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# Functional traits related to seedling performance in the Mediterranean leguminous shrub *Retama sphaerocarpa*: Insights from a provenance, fertilization, and rhizobial inoculation study

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## ABSTRACT

We studied functional traits related to survival and growth in seedlings of the Mediterranean leguminous shrub *Retama sphaerocarpa* (L.) Boiss. Plants from five provenances were grown at two contrasting fertilization rates. In one of the provenances, low-fertilized plants were also inoculated with a *Bradyrhizobium* strain to assess the influence of nodulation on seedling performance. Seedlings were transplanted onto an abandoned cropland and their growth and survival rates were measured for two years. Additionally, the persistence of the inoculated strain in the field was tracked using genetic methods. Fertilization produced two phenotypes of contrasting performance. High fertilization produced large plants with high nutrient concentration, photosynthetic rate, and root growth capacity. Plants with this phenotype had higher transplanting survival, growth, and water use efficiency than the plants of the low-fertilized phenotype, which were small and had low nutrient concentration, photosynthetic rate and root growth capacity. Provenances differed in relative growth rate (RGR) under optimal growing conditions and these differences were negatively related to the length of the growing season and positively related to the precipitation at the places of origin of seeds. Across provenances, transplanting survival and growth was positively related to the shoot carotenoid concentration. However, this relationship was only observed among low-fertilized seedlings. Among low-fertilized plants, nodulation did not increase either transplanting survival or growth significantly. The *Bradyrhizobium* strain used to inoculate seedlings survived at least two years in transplanted inoculated plants in spite of the presence of other native rhizobial strains in the field. In conclusion, high transplanting performance of *R. sphaerocarpa* seedlings is linked to a suite of attributes that promote fast seedling establishment during the wet season, which probably enhances drought avoidance during the dry season and helps avoid photoinhibition during the summer drought.

## 1. Introduction

Water and nutrient stress are important constraints for plant life in Mediterranean ecosystems and seedlings represent the most vulnerable stage of a plant's life cycle (Castro et al., 2004). Many studies have assessed the physiological and structural adaptations and acclimation mechanisms of plants to these factors (see Levitt, 1980; Chapin et al., 1993 and references therein). It is commonly accepted that certain functional attributes increase resistance to drought and nutrient stress. For instance, high allocation of biomass to roots at the expense of shoot growth (low shoot to root mass ratio) or the production of extensive and deep roots are mechanisms that enhance water acquisition and utilisation efficiency and therefore play an important role for plant survival in dry climates. However, few studies have analysed which functional attributes are related to seedling establishment in dry climates and results are frequently contradictory. In an interspecific comparison of Mediterranean chamaephytes and small shrubs in the field, species with seedlings that had low shoot to root mass ratio had greater survival than species with seedlings that had high shoot

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**Table 1**
Climate, duration of growing season and altitude of the sites where the *Retama sphaerocarpa* seeds of the studied provenances were recollected

| Location of seed sources | Annual rainfall (mm) | $T_{min}$ January (°C) | $T_{max}$ July (°C) | Duration of growing season (months) | Altitude (m) |
|---|---|---|---|---|---|
| Tres Cantos (Madrid) (40°36′N, 3°42′W | 585 | 0.9 | 31.8 | 2.3 | 704 |
| Navas del Rey (Madrid) (40°24′N, 4°17′W) | 560 | 1.8 | 33.1 | 2.7 | 700 |
| Alarilla (Guadalajara) (40°50′N, 3°5′W) | 490 | 0.2 | 31.6 | 2.6 | 708 |
| Almunia de Doña Godina (Zaragoza) (41°27′N, 1°22′W) | 375 | 2.3 | 32.2 | 3.2 | 400 |
| Rambla Honda (Almería) (37°7′N, 2°22′W) | 250 | 3.4 | 34.4 | 4.0 | 500 |

The duration of growing season was calculated as the period in which mean temperature is higher than 10 °C before summer drought. Onset of summer drought is the moment when temperature line crosses over precipitation line in the climatic diagram. Climatic data were obtained from Ninyerola et al. (2005).

to root mass ratio (Lloret et al., 1999). However, Valladares and Sánchez-Gómez (2006) observed the reverse trend among Iberian trees in a pot experiment. They also concluded that seedling survival was greater in species that had small seedlings than in species with large seedlings. In within-species studies, some authors have found that small seedlings and seedlings that had low shoot to root mass ratio had greater survival than the seedlings with the opposite traits (Tuttle et al., 1988; van den Driessche, 1991). However, other authors have observed greater survival in large seedlings and seedlings with high shoot to root mass ratio than in small seedlings or seedlings with low shoot to root ratio (Cook, 1980; van den Driessche, 1992; Villar-Salvador et al., 2004). These variable results may reflect species-specific responses or may be due to the wide range of experimental conditions differing among studies.

Many leguminous woody species are pioneer species in arid conditions and they have been successfully used for the revegetation of arid and nutrient deficient ecosystems (Forti et al., 2006). The greater competitive ability of leguminous species in sites with low resources can be in part attributed to their ability to form symbiosis with N fixing bacteria. The inoculation of rhizobial symbionts is a well-known technique in agronomy that enhances growth in leguminous species and neighbour non-leguminous species in oligotrophic soils (Newton, 2000). Inoculation success can be due to the ability of the strain to compete and persist with symbiotically efficient indigenous strains (Howieson, 1995). Both inoculation with mycorrhizal fungi exclusively and dual inoculation with mycorrhizal fungi and *Rhizobium* enhance transplanting performance of leguminous woody species in semiarid and arid areas (Requena et al., 2001; Caravaca et al., 2003). However, the effect of exclusive rhizobial inoculation on transplanting performance in woodland leguminous shrubs has seldom been tested (see Thrall et al., 2005).

The general objective of this study was to identify which functional traits are related to the transplanting survival and growth of seedlings of the Mediterranean leguminous shrub *Retama sphaerocarpa* (L.) Boiss. (retama). Additionally, we assessed if nodulation enhances its transplanting performance. We chose *R. sphaerocarpa* because it is widely used in revegetation projects (Caravaca et al., 2003). Retama is a broom-like pioneer shrub distributed in the western Mediterranean basin. It has photosynthetic stems (cladodes) and lives on a great variety of soil types and under an ample range of climatic conditions. Retama plays an important role in the dynamics of herb communities (Pugnaire et al., 1996).

We addressed the following specific hypotheses: (1) Large plants with high nutrient concentration and plants with high shoot to root mass ratio have greater out-planting performance than seedlings with the opposite attributes as shown by Villar-Salvador et al. (2004). (2) Provenances from drier locations grow less under optimal growing conditions but have higher transplanting performance under harsh conditions than provenances from wetter locations. This is based on the observation that physiological and structural mechanisms that confer stress resistance may reduce growth capacity, leading to a trade-off between growth and survival (Loehle, 1998; Sugiyama, 2006). (3) Inoculated seedlings have higher transplanting performance than non-inoculated plants as observed by Thrall et al. (2005). To address these hypotheses we grew plants from five provenances under two contrasting fertilization rates. In one of the provenances, low-fertilized plants were also inoculated with a *Bradyrhizobium* strain. At the end of the cultivation period, the functional features of the plants were measured and seedlings were then transplanted onto an abandoned cropland in a Mediterranean continental site. Their growth and survival were followed for two years, and the persistence of the inoculated strain in the field on the inoculated plants was tracked genetically using random amplification of polymorphic DNA (RAPD).

## 2. Material and methods

### 2.1. Plant cultivation

Seeds of *R. sphaerocarpa* were collected during autumn 1999 in five locations, which differed in rainfall, winter temperature and soil type (Table 1). Rambla Honda provenance was located in Europe's most arid region, where summer drought lasts 5–6 months and most rainfall falls between late autumn and mid-spring. Winters are very mild, and the mean air temperature during the cold season remains above 11 °C. The other provenances had typical semiarid (Almunia de D. Godina) and dry (remaining provenances) Mediterranean continental climate, which is characterized by very cold winters and hot and dry summers. Dry climate provenances had colder winters than the semiarid provenance. The duration of the growing season, defined as the number of months with mean air temperature above 10 °C before summer drought, differed among locations. Rambla Honda had the longest growing season, the three dry climate provenances had the shortest, and the semiarid provenance had an intermediate growing season. Soils in Almunia de D. Godina and Alarilla were basic while those of the other provenances were acid.

Seeds were sown in March 2000 in 44 Forest Pot™ containers (Nuevos Sistemas de Cultivo S.L., Girona, Spain) filled with non-sterilized Sphagnum moss peat. This container is a tray with 50 cavities of 300 mL. Three or four seeds were sown per cavity but only one plant per cavity was left after germination. Plants were grown in a glasshouse until mid-May 2000 to avoid frost damage and then cultivated outdoors under full sun until the end of cultivation period in mid November 2000.

An experiment was designed to test the effects of fertilization rate, provenance, and nodulation on the functional and transplanting performance of retama seedlings. To achieve this, half of the plants from each provenance received a high fertilization regime (F+) and the other half a low fertilization regime (F−). In addition, half of the F− plants in the Alarilla provenance were inoculated with a *Bradyrhizobium* strain (named RST-1). The number of treatments was 11 (two fertilization rates × five provenances, plus one low-fertilized *Rhizobium* inoculated treatment). Each treatment was replicated in four containers that were randomly arranged in space. Fertilizer was applied from late May 2000 until early October

2000. Nutrient solutions were supplied via sprinkling fertirrigation once a week. The fertilizer solution had nitrogen, phosphorus and potassium that were supplied as ammonium nitrate (N 33.5%), phosphoric acid (P 75%) and potassium sulphate (K 50%), respectively. At the end of the experiment, each F+ plant had received 100, 30, and 10 mg of N, P, and K respectively, whereas each F− seedling had received 10, 5, and 2 mg of N, P, and K respectively. Both F+ and F− nutrient solutions were enriched with 1 ppm of micronutrient mixture (Kanieltra, Hydro Agri, Oslo, Norway). Plants were kept well watered by irrigating all treatments every 1–3 days with the same amount of water, except on approximately five days during summer when high-fertilized plants received excess irrigation to help ensure constant media water content across treatments.

Rhizobial inoculation was applied to the seedlings of the Alarilla provenance because it was the closest one to both the site from where the inoculated strain was obtained and the site where seedlings were subsequently transplanted. High-fertilized (F+) plants were not inoculated because high fertilization inhibits nodulation (Valladares et al., 2002) and because our aim was to compare the performance of inoculated F− plants against non-inoculated F+ and F− seedlings. The inoculant was isolated from nodules collected from an individual of *R. sphaerocarpa* from Torrelaguna (Madrid, Spain). This inoculant has high nodulation success under operational nursery conditions, and it was prepared following the methodology explained in Valladares et al. (2002). Seedlings were inoculated during the first week of May with 1 mL of strain suspension per plant containing $10^8$ colony forming units $mL^{-1}$. At this date most seedlings were at the cotyledon stage and shoots were starting to elongate.

### 2.2. Morphology, nutrient concentration, and growth measurements

Plants from each treatment were sampled twice during the experiment. The first harvest was done when seedlings were at the cotyledon stage and the incipient shoots were visible (April 27, day 0). The second harvest was done at the end of the cultivation phase on November 15 (day 202). Five (first harvest) and 16 (second harvest) plants per treatment were randomly sampled at each date. However, in the case of inoculated plants, only nodulated seedlings were considered for morphological and nutrient concentration analyses in the second harvest. Plants were divided into shoots and roots and the number of nodules was counted. Root plugs were then washed from the growing medium and roots and shoots were rinsed in distilled water and dried for 48 h at 60 °C. Plant and nodule mass, the shoot to root mass ratio (S/R) and relative growth rate (RGR) were determined. RGR was calculated as:

$$\frac{\ln(\text{plant mass at day 202}) - \ln(\text{plant mass at day 0})}{(\text{day 202} - \text{day 0})}$$

$(g\,g^{-1}\,day^{-1})$

To assess nutrient concentration, shoots from the second harvest in each group were pooled separately and ground. N concentration was determined by Kjeldahl analysis with $SeSO_4$-$K_2SO_4$ as catalyst in a Tecator 20 digestion system and a Kjeltec-auto 1030 analyser (Tecator, Sweden).

Total P and K were determined by emission spectrometry in inductively coupled plasma (PerkinElmer ICP5500, USA) after digesting samples in a mixture of $HNO_3$ and $HClO_4$ in a warm sand bath at ambient pressure.

At the end of the experiment two root growth capacity (RGC) tests were done. The first one compared F+ and F− plants from all provenances. The second test compared F+ and F− non-inoculated plants with inoculated F− seedlings. RGC measures the capacity of plants to produce new roots under specific conditions (Burdett, 1987). Fifteen seedlings from each treatment were transplanted into 3 L pots (one plant per pot) containing perlite. All inoculated F− seedlings had nodules. Plants were randomly arranged and placed in a heated greenhouse with a mean minimum temperature of 14–15 °C and a mean maximum temperature of 20–22 °C. Radiation was half the outside radiation and relative humidity ranged between 40% and 70%. Seedlings were watered every 3 or 4 days. After 24 days, plants were lifted, shoots excised and root plugs cleaned from the potting medium. All new roots that protruded 1 cm out of the root plug were cut and oven dried at 60 °C new roots longer than 1 cm protruding out of the root plug were cut and oven dried at 60 °C for 48 h together with the shoots and weighed. Root growth capacity of each plant was determined as the total mass of new roots.

### 2.3. Net photosynthetic rate and photosynthetic pigment concentration

Maximum net photosynthetic rate ($A_{max}$) was measured on cladodes of five plants per treatment with a portable open gas exchange system (LCA4, Analytical Development Co., Hoddesdon, UK). Incident PPF was saturating (always >1900 $\mu mol\,m^{-2}\,s^{-1}$), which was supplied by a halogen lamp. Temperature during the measurements was kept ca. $28 \pm 2$ °C. Plants were watered the afternoon before measurements, which were taken between 9:30 and 12:00 (solar time) in the glasshouse. Measurements were done within one month after the end of cultivation.

Five plants per provenance and treatment were used for chlorophyll and carotenoid assessment. Portions of cladodes of 0.05 g were incubated in 3 mL dimethyl sulfoxide for 2 h in a dark oven at 65 °C. Optical density of the extracts was measured with a spectrophotometer at 663.0, 646.8, 480.0, 435.0 and 415.0 nm and concentrations calculated according to Wellburn (1994). Total chlorophyll and carotenoid concentration on a mass basis were measured and the carotenoid/chlorophyll ratio was calculated.

In the inoculated treatment, both $A_{max}$ and photosynthetic pigment concentration were measured in nodulated plants exclusively.

### 2.4. Transplanting and field performance

Transplanting was done in December 2000 in Marchamalo (Guadalajara, Centre Spain, 40°40′N, 3°12′W, altitude = 700 m). The site has typical Mediterranean continental climate. The historical mean annual rainfall and temperature are 410 mm and 13.2 °C, respectively. Mean maximum and mean minimum temperature of the hottest and coldest months are 32.1 °C and −1.2 °C, respectively, and summer drought typically lasts 3–4 months. The soil has a clay loam texture and pH is around 7.5. From an environmental point of view, the provenance of Alarilla was the most similar and the closest one to the planting site. Each treatment had three replications that were randomly arranged in space. Each repetition consisted of a row of 15 seedlings. Distance between seedlings within a row and between rows was 2 m. Prior to planting, soil was subsoiled to 60 cm in depth and during the experiment the plot was not irrigated and weeds around each plant were periodically controlled by hoeing. In the inoculated treatment, only plants with nodules were planted. Mortality was recorded twice throughout two years. At the end of the second growing season (2002), shoots of all living plants were excised at ground level and dried in a greenhouse for several days. Shoot growth was determined by both the difference between field shoot mass and treatment shoots mass at the end of nursery cultivation and RGR.

**Table 2**

Functional features of five *Retama sphaerocarpa* provenances cultivated at two fertilization rates at the end of nursery cultivation

| | Provenance | | | | | Fertilization rate | |
|---|---|---|---|---|---|---|---|
| | Tres Cantos | Navas del Rey | Alarilla | Almunia D. Godina | Rambla Honda | Low | High |
| Mass of 10 seeds (mg) | 728 ± 15 b | 709 ± 18 b | 726 ± 13 b | 847 ± 15 a | 815 ± 17 a | | |
| Shoot to root mass ratio | 1.08 ± 0.06 a | 0.95 ± 0.13 a | 1.03 ± 0.06 a | 1.07 ± 0.06 a | 0.96 ± 0.13 a | 0.76 ± 0.02 | 1.27 ± 0.05 * |
| RGC (mg) | 34.1 ± 4.9 a | 28.5 ± 5.0 a | 34.0 ± 4.9 a | 37.1 ± 5.0 a | 30.7 ± 4.9 a | 44.5 ± 5.0 | 21.2 ± 5.0 * |
| Plant N concentration (mg $g^{-1}$) | 14.8 ± 0.53 a | 16.0 ± 0.61 a | 15.3 ± 0.78 a | 14.1 ± 0.43 a | 14.0 ± 0.81 a | 13.9 ± 0.34 | 15.8 ± 0.38 * |
| Plant P concentration (mg $g^{-1}$) $10^{-1}$ | † | 5.7 ± 1.5 a | 5.9 ± 1.7 a | 5.5 ± 1.4 a | 6.3 ± 0.18 a | 1.9 ± 0.17 | 9.8 ± 0.46 * |
| Plant K concentration (mg $g^{-1}$) | 4.8 ± 0.3 a | 3.6 ± 0.4 c | 4.1 ± 0.3 b | 4.4 ± 0.2 ab | 4.3 ± 0.2 ab | 3.7 ± 0.2 | 4.9 ± 0.1 * |
| Chlorophyll (mg $g^{-1}$) | 2.6 ± 0.3 a | 2.3 ± 0.2 a | 2.4 ± 0.2 a | 2.4 ± 0.2 a | 2.1 ± 0.2 a | 1.9 ± 0.1 | 2.9 ± 0.1 * |
| Carotenoid (mg $g^{-1}$) | 0.71 ± 0.04 a | 0.50 ± 0.08 bc | 0.45 ± 0.05 c | 0.68 ± 0.04 ab | 0.64 ± 0.04 abc | 0.72 ± 0.04 | 0.48 ± 0.03 * |
| Carotenoid/chlorophyll ratio | 0.32 ± 0.05 a | 0.23 ± 0.05 b | 0.22 ± 0.05 b | 0.31 ± 0.04 ab | 0.34 ± 0.05 a | 0.40 ± 0.02 | 0.17 ± 0.02 * |
| $A_{max}$ ($\mu$mol $m^{-2}$ $s^{-1}$) | 4.0 ± 0.7 a | 4.7 ± 0.9 a | 5.0 ± 0.9 a | 4.9 ± 0.6 a | 4.0 ± 0.6 a | 2.8 ± 0.2 | 6.3 ± 0.4 * |

Data are means ± 1S.E. $A_{max}$, maximum net photosynthetic rate; RGC, root growth capacity. RGC data are adjusted means, after removing differences accounted for by the shoot mass covariate. Provenances with different letter indicate significant differences. Significant differences between fertilization treatments are indicated with an asterisk.

† Problems with samples in the laboratory prevented P concentration determination in the population of Tres Cantos.

In spring the first year, photochemical efficiency of photosystem II ($F_v/F_m$) was measured at mid-day on six plants per treatment with a portable fluorimeter (FMS2, Hansatech, UK) after darkening cladodes for 30 min.

Carbon isotopic composition ($\delta^{13}C$) was used as a surrogate of water use efficiency. We studied this in two provenances of contrasting climates: Rambla Honda and Alarilla (see Table 1). Current year shoots were harvested and finely ground at the end of the cultivation ($n = 5$) and from surviving plants in the field ($n = 6$) two years after planting. $^{13}C/^{12}C$ ratios were determined on 0.5–2 mg subsamples after combustion at 1020 °C in an elemental analyser (EA1500 NC, Carlo Erba, Milan, Italy) on-line with a mass spectrometer (Finnigan Delta Plus XL). Analytical precision is about ±0.1‰. The isotopic composition was expressed in delta notation and calculated as:

$$\delta^{13}C(‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000$$

where $R_{sample}$ and $R_{standard}$ are the $^{13}C/^{12}C$ ratios of each sample and of the classical calcite standard from Pee Dee Belemnite (PDB), respectively (Sulzman, 2007).

### 2.5. Isolation, genetic characterization, and nitrogenase activity of rhizobia in the transplanting plot

In spring 2003, two years after planting, we searched for nodules in seedlings of the Alarilla provenance that were nodulated when planted (inoculated F−) and in seedlings that were not nodulated at planting (non-inoculated both F− and F+). Four to six plants were excavated per treatment and the nodules attached to roots were placed in a portable fridge and taken immediately to the laboratory where they were surface sterilized with ethanol 95% (v/v) and $HgCl_2$ 0.1% (v/v) and washed with sterilized, distilled water. Nodules were cut and a loopful was transferred to a solid growth medium according to Vincent (1970). Two different strains were obtained: FIELD 1 obtained from the inoculated plants that were nodulated when planted and FIELD 2, obtained from the plants that were not inoculated in the nursery and were not nodulated when planted. This implies that the latter strain is native of the study site. We performed RAPD (Williams et al., 1993) to test whether the FIELD 1 strain was the same as the strain inoculated in the nursery (RST-1). Two *Bradyrhizobium* strains, RST-1 and RST-2, isolated from *R. sphaerocarpa* (Valladares et al., 2002) were used for the genetic study. RST-2 was a control strain used to set up the RAPD technique and to check reproducibility. DNA was prepared as described in Ausubel et al. (1992). Genomic DNA was purified from the lysate by repeated phenol–chloroform extractions and ethanol precipitations and was then treated with RNase A and proteinase K (Sambrook et al., 1989). The concentration and integrity of DNA was analysed by electrophoresis through 0.8% agarose gels, subsequently stained with ethidium bromide and compared with known amounts of phage lambda DNA (Sambrook et al., 1989). Five different oligonucleotide primers, obtained from Genotek (Barcelona, Spain), were arbitrarily chosen for DNA amplification in independent reactions: OPB-10, OPB-12 (Operon Company), P4 (Sikora et al., 1997), W60A, W60 (Williams et al., 1990). Final concentrations of PCR components were: 200 μM each of dATP, dCTP, dGTP and dTTP; 3 mM $MgCl_2$; 1 μM of primer; 1.25 units of *AmpliTaq* Gold DNA polymerase (Applied Biosystems, Madrid, Spain); 15 mM Tris–HCl (pH 8.0) −50 mM KCl as buffer; and 25 ng of template DNA, in 50 μL of final volume. Amplification was performed in a thermal cycler (PCR Express, Hybaid Limited, UK). Initial denaturing lasted 5 min at 95 °C, followed by 45 cycles consisting of denaturation (1 min, 94 °C), annealing (1 min, 36 °C), and DNA chain extension (2 min, 72 °C) (Williams et al., 1993). Final extension lasted 10 min at 72 °C. Blank control tubes containing all reagents except the template DNA or primer were also included in each run. The amplified products were electrophoresed through 1.2% agarose gels for 4 h at 3.5 V $cm^{-1}$, and the ethidium bromide-stained products were photographed under UV light (Sambrook et al., 1989). PCR profiles were compared visually, and those isolates showing patterns with identical bands were recorded as being the same type.

Measurement of the nitrogen fixation capacity of nodules was performed according to Fernández-Pascual et al. (1988).

### 2.6. Data analyses

As inoculation was applied only to F− plants of one of the provenances, the design was not full factorial. Therefore, to test the effect of fertilization and provenance on seedlings performance at the end of nursery cultivation and on out-planting growth, we used a mixed ANOVA model. Repetition was a random factor and it was nested within fertilization and provenance, which were fixed factors. In the case of RGC, as root growth depends on shoot size we used the shoot mass of plants used in RGC tests as covariate and calculated adjusted RGC means. To compare the performance of inoculated F− plants against non-inoculated F− and F+ seedlings of the Alarilla provenance we used a one-way ANOVA. Several variables were log-transformed to achieve homocedasticity (plant mass and carotenoid/chlorophyll ratio at the end of the cultivation phase and field shoot growth). Survival data were analysed by using a generalised linear model with a binomial distribution and a logit link function. The relationship between variables was analysed by using correlation analysis. We considered results significant when $P$-values were $\leq 0.05$. Classification of provenances was made by cluster analysis using the performance variables measured at the end of the cultivation period (see Table 2 and Fig. 2) and in the field (survival and shoot growth). Euclidean distance was used as distance measure and Ward's method as the linkage rule. Statistical analyses were performed with STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK, USA).

## 3. Results

### 3.1. Morphological and physiological differences at the end of the cultivation

Fertilization had a greater effect on functional attributes than provenance. F+ plants had greater plant mass, shoot to root mass ratio, RGR, RGC, $A_{\max}$, and nutrient and chlorophyll concentration than F− plants ($P < 0.01$). On the contrary, F− seedlings had higher carotenoid concentration and carotenoid/chlorophyll ratio than F+ plants ($P < 0.001$) (Fig. 1, Table 2).

Provenances differed in seed mass, K and carotenoid concentration ($P < 0.003$), the carotenoid/chlorophyll ratio ($P < 0.01$), plant mass, and RGR ($P < 0.05$). Seeds from the arid and semiarid locations (Rambla Honda and Almunia de D. Godina, respectively) had greater mass than those from the other provenances, which did not differ among them (Table 2). Plant mass and RGR differences among provenances depended on fertilization rate (provenance × fertilization interaction, $P = 0.05$). With respect to RGR, no differences among provenances were observed at the low fertilization level. However, under high fertilization, Navas del Rey and Tres Cantos had the highest RGR, Rambla Honda had the lowest, and Alarilla and Almunia D. Godina had intermediate RGR. With respect to plant mass, the interaction of provenance with fertilization consisted in changes in provenance ranking depending on fertilization regime (Fig. 1). Among provenances, plant mass of F− seedlings was not correlated with plant mass of F+ seedlings. A similar lack of correlation was observed for RGR. No significant correlation existed between RGR, both at high and low fertilization, and seed mass ($r = -0.65$, $P = 0.24$ and $r = -0.09$, $P = 0.89$ for F+ and F− plants, respectively). In contrast, provenance RGR at a high fertilization rate was positively related with the mean rainfall ($r = 0.90$, $P = 0.038$) and negatively related to the duration of the growing season at seed-source sites (Fig. 1).

The provenance of Tres Cantos had the highest K concentration, Navas del Rey the lowest and the remaining provenances had intermediate K concentration (Table 2). Tres Cantos and Rambla Honda had the highest carotenoid/chlorophyll ratio, Alarilla and Navas del Rey the lowest, and Almunia de D. Godina had an intermediate carotenoid/chlorophyll ratio. Differences in carotenoid/chlorophyll ratio were due to differences in carotenoid concentration but not in chlorophyll concentration. Neither K nor carotenoid concentration or the carotenoid/chlorophyll ratio, were correlated with any climatic variable at the seed-source location (data not shown).

Massive nodulation in the nursery was only observed in the inoculated F− treatment, where 94% of seedlings had nodules. The mean number of nodules per plant and nodule mass per seedling were $10.8 \pm 2.1$ and $3.4 \pm 0.53$ mg ($n = 32$), respectively. Plant mass in inoculated F− plants was positively correlated with nodule mass ($r = 0.59$, $P < 0.001$). Only 6% of non-inoculated F− plants from the Almunia de D. Godina and Navas del Rey provenances produced nodules. Neither F+ seedlings nor non-inoculated F− plants from other provenances produced nodules.

Inoculated F− plants had an intermediate N concentration with respect to non-inoculated F+ and F− seedlings. Similarly, inoculated F− plants had slightly higher $A_{\max}$ than non-inoculated F− plants but lower $A_{\max}$ than non-inoculated F+ seedlings (Table 3). For most of the remaining variables, inoculated and non-inoculated F− plants were similar and had lower values than F+ non-inoculated plants. RGC and plant K and chlorophyll concentration did not

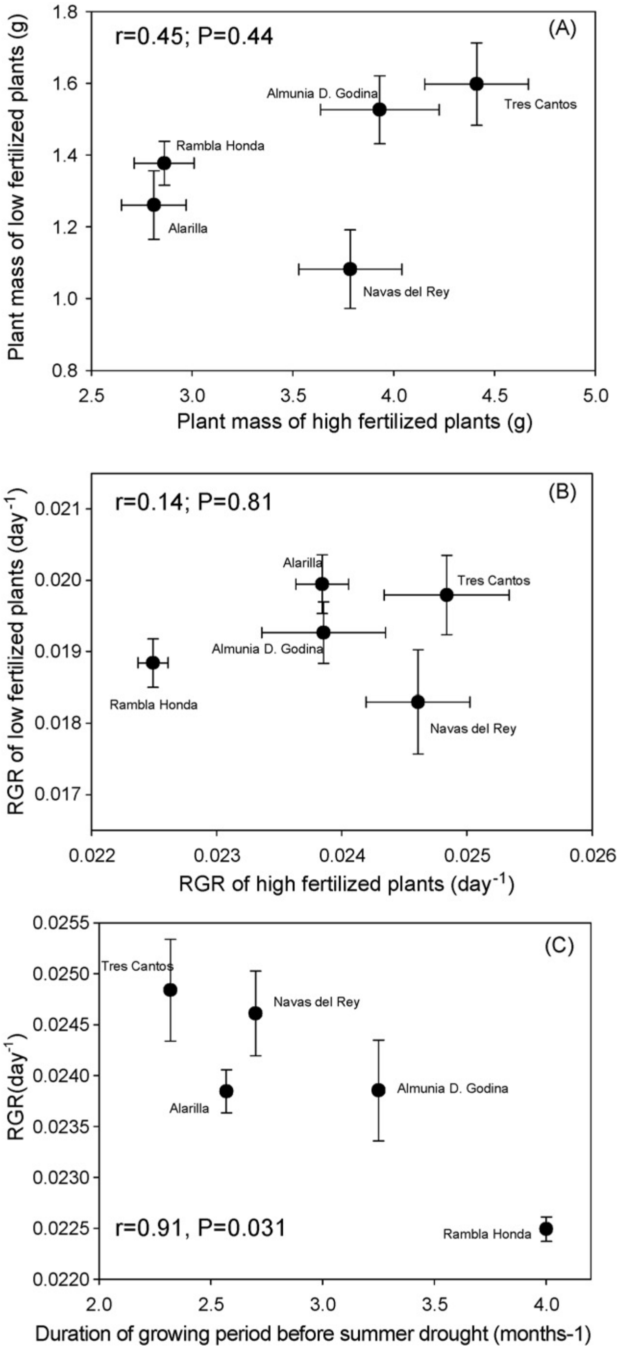

**Fig. 1.** Relationships between the plant mass (A) and RGR (B) of low-fertilized plants *versus* plant mass and RGR of high-fertilized seedlings across five *Retama sphaerocarpa* provenances. Figure (C) represents the relationship between RGR of high-fertilized plants from five *R. sphaerocarpa* provenances and the duration of the growing season of seed sources. Each point is the mean of 16 plants ± 1S.E.

**Table 3**
Functional differences among inoculated and non-inoculated low-fertilized *Retama sphaerocarpa* seedlings and non-inoculated high-fertilized seedlings

| | Low-fertilized | | High-fertilized |
|---|---|---|---|
| | Non-inoculated | Inoculated | Non-inoculated |
| Plant mass (g) | 1.26 ± 0.1 b | 1.27 ± 0.09 b | 2.62 ± 0.19 a |
| Shoot to root mass ratio | 0.80 ± 0.05 b | 0.71 ± 0.03 b | 1.26 ± 0.08 a |
| RGR (month$^{-1}$) × $10^{-3}$ | 20.0 ± 0.41 b | 19.90 ± 0.36 b | 23.51 ± 0.62 a |
| RGC (mg) | 47.5 ± 4.8 a | 40.6 ± 4.2 a | 40.4 ± 6.0 a |
| Plant N concentration (mg g$^{-1}$) | 13.9 ± 0.8 b | 15.5 ± 0.5 ab | 16.7 ± 1.0 a |
| Plant P concentration (mg g$^{-1}$) | 0.18 ± 0.01 b | 0.18 ± 0.01 b | 1.0 ± 0.13a |
| Plant K concentration (mg g$^{-1}$) | 3.5 ± 0.3 a | 4.0 ± 0.3 a | 4.8 ± 0.1 a |
| Chlorophyll (mg g$^{-1}$) | 2.2 ± 0.2 a | 2.7 ± 0.2 a | 2.7 ± 0.2 a |
| Carotenoid (mg g$^{-1}$) | 0.61 ± 0.07 a | 0.75 ± 0.02 a | 0.28 ± 0.05 b |
| Carotenoid: chlorophyll | 0.33 ± 0.05 a | 0.28 ± 0.02 a | 0.10 ± 0.02 b |
| $A_{max}$ (μmol m$^{-2}$ s$^{-1}$) | 2.9 ± 0.4 b | 3.7 ± 0.4 ab | 7.1 ± 1.3 a |

Data are means ± 1S.E. Means with different letter indicate significant differences. $A_{max}$, maximum net photosynthetic rate; RGC, new root growth capacity. RGC data are adjusted means after removing differences accounted for by the shoot mass covariate. Measurement of traits in the inoculated treatment was done only in nodulated plants.

differ among treatments while carotenoid concentration and the carotenoid/chlorophyll ratio, were lower in non-inoculated F+ plants than in the inoculated and non-inoculated F− plants.

### 3.2. *Transplanting performance, persistence of the inoculated Rhizobium strain and nitrogenase activity*

Two years after transplanting F+ plants had 43% greater survival than F− plants ($P<0.001$) (Fig. 2). This difference could already be observed at the end of the first summer ($P<0.001$). After the first summer, provenances differed in survival ($P<0.024$). Alarilla and Navas del Rey had the lowest survival, Tres Cantos and Rambla Honda the highest and the survival of Almunia de D. Godina was intermediate between both groups (data not shown). After two years in the field, the Alarilla provenance still had the lowest survival, while the other provenances had similar survival ($P=0.040$). Shoot mass of F+ plants increased more than in F− plants ($P<0.001$). Provenances differed in shoot mass increase, although differences were only noticeable among F+ plants but not among the F− ones (interaction provenance × fertilization, $P=0.004$) (Fig. 2). No significant differences in RGR and $F_v/F_m$ existed either between fertilization rates or among provenances (data not shown).

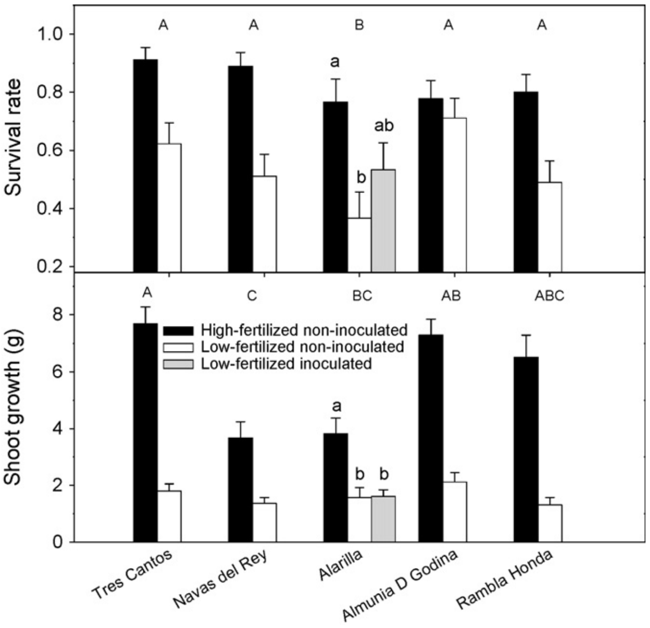

**Fig. 2.** Survival and shoot growth (means ± 1S.E.) two years after transplanting of *Retama sphaerocarpa* seedlings from five provenances that were cultivated in the nursery at high and low fertilization rates. In the Alarilla provenance, survival and aboveground biomass production of inoculated low-fertilized seedlings in the nursery is compared with that of low- and high-fertilized non-inoculated plants. Statistical differences among provenances are indicated with upper case letters, whereas differences among the low fertilized plants, both inoculated and non-inoculated, and non-inoculated high-fertilized seedlings are indicated with lower case letters. All transplanted inoculated F− plants were nodulated.

Inoculated F− plants had greater survival than non-inoculated F− plants, but lower survival than non-inoculated F+ plants ($P=0.037$). Survival differences between inoculated F− and non-inoculated F− plants were not apparent after the first summer (data not shown). No differences in shoot mass increase were observed between inoculated and non-inoculated F− plants. These two groups had lower shoot growth than non-inoculated F+ plants (Fig. 2).

Among provenances, survival of low fertilized plants and shoot growth was positively related to carotenoid concentration and $F_v/F_m$. No relationship existed between the survival of high-fertilized seedlings and carotenoid concentration. $F_v/F_m$ was positively related to carotenoid concentration. Field survival of retama provenances was not related to RGR measured under optimal growing conditions in the nursery (Fig. 3).

Rambla Honda had higher $\delta^{13}C$ than Alarilla ($P=0.038$) and F+ plants had higher $\delta^{13}C$ than F− seedlings ($P=0.014$), both at the end of the cultivation and two years after planting. At the end of the cultivation period, inoculated and non-inoculated F− plants had lower $\delta^{13}C$ than non-inoculated F+ plants. However, in the field, non-inoculated F− retama plants had lower $\delta^{13}C$ than inoculated F− and non-inoculated F+ plants (interaction treatment × site $P=0.024$) (Fig. 4).

All inoculated F− and non-inoculated F+ plants of the Alarilla provenance excavated in the field had nodules. In contrast, only one out of four F− non-inoculated plants had nodules. RAPD has never been applied to track the genetic variability of *Bradyrhizobium* sp. isolated from retama. Five oligonucleotide decamers (OPB-10, OPB-12, P4, W60A and W60) were tested for their ability to generate RAPDs markers from genomic DNAs. OPB-12 primer yielded clear and reproducible patterns. This primer (OPB-12) was chosen for further analysis of Bradyrhizobium isolates (RST-1, RST-2, FIELD-1, FIELD-2). The OPB-12 patterns were reproducible between the three different DNA preparations from the same strain as well as between several electrophoresis when samples were ran a second or a third time. The unrelated isolates RST-1 and RST-2 gave

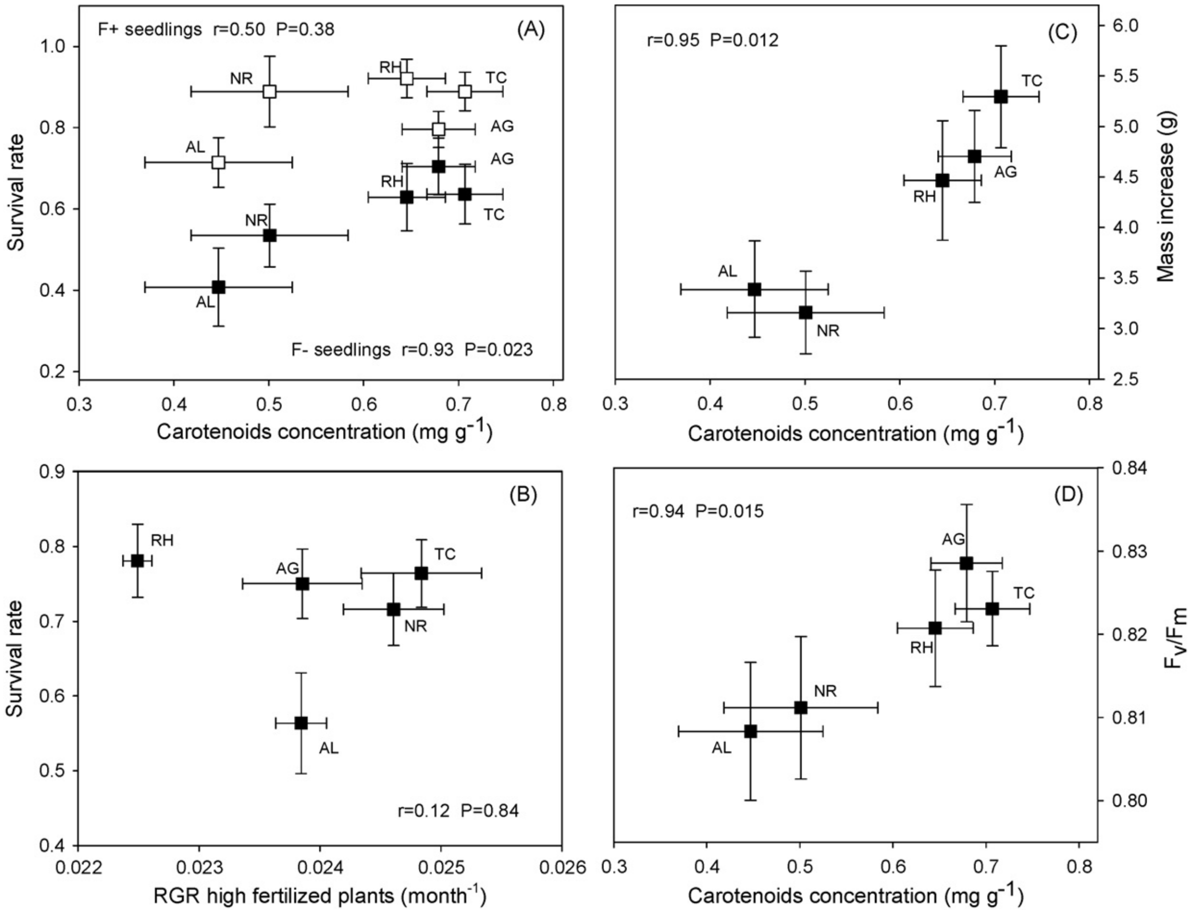

**Fig. 3.** (A) Relationship between transplanting survival of high- and low-fertilized plants (open and closed squares, respectively) and the mean provenance carotenoid concentration. (B) Relationship between transplanting survival and the RGR of seedlings during nursery cultivation at high fertilization. (C) Relationship between transplanting growth and mean provenance carotenoid concentration. (D) Relationship between $F_v/F_m$ and mean provenance carotenoid concentration. In (B) and (C) carotenoid concentration for provenances are averaged over fertilizer levels. Survival and shoot mass increase was measured two years after transplanting, $F_v/F_m$ was measured during the spring of the first year and carotenoid concentration was determined at the end of nursery cultivation period. Bars are one standard error. AG, Almunia D. Godina; AL, Alarilla; NR, Navas del Rey; RH, Rambla Honda; TC, Tres Cantos.

a reproducible and distinct profile in the RAPD analysis (Fig. 5). The strain isolated in the field from the plants that were nodulated in the nursery (FIELD-1) had a different RAPD pattern than the strain isolated in the field from the non-inoculated plants (FIELD-2). The FIELD-1 strain had the same molecular pattern to the one used to inoculate the F− seedlings in the nursery (RST-1) and thus, were considered identical. The molecular pattern of strain FIELD-1 was the only genetic pattern recovered from inoculated plants in the field and the pattern of FIELD-2 was the only one recovered from the non-inoculated plants (Fig. 5).

Nitrogenase activity of the nodules obtained from excavated inoculated F− plants was $13.4 \pm 6.8\,\mu\text{mol g}^{-1}\,\text{h}^{-1}$ whereas the nitrogenase activity of non-inoculated plants that had nodulated in the field (F+ and F− plants pooled) was $6.4 \pm 4.0\,\mu\text{mol g}^{-1}\,\text{h}^{-1}$. These differences were not statistically significant (Mann–Whitney $U$ test = 16.0, $Z = -0.71$ $P = 0.47$).

### 3.3. Provenance classification

Cluster analysis revealed two main groups. The first one was formed by the Alarilla and Navas del Rey provenances. This group had the lowest field performance, carotenoid and K concentration, and the lowest carotenoid/chlorophyll ratio. The second group was composed by the rest of the provenances and had the opposite performance attributes to the first group. In this second group, Tres Cantos and Almunia de Doña Godina provenances had more affinity between them than with Rambla Honda (data not shown).

## 4. Discussion

### 4.1. Functional traits related to transplanting performance: integrating the fertilization and provenance results

Fertilization in the nursery affected more attributes, induced stronger changes in attributes, and affected transplanting performance more than provenance and rhizobial inoculation. Fertilization produced two phenotypes with contrasting functional attributes that had distinct transplanting performance. In agreement with our first hypothesis, large seedlings with high shoot to root mass ratio and high nutrient concentration (high-fertilized seedlings) had greater transplanting performance (Fig. 2) than the seedlings with the opposite traits (low-fertilized seedlings) although the former attributes have been traditionally considered to reduce drought resistance (van den Driessche, 1991; Lloret et al., 1999). Similar to our findings, transplanting performance in several Mediterranean species has also been positively related to plant size, shoot to root ratio and nutrient content when planted (Puértolas et al., 2003; Villar-Salvador et al., 2004; Oliet et al., 2005; Tsakaldimi et al., 2005). Survival in dry environments depends on the formation of new roots during the wet season and high pho-

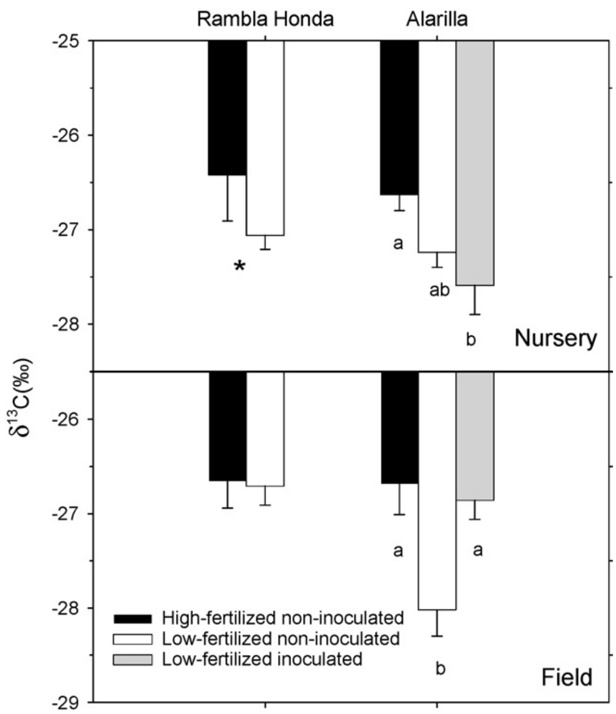

**Fig. 4.** $\delta^{13}$C of two *Retama sphaerocarpa* provenances at the end of nursery cultivation period (upper figure) and two years after transplanting (lower figure). In the Alarilla provenance, the $\delta^{13}$C of inoculated low-fertilized seedlings is compared with the $\delta^{13}$C of the non-inoculated low- and high-fertilized plants. Treatments in the Alarilla provenance with different letters have significant differences. Data are means ± 1S.E. Asterisk indicates significant differences between high- and low-fertilized plants.

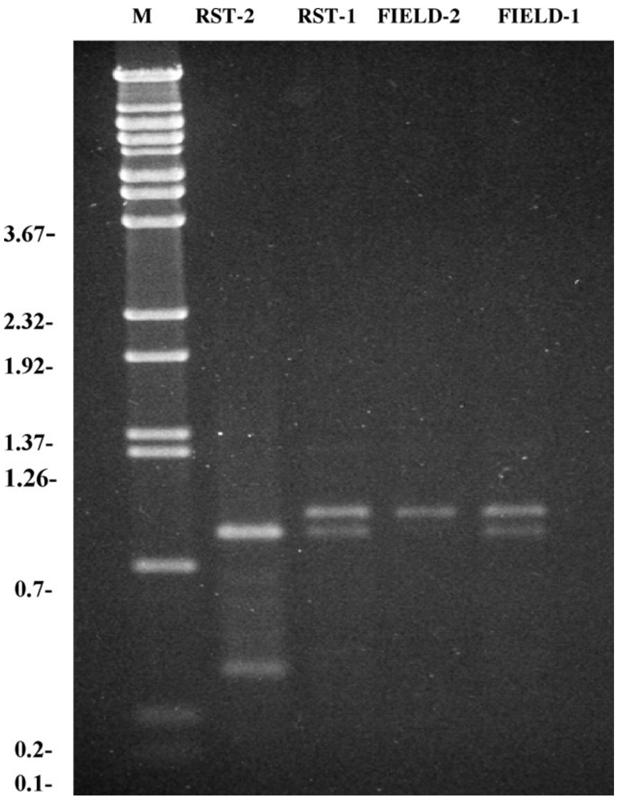

**Fig. 5.** RAPD patterns of the *Bradyrhizobium* strains analysed. OPB-12 was employed as primer and RST-2 was the reference strain. Lane M, size markers [Lambda DNA-BstE II digest (New England Biolabs)] with sizes in kilobase pairs shown on the left of the panel. RST-1 was the strain used to inoculate seedlings in the nursery, FIELD 1 was the strain isolated in the field from inoculated F− plants, and FIELD 2 was the strain isolated in the field from non-inoculated plants and this implies that it is native of the study site.

tosynthesis enhances root production (van den Driessche, 1987; Kaushal and Aussenac, 1989). Large seedlings produce larger root systems and have higher nutrient content, which can enhance seedling establishment in dry sites and on oligotrophic soils (Cook, 1980; Leishman and Westoby, 1994; Westoby et al., 1996). Further, plants with high N concentration can potentially remobilize larger amounts of N to support new growth than seedlings with low N concentration (Salifu and Timmer, 2003). In agreement with this, high-fertilized phenotypes were bigger, had greater new root growth capacity, photosynthetic rate, and had higher N concentration than low-fertilized phenotypes. Superior transplanting performance of high-fertilized seedlings compared to low-fertilized plants may also be explained by their higher water use efficiency as inferred by their higher $\delta^{13}$C (Livingston et al., 1999).

Provenances differences can affect multiple functional attributes relevant for plant fitness (Cregg, 1994; Chunyang et al., 2000). Provenances differed in RGR and consequently on final plant mass but these differences were only apparent under high nutrient availability (Fig. 1). Differences in RGR can be attributed to maternal effects (seed size) and to genetic differences or both. In our study, no relationship was observed between RGR and seed size, supporting the hypothesis that RGR differences among provenances have a genetic basis. However, contrary to our second hypothesis, provenances of low RGR did not have greater survival than fast growing provenances suggesting no trade-off between RGR and survival in this species.

Functional differences among provenances are frequently related to the ecological conditions of seed sources (Balaguer et al., 2001; Aranda et al., 2005). Only RGR and $\delta^{13}$C were related to the climate of seed sources. The provenance of the driest and hottest site, Rambla Honda, had higher water use efficiency than the Alarilla provenance, which had more humid and colder climate, as inferred by its higher $\delta^{13}$C (Zhang and Marshall, 1993) (Fig. 4). Similarly, RGR differences among provenances were negatively related to the length of the growing season (Fig. 1) and positively related to rainfall, which in part supports our second hypothesis. This suggests a selection for genotypes capable of early fast growth in sites with shorter growing season and humid climate. Similar to our findings, ecotypes of *Medicago polymorpha* from sites with higher rainfall and longer growing season have lower RGR than ecotypes from drier sites (Del Pozo et al., 2002). The length of growing season in Mediterranean climates is determined by the duration of low temperature in winter and summer drought (Mitrakos, 1980). Seedlings that emerge in spring need to establish rapidly before the summer drought begins. Therefore, seedlings with a high RGR may have an advantage for establishing in sites with short growing season, as it occurs in areas with continental Mediterranean climate, than seedlings with low RGR.

Very interestingly, provenances that concentrated more carotenoids had higher survival and growth in the field than the provenances that had lower carotenoid concentration. Carotenoids protect the photosynthetic apparatus against photoinhibition, a process that frequently reduces plant carbon assimilation when plants are exposed to high radiation and drought simultaneously (Hikosaka et al., 2004; Flexas and Medrano, 2002). Retama reduces photodamage through structural avoidance of light interception (Valladares and Pugnaire, 1999). Our results suggest that carotenoids also play an important photoprotection role in retama and that the provenances with higher carotenoid concentration had a potentially better capacity to avoid photoinhibition, which may explain their higher survival and growth. The positive relationship between $F_v/F_m$ and carotenoid concentration supports this suggestion. Although provenances $F_v/F_m$ values were high, minor reductions in $F_v/F_m$ from optimum values (≈0.83) can be very significant for plant performance in Mediterranean shrubs (Aragón et al., 2008).

The importance of carotenoids for retama survival, however, may be context-dependent. This hypothesis is supported by the fact that survival of provenances with higher carotenoid concentration was only apparent among low fertilized seedlings but not among high-fertilized plants (Fig. 3). Thus, a high carotenoid concentration would be less important for seedlings that experience low water stress, as is probably the case of high-fertilized seedlings because they have higher drought avoidance capacity than low fertilized seedlings. The higher survival of high-fertilized seedlings compared to low-fertilized seedlings in spite of their lower carotenoid concentration does also support this idea.

### 4.2. *Effect of rhizobial inoculation*

Nodulation of inoculated plants was high and comparable to results from a previous study with the same rhizobial strain (Valladares et al., 2002). However, compared to fertilization, inoculation had a small effect on most plant functional attributes at the end of nursery cultivation. High-fertilized non-inoculated plants were superior to inoculated low-fertilized plants. Similarly, differences between inoculated plants and non-inoculated low-fertilized seedlings were small (Table 3), which is in contrast with a previous study in which inoculation had a marked effect on retama performance (Valladares et al., 2002). This suggests that nodules in the present study had low nitrogenase activity during the cultivation phase, which may be explained either by the different provenances used in both studies, by the lower fertilization rate in the present study, or both (Woldemeskel and Sinclair, 1998; Thrall et al., 2000; Gan et al., 2005). However, some effects of inoculation were observed in our study. First, inoculated plants tended to concentrate more N and had higher photosynthesis than non-inoculated low-fertilized seedlings, which was consistent with the higher $\delta^{13}C$ in the former treatment. Second, growth of inoculated plants was positively correlated with nodule mass.

Field survival of inoculated plants was 20% higher than that of non-inoculated low-fertilized seedlings, although differences were not statistically significant. However, field growth was not enhanced by inoculation. In several *Acacia* species rhizobial inoculation increased seedling survival but, similar to our findings, it had no clear effect on growth (Thrall et al., 2005). Two years after transplanting, inoculated seedlings still maintained higher water use efficiency as inferred by their more positive $\delta^{13}C$, which can probably be attributed to greater photosynthetic capacity rather than to differences in stomatal sensitivity to environmental conditions (Zhang and Marshall, 1993; Livingston et al., 1999). However, higher water use efficiency did not translate into greater transplanting performance. The lack of significant superior transplanting performance in inoculated seedlings cannot be explained by loss of the inoculated strain or by low nitrogenase activity. Inoculated plants still maintained a symbiotic relationship with the inoculated strain (RST-1) two years after transplanting and had high nitrogenase activity. This indicates that the inoculated strain is a good competitor against native rhizobial strains. The lack of differences in transplanting performance between inoculated and non-inoculated F− seedlings may be explained by the nodulation of non-inoculated seedlings with native rhizobial strains (FIELD-2) after transplanting. These native strains had similar nitrogenase activity to the strain used for the inoculated F− plants.

## 5. Concluding remarks

Transplanting performance of retama seedlings is linked to a suite of attributes that promote fast seedling establishment during the wet season, probably enhancing drought avoidance during the first dry season. Large plants, with higher nutrient concentration and root growth capacity had a greater field performance than seedlings with the opposite traits. Similarly, transplanting performance was higher in provenances with higher carotenoid concentrations, suggesting a greater capacity to minimize photoinhibitory damage. Performance differences among provenances were not related to the climate of seed source, except RGR, which was higher in provenances with a short growing season and high rainfall. Rhizobial inoculation had small effects on the performance of retama seedlings although the strain used in this study had high field persistence. RAPD analysis has a high potential for strain identification in studies of rhizobial symbiosis persistence.

Our study demonstrates that high fertilization during nursery cultivation enhances survival and growth in retama seedlings. However, high fertilization creates potential for negative environmental impacts and health risks. Therefore, future research should explore other fertilization regimes and nutrient combinations (especially N *versus* P) to maximise plant nutrient loading and rhizobial symbiosis, and minimise fertilizer leaching from nurseries (see Quoreshi and Timmer, 1998).

## Acknowledgements

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