INTERACCIONES ENTRE PLANTAS Y MICROORGANISMOS DEL SUELO
Consecuencias para la dinámica de comunidades vegetales

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Interacciones entre plantas y microorganismos del suelo: Consecuencias para la dinámica de comunidades vegetales

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RESUMEN

En esta tesis doctoral pretendemos aclarar aspectos fundamentales relacionados con la interacción entre plantas y microorganismos del suelo y sus consecuencias para la dinámica de las comunidades vegetales. Pretendemos comprobar si las comunidades microbianas están determinando interacciones negativas y/o positivas entre plantas; si tras el abandono de tierras dedicadas al cultivo, las plantas y los microorganismos del suelo siguen patrones sucesionales, y si los microorganismos asociados a las especies vegetales son los que determinan su éxito competitivo en una comunidad vegetal. Para evaluar esto realizamos diferentes experimentos en campo y en invernadero apoyados por una serie de análisis físicos, químicos, biológicos y moleculares del suelo y de sus comunidades microbianas.

En el primer capítulo de esta tesis buscábamos separar, dentro de los procesos de facilitación entre plantas, el efecto del suelo y de la copa del arbusto facilitador sobre el desarrollo de la comunidad de herbáceas que vive bajo el mismo. Para ello, en campo (Rambla del Saltador, Tabernas, Almería) seleccionamos individuos de copa pequeña, mediana y grande del arbusto facilitador *Retama sphaerocarpa* (*Retama*) e intercambiamos bloques de suelo tomados bajo cada una de estas copas. Encontramos que existe un fuerte efecto del suelo sobre la diversidad, la biomasa y la abundancia de la comunidad de herbáceas, el cual es independiente e incluso más importante que el conocido efecto de copa.

Con base a estos resultados, en el segundo capítulo buscábamos indagar en mayor profundidad sobre aquellos factores, en especial de la matriz del suelo, que influyen sobre el desarrollo de la comunidad de herbáceas facilitadas. Establecimos un experimento en campo (Rambla del Saltador) usando como factores de estudio los microorganismos, el microhabitat y las propiedades físico-químicas del suelo asociados a los arbustos de *Retama* y a los claros de suelo desnudo entre arbustos. Evaluamos la respuesta en la germinación y desarrollo de especies vegetales que típicamente se desarrollan en claro o bajo *Retama,* y encontramos que los microorganismos del suelo asociados a *Retama* afectan positivamente a la germinación y desarrollo inicial de algunas especies pero negativamente a los de otras. Así mismo, la copa favorece la germinación de las plantas pero tiene un efecto neutro en cuanto a su desarrollo en biomasa, mientras que las propiedades del suelo tienen un efecto positivo sobre la germinación y el desarrollo de las plantas convirtiéndolo en el principal promotor de los procesos de facilitación entre plantas.

Dentro de la dinámica de las comunidades vegetales, otro proceso de relevancia es la sucesión secundaria. En el tercer capítulo buscábamos conocer si los microorganismos del suelo sufren un proceso de sucesión secundaria similar al de las plantas y si existen vínculos entre ambas sucesiones. Para esto, seleccionamos una extensa área de campos abandonados en diferentes fechas desde hace aproximadamente 100 años en el municipio de Tabernas (Almería). Usando sistemas de información geográfica, establecimos una cronosecuencia con 5 fechas de abandono distintas y en cada etapa caracterizamos las comunidades vegetales (cobertura y diversidad) y microbianas del suelo (biomasa, actividad y composición microbiana), así como las propiedades físico-químicas del suelo. Encontramos que los campos abandonados sufren procesos de sucesión secundaria tanto en lo referente a los microorganismos del suelo como a las comunidades vegetales, caracterizados por cambios en las propiedades del suelo, en la biomasa, actividad y composición microbiana, así como por cambios en la cobertura y diversidad de plantas. De esta manera, nuestros resultados sugieren que la sucesión microbiana sigue en el tiempo a la sucesión de plantas.

En esta misma zona, también encontramos áreas que durante estos 100 años de estudio no han presentado ningún cambio en cuanto a la composición y estructura de las comunidades vegetales, es decir, que no han sufrido un proceso de sucesión secundaria. Estas áreas son dominadas por la gramínea *Lygeum spartum (Lygeum)* e impiden la entrada de otras especies típicas de la zona como *Salsola oppositifolia (Salsola).* En el cuarto capítulo pretendíamos profundizar en las razones que han llevado a esta detención de la sucesión secundaria, determinando si la habilidad competitiva de esta especie está vinculada a las comunidades microbianas del suelo, a las propiedades del suelo o a estrategias de la planta a lo largo de su etapa de desarrollo. Bajo condiciones controladas, realizamos 3 experimentos donde usamos semillas, plantas jóvenes o plantas adultas, respectivamente, de *Lygeum* y *Salsola* creciendo en interacción intra o inter-específica, con suelos procedentes de bajo la copa de *Lygeum* o *Salsola* y con o sin microorganismos. Encontramos que los microorganismos de los suelos pertenecientes a *Lygeum* facilitan el desarrollo de *Salsola* tanto en estado juvenil como en estado adulto, pero las semillas de *Lygeum* germinan mucho más rápido que las de *Salsola,* lo que sugiere una rápida ocupación del espacio por parte de *Lygeum*. Esto sumado a su forma de crecimiento clonal, la convierte en una especie muy competitiva y con una gran dominancia, pudiendo incluso detener el avance de la sucesión secundaria.

La información obtenida con el desarrollo de esta tesis es relevante de cara a realizar predicciones de los efectos del cambio global en ecosistemas semiáridos ya que, al incluir tanto el estudio de comunidades vegetales como microbianas, proporciona un mejor conocimiento de los cambios funcionales y estructurales que pueden ocurrir en dichos ecosistemas. Esto permitiría anticipar las respuestas ante los cambios ambientales y, por tanto, mejorar su gestión y conservación.

INTRODUCCIÓN GENERAL

La biodiversidad global está siendo sometida a una tasa de cambio sin precedentes (Pimm et al. 1995) como respuesta a los múltiples factores antropogénicos que inciden en el medio ambiente (Vitousek 1994). Entre los principales vectores que afectan el funcionamiento y la biodiversidad de los ecosistemas se destacan en orden, el cambio de uso del suelo y el cambio climático (Sala et al. 2000). Uno de los más importantes cambios en el uso del suelo ha tenido lugar en áreas dedicadas a la agricultura. La intensificación de los cultivos y la severa desertificación relacionada con el calentamiento global y la sequía, han incrementado el abandono de tierras en zonas áridas (Zhao et al. 2005, Lasanta 2012). Los ecosistemas áridos comprenden cerca del 41% de la superficie terrestre y son habitados por más del 38% de la población mundial (Reynolds et al. 2007), por lo que la degradación de tierras afecta aproximadamente a más de 2500 millones de personas en el mundo, cifra que aumentará en el futuro cercano debido al cambio climático y al rápido crecimiento poblacional (MEA 2005, FAO 2010). Por otra parte, el clima y la composición de la atmósfera están cambiando rápidamente, registrándose aumentos significativos en la temperatura global y la concentración de dióxido de carbono que desencadenan alteraciones en el ciclo hidrológico global y cambios en los patrones regionales de precipitación (IPCC 2007).

Las interacciones planta-planta son una parte principal de los mecanismos que gobiernan la respuesta de las especies y comunidades vegetales a estos cambios ambientales (Brooker 2006); pueden ser positivas o negativas, y el balance neto de la interacción será de facilitación (si es positivo) o de competencia (Armas and Pugnaire 2005). Aunque por mucho tiempo la teoría ecológica y los modelos de interacción entre plantas han estado centrados en la competencia, existe gran evidencia experimental sobre las interacciones positivas entre plantas (Pugnaire et al. 1996b, Callaway and Pugnaire 2007, Brooker et al. 2008), las cuales toman gran relevancia en zonas de elevado estrés ambiental (Callaway et al. 2002) tales como los ecosistemas áridos (Tirado and Pugnaire 2005).

Bajo las extremas condiciones ambientales de estos ecosistemas, el efecto nodriza, un clásico ejemplo de facilitación donde una especie facilita el establecimiento y crecimiento de otras mediante el mejoramiento de las condiciones físicas bajo su copa (Flores and Jurado 2003, Callaway 2007), es un mecanismo clave para mantener la biodiversidad global, ya que promueve tanto la diversidad de especies como el aumento de la productividad del sistema (Callaway 1995, Pugnaire et al. 1996a, Pugnaire et al. 1996b, Brooker et al. 2008). Se ha visto que plantas nodriza,

como es el caso de *Retama sphaerocarpa* (L) Boiss., protegen a las plantas beneficiarias de los altos niveles de irradiación y temperatura (Moro et al. 1997, Yang et al. 2009, Jankju 2013), contribuyen al aumento de la disponibilidad hídrica por acción de la sombra o del levantamiento hidráulico (Zou et al. 2005, Prieto et al. 2012) e incrementan la disponibilidad de nutrientes debido a la acumulación de hojarasca (Rodríguez-Echeverría and Pérez-Fernández 2003). Las grandes modificaciones inducidas por las plantas nodriza sobre las propiedades del suelo y las condiciones microclimáticas han sido tradicionalmente identificadas como el principal mecanismo de facilitación en ambientes áridos. Sin embargo, datos recientes han sugerido un posible papel de los microorganismos del suelo en este proceso (Hortal et al. 2013, Rodríguez-Echeverría et al. 2013), que se ha explorado para las asociaciones de micorrizas (Van Der Heijden and Horton 2009, Van der Putten 2009), pero que se ha obviado para las comunidades de bacterias a pesar de la fuerte influencia que tienen para el desarrollo de las plantas.

La mayoría de los modelos actuales consideran la disponibilidad de luz y nutrientes como la fuerza que gobierna las interacciones entre plantas (Grime 1983, Tilman 1988) y, por tanto, la composición y estructura de las comunidades vegetales. Sin embargo, las interacciones entre plantas y organismos del suelo pueden tener consecuencias importantes para la dinámica de la vegetación (Van der Putten and Peters 1997) porque, aunque la naturaleza de las interacciones suelo-planta es todavía en gran parte desconocida, se sabe que la identidad de las especies vegetales influye notablemente en la comunidad de organismos del suelo, y que éstos dejan un legado que condiciona el desarrollo posterior de la comunidad vegetal. Este legado vendría mediado por la acumulación en la rizosfera de microorganismos cuyos efectos pueden ser positivos (Bever 2003, Rodríguez-Echeverría et al. 2013) o negativos (Klironomos 2002) afectando al resultado de la interacción con otras especies de plantas (Van der Putten and Peters 1997, Bonanomi and Mazzoleni 2005).

Otro mecanismo que promueve la biodiversidad y en el que los microorganismos pueden también jugar un papel importante, es la sucesión secundaria que ocurre tras una fuerte perturbación como el cambio de uso del suelo o el abandono de tierras dedicadas al cultivo. Los fenómenos de sucesión secundaria se desarrollan a lo largo del tiempo tendiendo a la restauración natural de los ecosistemas perturbados. El estudio de la sucesión secundaria en ecosistemas áridos ha estado principalmente centrado en la dinámica de las comunidades de plantas, con escasa atención a las comunidades microbianas del suelo. En otros ecosistemas como dunas arenosas de interior se han observado cambios en las comunidades microbianas a lo largo del tiempo (Tarlera et al.

2008). Así mismo, tras la restauración de ecosistemas forestales se han observado tendencias sucesionales en la comunidad de bacterias (Banning et al. 2011), mientras que otros autores (Kuramae et al. 2011) encontraron cambios en las comunidades microbianas pero únicamente en etapas tempranas de sucesión. Aun así, en comparación con las comunidades de plantas, hay una escasa evidencia que demuestre patrones sucesionales en comunidades microbianas y menos aún sobre su cambio respecto a la composición de las comunidades de plantas. Sin embargo, cambios en los microorganismos del suelo pueden resultar en efectos lineales sobre la tasa de crecimiento de las plantas (Bever 2003), y aunque la escasa evidencia experimental existente ha sido principalmente desarrollada bajo condiciones controladas, los datos disponibles sugieren que las comunidades microbianas pueden jugar un importante papel en la sucesión secundaria. El escaso conocimiento actual nos muestra que, dependiendo de la posición sucesional de las especies de planta implicadas, las interacciones negativas planta-suelo pueden promover el avance sucesional favoreciendo el reemplazo de especies (Klironomos 2002, Bever 2003, Bonanomi et al. 2005, Kardol et al. 2006) o, por el contrario, retroalimentaciones positivas pueden conducir a la dominancia de una especie, reduciendo la tasa de sucesión (Bever 2003, Kardol et al. 2006).

Parece probado que las plantas pueden modificar la estructura de la comunidad microbiana asociada a la rizosfera (Chanway et al. 1991, Kowalchuk et al. 2002, Hortal et al. 2013) y que estos cambios influyen en su propio funcionamiento (Bever 1994, Bever et al. 1997), lo que puede hacer que aumente o disminuya su tasa de crecimiento. No obstante, el papel de tales interacciones en la dinámica temporal de la comunidad vegetal ha recibido poca atención (Bardgett et al. 2005, Kardol et al. 2006). No está claro, por ejemplo, cómo cambios específicos en la comunidad de microorganismos del suelo contribuyen a la dinámica de la comunidad vegetal y sus tasas de reemplazo sucesional, o cuánto tiempo permanecen operacionales estos efectos (Van der Putten 2003). Lo que sí parece claro es que cuando la interacción suelo-planta reduce la capacidad competitiva de una especie pionera, también afecta de forma decisiva el resultado de competencia con especies sucesionalmente más tardías (Bever 2003), de forma que la influencia de las especies pioneras sobre el suelo influye en la dinámica posterior de la comunidad vegetal.

Las dificultades tradicionalmente asociadas al estudio de los microorganismos han hecho que exista un gran desconocimiento sobre su influencia en la dinámica de las comunidades vegetales. Recientemente, los avances asociados al uso de técnicas moleculares han facilitado acceder a esta caja negra y comprender mejor cómo funcionan las comunidades microbianas. Está

demostrado, por ejemplo, que la diversidad y composición de las comunidades microbianas del suelo influyen en el crecimiento y la productividad de las plantas (Wardle et al. 2004) y en la abundancia de especies (Klironomos 2002, Hortal et al. 2013). El efecto de los microorganismos del suelo en las comunidades de plantas es el resultado combinado de la interacción entre diferentes grupos microbianos así como entre plantas y organismos patógenos, parásitos, mutualistas y descomponedores (Van der Putten and Peters 1997, Kulmatiski and Beard 2008, Rodríguez-Echeverría et al. 2013). Por tanto, la integración de las comunidades microbianas del suelo en el marco de las interacciones entre plantas y de la sucesión secundaria nos debería proporcionar una imagen más realista de estos procesos.

OBJETIVOS GENERALES

Explicar aspectos fundamentales relacionados con la interacción entre plantas y microorganismos del suelo y sus consecuencias para la dinámica de las comunidades vegetales.

Específicamente, los objetivos que se abordarán en los diferentes capítulos de esta tesis son:

- Determinar si en los procesos de facilitación en ecosistemas semiáridos, los suelos de las especies nodriza afectan la productividad y diversidad de las plantas a las que benefician, en un proceso independiente del efecto de copa.
- Diferenciar el efecto de los microorganismos del suelo, del microhabitat y de las propiedades del suelo en los procesos de facilitación en ecosistemas semiáridos.
- Evaluar si las interacciones entre plantas, suelo y microorganismos del suelo definen la sucesión secundaria en ecosistemas semiáridos (capítulo 3).
- Definir si la interrupción de la sucesión es el resultado de la interacción entre plantas y microorganismos del suelo.

1. SOILS UNDER NURSE SPECIES AFFECT PLANT PRODUCTIVITY AND DIVERSITY INDEPENDENTLY OF THE CANOPY EFFECT

Lozano Y.M., Hortal S., Armas C. and Pugnaire F.I. Soils under nurse species affect plant productivity and diversity independently of the canopy effect. *In preparation*.

1.1. ABSTRACT

The nurse plant effect has been mainly explained by the positive effect of the canopy on the amelioration of microclimatic conditions while the direct effect of the complex soil matrix on this facilitation process has received less attention. We examined the effects of both canopy and soil of the nurse shrub *Retama sphaerocarpa* on changes occurring in aboveground biomass, abundance, diversity and richness of the understorey plant community. We hypothesized that, in addition to the positive effect of the canopy associated to size, there will be a strong and independent effect of soil on these changes. For this, we collected three soil blocks 20 cm x 20 cm x 15 cm (length, width, depth) under the canopy of eighteen *Retama* shrubs grouped into three different canopy sizes (small, medium, large) and randomly distributed it back into the extraction holes under the shrubs, such that each shrub had three soil blocks, each of them from a different soil origin (collected under small, medium or large shrubs). We found that both soil origin and canopy size of the nurse shrub independently affected the total aboveground biomass and plant diversity. Regarding canopy effect, both biomass and diversity increased in communities growing under large *Retama* canopies promoted by a decrease in irradiance and temperature related to deeper shade. However, regarding soil origin, we recorded an inverse relation between them so that aboveground biomass increased and plant diversity decreased under soils from large *Retama* shrubs. The positive effects of soil organic matter and the increase in nutrients added to soil microbial and activity could promote a higher biomass under soils from large *Retama* shrubs. On the other hand, factors linked to low soil seed bank diversity due to limitations in seed dispersal, temporally variable environment or positive plant interactions of some species with soil microorganisms or nutrients that could cause competitive exclusion may explain the reduced plant diversity observed in soils from under large shrubs. The effect of soil origin on biomass and abundance per species was larger than the canopy effect, thus playing a key role in determining the structure of understorey communities. Overall, complex processes that occur in the soil matrix need more attention to increase our understanding of the mechanisms that regulate facilitation among plants.

1.2. INTRODUCTION

Although models for plant interactions have mainly emphasized competition (Went 1973, Tilman 1982, Aerts 1999, Keddy 2001, Grime 2002), plant species may also provide benefits to neighbors through facilitation processes (Callaway 1995, 2007, Callaway and Pugnaire 2007). Facilitation, defined as the interaction where one plant species benefits another neighboring species in terms of germination, survival or growth, is a well-known phenomenon described in many ecosystems around the world (Callaway 2007). A particular case of facilitation is the nurse plant effect (Niering et al. 1963), in which a species develops several mechanisms to facilitate the establishment and growth of others species beneath their canopy (Pugnaire et al. 1996a, Callaway 2007). The nurse effect has great relevance in extreme conditions, such as arid environments (Flores and Jurado 2003), where nurse plants mitigate high temperatures and irradiance (Moro et al. 1997, Yang et al. 2009, Jankju 2013) through shade and increased water availability due to shading or hydraulic lift (Zou et al. 2005, Prieto et al. 2012). Nurse plants also increase nutrient availability and cycling through the accumulation of litter and increased decomposition due to higher soil moisture (Rodríguez-Echeverría and Pérez-Fernández 2003, Armas et al. 2012) and microbial activity and biomass (Hortal et al. 2013). All these mechanisms promote the establishment of a plant community under the canopy of nurse plants including species that would otherwise be absent without facilitator species (Armas et al. 2011).

The nurse effect has been mainly linked to plant age and canopy size and its buffering of physical conditions underneath (Pugnaire et al. 1996a, Pugnaire et al. 1996b, Moro et al. 1997, Callaway 2007). As a consequence, understorey plant diversity and productivity increases with increasing canopy size of the nurse (Pugnaire et al. 1996b, Schöb et al. 2013). Canopies also affect soil chemical and physical properties underneath, increasing fertility under the canopy of nurse shrubs compared to gaps (Pugnaire et al. 2004). Although changes promoted by nurse plant canopies on soil properties and microclimatic conditions have been recognized as the main mechanism behind the facilitative effects of nurses in arid conditions, the role of soil has been scantly addressed and mostly considered a consequence not independent from canopy effects. Recent reports have shown, however, that soil biota changes in composition, biomass and activity with shrub age (Hortal et al. 2013) suggesting a potential role of soil microbial communities in the facilitation process (Rodríguez-Echeverría et al. 2013). Moreover, the complex soil matrix under a nurse plant species has intrinsic properties that may directly affect the development of understorey communities. For instance, soil texture, soil organic matter contents and aggregation have an

effect on water dynamics and soil oxygenation, affecting the availability of dissolved nutrients and root development (Porta-Casanellas et al. 2003, Rucks et al. 2004).

In an experiment in the field we addressed these points trying to tell apart the effects of soil origin (collected under small, medium or large nurse shrubs) and canopy (small, medium or large canopies) of the facilitator shrub species *Retama sphaerocarpa* on the plant community growing underneath this shrub species.

1.3. OBJECTIVES

Our main objectives were 1) to determine whether there is an effect of soil on plant communities growing under *Retama sphaerocarpa*, and whether this effect is independent of the canopy; and 2) to assess the contribution of both soil origin and canopy size on plant diversity and productivity of the understorey plant community.

1.4. HYPOTHESES

We hypothesized that, in addition to the well-known positive effect of the canopy associated to size, there will be a strong effect of soil independent from the canopy influencing understorey communities. We expected an increase in plant productivity, diversity and richness under the canopy of large *Retama* shrubs compared to small shrubs, an in soils collected under large *Retama* shrubs compared to soils collected under small shrubs, as well as a strong and independent effect of soil on plant biomass and abundance per species.

1.5. METHODS

1.5.1. Field site and species

The field site was located in the Rambla del Saltador, a dry valley on the southern slope of the Sierra de los Filabres mountain range, Almería, southeast Spain (37º08´N, º22´W; 630 m altitude). The climate is semiarid Mediterranean, with a mean annual temperature of 16º C and mean annual rainfall of 300 mm (Puigdefabregas et al. 1999). The soil is of alluvial origin, with a loamy sand texture, characterized by low values of water holding capacity, electrical conductivity, cation exchange capacity, organic matter, nutrient concentrations, and a moderate alkaline pH (Pugnaire

et al. 1996b, Puigdefábregas 1996). The plant community is dominated by the leguminous species *Retama sphaerocarpa* (L.) Boiss. (*Retama* hereafter, Fig. 1.1), a tall shrub with an open canopy with photosynthetic stems and a dimorphic root system with shallow lateral roots and tap roots that can reach 30 m deep (Haase et al. 1996). Thanks to its root system, shrubs can lift water from deep, wet soil layers and release it to shallow, dry soil layers via hydraulic lift (Prieto et al. 2010), contributing to its nurse effect (Prieto et al. 2012). *Retama* facilitates the establishment and growth of many other plant species under their canopy (Pugnaire et al. 1996b) compared to gaps among shrubs, having an overall positive impact on local community diversity in semiarid environments (Armas et al. 2011). Plant biomass and species richness under *Retama* increase with shrub size and age (Pugnaire et al. 1996b).

Fig.1.1. Field site in the Rambla del Saltador dominated by the leguminous shrub *Retama.* Patches of vegetation underneath *Retama* contrasted with almost bare soil in gaps among shrubs.

1.5.2. Experimental design

Eighteen *Retama* shrubs grouped into three different canopy sizes (small, medium, large) were randomly selected in a ~2 ha homogeneous plot. Mean shrub height (±SE) in each size category was 1.04±0.06; 2.12±.0.08; 3.29±0.22 m, and mean projected canopy area (calculated as the area of an ellipse; [Pugnaire et al. 1996b]) was 0.95±0.12; 7.19±0.66; 37.43±3.13 m^2, respectively (Hortal et al. 2013). Shrub height and canopy area were significantly different among size classes and corresponded to different shrub ages, *i.e.*, <10, 10-25, >25 years old, respectively, according to data from the same field site (Pugnaire et al. 1996b, Hortal et al. 2013). In November 2010, before the onset of the germination and growth of the herbaceous community, three soil blocks 20 cm x 20 cm x 15 cm (length, width, depth) were collected using a square shovel to maintain its structure at the northern aspect of each shrub and at an intermediate distance between the trunk

and the edge of the canopy, where microclimatic conditions, soil chemical fertility and soil seed bank lead to high levels of productivity and diversity (Moro et al. 1997).

The 54 soil blocks were randomly distributed back into the extraction holes under the 18 shrubs (six per canopy size) such that each shrub had three soil blocks, each of them from a different soil origin, i.e. collected under small (SS), medium (MS) or large shrubs (LS) (Fig. 1.2). Soil properties, soil microbial biomass and activity, and community composition under the 18 shrubs were characterized at sampling time (Hortal et al. 2013). Soil blocks were placed into plastic containers to avoid mixing with adjacent soil, with small punctuations in the bottom to allow for drainage (Fig. 1.3). Therefore, seeds from three different soil origins emerged and grew under three different canopy sizes (referred as SC, MC and LC for small, medium and large canopies). This experimental design allows separating microclimate from soil and seed bank effects as causes of changes in diversity and productivity of plant communities under *Retama* shrubs. All soil blocks were covered with a wire mesh to prevent seed predation and grazing. In April 2011, six months after establishment and at peak of plant growth, plants were harvested, sorted by species, and counted. Plant material was dried at 70ºC during 48 h and weighted to obtain aboveground dry mass. Plant diversity was assessed through the Shannon's diversity index. All taxa were identified to the species level.

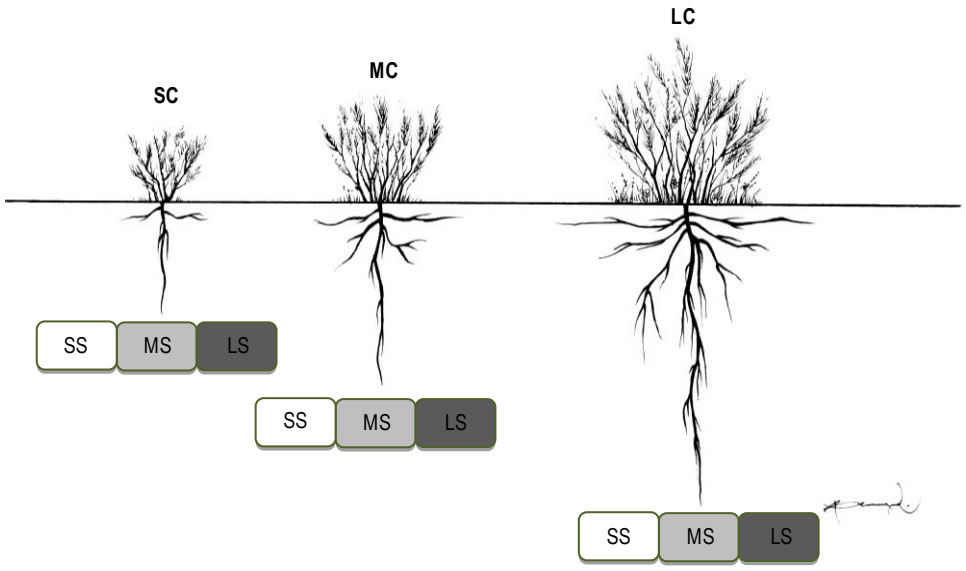

Fig. 1.2. Experimental design. Soil blocks collected under small (SS), medium (MS), and large (LS) *Retama* shrubs were randomly assigned to grow under small (SC), medium (MC) and large (LC) shrubs. Drawing by Josep Berenguel.

Fig. 1.3. Field experiment. Soil blocks translocated under a large *Retama* shrub (a), and detail of the experimental unit: a plant community growing in a soil block in a plastic container (b).

1.5.3. Statistical analyses

The experimental design was a split plot factorial where each shrub (small, medium and large) was the main plot, and each soil block (from under small, medium and large *Retama* shrubs) was the sub-plot. Canopy size and soil origin were considered as fixed factors and main plots and subplots where included as random effects. Aboveground mass and plant diversity were analyzed using linear mixed models. Violations of normality and/or homoscedasticity were checked and the best model was selected by comparison using the Akaike information criterion (Akaike 1974). Species richness was analyzed using Generalized Linear Mixed Models with a poisson distribution, a logistic link function and the maximum likelihood (ML) criterion. Significance was established at $p<0.05$. Post-hoc comparisons were performed using Fisher's LSD test. We also carried out a principal component analysis (PCA) of the abundance and aboveground biomass per species and only on those species that have a minimum frequency ≥ 20% (i.e., in at least 10 soil blocks). Differences were evaluated through NPMANOVA with 9999 permutations using Past v 2.12 software (Hammer et al. 2001). The rest of the data was analyzed with the InfoStat software package (Di Rienzo et al. 2013).

1.6. RESULTS

1.6.1. Aboveground productivity

Understorey plant biomass increased with canopy size independently from soil origin (Table S1.1). Total aboveground biomass was three times larger in blocks growing under large shrubs than in blocks growing under small shrubs (Fig. 1.4a, Table S1.1). Soil origin also had a significant effect

on total aboveground biomass, being higher in soils collected under large and medium *Retama* shrubs than in soils collected under small *Retama* shrubs (Fig. 1.4b, Table S1.1).

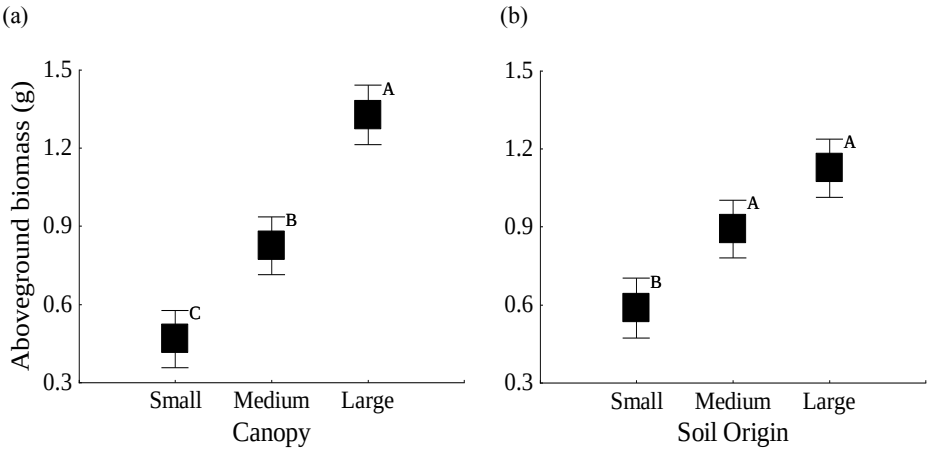

Fig. 1.4. Total aboveground biomass of plants growing under *Retama* shrubs of different canopy size (a), and with soils collected from different *Retama* canopy sizes (b). Different letters indicate significant differences among treatments after post-hoc comparisons at a significance level of 0.05.

Regarding aboveground biomass per species, the ordination analysis showed a positive correlation between the aboveground biomass of several species such as *Avena sterilis, Bromus rubens, Brachypodium distachyon, Lagurus ovatus and Geranium molle* with soils collected under large *Retama* shrubs, while biomass of *Minuartia hibrida*, *Rumex bucephalophorus*, *Medicago minima, Paronychia argentea, Sisymbrium erysimoides* and *Stipa capensis* were positively correlated with soils obtained under small or medium *Retama* shrubs (Fig. 1.5, Table S1.2). We only found significant differences in aboveground biomass per species in relation to soil origin, so that biomass of species grouped in soils from large *Retama* shrubs was significantly different to that found in soils from small or medium *Retama* shrubs ($F_{2,51}$ = 1.97, p<0.001, NPMANOVA, Table 1.1). No differences were found regarding canopy size or interaction between soil origin and canopy size ($F_{2,51}$ = 1.37, p=0.07; $F_{4,45}$ = 0.73, p = 0.96, respectively, NPMANOVA).

	Aboveground biomass			Abundance		
Soil Origin	Small (SS)	Medium (MS)	Large (LS)	Small (SS)	Medium (MS)	Large (LS)
Small (SS)		ns	**2.31***		ns	**3.11***
Medium (MS)			**2.23***			**2.87***
Large (LS)						

Table 1.1. *F* values of pairwise comparisons of Bray-Curtis similarity index of aboveground biomass and abundance per species using one-way NPMANOVA analysis. Asterisks and bold values indicate significant differences among soil origin after post-hoc comparisons at a significance level of 0.05. ns= non significant; n=9999 permutations.

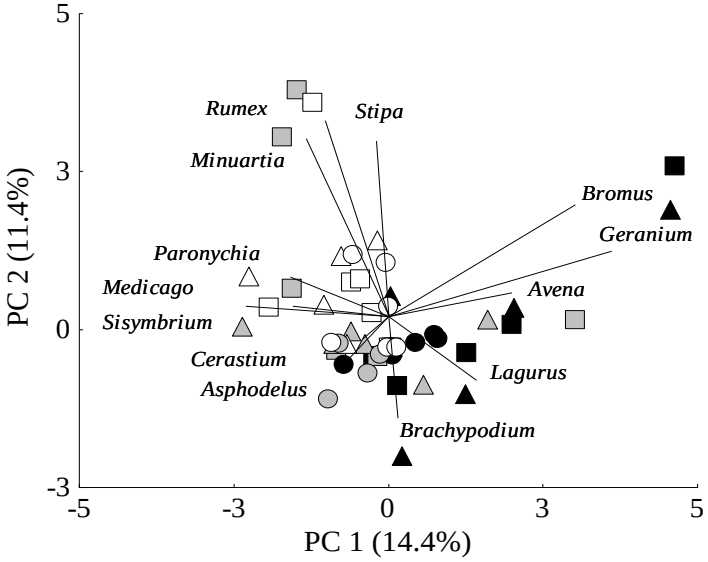

Fig. 1.5. Principal component analysis of aboveground biomass per annual/herbaceous plant species (n=54 soil blocks). Colours indicate soil origin from small (white), medium (grey) and large (black) *Retama* shrubs. Symbols indicate small (circle), medium (triangle) and large (square) *Retama* canopy size.

1.6.2. Species abundance

Soil origin influenced the number of individuals per species in soil blocks. PCA ordination based on species abundance rendered a clear separation of soil blocks along the first axis (Fig. 1.5). Plant community composition -in terms of plant abundance per species- grouped by soil origin rather than by canopy size (Fig. 1.6) so that the number of individuals per species in soils collected under large *Retama* shrubs were significantly different to soils collected under small or medium *Retama* shrubs ($F_{2,51}$ = 2.30, p<0.001, NPMANOVA; Table 1.1). The ordination analysis showed a positive correlation between several species such as *Minuartia hibrida*, *Rumex bucephalophorus*,

Medicago minima, Paronychia argentea, Sisymbrium erysimoides and *Stipa capensis* with soils collected under small or medium *Retama* shrubs, while*, Avena sterilis, Bromus rubens, Brachypodium distachyon, Lagurus ovatus and Geranium molle* were negatively correlated with these soils (Fig. 1.6, Table S1.2). Canopy size or interaction between soil origin and canopy had no significant effects on plant communities in terms of plant abundance per species ($F_{2,51}$ = 1.38, p = 0.08; $F_{4,45}$ = 0.76, p = 0.91, respectively, NPMANOVA).

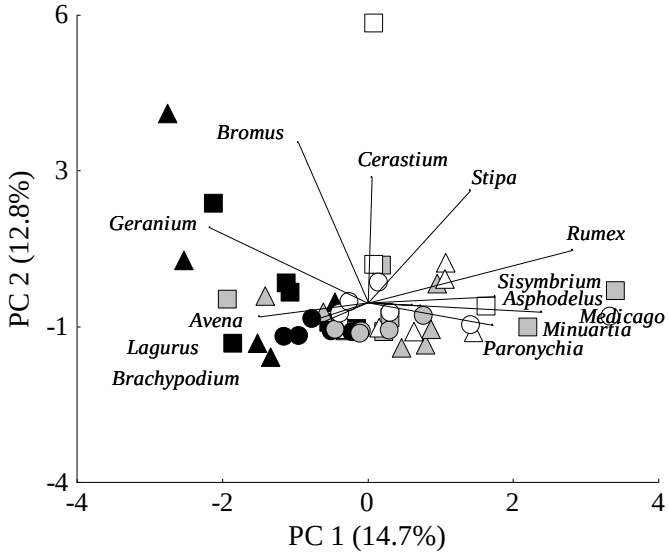

Fig. 1.6. Principal component analysis of plant community composition based on plant abundance (number of individuals per species). Colours indicate soil origin from small (white), medium (grey) and large (black) *Retama* shrubs. Symbols indicate small (circle), medium (triangle) and large (square) *Retama* canopy size.

1.6.3. Plant diversity and species richness

Similarly to aboveground biomass, plant diversity (Shannon index) was independently affected by canopy size and by soil origin with no significant interaction between them (Table S1.1). Plant diversity was higher under large *Retama* canopies than under small or medium canopies (Fig. 1.7a), as well as in soils collected from small and medium *Retama* than in soils from large *Retama* shrubs (Fig. 1.7b).

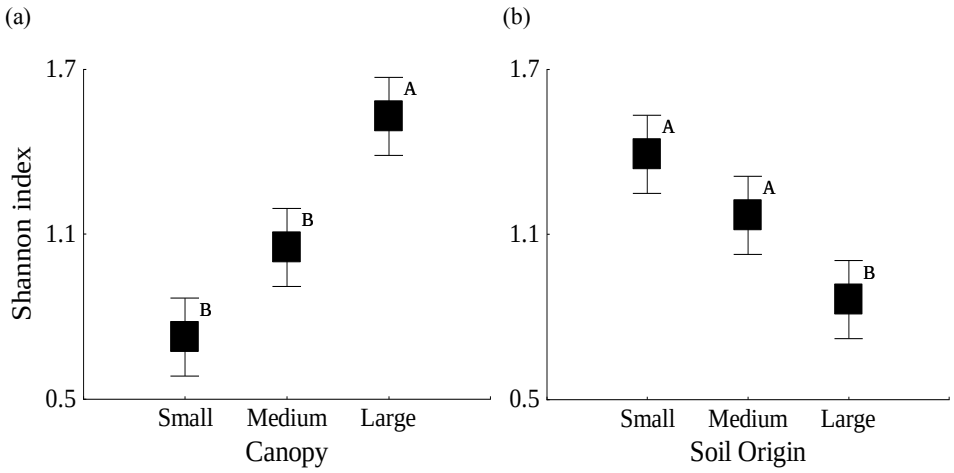

Fig. 1.7. Diversity of communities growing in soil blocks placed under *Retama* shrubs of different canopy size (a) and collected from under different *Retama* shrubs (b). Different letters indicate significant differences among treatments after post-hoc comparisons at a significance level of 0.05.

Species richness was affected by the interaction between canopy size and soil origin (Table S1.1), being highest under large *Retama* canopies for any soil origin and in particular in soils collected under small and medium *Retama* shrubs. Richness in blocks from small and from large *Retama* shrubs followed the same pattern being lower in blocks placed under small and medium *Retama* canopies than under large canopies. In soils from medium *Retama* shrubs, richness was lowest under small *Retama* canopies, intermediate under medium canopies and highest under large *Retama* canopies. Species richness was higher in soils collected from small and medium *Retama* shrubs than in soils from large *Retama* shrubs except under small *Retama* canopies where soils from large and medium *Retama* shrubs showed similar richness (Fig.1.8, Table S1.3).

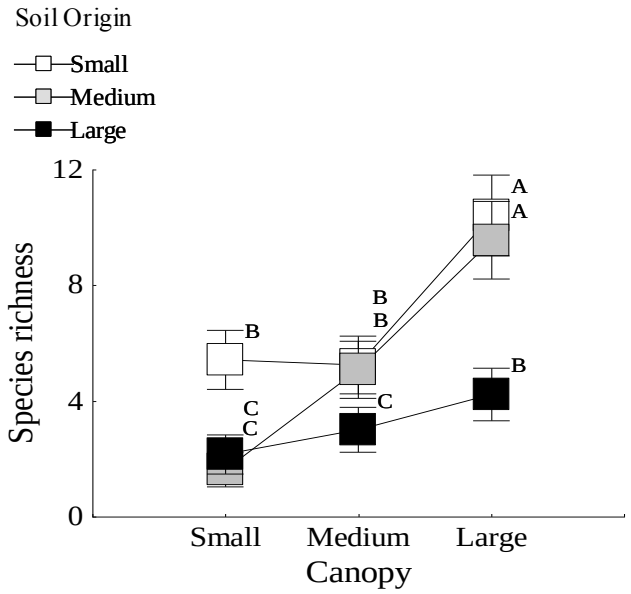

Fig. 1.8. Species richness of soil blocks collected under different *Retama* shrubs and growing under *Retama* shrubs of different canopy size. Different letters indicate significant differences among treatments after post-hoc comparisons at a significance level of 0.05.

1.7. DISCUSSION

Both soil origin and canopy size of the nurse shrub *Retama sphaerocarpa* affected, but with independent effects on, total aboveground biomass and plant diversity. Total aboveground biomass increased in communities growing under large *Retama* canopies and in soils collected from under large *Retama* shrubs; however, plant diversity and species richness increased under large *Retama* canopies but decreased in soils collected from large *Retama* shrubs. Soil origin showed an overall larger effect than canopy size on species plant abundance and biomass.

1.7.1. Aboveground productivity and species abundance

We found an increase in plant productivity parallel to the increase in canopy size related to deeper shade and overall better microclimatic conditions under the shrub canopy (Pugnaire et al. 1996b, Moro et al. 1997, Callaway 2007). Bigger canopies promote a decrease in temperature and irradiance at soil level (Moro et al. 1997, Yang et al. 2009, Jankju 2013) having a positive effect on understorey plants. Many species from arid environments reach their maximum photosynthetic rates at photosynthetically active radiation (PAR) levels far below the general natural maximum, ~2000 μmol m^{-2} s^{-1} (Callaway and Pugnaire 2007). Extreme temperatures typical of arid environments limit plant productivity through negative effects on plant tissues (Fischer and Turner

1978) and lead to greater water lost to transpiration as vapor pressure deficits increase with temperature (Anderson 1936, Day 2000). *Retama* canopies decrease understorey temperatures and daily amplitudes (Pugnaire et al. 2004) as well as soil water evaporation (Domingo et al. 1999), with an overall positive effect on aboveground biomass.

On the contrary, the poor aboveground biomass observed under small *Retama* shrubs was likely due to the fact that their canopies do not have a shade as deep as large *Retama* canopies and thus microclimatic conditions may be more similar to the dry, open spaces. Many species which showed a suitable development with low shade, such as *Stipa capensis* or *Paronychia argentea* (Pugnaire et al. 1996b, Boeken et al. 2004), increased their biomass when moved to better microclimatic conditions such as medium and large *Retama* canopies. Previous studies under *Retama* have also found higher mass of herbs under large *Retama* canopies than under small *Retama* canopies also linked to a different plant composition (Pugnaire et al. 1996b, Schöb et al. 2013).

Although shade is considered a major facilitation factor in arid ecosystems (Moro et al. 1997, Holmgren 2000), our results showed that soil effects on aboveground biomass of herbaceous plant communities was independent of canopy effects. Total aboveground biomass was higher in soils blocks collected under large *Retama* than in soils collected under small *Retama* shrubs. A recent survey in the same *Retama* shrubs has shown an increase in soil nutrients, soil microbial biomass and activity, as well as different soil microbial composition under large *Retama* than under small or medium *Retama* shrubs (Hortal et al. 2013). This, added to the increase in soil moisture under large shrubs (Pugnaire et al. 2004) and the increase in organic matter and nitrogen with age (Pugnaire et al. 1996b), generate better soil conditions that promoted the highest total aboveground biomass of understorey plants in soils from under large *Retama* shrubs.

Aboveground biomass and abundance per species were linked to soil origin rather than canopy size. We observed that plant communities grown in soils from large *Retama* shrubs were clearly different to plant communities grown in soils from medium and small *Retama* shrubs. For example, *Sisymbrium erysimoides,* a species typical from low shade microhabitats (Pugnaire and Lázaro 2000) showed similar aboveground biomass under any *Retama* canopy size, but showed higher aboveground biomass and abundance in soils from under small *Retama* than in soils from large *Retama* shrubs; while *Avena sterilis* or *Geranium molle*, species typical from understorey communities under *Retama* (Armas et al. 2011*)* were strongly facilitated by soil from large *Retama*

shrubs rather than *Retama* canopies. The soil origin effect could be explained because the presence of *Retama* strongly modifies soil resources underneath its canopy (Pugnaire et al. 2004), and soil characteristics such as the abundance and composition of soil seed bank (Aguiar and Sala 1999, Díaz-Villa et al. 2003). It has positive effects on soil water holding capacity, soil temperature (Moro et al. 1997, Pugnaire et al. 2004), porosity, aeration and buffering soil pH (Porta-Casanellas et al. 2003) through the accumulation of soil organic matter. The presence of *Retama* shrubs is also linked to an increase in soil nutrients (Rodríguez-Echeverría and Pérez-Fernández 2003), soil microbial biomass and activity (Hortal et al. 2013) as well as an increase in clay content (Sessitsch et al. 2001, Porta-Casanellas et al. 2003, Chapter II). All these factors together could explain the observed increase in both aboveground biomass and abundance of species in understorey plant communities irrespective of shrub canopy size.

1.7.2. Plant diversity and species richness

Similarly to different studies that have shown a positive relationship between canopy size and plant diversity or species richness (Pugnaire et al. 1996b, Maestre and Cortina 2005) we also found that plant diversity and richness significantly increased under large *Retama* canopies. However, we found significantly lower diversity and richness in soil blocks collected from under large *Retama* shrubs than in soil from under other origins. Our results showed that plant diversity was independently affected by canopy size and soil origin while species richness was affected by the interaction among them.

The increase in plant diversity and species richness under large *Retama* shrubs was linked to the amelioration of microclimatic conditions under large *Retama* canopies. Under these large canopies there are gradients of radiation and temperature that interact with gradients of litter accumulation and nutrient distribution (Moro et al. 1997, Amarasekare and Possingham 2001) promoting microhabitat heterogeneity and thus niche differentiation (Lambers et al. 2004). This phenomenon may facilitate seed germination of more species and, thus, plant diversity and species richness (Pugnaire et al. 1996b, Moro et al. 1997, Maestre and Cortina 2005). We suggest that seeds from greater number of plant species might find their germination optimum under the more diverse microhabitat conditions beneath large *Retama* shrubs leading to higher number of species that germinate here compared to other more homogeneous microhabitats (smaller shrub canopy sizes). In accordance, our results show that seeds of species such as *Desmazeria rigida, Diplotaxis virgata or Erodium cicutarium* were present in soils from small or medium *Retama*

shrubs, which were much more diverse and species rich than soils from large *Retama* shrubs, but they only germinated under large *Retama* shrubs. The positive canopy effect was also evident in species richness of soils from medium *Retama* shrubs, which, unlike plant diversity, significantly decreased under the hostile conditions of small *Retama* canopies compared to larger canopies.

Parallel to the increase in canopy size, the increase in microhabitat heterogeneity promoted seed germination of both rare and abundant species and therefore a lower species evenness under large canopies. Rare species such as *Limonium lobatum, Valantua muralis*, *Calendula tripterocarpa* or *Galium spurium* as well abundant species such as *Cerastium dichotomum, Paronychia argentea or Medicago minima* grew under better microhabitat conditions provided by medium and large *Retama* shrubs. This low species evenness in soils from medium and large *Retama* shrubs growing under medium and large *Retama* canopies was opposite to the observed high species evenness in the same soils growing under small canopies. The more homogenous microhabitat under small canopies promoted low species richness but high species evenness explaining the similar plant diversity between small and medium *Retama* canopies.

Although we found high total aboveground biomass in soils collected under large *Retama,* plant diversity and species richness showed an opposite pattern being lowest in soils from large *Retama* shrubs. We expected that the better properties in soils from large *Retama* shrubs (higher soil nutrient content, soil microbial biomass and activity) than in other soil origins (Pugnaire et al. 2004, Hortal et al. 2013), should have promoted high plant diversity and species richness. However, other factors linked to soil seed bank, seed dispersal, temporal climatic variation, conditions or even soil biota may have caused the low levels of plant diversity and species richness in large *Retama* soils.

It has been suggested that species composition of soil seed banks under *Retama* shrubs does not change with shrub age (Pugnaire and Lázaro 2000). However, our results suggest the opposite. We observed that species such as *Hypochaeris glabra, Rumex bucephalophorus* or *Valantia muralis* and other 32 species (62% of total species richness) were not present in any of the soils from large *Retama* shrubs as they did not germinate irrespective of microclimatic conditions (i.e, *Retama* canopy size), whereas they germinated in soils from other origins. This suggests low richness of soil seed bank from soil under large *Retama* shrubs, hostile conditions for seed germination and, probably, positive plant-soil feedbacks with some dominant species. Soil seed bank can sometimes show low similarity with aboveground plant diversity (Díaz-Villa et al. 2003)

since suitable places for seed deposition may not be suitable for seed germination and growth (Rey and Alcántara 2000). However, in our system, the presence of large shrubs strongly modifies seed dispersal patterns (Pulliam 1996, Aguiar and Sala 1999) hindering seed dispersal by wind underneath large *Retama* shrubs. This would lead to a low species diversity and richness of the soil seed bank under large *Retama* shrubs compared to a high diversity and richness in soils under small and medium *Retama* shrubs that would translate into similar observed aboveground diversity patterns.

We observed that some species increased their aboveground biomass and abundance in soils from large *Retama* shrubs suggesting positive interactions with soil microorganisms (Bever 2003, Van der Putten et al. 2013) or with soil nutrients (*e.g.*, nitrophilous species *Avena sterilis* (Ruiz Téllez et al. 2007)) that could cause competitive exclusion of other plant species (Callaway et al. 2002) reducing plant diversity and species richness. Finally, seed germination can also be affected by a temporally variable environment (i.e., dry versus wet years) (Espigares and Peco 1993, Pugnaire and Lázaro 2000). Our results suggest an important role of soil characteristics on seed germination and plant recruitment, being this effect even greater than the canopy effect.

Overall these results suggest a parallel increase in plant productivity and diversity (Tilman et al. 1996) in relation to canopy size. Different microhabitat conditions under large *Retama* shrubs (Moro et al. 1997) may have increased germination of more species and better conditions for plant growth, increasing plant diversity and thus the likelihood that productive species were present (i.e., positive selection) (Loreau and Hector 2001, Lambers et al. 2004). However, our results also showed an inverse relationship between plant productivity and diversity (Brooker and Callaghan 1998) in soils from different origin, which may happen in this nurse system as there is an independent effect of the shrub canopy and soil on plant productivity and diversity. As discussed above, total productivity in soils from large *Retama* increased due to the positive effects of soil organic matter, nutrients, moisture, temperature and biota on productivity. However, limitations in seed dispersal, temporally variable environment or competitive exclusion due to positive plant-soil feedbacks with some species could explain the observed decrease in plant diversity in soils from under large *Retama* shrubs.

1.8. CONCLUSIONS

We conclude that both canopy size and soil origin of the nurse shrub independently affected total aboveground biomass, plant diversity and species richness of the understorey herbaceous community. Total aboveground biomass increased under large *Retama* canopies and in soils from under large *Retama* shrubs but, contrary to our expectations, plant diversity and species richness decreased in soils collected from under large *Retama* shrubs. These results suggest both a parallel and inverse relationship between productivity and diversity due to the independent effects of canopy and soil on them. Nonetheless, soil origin had a larger influence than canopy size on species aboveground biomass and abundance linked to soil seed bank composition, soil nutrients and organic matter and soil biota. There is an array of complex processes that occur in the soil matrix that need more attention to increase our understanding of the mechanisms that regulate facilitation among plants.

1.9. APPENDIX

Effect	Aboveground biomass	Shannon index	Species richness
Canopy	**14.75*****	**8.11*****	**19.46*****
Soil origin	**5.68****	**3.50****	**24.86*****
Canopy*Soil origin	0.60	1.31	**9.27***

Table S1.1. *F* values of the linear mixed model for aboveground biomass, plant diversity (Shannon index) and chi-square values of the generalized mix model for species richness under different treatments. Values in bold denote a significant effect of that factor on the dependent variable (*** $P<0.001$, ** $P<0.01$, and * $P<0.05$).

	Aboveground biomass		Abundance	
Species	E1	E2	E1	E2
Asphodelus tenuifolius	-0.13	-0.15	0.11	-0.01
Avena sterilis	0.31	0.06	-0.26	-0.06
Brachypodium distachyon	0.02	-0.27	-0.14	-0.10
Bromus rubens	0.47	0.30	-0.17	0.63
Cerastium dichotomum	-0.06	-0.06	0.01	0.50
Geranium molle	0.56	0.18	-0.38	0.30
Lagurus ovatus	0.22	-0.17	-0.15	-0.08
Minuartia hibrida	-0.20	0.48	0.13	-0.01
Medicago minima	-0.24	0.03	0.42	-0.04
Paronychia argentea	-0.24	0.10	0.31	-0.09
Rumex bucephalophorus	-0.16	0.53	0.50	0.21
Stipa capensis	-0.03	0.47	0.31	0.02
Sisymbrium erysimoides	-0.36	0.03	0.25	0.44

Table S1.2. Eigenvectors of principal component analysis for plant community based on aboveground biomass and abundance per species

Species /Soil Origin	Small canopy (SC)			Medium canopy (MC)			Large canopy (LC)		
	Small (SS)	Medium (MS)	Large (LS)	Small (SS)	Medium (MS)	Large (LS)	Small (SS)	Medium (MS)	Large (LS)
Andryala ragusina	–	–	–	1	–	–	1	1	–
Asphodelus tenuifolius	3	2	2	2	2	1	3	2	1
Asterolinon linum-stellatum	–	–	1	1	1	1	–	1	–
Avena sterilis	1	–	2	–	1	2	1	–	2
Brachypodium distachyon	–	–	2	–	1	1	1	2	3
Bromus diandrus	1	–	1	–	–	–	1	–	3
Bromus rubens	2	–	–	–	1	2	4	3	2
Calendula arvensis	–	–	–	–	–	–	1	1	–
Calendula tripterocarpa	–	–	–	–	–	–	1	–	–
Campanula erinus	1	–	–	–	–	–	–	2	–
Centaurea melitensis	–	–	–	–	–	–	1	–	–
Cerastium dichotomum	2	1	–	1	1	1	3	2	1
Crucianella angustifolia	1	–	–	–	–	–	–	–	–
Desmazeria rigida	–	–	–	–	–	–	1	1	–
Diplotaxis virgata	–	–	–	–	–	–	–	2	–
Erodium cicutarium	–	–	–	–	–	–	–	2	–
Galium spurium	–	–	–	–	–	–	–	1	–
Geranium molle	1	–	1	–	1	2	–	1	4
Hedypnois cretica	1	–	–	–	–	1	2	1	–
Hypochaeris glabra	1	–	–	–	2	–	3	1	–
Lagurus ovatus	–	1	–	–	2	2	–	2	2
Leontodon hispidus	–	–	–	1	1	1	1	2	–
Leysera leyseroides	–	1	–	–	–	–	1	–	–
Limonium lobatum	–	–	–	–	1	–	–	–	–
Logfia minima	1	–	–	1	–	–	2	1	–
Lolium rigidum	1	–	–	–	–	–	1	–	–
Malva parviflora	–	–	–	–	–	–	–	1	–
Medicago minima	2	–	–	3	4	1	3	3	2
Medicago truncatula	–	–	–	1	–	–	3	1	–
Minuartia funkii	–	–	–	1	–	–	–	–	–
Minuartia hybrida	2	–	–	3	–	–	3	2	–
Paronychia argentea	2	–	–	2	3	–	4	4	–
Plantago albicans	2	–	–	1	1	–	1	–	1
Plantago lagopus	–	–	–	–	1	–	2	3	–
Polycarpon tetraphyllum	–	–	–	1	–	–	2	–	–
Reichardia intermedia	–	–	1	–	2	1	–	–	–
Reichardia picroides	–	–	–	–	–	–	–	1	–
Reichardia tingitana	–	2	–	–	–	–	1	1	–
Rostraria pumila	–	–	–	2	1	–	1	–	–
Rumex bucephalophorus	1	1	–	2	1	–	2	2	–
Silene decipiens	1	–	–	–	–	–	1	2	–
Silene sclerocarpa	1	–	–	1	–	–	–	1	–
Sisymbrium erysimoides	4	3	1	3	3	–	3	3	2
Sonchus oleraceus	–	–	1	–	–	–	–	–	–
Sonchus tenerrimus	–	–	1	–	–	1	–	–	1
Spergularia rubra	–	1	–	–	–	–	–	–	–
Stipa capensis	2	–	–	3	1	1	2	3	1
Trigonella monspeliaca	–	–	–	–	–	–	1	–	–
Trigonella polyceratia	–	–	–	1	–	–	1	–	–
Valantia muralis	1	–	–	–	1	–	1	1	–
TOTAL SPECIES	22	8	10	19	21	14	33	32	13

Table S1.3. List of species under small, medium and large *Retama* canopies growing with soil blocks from small, medium and large *Retama* shrubs. Numbers indicate the frequency of the species in each treatment, *i.e.* number of replicates in which the species was present (Maximum frequency = 6). A ´–´ symbol indicates the absence of a plant species. Species used for PCA analyses are highlighted in bold.

2. ISOLATING NOVEL FROM WELL-KNOWN FACILITATION MECHANISMS: EFFECTS OF SOIL MICROORGANISMS, MICROHABITAT AND SOIL PROPERTIES IN A NURSE SYSTEM IN A SEMIARID ENVIRONMENT

Lozano Y.M., Armas C., Hortal S., and Pugnaire F.I. Isolating novel from well-known facilitation mechanisms: effects of soil microorganisms, microhabitat and soil properties in a nurse system in a semiarid environment. *In preparation*.

2.1. ABSTRACT

Nurse plant species promote the establishment of other species under their canopies. Two mechanisms have been identified as key drivers of facilitation in arid environments: the improvement of microclimatic conditions and of soil properties by the nurse. However, soil biota may play an important role as well, but an analysis to tell apart the effects of these drivers is lacking. We addressed whether soil biota, soil properties, and shrub size had differential effects on understory plants depending on their life stage, hypothesizing that soil microbial communities associated to the nurse promote seed germination and plant growth of beneficiary species. We selected 3 microhabitats under field conditions (in gaps, under small, and under large *Retama sphaerocarpa* shrubs) and distributed 6 microcosms filled with sterile soil added with either alive or sterile inocula extracted from gap soils and from under small and large *Retama* shrubs. In each microcosm we sowed 50 seeds of each of six plant species, three usually found under *Retama* shrubs (*Lagurus ovatus*, *Medicago minima* and *Asphodelus tenuifolius*) and three preferentially found in gaps (*Stipa capensis*, *Sisymbrium erysimoides*, and *Andryala ragusina*). We monitored seed germination and measured aboveground biomass and individual number 5 months after sowing. Soil microbial communities under *Retama* shrubs and in gaps were different, and had significant effects on seed germination, ranging from positive to neutral to negative depending on species identity. High nutrient and soil organic matter content under *Retama* shrubs promoted seed germination and increased aboveground biomass and plant density, while amelioration of microclimatic conditions under *Retama* shrubs promoted seed germination but, contrary to our expectations, had no significant effect on plant performance. Our results showed that the effect of soil biota and soil properties were more decisive than canopy effects on plant establishment under *Retama* shrubs, evidencing that soil is a major driver of facilitation processes in this arid environment.

2.2. INTRODUCTION

The structure and composition of plant communities are influenced by positive and negative interactions among plants (Brooker et al. 2008, Armas and Pugnaire 2009). Facilitation, described as the positive, and at least one-way interaction where one plant species benefits another neighboring species in terms of germination, survival or growth, is a well-known phenomenon in many ecosystems around the world (Callaway 2007). A classic and frequent case of facilitation in plant communities is the nurse plant syndrome (sensu Niering et al. 1963, Franco and Nobel 1989), in which an individual of one species facilitates the establishment and growth of other species (Flores and Jurado 2003). In arid ecosystems, the most common mechanism behind such nurse effect are the amelioration of microclimatic conditions and soil properties under the canopy (Pugnaire et al. 1996a, Flores and Jurado 2003, Maestre and Cortina 2005, Callaway 2007, Pugnaire et al. 2011).

The nurse effect is especially important in arid environments (Flores and Jurado 2003) where shade provided by nurse plants can protect understory plants from high temperatures and irradiance (Moro et al. 1997, Jankju 2013). Although not always, water availability can be also higher under the canopy, either as a result of shading and higher soil water holding capacity or through hydraulic lift (Pugnaire et al. 2004, Armas and Pugnaire 2005, Zou et al. 2005, McCluney et al. 2012, Prieto et al. 2012). Nurse plants usually increase nutrient availability under the canopy through litter fall and enhance cycling because increased soil moisture speeds decomposition (Rodríguez-Echeverría and Pérez-Fernández 2003, Armas et al. 2012). Overall, all these processes improve soil chemical and physical conditions in the understory of nurses (Pugnaire et al. 1996a, 2004, 2011). Although the large modifications induced by nurse plants have traditionally been identified as the main mechanisms behind facilitation, recent reports evidenced the important impact of soil biota on plant-plant facilitation (Hortal et al. 2013) through processes independent on the nurse canopy or soil quality (Rodríguez-Echeverría et al. 2013).

Soil biota can influence plant performance either positively, e.g., through mycorrhizal associations (Van Der Heijden and Horton 2009, Smith and Read 2010), or negatively, e.g., through the accumulation of soil pathogens that impair plant establishment (Van der Putten and Peters 1997, Bever 2003). Although it is already known that soil microbial communities have a strong influence on individual plant performance (Bever et al. 2010), plant community biodiversity (Wardle et al. 2004, van der Heijden et al. 2008) and ecosystem multifunctionality (Loreau 2001, Wagg et al.

2014), the effect of soil microorganisms on the outcome of plant-plant interactions has been less explored (Selosse et al. 2006, Kardol et al. 2007, Pendergast et al. 2013, Rodríguez-Echeverría et al. 2013) and experiments in natural field conditions are just lacking.

In a recent observational study, Hortal et al. (2013) showed that soil microbial activity, along with soil microbial biomass (fungi and bacteria) were much higher under the nurse shrub *Retama sphaerocarpa* (L.) Boiss (hereafter *Retama*) than in open spaces without shrubs (hereafter gaps) and that both soil microbial activity and biomass increased as the shrub aged. The relative abundance of different microbial groups also changed with shrub age, with an increase in groups such as *Bacteroidetes* and *Proteobacteria* and a decrease in *Actinobacteria* and *Firmicutes*. In fact, the composition, activity and biomass of soil microbial communities in gaps and under the canopy of large *Retama* shrubs were quite different (Hortal et al. 2013) and suggest that changes are behind the great positive impact that large *Retama* shrubs have on their understory plant community (Pugnaire et al. 1996b, Armas et al. 2011). In a greenhouse experiment, Rodríguez-Echeverría et al. (2013) found that soil biota associated to *Retama* shrubs had a positive effect on the biomass of an experimental plant community, as well as on the abundance and growth of numerous plant species that usually grow in the understory of this nurse species. All this suggests an important role of soil biota in addition to soil physico-chemical properties and canopy size on plant-plant facilitation.

2.3. OBJECTIVES

In an experimental setup in field conditions we tried to disentangle the impact of each driver (soil microorganisms, soil properties, and canopy) on the facilitation exerted by *Retama* on its understory plant community.

Our main objectives were 1) to analyze whether seed germination, number of plants and plant biomass were affected by soil microorganisms, soil properties or microhabitat; and 2) to determine whether these drivers had different effects depending on the life stage of beneficiary plants.

2.4. HYPOTHESIS

We hypothesized that soil microbial communities associated to the nurse shrub are integral part of the facilitation effect of *Retama*, promoting seed germination and growth of understory plant species, being as important as soil properties and microhabitat in this process.

2.5. METHODS

2.5.1. Field site and species

The field site was located in the Rambla del Saltador, a dry valley on the southern slope of the Sierra de los Filabres mountain range, Almería, southeast Spain (37º08´N, º22´W; 630 m altitude). The climate is semiarid Mediterranean, with a mean annual temperature of 16º C and mean annual rainfall of 300 mm (Puigdefabregas et al. 1999). The soil is of alluvial origin, with a loamy sand texture, characterized by low values of water holding capacity, electrical conductivity, cation exchange capacity, organic matter, nutrient concentrations, and a moderate alkaline pH (Pugnaire et al. 1996b, Puigdefábregas 1996)

The plant community is dominated by the leguminous shrub *Retama sphaerocarpa,* a tall shrub with an open canopy with photosynthetic stems and a dimorphic root system with shallow lateral roots and tap roots that can reach 30 m deep (Haase et al. 1996). *Retama* shrubs can lift water from deep, wet soil layers and release it into shallow, dry soil layers (Prieto et al. 2010), contributing to its nurse effect (Prieto et al. 2012). *Retama* shrub facilitates the establishment and growth of many other plant species under their canopy (Pugnaire et al. 1996b) compared to gaps among shrubs, having an overall positive impact on local community diversity in semiarid environments (Armas et al. 2011, Schöb et al. 2013). Aboveground biomass and plant species richness increase with *Retama* shrub size and age (Pugnaire et al. 1996b, Pugnaire and Lázaro 2000).

2.5.2. Soil sampling and inoculum preparation

In October 2011 we randomly selected 8 large *Retama* shrubs in a ~5 ha homogeneous plot. Near each large shrub we selected one small *Retama* shrub and a gap in between (*i.e.*, a total of 24 microhabitats). We collected soil from the top 10 cm in each microhabitat, combined the 8 samples

corresponding to the same microhabitat and sieved it through a 5-mm sieve obtaining 60 kg of soil per soil origin (*i.e.*, gaps, under small and under large *Retama* shrubs). Pooling soil samples reduces variability but allows testing for differences between soil origins and could be considered as technical replicates (Kardol et al. 2006, Ayres et al. 2009, Meisner et al. 2013, Rodríguez-Echeverría et al. 2013). Fifty of these 60 kg of soil per origin were autoclaved during 20 min at 120ºC to destroy all soil organisms and were used as microcosms substrate.

Following recommendations by Meisner et al. (2013), and Pendergast et al. (2013), we took apart 10 kg from each soil origin and divided it in halves; one was left intact to prepare the "alive" soil inoculum and the other half was autoclaved at 120 ºC during 20 min to prepare the sterile soil inoculum. In both cases, the soil was stirred in distilled, autoclaved water in a proportion 1:2 (v:v) and then filtered through a 0.5 mm sieve to remove soil particles but allowing the pass of fungal spores, hyphae, soil bacteria and microfauna (Van de Voorde et al. 2012). These two types of inocula (sterile and alive) per soil origin were used to inoculate soil microcosms.

2.5.3. Experimental design

In each microhabitat we randomly distributed 6 microcosms (pots of 17 cm of diameter and 18 cm height; 1 L volume) filled with one of the three types of autoclaved soil and watered with either alive or sterile inocula of its respective soil origin. The volume of inoculum added to each microcosm was adjusted to have a density of 20% (v:v), i.e., 200 ml per microcosm. We thus had a total of 144 microcosms (3 microhabitat x 3 soil origins x 2 inocula x 8 replicates). In each microcosm we sowed 300 seeds of six different plant species (50 seeds per species), three of them usually found under *Retama* shrubs (*Lagurus ovatus* (L.), *Medicago minima* (L.) Bartal., and *Asphodelus tenuifolius* (Cav), hereafter *Lagurus*, *Medicago* and *Asphodelus*) and the other three species preferentially found in gaps (*Stipa capensis* (Thunb), *Sisymbrium erysimoides* (Desf), and *Andryala ragusina* (L.), hereafter *Stipa*, *Sisymbrium* and *Andryala*) (Pugnaire et al. 1996b, Pugnaire and Lázaro 2000, Boeken et al. 2004, Pugnaire et al. 2004). Prior to sowing, seeds surface was sterilized by submerging them in 75% ethanol for 2 minutes. All microcosms were covered with a wire mesh to prevent predation and herbivory (Fig. 2.1a)

Fig. 2.1 Field experiment: Microcosms covered with a wire mesh distributed under large *Retama* shrub (a), and detail of the plants grown in a microcosm (b).

Sowing took place in November 2011 and the number of germinated seeds was tallied twenty days later (Fig 2.1b). Microcosms were left in the field for 5 months, and then plants in each microcosm were counted, harvested, and separated by species. Shoots were dried at 70ºC during 48 h and weighted. Soil from each microcosm was also collected and divided in two halves; one was kept at 4ºC for physical and chemical analyses and another was kept at -80ºC for microbial analyses. Although samples were stored a couple of months, we were more interested in analysing relative differences among treatments than on the absolute values, we assumed these comparisons were valid since all samples were stored in the same conditions.

2.5.4. Soil analyses

Soil properties were measured in soils from each origin after inoculation with either alive or sterile inocula at the start of experiment (n=3). Soil electrical conductivity (EC) and pH were obtained using an aqueous solution of 1:5 for EC and 1:2.5 for pH (w:v), with a conductivity- and pH-meters (Crison, BA, Spain), respectively. Total soil carbon (C), organic C after removal of inorganic C with HCL 2N (Schumacher 2002) and total nitrogen (N) content were determined using a C/N analyzer (LECO Truspec, MI, USA). Anion phosphate (PO_4^{3-}), nitrate (NO_3^-), and sulphate (SO_4^{2-}) concentrations in water extract (1:10 soil:water) were analyzed by HPLC (Metrohm, HE, Switzerland). Percentage of clay, sand and silt were measured by the Robinson method.

2.5.5. Soil microbial community composition

Molecular analyses were performed on autoclaved soil samples from gaps and from under large *Retama* shrubs that were watered with their respective alive inoculum at the beginning of the

experiment (2 soil origin x 1 inoculum x 3 replicates). Moreover, at the end of the experiment (five months after sowing), we collected soil samples from gap soils placed in gaps and from large *Retama* soils placed under large *Retama* canopies inoculated at the beginning of the experiment with either alive or sterile inoculum (2 soil origin x 2 inocula x 3 replicates). DNA was extracted from 0.25 g of homogenized soil from each of the 18 soil samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) following the manufacturer's directions. A 16S rDNA gene fragment corresponding to V3 to V6 regions was amplified using primers 357F with Roche adaptor B (5`CTATCCCCTGTGTGCCTTGGCAGTCTCAGCCTACGGGAGGCAGCAG 3`) and 926 Rb (5` CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNNNCCGTCAAT T**Y**MTTTRAGT 3`) that included Roche adaptor A and 12 base-pair barcode (Sim et al. 2012). Each sample was amplified in quadruplicate to reduce random mispriming bias (Polz and Cavanaugh 1998). Amplicons were combined in a single tube in equimolar concentrations and the pooled amplicon mixture was purified twice (AMPure XP kit, Agencourt, Takeley, United Kingdom). DNA concentration was quantified using the PicoGreen® assay (Sim et al. 2012). This pool was prepared and pyrosequenced in a Roche Genome Sequencer FLX System (Roche, Basel, Switzerland) using 454 Titanium chemistry at Lifesequencing lab (Valencia, Spain).

Sequences were trimmed for primers, filtered and demultiplexed using the pyrosequencing pipeline from the Ribosomal Database Project (RDP, Michigan State Univ., USA). Sequences shorter than 150 bp, with quality scores <20 or containing any unresolved nucleotides were removed from the dataset. We used Acacia software version 1.52 (Bragg et al. 2012) for pyrosequencing noise removal using default parameters for error correction. We established a minimum average quality threshold of 30 and all sequences were trimmed to a maximum length of 570 bp. Chimeras were identified using Uchime tool (Edgar et al. 2011) from RDP pipeline and removed from the dataset. Retained sequences were aligned using the Aligner tool from the RDP pipeline and then were clustered into operational taxonomic units (OTUs) defined at 97% similarity cutoff using the complete linkage clustering tool of the RDP pipeline. Taxonomic assignation of sequences was performed using the RDP naïve Bayesian classifier (Wang et al. 2007) at a confidence level of 80 %, and relative abundances of the different phyla, classes, subclasses, order and main genus per each of the 18 samples were calculated.

2.5.6. Statistical analyses

The experimental design was a split plot factorial design where each replicate of each microhabitat was the main plot (a total of 24), and each microcosm (pots with one type of soil origin and one respective soil inoculum) was the subplot (a total of 6). Microhabitat type (gaps, under small and under large *Retama* shrubs), soil origin (from gaps, under small and under large *Retama* shrubs), type of inoculum (alive and sterile), and plant species (*Lagurus, Sisymbrium* and *Stipa*; 3 out of the 6 initial species sown, see results) were considered as fixed factors, and main plots and subplots where included as random effects. Aboveground biomass per plant was square root-transformed (Ramette 2007) and analyzed using General Linear Mixed Models that included number of neighbors as covariate. Spatial correlation was modeled with a compound symmetry correlation structure (corCompSymm) that considers a uniform correlation among seeds within a pot (the 300 seeds within each pot were sown at random). We tested several variance structures to avoid heteroscedasticity and selected the best one (varIdent) by comparison using the Akaike information criterion (Akaike 1974). VarIdent represents a variance structure with different variances for different strata (Gałecki and Burzykowski 2013). To select the most parsimonious model with lowest AIC, we compared the models using the likelihood ratio test and performed a graphical inspection of their residues distribution. The maximum likelihood estimation was calculated using the restricted maximum likelihood (REML) criterion. The total number of plants that survived at the end of the experiment with neighbors as covariate, the number of seeds that germinated and the relative abundance of microbial groups were analyzed using Generalized Linear Mixed Models with a binomial distribution, a logistic link function and the maximum likelihood (ML) criterion. Significance was established at $p<0.05$. Post-hoc comparisons were performed using DGC test (Di Rienzo et al. 2002). Differences in soil properties at the beginning of the experiment were evaluated by using linear models. Analyses were done with InfoStat-Statistical Software (Di Rienzo et al. 2013). Results shown throughout the text and figures are mean values ± 1 SE.

Similarity among bacterial community composition (with OTUs showing at least 5 reads in the overall dataset) were analyzed with principal coordinates analysis (PCoA) using Bray-Curtis similarity index. Differences among treatments were evaluated performing a NPMANOVA with 9999 permutations using Past v 2.12 software (Hammer et al. 2001) and multivariate mean comparisons (gDGC test) based on cluster analysis, using diagonal covariance matrix with a single linkage and Monte Carlo simulation with 500 permutations (Valdano and Di Rienzo 2008).

Shannon's diversity index of OTUs per sample was calculated with Past software after excluding singletons (OTUs only showing 1 read in the overall dataset) to reduce the overestimation of diversity (Tedersoo et al. 2010).

2.6. RESULTS

2.6.1. Seed germination

There was no germination of *Medicago* or *Andryala* seeds in any of the microcosms 20 days after sowing, and only few *Asphodelus* seeds germinated (less than 1.2 ± 0.1 seeds per pot with 52% of pots lacking any germination). We thus discarded these three species from further analyses.

Alive soil inoculum (i.e., with soil microorganisms) had a significant effect on seed germination but its effects were mediated by the other experimental factors (i.e., there was a significant "species x microhabitat x soil origin x inoculum" interaction, Table S2.1). Germination was greatest in soils from under *Retama* shrubs and lowest in soils from gaps (Figs. 2.2 and S2.1) in any microhabitat. The number of germinated *Lagurus* and *Sisymbrium* seeds was higher with alive than with sterile inocula irrespective of soil origin (Fig. 2.2a, b). On the contrary, alive inocula from gaps or from small *Retama* soils had a negative effect on germination of *Stipa* seeds, whereas there were no differences between sterile or alive inocula from soils collected under large *Retama* shrubs (Fig. 2.2c). Germination was also affected by microhabitat, being lowest in gaps for all plant species. For both *Lagurus* and *Sysimbrium*, germination was highest under large *Retama* canopies and intermediate under small *Retama* canopies (whit alive inocula) while *Stipa* germination was similar under the two *Retama* canopies (Fig. S2.1).

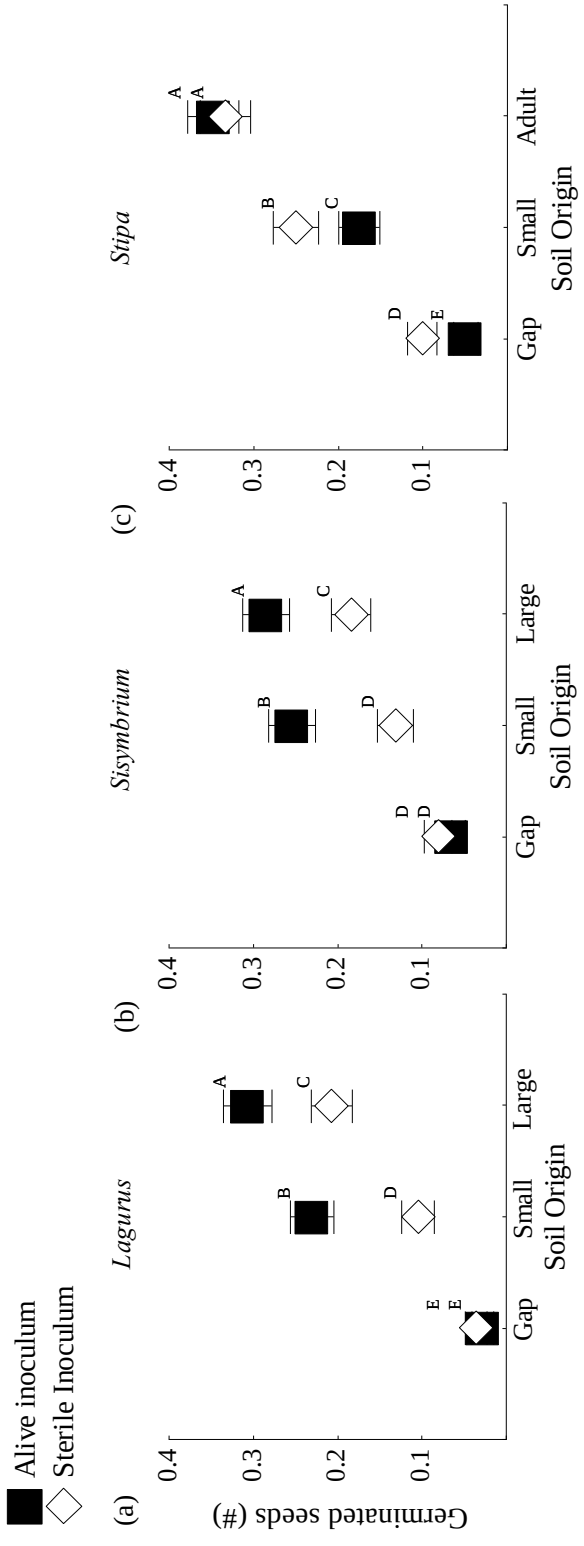

Fig.2.2. Results of generalized linear mix models of mean number of germinated seeds of *Lagurus* (a), *Sisymbrium* (b) and *Stipa* (c) sown in soils from gaps, small or large *Retama* shrubs, and watered with either and alive inoculum of soil microorganisms or sterile inoculum. Data are mean ± 1 SE, n = 8. Symbols with different letters indicate significant differences among treatments after post-hoc comparisons at a significance level of 0.05.

2.6.2. Aboveground biomass and number of plants

Five months after sowing we had an average of 5.62 ± 0.55 *Lagurus* plants, 3.01 ± 0.28 *Sisymbrium* and 10.92 ± 0.84 *Stipa* per microcosm. Maximum individual aboveground biomass was 0.86, 2.97 and 2.46 g for *Lagurus, Sisymbrium* and *Stipa,* respectively. Alive and sterile inocula from each soil origin had similar effects on plant biomass and number of established plants. The soil origin where plants grew had a significant impact on plant biomass but its effect was influenced by microhabitat and species (significant "species x soil origin x microhabitat" interaction, Table S2.1). *Lagurus* showed more biomass in soils from large than from small *Retama* shrubs or gaps regardless of the microhabitat they grew under. *Sisymbrium* plants had more biomass when grown in soils from under small *Retama* shrubs in any microhabitat, while *Stipa* showed more biomass when grown in soils from under either small or large *Retama* shrubs, in particular under small canopies with soil from large *Retama* shrubs (Fig. 2.3).

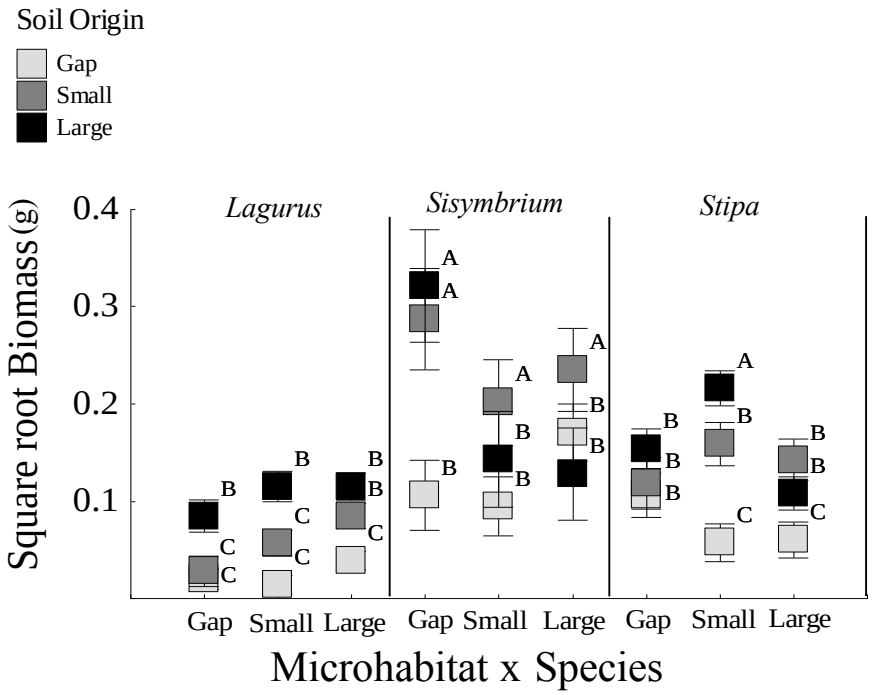

Fig.2.3. Mean aboveground individual plant mass of *Lagurus, Sisymbrium* and *Stipa* growing in microcosms with soils from gaps, small or large *Retama* shrubs, and growing either in gaps, under small or large *Retama* shrubs (microhabitat) 5 months after sowing. Data are mean ± 1 SE, n = 8. Symbols with different letters indicate significant differences among treatments after post-hoc comparisons at a significance level of 0.05.

Soil origin had a significant effect on the number of plants depending on the species identity (significant "soil origin x species" interaction, Table S2.1). Plant abundance for *Lagurus, Stipa* and

Sisymbrium was lowest in gaps and intermediate in soils from small *Retama* shrubs; for *Lagurus* and *Stipa*, it was highest in soils from large *Retama* shrubs (Fig. 2.4). Overall, the number of plants was higher under large *Retama* canopies than under small canopies or gaps (Fig. S2.2).

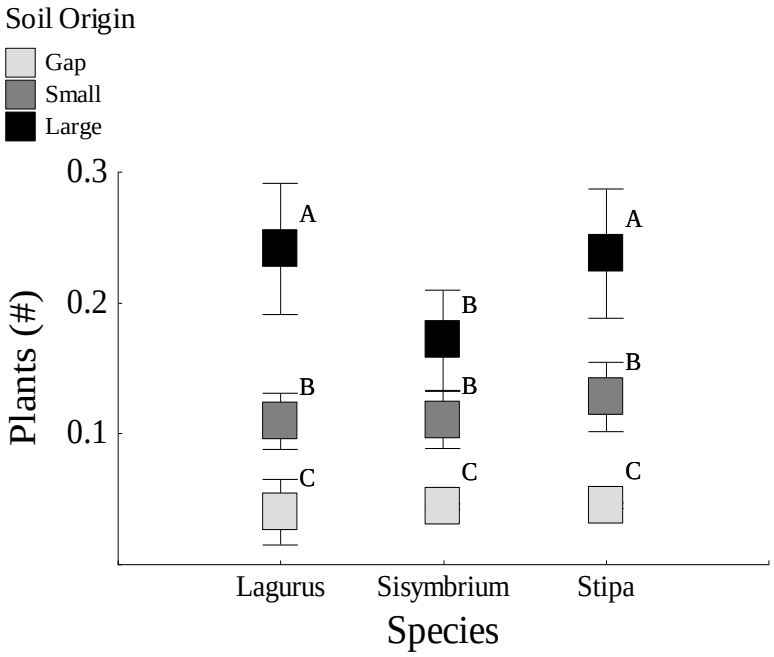

Fig. 2.4. Results from generalized mix model of number of *Lagurus, Sisymbrium* and *Stipa* individuals established per microcosm in soils from gaps, small or large *Retama* shrubs after 5 months from sowing (total of 300 seeds per microcosm). Data are mean ± 1 SE, n = 8. Symbols with different letters indicate significant differences among treatments after post-hoc comparisons at a significance level of 0.05.

2.6.3. Soil properties

Properties of soils collected at the beginning of the experiment were different depending on their origin, but there were no differences depending on whether they were watered with alive or sterile inocula (Table 2.1). Total C, organic C, and total N contents increased gradually from gaps to small to large *Retama* shrubs. Clay and SO_4^{-2} contents were higher in soils from large and small *Retama* shrubs than in gaps. Silt and NO_3^- contents were higher in soils from small *Retama* shrubs than in other soils. Sand content was lowest in small *Retama* shrubs, intermediate in large shrubs and highest in gaps. Soil electrical conductivity was highest in soils from large *Retama* shrubs and there were no differences in pH among soils. Soil PO_4^{3-} and NO_2^- were very low, well under the detection threshold of the HPLC.

2.6.4. Soil bacterial community composition

We obtained a total of 63857 sequences after filtering and removing chimeras. The mean number of retained sequences per sample was 3548 ± 231, with no differences among treatments. Average length of retained sequences was 550 ± 8 bp. We identified 8489 distinct operational taxonomic units (OTUs) at 97% similarity. Ordination de OTUs with a minimum of 5 reads within the overall dataset (1804) showed marked differences in bacterial community composition in soils with alive inocula from gap or large *Retama* shrubs both at the start (T0) and at the end of the experiment (T5). Communities from T0 compared to T5 were also markedly different (Fig. 2.5). Moreover, within each soil origin, soils with alive compared to sterile inocula were also different at the end of the experiment ($F_{2,17}$= 3.25, p<0.001, NPMANOVA and hierarchical cluster (Fig. S2.3)).

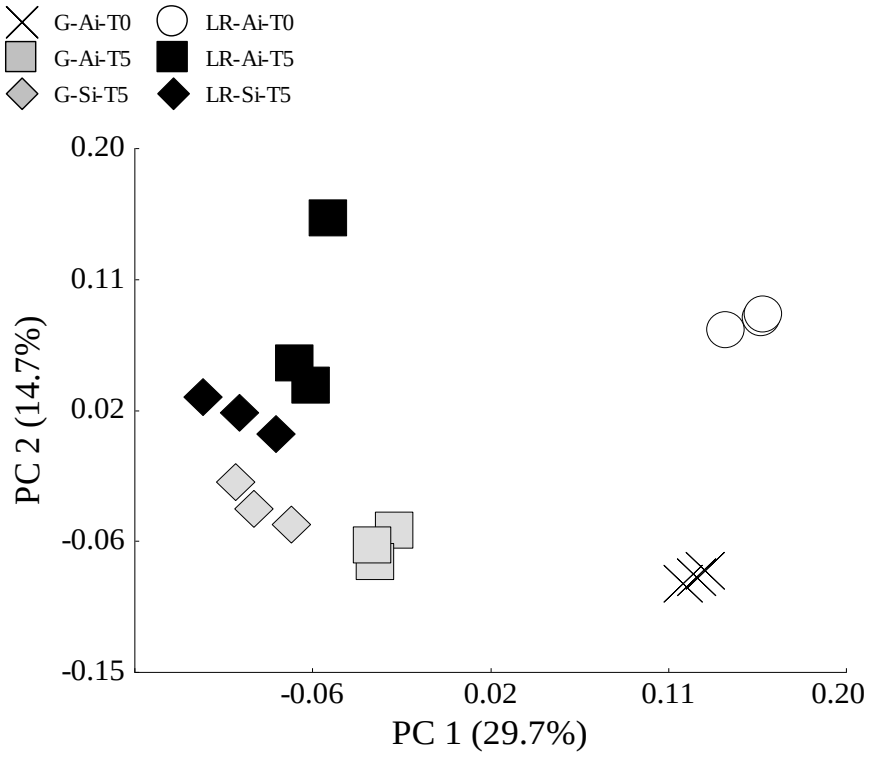

Fig. 2.5. Principal coordinates analysis (PCoA) of soil bacterial communities based on operational taxonomic units (OTUs) using Bray–Curtis similarity index. Crosses and circles indicate soil watered with alive inoculum from gaps and from under large *Retama* soils, respectively, at the start of the experiment (T0). Grey symbols represent soils from gaps (G) and black symbols soils from under large *Retama* shrubs (LR) at the end of the experiment (T5). Squares correspond to soils initially watered with alive inoculum (Ai) and diamonds with sterile inoculum (Si).

Soil properties	Gap		Small *Retama*		Large *Retama*		
	Alive inoculum	Sterile inoculum	Alive inoculum	Sterile inoculum	Alive inoculum	Sterile inoculum	*F values*
pH	8.04±0.11^{a}	8.17±0.11^{a}	7.86±0.11^{a}	8.29±0.11^{a}	8.01±0.11^{a}	7.90±0.11^{a}	1.01^{ns}
EC (µS/cm)	183.27±71.91^{a}	201.15±71.91^{a}	334.33±71.91^{a}	267.80±71.91^{a}	455.33±71.91^{b}	470.50±71.91^{b}	**7.18****
OC (g kg $^{-1}$)	4.18±0.11^{a}	4.29±0.11^{a}	7.38±0.63^{b}	8.58±0.63^{b}	13.73±0.24^{c}	14.00±0.24^{c}	**1350.06*****
N (g kg $^{-1}$)	0.52±0.10^{a}	0.48±0.10^{a}	0.77±0.10^{b}	0.90±0.10^{b}	1.37±0.10^{c}	1.39±0.10^{c}	**37.33*****
C (g kg $^{-1}$)	4.45±0.08^{a}	4.68±0.08^{a}	8.88±0.93^{b}	10.83±0.93^{b}	13.88±0.28^{c}	14.45±0.28^{c}	**1139.10*****
NO_3^- (mg/kg)	17.49±1.92^{a}	15.86±1.92^{a}	66.91±1.92^{c}	70.97±1.92^{c}	46.72±1.92^{b}	51.00±1.92^{b}	**377.84*****
SO_4^{2-} (mg/kg)	1.20±0.03^{a}	1.21±0.03^{a}	3.44±0.13^{b}	3.69±0.13^{b}	4.02±0.15^{b}	3.55±0.15^{b}	**560.02*****
Sand (%)	88.03±0.89^{c}	86.66±0.89^{c}	82.37±0.89^{a}	82.43±0.89^{a}	83.61±0.89^{b}	86.83±0.89^{b}	**15.46*****
Silt (%)	9.49±0.25^{a}	9.99±1.07^{a}	14.52±014^{b}	14.00±0.51^{b}	12.31±1.81^{a}	9.32±0.27^{a}	**31.48*****
Clay (%)	2.66±0.22^{a}	3.27±0.22^{a}	3.21±0.22^{b}	3.57±0.22^{b}	3.62±0.22^{b}	3.52±0.22^{b}	**4.14***

Table 2.1. Properties of soils from gaps, small and large *Retama* shrubs watered with their respective alive or sterile inocula at the start of the experiment. EC= Soil electrical conductivity and OC = Organic carbon. Values are mean ± 1 SE (n=3). Different letters in a row indicate significant differences ($p<0.05$) after DGC test. The last column shows *F* values of the general linear mixed model and significance (*, **, ***, at $p<0.05$, 0.01, 0.001,respectively; all significant values in bold; ns = non-significant)

Sequences mostly belonged to five different phyla, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria*. Other minor phyla (with relative abundance lower than 2%) were *Verrucomicrobia*, *Gemmatimonadetes*, *Chloroflexi*, *Cyanobacteria, Nitrospira*, *Armatimonadetes*, and *Planctomycetes*. Relative abundances of the different taxa differed among treatments (Fig. 2.6). We observed at the beginning of the experiment a higher abundance of *Actinobacteria* in soils from gaps than in soils from large *Retama* shrubs, while *Alphaproteobacteria* and *Gammaproteobacteria* were higher in large *Retama* soils than in gaps (Fig. 2.6). Comparing alive soils at the beginning and at the end of the experiment the abundance of *Actinobacteria* and *Acidobacteria* decreased in both soils from gaps and from under *Retama* shrubs while *Firmicutes, Bacteroidetes* and *Betaproteobacteria* increased in their relative abundances. Thus, at the end of the experiment, the abundance of *Bacteroidetes* was higher in soils under *Retama* than in soils from gaps while the abundance of *Firmicutes* was higher in gaps than in soil under *Retama* shrubs. There were no differences at the end of the experiment between soils initially watered with alive or sterile inoculum except for the relative abundances of *Betaproteobacteria* that increased even more in soils watered with sterile than with alive inoculum and *Deltaproteobacteria* that decreased in soils with sterile inoculum irrespective of the soil origin (Fig. 2.6). Among minor phyla, the relative abundance of *Verrucomicrobia* increased in alive soils from gaps at the end compared to the start of the experiment while the abundance of *Cyanobacteria* was higher in alive than in sterile soils irrespective of soil origin (data not shown). There were no differences in the relative abundance of other minor phyla. Abundance of unclassified bacteria was lowest in soils from *Retama* shrubs at the end of the experiment irrespective of inoculum type (data not shown). Bacterial diversity (Shannon's index) was 5.85 ± 0.13. Microbial diversity was higher in soils from large *Retama* shrubs (6.09 ± 0.16) at the start of the experiment than in the other soils either at the start or at the end of the experiment ($F_{1,12}$= 5,28, p=0.04).

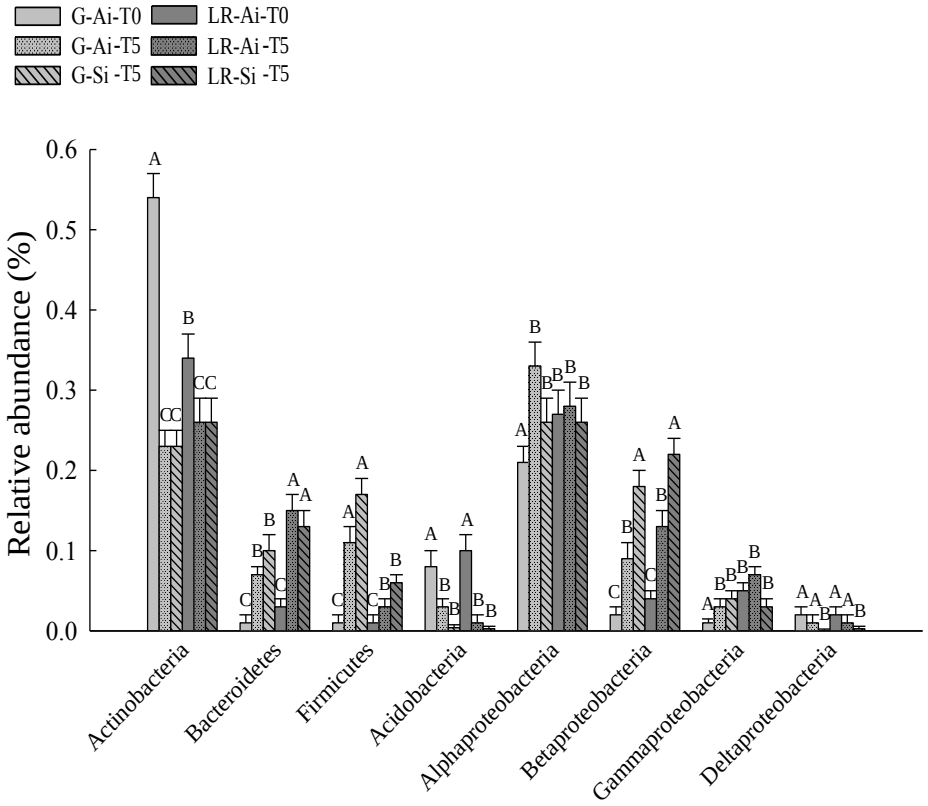

Fig. 2.6. Results from generalized linear mixed models of mean relative abundance (± 1 SE) of the most abundant bacterial taxonomic groups, i.e. phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* (within *Proteobacteria* phylum). Light grey bars represent soil collected from gaps (G) and dark grey bars represent soil from under large *Retama* shrubs (LR) watered with either alive (Ai) or sterile inoculum (Si). Soil was collected at the start (T0) and at the end of the experiment (T5). Different letters within a bacterial group indicate significant differences ($p < 0.05$) among treatments after DGC test; n = 3 microcosms.

2.7. DISCUSSION

Our data show once again that *Retama sphaerocarpa* shrubs are strong facilitators of plant communities in their understory, and disentangle some of the mechanisms involved in the process. We found that soil microorganisms affect seed germination, with effects ranging from positive to negative depending on species identity, soil characteristics, and microhabitat. Both plant growth and abundance were strongly affected by soil origin, with positive or neutral effects of microhabitat. The effect of soil microorganisms on plant growth and abundance was no evident. Overall, our results suggest that soil properties mediated facilitation in this system.

2.7.1. The positive effect of Retama soils on seed germination and plant performance

Our data show that soil from under *Retama* shrubs positively affected seed germination, biomass, and abundance of *Lagurus, Sisymbrium* and *Stipa*. Compared to gaps, soils from under *Retama* shrubs usually show a higher moisture (Pugnaire et al. 2004), have higher organic matter and nutrient content as well as higher silt and clay content. Accretion of soil organic matter under *Retama* soils increases water holding capacity, buffer soil temperature changes (Moro et al. 1997, Pugnaire et al. 2004), and increases soil oxygenation (Porta-Casanellas et al. 2003) which promoted seed germination and plant growth. Moreover, different chemical properties from *Retama* soils compared to gap soils also promoted seed germination and plant performance. For example, an increase in soil N content related to active N fixation from *Retama* shrubs (Rodríguez-Echeverría and Pérez-Fernández 2003) or changes in microbial activity that promote the release of available N for plants (Tortora et al. 2007), as well as an increase in anion content (Roem et al. 2002) might have promoted seed germination and plant performance in *Retama* soils. Added to this, the positive effect of *Retama* soil properties on plant performance at the end of the experiment also included the effect of the particular soil microbial community that was established in these soils along the experiment.

2.7.2. The effect of soil microorganisms on seed germination was species specific

Microbial communities associated to *Retama* soils and gaps were different, affecting seed germination in different ways, and were critical to determine the emergence of a specific understory community in each soil.

Germination of *Lagurus*, a species whose typical microhabitat is *Retama* understories (Pugnaire et al. 1996b), was, accordingly to its preferred natural microhabitat, positively affected by *Retama* soil microorganisms, while *Sisymbrium,* which prefers gaps (Pugnaire and Lázaro 2000), germinated more in soils from under *Retama* with alive inoculum than in soils from gaps. *Stipa* is a species mainly found in gaps (Boeken et al. 2004) but, interestingly, microorganisms from gap soils negatively affected its germination. Differences in seed germination of each species were strongly linked to differences between the microbial communities associated to soils from under *Retama* shrubs or to gaps.

Soils under *Retama* shrubs had, at the beginning of the experiment, higher abundance of *Alphaproteobacteria* and *Gammaproteobacteria* than soils from gaps, as well as higher abundance of key groups within these classes. For example, members of the abundant *Rhizobium* and *Bradyrhizobium* genera (*Alphaproteobacteria*) establish symbiotic root associations with legumes, as *Retama*, having a key role in atmospheric nitrogen fixation (Kersters et al. 2006). Similarly, free-living nitrogen fixers such as *Azomonas* (Kersters et al. 2006) or *Pseudomonas* (*Gammaproteobacteria* class) are known plant growth promoters (Saharan and Nehra 2011). On the other hand, members of *Actinobacteria,* commonly found in gaps (Suela Silva et al. 2013) are considered to be drought-resistant (Köberl et al. 2013), and in our experiment they were more abundant in gaps than in *Retama* soils.

Although most research on the effect of soil microbial communities on plants has not analyzed the effect of soil microorganisms on seed germination and the functions and identities of most seed-associated bacteria are currently unknown (Lopez-Velasco et al. 2013), it is known that the seed testa is affected by soil bacteria (Buyer et al. 1999). In turn, soil bacteria are affected by seed exudates and their components (Nelson 2004). Soil microorganisms from under *Retama* shrubs established positive seed-microbe interactions that promoted seed germination in *Lagurus* and *Sisymbrium,* while soil microorganisms from under small *Retama* shrubs or from gaps established negative seed-microbe interactions with *Stipa*, hindering its germination. This low germination of *Stipa* with soil microorganisms from gaps, its preferred habitat, could be due to several reasons. Among them, the presence of soil pathogens (Van der Putten et al. 2013), which produce enzymes and phytotoxines that can kill seeds before germination (Kremer 1993) or inhibit germination of viable seeds (Roberts and Feast 1972). In addition, some soil microorganisms promote seed dormancy (Miransari and Smith 2014) allowing seeds to wait for better conditions to germinate. Although data on seed-microbe interactions are still very scarce (Coombs and Franco 2003), our results showed that these associations could range from positive to negative depending on species identity and that these seed-microbe interactions were influenced by the particular properties of each soil.

Apart from the role of interactions between plants and soil microorganisms in determining the outcome of plant-plant interactions (Pendergast et al 2013; Rodriguez-Echeverría et al. 2013), competitive interactions among plants are also important. Thus, although soil microorganisms from under *Retama* shrubs facilitated germination of *Sisymbrium,* competition among species under

Retama canopies is quite intense (Schöb et al. 2013) and may exclude this species from under *Retama* shrubs limiting its development and explaining why this species is naturally found in gaps.

2.7.3. Soil microorganisms may contribute to the positive effect of soil on plant performance

Five months after sowing, the effect of soil microorganisms on plant performance was neutral or not evident, probably due to the fact that microbes were able to colonize soils initially inoculated with either sterile or alive inocula, and conformed soil microbial communities that were more homogeneous in their composition than soil microbial communities at the beginning of the experiment. Although at the end of the experiment we observed differences in composition between soil communities that received either sterile or alive inocula, the relative abundance of main microbial groups was similar among microcosms, and only showed differences in *Betaproteobacteria* and *Deltaproteobacteria*, irrespective of soil origin.

Microbial communities changed their composition in alive soils from both gap and large *Retama* shrubs between the beginning and the end of the experiment, likely because of microbial colonization and changes in weather (Fierer et al. 2010) in addition to modifications induced by plants over time (Van der Putten et al. 2013).

At the end of the experiment, microbial communities in *Retama* soils that received either alive or sterile inocula at the beginning of the experiment were more similar among them than microbial communities from gaps that received either alive or sterile inoculum 5 months earlier, suggesting a strong effect of soil origin properties on final microbial composition. At the end of the experiment, members of *Bacteroidetes* were more abundant in *Retama* soils than in soils from gaps. Bacteria in this phylum have the ability to rapidly explore organic matter (Acosta-Martinez et al., 2010), and are associated to high soil C availability as that in *Retama* soils. As suggested by Hortal et al. (2013), an increase in *Bacteroidetes* could have stimulated plant growth in soils from *Retama* shrubs. On the other hand, the abundance of *Firmicutes* was higher in gaps than in *Retama* soils, which corresponds with their ability to survive extreme environmental conditions and stand low substrate availability (Acosta-Martínez et al. 2010) as in gaps.

2.7.4. Positive and neutral effect of microhabitat on seed germination and plant performance

Microclimate under *Retama* positively affected seed germination and plant abundance since *Retama* canopies buffer high temperatures, decrease incident irradiance (Moro et al. 1997), and decrease soil water evaporation (Domingo et al. 1999) through shade. However, there was an overall neutral effect of microhabitat on aboveground plant biomass, and the better properties of soils under *Retama* shrubs appeared as the main responsible of the higher plant biomass.

2.8. CONCLUSIONS

We conclude that soil origin, microhabitat and soil microorganisms play a role in determining plant community structure under the nurse shrub species. The effect of soil microorganisms from *Retama* shrubs on seed germination ranged from positive to neutral to negative depending on plant species identity, while soil properties and microhabitat amelioration under *Retama* shrubs promoted seed germination of all species. The abundance and growth of plants were more affected by soil properties and the microbial community than by microhabitat under *Retama* shrubs. Thus, our results showed that soil and its microorganisms are major drivers of facilitation processes in this arid environment.

2.9. APPENDIX

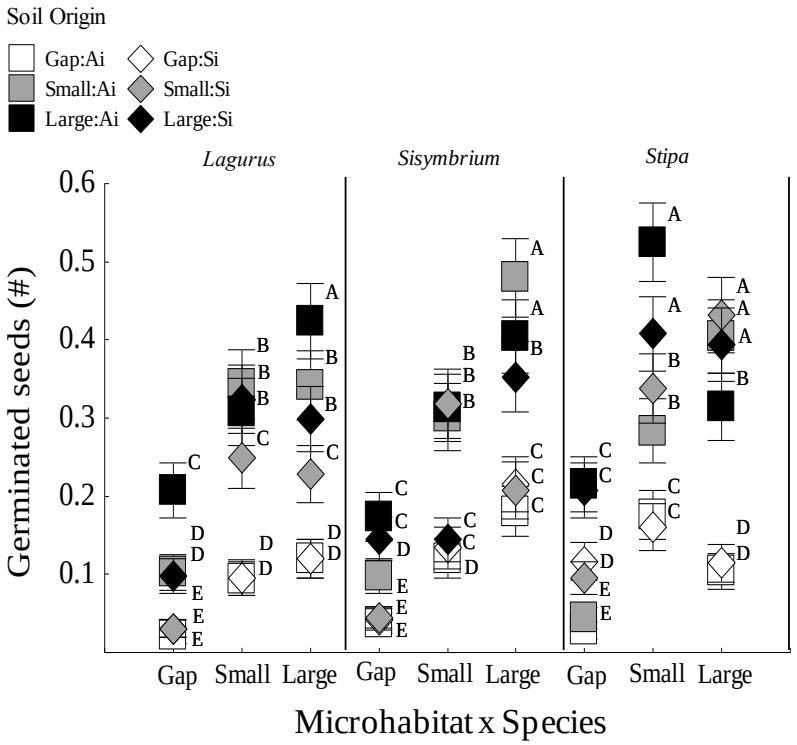

Fig. S2.1. Results from generalized mix model of mean number of *Lagurus, Sisymbrium* and *Stipa* germinated seeds in gaps or under small and large *Retama* shrubs, and growing on soils from gaps, small or large *Retama shrubs.* Ai= alive inoculum, Si = sterile inoculum. Data are mean ± 1 SE, n = 8. Symbols with different letters indicate significant differences among treatments after post-hoc comparisons at a significance level of 0.05.

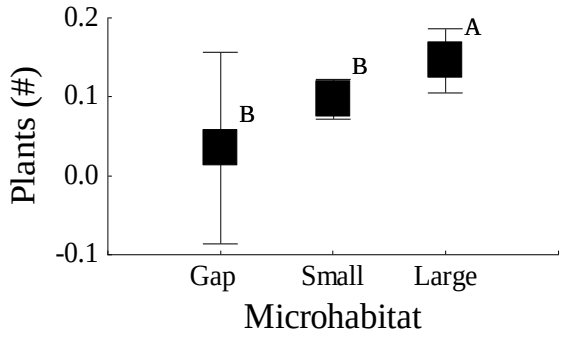

Fig. S2.2. Results of generalized mix model of mean number of plants established per microcosm in gaps and under small or large *Retama* shrubs after 5 months from sowing (total of 300 seeds per microcosm). Data are mean ± 1 SE, n = 8. Symbols with different letters indicate significant differences among treatments after post-hoc comparisons at a significance level of 0.05.

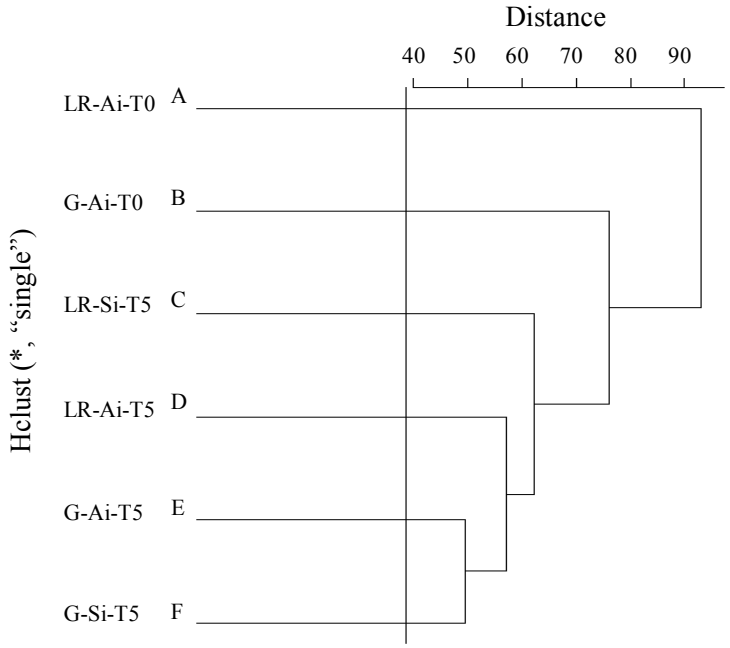

Fig. S2.3 Hierarchical cluster analysis of the OTUs of bacterial community in soils collected from gaps (G) and from under large *Retama* shrubs (LR) watered with alive (Ai) or sterile (Si) inoculum at the beginning (T0) and end of the experiment (T5) using multivariate mean comparisons. Cut off criterion obtained with the gDGC test is indicated with a vertical line. Different letters indicate groups statistically differing at a significance level of 0.05.

Fixed factors	Chi-square	df	p-value
(a) Number of germinated seeds			
Microhabitat (M)	22.5	2	**<0.0001**
Soil Origin (So)	16.44	2	**0.0003**
Inoculum (I)	1.87	1	0.1717
Plant Species (Ps)	56.98	2	**<0.0001**
M x So	78.75	4	**<0.0001**
M x I	0.53	2	0.768
M x Ps	37.17	4	**<0.0001**
So x I	14.47	2	**0.0007**
So x Ps	26.94	4	**<0.0001**
I x Ps	39.81	2	**<0.0001**
M x So x I	24.03	4	**0.0001**
M x So x Ps	70.23	8	**<0.0001**
M x I x Ps	24.01	4	**0.0001**
So x I x Ps	16.31	4	**0.0026**
M x So x I x Ps	41.75	8	**<0.0001**
(b) Number of plants			
M	16.45	2	**0.0003**
So	62.73	2	**<0.0001**
I	0.03	1	0.861
Ps	914.14	2	**<0.0001**
Number of neighbors	806.76	1	**<0.0001**
M x So	2.88	4	0.5774
M x I	7.10E-04	2	0.9996
M x Ps	8.35	4	0.0797
So x I	3.9	2	0.1423
So x Ps	25.93	4	**<0.0001**
I x Ps	0.55	2	0.7596
M x So x I	7.22	4	0.1249
M x So x Ps	10.39	8	0.2386
M x I x Ps	3.43	4	0.4891
So x I x Ps	1.94	4	0.7468
M x So x I x Ps	9.3	8	0.3177

Fixed-factors	F	df	p-value
(c) Aboveground biomass			
M	0.58	2/21	0.5674
So	17.19	2/105	**<0.0001**
I	0.07	1/105	0.7934
Ps	54.4	2/250	**<0.0001**
Number of neighbors	12.58	1/250	**0.0005**
M x So	1.82	4/105	0.1312
M x I	0.67	2/105	0.5163
M x Ps	5.89	4/250	**0.0002**
So x I	1.74	2/105	0.18
So x Ps	2.38	4/250	**0.0521**
I x Ps	1	2/250	0.3707
M x So x I	1.21	4/105	0.3107
M x So x Ps	2.52	8/250	**0.0118**
M x I x Ps	1.03	4/250	0.3933
So x I x Ps	0.56	4/250	0.6906
M x So x I x Ps	1.68	8/250	0.1026

Table S2.1. Results from generalized linear mix models on seed germination twenty days after sowing (a) and number of plants established at the end of the experiment (b); and general linear mixed model of aboveground biomass (c) at the end of the experiment (five months after sowing). Microhabitat (M), Soil Origin (So), Soil microbial inoculum (I) and Plant Species (Ps) and their interactions were considered fixed factors. All significant results are marked in bold.

3. INTERACTIONS AMONG SOIL, PLANTS, AND MICROORGANISMS DRIVE SECONDARY SUCCESSION IN A DRY ENVIRONMENT

Lozano Y.M., et al., (*in press*) Interactions among soil, plants, and microorganisms drive secondary succession in a dry environment. *Soil Biology & Biochemistry* (2014)

3.1. ABSTRACT

Secondary succession studies have mainly focused on plants, but little is known about the fate of soil microbial communities and their relationship with plant succession after disturbance, particularly in dry ecosystems. We examined changes in soil properties and of plant and soil microbial communities across a chronosequence of abandoned arable fields that included five successional stages according to time of abandonment stretching near a century. We hypothesized the existence of a parallel secondary succession above- and below-ground and explored the possible linkages between plant and microbial communities as well as the role of changes in soil properties over the successional gradient. Soil microbial communities were characterized by PLFAs analysis, enzymatic activities, and pyrosequencing of the 16S rDNA. We found clear patterns of plant and microbial secondary succession characterized by an increase in organic C, NH_4^+, and silt content as well as in soil microbial biomass and activity along the successional stages, linked to an increase in plant productivity and diversity. Plant and microbial composition were significantly different among successional stages, although no distinct microbial communities were observed in the two initial stages, suggesting that microbial succession may lag behind plant succession. However, the degree of change in the composition of soil microbial communities and plant communities across our chronosequence evidenced that above- and below-ground secondary succession developed with similar patterns and correlated with changes in multiple ecosystem functions such as increases in above-and below-ground productivity, diversity and nutrient accumulation as plant and microbial succession progressed.

3.2. INTRODUCTION

Plant secondary succession has been considered as a process of little applicability in arid environments (Cramer and Hobbs 2007) or even non-occurring (Rowlands 1980). Several reports have shown that plant secondary succession does actually occur in these extreme habitats (e.g. Bonet 2004, Scott and Morgan 2012) but our knowledge on secondary processes in arid environments is still poor, especially in comparison to more temperate regions (Abella 2010) where plant succession often shows a relatively rapid and predictable trajectory in species diversity and composition (e.g., Foster and Tilman 2000).

Soil microbial communities also change over time as it has been shown in different environments, from soils deglaciated only 20 years ago (Nemergut et al. 2007), to of *ca.* 77000 years old inland dunes (Tarlera et al. 2008). Changes in microbial community composition with time are influenced by factors such as carbon inputs, plant-microbial interactions (Tarlera et al. 2008) competition (Nemergut et al. 2007), soil variables such as pH, C, N and P concentrations (Banning et al. 2011) or land use history (Jangid et al. 2011). It has been suggested that plant community composition and soil chemistry explain different parts of the variation in soil microbial communities (Mitchell et al. 2012), but still little is known about the links between below-ground and above-ground succession processes.

The few existing studies on secondary succession in arid ecosystems have almost exclusively focused on the dynamics of plant communities, with little attention to soil microorganisms. However, interactions between plants and soil microorganisms may have important consequences for plant community dynamics, becoming key factors for community assemblage and ecosystem functioning (Kardol et al. 2013). We know that soil organisms influence plant community composition (Van der Putten et al. 2013), affecting plant performance either positively (Bever 2003, Rodríguez-Echeverría et al. 2013) or negatively (Klironomos 2002); and that plants in turn influence microbial communities and drive changes in physico-chemical soil properties (Van der Putten et al. 2013). Published reports on soil microbial changes with time concerned temperate ecosystems but never, to our knowledge, dry environments. Such reports, however, lacked enough information (e.g., microbial biomass, activity and composition) to help explain such variations.

3.3. OBJECTIVES

Our specific objectives were 1) to characterize changes in plant communities, soil properties and soil microbial communities at different times after agricultural abandonment, and evaluate whether changes follow a successional pattern; 2) to elucidate whether changes in plant and microbial community mirrored each other and; 3) to explore linkages among soil properties and plant and microbial communities along the chronosequence.

3.4. HYPOTHESIS

We hypothesized the existence of a parallel secondary succession above- and below-ground and we expected to find a process of succession in below-ground soil communities intimately linked to above-ground plant succession in plants.

3.5. METHODS

3.5.1. Study area

The field site was located at Llanos de Rueda (37.05º N, 2.22º W, 503 m altitude) a flat piedmont of approximately 120 ha in the Tabernas Basin, Almería, Spain. The climate is semiarid with a mean annual precipitation of 235 mm, mild winter temperatures (mean minimum temperature of 4.1ºC) and hot summers (average maximum temperature of 34.7ºC) (Lázaro et al. 2001). Extreme air temperatures above 45ºC and below freezing temperatures are not uncommon in the hottest and coldest months, respectively (Spanish National Meteorological Institute 2012). Soil parent material is a gypsum siltstone. Soils are orthic solonchacks with inclusions of calcic regosols, characterized by very low water holding capacity, low organic matter content, moderately alkaline pH (8.5) and low electrical conductivity (Pérez Pujalte et al. 1987).

The plant community is a sparse and short shrubland with low cover dominated by shrubs like *Artemisia barrelieri* (Besser), *Hammada articulata* (Moq.) O. Bolòs & Vigo, *Helianthemun almeriense* (Pau), *Salsola oppositifolia* (Desf.), *Thymelaea hirsuta* (L.) Endl, and perennial grasses as *Stipa tenacissima* (L.) and *Lygeum spartum* (L.) Kunth (Peinado et al. 1992).

3.5.2. Chronosequence selection

To define the chronosequence we used information from three different sources, (i) land use maps of the study area from 1928 (scale 1:25000) and 1949 (scale 1:5000) (Geographic and Cadastral Institute of Spain); (ii) orthophotos from 1956, 2000 and 2009, registered in the Environmental Information Network of Andalucía (REDIAM) with spatial reference ETRS89_30 and a geometric resolution of 1.0 m (years 1956 and 2009) or 0.5 m (year 2000) (Fig. 3.1); and (iii) direct field assessment performed in 2012.

Both land use maps and orthophotos were digitized with ArcGIS 10.0 (ESRI, Redlands, California, USA) using clustering analysis to group objects with similar features. Objects were attributed to land uses taking into account shadow tone, color, shape, texture features and geometric resolution (Mitchell 1999). Attributed land uses were checked by field assessment, available information in the literature, historical records from the Almería Historic Archive, and confirmed by interviews with people with first-hand knowledge of the area.

All digitized maps were overlapped in order to identify changes in land use. We recorded areas used as croplands anytime in the past and recorded their dates of abandonment (Fig. S3.1). Each of the identified areas was assigned into one of the following five categories according to the date of abandonment, i.e. the last 3, 12, 56, 63 years and >84 years or native grasslands. The most recently abandoned fields were marginally cultivated to sustain game bird populations, while traditional agricultural use consisted on non-irrigated crops for human subsistence. We considered native grasslands as the endpoint of succession, and grouped in this category any areas not cultivated after 1928 (i.e. areas abandoned more than 84 years ago).

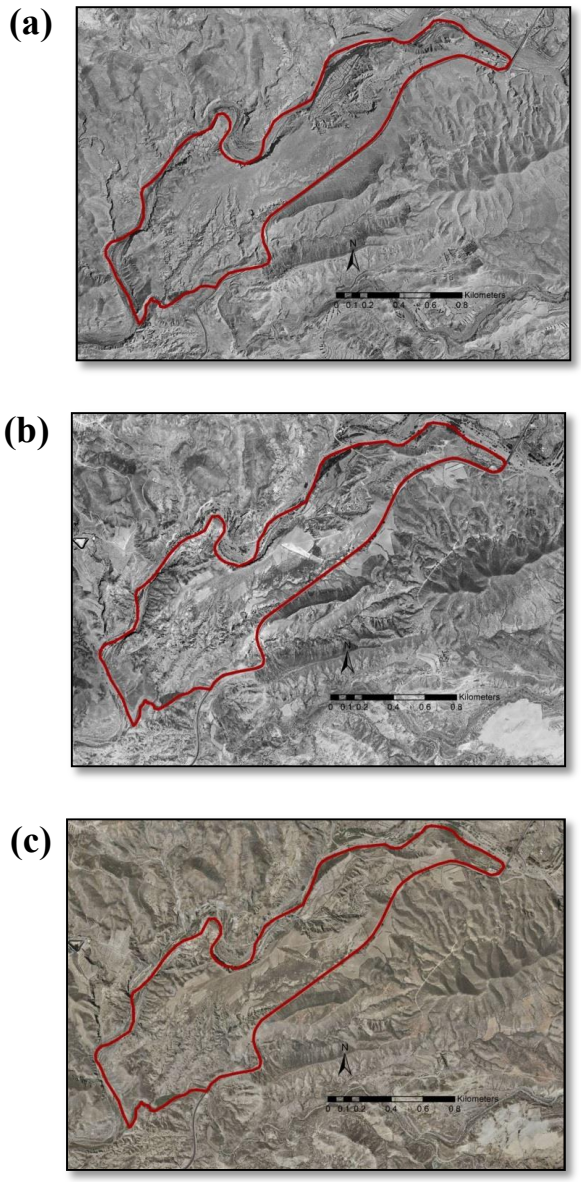

Fig. 3.1. Orthophotos of the study area in 1956 (a), 2000 (b), and 2009 (c). The boundary limits of the field site are shown in red.

Five 30 m^2 plots were randomly selected in each identified stage, giving a total of 25 plots. All plots were located as close as possible (maximum distance of 1 Km), and shared similar soil, climatic conditions and topographic position.

3.5.3. Plant community composition and soil sampling

We surveyed plant communities in May 2012 using transects. In each of the 25 plots we randomly placed five transects 25 m in length. We identified species found along transects and measured the length of intercepted segments (*i.e.* the transect length occupied by a given species). For each perennial species we calculated the percent cover by transect and then the average percent cover by plot. We also recorded the number of individuals for each perennial species in each plot as the sum of individuals recorded in the five transects per plot, and assessed plant diversity using the Shannon's diversity index. All taxa were identified to species level.

Seven soil cores, 4.5 cm in diameter and 10 cm deep, were collected at regular distances along each transect, combined, homogenized and sieved through 2 mm mesh to form one composite soil sample per plot. Each of the 25 composite soil samples collected was divided in two subsamples, one (approximately 100 g) was stored at -20ºC for soil microbial molecular analyses following and Tscherko et al. (2005), Hortal et al. (2013), and the other (approximately 400 g) was kept at 4ºC for physical and chemical analyses. Samples were processed within four weeks after collection.

3.5.4. Soil analyses

Soil electrical conductivity (EC) and pH were measured in each soil sample using a 1/10 (w/v) aqueous solution with a conductivity- and pH-meters (Crison, BA, Spain), respectively. Total soil carbon (C), organic C after removal of inorganic carbon with HCL 2N (Schumacher 2002), and total nitrogen (N) content were determined using a C/N analyzer (LECO Truspec, MI, USA). Anion phosphate (PO_4^{3-}), nitrate (NO_3^-), and sulphate (SO_4^{2-}) concentrations in water extract (1:10 soil:water) were analized by HPLC (Metrohm, HE, Switzerland). Soil ammonium content (NH_4^+) was calculated from the urease activity (below). Percentage of clay, sand and silt were measured by granulometry.

3.5.5. Bacterial composition: pyrosequencing

DNA was extracted from 0.25 g of homogenized soil from each of the 25 soil samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) following the manufacturer's directions. Following Hortal et al. (2013) a 16S rDNA gene fragment corresponding to V1 and V2 regions was amplified. The forward primer (5´-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNAGAGTTTGATCMTGGCTCAG-3´) contain Roche pyrosequencing adapter A (underlined), a Roche recommended 10 bp barcode 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. Each sample was amplified in triplicate, pooled, purified and DNA concentration determined following Hortal et al. (2013). An equimolar pool was prepared and pyrosequenced in a Roche Genome Sequencer FLX System (Roche, Basel, Switzerland) using 454 Titanium chemistry at Lifesequencing lab (Valencia, Spain).

Sequences were trimmed for primers, quality filtered and demultiplexed using the pyrosequencing pipeline tools from the Ribosomal Database Project (RDP, Michigan State Univ., USA). Sequences shorter than 150 bp, with quality scores <20 or containing any unresolved nucleotides were removed from the dataset. 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. 2012) and removed from the dataset. Sequences were aligned using the Aligner tool from the RDP 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. Taxonomic assignation of sequences was performed using the RDP naïve Bayesian classifier trained on the 16S training set 9 (Wang et al. 2007) at a confidence level of 80 %, and relative abundances of the different phyla, classes, subclasses and order per each of the 25 samples were calculated.

3.5.6. Microbial biomass and activity

Phospholipids fatty acids (PLFAs) were analyzed following Hortal et al. (2013). The $Gram^+$ specific fatty acids i15:0, i16:0, a15:0, i17:0, a17:0 and the $Gram^-$ specific fatty acids 16:1ω, 18:1ω9c, 18:1ω9t, cy17:0, cy19:0 were taken as a measure of the ratio between the $Gram^+$ and $Gram^-$

bacterial biomass. Fatty acids i15:0, i16:0, 18:1ω9c, 18:1ω9t, cy17:0 and cy19:0 were chosen to represent the bacterial biomass and 18:2ω6 was taken to indicate fungal biomass (Dungait et al. 2011).

Soil basal microbial respiration was analyzed by placing 15 g of soil moistened to 40–50% of its water holding capacity (water potential: -0.055 MPa) in hermetically sealed flasks and incubated for 24 days at 28°C. We measured the CO_2 accumulated in the air every day for the first 10 days and then weekly using an infrared gas analyzer (Toray PG-100, Toray Engineering Co. Ltd., Japan). Results were added to give a cumulative amount of CO_2 released after 24 days of incubation, and basal respiration was expressed as mg CO_2–C kg^{-1} soil day^{-1}. Soil dehydrogenase activity was determined on 1 g of soil, and the reduction of p-iodonitrotetrazolium chloride (INT) to p-iodonitrotetrazolium formazan (INTF) was measured by a modification of the method reported by Vonmersi and Schinner (1991) and was expressed as μg INTF g^{-1} soil h^{-1}. Urease activity was determined as the $NH4^+$ released in the hydrolytic reaction using urea as substrate and borate buffer (pH 10) (Kandeler and Gerber 1988). Alkaline phosphatase and β-glucosidase activities were determined following methods modified by Lucas-Borja et al. (2011), adding 2 ml of modified universal buffer (MUB) pH 11 and 0.5 ml of 0.115 M p-nitrophenyl phosphate (for the phosphatase assay) or 2 ml of MUB pH 6 and 0.5 ml of 0.025 M p-nitrophenyl phosphate β-D-glucopiranoside (for β-glucosidase assay) to 0.5 g of soil. The mixtures were then incubated at 37ºC for 1 h and, after that; enzymatic 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 phosphatase) or 2 ml of 0.1 M Tris–hydroxymethylaminomethane–sodium hydroxide (THAM-NaOH) pH 12 (for β-glucosidase) were added. Substrates were added before (in samples) and after (in controls) the addition of $CaCl_2$, NaOH and THAM. Microbial activity per unit of biomass was calculated using the ratio of basal respiration to total PLFAs.

3.5.7. Statistical analyses

Differences in plant cover, plant diversity, soil properties, microbial activity (basal respiration and enzymatic activities), microbial biomass (PLFAs groups), bacterial diversity, and relative abundance of bacterial taxa along successional stages were evaluated using general linear models. Significance was established at $p<0.05$. Post-hoc comparisons were performed using Fisher's LSD test. Results are presented as mean values ±1 SE throughout the text. Similarity among treatments in plant community composition (perennial plant species) or bacterial

community composition (with OTUs showing at least 5 reads in the overall dataset) were analyzed with Non-metric multidimensional scaling (NMDS) using Bray-Curtis similarity index, and differences among chronosequence stages were evaluated performing NPMANOVA with 9999 permutations using Past v 2.12 software (Hammer et al. 2001). The correlation between the first coordinates of each NMDS on microbial and plant community composition was analysed using Pearson's coefficient. Shannon's diversity index (H') of OTUs per sample was calculated with Past software after excluding singletons (OTUs only showing 1 read in the overall dataset) to reduce the overestimation of diversity (Tedersoo et al. 2010). We performed hierarchical cluster analyses of the relative abundance of bacterial community genera in each treatment using Bray-Curtis similarity index. We also performed a principal component analysis (PCA) and correlation matrix analysis of plant cover, soil properties, plant and microbial composition, microbial biomass and activity. Data were analyzed with InfoStat (Di Rienzo et al. 2013).

3.6. RESULTS

3.6.1. Plant community composition

Plant cover significantly increased with increasing time of abandonment, ranging from 0.39% just after agriculture cessation to 79.50% in native grasslands (Table 3.1). Diversity of perennial species showed a similar trend, and increased with increasing time of abandonment (Table 3.1). A total of 20 perennial species were found along the different stages. In each successional stage (ranked in increasing time since abandonment) we found 4, 9, 12, 15 and 17 species, respectively (Table 3.1).

The set of dominant perennial species changed over time (Table 3.1). *Launaea fragilis* and *Cynodon dactylon* were the dominant species during the first successional stage (3 years). In the second (12 years), *Thymelaea hirsuta* became dominant along with *Artemisia barrelieri*. In the 56 years stage, *Artemisia barrelieri* was highly dominant, covering 17.96%, which represented more than half the total cover (25.92%). *Salsola oppositifolia* and *Hammada articulata* were the dominant species in the 63 years stage. In native grasslands, the grass species *Stipa tenacissima* and *Lygeum spartum,* and the shrub species *Helianthemun almeriense* and *Anthyllis cytisoides* were dominant. Ordination of plant communities (Fig. 3.2a) showed clear differences among successional stages in species composition ($F_{4,20}$=17.46, p<0.001, NPMANOVA).

3.6.2. Soil properties

Differences in soil chemistry and texture were observed along the successional gradient (Table 3.2). Several parameters, *i.e.* soil EC, NH_4^+/NO_3^- ratio, organic C, NH_4^+, and silt contents increased gradually along the chronosequence while soil NO_3^- content decreased in the first three successional stages and then remained steady. Total N and NO_2 content were higher in native grasslands than in the other previous stages. On the contrary, soil SO_4^{2-} and sand content decreased towards the latest successional stages. No significant differences were found regarding C/N ratio, C and clay content. Mean soil pH values ranged 8.32-8.73. Soil PO_4^{3-} content was very low in all samples, well under the detection threshold of the HPLC.

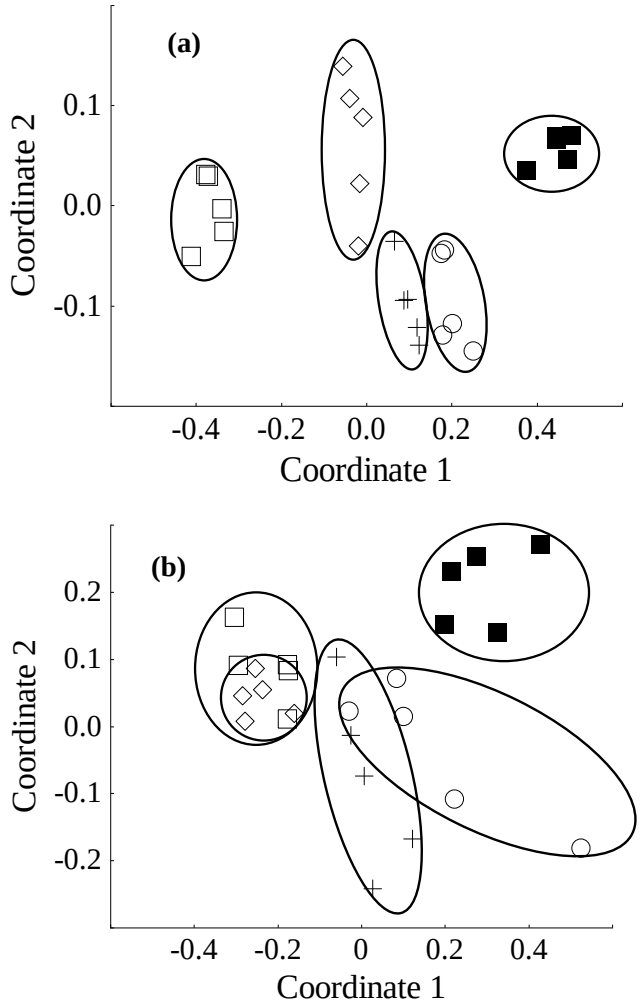

Fig. 3.2. Ordination of plant (cover of different perennial species) (a) and soil bacterial communities (OTUs abundance) (b) along successional stages by non-metric multidimensional scaling using Bray-Curtis similarity index. Only OTUs with at least 5 reads were included in the analysis. Field plots are coded by successional stage: Square (3 years), diamond (12 years), cross (56 years), circle (63 years) and solid square (native grassland). Stress value = 0.13 (a), 0.15 (b).

	Time of abandonment (years)					
Cover (%)	3	12	56	63	Native	$F_{4,20}$
Anthyllis cytisoides	–	–	–	0.24±0.24^{a} (1)	5.79±1.56^{b} (5)	**13.16*****
Artemisia barrelieri	0.02±0.02^{a} (1)	2.35±0.89^{a} (5)	17.96±3.28^{b} (5)	4.07±1.30^{a} (5)	1.33±0.84^{a} (5)	**19.17*****
Asparagus horridus	–	–	–	0.10±0.07^{a} (2)	0.07±0.02^{a} (4)	2.05^{ns}
Cynodon dactylon	0.12±0.08^{a} (2)	0.09±0.05^{a} (3)	0.13±0.13^{a} (1)	–	–	0.75^{ns}
Hammada articulata	–	0.02±0.02^{a} (1)	4.91±1.28^{b} (4)	11.01±1.70^{c} (5)	1.09±0.40^{a} (4)	**23.56*****
Helianthemun almeriense	–	–	0.54±0.35^{a} (2)	2.26±1.38^{a} (3)	17.38±1.16^{b} (5)	**83.30*****
Launaea arborescens	–	–	–	0.84±0.63^{a}(2)	0.22±0.22^{a} (1)	1.49^{ns}
Launaea fragilis	0.23±0.04^{a} (5)	1.30±0.67^{a} (5)	0.54±0.16^{a} (4)	0.48±0.29^{a} (2)	–	2.17^{ns}
Limonium insigne	–	–	–	–	0.70±0.29^{b} (5)	**5.84****
Lygeum spartum	–	–	–	–	7.08±3.51^{b} (5)	**4.08***
Phagnalon rupestre	–	–	0.09±0.06^{a} (3)	0.29±0.13^{b} (4)	0.02±0.02^{a} (1)	**3.79***
Phagnalon saxatile	–	0.02±0.02^{a} (1)	0.55±0.22^{a} (4)	2.57±0.82^{b} (5)	0.25±0.16^{a} (4)	**7.82*****
Rhamnus lycioides	–	–	0.02±0.02^{a} (1)	0.29±0.178^{a} (3)	0.04±0.04^{a} (1)	2.43^{ns}
Salsola genistoides	–	–	0.16±0.16^{a} (1)	0.82±0.51^{ab} (2)	1.95±0.91^{b} (5)	**3.12***
Salsola oppositifolia	0.02±0.01^{a} (3)	0.33±0.31^{a} (2)	0.57±0.42^{a} (3)	10.42±2.86^{b} (5)	0.71±0.38^{a} (4)	**11.69*****
Sedum sediforme	–	–	–	–	0.03±0.03^{a} (1)	1.00^{ns}
Stipa tenacissima	–	–	–	0.03±0.03^{a} (1)	42.38±3.33^{b} (5)	**161.94*****
Teucrium lusitanicum	–	0.03±0.03^{a} (1)	–	–	0.31±0.19^{a} (2)	2.53^{ns}
Thymelaea hirsuta	–	3.83±1.47^{b} (4)	0.30±0.30^{a} (1)	0.45±0.23^{a} (3)	–	**5.80****
Thymus hyemalis	–	0.01±0.01^{a} (1)	0.15±0.15^{a} (1)	0.02±0.02^{a} (1)	0.16±0.07^{a} (4)	1.11^{ns}
TOTAL	0.39±1.96^{a}	7.97±1.96^{b}	25.92±1.96^{c}	33.88±1.96^{d}	79.50±1.96^{e}	**249.19*****
Species richness	4	9	12	15	17	
Shannon index	0.37±0.12^{a}	0.97±0.12^{b}	1.01±0.12^{b}	1.62±0.12^{c}	1.67±0.12^{c}	**20.55*****

Table 3.1. Percent cover of plant species along the different successional stages. Data are mean ± 1 SE (n =5). '–' symbols indicate the absence of a plant species in a given successional stage. Last two rows show species richness and Shannon diversity index at each successional stage. Different letters in a row indicate significant differences among successional stages after Fisher's LSD test ($p<0.05$). The number of samples by successional stage where each particular species was present is included in parenthesis (maximum value = 5). The last column shows *F* values of the general linear model and significance (*, **, ***, at $p<0.05$, 0.01, 0.001, respectively; significant values in bold; ns = non-significant)

Soil properties	Time of abandonment (years)					$F_{4,20}$
	3	12	56	63	Native	
pH	8.43±0.15^{ab}	8.65±0.02^{a}	8.43±0.07^{b}	8.73±0.06^{a}	8.32±0.10^{b}	**5.75****
EC (µS/cm)	160.82±1.88^{a}	165.20±1.88^{ab}	172.64±1.88^{c}	168.65±1.88^{bc}	179.05±1.88^{d}	**13.86*****
OC (g kg $^{-1}$)	4.69±0.22^{a}	4.93±0.29^{ab}	7.55±1.28^{bc}	5.50±0.14^{b}	9.55±0.33^{c}	**42.63*****
N (g kg $^{-1}$)	0.65±0.06^{a}	0.40±0.06^{a}	0.70±0.18^{a}	0.55±0.17^{a}	1.14±0.07^{b}	**17.58*****
C (g kg $^{-1}$)	16.39±1.42^{a}	19.44±0.39^{a}	21.75±2.20^{a}	18.54±1.43^{a}	21.48±1.24^{a}	2.2^{ns}
C/N ratio	26.34±4.10^{a}	52.21±7.30^{a}	41.07±10.95^{a}	62.81±23.60^{a}	19.29±2.13^{a}	6.3^{ns}
NH_4^+ (mg/kg)	5.07±0.40^{a}	5.28±0.40^{ab}	5.72±0.40^{ab}	6.40±0.40^{b}	8.23±0.40^{c}	**10.03*****
NO_3^- (mg/kg)	1.87±0.19^{a}	1.28±0.19^{b}	0.63±0.19^{c}	0.68±0.19^{c}	0.82±0.19^{bc}	**7.33*****
NH_4^+/NO_3^-	2.84±0.66^{a}	4.49±0.66^{a}	9.21±0.66^{b}	9.55±0.66^{b}	10.44±0.66^{b}	**26.72*****
NO_2^- (mg/kg)	0.12±0.04^{a}	0.12±0.04^{a}	0.22±0.04^{ab}	0.12±0.04^{a}	0.31±0.04^{b}	**4.89** **
SO_4^{2-} (mg/kg)	14.01±0.64^{a}	13.62±0.64^{a}	15.29±0.64^{a}	10.07±0.64^{b}	11.13±0.64^{b}	**11.28*****
Sand (%)	76.77±2.03^{a}	74.92±2.03^{a}	71.32±2.03^{a}	73.97±2.03^{a}	63.69±2.03^{b}	**6.35****
Silt (%)	10.32±1.69^{a}	16.61±1.69^{b}	19.42±1.69^{b}	16.94±1.69^{b}	26.08±1.69^{c}	**11.27*****
Clay (%)	12.87±3.73^{a}	8.47±0.59^{a}	9.25±0.66^{a}	9.09±0.51^{a}	10.22±0.47^{a}	1.71^{ns}

Table 3.2. Soil properties along different successional stages, including soil electrical conductivity (EC) and organic carbon (OC). Values are mean ± 1 SE (n = 5). Different letters in a row indicate significant differences ($p<0.05$) among successional stages after Fisher's LSD test. The last column shows *F* values of the general linear model performed and its significance (*, **, ***, at $p<0.05$, 0.01, 0.001, respectively; all significant values in bold; ns = non-significant)

3.6.3. Microbial biomass and activity

We found an overall increase in microbial biomass with increasing time since abandonment (Table 3.3). Total microbial biomass showed the lowest values at the first (3 years) and second (12 years) stages and constantly increased afterwards. Fungal PLFAs were intermediate at 3 years, low at 12 years stage and high after 56 years of abandonment. Lowest values of bacterial biomass were also found at the 12 years stage, while the highest values were recorded in native grasslands. The highest ratio of fungi/bacteria was recorded 56 years after abandonment while the lowest values corresponded to the first two successional stages. There were no significant differences in the ratio $Gram^+$ / $Gram^-$ across successional stages. We also found an overall increase in soil microbial activity with increasing time since abandonment, with the highest values of phosphatase, β-glucosidase and urease activities recorded in native grasslands (Table 3.3). Soil basal respiration was significantly higher after 56 years of abandonment than in the first 12 years of abandonment. Dehydrogenase activity was similar across successional stages.

	Time of abandonment (years)					
	3	12	56	63	Native	$F_{4,20}$
Microbial biomass						
Fungi	0.33±0.04^{b}	0.12±0.01^{a}	0.62±0.13^{c}	0.66±0.14^{c}	0.79±0.06^{c}	**44.06*****
Bacteria	1.93±0.26^{b}	1.06±0.26^{a}	2.11±0.26^{b}	2.58±0.26^{b}	3.73±0.26^{c}	**14.67*****
Gram+	0.77±0.08b^{c}	0.43±0.08^{a}	0.66±0.08^{b}	0.92±0.08^{c}	1.21±0.08^{d}	**14.35*****
Gram-	1.16±0.20^{ab}	0.63±0.20^{a}	1.45±0.20^{b}	1.66±0.20^{b}	2.52±0.20^{c}	**11.86*****
Total	8.21±0.37^{a}	7.21±0.37^{a}	10.77±0.37^{b}	12.53±0.37^{c}	16.65±0.37^{d}	**102.19*****
Gram+/Gram-	0.71±0.12^{a}	0.86±0.12^{a}	0.50±0.12^{a}	0.57±0.12^{a}	0.48±0.12^{a}	1.93^{ns}
Fungi/Bacteria	0.18±0.02^{ab}	0.13±0.02^{a}	0.28±0.02^{d}	0.25±0.02^{cd}	0.21±0.02^{bc}	**7.98*****
Microbial activity						
Basal respiration (mg CO_2-C kg^{-1} day^{-1})	4.20±0.58^{a}	5.73±0.58^{a}	9.08±0.58^{b}	9.18±0.58^{b}	8.37±0.58^{b}	**14.77*****
Phosphatase (µmol PNP g^{-1} h^{-1})	0.73±0.12^{a}	1.19±0.12^{b}	1.27±0.12^{b}	1.28±0.12^{b}	2.48±0.12^{c}	**29.38*****
β-glucosidase (µmol PNP g^{-1} h^{-1})	0.38±0.05^{a}	0.48±0.05^{ab}	0.57±0.05^{b}	0.53±0.05^{ab}	0.75±0.05^{c}	**6.21****
Urease (µmol NH_3 g^{-1} h^{-1})	0.82±0.12^{ab}	0.74±0.12^{ab}	0.62±0.12^{a}	0.97±0.12^{b}	1.65±0.12^{c}	**12.94*****
Dehydrogenase (µmol INTF g-1 h^{-1})	0.63±0.10^{a}	0.81±0.10^{a}	0.80±0.10^{a}	0.67±0.10^{a}	0.56±0.10^{a}	1.10^{ns}
BR/total PLFA	0.51±0.06^{b}	0.80±0.06^{a}	0.85±0.06^{a}	0.74±0.06^{a}	0.50±0.06^{b}	**8.50** ***

Table 3.3. Microbial biomass (PLFA groups) and activity (basal respiration [BR] and enzymatic activities) along the successional stages. Microbial biomass results are in nmol g^{-1}. Values are mean ± 1 SE. Different letters in a row indicate significant differences ($p<0.05$) among successional stages after Fisher's LSD test; n =5. Last column shows *F* values of the general linear model performed and its significance (*, **, ***, at $p<0.05$, 0.01, 0.001, respectively; all significant values in bold; ns = non-significant)

3.6.4. Soil bacterial community composition

We obtained a total of 71524 sequences after filtering and removing chimeras. The mean number of retained sequences per sample was 2860 ± 135 (mean ± SE), with no significant differences among successional stages ($F_{4,20}$=2.09, p=0.12, ANOVA). Average length of retained sequences was 322 ± 0.23 bp. The slope of rarefaction curves was similar for all samples regardless of the treatment (Fig. S3.1). Sequences mostly belonged to five different phyla, *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, *Bacteroidetes* and *Gemmatimonadetes.* Other minor phyla (with relative abundance lower than 1%) were *Chloroflexi*, *Firmicutes*, *Armatimonadetes*, *Cyanobacteria*, *Nitrospira* and *TM7.*

Relative abundances of the different taxa differed among successional stages (Fig. 3.3). *Actinobacteridae* decreased in the last two successional stages and *Bacteroidetes* and *Deltaproteobacteria* showed the lowest relative abundance in native grasslands. On the contrary, *Acidobacteria* increased in the last two successional stages and *Gemmatimonadetes* showed the highest relative abundance in native grasslands. *Betaproteobacteria* was higher at the intermediate (56 years) stage than at the stages immediately before and after (12 and the 63 years). Among minor phyla, *Firmicutes* decreased with time while relative abundance of both *Cyanobacteria* and *Armatimonadetes* were significantly higher at the 12 and 63 years stage than at the others (data not shown). There were no significant changes in the relative abundance of *Acidimicrobidae, Rubrobacteridae* (subclasses within *Actinobacteria*), *Alphaproteobacteria, Gammaproteobacteria*, *Chloroflexi, Nitrospira, TM7* or unclassified bacteria. Bacterial diversity (Shannon's index) was 6.68 ± 0.02, without significant differences among successional stages.

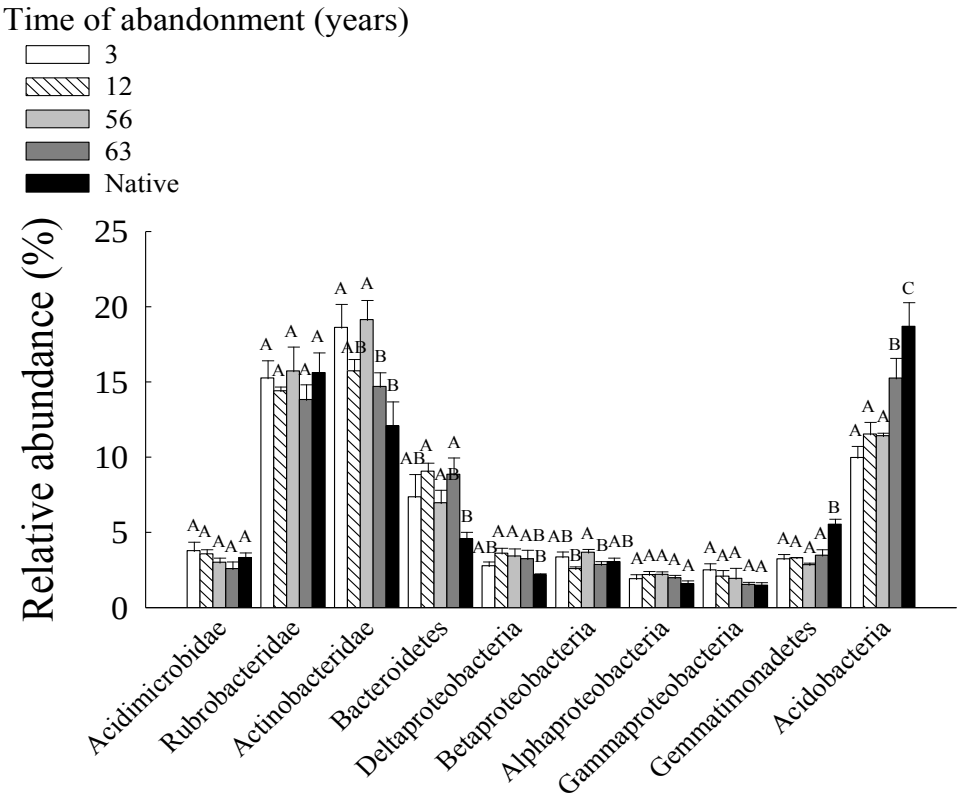

Fig. 3.3. Mean relative abundance (± 1 SE) of bacterial taxonomic groups, i.e. phyla *Bacteroidetes*, *Gemmatimonadetes* and *Acidobacteria*, classes *Deltaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* (within *Proteobacteria* phylum), subclasses *Acidimicrobidae*, *Rubrobacteridae* and *Actinobacteridae* (within *Actinobacteria* phylum) along different successional stages. Different letters within a bacterial group indicate significant differences ($p < 0.05$) among treatments after Fisher's LSD test; n=5.

We identified 10034 distinct operational taxonomic units (OTUs) at 97% similarity. Ordination of (OTUs) (Fig. 3.2b) with a minimum of 5 reads within the overall dataset (3108) and cluster hierarchical analysis of relative abundance of bacterial genera (>1%; Fig. S3.2) showed marked differences in bacterial community composition among successional stages ($F_{4,20}$=2.02, p<0.001, NPMANOVA), except for the first two which were similar. Same results were obtained when including all reads (data not shown).

3.6.5. Linkages among soil properties, plant and microbial communities

Ordination based on specific composition of both plant and microbial communities (Fig. 3.2a,b) showed that communities belonging to the same succesional stage clustered and that there were clear differences among successional stages, mainly spreading along coordinate 1. There was a strong correlation between coordinates 1 of microbial and plant communities' NMDS suggesting that both communities changed in parallel with time of land abandonment (r= 0.79; p<0.001).

Ordination of samples by PCA based on plant community composition, soil properties, microbial composition, biomass and activity showed a clear separation of successional stages along the first axis (Fig. 3.4), with the first two axes explaining 53.4% of total variance. The ordination stressed the trends observed in Tables 3.2 and 3.3, showing a strong and positive correlation of some soil properties, microbial activity and biomass with increasing time after abandonment. Of particular interest, we found positive correlations among the increase in plant cover with time, organic carbon and NH_4^+ contents, EC, silt content, microbial biomass, enzymatic activities, the relative abundance of the microbial groups *Gemmatimonadetes* and *Acidobacteria* and plant cover of the species *Stipa* and *Anthyllis.* On the other hand, sand content was positively correlated with the relative abundance of *Bacteroidetes* and *Deltaproteobacteria* while SO_4^{2-} content was positively correlated with the relative abundance of *Actinobacteridae*. (Fig 3.4, Table S3.1).

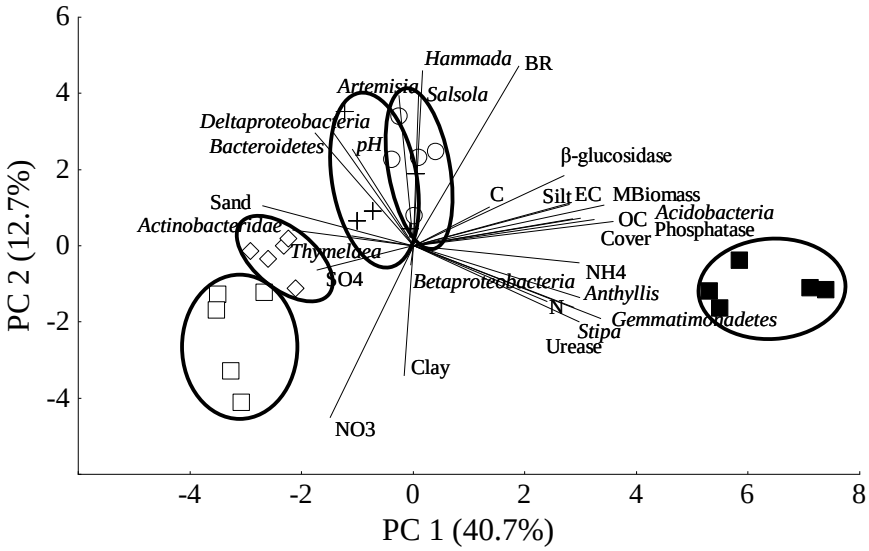

Fig. 3.4 Principal component analysis of plant cover, soil properties (electrical conductivity [EC], clay, sand, silt, SO_4^{-2}, NO_3^-, NH_4^+ pH, total nitrogen [N], organic carbon [OC], total carbon [C]), microbial composition (*Actinobacteridae, Bacteroidetes, Deltaproteobacteria, Betaproteobacteria, Acidobacteria, and Gemmatimonadetes*), total microbial biomass (M Biomass), microbial activity (Urease, Phosphatase, β-glucosidase, basal respiration [BR]) and plant composition (*Thymelaea hirsuta, Artemisia barrelieri, Hammada articulata, Salsola oppositifolia, Anthyllis cytisoides* and *Stipa tenacissima*), along the successional stages. The first two PCA axes explain 53.4 % of total variance. Field plots coded as in Fig. 3.1.

3.7. DISCUSSION

Our results evidence that secondary succession in arid environments did occur both below- and above-ground, in a process characterized by changes in soil microbial composition, biomass and activity that mirrored changes in plant species composition, diversity and cover. Plant and microbial communities changed with time of abandonment increasing ecosystem functions such as above- and below-ground productivity, diversity and soil nutrient accumulation as succession progressed.

3.7.1. Plant and microbial secondary succession

Plant communities in this semiarid environment undergo a secondary succession process characterized by an increase in species richness, diversity and productivity. Our results show a continuous increase in both species diversity and cover, in agreement with Bonet (2004). By contrast, other studies in dry environments have reported an initial increase in species richness and plant cover which remained steady or even decreased afterwards (e.g. Dana and Mota 2006, Otto et al. 2006, Scott and Morgan 2012). The continuous increase recorded in our field site may reflect that, although 84 years were enough to observe clear patterns of secondary succession, most likely they were not enough to re-assemble the native community due to extreme climatic conditions.

Our data also evidenced a clear pattern of succession in soil microbial communities including changes in species composition, biomass and activity. While Kuramae et al. (2011) showed a large overlap of microbial communities with no clear separation between successional stages in a chronosequence in chalk grasslands, our data show changes with time in relative abundance of several microbial groups. Microbial succession in our field site was also characterized by an increase in both fungal and bacterial biomass as well as in microbial activity over time, as reported for agricultural (Jangid et al. 2011) and moorland systems (Mitchell et al. 2012).

Our results showed that changes in composition of plant and microbial communities displayed a similar pattern along the successional stages (the multidimensional space in the ordination analyses). We found clear separation in plant and soil microbial communities among different successional stages, suggesting a successional process in both plant and microbial communities, and that both followed a similar path. Moreover, the set of 25 plot values in the first axes of the

nMDS of plant and soil communities were strongly correlated. That is, the degree of change in the composition of the soil microbial community with time mirrored that of the plant community, suggesting that plant and microbial successions might follow parallel processes.

3.7.2. Relationships among plant, microbial succession and soil properties

Our results showed that as both plant and microbial succession progressed, net primary productivity (measured through plant cover), soil microbial biomass and activity, and C and N content increased. In particular, plant and microbial succession in our field site were linked to an increase in soil organic C, NH_4^+ and silt contents. The increase in plant cover with time enhances the accretion of organic material such as litter and roots in soils, and consequently favours the accumulation of soil organic C (Pugnaire et al. 2004, Yang et al. 2009). Soil organic C reduces soil temperature amplitudes, increases soil water holding capacity and thus can enhance soil moisture under perennial plants (Chancellor et al. 1994, Duchaufour 1995). In addition, the increase in plant cover usually allows for better microclimatic conditions (temperature and soil moisture) under shrub canopies (Pugnaire et al. 2004), overall improving soil conditions.

Better soil conditions, promoted by the increase in plant cover, positively affected microbial communities in terms of biomass and activity, as reported for primary succession (Tscherko et al. 2005). Plant cover, through its effects on soil organic C and microclimatic conditions, promoted microbial growth and hence microbial biomass (Tortora et al. 2007). Plant cover may have also promoted higher soil respiration rates in later successional stages compared to initial stages where cover was lowest, in agreement with Conant et al. (2004) who suggested that soil respiration in semiarid areas is affected by temperature and soil moisture. The increase in phosphatase with time could be attributable to soil microbes trying to obtain the scarce phosphorus from organic sources (Tarafdar and Claassen 1988), while soil urease and β–glucosidase levels could be explained by an increase in plant residues and organic carbon returned to soil along the successional chronosequence.

Cover of dominant plant species in the last two successional stages, *Stipa* and *Anthyillis*, were positively correlated with microbial biomass, enzymatic activities silt, organic C contents and an abundance increase of *Gemmatimonadetes* and *Acidobacteria.* These results suggest that plant identity affects microbial biomass and community composition by modifying soil properties. Kowalchuk et al. (2002), and Martínez-García et al. (2011) found that plant species identity

determined the type of microorganisms found in the rhizosphere. *Acidobacteria* abundance increased with successional time as reported elsewhere (Tarlera et al. 2008) and *Gemmatimonadetes* were more abundant in native grasslands than in the other successional stages, as in other undisturbed and non-grazed semiarid pastures (Acosta-Martínez et al. 2010). Our results suggest that bacterial community composition and biomass were significantly affected by soil particle size, as reported by Sessitsch et al. (2001) who found higher biomass and abundance of *Acidobacteria* microbes in silt and clay than in sandy soils, while other microbial groups such as *Actinobacteria* were negatively correlated with silt content, as in our case. Agricultural practices may cause an accelerated loss of soil silt due to leaching (Urich 2002) and thus may indirectly increase the amount of sand in soils. However, plant colonization during secondary succession allows for an increase in soil retention thanks to a larger amount of roots (Carrick and Krüger 2007) and soil organic carbon, overall promoting the accretion (and retention) of silt particles in the soil.

Actinobacteria was the most abundant phylum across successional stages, and our results also showed an abundance of *Actinobacteridae* higher in the initial stages than in the last ones. *Actinobacteria* were positively correlated with SO_4^{-2} as reported by Jiang et al. (2010) in freshwater ecosystem. Members of *Actinobacteria* play an important role in cycling soil organic compounds and have evident impact on soil N and C. Species of *Streptomyces,* for example, were commonly found in our field site. They are known to be drought resistant (Köberl et al. 2013), can use many organic carbon compounds (Schlatter et al. 2013), and, although their role is likely minor relative to fungi (Rayner and Boddy 1988), they have a role in wood decomposition (Bontemps et al. 2013). The dominance by *Actinobacteria* in the first and intermediate stages correlates with their ability to colonize bare soil (Suela Silva et al. 2013), while their decrease at latter stages agrees with reports showing that *Actinobacteridae* are less abundant under shrubs than in patches without vegetation (Hortal et al. 2013).

On the contrary, *Bacteroidetes* and *Deltaproteobacteria* were less abundant in native grasslands than in early successional stages. Although *Bacteroidetes* exhibit copiotrophic attributes (Fierer et al. 2007) and *Mixococcales* (our most common *Deltaproteobacteria* order) inhabit soils on decaying organic material (Kersters et al. 2006) we did not find any positive correlation with soil organic carbon. Their abundance however was positively correlated with sand content. Soil texture is determinant in bacterial community structure (Girvan et al. 2003) and could explain changes in abundance of both groups here.

Agricultural practices (with large inputs of N into the system) also strongly affect biogeochemical cycles (Austin et al. 2006a) and, consequently, the trajectory of secondary succession. We recorded a decrease in NO_3^- content with successional time linked to land abandonment, with a parallel increase in NH_4^+ content and plant cover. Although Austin et al. (2006b) negatively correlated NH_4^+ with plant cover in arid Patagonia systems, our data show that larger levels of NH_4^+ could potentially positively affect C storage by plants (increasing net primary productivity), probably as many species prefer NH_4^+ as nitrogen source (Boudsocq et al. 2012). Opposite to McLendon and Redente (1992), who suggested that in semiarid ecosystems succession could be controlled by N limitation, our data show increasing N availability with time.

3.7.3. Microbial succession follows plant succession

Although our results suggest that plant and microbial succession processes developed in parallel, they also suggest that plant succession proceeded faster than microbial succession. We recorded changes in plant cover and diversity in the two first successional stages but not changes in soil microbial composition, suggesting that microbial succession might lag behind plant succession. Although it is assumed that plant and soil communities vary over similar time-scales (Bever 2003), the starting point of plant and microbial communities differs in abandoned agricultural fields. While plant secondary succession usually starts from disturbed, nude soil easy to colonize, microbial secondary succession starts from an established microbial community already present in former agricultural fields. Thus, the influence of plants on the established soil microbial community might have been low during the first successional stages when plant cover was low. This result contrasts to what happened in a temperate grassland (Kuramae et al. 2011), where the first stages but not intermediate and late stages showed microbial variation. As argued above, the increase in plant cover along the chronosequence would have influenced soil microbial community through the input of soil nutrients and organic C, buffering of temperature, improved soil moisture conditions, and accretion of small soil particles. As time since land abandonment increased, microbial communities might also have influenced plant performance either positively (Rodríguez-Echeverría et al. 2013) or negatively (Klironomos 2002) thereby establishing a feedback among plant and soil microbial communities as succession progressed.

3.8. CONCLUSIONS

Our data suggest that abandoned agricultural fields in this dry ecosystem undergo processes of secondary succession both below- and above-ground characterized by changes in soil properties, plant cover, microbial biomass and activity, plant and microbial community composition. The timing of changes in plant and microbial community composition suggest that microbial succession may follow plant succession. Thus, we suggest that secondary succession in dry environments is driven by the interaction between plants and soil microbial communities, and both their interplay and consequences warrant more attention.

3.9. APPENDIX

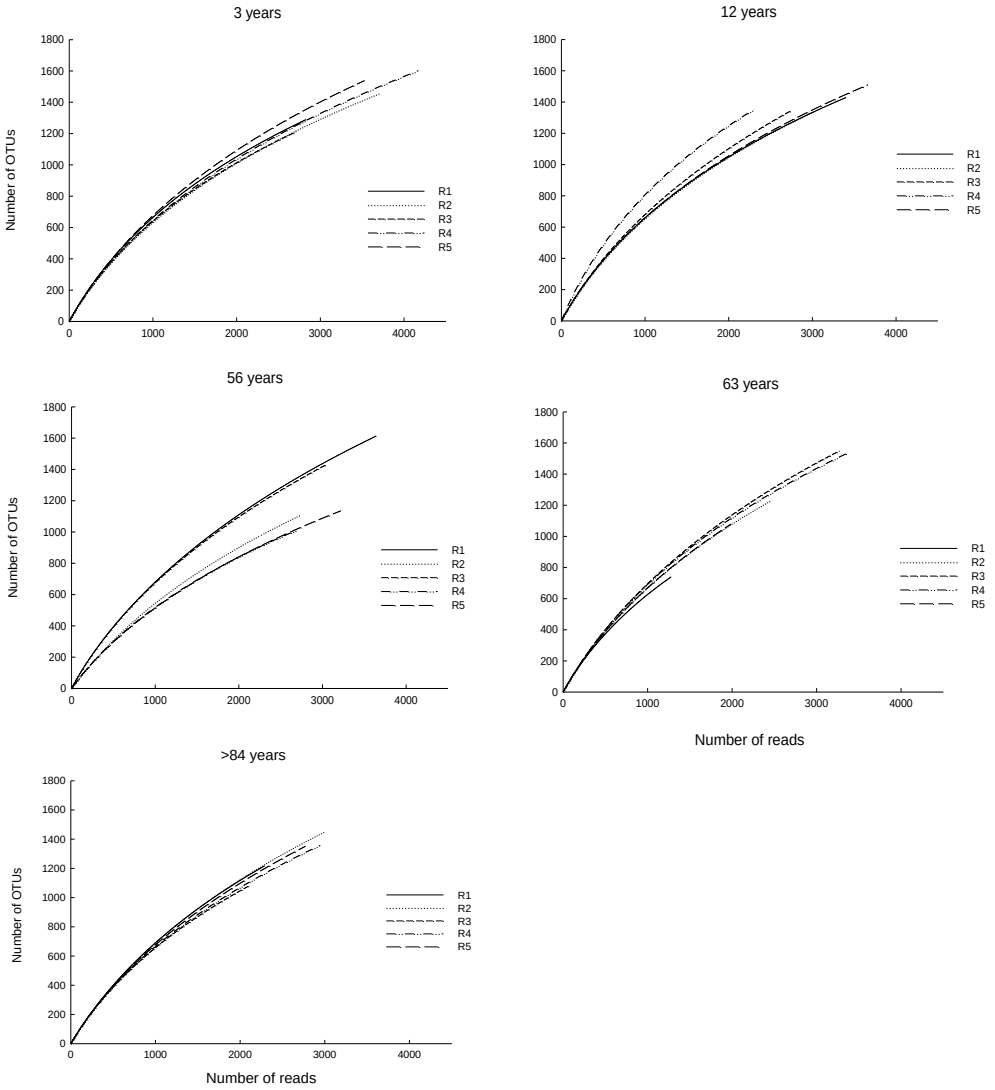

Figure S3.1 Rarefaction curves for each successional stage. R1-R5 indicate number of replicates (n=5). Operational taxonomic units (OTUs) were defined at 97% of similarity.

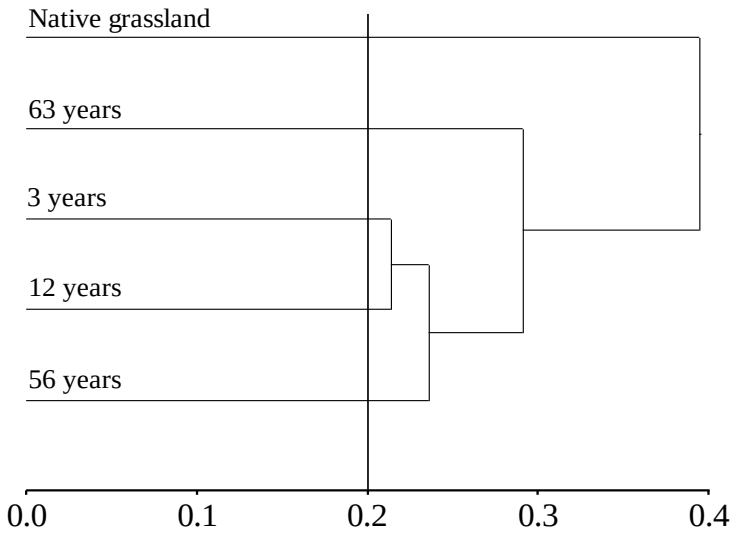

Figure S3.2. Hierarchical cluster analysis of the relative abundance of bacterial community genera in each treatment using Bray-Curtis similarity index. Cut off criterion is indicated with a vertical line. Cophenetic correlation=0.94.

	pH	EC	OC	N	C	NH_4^+	NO_3^-	SO_4^{2-}	Sand	Silt	Clay	BR	Phos	β-gluc	Urease
EC	ns														
OC	**-0.40***	**0.59****													
N	**-0.43***	ns	**0.69*****												
C	ns	ns	**0.56****	**0.41***											
NH_4^+	ns	**0.53****	**0.60****	**0.51****	ns										
NO_3^-	ns	**-0.52****	ns	ns	**-0.40***	ns									
SO_4^{2-}	ns	ns	ns	ns	ns	**-0.54****	ns								
Sand	ns	**-0.68*****	**-0.60****	**-0.65*****	**-0.55****	**-0.52****	ns	ns							
Silt	ns	**0.73*****	**0.61****	**0.48***	**0.60****	**0.44***	**-0.56****	ns	**-0.80*****						
Clay	ns	ns	ns	ns	ns	ns	**0.63*****	ns	ns	ns					
BR	ns	**0.50***	**0.52****	ns	ns	**0.49***	**-0.60****	ns	ns	ns	ns				
Phos	ns	**0.66*****	**0.74*****	**0.49***	ns	**0.77*****	ns	ns	**-0.56****	**0.65*****	ns	**0.56****			
β-gluc	ns	**0.48***	**0.69*****	ns	ns	**0.65*****	ns	ns	ns	**0.46***	ns	**0.71*****	**0.88*****		
Urease	ns	**0.46***	**0.45***	**0.49***	ns	**0.74*****	ns	**-0.54****	**-0.48***	**0.44***	ns	ns	**0.70*****	**0.57****	
Mbiomass	ns	**0.71*****	**0.73*****	**0.57****	ns	**0.76*****	**-0.47***	**-0.53****	**-0.65*****	**0.69*****	ns	**0.60****	**0.82*****	**0.70*****	**0.74*****
Actinob	ns	**-0.44***	ns	ns	ns	**-0.42***	ns	**0.60****	ns	**-0.49***	ns	ns	**-0.42***	ns	**-0.63*****
Betap	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Deltap	ns	ns	ns	ns	ns	ns	ns	ns	**0.41***	ns	ns	ns	ns	ns	ns
Bacter	ns	**-0.54****	ns	ns	ns	**-0.40***	ns	ns	**0.61****	**-0.41***	ns	ns	ns	ns	ns
Gemm	**-0.11***	**0.45***	**0.53****	**0.50***	ns	**0.66*****	ns	ns	**-0.45***	**0.46***	ns	ns	**0.71*****	**0.63*****	**0.87*****
Acidob	ns	**0.50***	**0.54****	**0.50***	ns	**0.68*****	ns	**-0.68*****	**-0.48***	**0.54****	ns	**0.49***	**0.69*****	**0.66*****	**0.76*****
Cover	ns	**0.80*****	**0.76*****	**0.59****	ns	**0.80*****	**-0.47***	**-0.48***	**-0.70*****	**0.75*****	ns	**0.56****	**0.9*****	**0.75*****	**0.76*****
Thymelaea	ns	ns	ns	**-0.42***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Artemisia	ns	ns	ns	ns	ns	ns	**-0.43***	**0.47***	ns	ns	ns	**0.52***	ns	ns	**-0.48***
Hammada	ns	ns	ns	ns	ns	ns	**-0.46***	**-0.40***	ns	ns	ns	**0.48***	ns	ns	ns
Salsola	**0.48***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.48***	ns	ns	ns
Anthyllis	ns	**0.62****	**0.64*****	**0.48***	ns	**0.65*****	ns	ns	**-0.50***	**0.52***	ns	ns	**0.74*****	**0.62****	**0.72*****
Stipa	ns	**0.63*****	**0.70*****	**0.63*****	ns	**0.75*****	ns	ns	**-0.68*****	**0.66*****	ns	ns	**0.87*****	**0.66*****	**0.84*****

	Mbiomass	Actinob	Betap	Deltap	Bacter	Gemm	Acidob	Cover	*Thymelaea*	*Artemisia*	*Hammada*	*Salsola*	*Anthyllis*
Actinob	**-0.47***												
Betap	ns	**0.43***											
Deltap	ns	ns	ns										
Bacter	**-0.41***	ns	ns	**0.59****									
Gemm	**0.69*****	**-0.67****	ns	ns	ns								
Acidob	**0.76*****	**-0.73*****	ns	ns	ns	**0.71*****							
Cover	**0.94*****	**-0.59****	ns	ns	**-0.45***	**0.76*****	**0.81*****						
Thymelaea	**-0.42*****	ns	ns	ns	**0.41***	ns	ns	ns					
Artemisia	ns	**0.40***	ns	**0.39***	ns	ns	ns	ns	ns				
Hammada	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
Salsola	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.58****		
Anthyllis	**0.75*****	**-0.42***	ns	**-0.41***	**-0.41***	**0.71*****	**0.60****	**0.78*****	ns	ns	ns	ns	
Stipa	**0.81*****	**-0.57****	ns	**-0.45***	**-0.53****	**0.86*****	**0.70*****	**0.89*****	ns	ns	ns	ns	**0.81*****

Table S3.1. PCA correlation matrix of soil microbial biomass, relative abundance of main bacterial groups, plant species cover, soil properties and microbial activity. (MBiomass: total microbial biomass; Actinob: Actinobacteridae; Betap: Betaproteobacteria; Deltap: Deltaproteobacteria; Bacter: Bacteroidetes; Gemm: Gemmatimonadetes; Acidob: Acidobacteria; Cover: Plant cover; EC: electrical conductivity; OC: organic carbon; BR: basal respiration; Phos: Phosphatase; β-gluc: β-glucosidase).The matrix is shown in two parts due to its size. Values correspond to Pearson correlations and their significance (*, **, ***, at $p<0.05$, 0.01, 0.001, respectively, all values in bold; ns = non-significant).

4. ARRESTED SUCCESSION AS OUTCOME OF THE INTERACTION BETWEEN PLANTS AND SOIL MICROORGANISMS

Lozano Y.M., Armas C., Hortal S., and Pugnaire F.I. Arrested succession as outcome of the interaction between plants and soil microorganisms. *In preparation*.

4.1. ABSTRACT

Causes of arrested succession, i.e., when few species dominate the community in such a way that succession seems stopped or delayed beyond reasonable time periods, are still unclear although a role for soil microbial communities is suspected in this phenomenon. The tussock grass *Lygeum spartum* frequently dominates extensive areas of abandoned fields in what seems arrested succession in an arid environment. We evaluated whether the competitive ability of this species is linked to its associated soil microbial community and soil properties or whether it is a consequence of the plant´s life strategy. We grew plants of this species along with individuals of *Salsola oppositifolia,* a shrub dominant in late successional stages, to release intra- and inter-specific interactions on sterile soil inoculated with either alive or sterile soil extracts collected under each of the two species. We established three parallel experiments according to different life history stages using seeds, saplings, and young adult individuals. At harvest, soil nutrient content and shoot mass were determined and microbial communities were characterized by pyrosequencing of the 16S rDNA. We found that both soil microbial communities and soil properties associated to *Lygeum* did not prevent but rather facilitated *Salsola* establishment. The competitive ability of *Lygeum* was linked to a positive effect of its own soil extracts on saplings and adult individuals and to a high and quick germination rate of *Lygeum* seeds in contrast to the slow pace and low germination rate of *Salsola* seeds. In conclusion, soil properties and soil microbial communities associated to *Lygeum* have an important influence on seed germination and initial growth of *Lygeum* individuals. Added to its clonal growth strategy, positive feedbacks with soil microroganisms allow for a quick dominance of this species and apparent arrested succession.

4.2. INTRODUCTION

Plant secondary succession takes place after disturbance, from tropical to temperate, and from savanna to artic or desert ecosystems (De Mera and Orellana 2007). Although for years it was thought that succession did not occur in arid environments due to extreme climatic conditions (Muller 1940, Rowlands 1980), several reports have shown that plant succession does also occur in such environments (Bonet 2004, Scott and Morgan 2012, Chapter III of this thesis). Arrested or truncated succession, which occurs when some species dominate the community in such a way that succession seems stopped or delayed beyond reasonable time periods (Young and Peffer 2010) has been described in tropical (Griscom and Ashton 2003, Boyes et al. 2011), temperate (Hill and Silander 2001, Mallik 2003), Mediterranean (Acácio et al. 2007), and arid (Walker et al. 2007) ecosystems.

Although the causes of strong dominance of a species could be manifold, species that lead to arrested succession show in most cases rapid vegetative spread (Royo and Carson 2006) and long lifespan (Young and Peffer 2010) that help them to become dominant in the plant community for long periods of time. Their dominance could also be enhanced as other species undergo dispersal limitation (Jordano and Godoy 2002) or seedling mortality due to herbivory (Boyes et al. 2011). Soil microorganisms could also play a role in this process as they can promote or hinder seed germination and plant performance (chapter II). While negative feedback associations, influenced by the accumulation of soil pathogens, enhance succession (Van Der Putten et al. 1993) avoiding the extreme dominance of a few species, soil microorganisms could establish positive feedback associations with plants contributing to their persistence over time (Bever 2003) as with late successional species (Kardol et al. 2006). These and other factors facilitate the extreme dominance of a single species and may cause arrested succession.

Different plant species are known to cause arrested succession in different ecosystems. For example, ferns as *Dennstaedtia punctilobula* in temperate environments (Hill and Silander 2001), bamboo species such as *Guadua sarcocarpa* in tropical systems (Griscom and Ashton 2003), *Cistus* species in Mediterranean environments (Miranda et al. 2004) and many other herbaceous and shrub species like *Acanthus pubescens* or *Isoglossa woodii* (Chapman et al. 1999, Griffiths et al. 2007). In addition, species that exhibit clonal growth are often involved in arrested succession (Royo and Carson 2006) as they form structures such as creeping stems, root suckers or rhizomes (Stuefer et al. 2001). Such clonal growth promote high stem and foliage densities that reduce light

(Pugnaire and Haase 1996, Griffiths et al. 2007) which, added to their ability to invade space and form dense thickets, prevent the establishment of other species.

Plant secondary succession in extreme conditions such as arid environments (Bonnet 2004, Scott and Morgan 2012, Chapter III) is characterized by a change in species dominance. For example, in arid SE Spain *Thymelaea hirsuta* (L.) dominates early successional stages, *Artemisia barrelieri* (Besser), *Salsola oppositifolia* (Desf.) and *Hammada* articulata (Moq.) O. Bolòs & Vigo in intermediate stages and *Stipa tenacissima* (L.) in late stages (Chapter III). However, there are also abandoned fields that do not undergo secondary succession and are dominated by a single plant species for long time periods. Although many factors could influence this phenomenon, we know that high soil salinity (Hasegawa et al., 2000), low nutrient content (Roem et al. 2002), soil microorganisms (Van der Putten 2013, Chapter II) as well as different competition strategies (Tilman 1982, Went 1973) prevent survival of some species while favor other species leading to arrested succession.

The perennial tussock grass, *Lygeum spartum* (L.) Kunth (*Lygeum* hereafter), is a species typically abundant in areas of high soil salinity in SE Spain where frequently dominates extensive areas of abandoned fields for long periods of time, decreasing plant diversity. This species is well adapted to high soil salinity, high irradiance and temperatures, as well as to low water holding capacity and organic matter content (Pérez Pujalte et al. 1987, Lázaro et al. 2001). Under similar microclimatic conditions, the halophytic species *Salsola oppositifolia* (*Salsola* hereafter), a shrub dominant in late successional stages (chapter III), can be found at some points growing along *Lygeum* individuals (Peinado et al., 1992). Here, we evaluated whether the competitive ability of the dominant tussock grass species, *Lygeum spartum,* in interaction with *Salsola oppositifolia* depends on soil properties, on its associated soil microbial community, or is rather just a consequence of its functional strategy.

4.3. OBJECTIVES

By growing *Lygeum* and *Salsola* from seeds and using saplings and young adults in either intra- or inter-specific interaction we tested whether 1) the soil microbial community associated to *Lygeum* positively affected seed germination and plant growth; 2) the soil microbial community associated to *Lygeum* had a negative effect on *Salsola* germination and shoot mass; 3) factors such as soil

nutrients, salinity or texture promoted *Lygeum* establishment but prevented *Salsola* establishment; and finally, 4) to what extent *Lygeum* life history strategies determined its competitive success.

4.4. HYPOTHESES

We hypothesized that soil properties and soil microorganisms naturally associated to *Lygeum* would positively affect germination rate, establishment and growth of this species, and that they would negatively affect individuals of other plant species such as *Salsola*. We also expected that rapid seed germination mediated by soil microorganisms and clonal growth would explain the extreme dominance of *Lygeum* in large patches of these arid environments. Ultimately, we expected that both soil microorganisms and plant strategies would explain arrested succession in this environment.

4.5. METHODS

4.5.1. Field site and species

We selected two native perennial species occurring in semiarid environments in south-east Spain. *Lygeum spartum* (L.) Kunth is a rhizomatous tussock grass (Nedjimi 2013) well adapted to extreme conditions of aridity, salinity, and high temperatures which is widespread over uncultivated land and abandoned fields in these arid ecosystems (Pugnaire and Haase 1996, Nedjimi 2013). *Salsola oppositifolia* (Desf.) is a succulent evergreen Chenopodiaceae shrub typical of the same extreme conditions as *Lygeum,* which successfully colonizes disturbed areas (Peinado et al. 1992, Pugnaire et al. 2004) and dominate advanced stages of succession (See chapter III). Seeds, saplings, and young adult (hereafter adults) individuals of these species used in these experiments were provided by Viveros Muzalé (Murcia, Spain).

4.5.2. Soil sampling and inoculum preparation

In March 2013 we collected soil from Llanos de Rueda (37.05º N, 2.22º W, 503 m altitude) in the Tabernas Basin, Almería, Spain from the top 10 cm under the canopy of 30 randomly selected *Lygeum* tussocks and 30 *Salsola* shrubs in a ~3 ha homogeneous plot. Soil samples collected under the same plant species were combined, homogenised and sieved through 5-mm mesh to give a 170 kg composite soil sample per soil origin (i.e. collected under *Lygeum* or under *Salsola*

canopies). Pooling soil samples within soil type reduces variability but allows testing for differences between soil types, and could be considered as technical replicates (Kardol et al. 2006, Ayres et al. 2009, Meisner et al. 2013, Rodríguez-Echeverría et al. 2013). 136 out of these 170 Kg were autoclaved during 20 min at 120 ºC to kill all soil biota and then used as microcosms substrate.

Following standard procedures (Kardol et al. 2007, Meisner et al. 2013, Pendergast et al. 2013, Gundale et al. 2014), we took apart the other 34 kg from each soil origin to prepare 2 types of inoculum; 17 kg were left intact to prepare the "alive" soil inoculum and the remaining 17 kg were autoclaved at 120 ºC during 20 min to prepare the sterile soil inoculum. Each soil subsample was stirred in distilled, autoclaved water in a proportion 1:2 (v:v) and then filtered through a 0.5 mm sieve to remove soil particles but allowing the passing of fungal spores, hyphae, soil bacteria and microfauna (Van de Voorde et al. 2012). These two types of inocula (sterile and alive) per soil origin (under *Lygeum* or *Salsola* canopies) were used to inoculate soil microcosms. Soil samples were stored at 4 ºC for a maximum of 1 month for physical and chemical analyses (Cernohlavkova et al. 2009) and at -80 ºC for soil microbial molecular analyses.

4.5.3. Experimental design

Growth chamber experiments: seeds and saplings

In March 2013, we established two similar experiments in a controlled growth chamber, one with seeds and another with ca. 3-month old saplings (Fig. 4.1). For both experiments, we filled half the microcosms (17 cm in diameter, 18 cm tall pots, 1 L in volume) with soil from each of the two autoclaved soil origins (under *Lygeum* or *Salsola*) mixed with perlite in a proportion 1:1 (v:v) to avoid soil compaction. We then supplied half the microcosms of each soil type with one of the two inocula (alive or sterile). The volume of soil inoculum added to each microcosm was adjusted to 20% (v:v), *i.e.,* 200 ml per microcosm. The design included two levels of plant-plant interaction per species, intra- and inter-specific (*i.e., Salsola*+*Salsola*, *Lygeum*+*Lygeum* and *Lygeum*+*Salsola*) totalling 96 microcosms (3 plant interaction levels x 2 soil origin x 2 inocula x 8 replicates) per experiment.

For the seed experiment, we randomly sowed 11 seeds of each species in the inter-specific interaction treatment and 22 seeds of the same species in the intra-specific interaction treatment to keep constant the number of seeds per microcosm. Seeds were previously surface-sterilized with

ethanol 75% for 2 minutes and washed with sterile water for 1 minute. Seeded microcosms were kept in the dark for the first five days. For the experiment with saplings, we grew two individuals of the same or different species in each microcosm according to the different plant interaction treatments. Plants of both species had similar height and above-ground mass at the start of the experiment, *Lygeum* plants having 5 stems. Throughout the experiments, the day light period was set at 13 h, and the temperature regime at 25/18 ºC day/night with relative humidity of ~70%. Microcosms were watered once a week with 100 ml distilled water and were kept for 5 months.

Fig. 4.1. Growth chamber experiment: Saplings grown in microcosms

Greenhouse experiment: Adult plants

Simultaneously to the growth chamber experiments, we established a similar experiment with adult individuals (~1 year old) in a greenhouse under natural temperature and radiation conditions. We used pairs of microcosms (23 cm diameter pots, 21 cm in height; 4 L in volume) each filled with one of the two autoclaved soil origins and watered with one of the two inocula, alive or sterile, in a factorial design (Fig. 4.2). The volume of soil inoculum added to each microcosm was adjusted to 20% (v:v), *i.e.,* 800 ml per microcosm. The experimental design was the same as the above chamber experiments but with 7 replicates, giving a total of 84 microcosms (3 plant interaction levels x 2 soil origin x 2 inocula x 7 replicates). Plants of both species had similar height and above-ground mass at the start of the experiment, and all *Lygeum* plants had 10 stems. Mean daily temperature during the course of the experiment was 25 ºC and relative humidity ca. 70%. Microcosms were watered twice a week with 500 ml of distilled water and were kept for 5 months.

4.5.4. Harvesting and soil sampling

All microcosms were randomly distributed and their position shifted twice a week to homogenize environmental conditions along the experiment. All saplings or adult plants dead within the first week of the experiment were replaced.

In the seed experiment, the number of germinated seeds was recorded 15 days after sowing and at the end of the experiment. After 5 months, all plants (germinated from seeds, saplings, and adult individuals) were harvested, and shoot dry mass determined after drying at 70 ºC during 48 h. Samples of soil from each microcosm were collected in the centre (seed experiment) or in the space between the two individuals (saplings and adult experiments) and stored at 4 ºC for a maximum of 1 month for physical and chemical analyses. A subsample of soil from the adults experiment was also stored at -80 ºC for microbial molecular analyses.

Fig 4.2. Greenhouse experiment: Adult plants grown in microcosms.

4.5.5. Soil chemical and physical analyses

Several soil properties were measured in *Lygeum* and from *Salsola* soils after inoculation with either alive or sterile inocula at the start of the experiment (n=3). Soil electrical conductivity (EC) and pH were measured using a 1/10 (w/v) water solution with a conductivity- and pH-meter (Crison, BA, Spain), respectively. Total soil carbon (C), organic C after removal of inorganic carbon with HCL 2N (Schumacher 2002), and total nitrogen (N) content were determined using a C/N analyzer (LECO Truspec, MI, USA). Anion phosphate (PO_4^{3-}), nitrate (NO_3^-), nitrite (NO_2^-) and sulphate (SO_4^{2-}) concentrations in water extract (1:10 soil:water) were analyzed by HPLC

(Metrohm, HE, Switzerland). Percent of clay, sand and silt were measured by the Robinson method (Porta-Casanellas et al. 1985).

4.5.6. Soil bacterial community composition

Molecular analyses were performed on soil samples collected in the field under *Lygeum* or *Salsola* individuals that were sterilized and then watered with their respective alive inoculum at the start of the experiment (2 soil origin x 1 inoculum x 3 replicates) as well as on soil samples collected in the microcosms 5 months after the inoculation and set up of the experiment (3 plant interaction levels x 2 soil origin x 2 inocula x 3 replicates). DNA was extracted from 0.25 g of homogenized soil from each of the 42 soil samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) following the manufacturer's instructions. The V3-V6 regions of the bacterial 16S rDNA gene were amplified using primers 357F with Roche adaptor B (5`CTATCCCCTGTGTGCCTTGGCAGTCTCAGCCTACGGGAGGCAGCAG 3`) and 926 Rb (5` CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNNNCCGTCAATT**Y**MTTTRAGT 3`) that included Roche adaptor A and 12 base-pair barcode (Sim et al. 2012). Each sample was amplified in quadruplicate to reduce random mispriming bias (Polz and Cavanaugh 1998). Amplicons were combined in a single tube in equimolar concentrations and the pooled amplicon mixture was purified twice (AMPure XP kit, Agencourt, Takeley, United Kingdom). DNA concentration was quantified using the PicoGreen® assay (Sim et al. 2012). This pool was prepared and pyrosequenced in a Roche Genome Sequencer FLX System (Roche, Basel, Switzerland) using 454 Titanium chemistry at Lifesequencing lab (Valencia, Spain).

Sequences were trimmed for primers, quality filtered and demultiplexed using the pyrosequencing pipeline tools from the Ribosomal Database Project (RDP, Michigan State Univ., USA). Sequences shorter than 150 bp, with quality scores <20 or containing any unresolved nucleotides were removed from the dataset. We used Acacia software version 1.52 (Bragg et al., 2012) (Bragg et al. 2012)(Bragg et al. 2012)(Bragg et al. 2012)for pyrosequencing noise removal using default parameters for error correction. We established a minimum average quality threshold of 30 and all sequences were trimmed to a maximum length of 570 bp. Chimeras were identified using Uchime tool (Edgar et al., 2011) from RDP pipeline and removed from the dataset. Retained sequences were aligned using the Aligner tool from the RDP pipeline and then were clustered into operational taxonomic units (OTUs) defined at 97% similarity cutoff using the complete linkage clustering tool of the RDP pipeline. Taxonomic assignation of sequences was performed using the RDP naïve

Bayesian classifier (Wang et al., 2007) at a confidence level of 80 %, and relative abundances of the different phyla, classes, subclasses, orders and main genera per each of the 42 samples were calculated.

4.5.7. Statistical analyses

For each of the experiments (with seeds, saplings and adult individuals), differences among treatments were analyzed using interaction (intra- and inter-specific), soil origin (from *Lygeum* and *Salsola*) and inoculum (alive and sterile) as fixed effects. At the end of the seed experiment, shoot mass was analyzed with General Linear Models using the ratio of germination (*i.e.,* number of seedlings at the end of experiment / number of sowed seeds) as covariate. For the experiments with saplings and adult plants, we accounted for the spatial correlation between the two individuals in the same microcosm and corrected the model using a compound symmetry correlation structure (corCompSymm). Violations of normality and homoscedasticity were checked for the three experiments. Heterocedasticity was corrected according to variance distribution using VarIdent for saplings and VarExp for adult plants. VarIdent represents a variance structure with different variances for different strata (Gałecki and Burzykowski 2013) while VarExp represents exponential variation of the residual variance (Pinheiro and Bates 2000). To select the most parsimonious model with the lowest AIC, we compared the models through likelihood ratio and graphical inspection of their residues distribution. The model fit was calculated using the restricted maximum likelihood (REML) criterion.

The number of germinated seeds and the total number of seedlings that survived till the end of the seed germination experiment were analyzed using Generalized Linear Model (GLM) with a binomial distribution with a logistic link function and the maximum likelihood criterion. Total number of seeds sown (22) and number of *Lygeum* seeds sown (11) were used as trials for germination and seedling survival analyses, respectively. The same GLM was used to check for differences in the relative abundance of microbial groups using 100 as trial. Significance was established at $p<0.05$. Post-hoc comparisons were performed using DGC (Di Rienzo et al. 2002) or Fisher test. All data were analyzed for each plant species separately with InfoStat Statistical Software (Di Rienzo et al. 2013). Results shown throughout the text and figures are mean values ± 1 SE.

Similarity on bacterial community composition among samples (with OTUs showing at least 5 reads in the overall dataset) was analyzed with principal coordinates analysis (PCoA) using Bray-

Curtis similarity index, and differences among treatments were evaluated performing NPMANOVA with 9999 permutations using Past v 2.12 software (Hammer et al. 2001). Shannon's diversity index of OTUs per sample was calculated with Past software after excluding singletons (OTUs only showing 1 read in the overall dataset) to reduce the overestimation of diversity (Tedersoo et al. 2010). We also performed a principal component analysis (PCA) and Pearson correlation coefficient matrix analysis of soil properties and microbial composition.

4.6. RESULTS

4.6.1. Seed germination

Salsola seed germination either under intra- or inter-specific interaction was minimum. We recorded less than 0.02 ± 0.2 and 0.08 ± 0.6 germinated seeds per pot, equivalent to 99-97% of pots without any germinated seed (intra- and inter-specific, respectively). We thus did not consider *Salsola* for further analyses on seed germination but took into account the presence of *Salsola* seeds in microcosms to analyse germination rates of *Lygeum* seeds.

Fifteen days after sowing, *Lygeum* seed germination was highest in soils from *Lygeum* when only seeds of *Lygeum* were present (intra-specific sowing) and in sterile *Salsola* soils under inter-specific interaction. In contrast, the lowest *Lygeum* seed germination was found in alive *Salsola* soils under inter-specific interaction (Fig. 4.3a). Five months after sowing, the highest number of *Lygeum* individuals was again found on sterile *Salsola* soil under the inter-specific treatment while no differences were found in other treatments (Fig 4.3b). Under inter-specific interaction, *Lygeum* shoot mass was higher on *Lygeum* soil with alive inoculum than in other soils and inocula combinations. However, under intra-specific interaction, *Lygeum* shoot mass was higher on sterile than on alive *Lygeum* soil or any *Salsola* soil inocula (Fig 4.3c, Table S4.1).

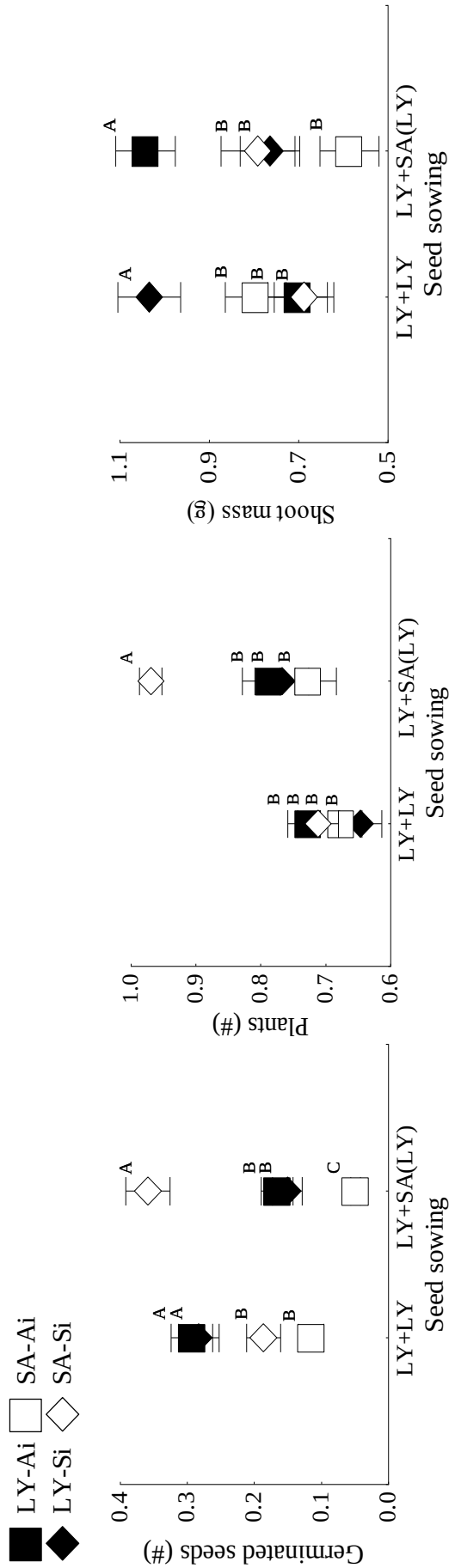

Fig. 4.3. Number of *Lygeum* seeds germinated per microcosm (out 11 seeds under inter-specific and 22 seeds under intra-specific interaction) fifteen days after the beginning of the experiment (a), number of plants that survived at harvest (b) and shoot mass of *Lygeum* individuals at the end of the experiment (c). Seeds were placed on sterile soils collected in the field under *Lygeum* (in black) or *Salsola* (in white) canopies, inoculated with alive (Ai) or sterile inoculum (Si). Microcosms had either only *Lygeum* seeds (intra-specific; LY+LY) or contained also *Salsola* seeds (interspecific; LY+SA(LY). Data are means ± 1 SE, n = 8. Symbols with different letters indicate significant differences between treatments after DGC comparisons at a significance level of 0.05.

Saplings experiment

Salsola individuals showed higher shoot mass than *Lygeum* individuals under both intra- and inter-specific interactions, with an average shoot mass of 0.98 ± 0.04 g vs. 0.66 ± 0.04 g, respectively. The statistical interaction between soil origin and plant interaction level had a significant effect on shoot mass (Table S4.1). Under both plant interaction levels, shoot mass of *Salsola* was higher on soils from *Lygeum* than from *Salsola* irrespective of soil inoculum, but were particularly higher under inter-specific interaction (Fig 4.4a).

Shoot mass of *Lygeum* saplings was influenced by the interaction among soil origin, inoculum and plant interaction level (Table S4.2). Under intra-specific interaction, *Lygeum* plants showed higher shoot mass in soils from *Lygeum* than in soils from *Salsola*, being higher with alive than with sterile inoculum in each soil origin, respectively. On the contrary, under inter-specific interaction, shoot mass was similar in soils from *Lygeum* irrespective of the inoculum. Thus, shoot mass in microcosms with alive inoculum from *Lygeum* soils was higher under intra- than under inter-specific plant interaction. In both plant interaction levels, shoot mass was lowest in soils from *Salsola* watered with sterile inoculum (Fig. 4.4b).

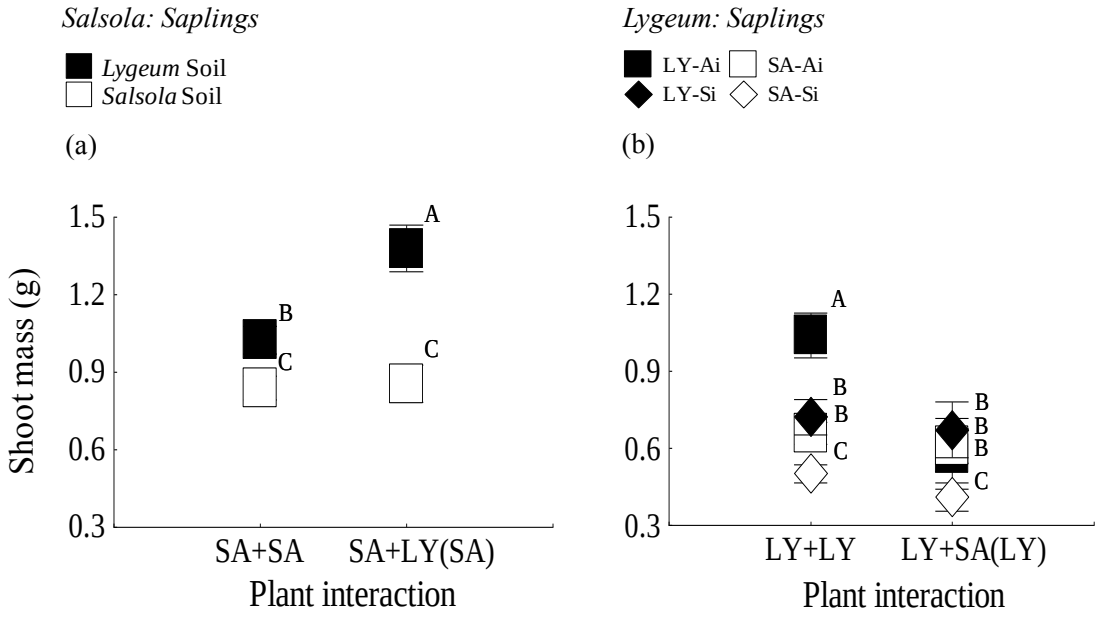

Fig. 4.4. Shoot mass (g) of sapling *Salsola* individuals grown in either soils from *Lygeum* or *Salsola* under intra- (SA+SA) or inter-specific (SA+LY(SA)) interaction (a); and shoot mass of sapling *Lygeum* individuals grown in soils from either *Lygeum* or *Salsola* with alive (Ai) or sterile (Si) inocula under intra- (LY+LY) or inter-specific (LY+SA(LY)) interaction (b). Data are means ± 1 SE, n = 8. Symbols with different letters indicate significant differences among treatments after DGC comparisons at a significance level of 0.05.

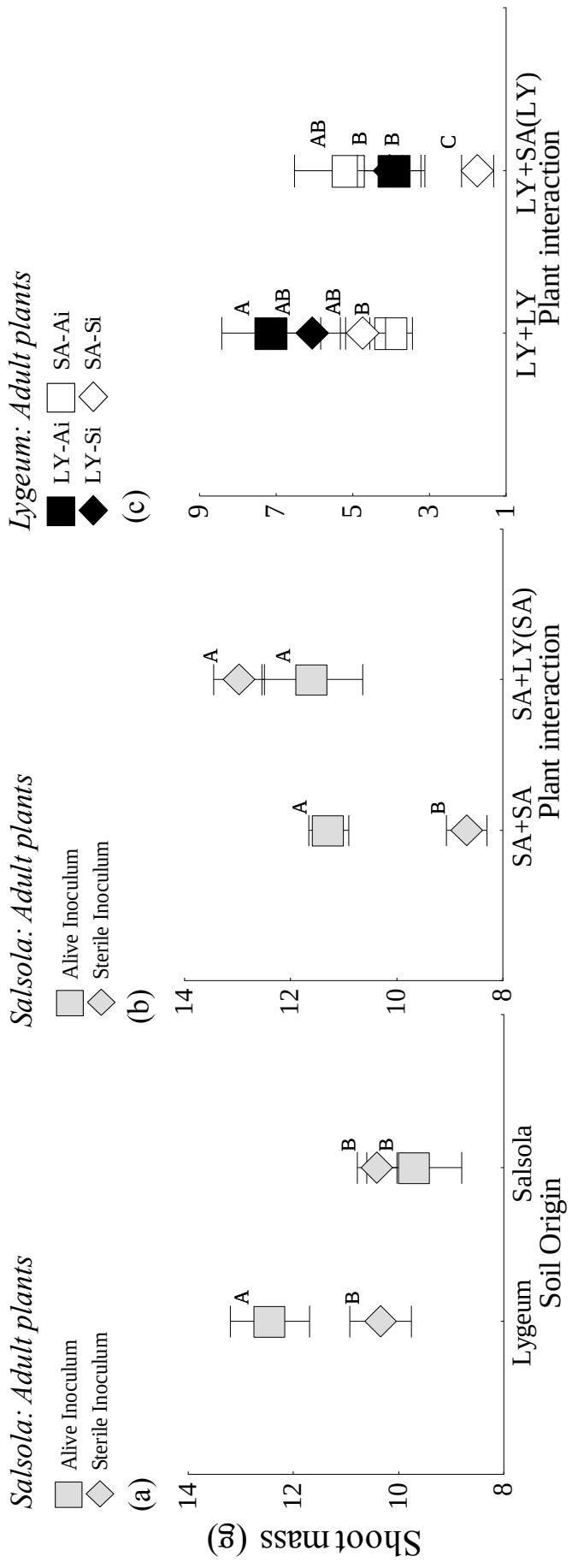

Fig 4.5. Shoot mass (g) of adult *Salsola* individuals grown in either soils from *Lygeum* or *Salsola* with alive or sterile inocula (a), under intra- (SA+SA) or inter-specific (SA+LY(SA)) interaction with alive or sterile inocula (b) and adult *Lygeum* plants grown under intra- (LY+LY) or inter-specific (LY+SA(LY)) interaction in soils from *Lygeum* (black) or *Salsola* (white), inoculated with their respective alive (Ai) or sterile (Si) inoculum (c). Data are means ± 1 SE, n = 7. Symbols with different letters indicate significant differences among treatments after Fisher

4.6.2. Adult plants

Similar to the sapling experiment, adult *Salsola* plants showed a higher shoot mass than *Lygeum* plants in either treatment, with average shoot mass of 10.59 ± 0.42 g and 4.53 ± 0.42 g in intra- and inter-specific interaction, respectively. Soil inoculum and soil origin along with plant interaction level significantly affected shoot mass of *Salsola* individuals (Table S4.2). Regarding soil origin, shoot mass was higher in soils from *Lygeum* with alive inoculum than with sterile inoculum, and in soils from *Salsola* with any inocula (Fig. 4.5a). Under intra-specific interaction, shoot mass was higher with alive than with sterile inoculum while no differences between inocula were found under inter-specific interaction (Fig. 4.5b).

Shoot mass of adult *Lygeum* individuals was influenced by the interaction among soil origin, soil inoculum and interaction treatment (Table S4.2), showing higher shoot mass under intra-specific than under inter-specific interaction. Under intra-specific interaction there were differences between alive soil inocula so that shoot mass was higher in alive soil inoculum from *Lygeum* than from *Salsola* soils, with intermediate values with sterile inocula. Under inter-specific interaction, and similar to saplings individuals, the lowest shoot mass was observed in sterile soil inoculum from *Salsola* soils with no differences among the other treatments (Fig. 4.5c).

In general, soils from *Lygeum* with either alive or sterile inoculum positively affected growth of saplings and adult *Lygeum* individuals compared to soils from *Salsola,* with a positive effect of alive soil inoculum being more evident in saplings than in adult plants and under intra-specific interaction (Fig 4.4b, 4.5c). Alive *Salsola* inoculum also had a positive effect on shoot mass of saplings and adult *Lygeum* individuals compared to sterile *Salsola* inoculum, in particular under inter-specific interaction. On the contrary, in *Salsola* individuals the effect was opposite; *i.e.,* shoot mass of *Salsola* plants was higher in soils from *Lygeum* than in soils from *Salsola* and, in the later, adult *Salsola* plants grew more with sterile than with alive *Salsola* inoculum (Fig S4.1).

4.6.3. Soil properties

We recorded differences in soil chemistry and texture between soils from *Lygeum* and from *Salsola* irrespective of whether they were inoculated with alive or sterile inoculum (Table 4.1). Soil C, organic C, total N, EC, SO_4^{-2}, and silt content were higher in *Lygeum* soils than in *Salsola* soils,

while pH and sand content were higher in *Salsola* soils than in *Lygeum* soils. No differences were found regarding NO_2^- and clay content. Soil PO_4^{3-} and NO_3^- were very low, well under the detection threshold of the HPLC.

Soil properties	Soil from *Lygeum*		Soil from *Salsola*		*F values*
	Alive inoculum	Sterile inoculum	Alive inoculum	Sterile inoculum	
pH	8.38±0.06^b	8.54±0.06^b	8.64±0.06^a	8.59±0.05^a	**7.71****
EC (mS/cm)	3.23±0.45^a	4.12±0.47^a	2.69±0.43^b	2.61±0.42^b	**5.21***
OC (g kg $^{-1}$)	7.60±0.33^a	6.66±0.33^a	3.82±0.33^b	3.75±0.33^b	**103.39*****
N (g kg $^{-1}$)	0.77±0.06^a	0.68±0.06^a	0.38±0.06^b	0.21±0.06^b	**48.57*****
C (g kg $^{-1}$)	16.70±0.23^a	16.17±0.23^a	12.55±0.23^b	12.70±0.23^b	**282.61*****
NO_2^- (mg/kg)	0.12±0.01^a	0.14±0.01^a	0.12±0.01^a	0.12±0.01^a	1.09^{ns}
SO_4^{2-} (mg/kg)	12.35±0.19^a	10.17±0.76^a	2.55±0.19^b	2.17±0.76^b	**254.72*****
Sand (%)	78.11±0.74^b	75.80±0.77^b	81.50±0.71^a	81.95±0.68^a	**43.33*****
Silt (%)	16.17±0.59^a	17.49±0.61^a	11.60±0.56^b	11.44±0.54^b	**84.66*****
Clay (%)	5.71±0.36^a	6.72±0.37^a	6.90±0.34^a	6.61±0.33^a	2.40^{ns}

Table 4.1. Properties of soils from *Lygeum* and *Salsola* watered with their respective alive and sterile inocula at the start of the experiment, including soil electrical conductivity (EC) and organic carbon (OC). Value are means ± 1 SE (n=3). Different letters in a row indicate significant differences ($p<0.05$) after Fisher's LSD test. The last column shows *F* values of the general linear model and significance (*, **, ***, at $p<0.05$, 0.01, 0.001,respectively; all significant values in bold; ns = non-significant).

4.6.4. Soil bacterial community composition

A total of 130679 sequences were obtained after filtering and removing chimeras. Average length of trimmed sequences was 548 ± 6 bp. In order to reduce any bias due to different sequencing effort among samples, we randomly selected a maximum of 3000 sequences per sample so that 40 samples had 2500-3000 sequences with 2 samples (belonging to different treatments) having less than 2500 sequences. The mean number of retained sequences per sample was 2797 ± 340 without significant differences in sequences number among treatments. We identified 15061 distinct OTUs at 97% similarity in the dataset. Ordination of OTUs with at least 5 reads (3151) showed differences on microbial composition between the two soils (from *Lygeum* and *Salsola*) and between soil inocula (alive vs sterile) irrespective of soil origin, at the end of the experiment ($F_{1,35}$=1.26, p=0.07; $F_{1,35}$=1.55, $p<0.05$, NPMANOVA, respectively). Marginal differences were found between alive soils from *Lygeum* and *Salsola* at the start of the experiment ($F_{1,5}$=.2.5, p=0.1,

NPMANOVA). There were no differences in soil microbiota composition between soils where the same plant species or the two target species were present (intra- vs inter-specific interaction) (Fig. 4.6).

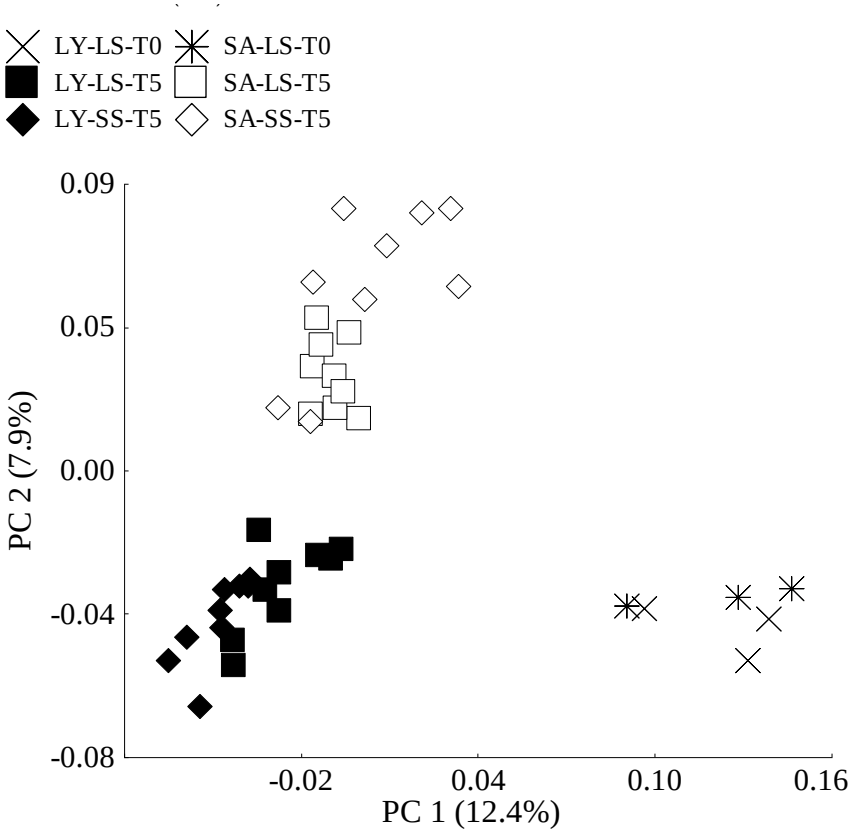

Fig. 4.6. Principal coordinates analysis (PCoA) of soil bacterial communities based on operational taxonomic units (OTUs) using Bray–Curtis similarity index. Soil collected at the end of the experiment of adult plants grown in soils from *Lygeum* (LY) or *Salsola* (SA) with their alive (square or Ai) or sterile (diamond or Si) inoculum. Asterisks indicate live soil inocula from *Lygeum* (⨯) or *Salsola* (✳). Soils were collected at the start (T0) and at the end (T5) of the experiment. n=9 at the end of the experiment, n=3 at the start of the experiment.

Soil bacterial communities were dominated by members of phyla *Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes, Cyanobacteria, Bacteroidetes, Gemmatimonadetes* and *Chloroflexi* (Fig. 4.7). Other less abundant phyla (with relative abundance less lower than 1 %) were *Nitrospira, Verrucomicrobia, Plantomycetes* and *TM7.* Soils from *Lygeum* and *Salsola* at the start of the experiment differed in relative abundance of several microbial groups. All four classes of *Proteobacteria* plylum were more abundant in *Salsola* than in *Lygeum* soils while the relative abundance of *Acidobacteria* was higher in *Lygeum* than in *Salsola* soils (Fig. 4.7, 4.8).

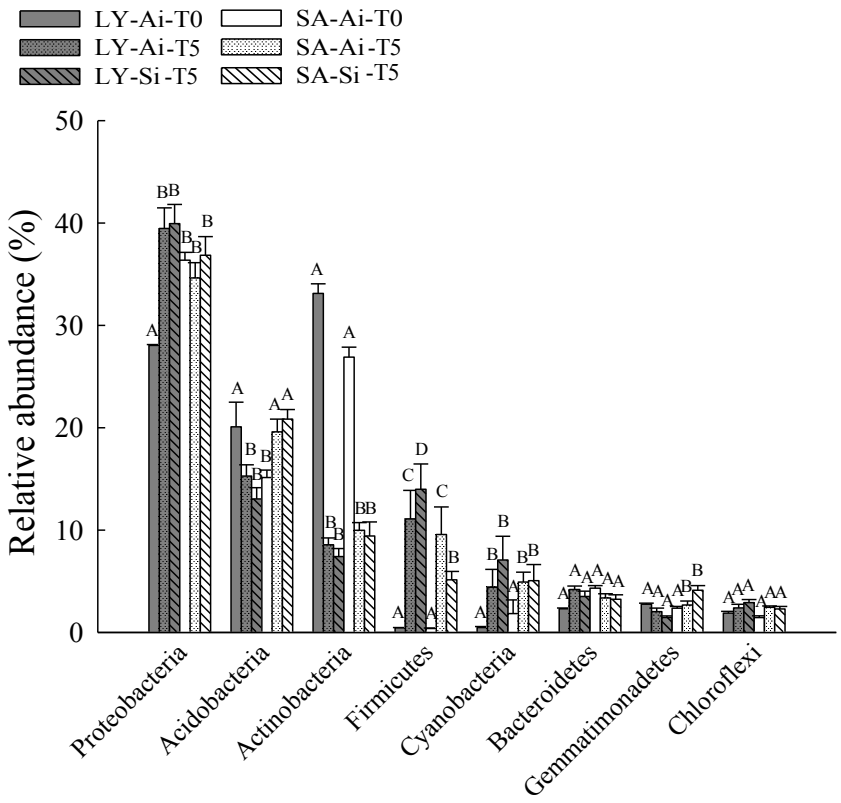

Fig. 4.7. Mean relative abundance (± 1 SE) of main bacterial taxonomic groups, *i.e.* phyla *Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes, Cyanobacteria, Bacteroidetes, Gemmatimonadetes* and *Chloroflexi*. Soil collected at the end of the experiment in microcosms where adult plants grew in *Lygeum* (LY) (gray bars) or *Salsola* (SA) soils (white bars), inoculated with their respective alive (Ai) or sterile (Si) inoculum. Soils were collected at the start (T0) and at the end (T5) of the experiment. Different letters within a bacterial group indicate significant differences ($p < 0.05$) among treatments after Fisher's LSD test. n=9 at the end of the experiment, n=3 at the start of the experiment.

Ordination of samples by PCA based on soil properties and soil microbial groups at the start of the experiment also showed a clear differentiation between *Lygeum* and *Salsola* soils along the first axis (Fig 4.8), with the first two axes explaining 78.4% of total variance. We observed a correlation among different soil properties and soil microbial groups (Fig. 4.8, Table S4.3). Members of *Actinobacteria* were more abundant in *Lygeum* than in *Salsola* soils and their abundance was positively correlated with the higher content of silt, N, C, organic carbon and SO_4^{2-} in soils from *Lygeum*, while *Proteobacteria* and *Bacteroidetes* were more abundant in *Salsola* than in *Lygeum* soil and their abundance negatively correlated with the above soil parameters.

In each soil, several microbial groups increased in abundance by the end of the experiment compared to the start of the experiment (*Proteobacteria* in *Lygeum* soils*, Acidobacteria* and *Gemmatimonadetes* in *Salsola* soils; and *Firmicutes* and *Cyanobacteria* in both) while other groups

decreased (*Acidobacteria* in *Lygeum* soils and *Actinobacteria* in both soils). Within *Proteobacteria*, members of *Alphaproteobacteria* were more abundant in *Salsola* soils at the start than at the end of the experiment or in *Lygeum* soils while *Betaprotebacteria, Deltaproteobacteria* and *Gammaproteobacteria* were more abundant in both soils at the end than at the start of the experiment (data not shown). The relative abundance of *Firmicutes* in *Lygeum* soils at the end of the experiment was higher in microcosms initially inoculated with sterile inoculum than in those inoculated with alive *Lygeum* inoculum while in *Salsola* soils their abundance was lower with sterile than with alive inoculum. There were no significant changes in *Bacteroidetes, Chloroflexi* or any minor phyla. Bacterial diversity (Shannon`s index) also differed among treatments (Table S4.4). It was higher in alive inoculum at the start (7.19 ± 0.79) than alive or sterile inoculum at the end of the experiment (6.05 ± 0.07, 6.41 ± 0.26, respectively), and in *Lygeum* than in *Salsola* soils (6.79 ± 0.44 vs 6.31 ± 0.31), and it was lower in *Salsola* intra-specific interaction (5.92 ± 0.08) than in the other plant interaction levels. No interaction among treatments was observed.

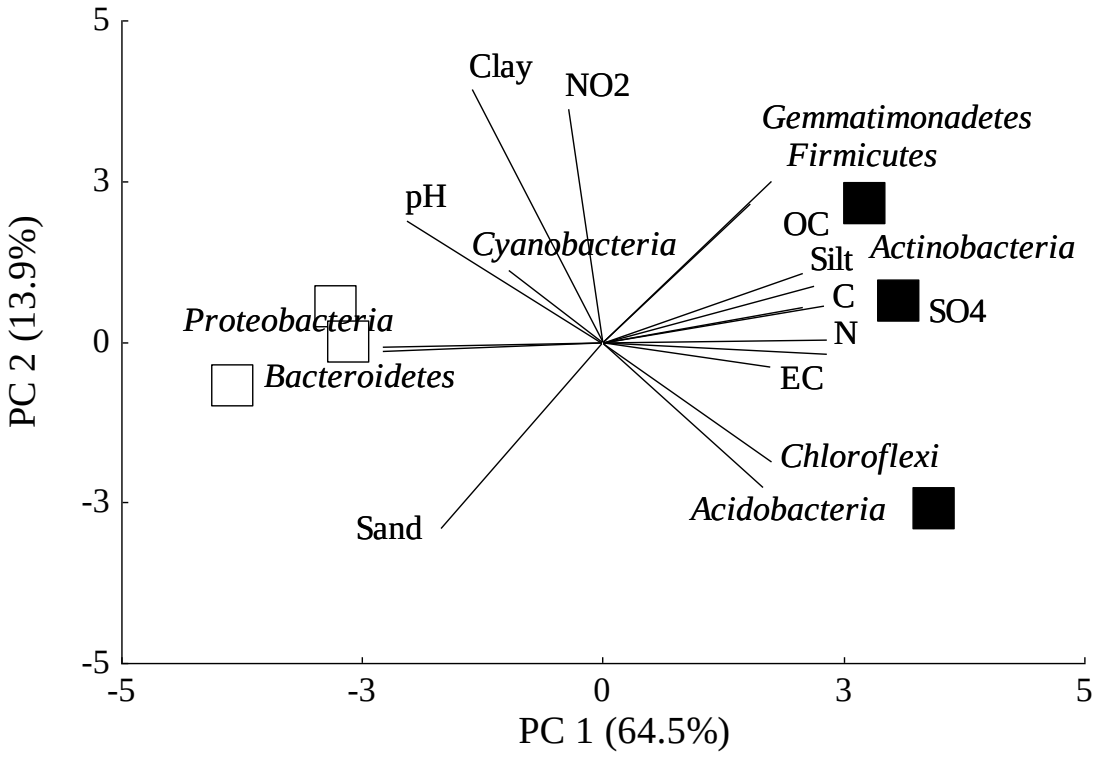

Fig. 4.8. Principal component analysis of soil properties (electrical conductivity [EC], clay, sand, silt, SO_4^{-2}, NO_2^-, pH, total nitrogen [N], organic carbon [OC], total carbon [C]) and microbial composition (relative abundances of *Actinobacteria, Chloroflexi, Acidobacteria, Bacteroidetes, Proteobacteria, Cyanobacteria, Gemmatimonadetes* and *Firmicutes*). The first two PCA axes explain 78.4 % of total variance. White and black squares correspond to samples from *Salsola and* Lygeum soils, respectively.

4.7. DISCUSSION

Contrary to our expectations, soil properties and microbial communities associated to *Lygeum* facilitated the development of both *Lygeum* and *Salsola* plants. Although *Salsola* individuals were more competitive than *Lygeum* in the saplings and adult life stages, *Lygeum* individuals were more competitive than *Salsola* in key initial life stages (seed germination and seedling growth). Our results showed that soil properties and soil microbial communities associated to *Lygeum* had an important influence on seed germination and initial growth of *Lygeum* individuals, playing perhaps a role in *Lygeum* dominance and arrested succession throught positive plant-soil feedbacks.

4.7.1. Positive effect of Lygeum soil properties and microbial communities

Plant growth, especially at initial stages, and seed germination (for *Lygeum* only) were in general higher in *Lygeum* than in *Salsola* soils. *Lygeum* soils were characterized by total C, organic C, N and silt contents higher than *Salsola* soils, therefore contributing to the enhanced plant performance in these soils. *Lygeum* soils also had higher salinity than *Salsola* soils but, although high soil salinity can limit plant performance (Hasegawa et al. 2000), both *Lygeum* and *Salsola*, as halophytic species, have developed survival strategies under these conditions. These strategies include selective ion uptake or accumulation in vacuoles to keep homeostasis or low osmotic potential (Flowers et al. 1977, Zhu 2001, Porta-Casanellas et al. 2003) or, in the case of *Salsola,* by notably increasing rooting depth to access deep water storages (Padilla and Pugnaire 2007).

The soil microbial community associated to *Lygeum* positively affected *Lygeum* seed germination and growth of saplings and adult plants. However, contrary to our hypothesis, *Lygeum* soil microbes did not negatively affect, but instead promoted, *Salsola* growth. In fact, soil microorganisms from *Lygeum* soils were more positive for *Salsola* individuals than soil microorganisms from under *Salsola* shrubs. Soil microbial communities from *Lygeum* and *Salsola* soils showed little differences in composition at the beginning of the experiment but still they differed in the relative abundance of certain microbial groups and, most likely, in microbial activity and biomass. Soil microbial composition was influenced by soil properties such as texture, soil organic C and SO_4^{2-} content. At the beginning of the experiment, *Acidobacteria* species were more abundant in *Lygeum* than in *Salsola* soils while *Proteobacteria* were higher in *Salsola* than in *Lygeum* soils, a pattern that could be related to soil silt content (Sessitsch et al. 2001). Members of the *Burkholderiales* order (*Betaproteobacteria*), described as potential plant-growth promoters

(Acton 2012) were more abundant in *Salsola* than in *Lygeum* soils at the beginning of the experiment but, like most members of *Proteobacteria,* increased their abundance with time in *Lygeum* soils, suggesting positive feedbacks whith these microorganisms.

A similar increase with time in *Lygeum* soils was observed for members of the *Rhodobacter* order (*Alphaproteobacteria*), known for their abundance in halophytic environments (Kersters et al. 2006). Several reports suggest that salinity affects microbial composition (Dupont et al. 2014) and that microorganisms inhabiting roots and leaves of halophytic species may contribute significantly to lower their stress and increase their salinity tolerance (Ruppel et al. 2013). In fact, halophytic species as *Salicornia brachiate* or *Halocnemum strobilaceum* are associated with many halotolerant microorganisms such as *Mesorhizobium* or *Halomonas* (Jha et al. 2012, Shi et al. 2012). We found that abundance of *Mesorhizobium* was higher in *Lygeum* than in *Salsola* soils, and that *Halomonas, Bacillus* and *Enterobacter,* which can also be halotolerant (Upadhyay et al. 2011, Shi et al. 2012), were found only in *Lygeum* soils. On the contrary, the halotolerant *Litoribacter* and *Microbacterium* (Upadhyay et al. 2011, Shi et al. 2012) were only found in *Salsola* soils. Therefore, our results show that *Salsola* and *Lygeum* soils differ in composition of halotolerant microbes, and that these are more abundant and show higher richness in *Lygeum* than in *Salsola* soils, which may contribute to the better performance of *Lygeum* and *Salsola* plants in *Lygeum* soils. Added to microbial composition, higher C and N content in *Lygeum* than in *Salsola* soils suggested a higher microbial activity and biomass linked with increased nutrient availability which may have positively influenced plant growth.

4.7.2. Soil microbes effects change with plant age and interactions

Our results showed that, overall, soil microorganisms (supplied *via* alive inocula) associated to *Lygeum* positively affected plant growth in both species, while soil microbes associated to *Salsola* had different effects on *Lygeum* plants depending on their life stage. *Salsola* inocula had a negative effect on *Lygeum* seed germination while it had a neutral or positive effect on later stages of *Lygeum* plant development. Therefore, *Lygeum* seeds germinated better when *Salsola* soil microbes were not present while, later on, *Lygeum* plants often grew less when *Salsola* microbes were removed.

On *Lygeum* soils the presence of *Salsola* seeds negatively affected *Lygeum* germination compared to intra-specific interaction; while on sterile *Salsola* soils the effect was the opposite, and

the maximum *Lygeum* seed germination rate and establishment did occur under inter-specific interaction. It has been suggested that seeds could recognize potential competitors presence and defer germination (Renne et al. 2014) a process that may have influenced both *Lygeum* and *Salsola* germination. *Lygeum* germination rate and establishment under inter-specific interaction were negatively affected by *Salsola* soil microorganisms and positively by *Lygeum* soil microorganisms, evidencing that changes in relative abundance of microbial groups affected initial stages of plant development. Negative plant-soil interactions (Klironomos 2002) also influence seed germination and initial growth stages. Contrary to what was found by (Pendergast et al., 2013) who suggested that plant invasion is promoted by the better development of a species on soil of its competitors, we found that both saplings and adult *Lygeum* individuals grew better on its own soil than on *Salsola* soil. In fact, soil microorganisms associated to *Lygeum* also facilitated *Salsola* growth evidencing a positive effect of *Lygeum* soil microorganisms on *Salsola* plants. Thus, the invasive nature of *Lygeum* plants may be given by its growth strategy and positive plant-soil feedbacks in own soils.

4.7.3. Soil microbial communities change with time

Although at the beginning of the experiment soil microbial communities associated to *Lygeum* and *Salsola* soils were quite similar, differences between soil origins increased with time. In the field, both soils were collected near the rhizosphere and both shared the same extreme semiarid climatic conditions (i.e., high temperatures and irradiation, low precipitation) that likely tended to homogenize microbial communities. After five months of experiment, the interactions among plants, soil microorganisms, and soil properties led to a clear differentiation between soil microbial communities. Most microbial groups increased their abundance with time, while other as *Acidobacteria* in *Lygeum* soils, or *Actinobacteria* in both soils, decreased. Soil microbial communities could change due to seasonal changes (Lipson et al. 2002) or due to new conditions. Moreover, changes in microbial abundance over time could be linked to the plant ability to select some microorganisms in the rhizosphere Kowalchuk et al. (2002), and Martínez-García et al. (2011), and Rodríguez-Echeverría et al. (2013).

4.7.4. Soil microorganisms and arrested succession

Our results suggest that the lack of species such as *Salsola* in communities dominated by *Lygeum* is linked to positive plant-soil feedbacks between soil microorganisms and *Lygeum*, even though

soil microbial communities in *Lygeum* soils and the presence of *Lygeum* individuals enhanced performance of saplings and adult *Salsola* individuals in inter-specific interaction.

Although seed viability tests showed similar germination rates in both species, *Lygeum* germination rate after 15 days of sowing far exceeded *Salsola's,* and benefited more from *Lygeum* than from *Salsola* soil extracts. We also observed that microorganisms from *Salsola* soils negatively affected *Lygeum* germination, showing the determinant role of soil communities on the initial life stages of plants. *Salsola* plants grew better in *Lygeum* than in its own soil, suggesting a negative feedback with their own soil microorganisms (Klironomos 2002). Under inter-specific competition, *Salsola* performed better in sterile than in alive soils, likely due to presence of pathogens that negatively affected plant growth (Klironomos 2002). This is in agreement with the fact that *Salsola* is a late-successional species (chapter III) and these negative interactions could easily promote secondary succession as suggested in chapter III. On the contrary, the positive feedbacks observed between *Lygeum* plants and their soil microbes are typical of late succession species (Kardol et al., 2006). Results suggest that the negative effect of *Lygeum* soils on the establishment of *Salsola* individuals is key to determine the later success of *Lygeum* individuals as adult plants.

Salsola individuals were more competitive than *Lygeum* individuals under both intra- and inter-specific interactions. C_4 species (*Salsola*) can reduce photorespiration at high temperatures and irradiance, therefore increasing photosynthetic efficiency (Padilla et al. 2009, González and Chueca 2010) and water use efficiency above that of C_3 species (*Lygeum)* (Sage 2004, González and Chueca 2010). Although *Lygeum* showed a low competitive success with C4 species such as *Salsola* or C3 species such as *Limonium insigne* (Armas and Pugnaire 2011), *Lygeum* success ability in initial life stages, its clonal growth strategy and the positive feedbacks with soil microorganisms combine to promote its local dominance and play a important role in arrested succession.

4.8. CONCLUSIONS

Our results show that *Lygeum* competitive ability was linked to positive feedback effects of its own soil microorganisms, which enhanced seed germination and establishment promoting early occupation of space which, in addition to its clonal growth habit, may prompt its dominance over time in a seemingly arrested succession.

4.9. APPENDIX

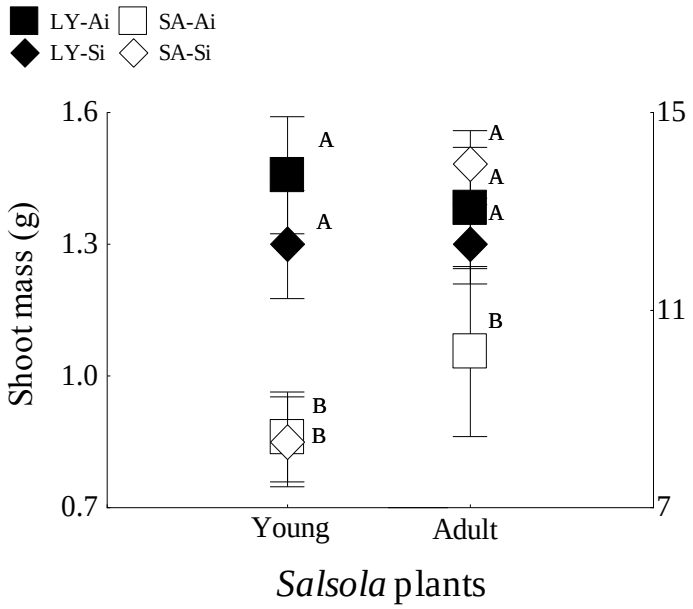

Fig. S4.1. Shoot mass (g) of saplings and adult plants of *Salsola* grown under inter-specific (SA+LY(SA)) interaction in soils from *Lygeum* (in black) or *Salsola* (in white), inoculated with their respective alive (Ai) or sterile (Si) inoculum. Data are mean ± 1 SE, n =8 for saplings and 7 microcosms for adult. Symbols with different letters indicate significant differences among treatments after post-hoc comparisons at a significance level of 0.2 and 0.18, respectively.

Source of variation	Chi-square	df	p-value
Seed germination : *Lygeum*			
Number of germinated seeds			
Plant interaction (Pi)	237.46	1	**0.0392**
Soil Origin (So)	231.31	1	**0.0132**
Inoculum (I)	207.79	1	**<0.0001**
Pi x So	118.97	1	**<0.0001**
Pi x I	170.20	1	**<0.0001**
So x I	136.10	1	**<0.0001**
Pi x So x I	118.97	1	**<0.0001**
Number of plants			
Plant interaction (Pi)	117.86	1	**<0.0001**
Soil Origin (So)	116.54	1	0.2497
Inoculum (I)	115.76	1	0.3784
Pi x So	113.66	1	0.1472
Pi x I	105.46	1	**0.0042**
So x I	92.56	1	**0.0003**
Pi x So x I	84.46	1	**0.0044**
	F	**Num df**	**p-value**
Shoot mass			
Plant interaction (Pi)	0.03	1	0.0636
Soil Origin (So)	12.86	1	**0.0007**
Inoculum (I)	0.62	1	0.4345
% Number plants- number of seeds	8.87	1	**0.0434**
Pi x So	0.88	1	0.3515
Pi x I	2.30	1	0.1351
So x I	0.05	1	0.8317
Pi x So x I	23.74	1	**<0.0001**

Table S4.1. Results from generalized linear models on seed germination experiment fifteen days after sowing and number of plants established at the end of the experiment (five months after sowing), and results from general linear mixed models on shoot mass of germinated seeds at the end of the experiment. Plant interaction (Pi), Soil Origin (So), Soil microbial inoculum (I) and their interactions were considered fixed factors. All significant results are marked in bold.

Source of variation	F	Num df	p-value
Saplings: *Salsola*			
Plant interaction (Pi)	7.40	1	**0.01**
Soil Origin (So)	27.05	1	**<0.001**
Inoculum (I)	0.02	1	0.89
Pi x So	6.10	1	**0.02**
Pi x I	1.89	1	0.17
So x I	0.03	1	0.87
Pi x So x I	1.54	1	0.22
Saplings: *Lygeum*			
Plant interaction (Pi)	7.95		**0.01**
Soil Origin (So)	13.02		**0.001**
Inoculum (I)	6.39		**0.01**
Pi x So	2.60		0.11
Pi x I	2.55		0.11
So x I	0.32		0.57
Pi x So x I	3.86		**0.05**
Adult plants: *Salsola*			
Plant interaction (Pi)	14.92	1	**0.001**
Soil Origin (So)	3.83	1	**0.05**
Inoculum (I)	1.05	1	0.31
Pi x So	0.96	1	0.33
Pi x I	11.13	1	**0.001**
So x I	4.28	1	**0.04**
Pi x So x I	1.83	1	0.18
Adult plants: *Lygeum*			
Plant interaction (Pi)	7.66	1	**0.01**
Soil origin (So)	4.68	1	**0.04**
Inoculum (I)	1.91	1	0.17
Pi x So	1.69	1	0.20
Pi x I	1.27	1	0.26
So x I	0.43	1	0.51
Pi x So x I	4.29	1	**0.04**

Table S4.2 Results from general linear models on shoot mass of sapling and adult plants of *Lygeum* or *Salsola* at the end of the experiments. Plant interaction (Pi), Soil Origin (So), Soil microbial inoculum (I) and their interactions were considered fixed factors. All significant results are marked in bold.

	pH	EC	Sand	Silt	Clay	N	C	OC	NO_2^-	SO_4^{2-}	Proteobacteria	Acidobacteria	Actinobacteria	Firmicutes	Cyanobacteria	Bacteroidetes	Gemmat
pH																	
EC	**-0.86***																
Sand	ns	ns															
Silt	ns	ns	**-0.92***														
Clay	ns	ns	ns	ns													
N	**-0.85***	ns	ns	**0.85***	ns												
C	ns	ns	**-0.82***	**0.95****	ns	**0.97*****											
OC	ns	ns	**-0.83***	**0.95****	ns	**0.92****	**0.97*****										
NO_2^-	ns	ns	ns	ns	ns	ns	ns	ns	ns								
SO_4^{2-}	**-0.83***	ns	ns	**0.88***	ns	**1.00*****	**0.99*****	**0.94****	ns								
Proteobacteria	ns	ns	ns	**-0.87***	ns	**-0.98*****	**-0.97*****	**-0.89***	ns	**-0.98*****							
Acidobacteria	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns						
Actinobacteria	ns	ns	**-0.85***	**0.94****	ns	**0.88***	**0.93****	**0.86***	ns	**0.90***	**-0.94****	ns					
Firmicutes	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns				
Cyanobacteria	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
Bacteroidetes	**0.87***	ns	ns	**-0.88***	ns	**-0.96****	**-0.97*****	**-0.97****	ns	**-0.97*****	**0.91***	ns	**-0.82***	ns	ns		
Gemmat	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.91****	ns	ns	
Chloroflexi	**-0.87***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**0.80***	ns	ns	ns	ns	ns

Table S4.3. PCA correlation matrix of soil properties and relative abundance of main bacterial groups. (Gemmat: Gemmatimonadetes; EC: electrical conductivity; OC: organic carbon). Values correspond to Pearson correlations and their significance (*, **, ***, at $p<0.05$, 0.01, 0.001, respectively, all values in bold; ns = non-significant).

Source of variation	F	df	p-value
Bacterial diversity			
Plant interaction (Pi)	6.94	2	**0.003**
Soil Origin (So)	3.96	1	**0.056**
Inoculum_Time(IT)	5.70	2	**0.008**
Pi x So	1.61	2	0.218
Pi x IT	2.40	1	0.108
So x IT	0.50	2	0.590

Table S4.4. Results from general linear models on soil bacterial diversity at the end of the greenhouse experiment. Plant interaction (Pi), Soil Origin (So), Inoculum_Time (IT) which integrated each soil inocula effect at the start and at the end of the experiment; and their interactions were considered fixed factors. All significant results are marked in bold.

CONCLUSIONES GENERALES

- El tamaño de copa del arbusto facilitador *Retama sphaerocarpa*¸ y el suelo bajo su copa influyen de manera positiva en el desarrollo de la comunidad de herbáceas que crece a su amparo, incrementándose tanto la biomasa como la abundancia de especies cuanto mayor sea la copa del arbusto.
- El origen del suelo (es decir, que provenga de arbustos de *Retama* grandes, medianos o pequeños) determina en gran medida la diversidad y productividad de la comunidad de especies facilitadas por *Retama,* siendo más decisivo que los efectos del microhabitat generado por el arbusto.
- La composición del banco de semillas, la disponibilidad de nutrientes y las interacciones entre plantas y comunidades microbianas del suelo afectan el desarrollo de la comunidad de herbáceas.
- Los microorganismos del suelo, las características del suelo y el microhabitat creado por la especie facilitadora arbustiva *Retama*, influyen tanto en la germinación de la comunidad de herbáceas bajo la copa del arbusto como en su crecimiento.
- El efecto de los microorganismos del suelo sobre las tasas de germinación de las especies de herbáceas es específico para cada especie, influyendo de forma positiva, neutra o negativa según la identidad de cada una. Las tasas de germinación también se ven afectadas positivamente por el microhabitat generado por la copa del arbusto facilitador y su suelo asociado.
- El efecto positivo de los suelos bajo el arbusto facilitador sobre el desarrollo de la comunidad de herbáceas se mantiene a lo largo del ciclo de vida de la especie facilitadora, de modo que el suelo (propiedades físicas, contenido en nutrientes y microbiota) es el principal promotor de los procesos de facilitación de *Retama sphaerocarpa* en zonas semiáridas.
- Tras el abandono de campos dedicados al cultivo, tanto las plantas como las comunidades microbianas del suelo siguen un proceso paralelo de sucesión secundaria.
- La sucesión secundaria en ecosistemas semiáridos está caracterizada por cambios en las propiedades del suelo, la cobertura vegetal, la biomasa y la actividad microbiana, así como por cambios en la composición de plantas y microorganismos del suelo.

- Teniendo en cuenta la velocidad de cambio de la composición de las comunidades vegetales y de las comunidades microbianas del suelo, los resultados sugieren que el proceso de sucesión microbiana sigue al de la sucesión en plantas.
- Respecto al proceso de sucesión detenida donde domina *Lygeum spartum*, la habilidad competitiva de *Lygeum* está vinculada a un efecto positivo de los microorganismos y nutrientes del suelo sobre su germinación y desarrollo.
- La retroalimentación positiva planta-suelo promueve una temprana ocupación del espacio que, sumada a la estrategia de crecimiento clonal de *Lygeum*, facilita su dominancia en el tiempo, causando una interrupción del avance sucesional.

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