that are grown under drought and low fertility conditions (Handley *et al*. 1999; Wheeler *et al*. 2000; Querejeta *et al*. 2006).

Although biomass and nutrient content of *Retama* seedlings were very similar between treatments at transplanting, total N, P, and K contents were 1.7, 2.5 and 1.7 times greater, respectively, in pre-inoculated than in control seedlings at harvest. Pre-inoculated *Retama* seedlings showed the classical response to AMF colonisation, characterised by greatly improved P status and enhanced growth (Allen *et al*. 2003). In contrast with *Pistacia*, shoot N concentration was strongly enhanced by pre-inoculation with native AMF in *Retama*. (significant shrub species × AMF interaction in Table 2). *Retama* exhibited $\delta^{15}N$ values around 0, which is typical of legumes obtaining their nitrogen mostly through atmospheric N_2 fixation (Shearer *et al*. 1983). Shoot N and $\delta^{15}N$ data indicate that AMF inoculation stimulated symbiotic nitrogen fixation by rhizobia in root nodules, and may have also enhanced soil nitrogen uptake (Tobar *et al*. 1994). Improved water status in pre-inoculated *Retama* seedlings appeared to be largely a nutrient mediated effect (Augé 2001), since shoot water content was strongly positively correlated with AM colonisation and with shoot N, P and K concentrations (Table 4). Unlike observed for *Pistacia*, improved nutrition in pre-inoculated *Retama* already lead to enhanced water status under mesic glasshouse conditions (Table 1), and differences with control shrubs simply increased further after field transplanting (Table 2).

Compared with *Pistacia* or other shrub species grown under identical environmental conditions (Querejeta *et al*. 2006), *Retama* exhibited remarkably negative shoot $\delta^{13}C$ values, revealing rather low water use efficiency. Low shoot $\delta^{13}C$ in leafless *Retama* might be related to the high intercellular concentration of carbon dioxide typically found in the mesophyll of photosynthetic stems in this shrub (Domingo *et al*. 2002). Shoot $\delta^{13}C$ was not influenced by plant mycorrhizal status in *Retama*, indicating that there was no significant difference in WUE between control and AMF-inoculated seedlings despite large differences in nutrient status. Therefore, faster growth in the latter treatment was the result of a roughly parallel upshift in both photosynthetic and transpiration rates (Scheidegger *et al*. 2000; Querejeta *et al*. 2003, 2006).

Shoot $\delta^{18}O$ was significantly more enriched in *Retama* than in *Pistacia* or other shrub species grown under identical field conditions (Querejeta *et al*. 2006). *Retama* is notorious for its deep rooting habit (Haase *et al*. 1996), so higher shoot $\delta^{18}O$ was almost certainly not due to shallower rooting pattern or more evaporated source water. Other characteristics of plant species aside from rooting pattern can greatly influence the $\delta^{18}O$ values of plant organic matter (Wang *et al*. 1998). For example, the shape, size and thickness of transpiring organs are acknowledged to play an important role in determining the $\delta^{18}O$ value of plant biomass (Sheshshayee *et al*. 2005). Also, Wang *et al*. (1998) reported that *Spartium junceum* (another Mediterranean leguminous shrub with photosynthetic stems) had the single most enriched cellulose $\delta^{18}O$ value in a collection of 90 plant species from all continents grown under homogeneous climatic conditions. Highly enriched shoot $\delta^{18}O$ in leafless *Retama* might reflect the fact that the Pèclet number (a dimensionless number related to the ratio of convection to diffusion in the transpiration stream) of photosynthetic stems is likely very different from that of 'normal' leaves (Wang *et al*. 1998). Highly enriched $\delta^{18}O$ in *Retama* might as well indicate an unusually large biochemical fractionation factor between stem water and shoot dry matter during tissue formation (depending on plant species, the fractionation factor of cellulose is 27 ± 4‰; De Niro and Epstein 1979; but see also Cernusak *et al*. 2004). In contrast with results obtained with *Pistacia*, or other dryland shrub species (Querejeta *et al*. 2006), shoot $\delta^{18}O$ was not affected by inoculation with native AMF in *Retama* (significant shrub species × AMF interaction in Table 2). Given that the water status of *Retama* was greatly improved by inoculation with native AMF (Table 2), we suggest that plant $\delta^{18}O$ might be less responsive to changes in stomatal conductance induced by AMF in species where transpiration occurs in stems rather than in leaves, although this hypothesis remains to be tested.

It is worthy to note that shoot $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in *Pistacia* and *Retama* shrubs grown under identical semiarid field conditions spanned a wider range of values when comparing AMF-inoculated seedlings than when comparing control ones (Fig. 1). Although control *Pistacia* and *Retama* seedlings showed partially overlapping distribution of isotopic values (closed symbols), pre-inoculated *Pistacia* and *Retama* seedlings showed distinctly different isotopic composition (open symbols). The data presented here support the view that diverging patterns of physiological response to AMF colonisation among

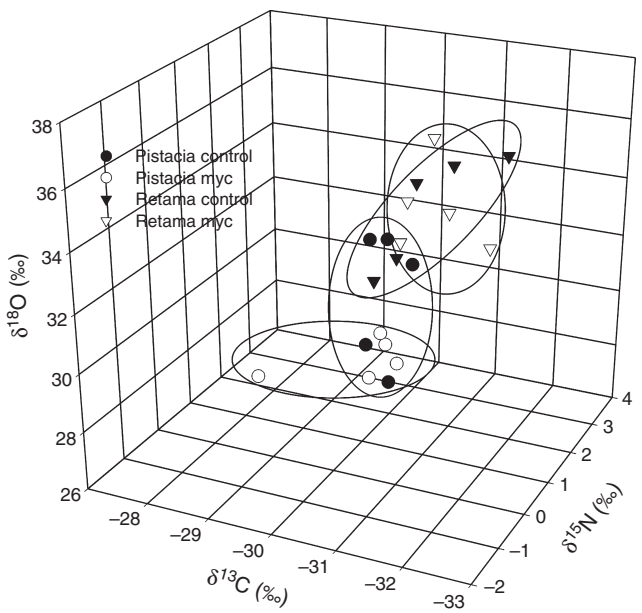

Fig. 1. Three-dimensional representation of the $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ isotopic composition of non-inoculated (control; $n = 5$) or AMF-inoculated (myc; $n = 5$) *Pistacia lentiscus* and *Retama sphaerocarpa* shrubs 12 months after field transplanting. Note the large difference in isotopic composition between *Retama* and *Pistacia* that were pre-inoculated with native AMF, and the much lesser difference between the controls.

co-occurring plant species may account for much of the interspecific variability in isotopic composition found in natural plant communities (Querejeta *et al*. 2003).

In conclusion, this paper shows that C, O and N isotope measurements can provide insight into the physiological mechanisms behind host plant growth stimulation by native AMF under semiarid conditions, particularly those involving water relations. In combination with DNA molecular techniques to characterise AMF diversity, stable isotope measurements of plant material might prove useful for screening and selecting optimal plant-AMF combinations based on ecophysiological criteria that are relevant to adaptation to local environmental conditions. The possibility to identify specific AMF strains that are capable of enhancing transpiration or water use efficiency in target host plants would have obvious applications to vegetation restoration in water-limited ecosystems.

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