

Stipa tenacissima Does not Affect the Foliar $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of Introduced Shrub Seedlings in a Mediterranean Semi-arid Steppe

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Abstract

Recent studies have shown that the tussock grass *Stipa tenacissima* L. facilitates the establishment of late-successional shrubs, in what constitutes the first documented case of facilitation of woody plants by grasses. With the aim of increasing our knowledge of this interaction, in the present study we investigated the effects of *S. tenacissima* on the foliar $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, nitrogen concentration, and carbon : nitrogen ratio of introduced seedlings of *Pistacia lentiscus* L., *Quercus coccifera* L., and *Medicago arborea* L. in a semi-arid Mediterranean steppe. Six months after planting, the values of $\delta^{13}\text{C}$ ranged between -26.9‰ and -29.6‰ , whereas those of $\delta^{15}\text{N}$ ranged between -1.9‰ and 2.7‰ . The foliar C : N ratio ranged between 10.7 and 53.5, and the nitrogen concentration ranged between 1.0% and 4.4%. We found no significant effect of the microsite provided by *S. tenacissima* on these variables in any of the species evaluated. The values of $\delta^{13}\text{C}$ were negatively correlated with predawn water potentials in *M. arborea* and were positively correlated with relative growth rate in *Q. coccifera*. The values of $\delta^{15}\text{N}$ were positively correlated with the biomass allocation to roots in the latter species. The present results suggest that the modification of environmental conditions in the area surrounding *S. tenacissima* was not strong enough to modify the foliar isotopic and nitrogen concentration of shrubs during the early stages after planting.

Key words: facilitation; *Medicago arborea*; *Pistacia lentiscus*; *Quercus coccifera*; stable isotopes; steppe; *Stipa tenacissima*.

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In arid and semi-arid ecosystems, vegetation is sparse and spatial distribution of soil properties is markedly patchy, lead-

ing to the development of “resource islands” under the discrete plant patches surrounded by relatively infertile soils (Schlesinger and Pilmanis 1998; Reynolds et al. 1999). These resource islands are points of high biological activity where facilitation may be the dominant interaction between the plant species forming the patch (Callaway 1995; Cheng et al. 2006). Facilitative interactions strongly influence the dynamics of plant patches in arid and semi-arid ecosystems, and are a key force shaping their structure and functioning (Whitford 2002).

Stable carbon and nitrogen isotope ratios ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively) are a powerful tool to evaluate the physiological status of plant, and are becoming increasingly used to understand plant-plant interactions in semi-arid areas (e.g. Williams et al. 1991; Ehleringer 1993; Gebauer et al. 2000). The $\delta^{13}\text{C}$ of plant leaf tissues is a useful index of long-term water use

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efficiency (WUE), defined as the ratio between photosynthesis per unit leaf area and stomatal conductance (Farquhar et al. 1989). As WUE increases, values of $\delta^{13}\text{C}$ become less negative and this relationship can be used to evaluate WUE during periods of stress (Ehleringer et al. 1992; Dawson et al. 2002). Despite the fact that the interpretation of the $\delta^{15}\text{N}$ of leaves is not straightforward (Handley et al. 1999), this variable can provide plant ecologists with useful information on the nitrogen sources used by plants, especially when the nitrogen supply rate is low in relation to plant demand (Högberg 1997).

Recent studies have shown that the tussock grass *Stipa tenacissima* L. facilitates the establishment of late-successional shrub species (*Pistacia lentiscus* L., *Quercus coccifera* L., and *Medicago arborea* L.) in semi-arid steppes of the Mediterranean Basin (Maestre et al. 2001, 2003a; Maestre and Cortina 2004). This interaction is of considerable interest because it is the first documented case of the facilitation of woody plants by grasses in semi-arid ecosystems. In these environments, studies have mainly described the facilitation of grasses by woody plants, or woody plant seedlings by adults, either conspecific or not (Callaway 1995; Maestre et al. 2005). With the aim of increasing our knowledge of this interaction, in the present study we investigated the effects of *S. tenacissima* on the foliar $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, nitrogen concentration, and the carbon : nitrogen (C : N) ratio of seedlings of *P. lentiscus*, *Q. coccifera*, and *M. arborea* in a semi-arid steppe of southeastern (SE) Spain. Soils under the canopy of *S. tenacissima* commonly have higher organic matter and nutrient contents than inter-tussock areas, as well as improved physical soil properties, such as lower surface compaction, higher infiltration rates, and higher water retention capacities after rainfall events (Puigdefábregas et al. 1999; Maestre et al. 2001, 2002; Armas and Pugnaire 2005). In the present study, we tested the hypothesis that seedlings growing in the vicinity of *S. tenacissima* will have lower $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, and higher nitrogen concentration, than those seedlings growing in bare ground areas devoid of vascular plants. Previous studies have found a strong negative relationship between foliar $\delta^{13}\text{C}$ and water availability (Stewart et al. 1995). Thus, we expected to find lower $\delta^{13}\text{C}$ values in seedlings growing in the vicinity of *S. tenacissima* because of increased water availability in this microsite. Lower $\delta^{15}\text{N}$ values, and higher nitrogen concentrations, were also expected because of the increase in overall nutrient availability and NO_3^- uptake, promoted by increased nitrogen mineralization in the vicinity of *S. tenacissima* tussocks (Djellali et al. 1985; Armas and Pugnaire 2005).

Results

During the first 6 months after planting, the precipitation at the

study site was only 55 mm, 51% of the 30 yr average at the nearest weather station (located 10 km from the study site; Pérez Cueva 1994). The average values of $\delta^{13}\text{C}$ ranged between -26.9‰ and -29.6‰ , whereas those of $\delta^{15}\text{N}$ ranged between -1.9‰ and 2.7‰ (Figure 1). The average values of the C : N ratio ranged between 10.7 and 53.5, whereas those of nitrogen concentration ranged between 1.0% and 4.4% (Figure 1). MANOVA analyses revealed significant and marginally significant differences between microsites in *M. arborea* and *P. lentiscus*, respectively, but not in *Q. coccifera* (Table 1). These were driven mainly by differences between the values obtained in the nursery with those from the field, because none of the variables evaluated differed between tussock and open microsites (Figure 1). Despite this, some differential responses to the microsite provided by *S. tenacissima* were observed. When compared with values obtained in the nursery, a significant increase in $\delta^{13}\text{C}$ was found for *M. arborea* in both tussock and open microsites. For *Q. coccifera*, this increase was only found in the tussock microsite. Similarly, a significant decrease in $\delta^{15}\text{N}$ was found for *M. arborea* in both tussock and open microsites, but for *P. lentiscus* this reduction was only found in the tussock microsite. Foliar nitrogen concentration decreased significantly in both *P. lentiscus* and *M. arborea* when passing from the nursery to the field. Compared with

Table 1. Results of MANOVA and ANOVA tests showing the effects of Microsite (nursery, tussock, and open) on the foliar carbon (C) and nitrogen (N) isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively), N concentration, and C : N ratio of shrub seedlings

Species	Pillai's trace	F	d.f.	P
<i>Medicago arborea</i>	1.010	2.54	8, 20	0.044
<i>Quercus coccifera</i>	0.651	1.21	8, 20	0.345
<i>Pistacia lentiscus</i>	0.969	2.35	8, 20	0.058
ANOVA results	Variable	F	df	P
<i>M. arborea</i>	$\delta^{13}\text{C}$	5.87	2, 12	0.017
	$\delta^{15}\text{N}$	16.39	2, 12	<0.001
	C : N ratio	18.79	2, 12	<0.001
	%N	17.42	2, 12	<0.001
<i>Q. coccifera</i>	$\delta^{13}\text{C}$	4.80	2, 12	0.029
	$\delta^{15}\text{N}$	1.24	2, 12	0.323
	C : N ratio	2.69	2, 12	0.108
	%N	3.11	2, 12	0.081
<i>P. lentiscus</i>	$\delta^{13}\text{C}$	0.683	2, 12	0.524
	$\delta^{15}\text{N}$	6.921	2, 12	0.010
	C : N ratio	5.551	2, 12	0.020
	%N	7.453	2, 12	0.008

Separate analyses were conducted for each species.

Significant values ($P < 0.05$) are in bold and indicate significant differences between microsites.

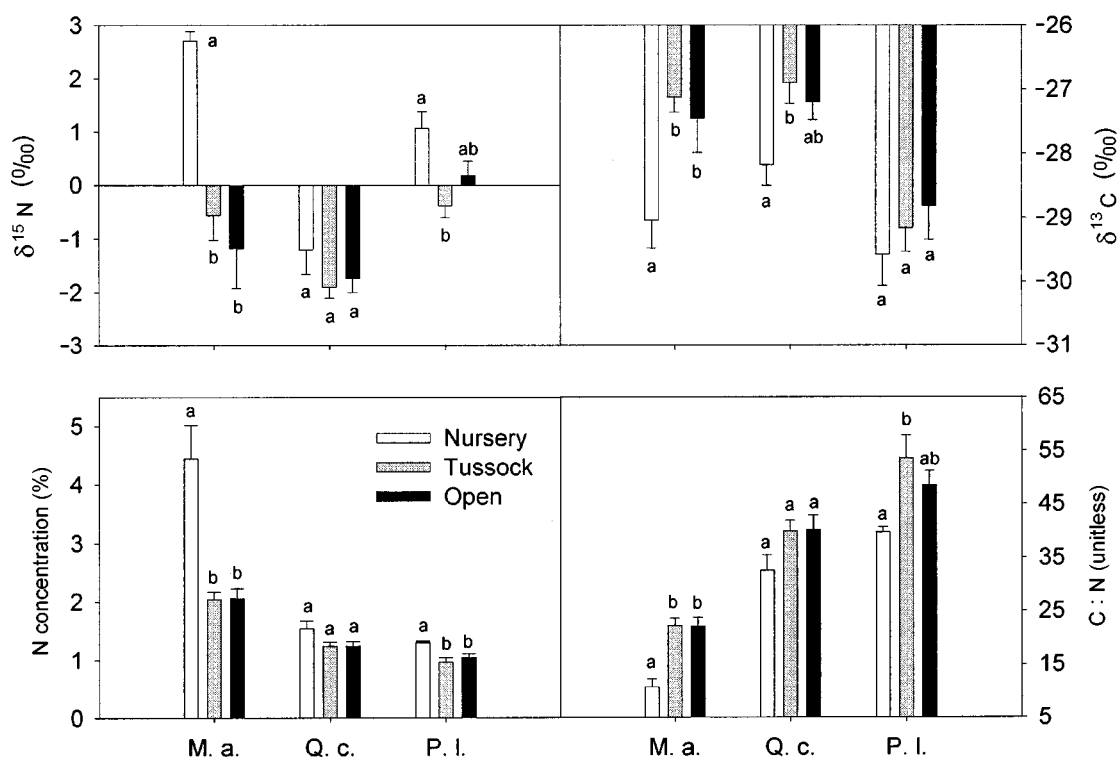


Figure 1. Values of foliar $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, nitrogen concentration (N), and carbon-to-nitrogen ratio (C : N) for seedlings in the nursery and in the field (tussock and open microsites).

Data are the mean \pm SE ($n = 5$). Different letters indicate significant differences between microsites ($P < 0.05$, Tukey's b-test). Post hoc results for N concentration are shown for arcsine-transformed data. M.a., *Medicago arborea*; Q.c., *Quercus coccifera*; P.l., *Pistacia lentiscus*.

values obtained in the nursery, a significant increase in the foliar C : N ratio was observed for *M. arborea* in both tussock and open microsites, but for *P. lentiscus* such an increase was only found in the tussock microsite. Foliar nitrogen concentration was not significantly correlated with either $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ in any of the species evaluated (data not shown). The species evaluated showed important differences in the relationships between their foliar isotopic composition and pre-dawn water potential (Ψ), relative growth rate (RGR), and the below : aboveground biomass (R : S) ratio (Figure 2). It was found that $\delta^{13}\text{C}$ and RGR were positively correlated in *Q. coccifera*, as well as $\delta^{15}\text{N}$ and the R : S ratio. It was found that $\delta^{13}\text{C}$ and Ψ were negatively correlated in *M. arborea*. Foliar nitrogen concentration was not related to either Ψ , RGR, or the R : S ratio in any of the species evaluated (Figure 3).

Discussion

Despite microsite-specific differences when comparing nurs-

ery and field values in *M. arborea* and *P. lentiscus*, we did not find significant differences between tussock and open microsites in any of the variables evaluated (foliar $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, N concentration, and C : N ratio) during the duration of the present study. Thus, our initial hypothesis was not supported by our results. The results contrast with previous studies reporting an increased performance (evaluated with variables such as survival and Ψ) of the shrub seedlings in the tussock compared with the open microsite (Maestre et al. 2001). These contrasting results are not fully surprising because the estimator of plant performance has a considerable influence on the observed outcome of a given plant-plant interaction (Hastwell and Facelli 2003; Maestre et al. 2005). The contrasting results also emphasize the need to use multiple estimators of plant performance when evaluating these interactions in arid and semi-arid environments (Maestre et al. 2005, 2006).

Despite the fact that we failed to find significant microsite effects on the variables evaluated, the use of stable isotopes in the present study provided new insights on the response of the different species evaluated during the first months after

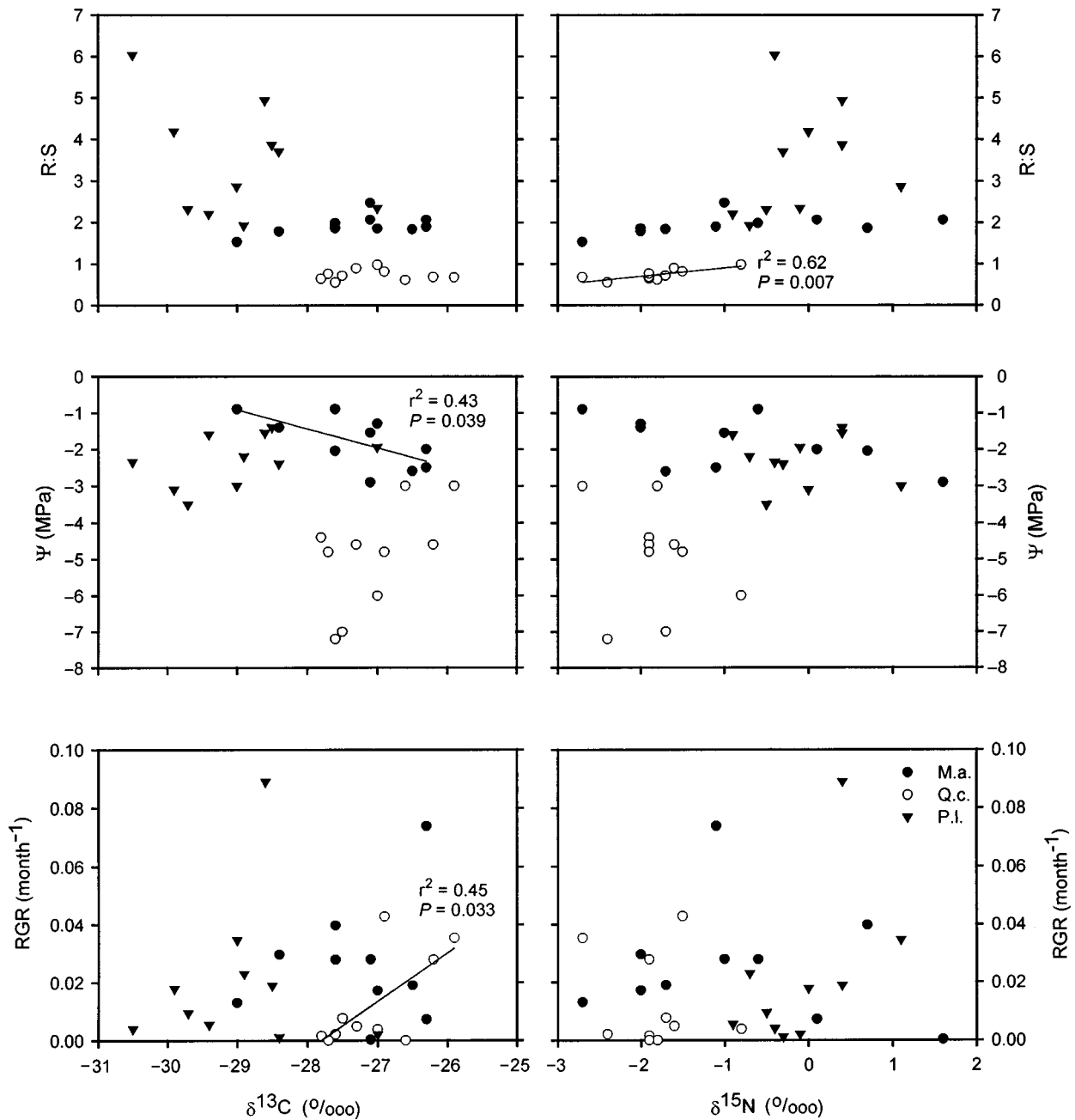


Figure 2. Relationships between foliar isotopic concentration ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and relative growth rate (RGR), predawn water potentials (Ψ), and aboveground/belowground biomass (R : S).

Data from both tussock and open microsites are included ($n = 10$). Only significant relationships ($P < 0.05$) are drawn. M.a., *Medicago arborea*; Q.c., *Quercus coccifera*; P.I., *Pistacia lentiscus*. Predawn water potential data are taken from Maestre et al. (2001).

planting. The values of $\delta^{13}\text{C}$ observed indicate that *M. arborea* and *Q. coccifera*, but not *P. lentiscus*, increased their WUE when passing from the nursery to the field. Such an increase suggests that these species were able to adjust their transpi-

ration rate during this transition (Vilagrosa et al. 2003). This agrees with the physiological strategies to cope with drought stress reported so far for these species. *Quercus coccifera* and *M. arborea* can reduce their stomatal conductance rates in

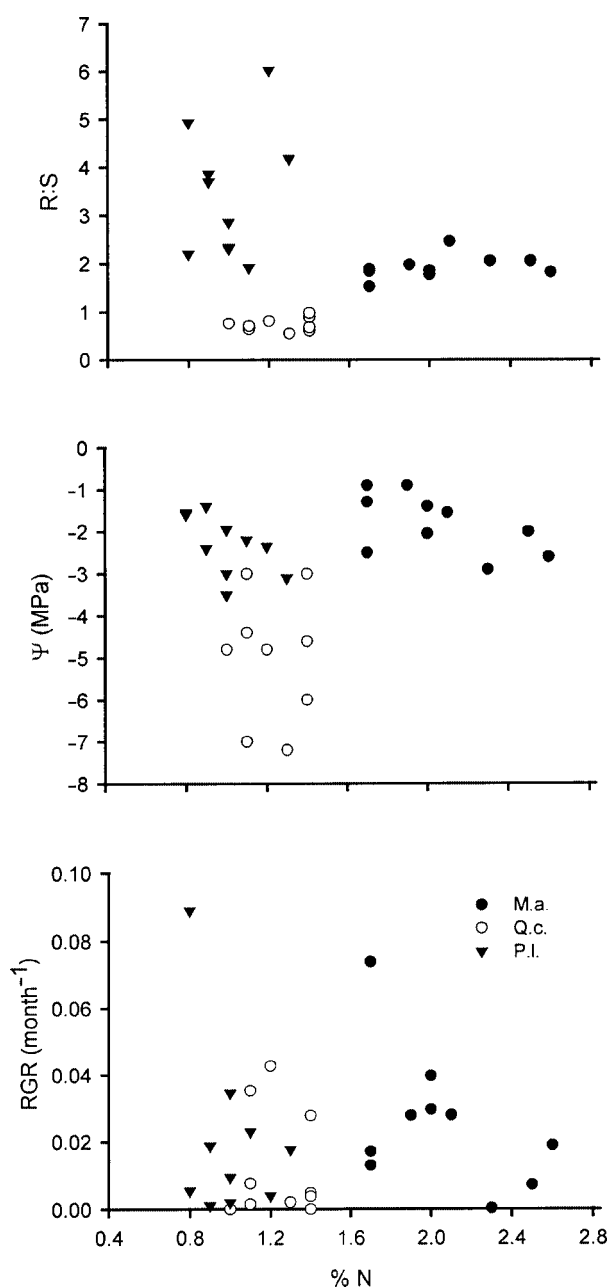


Figure 3. Relationships between foliar nitrogen concentration (%N) and relative growth rate (RGR), predawn water potentials (Ψ), and aboveground/belowground biomass (R : S).

Data from both tussock and open microsites are included ($n=10$). None of the relationships was significant ($P > 0.05$ in all cases). M.a., *Medicago arborea*; Q.c., *Quercus coccifera*; P.I., *Pistacia lentiscus*. Predawn water potential data are taken from Maestre et al. (2001).

response to drought (Noitsakis et al. 1991; Vilagrosa et al. 2003) and this strategy allows them to maintain an appropriate water status during periods of low water availability (drought avoidance by water saving; Levitt 1980). However, *P. lentiscus* follows a strategy of drought avoidance by water spending (Levitt 1980). This species has high stomatal conductance rates and an efficient system of water transport from roots to leaves, which allows it to maintain high transpiration rates while water is available and it drops some or all of its leaves during drought periods to reduce water losses (Vilagrosa et al. 2003). It is interesting to note that *P. lentiscus* was the species that showed the lowest survival during the first 6 months after planting (Maestre et al. 2001). When all the species were considered, seedling survival was positively related to average $\delta^{13}\text{C}$ values (Appendix 1). This result suggests that those species with higher WUE were more likely to survive during the first months after planting, a critical stage for shrub establishment in Mediterranean environments (Maestre et al. 2001, 2002, 2003b). However, this affirmation must be considered with caution owing to the low number of replicates and to the lack of a continuous gradient in the survival and $\delta^{13}\text{C}$ values observed. Similarly, we found a positive relationship between $\delta^{13}\text{C}$ and RGR in *Q. coccifera*, suggesting that seedlings with higher WUE (i.e. less negative $\delta^{13}\text{C}$ values) were able to increase their biomass allocation to roots. These results agree with those of Querejeta et al. (2003), who found a positive relationship between $\delta^{13}\text{C}$ and the growth of planted woody Mediterranean seedlings.

As reported by Lloret et al. (1999), who examined this relationship in a shrubland in NW Spain, we did not find significant relationships between $\delta^{13}\text{C}$ and the R : S ratio for any species. This result was unexpected, because a greater allocation to root biomass should increase WUE (Hilbert and Canadell 1995) and may be caused by the short length of the period studied, which could make insufficiently beneficial the selection for a particular WUE (Lloret et al. 1999). The negative correlation between leaf $\delta^{13}\text{C}$ and Ψ found in *M. arborea* suggests the existence of a relationship between isotope composition and the water constraint withstood by this species, as has been found in previously Mediterranean oaks, such as *Quercus ilex* L. and *Quercus pubescens* Willd. (Damesin et al. 1998).

Medicago arborea and *P. lentiscus* showed a reduction in their foliar $\delta^{15}\text{N}$ when passing from the nursery to the field, albeit in the latter species this reduction was found only in the tussock microsite. The present study cannot provide any evidence on the mechanisms involved in this depletion, because the $\delta^{15}\text{N}$ of leaves is affected by multiple causes. These include, but are not limited to, mineralization, nitrification, and ammonification processes, the proportion of ^{15}N present in the soil exploited by the roots, the activity of mycorrhizae and other

micro-organisms, and the depth reached by the roots (Lajtha and Milchener 1994; Dawson et al. 2002). We found a positive relationship between the R : S ratio and the foliar $\delta^{15}\text{N}$ in *Q. coccifera*. This result suggests that individuals of this species with larger root allocation are able to more efficiently exploit soil systems and to use more nitrogen from more nitrogen-saturated soil sites (Aber et al. 1989). Lloret et al. (1999) found a similar relationship when comparing seedlings of different shrub species in a Mediterranean shrubland. The present results suggest that even subtle changes in the R : S ratio may influence the patterns of N acquisition by *Q. coccifera* seedlings. However, it is interesting to note that the R : S ratio was not related to the foliar nitrogen concentration in this species. Thus, it is likely that the increase in the $\delta^{15}\text{N}$ values with the increase in the R : S ratio reflects a change in the relative importance of different nitrogen sources used by the seedlings (Aber et al. 1989; Lajtha and Milchener 1994), rather than an increase in the rate of nitrogen uptake.

Materials and Methods

Study area

The present study was conducted in a steppe located in the province of Alicante, in SE Spain (38°30' N, 0°23' W, slope 18°, orientation 140 SE, altitude 380 m above sea level). The climate is Mediterranean semi-arid, with a 30-yr average annual precipitation of 220 mm, falling mainly in autumn, and a mean annual temperature of 18 °C (Pérez Cueva 1994). Total plant cover is 54% and is dominated by the tussock grass *S. tenacissima*, interspersed with shrubs such as *Globularia alypum* L., *Ephedra fragilis* Desf., and *P. lentiscus*. *Stipa*-dominated steppes are widespread in the semi-arid areas of the Mediterranean Basin and derive from the degradation of open shrublands and woodlands by human activities performed over centuries (Le Houérou 2001). Nowadays, late-successional shrubs such as *P. lentiscus*, *Rhamnus lycioides* L., and *Q. coccifera* typically appear as isolated patches without symptoms of expansion and recovery (Maestre and Cortina 2005).

Experimental design

To investigate the effects of *S. tenacissima* on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of shrubs, we conducted a fully randomized factorial experiment with two factors: species and microsite. Seedlings of *M. arborea*, *Q. coccifera*, and *P. lentiscus* were introduced into two microsites: tussock and open. The tussock microsites were located upslope and adjacent to *S. tenacissima* tussocks (less than 15 cm from the edge of the tussock); the open microsites were located in the bare ground areas, at distances of 50–150 cm from the nearest *S. tenacissima* tussock (see

Table 2 for details on the soil properties at both microsites). Before starting the experiment, we randomly selected 60 replicated planting points per microsite and species (360 planting points in total). In each of these points we excavated a 25 cm × 25 cm × 25 cm hole and planted one seedling, during the 3rd week of December 1998. The seeds used in this experiment were collected from local provinces in the Valencia Region (eastern Spain) by the regional forest services (Generalitat Valenciana Forest Seed Bank, Quart de Poblet, Spain). Before transplantation, seedlings were grown in a nursery for 9 months in 330-mL containers (Roottrainer Ash, Roxburghshire, UK) with a mixture of peat, coconut fiber, and sand (50 : 45 : 5). Slow-release fertilizer (Osmocote Plus; Scotts, Columbus, OH, USA) was also included in the growing medium.

Seedling measurements and stable isotope analyses

Healthy leaves of five individuals per species were collected for isotopic ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and elemental (carbon and nitrogen concentration) analyses from the nursery during the week prior to planting. Sampled leaves came from the upper part of the canopy (20–40 randomly selected leaves were bulked for each seedling). The diameter of all seedlings planted was measured at the moment of planting and 6 months later (June 1999). At this date, we randomly selected five individuals per species and microsite for physiological, biomass, and stable isotope analyses. We measured Ψ *in situ* on small terminal shoots using a pressure bomb (Soilmoisture, Santa Barbara, California, USA). Measurements were performed 2 h before sunrise. Immediately after these measurements, we collected leaf samples for stable isotope analyses in the same manner as we did in the nursery. The seedlings selected for Ψ and isotope analyses were later harvested to quantify their above- and below-ground biomass. The RGR of these seedlings was also calculated accordingly to the formula $((\ln d_2 - \ln d_1)/(t_2 - t_1))$, where d_1

Table 2. Properties of soil (0–20 cm) in tussock and open microsites

Variable	Microsite	
	Tussock	Open
pH ^a	8.5 ± 0.0	8.5 ± 0.1
Organic matter (mg/g) ^a	36.9 ± 3.6	30.2 ± 2.8
Total nitrogen (mg/g) ^a	1.7 ± 0.1	1.5 ± 0.1
C : N ^a	12.2 ± 0.7	12.0 ± 0.4
Total CO ₃ (%) ^a	59.2 ± 3.7	66.8 ± 6.1
Sand (%) ^a	28.4 ± 2.2	28.4 ± 1.2
Silt (%) ^a	45.0 ± 2.5	44.4 ± 1.1
Clay (%) ^a	26.6 ± 2.0	27.1 ± 1.2
No. mycorrhizal propagules (/g dry soil) ^b	0.91	0.75

^aData are the mean ± SE ($n = 5$) and are taken from Maestre et al. (2001).

^bData are the mean and are taken from Azcón-Aguilar et al. (2003).

and d_2 are the diameter of seedlings at the moment of planting (t_1) and 6 months later (t_2), respectively.

Plant material collected was air dried at 65 °C until constant weight and was later ground to a fine powder in a ring mill (Herzog, Osnabrück Germany). Samples (2 mg) were transferred into 8 × 5 mm tin capsules (EMA part D1008; EMAL Tech USA, Mason, OH, USA) and injected into an elemental analyzer coupled to an isotope ratio mass spectrometer (IRMS; Finnigan MAT 252 or DELTAplus, San Jose, California, USA). The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the samples were calculated using the following equations:

$$\delta^{13}\text{C} = ((R_{\text{sample}} : R_{\text{standard}}) - 1) \times 1000 \text{ (‰ vs Pee Dee belemnite)}$$

$$\delta^{15}\text{N} = ((R_{\text{sample}} : R_{\text{standard}}) - 1) \times 1000 \text{ (‰ vs at-air)}$$

where R is the ratio of mass 45/mass 44 (carbon) and of mass 29/mass 28 (nitrogen), respectively. Isotopic analyses were conducted at the Stable Isotope Ratio Facility for Environmental Research at the University of Utah (<http://ecophys.biology.utah.edu/sirfer.html>).

Statistical analyses

Because the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, nitrogen concentration, and C : N values of each seedling were evaluated in the same sample, and thus these measurements cannot be considered to be independent of each other, multivariate analysis of variance (MANOVA) was used to analyze these data. Initially, two fixed factors were considered in this analysis: species (three levels: *P. lentiscus*, *Q. coccifera*, and *M. arborea*) and microsite (three levels: nursery, tussock, and open). "Nursery" was included as another level within the microsite factor to facilitate the comparison between the values observed in the nursery with those observed in the field. Because there was a significant Microsite × Species interaction (MANOVA; Pillai's trace = 0.894; $F_{16,144} = 0.259$; $P = 0.001$), separate analyses were conducted for each species. The MANOVA analyses were followed by univariate ANOVA and Tukey's b test for post hoc comparisons (Quinn and Keough 2002). Nitrogen concentration data were arcsine transformed (Quinn and Keough 2002) to achieve the homogeneity of variances required by MANOVA and ANOVA; the rest of data followed MANOVA assumptions. The MANOVA analyses conducted for each species followed the homogeneity of variance-covariance matrices assumption (Box's *M*-test; *M. arborea* : $F_{20,517} = 1.54$, $P = 0.064$; *Q. coccifera* : $F_{20,517} = 1.34$, $P = 0.148$; *P. lentiscus* : $F_{20,517} = 1.44$, $P = 0.099$). Regression analyses were also used to explore the relationships between isotopic data ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and RGR, Ψ and the R : S ratio. All statistical analyses were performed using the SPSS 9.0 package (SPSS, Chicago, IL, USA).

Acknowledgements

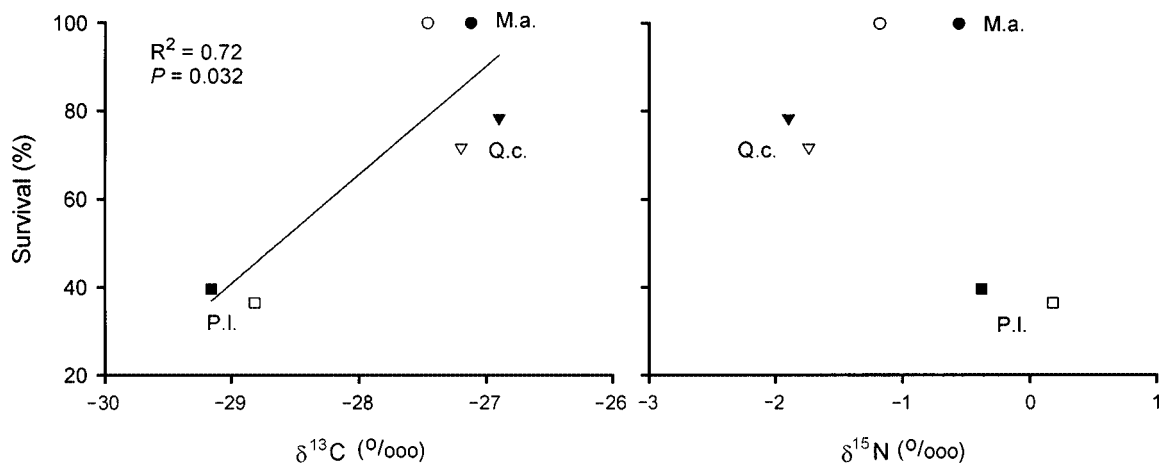
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Appendix 1. Relationships between foliar isotopic concentration ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and seedling survival 6 months after planting.

For stable isotope data, each point represents the average value obtained for a given combination of species and microsite (each average was obtained from five seedlings). Open and filled symbols correspond to data obtained from tussock and open microsites, respectively. Only significant relationships ($P < 0.05$) are drawn. M.a., *Medicago arborea*; Q.c., *Quercus coccifera*; P.I., *Pistacia lentiscus*. Survival data are taken from Maestre et al. (2001).

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