Soil Biology & Biochemistry 64 (2013) 139–146

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Soil microbial community under a nurse-plant species changes in composition, biomass and activity as the nurse grows

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ARTICLE INFO

Article history:
Received 25 November 2012
Received in revised form
9 April 2013
Accepted 25 April 2013
Available online 15 May 2013

Keywords:
Facilitation
Plant–Soil interactions
Semiarid
Pyrosequencing
Retama sphaerocarpa
Microbial community

ABSTRACT

Shrubs in semiarid ecosystems facilitate the establishment of other plant species under their canopies and promote changes in the understory plant communities as they grow. To better understand whether plant community dynamics are linked to changes in soil microbes, we characterized soil microbial communities in gaps (open spaces) and under *Retama sphaerocarpa* shrubs of three different size classes. Different methodological approaches including pyrosequencing of 16S rDNA, phospholipid-fatty acid (PLFA) analysis and microbial activity indicators were combined to characterize both the structure and function of soil microbial communities in the different treatments. Soil microbial communities under small shrubs showed higher microbial biomass and activity, as well as different relative abundance of several bacterial groups, than communities in gaps. Shrubs were associated with a higher relative abundance of *Bacteroidetes*, *Betaproteobacteria* and *Gammaproteobacteria* in detriment of *Actinobacteria* and *Firmicutes* without changes in overall bacterial diversity. Soil microbial community changed with shrub size, being most different in gaps and under the canopy of large shrubs. We suggest that changes in composition and function of soil microbial communities may promote the increase in the understory plant growth and species richness previously reported for this shrub species. Our data emphasize the importance of plant–soil interactions in defining the structure and composition of both plant and soil microbial communities and their impact on ecosystem functioning.

1. Introduction

Although interactions between plants and soil organisms have important consequences for ecosystem dynamics (Van Der Putten, 2003) and changes in plant communities with time occur in concert with changes in soil properties, the relationships between soil microbial community and plant community dynamics are not fully understood (van der Heijden et al., 2008). Plants are able to modify the structure of microbial communities in their rhizosphere (Kowalchuk et al., 2002; Berg and Smalla, 2009) while soil microbes are important regulators of plant productivity, both through direct effects and through regulation of nutrient availability (van der Heijden et al., 2008). However, the role of such interactions in plant community dynamics with time has received little attention (Bardgett et al., 2005; Bartelt-Ryser et al., 2005; Kardol et al., 2006).

Plant community changes in stressful environments can be driven by facilitation processes (Callaway et al., 2002; Butterfield et al., 2010), which also promote plant diversity (Butterfield et al., 2013). Under such extreme abiotic conditions facilitation by nurse species is common, particularly in dry environments (Flores and Jurado, 2003). Nurse species facilitate the establishment and growth of other plants and promote plant community changes in their understory (Pugnaire et al., 2011). A well-known example of nurse species is *Retama sphaerocarpa* (L.) Boiss., a large leguminous shrub widespread in North-west Africa and the Iberian Peninsula. Mature *R. sphaerocarpa* shrubs promote plant growth and species richness, which are at least 3 times higher under the canopy than in open areas (gaps) around shrubs (Pugnaire et al., 2004; Armas et al., 2011). Mechanisms of facilitation by *R. sphaerocarpa* shrubs include amelioration of adverse microclimatic conditions, increased nutrient mineralization rate, and accruement of organic matter, nutrients, and fine soil particles under its canopy (Pugnaire et al., 1996; Moro et al., 1997; Rodriguez-

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http://dx.doi.org/10.1016/j.soilbio.2013.04.018

Echeverria and Perez-Fernandez, 2003; Lopez-Pintor et al., 2006) as well as the positive effect of water lifted from deep soil storages and shed into upper soil layers (Prieto et al., 2010).

Both species richness and plant biomass under *R. sphaerocarpa* canopy increase as shrubs grow (Pugnaire et al., 1996; Pugnaire and Lazaro, 2000) so that *R. sphaerocarpa* shrubs of different size (assumed to differ in age, Pugnaire et al., 1996) host distinctive communities that largely differ in composition, richness and biomass (Pugnaire et al., 1996; Pugnaire and Lazaro, 2000). In particular, drought-resistant species typical of open spaces are found under small shrubs while large shrubs also host mesic species and subshrubs (Pugnaire et al., 1996). The frequency of some species increases with shrub size despite lack of differences in soil seed bank (Pugnaire and Lazaro, 2000). Overall, communities under large shrubs have higher species richness, more biomass and more perennial species than communities under small shrubs, which are rather similar to communities in gaps (Pugnaire et al., 1996; Pugnaire and Lazaro, 2000). The main mechanisms underlying this process are linked to changes in soil resources, mainly water and nutrients (Pugnaire et al., 1996, 2004; Moro et al., 1997). However, it remains unexplored whether plant communities under *R. sphaerocarpa* are also linked to different soil microbial communities.

It is thus well described that soil properties and plant communities under *R. sphaerocarpa* change as shrubs increase in size. Within this framework, we hypothesize that soil microbial communities under *R. sphaerocarpa* shrubs of different size would be also different, and would change in composition, biomass, and function. Moreover, soil microbial communities under *R. sphaerocarpa* would also differ from those of open areas. To test these hypotheses we characterized the microbial community in soils from gaps and under *R. sphaerocarpa* shrubs of three size classes. We combined different methodological approaches to characterize soil microbial communities including high-throughput pyrosequencing, PLFA analysis, and microbial activity indicators. Parallel to the amelioration of microclimatic conditions and increase in plant community biomass and richness as *R. sphaerocarpa* shrubs grow, we predicted an increase in soil microbial biomass, diversity and activity as well as changes in microbial community composition with increasing shrub size. In particular, according to the changes described for understory plant communities (Pugnaire et al., 1996; Pugnaire and Lazaro, 2000), we expected soil microbial communities under small shrubs to be more similar to communities in gaps than to communities under large shrubs. Overall, our objective was to obtain a detailed picture of belowground community structure and function that could allow us to discuss the potential links between soil microbial and plant communities along the ontogeny of this nurse species.

2. Materials and methods

2.1. Field site, soil sampling and chemical analyses

Our field site was at the bottom of the Rambla del Saltador valley, in the southern slope of Sierra de Filabres range in Almeria, southeast Spain (37°08′ N, 2°22′ W; 630 m altitude). The regional climate is semiarid with a pronounced dry season from May to September. Mean annual temperature is 17.9 °C and mean annual precipitation is 240 mm (data of a 30-year series from the nearest weather station in Tabernas, 490 m altitude, 9 km south of the field site). The valley bottom is filled with thick and poorly sorted alluvial deposits on mica-schist bedrock (Puigdefabregas et al., 1996). Soils are characterized by a very low water holding capacity, a pH ranging from slightly acidic (6.5) to moderately alkaline (8), low electrical conductivity, low cation exchange capacity (Puigdefabregas et al., 1996), low organic matter and low nutrient concentrations (Pugnaire et al., 1996). The field site is occupied by an open shrubland dominated by *R. sphaerocarpa*, a leafless leguminous shrub with evergreen photosynthetic stems and a very deep root system (20–30 m, Haase et al., 1996). Previous studies conducted at this same site have described the characteristics of the plant community growing under *R. sphaerocarpa* and in open spaces (Pugnaire et al., 1996; Pugnaire and Lazaro, 2000; Armas et al., 2011).

In March 2011, we randomly selected 24 *R. sphaerocarpa* shrubs and assigned them to three different size categories (small, medium and large) established according to shrub dimension ($n = 8$). Height and maximum and minimum canopy diameter were measured for each shrub. Mean shrub heights (±SD) were 1.04 ± 0.17; 2.12 ± 0.23 and 3.29 ± 0.62 m and mean projected canopy area (calculated as the area of an ellipse, Pugnaire et al., 1996) were 0.95 ± 0.34, 7.19 ± 1.87 and 37.43 ± 8.86 m^2 for small, medium and large *R. sphaerocarpa* shrubs, respectively. Shrub height and canopy area were significantly different among size classes. Shrubs in each size class are assumed to have different age, *i.e.* less than 10 years, 10–15 years and more than 25 years old, respectively, according to reported data from the same field site (Pugnaire et al., 1996).

Soil was sampled under the canopy of each shrub at an intermediate distance between the trunk and the edge of the canopy (where microhabitat should be most favourable for soil microorganisms, Moro et al., 1997) and in 8 gaps between shrubs in March 2011. For each sample, 4 soil cylinders 4.5 cm in diameter and 10 cm deep were collected, combined, homogenised and sieved through 2 mm mesh. A total of 32 composite samples were collected, eight for each of the four treatments (gap, small, medium and large shrubs). Each soil sample was divided into two subsamples, one (100 g approximately) was stored at −20 °C for molecular analyses and the other one (400 g approximately) was kept at 4 °C for a maximum of 1 month for chemical, microbial biomass and activity analyses as recommended by Cernohlavkova et al. (2009).

Total C and N content in each soil sample were determined using a LECO Truspec C/N analyser (St. Joseph, MI, USA). Due to the very low concentration of $CaCO_3$ in our field site (under 1%, Puigdefabregas et al., 1996) soil C measured in our analyses should mainly represent organic C. Soil pH and electrical conductivity (EC) were measured in 3 composite soil samples per treatment (obtained after pooling) by mean of a pH-electrode and conductivimeter (Crison Instrument S.A., Barcelona, Spain) in the water extract obtained after two hours by mechanical agitation of a soil:water (1:10 v/v) mix.

2.2. Microbial activity: enzyme activities

Microbial activity analyses were performed in 3 composite soil samples per treatment (obtained after pooling). Soil dehydrogenase activity (DA) was determined by using 1 g of soil, and the reduction of p-iodonitrotetrazolium chloride (INT) to p-iodonitrotetrazolium formazan was measured by a modification of the method reported by Von Mersi and Schinner (1991). Soil DA was expressed as μg INTF g^{-1} soil h^{-1}. Urease activity (UA) was determined as the NH_4^+ released in the hydrolytic reaction using urea as substrate and borate buffer (pH 10) (Kandeler and Gerber, 1988). The alkaline phosphatase (PA) and β-glucosidase (BA) activities were determined according to Tabatabai and Bremmer (1969) and Eivazi and Tabatabai (1977), respectively, adding 2 ml of modified universal buffer (MUB) pH 11 and 0.5 ml of 0.025 M *p*-nitrophenyl phosphate (for the PA assay) or 2 ml of MUB pH 6 and 0.5 ml of 0.025 M *p*-nitrophenyl β-D-glucopiranoside (for the BA assay) to 0.5 g of soil. The mixtures were then incubated at 37 °C for 1 h, after which the enzyme reactions were stopped by cooling on ice for 15 min. Then, 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH (for PA) or 2 ml of 0.1 M Tris-hydroxymethylaminomethane–sodium hydroxide (THAM–

NaOH) pH 12 (for BA) were added. In the control, the respective substrates were added before the addition of $CaCl_2$ and NaOH.

2.3. Microbial biomass: PLFAs

Phospholipids were extracted from 6 g of soil using a chloroform–methanol extraction method based on Bligh and Dyer (1959) in 3 composite soil samples per treatment. They were fractionated and quantified using the procedure described by Frostegard et al. (1993a) and Bardgett et al. (1996). Phospholipids were transformed by alkaline methanolysis into fatty acid methyl esters (FAMEs), which were quantified by a gas chromatograph (Trace GC Ultra Thermo Scientific) fitted with a 30-m capillary column (Thermo TR-FAME 30 m × 0.25 mm ID × 0.25 μm film), using helium as carrier gas. The initial temperature was 150 °C for 0.5 min and it was increased to 180 °C at 2 °C min^{-1} and then to 240 °C at 4 °C min^{-1}. The $Gram^+$ specific fatty acids i15:0, a15:0, i16:0 and i17:0 and the $Gram^-$ specific fatty acids 18:1ω9c, 18:1ω9t, cy17:0 and cy19:0 were taken as a measure of the ratio between the $Gram^+$ and $Gram^-$ bacterial biomass. The fatty acids i15:0, a15:0, 15:0, i16:0, i17:0, 18:1ω9c, 18:1ω9t, cy17:0 and cy19:0 were chosen to represent the bacterial biomass (Frostegard et al., 1993b; Bardgett et al., 1996; Dungait et al., 2011) and 18:2ω6 was taken to indicate the fungal biomass (Federle et al., 1986; Zelles et al., 1992; Baath, 2003). The ratio of bacterial to fungal PLFAs represents the ratio between the bacterial and fungal biomass (Bardgett et al., 1996).

2.4. Bacterial composition: pyrosequencing

Six soil samples per treatment (gap, small, medium and large) were randomly selected for molecular analyses. DNA was extracted from 0.25 g of homogenised soil per sample using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) following the manufacturer's instructions.

A 16S rDNA gene fragment corresponding to V1 and V2 regions was amplified. The forward primer (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNN AGAGTTTGATCMTGGCTCAG-3′) contained the Roche pyrosequencing adapter A (underlined), a Roche recommended 10 bp bar-code sequence (NNNNNNNNNN) used to tag each sample and the bacterial primer 27F. The reverse primer (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGCTGCCTCCCGTAGGAGT-3′) contained the Roche pyrosequencing adapter B (underlined) and the primer 338R. PCR amplifications were performed in 50 μl reaction volumes containing ultrapure H_2O, 2.5× 5 PRIME MasterMix including 1.5 mM Magnesium, 200 μM dNTPs, 1.25 U Taq polymerase (5 PRIME, Hamburg, Germany), 0.2 μM of primers and approximately 5–10 ng of template DNA. Fragments were amplified under the following conditions: initial denaturation at 94 °C for 3 min, followed by 30 cycles with denaturation at 94 °C for 40 s, annealing at 52 °C for 40 s and extension at 68 °C for 35 s, with a final extension at 68 °C for 7 min. Each sample was amplified in triplicate, pooled and purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany). Amplification was checked by electrophoresis in 2% agarose gels stained with SYBR® Safe DNA Gel Stain (Invitrogen™, Carlsbad, USA) and bands were visualized using UV light in a Gel Doc™ EZ Imager (BIO-RAD, Hercules, USA).

DNA concentration of each purified sample was determined as the mean of three lectures in NanoDrop 2000c (Thermo Scientific, Wilmington, USA). 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 the Centro Superior de Investigación en Salud Pública (Valencia, Spain).

2.5. Processing of pyrosequencing data

The 16S rDNA sequence data were processed using the pyrosequencing pipeline tools from the Ribosomal Database Project (RDP). Sequences were trimmed for primers, filtered and assigned to samples according to their tag. Sequences shorter than 95 bp, with quality scores <20 or containing any unresolved nucleotides were removed from the dataset. Most of the discarded sequences were shorter than 95 bp. 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 et al., 2011) and removed from the dataset. Sequences were aligned using the Aligner tool from the RDP pyrosequencing pipeline. Aligned sequences were clustered into operational taxonomic units (OTUs) defined at 97% similarity cutoff using the complete linkage clustering tool of the RDP pipeline. Rarefaction curves were constructed by using the Rarefaction tool from the RDP pipeline. Taxonomic assignation of the sequences was performed using the RDP naïve Bayesian classifier (Wang et al., 2007) at a confidence level of 50% (recommended for short sequences, Claesson et al., 2009). Relative abundances of the different phyla (or other taxonomic categories) in each of the 24 samples were calculated. We used Past software version 2.12 (Hammer et al., 2001) to calculate the Shannon diversity index (H′) for each sample after excluding the singletons from the overall dataset in order to reduce the overestimation of diversity (Tedersoo et al., 2010). Finally, sequences obtained for the 6 samples belonging to the same treatment were pooled and libraries were compared using RDP Library Compare tool (RDP pipeline).

2.6. Statistical analyses

Differences among treatments for soil C and N content, pH, EC, enzyme activities, PLFAs, relative abundance of the different taxa and Shannon diversity index were tested by ANOVA. Post-hoc comparisons were performed using Tukey HSD tests. Levene and Kolmogorov–Smirnov tests were used to check for homogeneity of the variance and normality, respectively. Variables were transformed when necessary. Relationships among soil C or N content and the relative abundance of the different taxa were evaluated by using linear models with C or N, treatment and the interaction among them as fixed factors. Principal coordinates analysis (PCoA) based on OTU composition using Bray–Curtis similarity measure was performed using data on OTUs showing at least 5 reads in the overall dataset. Similar results were obtained when using all data or excluding singletons. Differences in OTU composition among treatments based on Bray–Curtis distance were analysed using one-way NPMANOVA with 9999 permutations. All statistical analyses were performed using Infostat (Di Rienzo et al., 2012) except for PCoA and NPMANOVA that were implemented in Past software. Results are presented as mean values ± 1 SD throughout the text. Differences among treatments were considered significant at a significance level of 0.05.

3. Results

3.1. Soil properties

Content of C and N in soil were significantly different among treatments ($F_{3,28} = 24.67$, $p < 0.01$, $R^2 = 0.73$ for C; $F_{3,28} = 29.37$, $p < 0.01$, $R^2 = 0.76$ for N) being soil C and N low in gaps, intermediate under small and medium *R. sphaerocarpa* shrubs, and high under large shrubs (Table 1). Mean C/N ratio was 10.38 ± 2.42 and did not differ among treatments. Soil pH was neutral in all treatments and slightly higher under the canopy of small

Table 1
Mean values (±SD) of total carbon content (C), total nitrogen (N), pH, electrical conductivity (EC) and enzyme activities (dehydrogenase, urease, β-glucosidase and phosphatase) in soils from gaps and beneath *R. sphaerocarpa* shrubs of different sizes. Different letters in a row indicate significant differences ($p < 0.05$) among treatments by Tukey's comparison; $n = 8$ for C and N; $n = 3$ for pH, CE and enzyme activities.

	Gap	*R. sphaerocarpa*		
		Small	Medium	Large
C (g kg^{-1})	5.57 ± 0.83 a	9.02 ± 2.02 b	9.32 ± 2.67 b	14.49 ± 2.37 c
N (g kg^{-1})	0.51 ± 0.16 a	0.94 ± 0.30 b	0.90 ± 0.23 b	1.57 ± 0.20 c
pH	7.80 ± 0.09 a	8.02 ± 0.12 b	7.87 ± 0.02 ab	7.73 ± 0.05 a
EC (μS/cm)	40.87 ± 9.90 a	51.50 ± 2.42 a	58.80 ± 9.99 ab	72.83 ± 2.73 b
Dehydrogenase (μg INTF g^{-1} h^{-1})	5.56 ± 0.67 a	9.86 ± 0.75 b	9.72 ± 1.25 b	9.90 ± 0.57 b
Urease (μmol NH_3 g^{-1} h^{-1})	0.09 ± 0.01 a	0.33 ± 0.01 b	0.34 ± 0.02 b	0.63 ± 0.01 c
β-glucosidase (μmol PNP g^{-1} h^{-1})	0.18 ± 0.04 a	0.70 ± 0.03 b	1.17 ± 0.13 c	2.83 ± 0.39 d
Phosphatase (μmol PNP g^{-1} h^{-1})	0.45 ± 0.07 a	1.36 ± 0.09 b	1.73 ± 0.06 b	2.34 ± 0.33 c

R. sphaerocarpa shrubs ($F_{3,8} = 7.93$, $p < 0.01$, $R^2 = 0.75$; Table 1) than under larger shrubs and gaps. Soil EC was significantly higher under the canopy of large shrubs compared to gaps and small shrubs ($F_{3,8} = 10.24$, $p < 0.01$, $R^2 = 0.79$; Table 1).

3.2. Microbial activity

There were differences among treatments in all analysed enzyme activities, *i.e.* dehydrogenase ($F_{3,8} = 18.85$, $p < 0.01$, $R^2 = 0.88$), urease ($F_{3,8} = 18.64$, $p < 0.01$, $R^2 = 0.87$), β-glucosidase ($F_{3,8} = 94.20$, $p < 0.01$, $R^2 = 0.97$) and alkaline phosphatase ($F_{3,8} = 58.48$, $p < 0.01$, $R^2 = 0.96$). All microbial activity indicators showed higher values under *R. sphaerocarpa* shrubs than in gaps. Overall, enzyme activity values were significantly higher under large *R. sphaerocarpa* shrubs than under medium and small ones (Table 1), except in the case of dehydrogenase activity that was similar in all soils under shrubs. The largest increase was detected for β-glucosidase activity which was 4-times higher under large shrubs than under small ones.

3.3. Microbial biomass

There was an overall increase of all PLFA groups under the shrub compared to gaps and with increasing plant size (Table 2). Soil fungal, bacterial, $Gram^+$, $Gram^-$ and total PLFAs were significantly different among treatments ($F_{3,8} = 13.99$, $p < 0.01$, $R^2 = 0.84$; $F_{3,8} = 61.30$, $p < 0.01$, $R^2 = 0.96$; $F_{3,8} = 41.41$, $p < 0.01$, $R^2 = 0.94$; $F_{3,8} = 40.64$, $p < 0.01$, $R^2 = 0.94$ and $F_{3,8} = 137.50$, $p < 0.01$, $R^2 = 0.98$, respectively). Mean ratios fungi/bacteria and $Gram^+/Gram^-$ were 0.14 ± 0.02 and 0.79 ± 0.15 respectively and did not significantly differ among treatments.

3.4. Diversity and composition of bacterial communities

We obtained 130,690 sequences by pyrosequencing. A total of 30,767 sequences were retained after filtering. The mean number of retained sequences per sample was 1282, ranging from 1022 to 1628. Average length of retained sequences was 144 ± 2.93 bp (mean ± SD). Short reads in V1–V3 regions have been shown to be sufficient to characterize microbial community accurately, especially when using a confidence threshold of 50% in taxonomic assignation (Liu et al., 2007; Claesson et al., 2009; Jeraldo et al., 2011). All the sequences aligned correctly in the expected position of the 16S rDNA sequence of *Escherichia coli*. The slope of rarefaction curves was similar for all samples regardless of the treatment (Figure S1). In order to minimize any bias due to different sequencing effort among samples, we randomly selected 1000 sequences per sample to conduct further analyses (following Lauber et al., 2009; Fierer et al., 2011). Obtained results were similar when using all data.

We identified a total of 6384 distinct OTUs (at 97% similarity) in the dataset. Of these, 1055 OTUs were supported by 5 or more reads and 3026 corresponded to singletons. The number of OTUs per sample ranged from 605 to 708. Mean value of Shannon index after excluding singletons was 6.01 ± 0.09 (6.24 ± 0.07 when including all data) and did not significantly differ among treatments.

Sequences were taxonomically assigned using the RDP Classifier. The assigned sequences mainly belonged to 5 different phyla: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria* and *Firmicutes* (Fig. 1). Other identified minor phyla (with relative abundances lower than 1%) were *Nitrospira*, *TM7*, *Gemmatimonadetes*, *Verrucomicrobia*, *OP10* and *Chloroflexi*. Relative abundances of different phyla (and classes within *Proteobacteria*) differed among treatments (Fig. 1). Values were almost identical when excluding singletons from the overall dataset (data not shown). The relative abundance of *Actinobacteria* and *Firmicutes* was significantly lower under large shrubs than in gaps ($F_{3,20} = 3.58$, $p < 0.01$, $R^2 = 0.35$; $F_{3,20} = 3.04$, $p = 0.05$, $R^2 = 0.31$, respectively). On the contrary, increases in the relative abundance of *Bacteroidetes*, *Betaproteobacteria* and *Gammaproteobacteria* were detected. Thus, the relative abundances of *Bacteroidetes* and *Betaproteobacteria* were significantly higher under shrubs of any size than in gaps ($F_{3,20} = 5.57$, $p < 0.01$, $R^2 = 0.46$; $F_{3,20} = 13.61$, $p < 0.01$, $R^2 = 0.67$, respectively) while *Gammaproteobacteria* were relatively more abundant under large shrubs than in gaps ($F_{3,20} = 12.25$, $p < 0.01$, $R^2 = 0.65$). The relative abundance of unclassified bacteria was significantly lower under shrubs than in gaps ($F_{3,20} = 18.42$, $p < 0.01$, $R^2 = 0.73$). There were no significant changes in relative abundance among treatments in three taxonomic groups (Fig. 1), *i.e. Deltaproteobacteria*, *Alphaproteobacteria* and *Acidobacteria*. We observed no significant relationship between soil N or C and the relative abundance of the different bacterial groups neither as main effect nor in interaction with treatment.

When using the library comparison tool to compare changes in relative abundances among communities in gaps and large shrubs in detail, we found a significant decrease in the abundance of

Table 2
Mean values (±SD) on fungal, bacterial, $Gram^+$, $Gram^-$ and total PLFAs in soils from gaps and beneath *R. sphaerocarpa* shrubs of different sizes ($n = 3$). All results are given in nmol g^{-1}. Different letters in a row indicate significant differences ($p < 0.05$) among treatments by Tukey's comparison.

	Gap	*R. sphaerocarpa*		
		Small	Medium	Large
Fungi	0.13 ± 0.01 a	0.21 ± 0.03 ab	0.35 ± 0.05 bc	0.39 ± 0.09 c
Bacteria	0.94 ± 0.12 a	1.58 ± 0.16 b	2.12 ± 0.19 c	2.78 ± 0.21 d
$Gram^+$	0.37 ± 0.03 a	0.75 ± 0.09 b	1.00 ± 0.07 b	1.15 ± 0.14 c
$Gram^-$	0.57 ± 0.10 a	0.83 ± 0.07 ab	1.12 ± 0.20 b	1.63 ± 0.07 c
Total	2.27 ± 0.22 a	3.37 ± 0.32 b	4.86 ± 0.23 c	7.67 ± 0.52 d

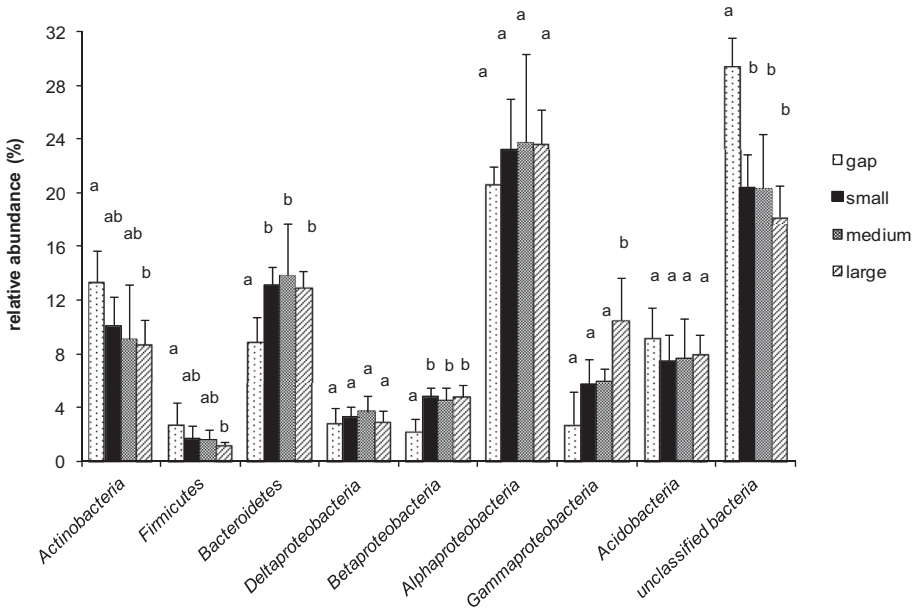

Fig. 1. Mean relative abundance (±SD) of the main identified bacterial taxonomic groups, *i.e.* phyla *Actinobacteria*, *Firmicutes*, *Bacteroidetes* and *Acidobacteria*, classes *Deltaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* (within *Proteobacteria* phylum), as well as of unclassified bacteria in soils from gaps and under *R. sphaerocarpa* shrubs of different size. All results are expressed as %. Different letters within a bacterial group indicate significant differences ($p < 0.05$) among treatments by Tukey comparison; $n = 6$.

subclasses *Rubrobacteridae* and *Actinobacteridae* (*Actinobacteria*) and class *Bacilli* (*Firmicutes*) under large shrubs. On the contrary, we found a significant increase of orders *Flavobacteriales* (*Bacteroidetes*), *Burkholderiales* (*Betaproteobacteria*), *Pseudomonadales* and *Xanthomonadales* (*Gammaproteobacteria*). We also found a significant decrease of *Acidobacteria* classes Gp4 and Gp16 while the abundance of class Gp6 significantly increased under large shrubs. Similarly, within *Alphaproteobacteria*, there was a significant increase in abundance of *Caulobacterales* and no global changes in *Rhizobiales* (the most abundant order within this class), which included groups that increased in abundance (like *Bradyrhizobiaceae*) and others that decreased (like *Methylobacteriaceae*).

Ordination of samples by PCoA based on their OTU composition and using Bray–Curtis similarity measure showed a separation of samples by treatments along the first coordinate (Fig. 2). NPMANOVA showed significant differences among treatments ($F_{3,20} = 1.80$; $p < 0.01$) being all pairwise comparisons except for small-medium shrubs significantly different (Table 3).

4. Discussion

Our results showed that microbial communities under small *R. sphaerocarpa* shrubs had higher biomass and activity as well as different composition than microbial communities in gaps, in sharp contrast with plant communities, which are very similar under small shrubs and in gaps (Pugnaire et al., 1996). We also found that the differences in soil microbial communities between gaps and under *R. sphaerocarpa* shrubs increased with shrub age. These results suggest that shrub presence promotes strong changes in the microbial community, most likely through root exudates, which could have deeper effects as shrubs age (Marschner et al., 2004). Symbionts colonizing shrub roots such as arbuscular mycorrhizal fungi (Martinez-Garcia et al., 2011) and *Rhizobiales* (Rodriguez-Echeverria and Perez-Fernandez, 2003) may also be indirectly contributing to microbial community changes. Factors such as root exudates produced by annual species in the understory of *R. sphaerocarpa* shrubs or microclimatic conditions would have a minor effect in the initial change of the microbial community since both of them are very similar between gaps and under the canopy of small shrubs (Pugnaire et al., 1996). Nevertheless, these factors could gain in importance as shrubs grow and the understory plant community changes. However, we can exclude the effect of soil pH, which has shown to be a key factor in regulating microbial communities in other systems (Lauber et al., 2009; Rousk et al., 2010), since it remained between 7.7 and 8.0 in our treatments.

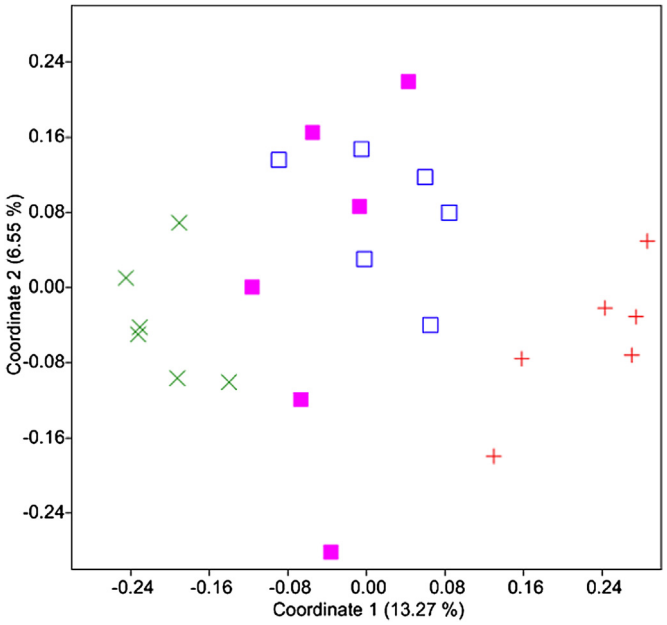

Fig. 2. Ordination of soil bacterial community composition by principal coordinates analysis (PCoA) using Bray–Curtis similarity index. Only OTUs with at least 5 reads were included in the analysis. Samples coded by treatment: + = gap, squares = small, filled squares = medium and, × = large shrubs; $n = 6$. Variance explained by the two first axes is showed in the graph.

Table 3
F values of pairwise comparisons of Bray–Curtis similarity index among treatments by one-way NPMANOVA analysis. Asterisks and bold values indicate significant *p*-values (<0.05); n.s. indicates non-significant differences; $n = 9999$ permutations.

	Gap	Small	Medium	Large
Gap		**1.76***	**1.82***	**3.19***
Small			n.s.	**1.74***
Medium				**1.31***
Large				

Soil bacterial communities were dominated by members of the phylum *Proteobacteria*, which play a key role in C, N and S cycling (Kersters et al., 2006). In particular, three of the five most abundant OTUs did belong to *Rhizobiales* (class *Alphaproteobacteria*), which establish symbiosis with legumes to fix N and should be more active under the canopy of *R. sphaerocarpa* shrubs (Rodriguez-Echeverria and Perez-Fernandez, 2003). *R. sphaerocarpa* shrubs were associated with a lower relative abundance of *Actinobacteria* and *Firmicutes*. The highest abundance of *Firmicutes* in gaps reflects well their ability to tolerate extreme conditions and support very low substrate availability (Acosta-Martinez et al., 2010). We also detected a significant decrease in unclassified bacteria in soil under plants, as reported by Uroz et al. (2010). Bacterial groups associated to plants have received much more attention than those from bulk soil, which may explain why bacteria in the rhizosphere are better classified. That points out that we still have much to learn about the microbial diversity reservoir that bulk soil may host, especially in arid ecosystems characterized by poor vegetation cover.

R. sphaerocarpa shrubs were also associated with a higher relative abundance of *Bacteroidetes*, *Betaproteobacteria* and *Gammaproteobacteria,* supporting the hypotheses that their abundance increases as communities mature (Fierer et al., 2007) and that they are copiotrophic, *i.e.* found in soils with high C availability (Fierer et al., 2007; Eilers et al., 2010). It is interesting to note that the increase in abundance of such groups could stimulate plant growth in soils under shrubs. *Bacteroidetes* have the ability to rapidly explore organic matter (Acosta-Martinez et al., 2010) and stimulate nutrient cycling while *Burkholderiales* and *Pseudomonadales* (two key groups within *Betaproteobacteria* and *Gammaproteobacteria* that increase as shrubs grow) have been described as potential plant-growth promoting organisms. On the one hand, *Burkholderiales* are efficient mineral weathering bacteria (Uroz et al., 2011) and it has been shown recently that they can also play a role in N_2 fixation (Garau et al., 2009; dos Reis et al., 2010) and confer resistance to water stress (Mayak et al., 2004). On the other hand, *Pseudomonadales* protect plants from fungal infection (Mendes et al., 2011), synthetize phytohormones, and contribute to nutrient uptake (Marilley and Aragno, 1999). These groups would thus play a particularly important role in extreme environments such as semiarid systems.

Although shrubs induced changes in bacterial community composition, Shannon's diversity index remained similar among treatments indicating that some bacterial phylotypes were replaced by others. Therefore, contrary to our expectations, the observed increase in plant diversity under *R. sphaerocarpa* as the shrub grows (Pugnaire et al., 1996; Pugnaire and Lazaro, 2000) was not parallelled with an increase in bacterial diversity in soil. Other studies have also reported a lack of increase in bacterial diversity with plant cover (Farias et al., 2009) or plant species richness (Kuramae et al., 2011). In fact, little is known about the interdependency of microbial diversity with plant diversity (Zak et al., 2003) and it appears that the effects of soil diversity on aboveground attributes can range from positive to negative depending on context (Wardle et al., 2004).

Unlike diversity, both bacterial and fungal biomass increased with shrub size, in parallel to the increase previously described for aboveground plant mass (Pugnaire et al., 1996). Bacterial mass significantly increased even under small shrubs. Closely related to this, we also found an overall increase in soil microbial activity as shrubs grew. The increase in soil nutrients and moisture, the amelioration of microclimatic conditions under the *R. sphaerocarpa* canopy, the use of root exudates as growth substrate (Haichar et al., 2008) and the accumulation of annual herbs with easily decomposable litter (Gallardo and Merino, 1993; Zhang et al., 2008) would explain this increase in soil microbial biomass and activity. We suggest that this stimulation of microbial activity and nutrient cycling under the canopy of *R. sphaerocarpa* shrubs could contribute to the facilitation effect of this species (Pugnaire et al., 1996, 2004).

4.1. Conclusions

Changes in soil bacterial composition as well as increases in soil microbial biomass and activity, but not diversity, do occur under the canopy of *R. sphaerocarpa* shrubs compared to gaps, with changes already starting in the understory of small shrubs and continuing as shrubs grow. We suggest that *R. sphaerocarpa* shrubs likely induce such changes through root exudates, which would act as environmental filters for soil microbes, in addition to contributions from the understory plant community, made up by species with an easy-to-decompose litter that would as well stimulate soil microbial activity and biomass. The combined effect would increase nutrient cycling and enhance plant growth in the understory of shrubs, contributing to the high facilitation effect already observed under large *R. sphaerocarpa* shrubs (Pugnaire et al., 1996), in a process that should be similar under other nurse species. Overall, our data on microbial community structure, biomass and activity suggest a complex interaction between soil microbial communities and plant communities with deep effects on ecosystem functioning.

Acknowledgements

This work was funded by the Junta de Andalucía Regional Government (grant P09-RNM-4821) and MICINN (grant CGL2010-17081). F. Bastida is grateful to CSIC and FSE for his JAE-DOC grant and Marie Curie Reintegration Grant (DYNOMIWAS, PERG07-GA-2010-263897). The funding sources did not have any involvement in the study design; collection, analysis and interpretation of data; writing of the report; or in the decision to submit the article for publication. We thank Christian Schöb, Jeff Powell and Amit Khachane for advice in data analysis. We also thank Stéphane Uroz, Christian Schöb and anonymous reviewers for critical reviews of the manuscript. We are grateful to Alex Mira and Stéphane Uroz for advice in preparation of samples for pyrosequencing. We thank Josep Berenguel, Olga Corona and Ester Campanario for field and technical assistance.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.04.018.

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