



Enhancing the early performance of the leguminous shrub *Retama sphaerocarpa* (L.) Boiss.: fertilisation versus *Rhizobium* inoculation

Fernando Valladares^{1,4}, Pedro Villar-Salvador², Susana Domínguez², Mercedes Fernández-Pascual¹, Juan Luis Peñuelas² & Francisco I. Pugnaire³

¹Centro de Ciencias Medioambientales, CSIC, Serrano 115 dpdo. E-28006 Madrid, Spain; ² Centro Nacional de Mejora Forestal 'El Serranillo', DGCONA, Ministerio de Medio Ambiente, Apartado de Correos 249, E-19004 Guadalajara, Spain; ³Estación Experimental de Zonas Áridas, CSIC, General Segura 1, E-04001 Almería, Spain

⁴Corresponding author*

Received 29 March 2001. Accepted in revised form 21 January 2002

Key words: fertilisation, leguminous shrubs, nitrogen use efficiency, revegetation, *Rhizobium* inoculation

Abstract

We have investigated the effect on growth of fertilisation versus biological nitrogen fixation by rhizobial nodules in *Retama sphaerocarpa* (L.) Boiss, a leafless leguminous shrub native to the Iberian Peninsula and North-West Africa that has generated interest for revegetation of dry Mediterranean habitats. Our main objective was to optimise the formation of root nodules under nursery conditions and to evaluate their influence on the first year of seedling growth in comparison with standard fertilisation. Seedlings of *R. sphaerocarpa* from two Spanish localities were grown under two levels of fertilisation, and half of each were inoculated with rhizobia isolated from adult *Retama*, *Cytisus* and *Adenocarpus* plants in the field. Although some promiscuity was observed, nodulation was significantly successful with specific rhizobia. At the end of the experiment, highly fertilised plants were taller and heavier and exhibited larger photosynthetic rates than either nodulated or non-nodulated plants under low fertilisation. High fertilisation enhanced seedling growth but inhibited both the nodulation and the nitrogenase activity of the nodules. Thus, physiological differences between nodulated and non-nodulated plants were observed in the low but not in the high fertilisation treatment. Nitrogen uptake and use was enhanced by root nodules, which translated into enhanced photosynthesis and growth. Since inoculation is simple, environmentally friendly and cheap, and nodulated plants are more likely to overcome transplant stress than non-nodulated ones, our results suggest that inoculation together with low, background fertilisation (instead of high fertilisation) should be used when producing high quality seedlings of this autochthonous Mediterranean shrub.

Introduction

Global change and the associated habitat degradation requires effective human interventions especially in dry and degraded areas. Among the most important interventions that will assist towards the recovery of these habitats are revegetation projects but their effectiveness is limited by the paucity of ecophysiological knowledge on native species. Leguminous shrubs are dominant in a range of arid and extreme habitats

(Bossard and Rejmanek, 1992; Nilsen, 1992; Pugnaire et al., 1996), and have been considered useful for revegetation of water deficient ecosystems that have low availability of nitrogen, phosphorus and other nutrients (Herrera et al., 1993). Biological nitrogen fixation is an alternative to artificial fertilisation that circumvents much of the negative effects (both environmental and sanitary) of fertiliser usage (Dileep Kumar et al., 2001; Newton, 2000), and is of especial interest in sustainable agriculture and land rehabilitation in adverse habitats (Barea et al., 1990; Franco et al., 2000). Despite intense research on biological nitrogen fixation by rhizobial nodules of legume crops (e.g., Dileep Ku-

* FAX No: +34-91-5640800.
E-mail: valladares@ccma.csic.es

mar et al., 2001; Newton, 2000; Streeter, 1988), very little is known regarding wild leguminous and non-leguminous nitrogen-fixing species, especially trees and shrubs (Dart, 1998; Huss-Danell, 1980; Schulze et al., 1991; Werner et al., 1998). Since the *Rhizobium*-legume symbiosis exhibits high specificity and the influence of nodulation on nutrient economy and plant growth is also very species specific (Pate, 1986; Santamaria et al., 1997), the knowledge obtained with legume crops cannot be directly extrapolated to wild shrub legumes.

Many leguminous shrubs exhibit a prominent stem net photosynthesis, which has a number of possible benefits: extension of period of carbon gain in environments with periodic droughts (Nilsen, 1992; Smith and Osmond, 1987), heat (Smith and Osmond, 1987) or excessive irradiance (Valladares and Pugnaire, 1999), and tolerance of herbivory (Bossard and Rejmanek, 1992). Stem photosynthesis helps meet different needs and provides a functional flexibility that can explain the invasive nature of certain leguminous shrubs (Bossard and Rejmanek, 1992).

We have investigated here the effect of fertilisation and biological nitrogen fixation by rhizobial nodules on seedling development in the practically leafless leguminous shrub retama (*Retama sphaerocarpa* (L.) Boiss). Our main objective has been to optimise the formation of root nodules under nursery conditions and to evaluate their influence on the first year of growth in comparison with a standard fertilisation practise. In particular, we had three questions: (i) What physiological and growth parameters are enhanced or affected by nodulation? (ii) How important is this enhancement in comparison with standard fertilisation? (iii) *Rhizobium* strains from which leguminous shrub (*Cytisus*, *Adenocarpus*, *Retama*) render the largest nodulation efficiency in Retama? Retama is native to the Iberian Peninsula and North-West Africa, and is frequent in a wide range of dry Mediterranean habitats. Retama has a notable ecological role due to the formation of 'islands of fertility' where the growth of a number of annuals and other woody species is favoured (Pugnaire et al., 1996). It has a remarkable capacity to withstand drought and the associated Mediterranean summer stresses due to its crown architecture and its deep root system (Haase et al., 1996, 1999; Valladares and Pugnaire, 1999). Retama formations have been intensively exploited, which has generated seminatural savanna-like systems of remarkable productivity (Gómez Sal et al., 1999). Retama seedlings can be produced in nurseries and there is increasing aware-

ness of its value in revegetation projects. However, studies on how seedling performance is affected by nutrient availability and/or by the presence of nodules in this species are lacking. Current nursery techniques do not involve inoculation and commercially available retama seedlings commonly lack nodules.

Materials and methods

Plant material and experimental design

Seeds of *Retama sphaerocarpa* were collected during the early autumn of 1998 from two wild populations, one located in the most arid region of Europe (Tabernas desert, Almería, Spain) and the other located in a dry continental site (Almunia de Doña Godina, Zaragoza, Spain). Seeds were sown in [®]Forest Pot containers (cavity volume of 300 cm³) filled with horticultural peat (pH ca. 7) on 17 March 1999. Peat was used because it enhanced the chances of not having nodules in the non-inoculated (control) plants, and because it is a standard substratum in many commercial nurseries. Plants were maintained in the nursery of the National Centre for Plant Genetic Improvement 'El Serranillo' (Ministerio de Medio Ambiente) in the vicinities of Guadalajara (Central Spain). Both the areas of seed origin and the area where the nursery is located have a Mediterranean-type climate with a dry and hot summer; most of the precipitation falls in autumn and spring. Three to four seeds were sown per cavity and after germination only one seedling was allowed to grow. Date of sowing was taken as day 0 of the experiment. The plants were well watered and kept outdoors under ambient irradiance, temperature and air humidity from spring to autumn 1999 (days 0 – 230). Plants were transferred in autumn (day 230) to a glass house and they were maintained under summer conditions (diurnal mean temperature of 25 °C, rising up to 35 °C at midday, ambient light supplemented with halogen 400 W Philips HLRG lamps rendering an average 500 ± 100 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) at the plant level) till the end of the experiment (day 295).

A factorial experiment was designed to test for main effects and interactions of fertilisation and *Rhizobium* inoculation on several morphological, physiological and growth variables. Factors were: two populations, two fertilisation regimes, and five inoculants plus a control (no inoculation). Half of the plants received a high fertilisation regime and the other half

a low fertilisation regime. Nutrient solutions contained 41, 12 and 4 mg per l of N (provided as $\text{NO}_3 \text{NH}_4$), P and K, respectively (high fertilisation) and 4, 2 and 1 mg per l of N, P and K respectively (low fertilisation). Both nutrient solutions were enriched with 1 ml per l of a solution of oligoelements containing 1 g of mg, 0.7 g of Ca, 0.7 g of S, 2 g of Mn, 2 g of B, 0.1 g of Cu, 0.1 g of Zn, 0.1 g of Na, 0.1 g of Mo, 0.5 g of Fe, 0.1 g of Ni, 0.1 g of Co, and 0.5 g of I per l of solution. Nutrient solutions were supplied via the irrigation system once a week. At the end of the experiment, each plant had received 200, 60, and 20 mg of N, P and K respectively in the high fertilisation regime, and 20, 10, and 4 mg per plant of N, P and K respectively in the low one.

Allometric and growth measurements

A minimum of 10 plants from each treatment were harvested at four different times during the experiment (days 30, 95, 180 and 295). Prior to harvest, plant height was measured in 20 plants per treatment. Harvested plants were divided into leaves (leaves are small in size and number and of a very short lifespan in *Retama sphaerocarpa*), cladodes (photosynthetic, green stems) and roots. Leaves and cladodes of each plant were scanned with a PC compatible desk scanner at 600 dpi resolution, and the area was estimated with a commercially available image analysis software (Sigmascan, Jaendel Scientific, USA). The projected area of the cladodes was multiplied by $\pi/2$ to correct for the fact that their surface corresponds to that of a hemi-cylinder. Plant material was then dried for 48 h at 65 °C and weighed. From this data we calculated root:shoot ratio, leaf area ratio (LAR, $\text{m}^2 \text{kg}^{-1}$, considering 'leaf area' as leaf plus cladode surface area) and relative growth rate (RGR, $\text{g g}^{-1} \text{day}^{-1}$, calculated as \ln mean dry weight at time 2 minus \ln mean dry weight at time 1 divided by time elapsed in days). Total plant biomass and root:shoot ratio were measured at the four harvesting times, LAR was calculated for days 30, 95 and 180. By day 30, most plants had germinated and were initiating stem growth with the cotyledons still functional. Since plants exhibited some ontogenetic and developmental variability at each sampling time even within the same treatment, only plants at the most frequent and representative ontogenetic stage were sampled each time. RGR was calculated for the following three periods: RGR_1 days 30–95, RGR_2 days 95–180, and RGR_3 days 180–295. In addition, cladode diameter was measured on 25

plants of each treatment on day 95 with a calliper of an instrumental error <0.01 mm.

Bacteria isolation and inoculant preparation

Five different inoculants were prepared from nodules collected from adult plants of different leguminous shrubs in the field. Inoculants RST-1 and RST-2 were obtained from two different individuals of *Retama sphaerocarpa* from Torrelaguna (Madrid, Spain), inoculant AH-EP1 was obtained from *Adenocarpus hispanicus* (Lam.) DC. (El Paular, Madrid, Spain), and inoculants CP-EP1 and CP-P1 were obtained from *Cytisus purgans* (L.) Boiss. (from El Paular, Madrid, and Piedralabes, Avila, respectively). According to Leon-Barrios et al. (1991), inoculants RST-1 and RST-2 were ascribed to *Bradyrhizobium* and inoculants AH-EP1, CP-EP1 and CP-PA1 to *Rhizobium*. The strains are kept with these reference codes in M. Fernández-Pascual collection. The surface of the nodules was sterilised with ethanol 95% (v/v) for 30 s and then washed five times with sterilised distilled water. Sterilised nodules were cut with a razor blade and a sample was transferred to Petri dishes containing solid Vincent (1970) medium. The inoculant was prepared by growing the strains from isolated colonies at 28°C with orbital shaking in liquid Vincent (1970) media. Seedlings were inoculated on day 57 with 1 ml per plant of strain suspension containing 10^8 colony forming units ml^{-1} .

Nitrogenase activity

Nitrogen fixation was measured by the acetylene reduction assay (ARA) on nodulated roots following the method described in Fernández-Pascual et al. (1988). Roots were enclosed in 100 ml tubes closed with rubber stoppers. Ten ml of air were removed from the tubes and the same amount of acetylene was added. Incubation temperature was 25 °C. Gas samples were taken after 15 min of acetylene exposition to eliminate the problems of plant disturbance and long-term exposure to acetylene (Sinclair and Serraj, 1995). Gas samples were analysed for ethylene and acetylene content in a Perkin-Elmer 8310 gas chromatograph equipped with a hydrogen flame ionisation detector and a column filled with Porapak R. Nitrogen was used as the carrier gas and the flow rate was set to 50 ml min^{-1} . Although a flow-through system for the assessment of nitrogenase activity (Fernández-Pascual et al., 1996; Minchin et al., 1992) was available, the relatively low number of nodules per root obtained with

inoculants AH-EP1, CP-EP1 and CP-PA1, their relatively low nitrogenase activity, and the elevated number of treatments and replicates made ARA a more viable method.

Photosynthetic measurements

Maximum photosynthetic (A_{\max}) and dark respiration rates of the cladodes were measured with a portable open gas exchange system (LCA4, Analytical Development Co., Hoddesdon, UK). The central unit recorded incident photosynthetic photon flux density (PPFD) and cuvette temperature simultaneously. Incident PPFD was either full sunlight during the measurements carried out in summer (days 90–95) or was supplied by an halogen lamp during the measurements carried out in winter (days 285–295); in both cases PPFD $\geq 1900 \mu\text{mol m}^{-2} \text{s}^{-1}$ was found to be saturating but not photoinhibitory and thus appropriate for rendering a quick measurement of A_{\max} . When temperature inside the cuvette exceeded the external air temperature by more than 4.0 °C the measurements were discarded. In all cases gas exchange was measured at 28 ± 5 °C, which were the natural summer temperatures; winter measurements were carried out in the heated glass house. Cladodes were naturally exposed to saturating sunlight for at least 1 h before entering the cuvette to ensure full induction of photosynthesis. Once A_{\max} was recorded, the cuvette containing the cladodes was darkened with a black piece of fabric and dark respiration was registered after allowing some minutes for stabilisation of the readings. Gas exchange measurements were carried out between 10:00 and 14:00 h, which was optimal for the determination of maximum rates due to the lack of stomatal and non-stomatal limitations during this period.

Nutrient content determinations

Nutrient analyses of plants harvested at the end of the experiment (day 295) were carried out at the Unit of Analysis of the Centre of Environmental Sciences (CSIC), Madrid, Spain. For determination of total phosphorus and potassium, samples were digested in a mixture of HNO_3 and HClO_4 in a warm sand bath at ambient pressure, and analysed by emission spectrometry in an inductively coupled plasma (Perkin-Elmer ICP5500, USA). Total nitrogen was determined by Kjeldahl analysis with SeSO_4 and K_2SO_4 as catalysts in a Digestion System 20 (Tecator, Sweden). N was automatically determined in a Kjeltec-auto

Table 1. Seed weight and stem diameter, leaf plus stem area ratio (LAR), root:shoot ratio and relative growth rate (RGR) of *Retama sphaerocarpa* from Almería (very dry site) and Zaragoza (dry site) at different times during the experiment

Variable ^a	Population		P
	Arid (Almería)	Semi-arid (Zaragoza)	
Seed weight (mg)	95	78	*
Stem diameter at day 95 (mm)	1.01	0.89	*
LAR at day 30 ($\text{m}^2 \text{kg}^{-1}$)	2.06	6.69	*
LAR at day 95 ($\text{m}^2 \text{kg}^{-1}$)	2.95	3.10	n.s.
LAR at day 180 ($\text{m}^2 \text{kg}^{-1}$)	2.19	2.35	n.s.
Root:shoot ratio at day 30	0.34	0.37	n.s.
Root:shoot ratio at day 95	0.62	0.77	*
Root:shoot ratio at day 180	0.76	0.83	n.s.
RGR ($\text{g g}^{-1} \text{day}^{-1}$) days 30–95	0.0509	0.0519	–
RGR ($\text{g g}^{-1} \text{day}^{-1}$) days 95–180	0.0126	0.0142	–
RGR ($\text{g g}^{-1} \text{day}^{-1}$) days 180–295	0.0033	0.0031	–

^a Values represent the mean of n independent samples; $n = 100$ for seed weight, $n = 20$ for stem diameter, for LAR and root:shoot ratio; RGR was calculated from pools of 20 seedlings from each population harvested at four different times during the experiment. Results of ANOVA are reported as * $P \leq 0.001$, n.s. (not significant) $P > 0.05$.

1030 analyser (Tecator, Sweden). N, P, and K contents were expressed on both dry weight and leaf area bases. Nitrogen uptake efficiency was estimated as the total plant N content versus the total N provided to the plant in each fertilisation treatment. Instantaneous N use efficiency was estimated as the maximum photosynthetic rate per g of N.

Statistical analyses

Analysis of variance (ANOVA, Tukey test, SYSTAT 6.0 Windows version 1996, SPSS Inc. Chicago, IL, USA) was used to test for differences among populations, and fertilisation and inoculation treatments. In all cases, the data met the assumptions of normality and homocedasticity. For the sake of simplicity, data from the two populations were pooled together when they did not differ significantly. Since the combination of high fertilisation plus inoculation rendered few true replicates (i.e., highly fertilised and nodulated plants), it was not included in the final analyses of most variables.

Results

Overview of the main effects on seedling performance

Seedling performance under the different treatments was very similar for the two populations (Almería, arid

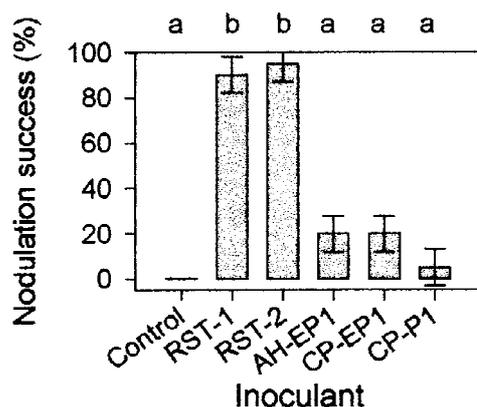


Figure 1. Nodulation success (% of total harvested plants exhibiting root nodules, mean \pm SD) with the different inoculants (RST-1 and RST-2 from *Retama sphaerocarpha*, AH-EP1 from *Adenocarpus hispanicus*, CP-EP1 and CP-P1 from *Cytisus purgans*, see Material and Methods) applied on day 57 to *Retama sphaerocarpha* seedlings versus the control (i.e., non-inoculated plants). Letter code indicates significantly different groups (ANOVA $P < 0.001$, Tukey test, $n \geq 20$ plants per harvest).

site, and Zaragoza, semi-arid site) of *Retama sphaerocarpha* examined, despite the significantly different weight of the seeds from each locality (Table 1). Significant differences between the two populations were observed only during the first 2 months of growth and only in LAR (at day 30), stem diameter and root:shoot ratio (at day 95, Table 1). Seedlings from heavier seeds (arid site) were initially taller and heavier (this trend was significant during the first 3 months and could be observed up to day 180 in the low fertilisation treatment). However, seedlings from the two populations exhibited the same relative growth rate (Table 1), and the differences between the two populations were not visible by the end of the experiment (day 295). The high fertilisation treatment enhanced plant growth, but only after 6 months. Morphological and physiological differences between inoculated and non-inoculated plants were observed in the low but not in the high fertilisation treatment.

Inoculation, nodulation and nitrogenase activity

Even though a few *Retama sphaerocarpha* seedlings inoculated with *Rhizobium* strains isolated from *Adenocarpus hispanicus* and *Cytisus purgans* (AH-EP1 and CP-EP1 respectively) produced nodules, the nodulation success (i.e., percentage of harvested seedlings with nodules on the roots) was not significantly different from non-inoculated seedlings (i.e., controls, which did not exhibit nodules, Figure 1). On the

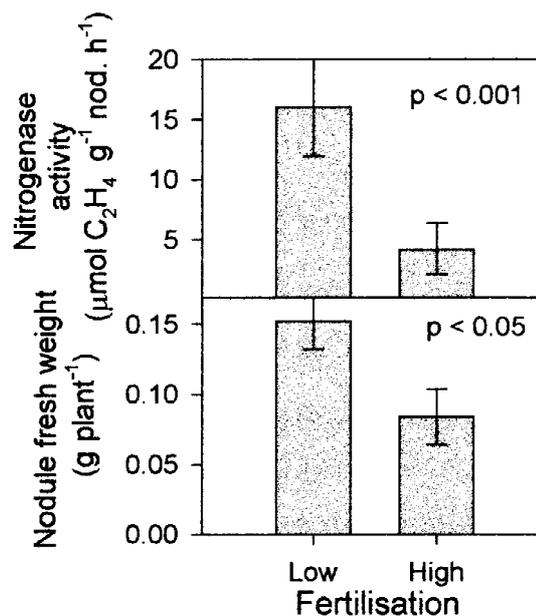


Figure 2. Root nodule fresh weight per plant and nitrogenase activity (mean \pm SD) of *Retama sphaerocarpha* seedlings under low and high fertilisation regimes at the end of the experiment (day 295). Only data for the nodules from inoculants RST-1 and RST-2 are shown (not enough material was obtained from the less successful inoculants). Nitrogenase activity was measured by acetylene reduction after 15 min. No significant differences were found for seedlings from the two populations or for inoculant RST-1 versus inoculant RST-2; thus the data were pooled. Significance of the mean differences (P value of the ANOVA) is provided for each variable ($n = 8$ plants).

contrary, *Rhizobium* inoculants from adult *Retama sphaerocarpha* shrubs (strains RST-1 and RST-2) had a very high nodulation success (Figure 1). Nitrogenase activity was detectable in nodules from strains RST-1 and RST-2 and high fertilisation treatment inhibited both the formation of nodules and the nitrogenase activity of the nodules (Figure 2).

Inoculation versus fertilisation: effects on growth

Fertilisation increased seedling relative growth rate, which translated into the largest plants at final harvest (Figure 3). Plants in the high fertilisation treatment but without nodules (nodulated plants were not included in this treatment) were significantly taller and heavier and their root:shoot ratio lower than in the case of low-fertilised plants, either with or without nodules (Figure 3). Nodulated plants exhibited higher relative growth rates and were heavier and taller than non-nodulated plants under low fertilisation (Figure 3). Nodulated plants exhibited root:shoot ratios higher than highly

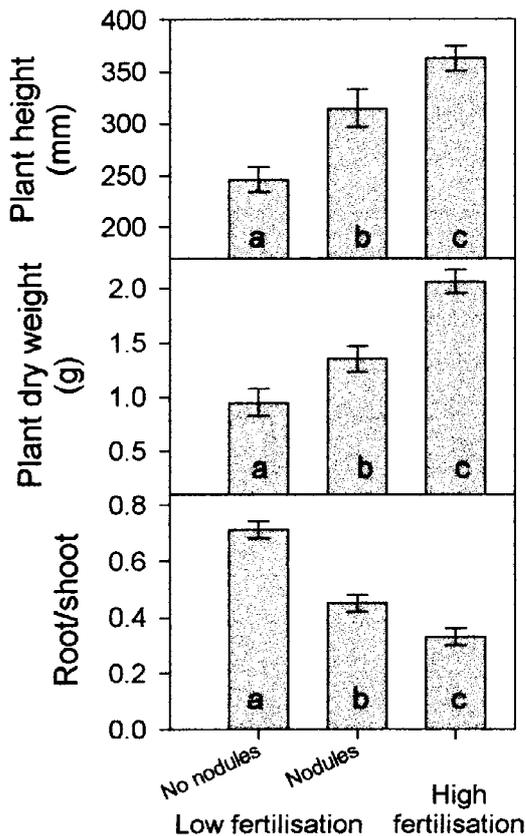


Figure 3. Plant height, plant dry weight and root:shoot ratio (mean \pm SD) of *Retama sphaerocarpa* seedlings with and without root nodules under low fertilisation and without nodules under high fertilisation (the treatment nodules + high fertilisation did not render enough replicates and was excluded from the analyses) at the end of the experiment (day 295). Only data for nodulated plants from inoculants RST-1 and RST-2 are shown (not enough material from the less successful inoculants was available). No significant differences were found for seedlings from the two populations or for inoculant RST-1 versus RST-2; thus the data were pooled. Letter code indicates significantly different groups (ANOVA $P < 0.001$, Tukey test, $n = 15$ plants, except for plant height measurements where $n = 40$ plants).

fertilised plants but lower than non-nodulated plants (Figure 3). In all cases, differences between treatments in plant dry weight were due to differences in shoot weight since root weight remained constant across treatments.

Inoculation versus fertilisation: effects on photosynthesis

Maximum rates of photosynthesis (A_{\max}) and respiration were measured at two different times during the experiment (days 85–95 and 285–295), with the second series of measurements exhibiting lower rates

than the first one. High fertilisation plants without nodules exhibited the same A_{\max} and respiration rates as nodulated plants under low fertilisation, and these rates were significantly higher than those of non-nodulated plants in low fertilisation (Figure 4). There were no differences among treatments in respiration rates measured at day 295.

Inoculation versus fertilisation: effects on nutrient uptake

Fertilisation significantly enhanced nitrogen and phosphorus concentration of both roots and shoots but had no significant effect on potassium concentration (Figure 5). In the low fertility treatment, nodulated plants had higher tissue N concentration than non-nodulated plants in both roots and shoots. Nodulated plants did not differ from non nodulated plants in their phosphorus and potassium concentrations (Figure 5). Nitrogen uptake efficiency (estimated as the fraction of the available nitrogen that was incorporated into the plant) was very poor in highly fertilised plants (Figure 6), while nodulated plants were very efficient (plants had more nitrogen than the amount supplied with the watering due to the nitrogenase activity of the nodules, Figure 6). Instantaneous nitrogen use efficiency (estimated by A_{\max} on a nitrogen content basis) was also very low in highly fertilised plants, and was also significantly larger in nodulated than in non nodulated plants under low fertilisation (Figure 6).

Discussion

We have found a significant and positive effect of root nodules on the physiology and growth of retama (*Retama sphaerocarpa*) seedlings (Figures 3 and 4). High fertilisation had also a positive effect on plant growth, but the effects of nodulation on growth of these plants could not be determined due to the inhibitory effects of nutrients on nodule formation (Figure 2). Thus, either high fertilisation or inoculation must be applied to enhance seedling growth and quality, but both methods are mutually exclusive. However, the use of fertilisers has important environmental and sanitary drawbacks (Newton, 2000), while inoculation is environmentally clean and safe (Dileep Kumar et al., 2001). Other advantages of nodulation and reduced fertiliser application were apparent. Nitrogen uptake efficiency and photosynthetic nitrogen use efficiency increased under low fertilisation (Figure 6; Nilsen, 1992). The

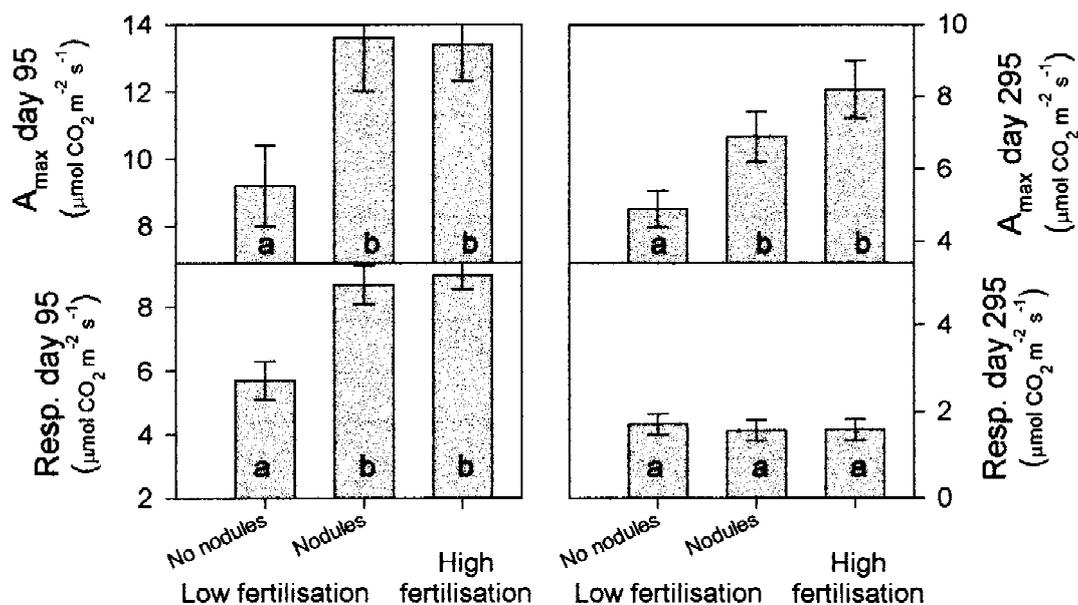


Figure 4. Maximum photosynthetic rate (A_{\max}) and dark respiration (mean \pm SD) of *Retama sphaerocarpa* seedlings with and without root nodules under low fertilisation and without nodules under high fertilisation (the treatment nodules + high fertilisation did not render enough replicates and was excluded from the analyses) at days 95 and 295 of the experiment. High respiration rates were due to high measurement temperatures (28 °C). Only data for the nodulated plants from inoculants RST-1 and RST-2 are shown (not enough material from the less successful inoculants was available). No significant differences were found for seedlings from the two populations or for inoculant RST-1 versus RST-2; thus the data were pooled. Letter code indicates significantly different groups (ANOVA $P < 0.001$, Tukey test, $n = 8$ plants).

lower root:shoot ratio of highly fertilised plants (Figure 3) makes them more vulnerable than nodulated plants not only to nutrient limitations but also to water shortage, a typical limiting factor for plant survival and growth in the Mediterranean region. Legumes seldom occur as pioneers in primary succession, which has been attributed to the lack of enough nitrogen in the seeds for nodule formation before the plant obtains a net gain in nitrogen through symbiotic fixation (Hanley and Fenner, 1997). Transplantation of nodulated plants eliminates this problem and legumes could then be used to accelerate plant succession. All these facts together point to inoculation as the recommended method to ensure the establishment of leguminous shrubs in arid and poor habitats.

The inhibition of biological nitrogen fixation by high fertilisation is attributable to the well-known inhibitory effects of nitrate on nodulation and nitrogenase activity (Streeter, 1988). Nodule formation and maintenance is energetically expensive so plants having access to combined nitrogen do not invest in nodules (Streeter, 1988). In very productive legume crops such as soybean, nitrogen fixation and soil nitrogen levels are usually not enough to maximise yield, and fertilisation renders better results than nodulation

(Newton, 2000; Streeter, 1988). Our results reinforce these findings since highly fertilised plants of retama exhibited larger photosynthetic and growth rates than nodulated plants under low fertilisation (Figures 3 and 4). But considering the moderate productivity of wild, leguminous shrubs, and the fact that most of these shrubs grow in water-limited environments, maximisation of yield in arid zones becomes less relevant than assuring plant survival. Herrera et al. (1993) found that inoculation with selected rhizobia the seedlings of two leguminous shrubs (*Anthyllis cytisoides* and *Spartium junceum*), to be used for revegetation of arid, Mediterranean sites improved outplanting performance and plant survival. The comparative effect of different fertilisation regimes was not investigated in their study, but the results vary according to the species (Herrera et al., 1993) and can be affected by the particular environmental characteristics of the site (Ovalle et al., 1996).

As a practically leafless shrub, photosynthetic carbon gain in retama is achieved by the stems (Haase et al., 1999). In our study, maximum photosynthetic rate at the end of the experiment exhibited a significant correlation with plant dry weight (Figures 3 and 4), as has been shown in other non-leguminous shrubs

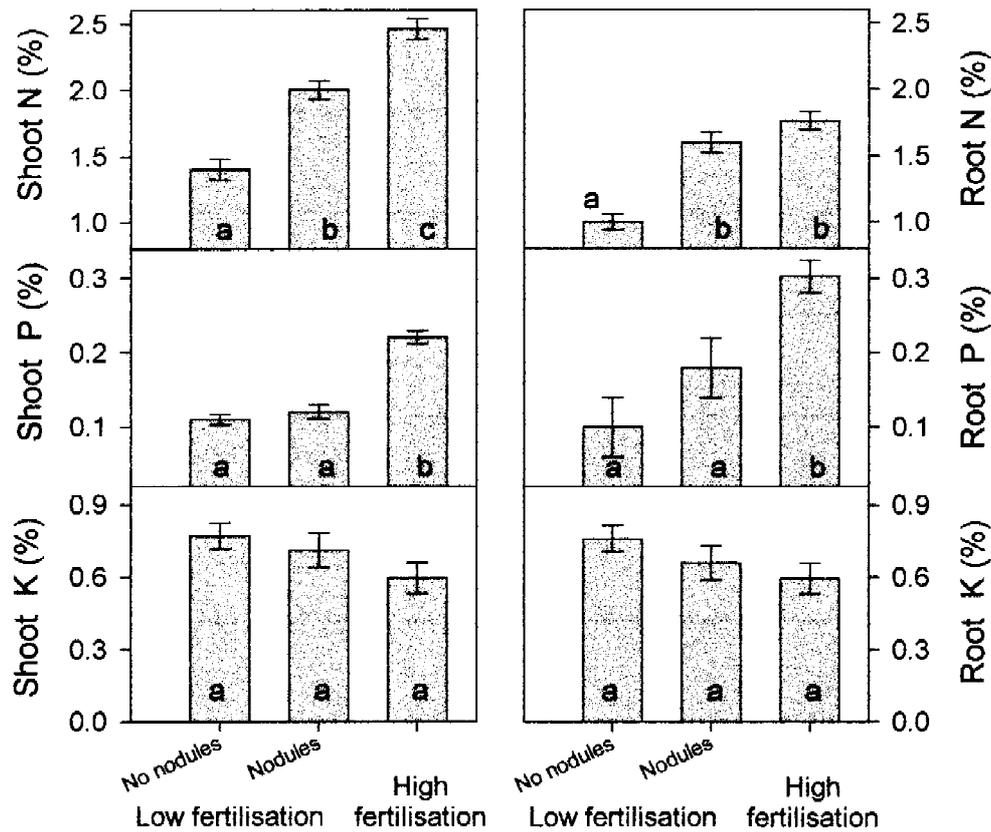


Figure 5. Nitrogen, phosphorus and potassium concentration (mean \pm SD) of shoots and roots of *Retama sphaerocarpa* seedlings with and without root nodules under low fertilisation and without nodules under high fertilisation (the treatment nodules + high fertilisation did not render enough replicates and was excluded from the analyses) at the end of the experiment (day 295). Only data for the nodulated plants from inoculants RST-1 and RST-2 are shown (not enough material from the less successful inoculants was available). No significant differences were found for seedlings from the two populations or for inoculant RST-1 versus RST-2; thus the data were pooled. Letter code indicates significantly different groups (ANOVA $P < 0.001$, Tukey test, $n = 15$ plants).

where carbon gain is achieved by leaves (e.g., Valladares et al., 2000). Even though green stems are photosynthetically not efficient in a number of woody species (e.g., Comstock and Ehleringer, 1988), photosynthetic activity of the cortical tissues of leguminous shrubs like *Retama*, *Spartium* or *Cytisus* is as high and efficient as reported for leaves (Bossard and Rejmanek, 1992; Haase et al., 1999; Nilsen, 1992). In fact, green, photosynthetic stems allow for an efficient tradeoff between irradiance capture and avoidance in arid environments (Valladares and Pugnaire, 1999) and confers a great physiological flexibility, which plays an important role in the ecological success of these leguminous shrubs (Bossard and Rejmanek, 1992). It has been argued that one important advantage of having photosynthetic stems is to guarantee whole-season supply of photosynthate for rhizobial nodules, since these nodules fix nitrogen down to 4 °C during

the winter (Wheeler et al., 1979). All these features, together with its remarkable drought tolerance and its capacity to fix nitrogen via rhizobial nodules, make *Retama sphaerocarpa* a well-suited species for revegetation projects in arid Mediterranean environments of low fertility.

When evaluating plant performance, it is essential to consider the influence of ontogeny (McConnaughay and Coleman, 1999). For instance, in agreement with our results with retama (Figure 4), photosynthetic characteristics have been shown to vary during the ontogeny of the plant (Altesor et al., 1992). Seed size and weight exerts a strong influence on plant morphology and physiology (Marañón and Grubb, 1993; Shipley and Peters, 1990), but our results show that the influence of seed weight in the performance of retama seedlings (i.e., larger seeds render larger seedlings) vanishes after 3 months (Table 1), which confirms

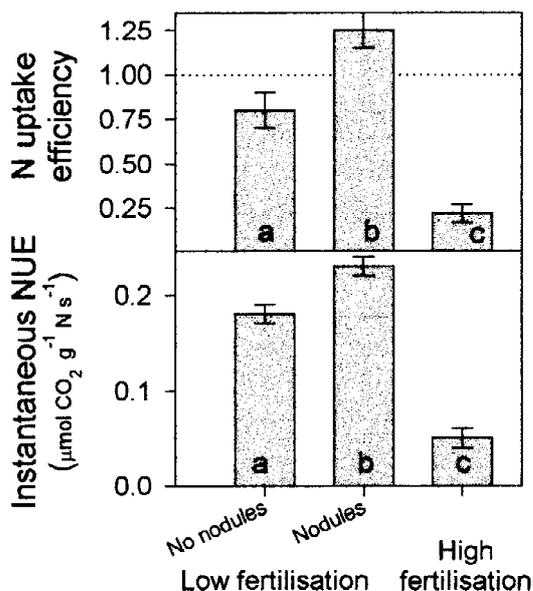


Figure 6. Nitrogen uptake efficiency (total plant N expressed as a fraction of N provided per plant in each fertilisation treatment, the reference value of 1 is indicated by the dotted line) and instantaneous N use efficiency (maximum rate of carbon gain per g of N) of *Retama sphaerocarpa* seedlings with and without root nodules under low fertilisation and without nodules under high fertilisation (the treatment nodules + high fertilisation did not render enough replicates and was excluded from the analyses). Values are expressed as mean \pm SD for plants at the end of the experiment (day 295). Only data for the nodulated plants from inoculants RST-1 and RST-2 are shown (not enough material from the less successful inoculants was available). No significant differences were found for seedlings from the two populations or for inoculant RST-1 versus RST-2; thus the data were pooled. Letter code indicates significantly different groups (ANOVA $P < 0.001$, Tukey test, $n = 15$ plants).

that ontogeny can change the outcome of comparative studies.

Rhizobium-legume symbiosis is known to be specific (Fisher and Long, 1992), but some rhizobia isolated from leguminous shrub were shown to be promiscuous (Santamaria et al., 1997). Although some promiscuity was observed in our experiment (i.e., rhizobia strains from *Cytisus* and *Adenocarpus* initiated some nodules in *Retama*), our results indicate a significantly larger nodulation success with specific rhizobia in *Retama*. Some preliminary studies with sterilised soils suggested that the seeds of *Retama* contain viable rhizobia that can produce nodules under artificial conditions (Pérez-Fernández and Lamont, 2000). However, current nursery techniques, which do not involve inoculation, most commonly produce non-nodulated *retama* seedlings.

In conclusion, nitrogen uptake and use was enhanced by the presence of rhizobial nodules in *retama* seedlings, which translated into enhanced growth under low fertilisation. Nodulation success was rather high using inoculants from adult *R. sphaerocarpa* plants. Inoculation is simple, environmentally friendly and cheap, and nodulated plants are more likely to overcome transplant stress than non-nodulated ones. Thus, our study suggests that inoculation together with low, background fertilisation (instead of high fertilisation) should be used when producing high quality seedlings of this autochthonous shrub for revegetation projects of dry and poor Mediterranean areas.

Acknowledgements

Thanks are due to Esteban Manrique for the kind loan of the gas exchange equipment, and to Celia Martínez-Alonso for help in the nitrogenase activity measurements. This research was supported by a grant of the Spanish CICYT (AMB98-1108-C04-01).

References

- Altesor A, Ezcurra E and Silva C 1992 Changes in the photosynthetic metabolism during the early ontogeny of four cactus species. *Acta Oecol.* 13, 777–785.
- Barea JM, Salamanca CP, Herrera MA and Roldán-Fajardo BE 1990 Microorganism-plant symbiosis in the establishment of a vegetal cover on degraded lands. In *Soil Degradation and Rehabilitation in Mediterranean Conditions*. Eds. J Albaladejo, MA Stocking, E Díaz. pp. 139–158. Consejo Superior de Investigaciones Científicas (CSIC), Madrid.
- Bossard CC and Rejmanek M 1992 Why have green stems? *Funct. Ecol.* 6, 197–205.
- Comstock JP and Ehleringer JR 1988 Contrasting photosynthetic behavior in leaves and twigs of *Hymenoclea salsola*, a green-twigged warm desert shrub. *Am. J. Bot.* 75, 1360–1370.
- Dart P 1998 Nitrogen fixation by tropical trees and shrubs. In *Biological Nitrogen Fixation for the 21st Century*. pp. 667–670. Ed. C Elmerich. Kluwer Academic Publishers, Netherlands.
- Dileep Kumar B S, Berggren I and Martensson M A 2001 Potential for improving pea production by co-inoculation with fluorescent *Pseudomonas* and *Rhizobium*. *Plant Soil* 229, 25–34.
- Fernández-Pascual M, de Felipe MR, Serra MT and Pozuelo JM 1988 Effects of cianazine and Linuron on chloroplast development, nodule activity and protein metabolism in *Lupinus albus* L. *J. Plant Physiol.* 133, 288–294.
- Fernández-Pascual MC, de Lorenzo C, Sixto H, Pozuelo JM, Guasch L and de Felipe MR 1996 Photosynthetic and nitrogenase activities in *Lupinus albus* plants treated with herbicides from different chemical groups. In *Proceedings of the International Symposium on Weed and Crop Resistance to Herbicides*. pp. 134–136. Eds. R de Prado, J Jorrin, L García Torres, G Marshall. University of Córdoba, Córdoba.

- Fisher RF and Long SR 1992 Rhizobium — plant signal exchange. *Nature* 357, 655–660.
- Franco AA, Campello FC, de Faria SM and Dias, LE 2000 The importance of biological nitrogen fixation on land rehabilitation. *In* Nitrogen Fixation: From Molecules to Crop Productivity. pp. 569–570. Ed. FO Pedrosa. Kluwer Academic Publishers, Netherlands.
- Gómez Sal A, Rey Benayas JM, López-Pintor A and Rebollo S 1999 Role of disturbance in maintaining a savanna-like pattern in Mediterranean *Retama sphaerocarpa* shrubland. *J. Veg. Sci.* 10, 365–370.
- Haase P, Pugnaire FI, Fernández EM, Puigdefábregas J, Clark SC and Incoll LD 1996 An investigation of rooting depth in the semi-arid shrub *Retama sphaerocarpa* (L.) Boiss. by labelling of ground water with a chemical tracer. *J. Hydrol.* 170, 23–31.
- Haase P, Pugnaire FI, Clark SC and Incoll LD 1999 Diurnal and seasonal changes in cladode photosynthetic rate in relation to canopy age structure in the leguminous shrub *Retama sphaerocarpa*. *Funct. Ecol.* 13, 640–649.
- Hanley ME and Fenner M 1997 Seedling growth of four fire-following Mediterranean plant species deprived of single mineral nutrients. *Funct. Ecol.* 11, 398–405.
- Herrera MA, Salamanca CP and Barea JM 1993 Inoculation of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified Mediterranean ecosystems. *Appl. Environ. Microbiol.* 59, 129–133.
- Huss-Danell K 1980 Nitrogen fixation and biomass production in clones of *Alnus incana*. *New Phytol.* 85, 503–511.
- León-Barrios M, Gutierrez-Navarro AM, Pérez-Galdona R and Corzo J 1991 Characterization of Canary Island isolates of *Bradyrhizobium* sp. *Chamaecytisus proliferus*. *Soil Biol. Biochem.* 23, 487–489.
- Marañón T and Grubb PJ 1993 Physiological basis and ecological significance of the seed size and relative growth rate relationship in mediterranean annuals. *Funct. Ecol.* 7, 591–599.
- McConnaughay KDM and Coleman JS 1999 Biomass allocation in plants: ontogeny or optimality? A test along three resource gradients. *Ecology* 80, 2581–2593.
- Minchin FR, Iannetta PPM, Fernández-Pascual M, de Lorenzo C, Witty JF and Sprent JI 1992 A New procedure for the calculation of oxygen diffusion resistance in legume nodules from flow-through gas analysis data. *Ann. Bot.* 70, 283–289.
- Newton WE 2000 Nitrogen fixation in perspective. *In* Nitrogen fixation: from molecules to crop productivity. pp. 3–8. Ed. FO Pedrosa. Kluwer Academic Publishers, Netherlands
- Nilsen ET 1992 Partitioning growth and photosynthesis between leaves and stems during nitrogen limitation in *Spartium junceum*. *Am. J. Bot.* 79, 1217–1223.
- Ovalle C, Longeri L, Aronson J, Herrera A and Avendaño J 1996 N₂-Fixation, nodule efficiency and biomass accumulation after two years in three Chilean legume trees and *Tagasaste Chamaecytisus proliferus* subsp. *palmensis*. *Plant Soil* 179, 131–140.
- Pate JS 1986 Economy of symbiotic nitrogen fixation. *In* On the Economy of Plant Form and Function. pp. 299–326. Ed. TJ Givnish. Cambridge University Press, New York.
- Pérez-Fernández MA, Lamont BB 2000 Competition between seedlings of Australian and Spanish legumes in Australian soils. *In* 9th International Conference on Mediterranean-Type Ecosystems: past, present and future. pp. 61–62. Book of Abstracts, Stellenbosch.
- Pugnaire FI, Haase P and Puigdefábregas J 1996 Facilitation between higher plant species in a semi-arid environment. *Ecology* 77, 1420–1426.
- Santamaria M, Corzo J, León-Barrios M and Gutierrez-Navarro AM 1997 Characterisation and differentiation of indigenous rhizobia isolated from Canarian shrub legumes of agricultural and ecological interest. *Plant Soil* 190, 143–152.
- Schulze ED, Gebauer G, Ziegler H and Lange OL 1991 Estimates of nitrogen fixation by trees on an aridity gradient in Namibia. *Oecologia* 88, 451–455.
- Shipley B and Peters RH 1990 The allometry of seed weight and seedling relative growth rate. *Funct. Ecol.* 4, 523–529.
- Sinclair TR and Serraj R 1995 Legume nitrogen fixation and drought. *Nature* 378, 344.
- Smith SD and Osmond CB 1987 Stem photosynthesis in a desert ephemeral, *Eriogonum inflatum*. Morphology, stomatal conductance and water use efficiency in field conditions. *Oecologia* 72, 533–541.
- Streeter JG 1988 Inhibition of legume nodule formation and nitrogen fixation by nitrate. *CRC Crit. Rev. Plant Sci.* 7, 1–23.
- Valladares F and Pugnaire FI 1999 Tradeoffs between irradiance capture and avoidance in semi-arid environments simulated with a crown architecture model. *Ann. Bot.* 83, 459–470.
- Valladares F, Wright SJ, Lasso E, Kitajima K and Pearcy RW 2000 Plastic phenotypic response to light of 16 congeneric shrubs from a Panamanian rainforest. *Ecology* 81, (7) 1925–1936.
- Vincent JM 1970 A Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook 15. Blackwell Scientific Publications Ltd., Oxford.
- Werner D, Vinuesa-Fleischmann P, Scheidemann P, Wetzell A and Redecker D 1998 Nitrogen fixing trees: research tasks in physiology, symbiotic interactions and ecology. *In* Biological Nitrogen Fixation for the 21st Century. pp. 677–678. Ed. C Elmerich. Kluwer Academic Publishers, Netherlands.
- Wheeler CT, Perry DA, Helgersson O and Gordon JC 1979 Winter fixation of nitrogen in Scotch broom (*Cytisus scoparius* L.). *New Phytol.* 82, 697–701.

Section editor: F.R. Minchin