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A role for below-ground biota in plant–plant facilitation

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Summary

1. Plant–plant facilitation is an important driver of plant diversity, which in turn maintains ecosystem multifunctionality and can buffer some negative effects of climate change. Facilitation is classically attributed to the amelioration of environmental extremes and resource availability. Integrating below-ground biota into the positive plant interactions framework should provide a more realistic understanding of this process, enabling us to gain insights into the dynamics of below–above-ground communities.

2. We tested the effect of below-ground biota on the performance of a plant community and of individual species using soil extracts from the understorey of a benefactor plant species and adjacent open spaces. Soil bacteria from extracts and experimental microcosms were analysed using pyrosequencing.

3. Soil biota had a significant effect on the abundance, growth, functional traits and reproductive output of beneficiary plant species through processes that are independent of the direct influence of the benefactor species. Different soil bacterial communities were associated with the benefactor species, the individual beneficiary plant species and the plant community, revealing complex below–above-ground links between plants and soil microbiota.

4. *Synthesis*. The below-ground biota cultivated by benefactor plant species play a fundamental role in positive interactions between plant species contributing to the preservation of diversity and the evolution of plant communities.

Key-words: nurse species, plant reproduction, plant–soil (below-ground) interactions, pyrosequencing, Retama, soil bacteria

Introduction

Plant–plant facilitation is the positive interaction between plants in which the presence of one species benefits neighbouring plants in terms of germination, survival and/or growth (Callaway 2007). Facilitation is an ecological process occurring in communities world-wide that is particularly important to maintain biodiversity in stressful environments (Pugnaire *et al.* 1996; Callaway *et al.* 2002; Valiente-Banuet & Verdú 2008). The mechanisms driving plant–plant facilitation in stressful environments are traditionally explained by the amelioration of environmental conditions (Callaway 2007 and references therein). Under the severe conditions of arid environments, the benefactor species, also known as nurse species, usually buffers extreme temperature, humidity and irradiation and increases water availability for the beneficiary (facilitated) species, which cannot establish outside the protection of the nurse species (Callaway 2007). Recent data have suggested a potential role of soil micro-organisms in facilitation that has been further explored for mycorrhizal associations (Van der Heijden & Horton 2009; Van der Putten 2009; Martínez-García & Pugnaire 2011; Montesinos-Navarro *et al.* 2012). However, the role that soil communities may play in plant facilitation has been largely neglected despite the strong influence of soil microbial communities on individual plant performance (Bever *et al.* 2010).

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Below-ground communities influence the diversity and composition of plant communities (Van der Heijden, Bardgett & Van Straalen 2008), plant growth and productivity (Wardle *et al.* 2004), plant species abundance (Klironomos 2002), nutrient availability and ecosystem functioning (Loreau 2001), and even evolution through the selection for certain suites of functional plant traits (Lau & Lennon 2011). Most evidence of the effect of soil communities on the structure and dynamics of plant communities has been obtained from plant–soil feedback approaches in which single plant species are grown in soil pre-conditioned by either conspecific or heterospecific individuals (Brinkman *et al.* 2010; Reinhart 2012). Although this approach has provided valuable insights into interaction dynamics, other methods are needed to better understand the complex interactions between below-ground and above-ground communities (Kulmatiski *et al.* 2008; Van der Putten *et al.* 2013). For example, the few studies that tested the role of below-ground biota on plant competition found that soil micro-organisms can change the intensity and even the outcome of interspecific competition (Casper & Castelli 2007; Kardol *et al.* 2007; Pendergast, Burke & Carson 2013). Integrating soil communities in the framework of positive plant interactions should, therefore, provide a more realistic picture of these processes. The large modification induced by benefactor plants on soil properties and microclimate has a positive effect on the understorey plant communities, but these changes also alter the composition and structure of soil communities beneath the canopy (Hortal *et al.* 2013). It is therefore possible that plant–plant facilitation might occur not only directly through the amelioration of environmental conditions but also indirectly through altered soil communities that have a positive effect on the facilitated species.

Here we tested if plant–plant facilitation is mediated by soil biota using the nurse shrub *Retama sphaerocarpa* (L.) Boiss. (herafter *Retama*) and associated plant communities. We performed a series of experiments analysing the role of soil biota associated with a nurse species on beneficiary plant species at two levels: individual single species and a plant community. *Retama* is a model nurse species that induces great modifications in the microclimate and soil properties under the canopy in semi-arid environments. These modifications are considered responsible for the large positive impact of this species on other plant species (Pugnaire *et al.* 1996; Armas, Rodríguez-Echeverría & Pugnaire 2011). Soil microbial activity, biomass and community composition are also significantly altered under *Retama* when compared to adjacent open gaps (Hortal *et al.* 2013). It remains unknown, however, whether these differentiated soil communities have any effect on the plant communities growing in the understorey. We expected that soil biota from open spaces (gaps) and from nurse shrub understories will distinctly affect a) an experimental plant community composed by the most frequently found species under *Retama* and b) single plant species with different microhabitat preferences in the field: gaps, under *Retama* or no preference (Pugnaire *et al.* 1996; Armas, Rodríguez-Echeverría & Pugnaire 2011) (Table 1). These experiments were also intended to assess whether plants exerted any effect on soil microbial communities. According to the plant–soil feedback theory (Bever, Westover & Antonovics 1997), we expected that plant growth would lead to the development of similar soil microbial communities for either each plant species or for the plant community.

Table 1. Plant species and number of seeds used in each experiment. In the plant community experiment, we sowed in each pot the sum of all seeds (191) from the 14 species. In the single-species experiment, only 3 seeds of one species per pot were sowed. Microhabitat refers to the preferred microhabitat where species are found in the field: under *Retama* shrubs, in open gaps, or in both. All seeds were collected in a semi-arid shrubland in Almería (Spain). The number of seeds in the plant community experiment reflects natural abundance in the field

	Family	Microhabitat	Seeds (#)
Plant community experiment			
Avena sterilis	Poaceae	Retama	3
Brachypodium distachyon	Poaceae	Retama	20
Medicago littoralis	Fabaceae	Retama	20
Silene decipiens	Caryophyllaceae	Indifferent	50
Asphodelus fistulosus	Asphodelaceae	Indifferent	5
Lagurus ovatus	Poaceae	Indifferent	20
Herniaria sp.	Caryophyllaceae	Indifferent	10
Malva parviflora	Malvaceae	Indifferent	5
*Marrubium vulgare**	Lamiaceae	Indifferent	20
Medicago minima	Fabaceae	Indifferent	8
Medicago truncatula	Fabaceae	Indifferent	5
*Paronychia argentea**	Caryophyllaceae	Indifferent	5
Reichardia tingitana	Asteraceae	Indifferent	10
*Retama sphaerocarpa**	Fabaceae	Indifferent	10
Single-species experiment			
Clypeola jonthlaspi	Brassicaceae	Gap	3
Stipa capensis	Poaceae	Gap	3
Avena sterilis	Poaceae	Retama	3
Capsella bursa-pastoris	Brassicaceae	Retama	3
Carrichtera annua	Brassicaceae	Retama	3
Geranium molle	Geraniaceae	Retama	3
Bromus rubens	Poaceae	Indifferent	3
Lagurus ovatus	Poaceae	Indifferent	3
Medicago truncatula	Fabaceae	Indifferent	3
Silene decipiens	Caryophyllaceae	Indifferent	3
Trigonella polycerata	Fabaceae	Indifferent	3

*Indicates perennial.

Materials and methods

SOIL SAMPLING AND INOCULA PREPARATION

Soil samples were collected in a dry arid valley in south-eastern Spain (37°08′N, 2°22′W, 630 m elevation). Patches of vegetation under *Retama* shrubs, the dominant species in this open shrubland, strongly contrast with bare soil in between, because *Retama* facilitates the establishment and growth of many other species under its canopy (Pugnaire *et al.* 1996; Rodríguez-Echeverría & Pérez-Fernández 2003; Armas, Rodríguez-Echeverría & Pugnaire 2011). A total of 60 kg of soil were collected from the top 15-cm layer under the canopy of 10 randomly selected *Retama* shrubs and adjacent gaps. Soil samples were pooled by soil type, gap or *Retama,* as described in other studies (Callaway *et al.* 2011; Meisner *et al.* 2013; Pendergast, Burke & Car-

son 2013). Pooling soil samples within each soil type reduces variability but allows testing for differences between soil types considering experimental replicates as technical replicates. Each composite soil sample was sieved through a 5-mm sieve and used to prepare a soil inoculum from both soil types (gap and *Retama*). Soil inocula were prepared by stirring 10 kg of soil in distilled autoclaved water in a proportion 1:2 (v:v). The suspension was filtered through a 0.5-mm sieve to remove soil particles but allowing the pass of fungal spores and hyphae, soil bacteria and microfauna. We used soil suspensions instead of whole sieved soil to minimize the possible differences in nutrient and organic matter contents between inocula. Soil filtrates have been used in other studies to separate the effects of different microbial groups (Klironomos 2002; Callaway *et al.* 2011), but we were interested in the whole soil community and therefore used the total soil suspension. This methodology has been proved adequate to test the effect of soil biota on plant performance, since filtering soil suspensions through smaller sieves (20–200 μm) can eliminate soil bacteria and fungi adsorbed to soil aggregates (Van de Voorde, Van der Putten & Bezemer 2012).

The two soil extracts (GI from gaps, RI from *Retama*) were used to inoculate pots filled with an autoclaved mixture of river sand and soil collected from *Retama* understories (1:2). Soil from *Retama* was used for the sterile common background soil because nutrient concentration in this soil is higher than in the gap soil (Pugnaire, Armas & Valladares 2004). By using a richer soil, we reduced any relative potential differential input of nutrients with inoculation. The volume of soil extract added to each experimental pot was adjusted to have an inoculum density of 20% (v:v). Immediately after inoculation, soil nutrients were determined in 3 replicates from both treatments, that is, sterilized soil inoculated with soil extract from *Retama* and sterilized soil inoculated with soil extract from gaps, at CEBAS ionomics laboratory (Spain); total C and N content were determined using a LECO Truspec C/N analyser (St. Joseph, MI, USA), and micronutrients were determined after acid digestion by inductively coupled plasma (ICP) emission spectrometry using an ICAP 6500 DUO Thermo (Thermo Scientific, Wilmington, DE, USA). There were no significant differences in nutrient concentration between inoculation treatments (Table 2).

Table 2. Nutrient content (mean ± SE, $n = 3$) in soils inoculated with soil extracts from gaps or from under *Retama.* *P*-values of *t*-test for each nutrient are shown; there are no significant differences between soils

	Gap	Retama	*P*
N (g per 100 g)	0.11 ± 0.02	0.11 ± 0.01	0.96
C (g per 100 g)	1.21 ± 0.06	1.12 ± 0.11	0.50
Al (g kg^{-1})	20.72 ± 0.61	19.66 ± 0.14	0.51
As (mg kg^{-1})	14.23 ± 1.92	9.66 ± 0.96	0.10
Be (mg kg^{-1})	0.58 ± 0.04	0.51 ± 0.01	0.21
Bi (mg kg^{-1})	11.20 ± 0.54	9.13 ± 1.56	0.28
B (mg kg^{-1})	1.57 ± 0.29	1.85 ± 0.14	0.43
Ca (g per 100 g)	0.38 ± 0.02	0.35 ± 0.07	0.78
Cr (mg kg^{-1})	29.85 ± 0.90	29.61 ± 2.81	0.94
Cu (mg kg^{-1})	31.13 ± 0.53	29.37 ± 0.87	0.16
Fe (g kg^{-1})	26.11 ± 0.27	23.91 ± 1.26	0.17
K (g per 100 g)	0.39 ± 0.02	0.37 ± 0.03	0.69
Li (mg kg^{-1})	39.18 ± 0.70	37.26 ± 2.11	0.44
Mg (g per 100 g)	0.34 ± 0.004	0.32 ± 0.02	0.42
Mn (mg kg^{-1})	284.56 ± 16.06	243.75 ± 17.74	0.16
Na (g per 100 g)	0.08 ± 0.01	0.08 ± 0.005	0.83
Ni (mg kg^{-1})	26.37 ± 0.59	25.06 ± 0.51	0.17
Pb (mg kg^{-1})	13.70 ± 0.60	12.59 ± 0.89	0.36
P (g per 100 g)	0.04 ± 0.001	0.03 ± 0.002	0.09
S (g per 100 g)	0.01 ± 0.0004	0.02 ± 0.004	0.34
Sr (mg kg^{-1})	32.71 ± 1.84	30.27 ± 4.06	0.61
Ti (mg kg^{-1})	106.96 ± 7.17	110.31 ± 7.87	0.77
Tl (mg kg^{-1})	15.52 ± 1.40	12.58 ± 3.23	0.45
V (mg kg^{-1})	37.54 ± 1.21	36.00 ± 2.23	0.58
Zn (mg kg^{-1})	52.01 ± 0.60	49.66 ± 2.28	0.38

PLANT COMMUNITY EXPERIMENT

Seeds of the 14 species most frequently found under *Retama* were sown in 1.5-L pots ($n = 10$) inoculated either with soil extracts from gaps or from under *Retama*, or inoculated with an autoclaved suspension of *Retama* soil extract as a control (treatments are identified as G, R and C, respectively). A total of 191 seeds were added per pot. Details on the plant species and number of seeds can be found in Table 1. All seeds were collected in the field, surface-disinfected by immersion in 10% bleach during 10 min and washed 3 times with autoclaved water. Ten additional pots were inoculated with soil extracts from *Retama,* but no seeds were added (RI_X). All pots were kept in a glasshouse with natural daylight from December to April with mean daily temperatures ranging between 5 and 25 °C, were watered regularly and rotated in a weekly basis to avoid any microsite differences within the glasshouse. Plants were harvested 4 months after seeding; we recorded plant species identity and abundance, calculated species richness, Shannon's diversity index and measured plant dry biomass in each pot. No seeds germinated in the RI_X pots. Plant material was oven-dried at 70 °C during 48 h and weighted. Above-ground dry mass was recorded for each species per pot separately. Root dry mass was recorded per pot, since it was not feasible to tell apart individual root systems.

SINGLE-SPECIES EXPERIMENT

The individual response of 11 annual plant species to *Retama* and gap soil extracts was tested in this experiment (Table 1). Three seeds were sown in 12 250 mL pots per plant species, 6 containing soil extracts from *Retama* understories and 6 with soil extracts from gaps. Seed disinfection, germination, experimental set-up and growth conditions were the same as in the whole-community experiment. At harvest, we measured individual plant mass, height, number of leaves, leaf dry mass, specific leaf area (SLA) and reproductive effort (number of fruits, mean seed mass calculated from 10 fruits randomly selected from each plant and reproductive fitness as the total seed mass produced by plant). SLA was measured following the protocol described by Cornelissen *et al.* (2003). Three fully expanded leaves of each plant were cut, rehydrated overnight in the dark and subsequently scanned the following morning. Leaf area was measured using the software Midebmp (Almería, Spain). Leaves were dried in the oven at 60 °C and weighted after 72 h. SLA was measured as the ratio between fresh leaf area and leaf dry mass.

MOLECULAR CHARACTERIZATION OF SOIL BACTERIAL COMMUNITIES

Molecular analyses were performed on samples from the two soil extracts collected at the beginning of the experiments (GI and RI), on soil samples of the whole-community experiment at the end of the experiment (G, R, C and RI_X), as well as on soils from two species (*Clypeola jonthlaspi* [Cly] and *Carrichtera annua* [Can]) of the single-species experiment inoculated with soil extracts from gaps or under *Retama* (G-Cly, R-Cly, G-Can and R-Can). For all treatments

$n = 3$, except $n = 1$ for RI_X which was a pooled sample of the DNA extracted individually from 3 soil pots and then pooled for PCR and pyrosequencing. *Clypeola jonthlaspi* (Cly) and *Carrichtera annua* (Can) were selected because these species have contrasted microhabitat preference (Table 1), and both are Brassicaceae, with overall low affinity to form associations with mycorrhizal fungi (Gerdemann 1968; Cooke, Ash & Groves 2012). All samples were randomly collected at the end of the experiments, except for GI and RI, and kept frozen at −20 °C until processing.

We extracted DNA from 0.25 g of homogenized soil per sample using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. A 16S rDNA gene fragment corresponding to V1 and V2 regions was amplified following Hortal *et al.* (2013). The forward primer (5′-CCATCT CATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNAGAGTTTG ATCM-TGGCTCAG-3′) contained the Roche pyrosequencing adapter A (underlined), a Roche recommended 10 bp barcode sequence (NN NNNNNNNN) used to tag each sample and the bacterial primer 27F. The reverse primer (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCA GGCTGCCTCCCGTAGGAGT-3′) contained the Roche pyrosequencing adapter B (underlined) and the primer 338R.

DNA concentration of each purified PCR product sample was determined as the mean of three lectures in NanoDrop 2000c (Thermo Scientific). Equal amounts of PCR product for each sample were combined in a single tube to obtain an equimolar pool. The pool was pyrosequenced in a Roche Genome Sequencer FLX System (Roche, Basel, Switzerland) using 454 Titanium chemistry at CSISP-CSIC laboratory (Spain).

The 16S rDNA sequence data were processed using the pyrosequencing pipeline tools from the Ribosomal Database Project (RDP, Michigan State U, USA). Sequences were trimmed for primers, filtered and assigned to samples according to their tag. Sequences shorter than 100 bp, with quality scores < 20 or containing any unresolved nucleotides, were removed from the data set. We used Acacia software, version 1.52 (Bragg *et al.* 2012) for pyrosequencing noise removal using default parameters for error correction. Chimeras were identified using Decipher's Find chimeras tool (Wright, Yilmak & Noguera 2012) and removed from the data set. Sequences were aligned using the Aligner tool from the RDP pipeline. Aligned sequences were clustered into operational taxonomic units (OTUs) defined at 97% similarity cut-off using the complete linkage clustering tool of the RDP pipeline. Rarefaction curves of soil microbial OTUs as a function of number of reads were constructed using the RDP pipeline (see Fig. S1 in Supporting Information). Taxonomic assignation of sequences was performed using the RDP naive Bayesian classifier (Wang *et al.* 2007) at a confidence level of 50% recommended for short sequences (Claesson *et al.* 2009) and calculated the relative abundances of the different phyla, classes and genera per sample.

STATISTICAL ANALYSES

Plants

Differences among treatments were analysed with one-way ANOVA, generalized linear models (GLZ with Poisson distribution and logarithmic link function) or nested ANOVA (SLA and leaf mass). Violations of normality and/or homoscedasticity were checked, and Tukey's *post hoc* tests were performed with Infostat (Di Rienzo *et al.* 2012). Single-sample *t*-tests were used to analyse whether plants that received gap soil extract differed in the date of flowering or fruiting to those that received *Retama* soil extract. We gave a value of 0 for time of flowering and fruiting for plants inoculated with *Retama* soil extract, and counted the number of days that plants inoculated with gap soil extract were delayed. We tested whether flowering or fruiting starting date for plants inoculated with gap soil extract differed from the mean value = 0 (same day of flowering or fruiting as plants with *Retama* soil extract). As these analyses are based on pairwise comparisons and selected plant species are of disparate families, we did not correct them by species phylogenies (Møller & Birkhead 1992).

Soil microbial communities

Shannon's diversity index (H') of OTUs per sample was calculated after excluding singletons (OTUs only showing 1 read in the overall data set) to reduce the overestimation of diversity (Tedersoo *et al.* 2010). Differences among samples in the microbial diversity were analysed using one-way ANOVA. Similarity in OTUs composition (with OTUs ≥ 5 reads) among treatments were analysed with Principal Coordinates Analysis using Bray–Curtis similarity index and NPMANOVA with 9999 permutations using Past v 2.12 (Hammer, Harper & Ryan 2001). We performed hierarchical cluster analyses of the relative abundance of bacterial genera in each treatment using Bray–Curtis similarity index in Infostat (Di Rienzo *et al.* 2012). Differences among treatments in relative abundance for each taxon were analysed using generalized linear models in R (R Development Core Team, 2011)

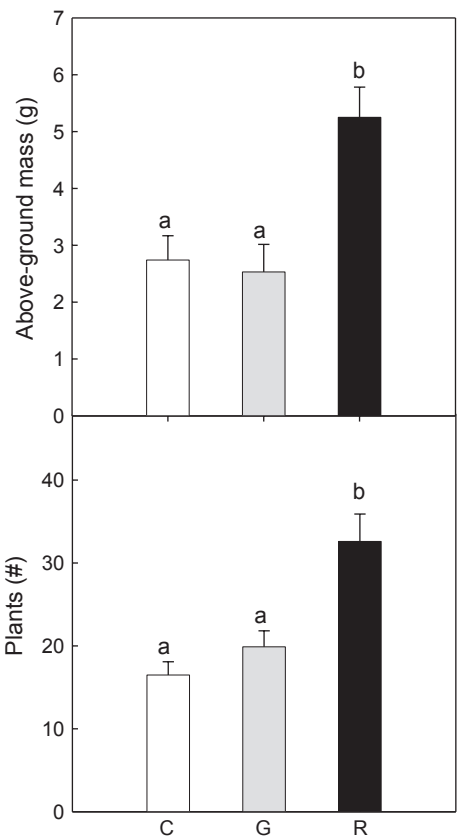

Fig. 1. Above-ground dry mass (g) and number of individual plants in the plant community experiment. Plants were grown in soil inoculated with soil extracts from gaps (G), from *Retama* understorey (R) or without inoculum (C). Data are mean ± SE, $n = 10$. Bars with different letters are significantly different after *post hoc* comparisons at a significance level of 0.05.

Results

In the plant community experiment, the number of individuals (Wald $F_{2,27} = 63.32$, $P < 0.001$) and above-ground mass ($F_{2,27} = 4.21$, $P = 0.03$) in pots inoculated with soil extracts from *Retama* understories almost doubled that of other treatments after 4 months, whereas no differences were found between control and soils inoculated with extracts from gaps (Fig. 1 and Table S1). No differences were found among treatments in total root mass ($F_{2,27} = 1.20$, $P = 0.31$), species richness ($F_{2,27} = 2.60$, $P = 0.09$) or plant diversity ($F_{2,27} = 1.12$, $P = 0.34$) (Table S2), but the relative contribution of some species (*Lagurus ovatus, Reichardia tingitana, Silene decipiens*) to above-ground mass was dependent on the soil extract received (Fig. S2).

In the single-species experiment, inoculation with soil extract from under *Retama* led to a significant increase in plant biomass and height, specific leaf area (SLA) and reproductive output in most species compared to individuals inoculated with gap soil extracts (Tables 3, S1, S3 and S4). Above-ground mass of *Clypeola jonthlaspi, Stipa capensis, Lagurus ovatus* and *Trigonella polycerata* was significantly higher in soils inoculated with *Retama* soil extract (Tables 3, S1 and S3). The same occurred with plant height of *S. capensis, L. ovatus* and *S. decipiens*. No differences were found in the final number of leaves for any of the studied species (Tables S1 and S3). SLA was higher in soils inoculated with *Retama* soil extracts for *Avena sterilis, Carrichtera annua, Geranium molle* and *Medicago truncatula,* but not for *C. jonthlaspi* (Tables 3, S1 and S3). Fruit number of *C. jonthlaspi, C. bursa-pastoris* and *T. polycerata* was significantly higher in plants from soils inoculated with *Retama* soil extracts (Tables S1 and S4). Significant differences were also found in seed mass for *C. bursa-pastoris*, *M. truncatula* and *S. decipiens*. The two former species had larger seeds in soils inoculated with *Retama* soil extracts, while *S. decipiens* produced larger seeds in inoculated with gap soil extracts. Reproductive fitness – mass of all seeds per plant – was significantly higher in soils with *Retama* soil extract for *C. jonthlaspi* and *M. truncatula* (Tables 3, S1 and S4). This outstanding effect of *Retama* soil extracts was independent from plant microhabitat preferences (data not shown). Inoculation with soil extract from *Retama* also led to a significantly faster flowering ($t_{10} = 4.00$, $P < 0.01$) and fruiting ($t_6 = 2.80$, $P < 0.05$), which started between 3 and 10 days earlier in most species (Fig. 2).

There were no differences in bacterial diversity between soil extracts (Shannon's index), or among soils in the plant community or single-species experiments (overall mean $H = 6.57 \pm 0.05$). We retained a total of 81 053 sequences after filtering and removing of chimeras and identified 14 475 distinct OTUs at 97% similarity. Ordination of operational taxonomic units (OTUs) of bacterial rDNA sequences (Fig. 3) and cluster hierarchical analysis of relative abundance of bacterial genera (Fig. S3) showed marked differences in bacterial community composition. Soil bacterial communities in extracts from gaps and under *Retama* were vastly different ($F_{1,18} = 1.32$, $P < 0.01$, NPMANOVA; Fig. 3a, Table S5). Plants also changed the soil bacterial community composition ($F_{2,18} = 3.01$, $P < 0.001$, NPMANOVA), while the absence of plants (RI_X treatment) led to a completely disparate bacterial community (Fig. 3a and Fig. S3). In the plant community experiment, soil bacterial communities were very variable within treatments, and thus differences among them could not be detected ($F_{2,6} = 1.20$, $P = 0.10$, NPMANOVA; Fig. 3b). In the single-species experiment, the analysis of soil microbial community composition was only performed for soils where *Carrichtera annua* and *Clypeola jonthlaspi* grew; soil extract origin ($F_{1,8} = 2.47$, $P < 0.001$, NPMANOVA) and plant species identity ($F_{1,8} = 2.25$, $P < 0.001$, NPMANOVA) affected soil microbial composition (Fig. 3c).

Irrespective of the experiment or treatment, soil bacterial communities were dominated by members of five different phyla (in decreasing abundance): Proteobacteria, Actinobacteria, Acidobacteria, Bacterioidetes and Firmicutes (Table S5). There were no differences in bacterial diversity between soil extracts, or among soils in the plant community or single-species experiments, but differences were detected in the

Table 3. Summary of soil extract effects on plant traits of different species (single-species experiment). R means that plants growing on soils inoculated with soil extracts from *Retama* understorey rendered a significantly higher mean value than plants of the same species growing on soils inoculated with gap soil extracts. G means higher values in plants that received gap soil extracts. Data means and statistical results are shown in Tables S1, S3 and S4. Biomass is above-ground dry plant mass, SLA is specific leaf area and reproductive fitness is the total seed mass per plant

Microhabitat	Species	Biomass	Height	SLA	Fruits #	Seed mass	Reproductive fitness
Gap	*Clypeola jonthlaspi*	R	–	G	R	–	R
	Stipa capensis	R	R	–	–	–	–
Retama	*Avena sterilis*	–	–	R	–	–	–
	Capsella bursa-pastoris	–	–	–	R	R	–
	Carrichtera annua	–	–	R	–	–	–
	Geranium molle	–	–	R	–	–	–
Indifferent	*Bromus rubens*	–	–	–	–	–	–
	Lagurus ovatus	R	R	–	–	–	–
	Medicago truncatula	–	–	R	–	R	R
	Silene decipiens	–	R	–	–	G	–
	Trigonella polycerata	R	–	–	R	–	–

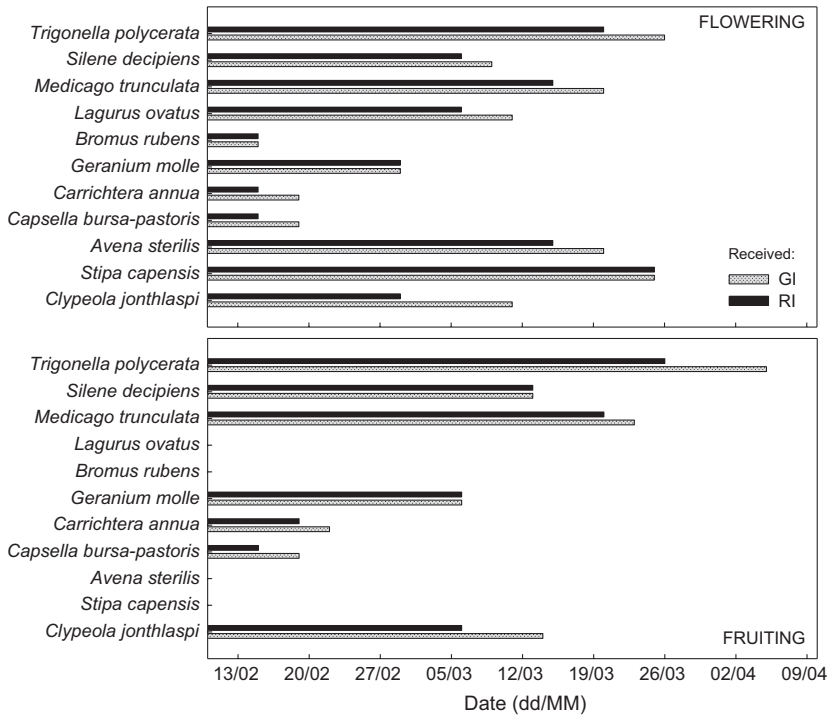

Fig. 2. Starting dates of flowering and fruiting for each plant species and soil extract inoculation in the single-species experiment. Plants were grown in soil inoculated with soil extracts from gaps (G) or from *Retama* understorey (R).

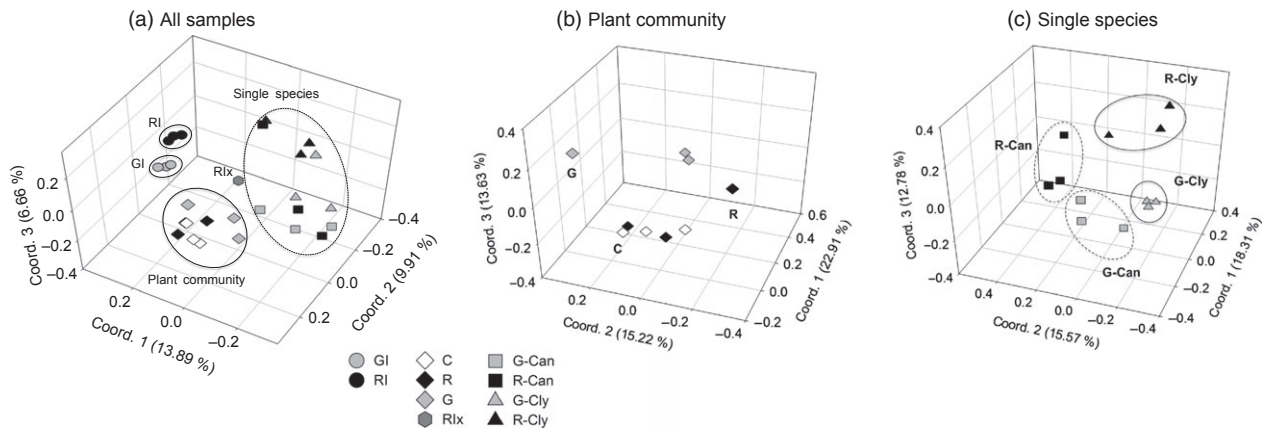

Fig. 3. Ordination of soil bacterial communities based on operational taxonomic units (OTUs) performing principal coordinates analysis (PCoA) using Bray–Curtis similarity index. Values of the sum of the variance explained by the first three coordinates of each PCoA analysis were 30.45% (a), 51.76% (b) and 46.66% (c). Legend: Soil extracts from gaps (GI) or under *Retama* (RI); soil bacterial communities after the plant community grew in pots that were not inoculated (C), inoculated with extracts from gaps (G), from under *Retama* (R) or with soil extract from under *Retama* but with no plants (RI_X); and soil bacteria after the growth of two single species (*Carrichtera annua* [Can] or *Clypeola jonthlaspi* [Cly]) inoculated with soil extracts from gaps (G-Can or G-Cly) or from under *Retama* (R-Can or R-Cly).

relative abundance of some taxa (Table S5). A total of 173 genera were identified from the obtained sequences. The relative abundance of each phylum was similar in the two initial soil extracts except for Bacteroidetes (greater in GI) and Cyanobacteria (greater in RI). Significant differences between extracts were found for Betaproteobacteria and Acidobacteria Gp4 that were more abundant in GI, and for Acidobacteria Gp6 and Gp7 that were more abundant in RI. Significant differences in the relative abundance of several genera were also found between the two initial soil extracts. The relative abundance of *Arthrobacter* was higher in RI than GI, whereas *Solirubrobacter* and *Sphingomonas* had a greater abundance in GI (Tables S5 and S6). Treatments in the plant community experiment differed in the relative abundance of Acidobacteria and Gemmatimonadetes that were significantly lower in G than in R, and in the relative abundance of Chloroflexi that was significantly higher in the pots inoculated with soil extract from *Retama* than in the control pots. Significant differences were found between treatments G and R for Deltaproteobacteria that were more abundant in the pots inoculated with soil extract from *Retama*, and for Acidobacteria Gp4 that were significantly lower in treatment G (Tables S5 and S6). In the single plant species experiment, plant species had a significant effect on the final relative abundance of the phylum

TM7 and the genus *Solirubrobacter* that had a higher abundance in the soil from *Carrichtera annua*. Plant species also had a significant effect on the relative abundance of the genus *Naxibacter,* which was higher for *Clypeola jonthlaspi.* Inoculation had a significant effect on the relative abundance of the class Acidobacteria Gp6 and the genus *Microvirga* that was higher for plants inoculated with soil extract from gaps. A significant interaction between plant species and inoculation was found for the relative abundance of the genus *Adhaeribacter*, showing the highest abundance in G-Cly and the lowest in R-Cly soils (Tables S5 and S6).

Discussion

Our results reveal a key role of below-ground biota as mediator of facilitation by nurse plant species. Soil biota associated with *Retama* led to increases in the number of plant individuals and productivity in the plant community experiment, and improved plant performance in the single-species experiment. Such effects were a consequence of the application of soil extracts and can only be explained by dissimilar soil communities under *Retama* and in gaps, as there were no differences in the initial chemical composition of inoculated soils. This positive effect of soil biota was also independent of the physical amelioration provided by the benefactor species. This indirect mechanism of facilitation by below-ground biota has, to our knowledge, not been shown before, although its existence in desert soils had been hypothesized (Sarig, Barness & Steinberger 1994; Callaway 2007). Interestingly, the positive effect of *Retama* soil biota on plant performance was independent of the preferred microhabitat in the field. Microhabitat preference is largely driven by environmental conditions and can change with location and shrub age (Armas, Rodríguez-Echeverría & Pugnaire 2011; Soliveres 2011) and therefore was not a good predictor of the effect of soil biota on plant growth or reproduction.

Soil extracts influenced not only plant growth, but also plant abundance, plant functional traits, phenology and reproductive effort, thus potentially modulating the development of plant communities (Lau & Lennon 2011). Other studies have shown differential growth responses of co-occurring plant species to soil microbes (Rodríguez-Echeverría & de la Peña 2009; de la Peña *et al.* 2010; Reinhart 2012), but soil biota effects on plant reproduction have seldom been explored or reported. An early flowering and fruiting, triggered by the *Retama* soil extract, could be advantageous for annual plants in semi-arid environments. A microbial-mediated selection of plant functional traits has recently been shown in species hosting microbial mutualists (Friesen 2011). Our data show that micro-organisms, as a whole, also affect functional plant traits, as shown by the different effect of gap and *Retama* soil microbiota on non-mycorrhizal, non-rhizobial plant species such as *Carrichtera annua* and *Clypeola jonthlaspi* (Gerdemann 1968; Cooke, Ash & Groves 2012). Such changes could be related to increased nutrient availability promoted by soil microbes and/or to molecular interactions between plant roots and microbes (Friesen 2011). Nutrient mineralization is probably the most important route by which soil microbes influence plant nutrient availability (Van der Heijden, Bardgett & Van Straalen 2008). Since microbial activity is higher under *Retama* than in gaps (Hortal *et al.* 2013), the soil communities associated with *Retama* used in our experiments could lead to an increase in nutrient availability. The observed differences in the composition of soil bacterial communities from *Retama* and gaps also imply that there might be differences in the identity and abundance of other important groups such as plant growth promoting bacteria, which produce phytohormones that can alter plant growth and performance (Friesen 2011). Although we only studied the composition of soil bacteria communities, this does not imply that other groups of soil microbiota, such as soil fungi, *Archaea* or microinvertebrates that passed through the 0.5-mm mesh, did not contribute to the observed differential effect of both inocula. For example, soil fungi can increase plant nutrient availability through the decomposition of organic matter (Wardle *et al.* 2004) and enhanced nutrient uptake through the mycorrhizal symbiosis (Van der Heijden, Bardgett & Van Straalen 2008). Since fungal biomass is higher under *Retama* than in gaps (Hortal *et al.* 2013), soil fungi could also contribute to faster nutrient cycling and to the observed positive effect of the *Retama* soil. Nonetheless, some of the selected beneficiary species are not mycorrhizal and, therefore, will not be affected by this particular group of soil organisms. With our experimental approach, we cannot tell the relative contribution of different soil microbes to plant performance apart. We were interested in the effect of soil microbiota as a whole as mediator of plant–plant interactions because the structure and function of soil communities depend on complex interactions between different soil microbial groups (Pendergast, Burke & Carson 2013). The molecular analysis was performed just on soil bacteria because this is the dominant microbial group in terms of biomass in these soils (Hortal *et al.* 2013) and because the rapid response of soil bacteria to both biotic and abiotic conditions.

In both plant community and single-species experiments, we found a remarkable plant influence on soil microbial composition, suggesting the existence of complex above–below-ground interactions probably occurring at the microsite scale in natural conditions (Pendergast, Burke & Carson 2013). Although Proteobacteria, Actinobacteria and Acidobacteria were the most abundant phyla in both soil extracts, as shown in other reports (Uroz *et al.* 2010; Nacke *et al.* 2011), communities in the two soil extracts differed – as previous data from the same field site had shown (Hortal *et al.* 2013). In the field, soil microbial communities under *Retama* and in gaps differ in species composition, biomass and activity (Hortal *et al.* 2013). These modifications seem to be driven by the presence of the shrub since they are already observed for small *Retama* shrubs with an understorey plant community similar to that found in gaps (Hortal *et al.* 2013). Our results suggest that such differences might contribute to the facilitation of plant establishment and growth **o**bserved in the understorey of *Retama.* Without the influence of *Retama*, the experimental plant community drove the development of bulk

soil microbial communities that were more similar among treatments at the end of the experiment than at the beginning. This result reflects the decisive influence of the composition of plant communities on the development of bulk soil communities as it had been previously found for rhizosphere communities (Berg & Smalla 2009). Interestingly, in the single-species experiment, the type of extract used and plant species identity had similar influence on the final composition of bacterial communities, overall suggesting that time and initial bacterial composition might determine how different plant species select their own soil community.

To conclude, we found that soil biota play a fundamental role in the facilitation effect of the nurse species *R. sphaerocarpa*. Soil communities that thrived under *Retama* led to an exceptional increase in plant number and biomass in the community of annual species and had an overall positive effect on individual plant performance and reproduction. In turn, single plant species and plant communities selected for different microbial groups, rendering different soil microbial communities. All data suggest that the soil microbial community and the understorey plant community can establish positive interactions that are an important mechanism to preserve plant biodiversity and ecosystem functioning in harsh environments.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Rarefaction curves for operational taxonomic units (OTUs) found in each treatment.

Figure S2. Relative contribution of each plant species to total biomass (plant community experiment).

Figure S3. Hierarchical cluster analysis of the relative abundance (%) of bacterial genera detected in the soil communities before and after the experiments.

Table S1. *F* values from ANOVA or GLZ, degrees of freedom (d.f.) and significance.

Table S2. Plant species richness, plant diversity (Shannon's Index) and below-ground mass (g) of the plant communities.

Table S3. Above-ground dry mass (g), height (cm), number of leaves and SLA (specific leaf area, m^2/kg^{-1}) of each studied plant species (single-species experiment).

Table S4. Reproductive effort (number of fruits and mean seed mass) and fitness (total seed mass per plant) of each studied plant species (single-species experiment).

Table S5. Mean relative abundance per treatment of the most abundant identified soil bacterial taxonomic groups.

Table S6. P-values obtained in the GLM analysis of relative abundance of the bacterial taxa showed in Table S5.