# *Efectos de la fragmentación del encinar en las interacciones planta-suelo-microorganismos*

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Effects of forest fragmentation on the plant-soil-microbial interactions

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Tesis Doctoral

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## Effects of forest fragmentation on the plant-soil-microbial interactions

**TESIS DOCTORAL**

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## Índice

**Abstract**

Forest fragmentation implies profound ecological transformations worldwide, mainly threatening aboveground biodiversity. However, the complex impacts of forest fragmentation on ecosystem processes are not yet well understood, especially taking into account their interactions with other global change drivers such as the increasing drought, particularly relevant in the Mediterranean area. The interaction of the soil-microbial system with the standing plants is crucial to fully understand the effects of forest fragmentation over ecosystem processes. The main objective of this thesis is to understand the impacts of forest fragmentation on the edaphic properties and on the structure of soil microbial communities, as well as on their capacity to decompose and metabolize soil organic matter. To fulfill this objective we have used different methodological approaches to study the physicochemical characteristics of soils and the structure and function of soil microbial communities, as influenced by holm oak trees and seedlings. The study has been carried out in fragmented holm oak forests immersed in an active agricultural matrix and located in two climatically different regions of Spain. We have also focused on the functional responses of the plant-soil-microbial system to drought, the most important climate change-related threat in Mediterranean ecosystems. Our results point out to a high complexity of the plant-soil-microbial system and reveal important responses of this system to forest fragmentation. Additionally, we have found a differential responsiveness of the soil-plant-microbial system to drought, depending on both the physicochemical characteristics of soils and the historical adaptation of soil microbial communities to specific bioclimatic conditions. We found that forest fragmentation has direct effects over the microbial community (structure and diversity), and indirect effects over the soil-microbial functioning (soil respiration, enzymatic activities and metabolic profile) mediated through

the influence of the tree size, which triggers in turn a cascade of causal-effect relations that stimulates soil microbial activity. Moreover, the interaction found between drought and fragment size suggests that depending on the local bioclimatic conditions, forest fragmentation could ameliorate to some extent the negative effect of drought by increasing the fertility and water holding capacity of soils, especially in soils with historic adaptation to drought.

**Keywords:** forest fragmentation, enzymatic activity, soil respiration, denaturing gradient gel electrophoresis, microbial community-level physiological profiles, microbial biomass, microbial alpha-diversity, microbial beta-diversity, structural equation models.

**Resumen**

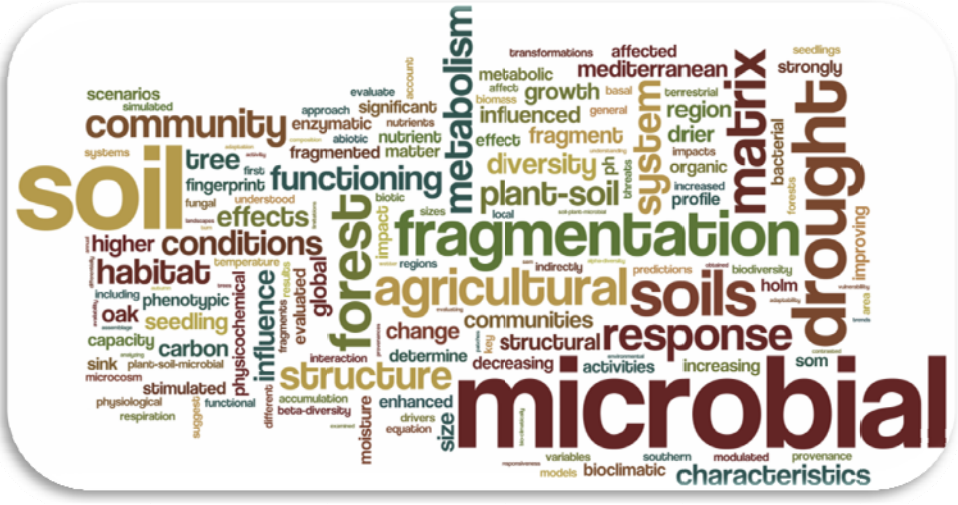
La fragmentación del hábitat implica profundas transformaciones ecológicas en todo el mundo, amenazando principalmente la biodiversidad de las comunidades sobre el suelo. Sin embargo, el complejo impacto de la fragmentación de los bosques en los procesos ecosistémicos todavía no se comprende bien, especialmente tomando en cuenta las interacciones con otros motores de cambio global como el incremento de la sequía, particularmente relevante en los ecosistemas Mediterráneos. La interacción del sistema suelo-microorganismos con las plantas es crucial para entender los efectos de la fragmentación del bosque sobre los procesos del ecosistema. El principal objetivo de esta tesis es comprender los impactos de la fragmentación del bosque en las propiedades edáficas y en la estructura de las comunidades microbianas del suelo, así como en su capacidad de descomponer y metabolizar la materia orgánica del suelo. Para cumplir con este objetivo se han utilizado diferentes enfoques metodológicos para estudiar las características físico-químicas del suelo y la estructura y funcionalidad de las comunidades microbianas del mismo, así como la influencia de árboles y plántulas de encinas. El estudio ha sido llevado a cabo en encinares fragmentados inmersos en una matriz agrícola activa, y localizados en dos regiones climáticamente distintas de España. También nos hemos centrado en la respuesta funcional del sistema planta-suelo-microorganismos a la sequía, la amenaza relacionada con el cambio climático más importante en los ecosistemas Mediterráneos. Nuestros resultados señalan la elevada complejidad del sistema planta-suelo-microorganismos y revelan respuestas importantes de este sistema a la fragmentación de los bosques. Adicionalmente, hemos encontrado una respuesta diferencial del sistema planta-suelo-microorganismos a la sequía, dependiendo tanto de las características físico-químicas del suelo y de la adaptación histórica de las comunidades microbianas a condiciones

bioclimáticas específicas. Hemos encontrado que la fragmentación del bosque tiene efectos directos sobre la comunidad microbiana (estructura y diversidad), y efectos indirectos sobre el funcionamiento del sistema suelo-microorganismos (respiración del suelo, actividades enzimáticas y perfil metabólico) mediadas a través de la influencia del tamaño del árbol, que desencadena a su vez una cascada de relaciones causa-efecto que estimula la actividad microbiana del suelo. Además, se ha encontrado una interacción entre sequía y tamaño del fragmento que sugiere que, dependiendo de las condiciones bioclimáticas locales, la fragmentación de los bosques podría aminorar, hasta cierto punto, el efecto negativo del aumento de las sequías mediante el incremento de la fertilidad y la capacidad de retención del agua de los suelos, especialmente en aquellos suelos con adaptación histórica a la sequía.

**Palabras clave:** fragmentación del bosque, actividades enzimáticas, respiración del suelo, electroforesis en gel con gradiente de desnaturalización, perfiles fisiológicos a nivel de comunidad microbiana, biomasa microbiana, diversidad alfa microbiana, diversidad beta microbiana, modelos de ecuaciones estructurales.

# Capítulo 1

## Introducción general



## Introducción

La intensa intervención humana es una característica común de los ecosistemas mediterráneos (Grove y Rackham, 2003). A lo largo de los siglos, se han producido talas selectivas o masivas, quemas, roturaciones, pastoreo, reforestaciones e incremento del urbanismo, que han determinado la estructura y dinámica de los bosques actuales, y que tendrán indudables repercusiones en su respuesta futura al cambio global (Valladares et al., 2004). Entre las transformaciones humanas que han contribuido a la modificación de los bosques mediterráneos, la fragmentación rápida e intensa del hábitat puede ser considerada como una de las más importantes (Alados et al., 2004; Blondel, 2010). La fragmentación de los bosques mediterráneos tiene importantes implicaciones para las estrategias de conservación y manejo (Saunders et al., 1991) y se ha convertido, cada vez más, en un importante motor de cambio global en la cuenca mediterránea (MEA, 2005). Esta fragmentación de los bosques en interacción con el pronosticado aumento de la sequía en los ecosistemas mediterráneos (Fig. 1.1; ver MEA 2005; Christensen et al., 2007), son en conjunto una amenaza creciente para estos ecosistemas.

La fragmentación se asocia a una modificación progresiva del paisaje (cambios en tamaño y distribución de los fragmentos del hábitat, en distancias entre fragmentos de un mismo hábitat, y en la conexión entre los fragmentos) y suele acompañarse, además, de procesos de alteración de la cubierta vegetal (Andrén, 1994). Como consecuencia, el impacto de la fragmentación del hábitat en la composición y funcionamiento de las comunidades dependerá, en primer lugar, de las condiciones bióticas y abióticas tanto de los fragmentos remanentes como de la matriz circundante y, en segundo lugar, del tamaño de los fragmentos resultantes, pues cuanto más pequeño es el fragmento mayor es la influencia de la matriz en la que está embebido (Fernández et al., 2002; Fischer y

Lindenmayer, 2007). El tamaño del fragmento no solo determina la cantidad de recursos disponibles, sino también la influencia de la matriz circundante, debido a la creciente importancia de los efectos borde a medida que la fragmentación aumenta (Fernández et al., 2002).

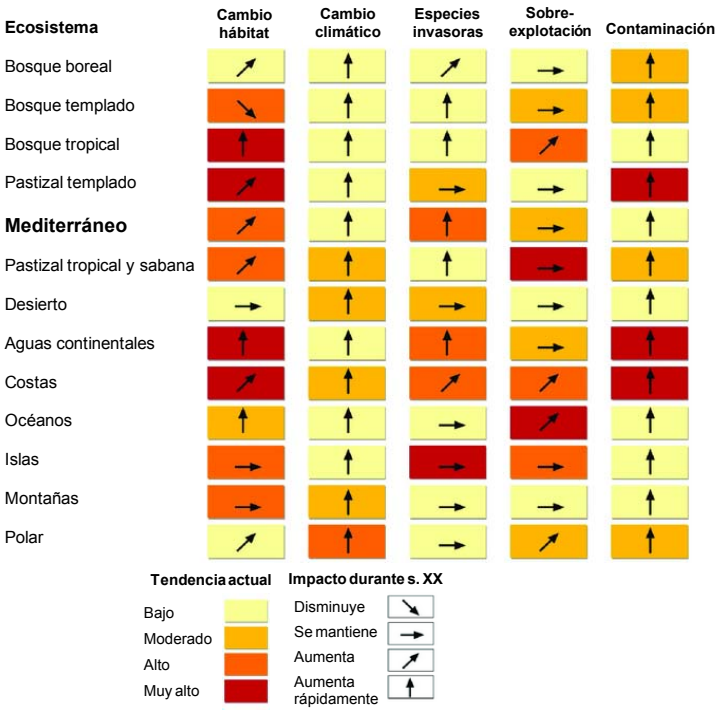



**Figura 1.1** Impacto de los principales motores de cambio global sobre la biodiversidad y los servicios de los distintos tipos de ecosistemas. Elaborado a partir de Millennium-Ecosystem-Assessment (2005)

Entre las alteraciones abióticas ocasionadas por la reducción de los fragmentos forestales se encuentran el incremento de la insolación y la exposición al viento, que repercuten notablemente sobre la humedad y temperatura del suelo y del aire (Saunders et al., 1991). Este conjunto de alteraciones podrían reducir la homeostasis climática de los paisajes fragmentados (Janzen, 1986; Saunders et al., 1991). Por otra parte, las alteraciones de procesos bióticos derivadas de la fragmentación han sido

ampliamente exploradas, principalmente las derivadas de la disminución del tamaño poblacional y la diversidad de los macroorganismos, así como del incremento eventual de las especies exóticas. De manera general, la fragmentación del hábitat tiene un impacto negativo sobre la biodiversidad (Saunders et al., 1991; Didham et al., 1996; Fahrig, 2003; Fischer y Lindenmayer, 2007), alterando el funcionamiento de los ecosistemas y afectando importantes servicios ecosistémicos (IPCC, 2007). Sin embargo, aún no se ha alcanzado un consenso sobre el signo de los efectos de la fragmentación del hábitat sobre la biodiversidad, habiéndose mostrado ausencia de efectos significativos (Wolff et al., 1997; Parker y Mac Nally, 2002; Rantalainen et al., 2008), o incluso efectos positivos sobre ciertas poblaciones (Dooley y Bowers, 1998; Díaz et al., 1999; Rantalainen et al., 2005), así como sobre la productividad de bellotas de las encinas (Morán-López et al., 2015).

La pérdida de biodiversidad, principalmente vegetal, como consecuencia de la reducción de los fragmentos forestales se ha asociado con la disminución de la variabilidad genética y de la adecuación biológica, lo cual se refleja en una menor supervivencia (Lienert, 2004; Aguilar et al., 2006). Como consecuencia, los suelos de paisajes fragmentados pueden enfrentarse a la pérdida de interacciones biológicas y a una reducción de los aportes de materia orgánica, disminuyendo la disponibilidad y ciclado de nutrientes (Garcia et al., 2002), y alterando los ciclos biogeoquímicos y otros procesos ecosistémicos clave para el funcionamiento del ecosistema (Lindenmayer y Fischer, 2006). Sin embargo, en el caso de paisajes fragmentados con influencia de una matriz agrícola, los cambios en la calidad del hábitat pueden concurrir debido a otros factores asociados con el tipo de uso de la matriz, como la fertilización de las áreas adyacentes, el arado intensivo, el apisonamiento y/o la erosión del suelo (Boutin y Jobin,

1998; Matesanz et al., 2009), alterando también la estructura del paisaje y la dispersión de semillas (Morán-López et al., 2015).

En este sentido, la fragmentación del hábitat podría alterar la estructura del suelo y su funcionalidad, en particular aquellos procesos relacionados con el ciclado de carbono. Esto es de especial interés debido a que los suelos son el principal almacén de carbono en los ecosistemas terrestres, albergando hasta dos terceras partes del carbono del mundo (Dixon et al., 1994; Schlesinger y Andrews, 2000).

### *1.1 Importancia del ciclo de carbono en los ecosistemas terrestres*

El contenido de carbono en suelo es el resultado de un balance entre las entradas de materia orgánica (compuesta en promedio de un 50% de C) originada a través de la producción primaria neta del sistema (aproximadamente el 50% de la producción primaria bruta; (Vicca et al., 2012) y que consiste principalmente en hojarasca, raíces, exudados y rizodeposición, y las salidas de carbono del sistema a través de procesos bióticos tales como la oxidación aeróbica de la materia orgánica, y abióticos como la foto-degradación o la lixiviación, entre otros (Bond-Lamberty et al., 2004; Houghton, 2005).

La respiración del suelo ($R_s$), que es el segundo flujo de carbono más importante del ecosistema después de la producción primaria bruta (Janssens et al., 2001), tiene un importante papel en la regulación de los balances netos de C en los sistemas terrestres. $R_s$ es el resultado de la contribución prácticamente igualitaria de la respiración heterotrófica, derivada de la actividad aeróbica de los microorganismos, y la respiración autotrófica, resultante de la respiración de las raíces de las plantas y sus microorganismos rizosféricos y simbióticos asociados (Fig. 1.2; Bond-Lamberty et al., 2004). Además, $R_s$ es un flujo fuertemente sensible a los cambios en las condiciones micro-climáticas del suelo (p. ej. temperatura y

humedad; ver Raich y Schlesinger, 1992; Reichstein et al., 2003; Cook y Orchard, 2008), así como en la productividad de la vegetación, responsable del suministro de carbono a las partes subterráneas del ecosistema (Martin et al., 2009). Concretamente en ecosistemas Mediterráneos, la regulación climática de la respiración del suelo está fuertemente modulada por la humedad del mismo, siendo el factor limitante de la actividad autotrófica y heterotrófica durante buena parte del año (Curiel Yuste et al., 2007; Matías et al., 2012).

Sin embargo, aún no hay suficiente conocimiento de cómo estos factores que regulan la respiración del suelo a las diferentes escalas espaciales y temporales se verán afectados por otros factores de cambio global tan importantes en la cuenca Mediterránea como la fragmentación del hábitat. En este sentido, las contribuciones al conocimiento enfocadas a determinar los mecanismos y factores responsables de la variabilidad de la respiración del suelo son de gran utilidad para reducir la incertidumbre en las estimaciones de emisiones de carbono en los ecosistemas terrestres (Meir et al., 2006).

Entre los procesos implicados en la respiración del suelo se encuentra la respiración heterotrófica, fruto de la actividad descomponedora de la materia orgánica por parte de los microorganismos del suelo. La descomposición de la materia orgánica del suelo depende enormemente de la naturaleza y composición química del sustrato (Gallardo y Merino, 1993; Cotrufo et al., 2013), las condiciones micro-ambientales (Schimel et al., 1999; Sierra et al., 2015) y la composición (diversidad y abundancia) de los organismos del suelo, tanto macro como microorganismos (Fig. 1.2)(Hättenschwiler et al., 2005; Curiel Yuste et al., 2007). Además, la descomposición de la materia orgánica por parte de las comunidades microbianas del suelo ocurre mayoritariamente a través de la liberación de enzimas extracelulares específicas para cada sustrato

(Nannipieri et al., 2003; Baldrian, 2014). Al ser el agente inmediato de la descomposición de la materia orgánica, las actividades enzimáticas, representan un eslabón clave en la retroalimentación entre el clima, los ecosistemas y las concentraciones de $CO_2$ en la atmósfera.

Existe un amplio conjunto de enzimas extracelulares producidas por los microorganismos del suelo (p. ej. bacterias y hongos) tales como enzimas responsables de oxidación de o carbohidratos de diferente complejidad (glucosidasas, oxidasas, hidrolasas o peroxidasas), y de pasos metabólicos asociados al ciclo del nitrógeno (proteasas, peptidasas, ureasa o quitinasa), del fósforo y del azufre (fosfatasas y arilsulfatasas) (Sardans y Peñuelas, 2005; Sardans et al., 2008; Sinsabaugh et al., 2008; Burns et al., 2013; Baldrian, 2014). Aunque existen estudios sobre la sensibilidad de algunas enzimas al cambio climático (Sardans y Peñuelas, 2005; Schimel et al., 2010; Gómez-Luna et al., 2012) e incluso a la fragmentación del hábitat (Lázaro-Nogal et al., 2012), estamos lejos de entender la resistencia y/o sensibilidad de las diferentes enzimas responsables de la descomposición de la materia orgánica al cambio global.

La descomposición de la materia orgánica del suelo también depende fuertemente de la cantidad y calidad de la materia orgánica a la que los microorganismos pueden acceder (Gallardo y Merino, 1993; Burns et al., 2013; Cotrufo et al., 2013). En este sentido, los compuestos orgánicos que constituyen la materia orgánica consisten en una variedad de sustratos que históricamente han sido divididos de una manera arbitraria en una fracción lábil integrada por compuestos orgánicos fácilmente consumidos por los microorganismos por su palatabilidad (p. ej. ácidos orgánicos, aminoácidos, almidones, bio-polimeros, celulosas, hemicelulosas, quitina, peptidoglicanos, entre otros) y/o accesibilidad (disueltos en la solución acuosa del suelo), y una fracción estable, constituida por compuestos orgánicos químicamente más complejos (p. ej. ácidos fúlvicos, ácidos

húmicos y huminas; (Cochran et al., 2007) o de difícil accesibilidad física o geoquímica (Van Veen y Kuikman, 1990; Christensen, 2001). Esta heterogeneidad estructural de los compuestos orgánicos del suelo requiere de la interacción de diversos tipos de enzimas, producidos por diferentes grupos funcionales microbianos (Romaní et al., 2006). En general, las bacterias se encargan de la mayor parte de la rápida descomposición de sustratos fácilmente disponibles, mientras que los hongos pueden degradar compuestos orgánicos más recalcitrantes (Wardle et al., 2004; Bardgett y Wardle, 2010) y son capaces de adaptarse mejor a las sequías (Curiel Yuste et al., 2011; Grigulis et al., 2013; Fuchslueger et al., 2014).

En este sentido, es importante entender la ecología de las comunidades microbianas del suelo, su composición, estructura y funcionamiento, ya que son responsables de la producción de enzimas y descomposición de la materia orgánica. La ecología de estas comunidades depende fuertemente de las propiedades del suelo, como el pH (Fierer y Jackson, 2006), la materia orgánica (Burns et al., 2013) o la disponibilidad de nutrientes (O'Donnell et al., 2001; Baldrian, 2014), entre otros. Además, la estructura de las comunidades microbianas está regulada por las condiciones ambientales, como las variaciones de temperatura (Pace, 1997; Criquet et al., 2002) y/o la precipitación histórica (Curiel Yuste et al., 2007; Evans y Wallenstein, 2012).

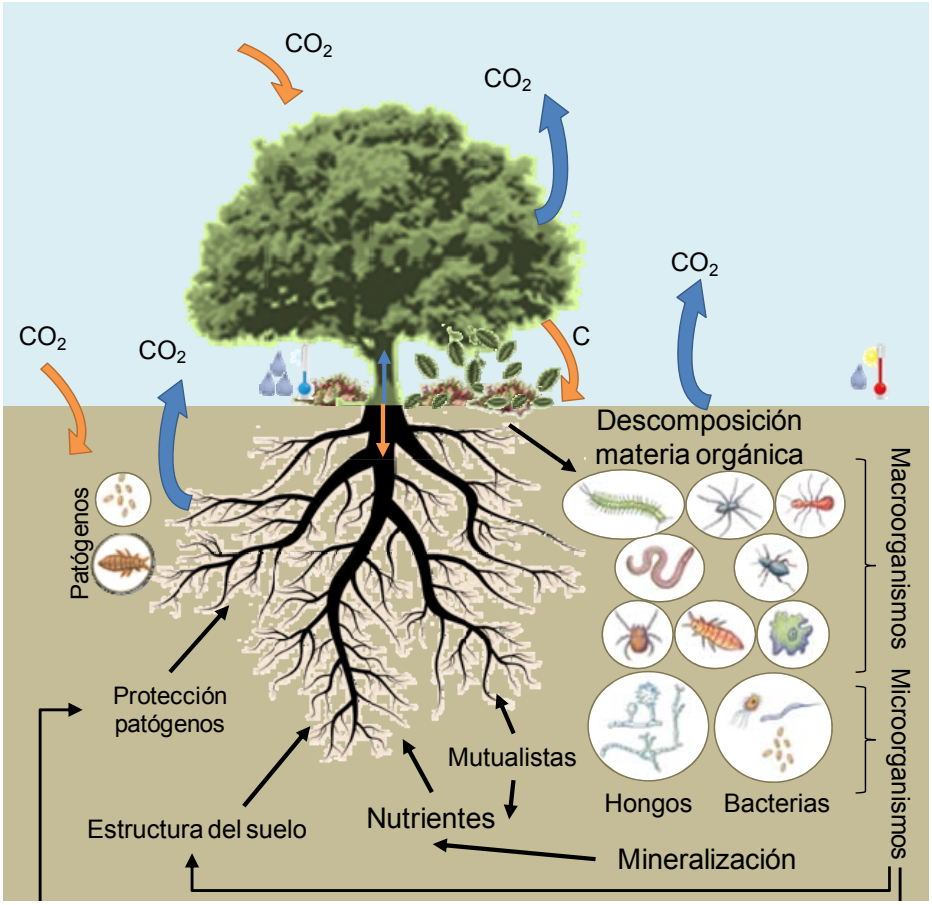



**Figura 1.2** Esquema de las interacciones planta-suelo-microorganismo. La planta proporciona el carbono en forma de materia orgánica muerta (hojas, ramas, raíces) y rizo-deposición y exudados, modificando a su vez las condiciones micro-ambientales del medio circundante (p. ej. aumentado la humedad del suelo y disminuyendo la temperatura). Los organismos del suelo se encargan de la descomposición de la materia orgánica y de su incorporación en la cadena trófica. Tanto la planta como los microorganismos dependen de las características fisicoquímicas del suelo. Adicionalmente, hongos y bacterias realizan procesos de mineralización de la materia orgánica, liberando nutrientes. Algunos hongos son capaces de establecer relaciones mutualistas con las plantas (micorrizas), estrechando la relación planta-microorganismos. Los organismos del suelo liberan nutrientes, modifican la estructura del suelo y ofrecen protección contra patógenos a las plantas, entre otros. En el esquema se muestran los flujos de carbono del sistema como entradas (flechas naranjas) y salidas (flechas azules) del mismo.

*1.2 El papel de las interacciones planta-suelo-microorganismo en los ciclos biogeoquímicos*

Por otra parte, las plantas y sus estrechas relaciones causa-efecto con los microorganismos, juegan un papel determinante en la composición, estructura y funcionamiento de estas comunidades subterráneas (Aponte et al., 2011; Sardans y Peñuelas, 2013; Rincón et al., 2014). En este sentido, la mayoría de las propiedades químicas y físicas del suelo que afectan a las que las comunidades microbianas (p. ej. disponibilidad de nutrientes, contenido de humedad, calidad y cantidad de materia orgánica, pH, entre otros) están fuertemente determinadas por la naturaleza de la cobertura vegetal (Fig. 1.2); de esta manera, cambios en las propiedades fisicoquímicas del suelo derivadas de cambios en la composición y/o vigor de la cobertura vegetal pueden provocar alteraciones en las comunidades microbianas que, a su vez, puede afectar a la capacidad de estas comunidades para reciclar los nutrientes necesarios para garantizar la supervivencia y crecimiento de la planta, creando una retroalimentación negativa con el consecuente colapso ecosistémico (Aponte et al., 2011; Fuchslueger et al., 2014).

La planta, además, provee substrato tanto a los organismos descomponedores de la materia orgánica (macro y microorganismos, Fig. 1.2), como a los organismos asociados a las raíces (rizosféricos y simbióticos), a través de los aportes en la parte aérea (hojarasca, ramas, etc.) y en la parte subterránea (raíces, exudados, rizodeposición, etc.). Al descomponer la materia orgánica de las plantas, los microorganismos regulan indirectamente el crecimiento de las mismas, pues determinan el suministro de nutrientes disponibles del suelo. Los organismos asociados a las raíces (p. ej. mutualistas y patógenos) influyen en la planta de una manera más directa, principalmente en la calidad, direccionalidad y flujo tanto de energía como de nutrientes (Fig. 1.2)(Wardle et al., 2004). Sin

embargo, estudios recientes indican que las interacciones planta-suelo-microorganismo son mucho más complejas que las estudiadas hasta este momento (Rincón et al., 2007; Bardgett y Wardle, 2010; Grigulis et al., 2013; Sardans y Peñuelas, 2013), especialmente si se tiene en cuenta su respuesta a estreses ambientales como los derivados del cambio global.

En este sentido, también se esperaría que la diversidad de los microorganismos sea importante para el mantenimiento de los procesos del suelo (incluyendo la descomposición de la materia orgánica), y que su reducción pudiera interrumpir o afectar negativamente al funcionamiento del sistema suelo y, por ende, del ecosistema en general (Wagg et al., 2014). Sin embargo, los estudios hasta ahora realizados respecto a la relación entre estructura (diversidad y ensamblaje de especies) y funcionalidad de las comunidades microbianas presentan resultados inconclusos y/o contradictorios (Griffiths et al., 2000; O'Donnell et al., 2001; Bell et al., 2005; Langenheder et al., 2010; Levine et al., 2011; Curiel Yuste et al., 2014; Mendes et al., 2015), por lo que es necesario diseñar nuevas estrategias de estudio para ampliar nuestro conocimiento de esta relación y así poder predecir su respuesta a las alteraciones asociadas al el cambio global.

Ante una alteración ambiental, se han descrito diferentes estrategias que las comunidades microbianas pueden mostrar: resistencia (la composición de la comunidad microbiana se mantiene igual), resiliencia (la composición microbiana es alterada pero regresa a su composición original), redundancia funcional (la composición de la comunidad microbiana es alterada pero funciona como la comunidad original), o la alteración de la composición de la comunidad que tiene como resultado un cambio en su funcionalidad (Allison y Martiny, 2008). De la respuesta de estas comunidades a diferentes motores de cambio global y/o su interacción dependerá, en gran medida, su capacidad para generar las

diferentes funciones específicas con las que los microorganismos proveen al ecosistema (Wagg et al., 2014).

Sin embargo, los estudios sobre la respuesta microbiana ante perturbaciones se han centrado mayoritariamente en los efectos de alteraciones climáticas sobre la diversidad de las comunidades microbianas (p. ej. sequía, rehidratación, frío, inundaciones etc., ver Schimel et al., 1999; Fierer et al., 2003; Schimel et al., 2007; Allison y Martiny, 2008; Rincón et al., 2008; Curiel Yuste et al., 2011; Zak et al., 2011; Evans y Wallenstein, 2012; Göransson et al., 2013; Zumsteg et al., 2013; Fuchslueger et al., 2014). Pocos estudios, sin embargo, se han centrado en los efectos sobre estas comunidades microbianas y la funcionalidad del suelo de otros motores de cambio global, tales como la fragmentación del hábitat, de amplia repercusión especialmente en las zonas Mediterráneas, (Zheng et al., 2005; Malmivaara-Lämsä et al., 2008; Rantalainen et al., 2008; Lázaro-Nogal et al., 2012; Riutta et al., 2012). Ya que las predicciones futuras para la cuenca Mediterránea son escenarios de mayor sequía y paisajes más fragmentados (IPCC 2007), resulta esencial entender los efectos de la fragmentación del bosque y su interacción con el incremento de la sequía sobre los ecosistemas Mediterráneos.

### *1.3 Efectos de la fragmentación del hábitat en la funcionalidad del suelo*

Resultados previos con sistemas experimentales como modelo de estudio de la fragmentación han mostrado que las comunidades de organismos descomponedores de materia orgánica pueden ser insensibles a la fragmentación del hábitat (revisado por Rantalainen et al., 2008)), encontrándose incluso un efecto positivo de la fragmentación sobre su composición (Rantalainen et al., 2005). Por el contrario, Malmivaara-Lämsä et al. (2008) encontraron que la cantidad de biomasa microbiana y la actividad (respiración basal) de comunidades microbianas de ecosistemas

Boreales fragmentados, fueron negativamente afectadas por el efecto borde, relacionados con una menor humedad del suelo. Asimismo en paisajes fragmentados, la funcionalidad de las comunidades microbianas, particularmente la descomposición de la materia orgánica, parece estar controlada por las alteraciones en las propiedades abióticas del suelo, como la temperatura (Zheng et al., 2005), la calidad del sustrato y la disponibilidad de nutrientes (Lázaro-Nogal et al., 2012), y la humedad del suelo (Riutta et al., 2012).

Con estos estudios queda evidenciada la importancia de cuantificar el efecto de la fragmentación del hábitat sobre la ecología de las comunidades microbianas, responsables de la descomposición de la materia orgánica y las emisiones de $CO_2$ de origen heterótrofo; entender su ecología en un mundo cambiante no sólo es crucial para poder elaborar predicciones más precisas en un escenario de cambio global, sino también para reducir las pérdidas de carbono de los suelos, e incrementar la capacidad de los sistemas edáficos para almacenar C que de otra manera sería emitido a la atmósfera en forma de $CO_2$ (Schlesinger y Andrews, 2000; Rotenberg y Yakir, 2010).

Como se mencionó anteriormente, es importante considerar la estrecha relación de las plantas con la estructura y el funcionamiento de las comunidades microbianas, así como evaluar de forma integrada el sistema planta-suelo-microorganismo en escenarios de perturbación ambiental. Concretamente, es importante ahondar en el efecto conjunto de varios motores de cambio global (p. ej. fragmentación del hábitat e incremento de la sequía) en este sistema, pues frecuentemente estos motores presentan efectos no aditivos, lo cual podría atenuar o exacerbar la respuesta del ecosistema a motores individuales de cambio global (Matesanz et al., 2009; Lázaro-Nogal et al., 2012).

## Estructura y objetivos de la tesis

El objetivo de la presente tesis doctoral fue evaluar el efecto de la fragmentación del bosque sobre la funcionalidad del sistema planta-suelo-microorganismo. En la tesis se exploró la importancia relativa de la respuesta de este sistema a dos de los motores de cambio global más importantes en el Mediterráneo continental: la fragmentación del hábitat y la sequía. Para ello, se analizaron múltiples indicadores de la funcionalidad del sistema planta-suelo-microorganismo como la respiración del suelo, la actividad enzimática y el perfil metabólico, la diversidad y estructura de las comunidades microbianas, y la productividad, fisiología y aclimatación de plántulas de *Quercus ilex* (Fig. 1.3), utilizando una aproximación multi-escala.

Por una parte se estudió la respuesta de la funcionalidad del suelo y las comunidades microbianas a la fragmentación del hábitat (Capítulo 2 y 3). Para ello, se realizó un muestreo de campo en encinares fragmentados de la región sur, en la submeseta mesomediterránea, en el cual se determinó la respiración del suelo *in situ* y algunas características estructurales de las encinas, además, se recolectaron muestras de suelo para la posterior determinación de parámetros fisicoquímicos, actividades enzimáticas y biomasa microbiana (Capítulo 2). Las muestras de suelo de este muestreo, así como los parámetros fisicoquímicos determinados, se utilizaron posteriormente como base para analizar del efecto específico de la fragmentación del encinar sobre la estructura y funcionalidad de las comunidades microbianas (bacterias y hongos), estudio en el que se evaluaron la diversidad, el ensamblaje y el perfil metabólico, así como el posible vínculo existente entre diversidad y función en estas comunidades (Capítulo 3).

En los siguientes capítulos se exploró la respuesta del sistema planta-suelo-microorganismo a la sequía, utilizando microcosmos con

plántulas de *Q. ilex* y suelos de encinares fragmentados como modelo de estudio (Capítulos 4 y 5). En estos experimentos, adicionalmente se estudió una segunda región climatológicamente contrastada con respecto a la anterior: la región norte en la submeseta supremediterrránea. En un primer experimento en condiciones controladas, se exploró la respuesta a la sequía crónica gradual (p. ej. verano) del sistema planta-suelo-microorganismo y a las primeras lluvias del otoño (rehidratación) del sistema suelo-microorganismo. Para ello, se examinó el intercambio neto del ecosistema a lo largo de la simulación, además de actividades enzimáticas, y la diversidad y estructura de las comunidades microbianas (bacterias y hongos), así como rasgos funcionales y fisiológicos de las plántulas (Capítulo 4). Finalmente, se evaluó el impacto de la plasticidad de las plántulas de encinas sobre la respuesta del sistema planta-suelo-microorganismo a sequía. Para ello, se realizó un segundo experimento en condiciones controladas, con plántulas de diferentes procedencias crecidas en presencia o no de microorganismos nativos de suelos procedentes de encinares fragmentados de las regiones anteriormente mencionadas, sometidas a una sequía crónica gradual. En este experimento, se evaluó el intercambio neto del ecosistema, así como rasgos funcionales y fisiológicos de las plántulas, y su capacidad de asociación con hongos ectomicorrícicos (Capítulo 5).

Los capítulos 2 al 5 de la tesis han sido escritos en inglés para su publicación en revistas internacionales y para facilitar la difusión de este trabajo. Al igual que la introducción general, la discusión y conclusiones generales se presentan en castellano. A continuación se detallan el título y objetivos específicos de cada capítulo.

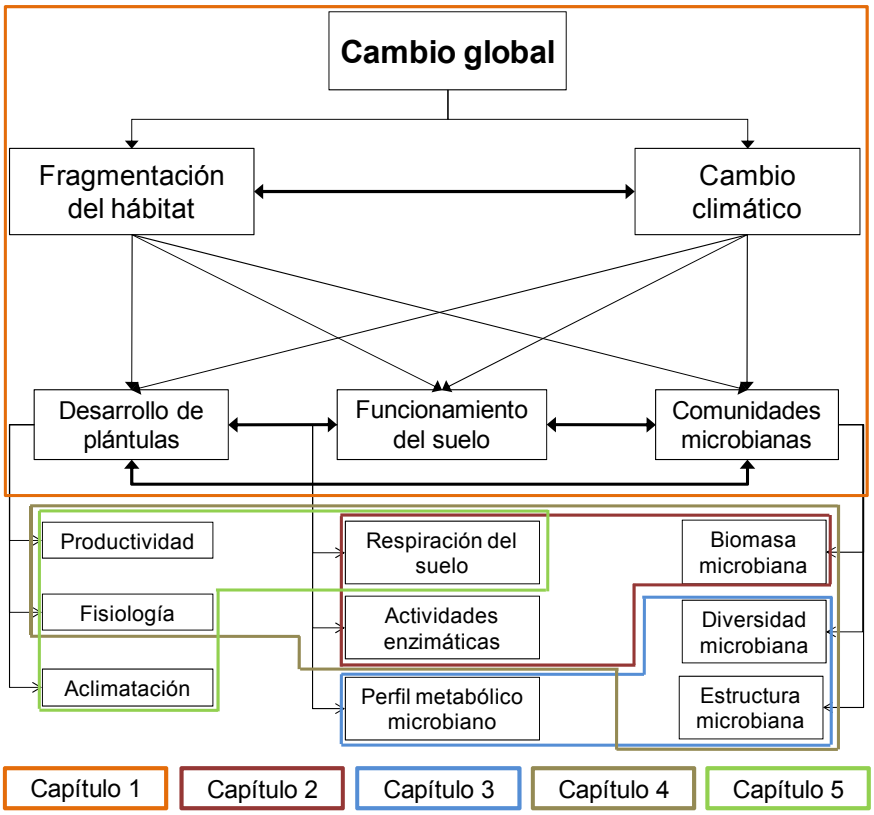



**Figura 1.3** Esquema conceptual de la tesis doctoral y de los aspectos abordados en cada capítulo.

### Capítulo 2. La fragmentación del bosque afecta indirectamente al sistema árbol-suelo y a la funcionalidad microbiana en encinares mediterráneos.

Forest fragmentation indirectly affects tree-soil system and microbial functioning in Mediterranean holm oak forests.

Dulce Flores-Rentería, Ana Rincón, Teresa Morán-López, Ana-Maria Hereş, Leticia Pérez-Izquierdo, Fernando Valladares, Jorge Curiel Yuste

Manuscrito enviado a la revista *Soil Biology and Biochemistry*.

El objetivo de este capítulo fue analizar la respuesta de la funcionalidad de las comunidades microbianas a la matriz agrícola circundante, en la que están embebidos los fragmentos de encinares, así como a la cobertura de las encinas. Además de identificar el papel y la importancia relativa de

factores bióticos y abióticos en el funcionamiento microbiano del suelo en una escala de paisaje. Para ello, se determinó la respiración del suelo de encinares fragmentados en campo, así como su actividad enzimática en el laboratorio, además diversos factores bióticos y abióticos de los mismos (materia orgánica, humedad, temperatura, nutrientes, pH).

**Capítulo 3. La matriz agrícola afecta directamente a la estructura e indirectamente al metabolismo de las comunidades microbianas de los suelos de encinares fragmentados.**

> Agricultural matrix directly affects the structure and indirectly the metabolism of microbial communities in fragmented Mediterranean holm oak forest soils
>
> Dulce Flores-Rentería, Ana Rincón, Fernando Valladares, Jorge Curiel Yuste.
>
> Manuscrito en preparación.

El objetivo de este capítulo fue analizar la respuesta de las comunidades microbianas del suelo a la influencia de la matriz agrícola circundante, a la par de identificar el papel e importancia relativa de factores bióticos y abióticos en el ensamblaje y diversidad de las comunidades microbianas. Para ello se determinó el perfil estructural y metabólico de las comunidades microbianas (bacterias y hongos) de suelos de encinares fragmentados. Además, se analizaron los posibles vínculos entre la estructura y el funcionamiento de las comunidades microbianas.

**Capítulo 4. La fragmentación del hábitat puede modular los efectos de la sequía sobre el sistema planta-suelo-microorganismo en encinares Mediterráneos (*Quercus ilex*) fragmentados.**

> Habitat fragmentation can modulate drought effects on the plant-soil-microbial system in Mediterranean holm oak (*Quercus ilex*) forests
>
> Dulce Flores-Rentería, Jorge Curiel Yuste, Ana Rincón, Francis Q. Brearley, Juan Carlos García-Gil, Fernando Valladares
>
> Manuscrito publicado en *Microbial Ecology* 1-15. doi: 10.1007/s00248-015-0584-9

El objetivo de este capítulo fue estudiar el impacto de la sequía y rehidratación en la estructura y funcionamiento de las comunidades microbianas del suelo, y evaluar la interacción potencial entre fragmentación y clima, explorando la respuesta fisiológica a la sequía de las plántulas de encina. Para lo cual se llevó a cabo un experimento en condiciones controladas usando suelo de las dos regiones estudiadas (norte y sur) y de tamaño de fragmento contrastado (grande y pequeño) y plántulas de *Q. ilex* de la región sur. Estos microcosmos fueron sometidos a una sequía crónica gradual y su posterior rehidratación.

**Capítulo 5. La influencia de la fragmentación del bosque y la región bioclimática sobre las características bio-fisicoquímicas del suelo se sobreponen al potencial efecto de la procedencia de las plántulas, en la resistencia a sequía del sistema planta-suelo-microorganismo**

> The influence of forest fragmentation and bioclimatic region over the bio-physicochemical soil characteristics overshadows potential seedling provenance effects in the resistance of the plant-soil-microbial system to drought.
>
> Dulce Flores-Rentería, Jorge Curiel Yuste, Fernando Valladares, Ana Rincón.
>
> Manuscrito en preparación.

El objetivo general de este capítulo fue evaluar el impacto de la sequía sobre el funcionamiento del sistema planta-suelo-microorganismo y evaluar cómo la procedencia de las plántulas y el origen de los suelos, con o sin microorganismos nativos pueden afectar este funcionamiento. Para ello se llevó a cabo un experimento en condiciones controladas usando suelo de las dos regiones estudiadas, norte y sur, con diferente influencia de la matriz agrícola circundante. Los suelos se utilizaron como inoculo (presencia o no de microorganismos nativos) para crecer plántulas de *Q. ilex* de tres procedencias de la península ibérica. Estos microcosmos fueron sometidos a una sequía crónica gradual.

## Regiones de estudio

Para este estudio se seleccionó la encina (*Quercus ilex* subsp. *ballota* (Desf.) Samp, de aquí en adelante *Q. ilex*), especie clave de los bosques Mediterráneos. La encina es un árbol-arbusto perenne ampliamente distribuido por toda la región mediterránea. Se distribuye en climas desde clima frío semiárido hasta mediterráneo húmedo templado, y es considerada tolerante a la sequía (Baquedano y Castillo, 2007). Es una especie monoica, que florece en primavera, de polinización anemófila y cuyos frutos (bellotas) son dispersados en el otoño por aves y mamíferos (Castroviejo et al., 1993).

Las regiones de estudio se escogieron entre las estudiadas previamente por Santos y Tellería (1998); Díaz et al. (1999) y Díaz y Alonso (2003), seleccionando áreas de encinares fragmentados en la submeseta Norte y en la submeseta Sur (Fig. 1.4). Previamente, se realizó un estudio del material cartográfico de ambas áreas, seleccionándose fragmentos de tamaños contrastados: fragmentos grandes con un área superior a 10 hectáreas y fragmentos pequeños embebidos en la matriz agrícola. En una segunda fase se visitaron todas las áreas preseleccionadas, y se procedió a la selección definitiva de los fragmentos de acuerdo a los siguientes criterios: i) ubicación de las áreas en terrenos llanos o de relieve suave; ii) la matriz circundante preferentemente de uso agrícola, dada la elevada influencia de este rasgo del paisaje sobre los factores bióticos y abióticos; iii) los fragmentos pequeños con al menos tres encinas y un máximo de 0.5 hectáreas. Se seleccionaron tres fragmentos grandes y entre tres y cinco fragmentos pequeños en cada una de las regiones (Fig. 1.5). Los fragmentos grandes, se subdividieron en interior (>30 metros desde el borde del fragmento) y borde (Fig. 1.6). En ambas regiones, el uso de la matriz agrícola, está destinada al cultivo intensivo de cereales, legumbres y algunos viñedos.

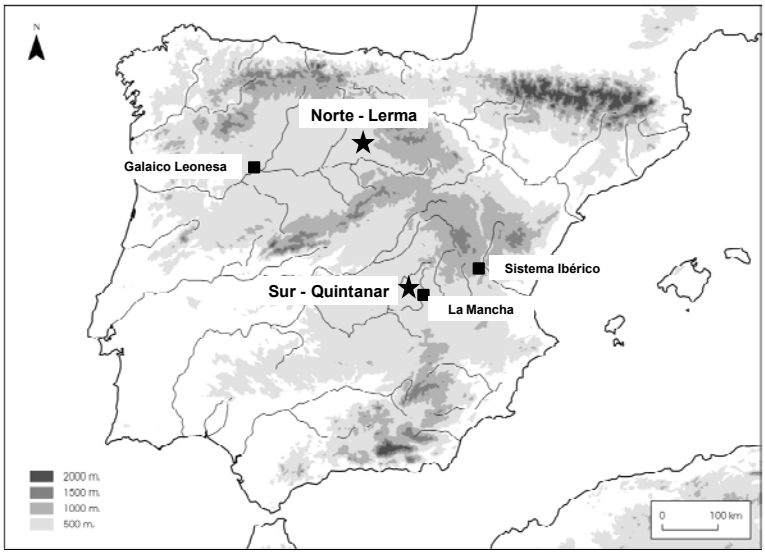



**Figure 1.4** Localización de las áreas de estudio en la península Ibérica. Se muestran las localidades correspondientes a las regiones bioclimáticas Norte-Lerma y Sur-Quintanar (estrellas), y las localidades de procedencia de bellotas (cuadrados) referidas en el Capítulo 5 (Galaico-Leonesa, La Mancha y Sistema Ibérico). La altitud está representada en variación de gris.

La región Norte se localiza en las cercanías de Lerma, en la provincia de Burgos, (41°58'-42°02'N, 03°45'-03°52'O; 930 msnm). Los fragmentos de encinar estudiados se localizan en un área de 1500 hectáreas, separados por al menos 50 m y un máximo de 11 km (Fig. 1.5a). Esta región pertenece a la zona bioclimática supramediterránea (Rivas-Martínez, 1981) y se caracteriza por 554 mm de precipitación anual y una temperatura media de 11 °C (Fig. 1.7). La cobertura arbórea está dominada por encinas (*Quercus ilex*), con quejigos aislados (*Quercus faginea* LAM) y sabinas (*Juniperus thurifera* L.), y un sotobosque compuesto por arbustos típicos de las zonas supremediterráneas húmedas y frías (p. ej. *Cistus laurifolius* L., *Genista scorpius* (L.) DC, *Thymus zygis* Loefl. ex L.)(Santos y Tellería, 1998; Díaz et al., 1999). Los suelos dominantes están clasificados como cambisoles (cálcicos) (WRB, 2007), con textura franco arenosa: 11% arena, 42% limo and 47% arcilla.

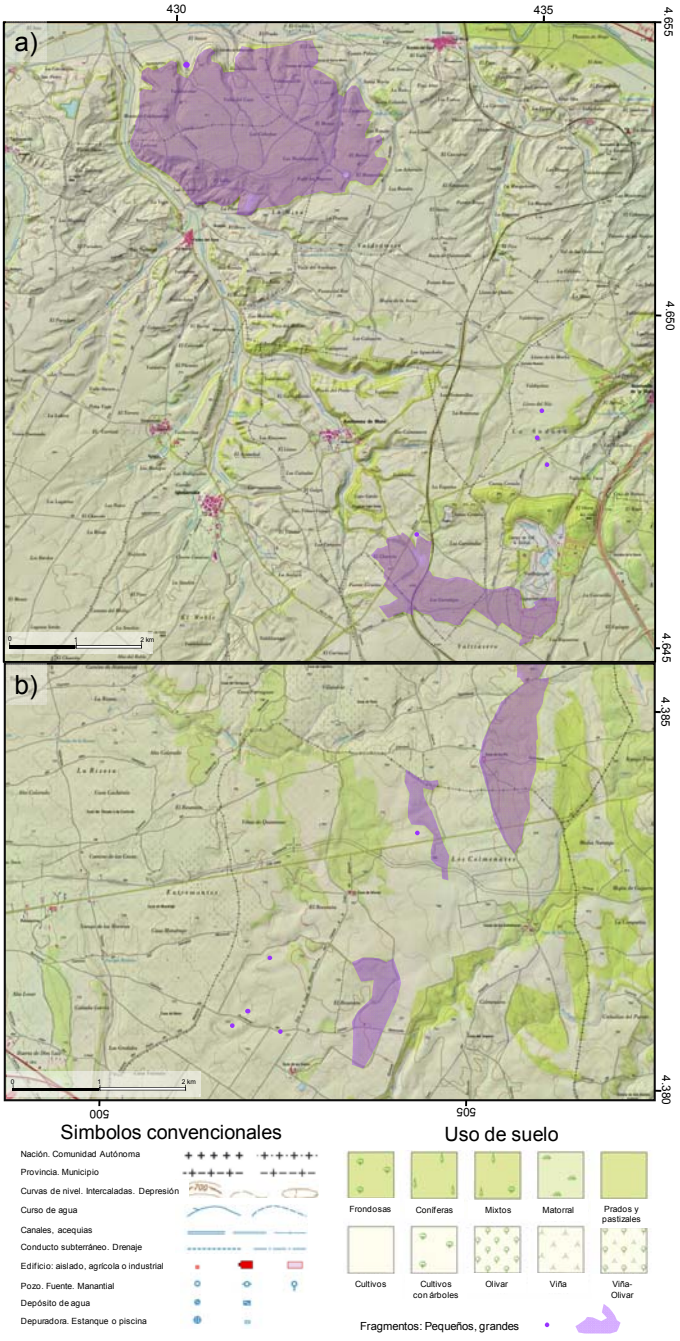



**Figura 1.5** Mapa de las regiones bioclimáticas Norte (a) y Sur (b) en España, mostrando los fragmentos de encinar (morado). Modificado de SIGPAC (http://www.sigpac.jcyl.es/visor). Zona UTM 30.


**Figura 1.6** Encinares mediterráneos fragmentados en Quintanar de la Orden, España. a) Interior de un fragmento grande (> 10 ha); b) borde de un fragmento grande (> 10 ha); c) fragmentos pequeños (< 0.5 ha); d) producción de bellota (cosecha) del año 2012 (*Quercus ilex* ssp. ballota). Las fotos a y c se tomaron en junio de 2011, b y d en noviembre de 2012 (fotos: Mario Díaz, tomadas de (Valladares et al., 2014).

La región Sur se localiza en las cercanías de Quintanar de la Orden, en la provincia de Toledo, (39°30'-39°35'N, 02°47'-02°59'O; 870 msnm). Los fragmentos de encinar estudiados se localizan en un área de 1000 hectáreas, separados por al menos 50 m y un máximo de 8 km (Fig. 1.5b). Esta región pertenece a la zona bioclimática mesomediterránea (Rivas-Martínez, 1981) y se caracteriza por 434 mm de precipitación anual y una temperatura media de 14 °C (Fig. 1.7). La cobertura arbórea está dominada por encinas (*Quercus ilex*), con algunas coscojas (*Quercus coccifera* L.), y un sotobosque compuesto por arbustos típicos de las xéricas mesomediterráneas (p. ej. *Asparagus acutifolius* L., *Cistus ladanifer* L., *Rhamnus alaternus* L., *Rhamnus lycioides* Brot) (Santos y Tellería, 1998;

Díaz y Alonso, 2003). Los suelos dominantes están clasificados como cambisoles (cálcicos) (WRB, 2007), con textura franco arenosa: 17% arena, 39% limo and 44% arcilla.

Ambas regiones se caracterizan por un verano seco pronunciado, que abarca desde julio a septiembre (Fig. 1.7).

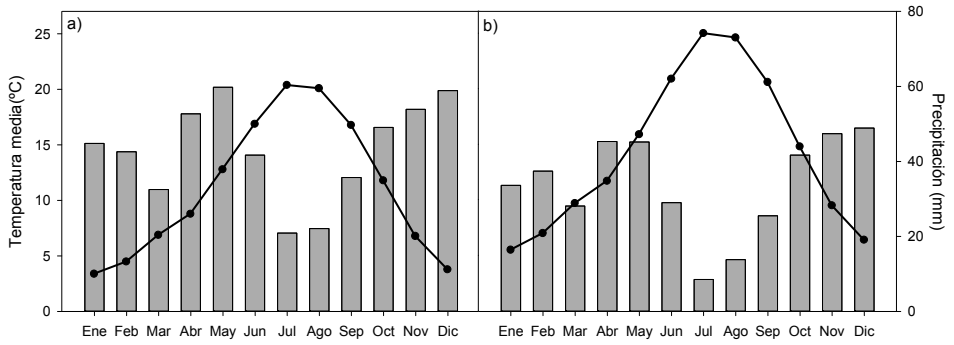



**Figura 1.7** Diagramas ombrotérmicos de las dos regiones de estudio: Norte, Lerma (a) y Sur, Quintanar de la orden (b). Eje izquierdo: temperatura, en línea continua y puntos; eje derecho: precipitación, en barras. Datos tomados de Ninyerola et al. (2005).

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# Capítulo 2

## Forest fragmentation indirectly affects tree-soil system and microbial functioning in Mediterranean holm oak forests

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## Abstract

Landscape transformations such as forest fragmentation affect the carbon sink capacity of soils and their vulnerability to other global change threats. The adverse effects of habitat fragmentation on biodiversity are well documented, but much less are known about their impact on ecosystem processes. We studied key mechanisms and drivers of soil microbial functioning in fragmented landscapes by analyzing soil respiration and microbial enzymatic activities in Mediterranean holm oak forest fragments of different sizes. For that, we evaluated the impact of forest fragmentation on soil microbial functioning by building models using structural equation models (SEM) including biotic (microbial biomass), abiotic (soil moisture, temperature, organic matter (SOM), pH, nutrients) and tree structural (basal area) variables. Our results indicates that the higher fertility of those fragmented Mediterranean forests strongly influenced by the agricultural matrix enhances tree growth, which triggered a cascade of causal-effect relations that indirectly stimulated soil microbial activities and nutrient cycling. The increment of SOM associated with tree growth also modified the pH, changed the microclimatic conditions (increasing soil moisture and buffering temperature) and stimulated microbial growth resulting in a significant increase in soil enzymatic activity and respiration under the trees most influenced by the agricultural matrix. Collectively, our results revealed that the increasing trends in forest fragmentation have profound effects over the functioning of the plant-soil-microbial system, whit deep impacts on the soil carbon and nutrient cycles. Forest fragmentation should therefore be considered in estimations of the carbon sink capacity under global change scenarios.

## Introduction

Mediterranean forests have been intensively transformed by humans for centuries. Among other human-related activities that have contributed to this transformation to the state we know Mediterranean forests today, the rapid and intense forest fragmentation might be considered as one of the most impacting ones, and consequently, a special attention has been paid out to it (Alados et al., 2004; Blondel, 2010). While the negative effects of forest fragmentation on biodiversity have been widely explored (Saunders et al., 1991; Fahrig, 2003; Fischer and Lindenmayer, 2007), little research has been conducted on its effects on ecosystem functioning (Turner, 2005). Fragmentation has important implications for forest conservation and management strategies (Saunders et al., 1991), becoming an increasingly important global change driver in the Mediterranean basin (Millennium-Ecosystem-Assessment, 2005). Forest fragmentation results in remnant areas of native vegetation typically surrounded by a matrix of agricultural lands, in which solar radiation, wind, water, and temperature are significantly altered (Saunders et al., 1991). As a consequence, the impact of forest fragmentation on ecosystem functioning (e.g. processes such as primary productivity and organic matter decomposition) will depend first on the environmental biotic and abiotic conditions of both the remnant areas and the surrounding matrix, and second on the size of the resulting fragments because the smaller the fragment, the greater the edge effect, and therefore, higher the influence of the matrix in which it is imbibed (Fernández et al., 2002; Fischer and Lindenmayer, 2007).

Forest fragmentation may alter the ecosystem's functioning at many levels, but the effects on soil processes and, in particular the ones related to carbon cycling, are of special interest, given that soils are the largest carbon pool in terrestrial ecosystems (Schlesinger and Andrews, 2000). This pool results from the balance between carbon inputs (i.e.

primary production, below ground allocation, litter fall) and outputs (i.e. organic matter decomposition, soil $CO_2$ efflux, among others) within the system, being regulated, among other factors, by soil respiration ($R_S$), which is the largest ecosystem flux after gross primary productivity (Janssens et al., 2001), and results from both heterotrophic (from the aerobic activity of microorganisms; $R_H$) and autotrophic respiration (roots respiration plus associated symbiotic microorganisms; $R_A$). Despite that changes in $R_S$, particularly in semi-arid systems, can result in strong modifications of the ecosystem source-sink capacity, there are only few studies that have evaluated the effect of forest fragmentation over this important component of the terrestrial carbon cycle (Qi et al., 2002; Baldocchi et al., 2006; Wang and Epstein, 2013). It is essential to understand the effects of forest fragmentation on $R_H$ because it is an important component of $R_S$, as it accounts for at least 50% of the total soil respiration (Bond-Lamberty et al., 2004). $R_H$ involves many processes such as the break-down of different soil organic matter (SOM) components and/or the mineralization of different essential nutrients by the action of specific extracellular enzymes, e.g. β-glucosidase to consume labile carbohydrates, or chitinase and phosphatase to mineralize nitrogen (N) and phosphorous (P), respectively. Additionally, as the proximate agents of organic matter decomposition in soil (Sinsabaugh et al., 2008), enzymatic activity represents a key link in feedbacks between climate, ecosystems and atmospheric $CO_2$ concentrations.

Soil microbial functioning is susceptible of being affected by forest fragmentation, but we do not know to which extent. For instance, previous studies have shown that $R_S$ in fragmented landscapes can be modulated by changes in soil temperature (Zheng et al., 2005) and water availability (Flores-Rentería et al., 2015), suggesting that the forest fragmentation effects may strongly interact with the well described effect of climate over

*Rs* and $R_H$, especially in semi-arid ecosystems (e.g. Curiel Yuste et al. (2007). On the other hand, it has been observed that different microbial enzymatic activities at forest edges can be largely affected by differences in litter quality and micro-environmental conditions caused by fragmentation (Lázaro-Nogal et al., 2012). Slight changes within the decomposer community across the edge-influence areas can lead to differences in soil functional properties (Malmivaara-Lämsä et al., 2008; Riutta et al., 2012; Flores-Rentería et al., 2015). Quantifying the effect of forest fragmentation over soil organic matter decomposition and $CO_2$ emissions is, therefore, crucial for reducing carbon losses from soils and increasing sequestration of atmospheric $CO_2$ in soil.

To evaluate the relative importance of key factors driving SOM decomposition in fragmented landscapes, we analyzed soil microbial functioning (i.e. enzymatic activity and *Rs*) in Mediterranean holm oak forests patches of different size imbibed in an agricultural matrix and differentially influenced by it. We evaluated whether the impact of forest fragmentation on soil microbial functioning could be explained through its effect on microhabitat characteristics.

Our objectives were: (1) to analyze the response of the soil microbial functioning to both the agricultural matrix in which forest fragments were imbibed, and the cover by tree canopies; and (2) to identify the role and relative importance of biotic and abiotic factors in soil microbial functioning at a landscape scale. Since holm oak forest fragmentation has been described to favor soil nutrient's content and microbial biomass (Flores-Rentería et al., 2015), and in view of the lack of studies on the effect of Mediterranean fragmented forest on soil functioning, we hypothesized three possible non-mutually exclusive way of how forest fragmentation affects soil functioning (Fig. 2.1): 1) positively, either directly and/or indirectly; 2) negatively, either directly and/or

indirectly or 3) neutral or negligible effects on soil functioning. The way, sign and magnitude of the forest fragmentation effect on soil functioning with the current knowledge is uncertain.

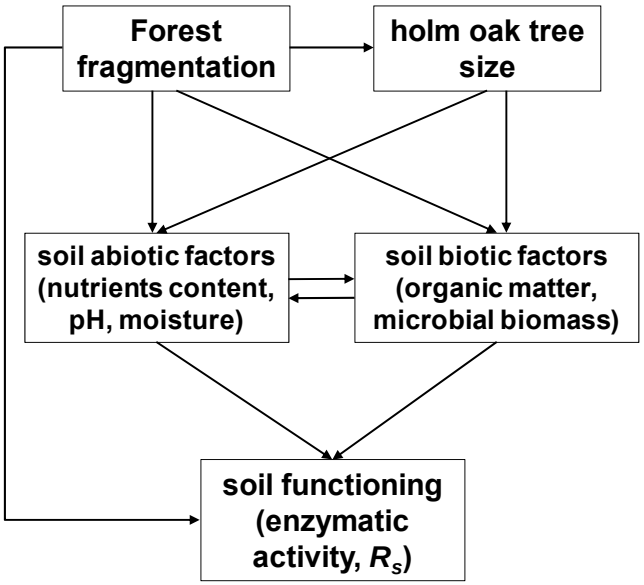


**Figure 2.1** Hypothesized scenarios of forest fragmentation effects over soil function, where affects are presented by solid lines, and with the available knowledge can be either positive (scenario 1) or negative (scenario 2); and a neutral or negligible scenario (3) the effects would not exist or would be insignificant.

## Material and methods

*Study area*

The study area was located near Quintanar de la Orden (39°30'-39°35'N, 02°47'-02°59'W; 870 a.s.l.), in Toledo, south-eastern Spain (Fig. 1.4 in Chapter 1). This area has a Mesomediterranean climate characterized by 434 mm of mean annual precipitation and 14 °C of mean annual temperature (Ninyerola et al., 2005), with a pronounced summer drought, usually lasting from July to September (Fig. 1.7 in Chapter 1). The landscape, a former predominant holm oak Mediterranean forest, is currently dominated by cereal and legume croplands, with scattered grape crops that complete the mosaic. The original forests are now highly fragmented in a variety of patch sizes, covering only the 28 % of their original area (Díaz and Alonso, 2003). The dominant tree is the holm oak (*Quercus ilex* L. ssp. ballota (Desf.) Samp), while the understory is mainly composed by shrubs of Kermes oak (*Quercus coccifera* L.) and, to a lesser extent, by species of *Genista*, *Asparagus*, and *Rhamnus* (for a full description of the study area see: (Santos and Tellería, 1998; Díaz and Alonso, 2003).

*Experimental design and sampling*

A total of three large (> 10 ha) and five small (< 0.5 ha; with at least three trees) forest fragments within an area of 1000 ha, separated of a minimum of 50 m to a maximum of 8 km, were studied (Fig. 1.5b in Chapter 1). Prevalent soils were Cambisols (calcics) (WRB, 2007), with sandy loam texture (17-39-44 % sand-silt-clay).

We defined the influence of the agricultural matrix on forest fragments by the factor "matrix influence" with three levels: (1) low influence, at the interior of large fragments (at least 30 m from the forest edge; coded as "forest interior"); (2) mid influence, at the edges of large fragments (coded as "forest edge"); and (3) high influence, in small

fragments (coded as "small fragments"; Fig. 1.6 in Chapter 1). Additionally, the factor "tree cover" was evaluated at two levels: (1) under holm oak canopy (halfway of the canopy, starting from the trunk; coded as "under canopy"), and (2) outside the canopy (1.5 m outside any canopy projection; coded as "open areas"). For each of the three large fragments, we selected five holm oak trees in the forest interior and five trees at the forest edge, while for each of the five small fragments we selected three holm oak trees (15 trees per matrix influence-fragmentation level), resulting in a total of 45 selected trees. For each of the selected trees, two coverage-sampling points were established: one under canopy and the other in open areas, resulting in a total of 90 soil samples.

*Field measurements*

The field campaign was conducted in spring 2013, during the rainy and growing season in Mediterranean ecosystems, when temperature and moisture were not limiting factors neither for plant growth nor for soil microbial functioning. Daily maximum temperature during the sampling days was of 24 °C, with 15 °C in average, and no precipitation was recorded.

Soil respiration ($R_s$) was measured at each sampling point with a portable dynamic closed chamber (SRC-1, PP-Systems, Massachusetts, USA) connected to an infrared gas analyzer (EGM-4, PP- Systems). The soil chamber was adjusted with 90 temporary external PVC collars (5 cm depth x 10 cm diameter) inserted into the soil, which ensured a tight seal. To minimize the effects of collar's placing, they were inserted into the soil 24 h before $R_s$ measurements on a depth of about 3 cm (Wang et al., 2005). $R_s$ measurements were carried out at maximum daily soil activity (13:30-16:30 h) (Matías et al., 2011), during three consecutive days. Immediately after $R_s$ was measured, soil temperature and field soil moisture were recorded at 10 cm depth by using a wireless multilogger thermometer

(Omega, Connecticut, USA), and a time domain reflectometer (TDR 300, Spectrum technologies, Illinois, USA), respectively. Then, soil cores (2 cm in diameter) were taken from a depth of 0-15 cm. Soils were sieved (< 2 mm) and stored at 4 °C for later analyses.

Height, basal area (based on stems ≥ 3 cm of diameter), and canopy projection were measured for each of the 45 selected holm oak multi-stem trees. A tree influence index was calculated for each sampling point, according to the following formula:

$Tree\ influence\ index = \frac{Basal area}{Distance from the trunk}$. Tree's basal area was selected to calculate this tree influence index, given its recognized direct relationship with soil functioning (Søe and Buchmann, 2005; Katayama et al., 2009; Barba et al., 2013).

*Soil properties*

Soil moisture was determined by weight lost of samples oven-dried at 105 °C for 48 h. Total C and N contents were measured on air-dried soil samples, using a C:N elemental analyzer (Flash EA 1112 Series, Thermo Fisher Scientific). Total concentrations of P, K, Ca, Na, S, Mg, Fe, Mn, Cu, Mo, and Zn were determined by digestion with $HNO_3$ and $H_2O_2$ (4:1, v:v), followed by inductively coupled plasma-optical emission spectrometry (ICAP-6500 Duo/Iris Intrepid II XDL, Thermo Fisher Scientific, Massachusetts, USA). Soil pH was determined on a 1:10 (w:v) aqueous suspension. Soil organic matter (SOM) was assessed by loss on ignition at 400 °C during 4 hours. Microbial biomass carbon content was determined by the chloroform fumigation-extraction method modified by Gregorich et al. (1990).

*Soil enzymatic activity*

We determined the polysaccharide-specific hydrolytic enzymes β-glucosidase and chitinase, and the phosphorus-mineralizing acid

phosphatase. Enzymatic assays were based on methylumbelliferone (MU) (fluorogenic substrate) release upon cleavage by enzymes (Mathieu et al. (2013): MU-β-d-glucopyranoside (MU-G) for β-glucosidase (EC 3.2.1.3), MU-N-acetyl-β-glucosaminide (MU-Q) for chitinase (EC 3.2.1.14), and MU-phosphate free acid (MU-P) for acid phosphatase (EC 3.1.3.2). Stocks 5 mM enzyme solutions were prepared in methoxy-ethanol, and enzyme substrates were diluted in sterile ultra-pure water to a final concentration of 800 µM for MU-P and 500 µM for MU-G and MU-Q assays. Stocks and calibration solutions, as well as diluted substrates were kept at −20 °C in the dark. Fluorogenic assays were performed by mixing 200 µl of soil supernatant (soil incubated over night with Tris-acetate buffer 10 mM, pH 4.5 in a horizontal shaker at 25 °C and 100 rpm), and 50 µl of the corresponding substrate with a final volume of 250 µl per well, using black 96-well microplates. Controls with soil supernatant heated at 100 °C for 10 min were also conducted separately for each sample. All reactions were performed at room temperature, applying a stirring of 500 rpm, in the dark and at different incubation times depending on the enzymatic test: 15 min (MU-P), 40 min (MU-G), and 60 min (MU-Q). After incubation, microplates were spin (3000 rpm for 3 min), and 100 µl of the reaction mix was added to 100 µl of stopping buffer (Tris 1 M, pH 10–11). Measurements were carried out with a Victor3 microplate reader (Perkin-Elmer Life Sciences, Massachusetts, USA), at 355/460 nm excitation-emission wavelengths. Experimental calibrations of known MU concentrations were performed, allowing estimating each enzymatic activity by extrapolating well fluorescence signals on the respective calibration regression lines. Blanks with buffer and fluorogenic substrates related to auto-fluorescence, and controls were subtracted from all measures. All enzymatic activities are expressed in pmol $min^{-1}$ $mg^{-1}$ of dry soil.

*Data analysis*

All statistical analyses were performed by using SPSS® and SPSS® AMOS 20.0 software's (IBM Corporation Software Group, Somers, NY).

Prior to analyses, all variables were tested for normality, and log transformations were applied to meet variance homoscedasticity when required. All data were first analyzed by two-way Analysis of Variance (ANOVA) considering the factors "matrix influence" (low at forest interior, mid at forest edge and high at small fragments), "tree cover" (under canopy and open areas), and their interaction (data not shown). Subsequently, and due to the high effect of tree cover factor, fragmentation effects within each coverage level, as well as coverage effects within each fragmentation level were separately evaluated by one-way ANOVA. Significant differences among treatments were determined by post-hoc multiple comparisons using the Tukey HSD test ($p < 0.05$).

A principal component analysis (PCA) was conducted to reduce the *n*-dimensional of nutrient's data into two linear axes explaining the maximum amount of variance, and its axes latter used as new variables (PC1 and PC2 nutrients). Linear correlations between all measured variables were tested using Pearson's *r* ($p < 0.05$).

Structural equation models (SEMs) were used to test the direct and indirect influence of both biotic and abiotic factors on measured soil microbial functioning variables (β-glucosidase, chitinase, phosphatase acid and $R_s$). Since the sample size was relatively small ($n = 90$), the number of predictors included in the model was thoroughly limited, as recommended by Shipley (2002). Our models considered a complete set of hypothesis based on literature, previous exploratory analyses (ANOVA, correlations), and our own experience (Flores-Rentería et al., 2015). Namely, we hypothesized that soil microbial functioning would be dependent on both abiotic and biotic conditions, such as pH (Nannipieri et al., 2002;

Sinsabaugh et al., 2008), SOM (Søe and Buchmann, 2005; Curiel Yuste et al., 2007; Sinsabaugh et al., 2008; Casals et al., 2009; Kivlin and Treseder, 2014), soil moisture (Reichstein et al., 2003; Sardans and Peñuelas, 2005; Curiel Yuste et al., 2007; Williams and Rice, 2007; Kivlin and Treseder, 2014), C:N ratio (Martin et al., 2009; Kivlin and Treseder, 2014), and microbial biomass (Gómez-Luna et al., 2012), and that all these variables would be affected by the tree influence index (Belsky and Canham, 1994; Søe and Buchmann, 2005; Barba et al., 2013). At the same time, we hypothesized that the microbial biomass would be affected by SOM, soil moisture, and the C:N ratio (Pugnaire et al., 2004; Fierer and Jackson, 2006; Williams and Rice, 2007; Sinsabaugh et al., 2008; Fierer et al., 2009). A correlation between soil temperature and soil moisture, both affecting $R_s$, was also taken into account (Curiel Yuste et al., 2003; Matías et al., 2012). Additionally, we included in our model causal relations and correlations among abiotic variables, i.e. SOM influence on soil physical properties related to soil moisture positively increasing the soil water-holding capacity (Abu-Hamdeh, 2001; Sinsabaugh et al., 2008), and buffering high temperatures (Pugnaire et al., 2004). Several models were run and the best-fitted ones were finally selected according to the covariance proximity between observed and expected data (goodness-of-fit $\chi^2$). Standardized path coefficients were estimated by using the maximum likelihood algorithm (Shipley, 2002). The degree of fit between observed and expected covariance structures was assessed by root mean square error of approximation statistic (RMSEA) (Steiger, 1990). RMSEA values < 0.08 indicate a good fit, between 0.08 to 0.10 provide a moderate fit, and > 0.10 suggest a poor fit (Maccallum et al., 1996). Model fit to data was additionally evaluated by the goodness-of-fit index (GFI) and the Bentler and Bonett's normed-fit index (NFI), both with values ranging between 0 and 1, and those > 0.9 indicating an acceptable fit (Iriondo et al., 2003).

## Results

*Effects of the agricultural matrix on tree-soil characteristics*

Structural characteristics of trees such as basal area, height and canopy projection were significantly affected by the influence of the agricultural matrix, with trees being significantly thicker and taller in small fragments and at the forest edge than in the forest interior (Fig. 2.2; Table S2.1).

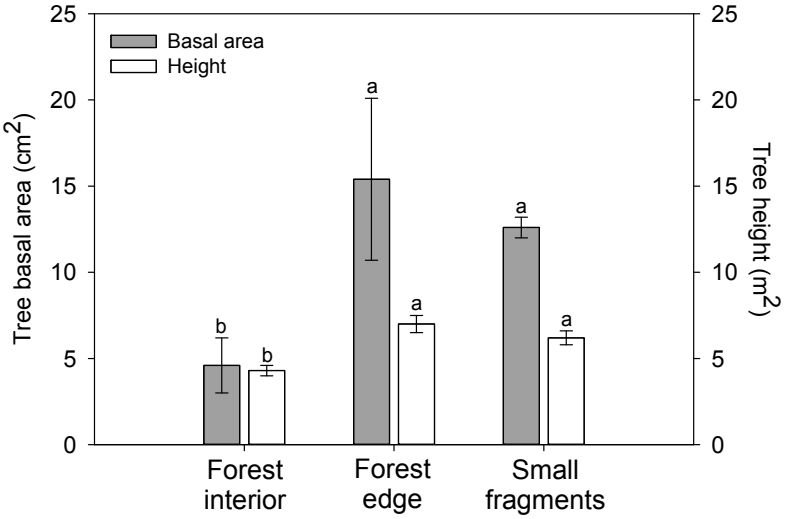


**Figure 2.2** Tree size from three matrix influence level (low: forest interior; mid: forest edge; and high: small fragment) of holm oak forests in Spain. Grey filled bars represent tree basal area and white bars tree height. Error bars are standard error. For each variable, different letters represent differences among matrix influence levels ($P< 0.05$, $n=45$), according to Tukey's post-hoc comparison (see Table S2.1 for more information).

Soil moisture was significantly affected by the agricultural matrix only in open areas ($F_{2, 42}=4.93$; $p=0.001$), with the highest values recorded at forest edge (Table 2.1; Table S2.1). Soil temperature was also significantly affected by the influence of the matrix, but contrary to soil moisture, at forest edge it showed the lowest values, both under canopy ($F_{2, 42}=3.41$; $p=0.04$) and in open areas ($F_{2, 42}=7.13$; $p<0.001$).

**Table 2.1** Characteristics of soil and trees in fragments with three matrix influence levels (low: forest interior; mid: forest edge; and high: small fragment) of holm oak forests in Spain. Capital letters represent differences among tree cover for a given matrix influence level, one way-ANOVA ($p < 0.05$, $n$=30), while lowercase letters represent differences among matrix influence for a given tree cover (under canopy or open areas), one way-ANOVA ($p < 0.05$, $n$=45). Data are means ± standard error.

| | Under canopy | | | Open areas | | |
|---|---|---|---|---|---|---|
| | **Forest interior** | **Forest edge** | **Small fragments** | **Forest interior** | **Forest edge** | **Small fragments** |
| Microbial biomass (mg C kg $^{-1}$) | 1170.7 ± 4.9$^{a,A}$ | 1576.3 ± 5.9$^{a,A}$ | 2438.0 ±12.7$^{a,A}$ | 635.4 ± 3.6$^{a,B}$ | 810.9 ± 4.3$^{a,B}$ | 769.0 ± 4.8$^{a,B}$ |
| Soil moisture (%) | 13.4 ± 0.5 $^{a,A}$ | 15.5 ± 0.7$^{a,A}$ | 19.0 ± 0.9$^{a,A}$ | 7.2 ± 0.3 $^{b,B}$ | 10.3 ± 0.5$^{a,B}$ | 8.4 ± 0.4$^{ab,B}$ |
| Field soil moisture (%) | 9.0 ± 0.5 $^{a,A}$ | 10.3 ± 0.6$^{a,A}$ | 11.4 ± 0.6$^{a,A}$ | 7.6 ± 0.4 $^{a,A}$ | 9.4 ± 0.5 $^{a,A}$ | 7.3 ± 0.4 $^{a, B}$ |
| Soil temperature (ºC) | 18.8 ± 0.3 $^{a,B}$ | 17.6 ± 0.3 $^{b,B}$ | 18.1 ± 0.3$^{ab,B}$ | 26.1 ± 0.4$^{a,A}$ | 22.7 ± 0.5$^{b,A}$ | 26.3 ± 0.4 $^{a,A}$ |
| pH | 8.0 ± 0.1 $^{a,B}$ | 8.0 ± 0.1$^{a,B}$ | 7.9 ± 0.1$^{a,B}$ | 8.2 ±0.1$^{a,A}$ | 8.2 ± 0.1$^{a,A}$ | 8.2 ± 0.1$^{a,A}$ |
| Soil organic matter (%) | 7.7 ± 0.5$^{a,A}$ | 9.9 ± 0.5$^{a,A}$ | 16.1 ± 1.0$^{a,A}$ | 3.5 ± 0.3$^{a,B}$ | 4.4 ± 0.4$^{a,B}$ | 4.7 ± 0.5$^{a,B}$ |
| C:N ratio | 10.4 ± 0.5$^{a,A}$ | 9.0 ± 0.3$^{a,A}$ | 9.5 ± 0.4$^{a,A}$ | 9.9 ± 0.5$^{a,A}$ | 9.3 ± 0.4$^{a,A}$ | 9.7 ± 0.5$^{a,A}$ |
| Tree influence index | 318.1 ± 3.1$^{b,A}$ | 603.3 ±4.2 $^{a,A}$ | 572.6 ± 4.9$^{a,A}$ | 109.1 ± 1.8$^{b,B}$ | 239.2 ± 2.8$^{a,B}$ | 219.0 ± 3.1$^{a,B}$ |

As drawn by plotting the two first PCA components (Fig. 2.3), both factors, agricultural matrix and tree cover, independently influenced soil nutrient variability with higher amounts of C, N and P and other cations, in small fragments and at forest edge than in the forest interior (PC1, Fig. 2.3), and a clear enrichment of nutrients, especially of N, C and P, under canopy (PC2, Fig. 2.3).

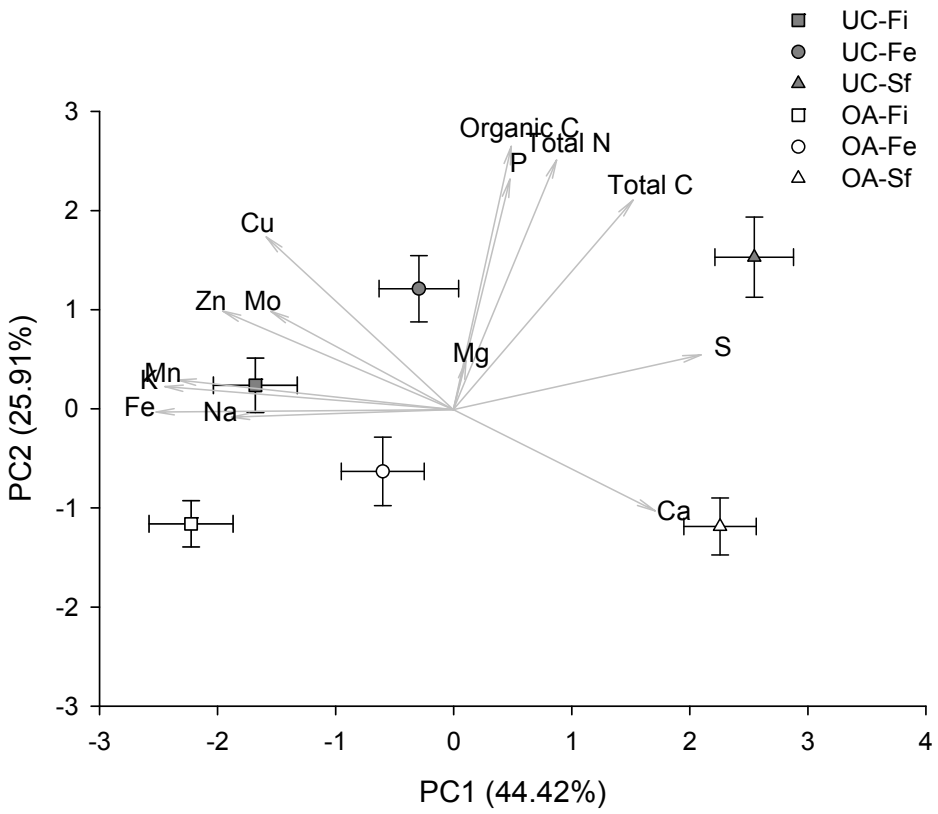


**Figure 2.3** Principal Component Analysis (PCA) of soil nutrients (scores and eigenvectors) from three levels of matrix influence (low: forest interior; mid: forest edge; and high: small fragment) of holm oak forests in Spain. Tree cover is represented by different colors: grey = under canopy (UC); white = open areas (OA). Soil provenances are represented by different symbols: squares = forest interior of large fragments (Fi); circles = forest edge of large fragments (Fe); triangles = small fragments (Sf). Error bars represent standard error.

As expected, soil biotic and abiotic variables were strongly influenced by the canopy cover. Under canopy, significantly higher values of microbial biomass, soil moisture, SOM, and lower values of soil temperature and pH, were found compared with open areas (Table 2.1; Table S2.1).

*Influence of the factors agricultural matrix and tree cover on soil microbial functioning*

Maximum β-glucosidase (14.62 pmol·mg$^{-1}$ min$^{-1}$), chitinase (2.87 pmol·mg$^{-1}$ min$^{-1}$) and phosphatase (12.57 pmol·mg$^{-1}$ min$^{-1}$) activities were obtained under trees in small fragments (Fig. 2.4a-c). The tree cover significantly and positively affected all enzymatic activities, independently of the level of influence of the matrix (Table S2.1; Fig, 2.4a-c). β-glucosidase activity was significantly higher at the forest edge and in small fragments than in the forest interior, both under canopy and in open areas (Fig. 2.4a). Chitinase activity was unaffected by the agricultural matrix influence under canopy, while in open areas, it was significantly higher at the forest edge and in small fragments than in the forest interior (Fig. 2.4b). In open areas, phosphatase activity was unaffected by the matrix influence, while under canopy, it was significantly higher in small fragments than in the forest interior (Fig. 2.4c). The agricultural matrix did not have a significant effect on $R_s$ in any case, while the tree cover did at the forest edge where $R_s$ was significantly higher in open areas than under canopy (Fig. 2.4d).

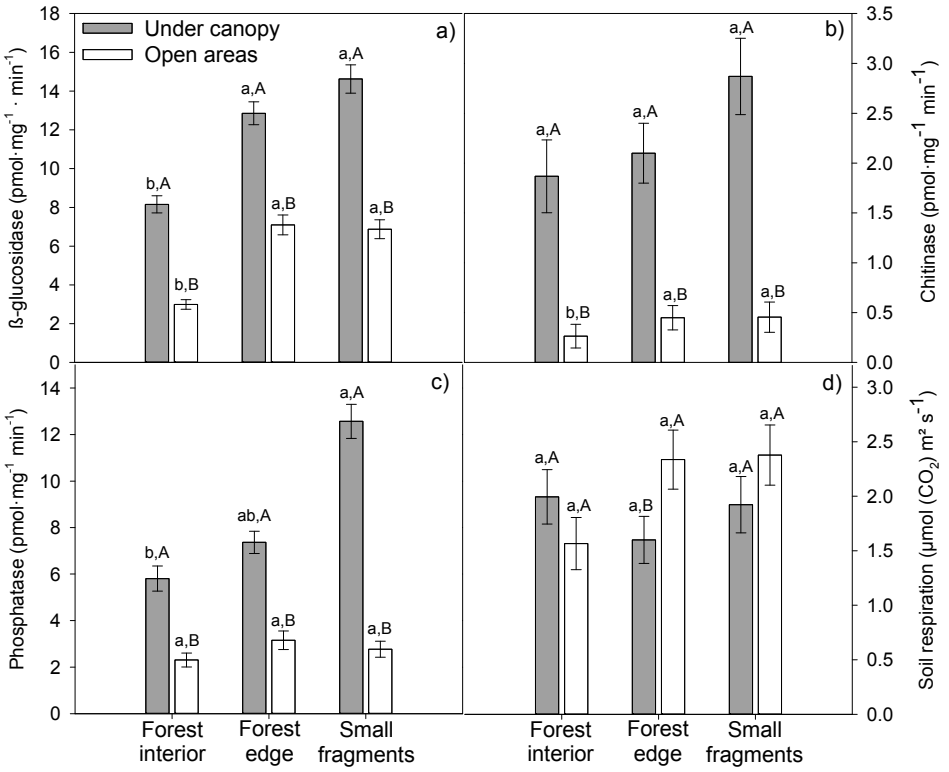


**Figure 2.4** Enzymatic activities and respiration ($R_s$) of soils from three matrix influence levels (low: forest interior; mid: forest edge; and high: small fragment) of holm oak forests in Spain. Grey filled bars represent under canopy and white bars open areas. Values are means ± standard error. Capital letters represent differences among tree cover (under canopy or open areas) for a given matrix influence level ($p$<0.05, $n$=30), while lowercase letters represent differences among matrix influence within each tree cover ($p$<0.05, $n$=45), according to Tukey's post-hoc comparison (see Table S2.1 for more information).

*Factors controlling soil microbial functioning*

Microbial biomass and all enzymatic activities correlated positively with the tree influence index and with some abiotic and biotic variables such assoil moisture (field and lab measures), SOM and the principal axes of nutrients PCA, and negatively correlated with some other soil characteristics such as temperature and pH (Table S2.2). Besides that, all the enzymatic activities were also highly correlated among them. As for $R_s$,

it was solely correlated with β-glucosidase, the enzyme more directly related to the C cycle (Table S2.2).

An overview of the proposed general fitted model (Figs. 2.5 and 2.6) showed that, as expected, and according to previous ANOVA's results, the agricultural matrix exerted a strong positive influence over the tree influence index (tree basal diameter weighted by the distance). In general, trees significantly and directly influenced many different soil properties such as SOM amount, soil pH and microbial biomass; however, trees had no direct influence on other variables such as soil moisture or C:N ratio that were rather indirectly affected via SOM (Figs. 2.5 and 2.6). SOM directly affected soil moisture and C:N ratio, but also pH and microbial biomass. In addition, the amount of microbial biomass was directly influenced by soil moisture and C:N ratio, but not by pH (Figs. 2.5 and 2.6).

The structural-equation model proposed for enzymatic activities (Fig. 2.5a), provided a good fit for all enzymes (Fig. 2.5), as indicated by the non-significant $f$ value ($\chi^2 = 7.72$; $p = 0.46$) and by the goodness-of-fit indices (RMSEA < 0.001; NFI and GFI > 0.98). Direct significant effects differed among enzymatic activities (Fig. 2.5; Table S2.3). Among the three enzymes under study, the agricultural matrix exerted a strong, positive and direct effect only over β-glucosidase activity (Fig. 2.5b; Table S2.3). The agricultural matrix had indirect effects over all enzymatic activities, i.e. via its positive effect over trees (tree influence index directly affecting β-glucosidase and chitinase activities, Fig. 2.5b and c), or over variables affected by trees (i.e. microbial biomass, soil pH). Specifically, trees had a strong and direct effect on pH affecting all enzymatic activities (Fig. 2.5b, c and d), and on microbial biomass, which in turn directly affected β-glucosidase and phosphatase activities (Fig. 2.5b and d).

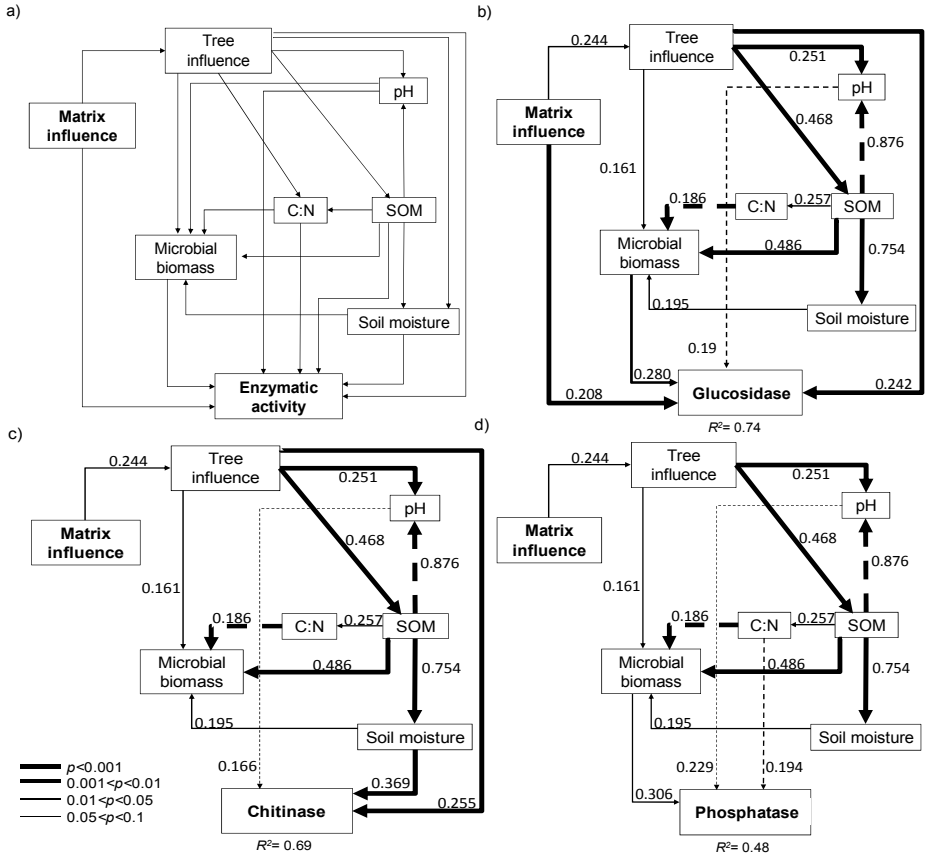


**Figure 2.5** Path diagrams representing hypothesized causal relationships among the agricultural matrix influence, biotic and abiotic variables and soil enzymatic activities. (a) General fitted model, and individual models for (b) β-glucosidase, (c) chitinase, and (d) phosphatase acid activities. Arrows depict causal relationships: positive effects are indicated by solid lines, and negative effects by dashed lines, with numbers indicating standardized estimated regression weights (SRW). Arrow widths are proportional to significance values according to the legend. Paths with coefficients non-significant are omitted (see more information in Table S2.2). Fit statistics of the model (NFI, GFI and RMSEA) and sample size (*n*) are given for all proposed models.

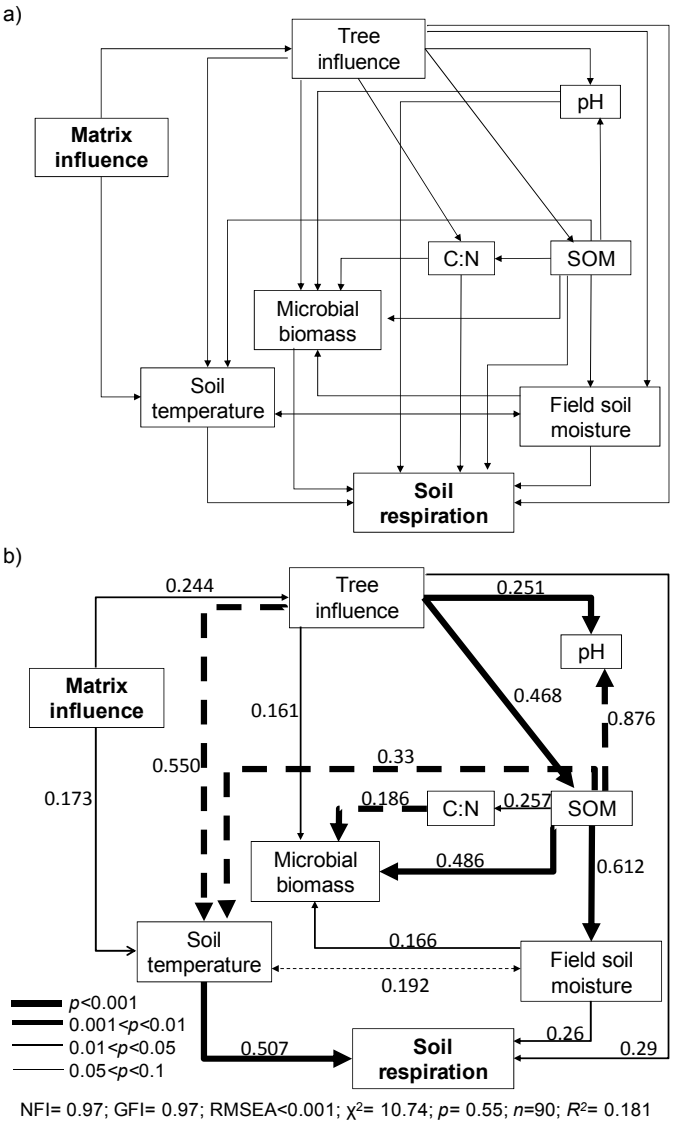


**Figure 2.6** (a) General Path Model, and (b) model fitted for soil respiration, representing hypothesized causal relationships among matrix influence, biotic and abiotic predictors and soil respiration. Arrows depict causal relationships: positive and negative effects are indicated by solid and dashed lines respectively, with numbers indicating standardized estimated regression weights (SRW). Arrow widths are proportional to *p* values according to the legend. Paths with coefficients non-significant are omitted (see more information in Table S2.2). Fit statistics of the model (NFI, GFI and RMSEA) and sample size (*n*) are given for the proposed model.

Contrarily to that we a priori expected, SOM quantity did not affect directly any enzymatic activity (Fig. 2.5b-d), although some indirect paths were observed, i.e. through its effect on microbial biomass affecting β-glucosidase and phosphatase activity, through its effect on soil moisture affecting chitinase activity (Fig. 2.5c), and through its effect on C:N ratio affecting phosphatase activity (Fig. 2.5d).

The structural-equation model proposed for $R_s$ (Fig. 2.6a) provided a low but significant goodness of fit of the data (Fig. 2.6b), denoted by a lower coefficient of determination with respect to those obtained for the three enzymatic activities, a significant RMSEA (<0.001), high NFI and GFI values (>0.97), and a non-significant $f$ value ($\chi^2$ = 10.74; $p$ = 0.55). Consistent and significantly positive effects of soil temperature, field soil moisture and tree influence index were observed on $R_s$, with the strongest effect caused by soil temperature (Table S2.3; Fig. 2.6b). Contrarily to what we first proposed (Fig. 2.6a), $R_s$ was not directly affected by SOM, C: N ratio, pH, microbial biomass, or agricultural matrix influence (Fig. 2.6b). Nevertheless, indirect effects of the agricultural matrix over $R_s$ were observed through its positive effect on soil temperature, and on the tree influence, which on its turn, positively affected soil moisture via SOM (indirect effect), and negatively affected soil temperature (direct effect) (Fig. 2.6b). As expected, soil temperature and moisture were negatively correlated (Fig. 2.6b).

## Discusion

*Effect of forest fragmentation on soil functioning*

The range of values of the soil functional variables measured (e.g. enzymatic activities and soil respiration) were similar to those reported in other Mediterranean ecosystems (Joffre et al., 2003; Sardans and Peñuelas, 2005; Waldrop and Firestone, 2006; Barba et al., 2013; Gómez-Rey et al., 2013; Pérez-Izquierdo et al., 2014). Overall, the surrounding agricultural matrix favored the accumulation of N, C, P, S and Ca in soils from small fragments and edges of large fragments, in agreement with previous findings (Flores-Rentería et al., 2015). This fertilization effect of the agricultural matrix in the small fragments and edges of large fragments was mainly associated to a significant increase of the size of the trees. The effects of agricultural matrix on tree size could have resulted from the high resource (nutrients, water and/or light) availability, leading to enhanced net primary productivity and increased carbon sequestration through an stimulation of the photosynthetic capacity of trees (Reichstein et al., 2014). The higher nutrient availability could be explained by the enhanced enzymatic activity and/or by the possible runoff of fertilizer from adjacent areas (Boutin and Jobin, 1998). While we cannot discard other factors (e.g. management, lower tree competition) to explain the higher size of trees in small fragments and at forest edges compared with forest interiors, similar patterns found in holm oak fragmented forest in the northern plateau of the Iberian Peninsula (i.e. height, basal diameter and canopy cover; Santos and Tellería, 1998) confirm the strong relation between fragmentation and tree size.

Unlike tree growth, which was clearly and strongly influenced by the agricultural matrix, only one out of the three enzymes (β-glucosidase activity) was, at some extent, directly and positively influenced by the agricultural matrix, independently of the tree cover. Based on the large differences in nutrient availability observed in the principal components analysis, the fertilization effect of the agricultural matrix over the activity of these enzymes was minor than expected, taking into account the strong effect that nutrients have over the capacity of microbes to decompose SOM (Malmivaara-Lämsä et al., 2008; Gómez-Luna et al., 2009; Baldrian, 2014).

Collectively, our results showed that the positive relation of forest fragmentation with microbial enzymatic activities was mainly mediated by the changes in micro-environmental conditions observed under trees influenced by the agricultural matrix. Structural Modeling Equations further indicated that the net effect of forest fragmentation on enzymatic activities was dependent on a complex cascade of direct and indirect interconnected causal-effects relations. For instance, the higher amount of SOM under the larger trees of small fragments and forest edges (i.e. with high agricultural matrix influence) indirectly and positively influenced microbial enzymatic activity by directly improving water availability and increasing microbial biomass.

Tree size and closeness was also the main controller of $R_s$, which integrates not only microbial (heterotrophic) but also plant (roots, autotrophic) metabolic activity. Indeed, the most important factors explaining the variability of $R_s$ were the micro-environmental (temperature and moisture) conditions, which were also strongly and directly influenced by trees. Additionally, the observed direct influence of the tree over $R_s$, that has been previously reported in similar ecosystems (Matías et al., 2012; Barba et al., 2013), might be partially attributed, among other things, to the strong influence of tree closeness over root respiration (Tang and

Baldocchi, 2005). Nonetheless, and despite the fact that the observed controls were very much in agreement with those observed for rates of key enzymes activities, the capacity of the designed models to explain the spatial variation of $R_s$ was very limited with respect to the predicted variability obtained for those enzymatic activities (18%, versus 48-74% of the variability, respectively). This is probably because $R_s$ accounts for many biological components (not only microbes and roots, but also mycorrhizas) controlled by different variables (Heinemeyer et al., 2007), which can greatly hinder the spatial interpretation of this flux (Søe and Buchmann, 2005; Barba et al., 2013).

Trees strongly influenced by the agricultural matrix, therefore, were also responsible for modifying their surrounding edaphic environment by improving the SOM accretion, increasing the availability of nutrients and the capacity of soils to retain water, further buffering soil temperature, and hence decreasing water evaporation rates (Belsky and Canham, 1994; Hastwell and Morris, 2013). To other recent studies which also found a strong influence of the canopy cover on the microbial functioning, in fragmented habitats (Lázaro-Nogal et al., 2012; Riutta et al., 2012) our study adds a mechanistic dimension, showing how the effect of forest fragmentation over soil activity results from a cascade of causal-effect relations triggered by its positive effect over tree growth. Since it is well known that trees supplies to the soil microbial communities with the necessary organic substrates (i.e. organic matter, litter, root exudates) to grow (Schlesinger and Andrews, 2000; Dakora and Phillips, 2002; Baldrian, 2014) studies should take into account the complexity of the plant-soil interactions in order to understand possible effects of forest fragmentation over the ecology and functioning of soil microbial communities.

*Interaction of factors directly and indirectly controlling soil functioning*

Structural equation model leaves us important clues about the cause-effect relationships and controls over the functioning of microbial community in these fragmented environments. SEM shows that, as already discussed above, trees exerted, directly or indirectly, the strongest control over most of the soil abiotic and biotic variables (i.e. soil pH, SOM, microbial biomass) measured, indirectly affecting the measured indicators of soil functioning. However, trees also influenced directly those indicators of soil functioning most closely related to the cycling of carbohydrates. Indeed, $R_s$ and β-glucosidase, were directly influenced by the closest tree, which could reflect in both cases the positive effect of root exudates on the microbial capacity to decompose SOM (priming effect; (Kuzyakov et al., 2000; Dakora and Phillips, 2002; Kuzyakov, 2010).

The strong control of trees over pH appeared to have also important implications for soil processes and specifically for microbial activity, since it was the only environmental factor directly affecting all three enzymatic activities. This observation reinforces the idea that microbial activities are generally very sensitive to even small variations in soil pH (Bååth and Anderson, 2003; Fierer and Jackson, 2006; Sinsabaugh et al., 2008; Legay et al., 2014). Soil pH can modify the active site conformation of enzymes, so that various enzyme isoforms can differentially perform in terms of efficiency at different pHs (Frankenberger and Johanson, 1982). The net effect of tree size and closeness over pH was negative (acidification) which could be mainly associated with the higher SOM accumulation and hence the accumulation of humic acids, or the acidifier effect that has been largely attributed to the rhizospheric environment (Dakora and Phillips, 2002). As a result, we could expect that with an increasing influence of the agricultural matrix,

the pH differences between open areas and under tree canopy would be increasingly intensified.

Another important fact drawn from our models were the mechanisms behind the influence of SOM amount over soil microbial functioning. Although the amount of SOM has generally been strongly correlated with rates of microbial enzymatic activities (Sinsabaugh et al., 2008; Burns et al., 2013; Baldrian, 2014), SEMs indicated that this strong effect was mainly indirect, through increasing microbial biomass and moisture availability, and decreasing C:N ratio and soil pH.

SEMs also shed light on the controls of the different soil metabolic pathways represented by the three different enzymes chosen for this study. For instance, the observed negative effect of C:N (associated with more SOM accumulation) over phosphatase may respond to the already observed inhibition of this enzymatic activity, associated with the acquisition of mineral forms of phosphate, when available N in soil decreases as C accumulates (Kivlin and Treseder, 2014). Moreover, microbial biomass, which was indirectly controlled by tree size and proximity (tree influence index) through SOM production, showed the strongest direct and positive effect overrates of β-glucosidase and phosphatase activities (Sinsabaugh et al., 2008; Nannipieri et al., 2011) but had no effect over chitinase, which is usually associated with fungal biomass (Baldrian, 2008; Sinsabaugh et al., 2008; Kivlin and Treseder, 2014), rather than with overall microbial biomass (Šnajdr et al., 2008).

## Conclusion

Our study emphasizes that forest fragmentation exerts important alterations on tree-soil-microbial interactions and soil functioning. Forest fragmentation positively affects tree growth and forest productivity (SOM accumulation), inducing changes in both abiotic (microclimate and pH) and biotic (stimulation of microbial growth) conditions that ultimately improve the conditions for microbial enzymatic activity. The increment of SOM associated with tree growth also modified the pH, changed the microclimatic conditions (increasing soil moisture and buffering temperature) and stimulated microbial growth resulting in a significant increase in soil enzymatic activity and respiration under the trees most influenced by the agricultural matrix. Collectively, our results showed that the holm oak forest fragmentation with a surrounding agricultural matrix have profound effects over the functioning of the plant-soil-microbial system, specially on its effects on the soil carbon and nutrient cycles. Forest fragmentation should, therefore, be considered in estimations of the carbon sink capacity under global change scenarios.

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## Supplementary material

**Table S2.1** Tree cover ($n$=45) and matrix influence ($n$=30) effects in soil biotic and abiotic properties and tree characteristics, of Holm oak forest fragments in Spain (one-way ANOVA). Significant effects ($p$ < 0.05) are noted in bold. MI = Matrix influence. C = tree cover. $R_s$ = soil respiration. SOM = soil organic matter. PC1 and PC2 = first and second component of Principal Component Analysis, respectively.

| | | Under canopy | Open areas | Forest interior | Forest edge | Small fragments |
|---|---|---|---|---|---|---|
| | | **MI** | **MI** | **C** | **C** | **C** |
| β-glucosidase | F | **6.53** | **13.80** | **57.34** | **14.40** | **17.11** |
| | *p* | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| Chitinase | F | 1.99 | **4.53** | **8.37** | **12.25** | **39.00** |
| | *p* | 0.15 | **0.02** | **0.01** | **<0.001** | **<0.001** |
| Phosphatase | F | **5.79** | 0.42 | **56.85** | **36.70** | **64.97** |
| | *p* | **0.01** | 0.66 | **<0.001** | **<0.001** | **<0.001** |
| $R_s$ | F | 0.84 | 2.89 | 1.70 | **4.81** | 1.34 |
| | *p* | 0.44 | 0.07 | 0.20 | **0.04** | 0.26 |
| Microbial biomass | F | 2.42 | 1.47 | **31.65** | **28.15** | **13.83** |
| | *p* | 0.10 | 0.24 | **<0.001** | **<0.001** | **<0.001** |
| Soil moisture | F | 1.27 | **4.93** | **35.62** | **4.57** | **19.52** |
| | *p* | 0.29 | **0.01** | **<0.001** | **0.04** | **<0.001** |
| Field soil moisture | F | 0.77 | 3.07 | 2.18 | 0.33 | **5.75** |
| | *p* | 0.47 | 0.06 | 0.15 | 0.57 | **0.02** |
| Soil temperature | F | **3.41** | **7.13** | **129.08** | **23.97** | **120.26** |
| | *p* | **0.04** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| pH | F | 0.75 | 1.19 | **12.89** | **7.50** | **7.78** |
| | *p* | 0.48 | 0.31 | **<0.001** | **0.01** | **0.01** |
| SOM | F | 1.81 | 0.89 | **28.99** | **21.93** | **14.62** |
| | *p* | 0.18 | 0.42 | **<0.001** | **<0.001** | **<0.001** |
| C:N | F | 0.98 | 0.15 | 0.09 | 0.17 | 0.01 |
| | *p* | 0.38 | 0.86 | 0.77 | 0.68 | 0.94 |
| PC1 Nutrients | F | **22.54** | **25.62** | 0.61 | 0.22 | 0.26 |
| | *p* | **<0.001** | **<0.001** | 0.44 | 0.64 | 0.61 |
| PC2 Nutrients | F | 2.02 | 0.82 | **14.94** | **8.49** | **14.69** |
| | *p* | 0.15 | 0.45 | **<0.001** | **0.007** | **<0.001** |
| Tree basal area | F | **8.79** | - | - | - | - |
| | *p* | **<0.001** | - | - | - | - |
| Tree height (m) | F | **10.09** | - | - | - | - |
| | *p* | **<0.001** | - | - | - | - |
| Tree canopy | F | **12.02** | - | - | - | - |
| | *p* | **<0.001** | - | - | - | - |
| Tree influence index | F | **5.08** | **5.80** | **29.01** | **23.77** | **12.58** |
| | *p* | **0.01** | **0.01** | **<0.001** | **<0.001** | **<0.001** |

**Table S2.2** Pearson's correlations of soil and tree characteristics in fragments with three matrix influence levels of Holm oak forests in Spain ($n$=90). Significant effects are noted in bold (*$p$<0.05; **$p$<0.001). $R_s$= soil respiration (µmol ($CO_2$) $m^2$ $s^{-1}$).

| | $R_s$ | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| β-glucosidase (pmol $min^{-1}$ $mg^{-1}$) (1) | **0.23*** | | | | | | | | | | | | |
| Chitinase (pmol $min^{-1}$ $mg^{-1}$) (2) | 0.13 | **0.78**** | | | | | | | | | | | |
| Phosphatase (pmol $min^{-1}$ $mg^{-1}$) (3) | 0.09 | **0.69**** | **0.56**** | | | | | | | | | | |
| Microbial biomass (mg C $kg^{-1}$) (4) | 0.08 | **0.76**** | **0.72**** | **0.62**** | | | | | | | | | |
| Soil moisture (%) (5) | 0.20 | **0.66**** | **0.74**** | **0.48**** | **0.70**** | | | | | | | | |
| Field soil moisture (%) (6) | 0.19 | **0.42**** | **0.45**** | **0.22*** | **0.46**** | **0.83**** | | | | | | | |
| Soil temperature (°C) (7) | 0.12 | **-0.51**** | **-0.64**** | **-0.37**** | **-0.53**** | **-0.61**** | **-0.40**** | | | | | | |
| pH (8) | -0.07 | **-0.62**** | **-0.57**** | **-0.59**** | **-0.63**** | **-0.55**** | **-0.36**** | **0.33**** | | | | | |
| SOM (%) (9) | 0.16 | **0.75**** | **0.74**** | **0.59**** | **0.78**** | **0.79**** | **0.56**** | **-0.57**** | **-0.76**** | | | | |
| C:N (10) | 0.08 | -0.05 | -0.01 | -0.15 | -0.05 | 0.13 | **0.27*** | -0.10 | -0.05 | **0.21*** | | | |
| PC1 Nutrients (11) | 0.18 | **0.37**** | **0.25*** | 0.18 | **0.21*** | 0.10 | -0.08 | -0.06 | -0.14 | 0.19 | -0.17 | | |
| PC2 Nutrients (12) | 0.17 | **0.69**** | **0.64**** | **0.57**** | **0.72**** | **0.79**** | **0.62**** | **-0.49**** | **-0.72**** | **0.92**** | 0.12 | -0.006 | |
| Tree influence index ($m/cm^2$) | 0.08 | **0.58**** | **0.56**** | **0.25*** | **0.49**** | **0.44**** | 0.2 | **-0.67**** | -0.16 | **0.47**** | 0.02 | **0.35**** | **0.34**** |

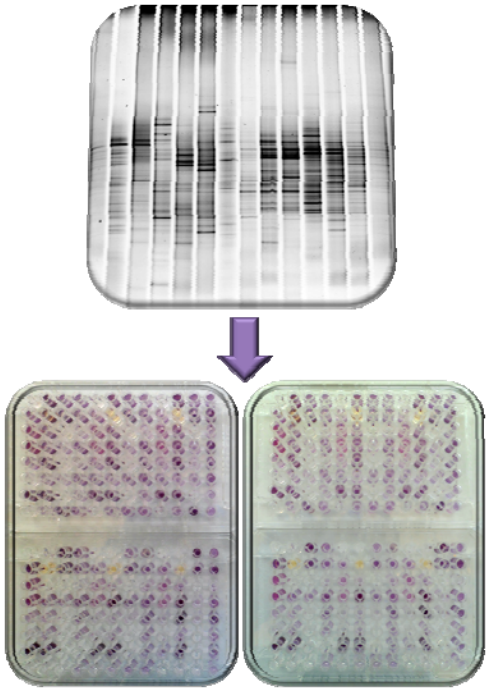
**Table S2.3** Standardized total (T), direct (D) and indirect (I) effects (based on standardized regression weights (SRW)) for each structural equation model (SEM) (Fig. 2.5 and 2.6). Significant direct effects are noted in bold ($n$=90). $R_s$ = soil respiration. SOM = soil organic matter.

| | β-glucosidase | | | Chitinase | | | Phosphatase | | | $R_s$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T | D | I | T | D | I | T | D | I | T | D | I |
| **Matrix influence** | 0.339 | **0.208** | 0.131 | 0.142 | 0.005 | 0.137 | 0.135 | 0.078 | 0.057 | 0.112 | 0 | 0.112 |
| **Tree influence** | 0.539 | **0.242** | 0.296 | 0.562 | **0.255** | 0.307 | 0.232 | -0.065 | 0.297 | 0.062 | **0.292** | -0.230 |
| **Microbial biomass** | 0.280 | **0.280** | 0 | 0.164 | 0.164 | 0 | 0.306 | **0.306** | 0 | -0.160 | -0.160 | 0 |
| **SOM** | 0.604 | 0.206 | 0.399 | 0.605 | 0.088 | 0.517 | 0.614 | 0.264 | 0.351 | 0.154 | 0.338 | -0.183 |
| **Soil moisture** [a] | 0.130 | 0.076 | 0.055 | 0.400 | **0.369** | 0.030 | 0.039 | -0.021 | 0.059 | 0.233 | **0.260** | -0.027 |
| **pH** | -0.229 | -0.190 | -0.039 | -0.188 | **-0.166** | -0.023 | -0.271 | **-0.229** | -0.42 | 0.088 | 0.066 | 0.023 |
| **C:N ratio** | -0.139 | -0.087 | -0.052 | -0.113 | -0.083 | -0.030 | -0.250 | **-0.194** | -0.056 | 0.016 | -0.018 | 0.034 |
| **Soil temperature** | - | - | - | - | - | - | - | - | - | 0.507 | **0.507** | 0 |

[a] soil moisture determined in the lab for enzymatic activities or field soil moisture in the case of $R_s$.

# Capítulo 3

## Agricultural matrix directly affects the structure and indirectly the metabolism of microbial communities in fragmented Mediterranean holm oak forest soils



Manuscript in preparation

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El capítulo III de la tesis doctoral: FLORES-RENTERÍA, Dulce. Efectos de la fragmentación del encinar en las interacciones planta-suelo-microorganismos = Effects of forest fragmentation on the plant-soil-microbial interactions (2016, está relacionado con el artículo: Agricultural matrix affects differently the alpha and beta structural and functional diversity of soil microbial communities in a fragmented Mediterranean holm oak forest. Soil Biology and Biochemistry 92: 79-90 (2016). http://hdl.handle.net/10261/151312

## Abstract

Given the increasing fragmentation of forest in the Mediterranean basin, understanding its impacts over the ecology of soil microbial communities, responsible for many ecosystem functions, and their capacity to metabolize different substrates from soil organic matter, is of upmost importance. We evaluated how the influence of the agricultural matrix, as one of the main consequences of forest fragmentation, may affect both the composition and the functioning of soil microbial communities in Mediterranean holm oak forests. We determined structural and functional alpha and beta-diversity of microbial communities, as well as microbial assemblages and metabolic profiles, by using a commonly used fingerprinting technique (Denaturing Gel Gradient Electrophoresis) and a community level physiological profiles technique (EcoPlate). The complex plant-soil-microbial connections, and key drivers of soil microbial structure and metabolism were evaluated by using structural equation models (SEM) and multivariate ordination (envfit) approaches. Our results indicated that forest fragmentation may exert an important, but opposite in sign, effect over local (alpha) and landscape (beta) structural and functional diversity of microbial communities suggesting that mechanisms and processes involved in the influence of fragmentation over the ecology of these communities were strongly scale-dependent. Both SEM and multivariate analyses confirmed the complexity behind the effect of forest fragmentation over the ecology of microbial communities and the functioning of the plant-soil-microbial system. The observed consistent relation between the structure and functional diversity of microbial communities further showed the important role that the diversity and assemblage of microbial communities might have over their functioning. Together, our results suggest that the forest fragmentation can affect the microbial communities through the complex interactions with the plant-soil system; affecting the nutrient cycling and functioning if forest soils.

## Introduction

In the Mediterranean basin, forest fragmentation, resource overexploitation, and poor management are the main drivers of forest degradation (FAO, 2011). Little research has been conducted to understand the effects of forest fragmentation on ecosystem functioning (Turner, 2005), despite the fact that it has important implications for forest conservation and management strategies (Saunders et al., 1991), as well as for ecosystem functioning, particularly taking into account its strong impact on the plant-soil-microbial system (Flores-Rentería et al., 2015). Within this framework, microbes are critical for driving ecosystem nutrient cycling, providing plants with the necessary nutrients to grow. Moreover, bacteria and fungi are responsible for about 90% of all organic matter decomposition (McGuire and Treseder, 2010; Ushio et al., 2013), and at least 50% of all $CO_2$ globally emitted from soils (Bond-Lamberty et al., 2004). However, very few studies have been designed to understand how forest fragmentation may affect the functioning of these microbial communities (Flores-Rentería et al., 2015).

Disturbance is generally detrimental to soil biodiversity, especially in agro-ecosystems (Walker, 2012). However, depending on the disturbance regime, changes in spatial environmental heterogeneity associated with fragmentation have been linked to either increases or decreases in soil biodiversity (Rantalainen et al., 2005; Flores-Rentería et al., 2015). For example, studies on forest fragmentation effects on microbial community structure have shown modest changes (Malmivaara-Lämsä et al., 2008; Flores-Rentería et al., 2015) or no changes (Rantalainen et al., 2005) in species composition. On the contrary, forest fragmentation can affect the functioning of microbial communities, as previously showed in Chapter 2 and other studies (Malmivaara-Lämsä et al., 2008; Riutta et al., 2012; Flores-Rentería et al., 2015). Furthermore, while it is often

hypothesized that diversity is important for the maintenance of soil processes, and that reductions in the richness of soil microbial communities will disrupt the functional capability of soils (Giller et al., 1997; Wagg et al., 2014), we are just beginning to address this question, and the results presented so far draw contradictory conclusions (Griffiths et al., 2000; O'Donnell et al., 2001; Bell et al., 2005; Langenheder et al., 2010; Levine et al., 2011; Curiel Yuste et al., 2014; Tardy et al., 2014; Mendes et al., 2015). More knowledge about microbial diversity and its function is therefore required for current and future predictions of ecosystem functioning in a changing world; much more empirical work is needed to define the functional consequences, at the ecosystem scale, of changes in microbial composition and their responses to disturbances and global change.

Diversity measurement is particularly challenging for microbial communities (Hughes et al., 2001; Koleff et al., 2003; Magurran, 2004; Lozupone and Knight, 2008; Haegeman et al., 2013). Commonly, microbial diversity has been characterized as the diversity within a given community (alpha- diversity) generally using the total number of operational taxonomic units (OTU's richness), their relative abundances (Shannon diversity), or indices that combine these two dimensions (evenness). Studies have generally used microbial alpha-diversity to explore the relationships between structure and functioning of microbial communities (e.g. Curiel Yuste et al., 2011), whereas beta-diversity, which analyses the biological diversity among communities along environmental gradients (Koleff et al., 2003; Lozupone and Knight, 2008; Maaß et al., 2014), has been probably less studied for these communities. However, patterns of microbial community structure and diversity at the landscape scale and in perturbation gradients may also add info on co-occurrence -examining which organisms sometimes or never occur together-, that may help us

understanding which conditions prefer or do not prefer (Fuhrman, 2009; Rincón et al., 2014). Several ecological processes potentially contribute to changes in co-occurrence patterns at the landscape scale, including competition, habitat filtering, historical effects and neutral processes (Horner-Devine et al., 2007; Maaß et al., 2014).

In this study, we used a molecular fingerprinting technique, Denaturing Gradient Gel Electrophoresis (DGGE), to characterize the structure of microbial communities (bacteria and fungi) coupled with the community level physiological profiles, using Biolog™ EcoPlates, as indicator of microbial functioning, in order to evaluate the influence of the agricultural matrix, as one of the main consequences of forest fragmentation, on soil microbial ecology (i.e. structure and functioning) in fragmented Mediterranean holm oak forests. More precisely, we evaluated if the impact of forest fragmentation on the capacity of soil microbial communities to metabolize different substrates (metabolic profile) could be explained through its effects on microbial structure (assemblage, alpha and beta diversity) and/or changes in microhabitat characteristics. Based on previous studies, we here hypothesized that the agricultural matrix will exert strong direct (via changes in nutrient availability) and indirect (via its influence over tree growth) effects over the microbial community structure, as well as over its capacity to metabolize different substrates. Secondly, we hypothesized that the metabolic activity of soil microbial communities will be largely influenced by the structure of these communities. Specifically, our objectives were: (1) to analyze the response of structural and functional diversity of soil microbial communities to the agricultural matrix influence; (2) to understand which biotic and abiotic factors associated with fragmentation (i.e. matrix influence) affect this diversity; and (3) to analyze causal relations between microbial community structure and its capacity to metabolize different substrates.

## Material and methods

*Study area*

The study area is located near Quintanar de la Orden (39°30'-39°35'N, 02°47'-02°59'W; 870 a.s.l.), in Toledo, southeastern Spain (Fig. 1.4 in Chapter 1). This area has a Mesomediterranean climate characterized by 434 mm of mean annual precipitation and 14 °C of mean annual temperature, respectively (Ninyerola et al., 2005), with a pronounced summer drought, usually lasting from July to September (Fig. 1.7 in Chapter 1). The landscape, a former predominant holm oak Mediterranean forest, is currently highly fragmented and surrounded by active croplands of cereals and legumes, with scattered grape crops that complete the mosaic. The original forests are in a variety of patch sizes, covering only 28 % of their original area (Díaz and Alonso, 2003). The dominant tree is the holm oak (*Quercus ilex* L. ssp. ballota (Desf.) Samp; Fagaceae), with the understory mainly composed by shrubs of Kermes oak (*Quercus coccifera* L.) and scattered *Genista*, *Asparagus*, and *Rhamnus* species (for a full description of the study area see: (Santos and Tellería, 1998; Díaz and Alonso, 2003).

*Experimental design and sampling*

A total of three large (> 10 ha) and five small (< 0.5 ha; with at least three trees) forest fragments within an area of 1000 ha, separated of a minimum of 50 m and a maximum of 8 km, were studied (Fig. 1.5b in Chapter 1). Prevalent soils were Cambisols (calcic) (WRB, 2007), with sandy loam texture (17-39-44 % sand-silt-clay).

We defined the influence of the agricultural matrix on forest fragments by the factor "matrix influence" with three levels: (1) low influence, at the interior of large fragments (at least 30 m from the forest edge; coded as "forest interior"); (2) mid influence, at the edges of large fragments (coded as "forest edge"); and (3) high influence, in small

fragments (coded as "small fragments"; Fig. 1.6 in Chapter 1). Additionally, the factor "tree cover" was evaluated at two levels: (1) under holm oak canopy (halfway of the canopy, starting from the trunk; coded as "under canopy"), and (2) outside the canopy (1.5 m outside any canopy projection; coded as "open areas"). For each of the three large fragments, we selected five holm oak trees in the forest interior and five trees at the forest edge, and three trees at five small fragments (15 trees per matrix influence-fragmentation level), resulting in a total of 45 selected trees. For each selected tree, two coverage-sampling points were established: one under canopy and the other in open areas, resulting in a total of 90 soil samples.

Height, basal area and canopy projection were measured for each of the 45 holm oak multi-stem trees. A tree influence index *(Tii)* was calculated at each sampling point, according to the formula: $Tii = \frac{Basal area}{Distance from the trunk}$ (Chapter 2). The basal area was selected to calculate this tree influence index given its recognized direct relationship with soil functioning (Barba et al., 2013; see Chapter 2). Soil moisture was determined by weight lost of samples oven-dried at 105 °C for 48 h. Total C and N contents were measured on air-dried soil samples, using a C:N elemental analyzer (Flash EA 1112 Series, Thermo Fisher Scientific). Total concentrations of P, K, Ca, Na, S, Mg, Fe, Mn, Cu, Mo, and Zn were determined by digestion with $HNO_3$ + $H_2O_2$ (4:1, v:v), followed by inductively coupled plasma-optical emission spectrometry (ICAP-6500 Duo/Iris Intrepid II XDL, Thermo Fisher Scientific, Massachusetts, USA). Soil pH was determined on a 1:10 (w:v) aqueous suspension. Soil organic matter (SOM) was assessed by loss on ignition at 400 °C, during 4 hours.

*Soil community structure*

The structure of soil bacterial and fungal communities was assessed by the DNA community fingerprinting technique of denaturing gradient gel

electrophoresis (DGGE). Soil DNA was extracted with the MoBio Power soil DNA isolation kit (Solana Beach, USA), and yields assessed by electrophoresis at 80 V on a 1.2 % agarose gel. The universal primers 338F/518R were used for amplification of the bacterial 16S rRNA gene (Muyzer et al., 1993). In the case of fungi, the internal transcribed spacer nrDNA region ITS-1 was PCR-amplified using the primer pair ITS1-F/ITS2 (Gardes and Bruns, 1993). A GC clamp was respectively added to the 5' end of forward bacterial (338F) and fungal (ITS1-F) primers to stabilize the melting behavior of the DNA fragments (Muyzer et al., 1993). PCRs were carried out on a Mastercycler® gradient Thermocycler (Eppendorf, Germany), with 50 µl final volume containing 10x $NH_4$ reaction buffer, 2 and 1.5 mM $MgCl_2$ (for fungi and bacteria, respectively), 0.2 mM total dNTPs, 2.5 U Taq (Bioline, London, UK), 1µM of each primer, 0.5 µl of 10 mg $ml^{-1}$ bovine serum albumin (BSA) and 50 ng of template DNA, determined using a NanoDrop 1000 (Thermo Scientific, USA). PCR cycling parameters were: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 or 45 s (fungi or bacteria, respectively), and 72 °C for 30 or 45 s (fungi or bacteria), with a final extension at 72 °C for 5 or 10 min (fungi or bacteria, respectively). Negative controls (containing no DNA) were included in each PCR run.

DGGE was carried out on a DCode universal mutation detection system (Bio-Rad, Hemel Hempstead, UK), using 10% polyacrilamide gels, with denaturant urea-formamide gradients of 10-50% for fungi (Anderson et al., 2003) and 30-60% for bacteria (Grossman et al., 2010), with the concentrations of 7 M urea and 40 % formamide (v/v) for the 100 % denaturant. Electrophoreses were run at 60 °C 75 V for 16 h, loading equal volumes of amplified DNA. Gels were stained with SYBR Gold nucleic acid stain (Molecular Probes, The Netherlands). DGGE fingerprint profiles were digitized and analyzed using a Kodak DC290 zoom digital camera with

KODAK 1D Image Analysis software (Kodak, NY, USA). Bands were adjusted with a Gaussian model with a profile width of 80%. Noise was eliminated by removing bands below a 10% band peak intensity threshold. Each band of the DGGE profile was hereafter referred to as an operational taxonomic unit (OTU). Gel bands were analyzed by using internal reference bands, and known reference markers loaded in lanes at either side of the gel. The number and pixel intensity of bands in a particular sample were considered comparative proxies of richness and relative abundance of fungal or bacterial OTUs, respectively (Cleary et al., 2012). Similar analysis of DGGE banding patterns have been previously used in other studies (Anderson et al., 2003; Gafan et al., 2005; Cleary et al., 2012; Suzuki et al., 2012; Vaz-Moreira et al., 2013; Flores-Rentería et al., 2015).

*Microbial metabolic profile*

Community level physiological profiles (CLPP) of cultivable microbial communities (both bacteria and fungi, those not inhibited by tetrazolium dye) were determined with Biolog™ EcoPlates (BIOLOG Inc., Hayward, CA). From here, we define "metabolic profile" as the identity and abundance of the substrates that microbial communities were able to metabolize, measured either by qualitative (presence/absence) or quantitative (abundance) approaches. We used the procedure adapted from (Garland and Mills, 1991). Briefly, 4 g (dry weight equivalent) of each soil sample was added to 36 ml of sterile 0.8% saline solution (NaCl). The mixture was then shaken on an orbital shaker for 20 min, and left to stand at room temperature, for 30 min. A volume of 250 µl supernatant was diluted into 24.75 ml of sterile saline solution. Only in the case of fungal plates, 25 µl streptomycin and 25 µl tetracycline (dilution 1:1000, w:v, in both cases) were added to 24.7 ml of sterile saline solution to limit the bacterial growth. Supernatant dilutions were mixed for 30s and left to stand for 10 min. A 100 µl aliquot of each diluted solution was added to each of 96 wells

in a Biolog™ EcoPlates (arranged by triplicate for each substrate). Plates were incubated at 28 °C in a humidity-saturated environment. Color formation in each well was monitored at monochromatic light (590 nm) absorbance using a Victor3 microplate reader (Perkin-Elmer Life Sciences, Massachusetts, USA). Measurements were performed once per day during 7 and 10 days for bacterial and fungal plates, respectively. A single time point absorbance was used to all posterior analyses at 96 and 168 h for bacterial and fungal plates, respectively, when the asymptote was reached (data not shown). Optical density (absorbance) value from each well, corrected by subtracting the blank well (inoculated, but without a substrate), was normalized by the color summation of the entire plate. Subsequently, we averaged the three values for each individual substrate within a plate. The EcoPlates system has been recognized as a useful tool for comparing microbial communities (Classen et al., 2003; Gomez et al., 2004; Weber et al., 2007; Weber and Legge, 2009; Frąc et al., 2012), since it can detect functional changes in microbial communities as a result of differing carbon availability in soil, its physiological basis has been considerate to provide an ecologically relevant overview as long as results are interpreted as a profile of phenotypic potential and not in terms of *in situ* activity (Gomez et al., 2004).

*Data analysis*

A principal component analysis (PCA) was conducted to reduce the *n*-dimensional of soil nutrients data into two linear axes explaining the maximum amount of variance (Fig. 2.3, in Chapter 2).

Structural alpha-diversity of both bacterial and fungal communities was estimated from the number and intensity of bands (OTUs): richness ($S$), Shannon ($H'$) and evenness ($E_H$) diversity indexes were calculated as follows:

Shannon $(H') = -\sum_{i=1}^{S}\left({}^{n_i}/_{N}\right) \cdot ln \cdot \left({}^{n_i}/_{N}\right)$ and Evenness$(E_s) = \frac{H'}{ln\, S}$, where $n_i$ is the band intensity, $N$ is the sum of all intensities of a sample and $S$ is the number of bands of a sample (richness). Similarly, the functional alpha-diversity was evaluated as functional richness (*SS*, total number of C substrates catalyzed), functional Shannon (*SH'*; using the optical density as abundance), and functional evenness ($SE_S$, substrate activity divided by *ln* substrate richness) (Classen et al., 2003; Grizzle and Zak, 2006).

Environmental variables and structural and functional alpha-diversity were analyzed by two-way Analysis of Variance (ANOVA) considering the factors matrix influence and coverage. Subsequently, and due to the high effect of tree coverage factor, fragmentation effects within each coverage level, as well as coverage effects within each fragmentation level were separately evaluated by one-way ANOVA. Tukey's HSD were used as post hoc test ($p< 0.05$). Linear correlations between all measured variables were tested using Pearson's $r$ with $p < 0.05$ significance threshold.

Microbial community assemblages and metabolic profiles (both bacterial and fungal) were explored by Nonmetric multidimensional scaling (NMDS) analysis, which provided graphical ordination of the community grouping, using the functions *metaMDS* and *isoMDS* in vegan and MASS R packages, Oksanen et al. (2013). For bacteria and fungi, these analyses were performed using both quantitative (abundance) and qualitative (presence/absence) data: we used the data of relative DGGE band intensity for microbial assemblage analyses, whereas for analyzing microbial metabolic profiles we used the normalized optical density data obtained in the EcoPlates. The dissimilarity matrices were built using the Bray-Curtis distance measure. Regarding NMDS, a measure of stress <5 provides an indication of an excellent fit of the model, hence suggesting that the structure of the community is well represented in reduced

dimensions, a measure of stress between 20 and 30 provides a good fit, and measures of stress above 30 provides a poor fit, and hence and indication o a poor representation in reduced dimensions. The preferred solution, based on the lowest stress and instability was three dimensional, although two dimension graphs were finally presented. To seek for differences among microbial assemblage and metabolic profile we applied a non-parametric multivariate analysis of variance (NPMANOVA), performed with Bray-Curtis distances as a measure of dissimilarity among treatments (Anderson, 2001), considering the factors matrix influence, coverage and their interaction. Significance was obtained from permutations of the raw data ($F$ test based in 1000 sums of squares). The agricultural matrix influence effects within each coverage level, as well as the coverage effects within each matrix influence level, were separately evaluated by subsequent NPMANOVAs.

As a measure of beta-diversity we used the multivariate dispersion, using the distance to the centroid (Anderson et al., 2006) calculating one centroid for each soil provenance (i.e. under canopy or open areas for each: forest interior, forest edge or small fragments), calculated using *betadisper* and *permutest* functions in the vegan R package (Oksanen et al., 2013). Beta-diversity was determined by using both quantitative (abundance) and qualitative (presence/absence) data, which may provide complementary information on the structural and functional response of these communities to disturbances (Lozupone and Knight, 2008; Maaß et al., 2014). In both cases, we used the Bray-Curtis dissimilarity matrix, considering the factors matrix influence and coverage. Subsequent multiple comparison of means was performed through Tukey's HSD test ($p < 0.05$).

*Controlling factors*

To determine which environmental variables explained most of the variation of the structure and function of the microbial communities, we

used two approaches including thev ariables: tree influence index, SOM, pH, soil moisture, C:N ratio, PC1 and PC2 of nutrients PCA's. In the first approach, the *envfit* function (vegan R package; Oksanen et al. (2013), was used to plot the vectors of variables that were significantly correlated ($p$<0.05) with the assemblage and metabolic profile of microbial communities on the NMDS ordination. The second approach consisted of structural equation modeling (SEM) to test not only the direct influence of biotic and abiotic factors on microbial functioning, but also their indirect effects, with an aprioristic model in which the causal relationships among measured variables were explicitly included (Shipley, 2002; Iriondo et al., 2003; Milla et al., 2009). SEM models were individually performed for each soil bacterial and fungal functional indicator (functional alpha and beta-diversity, and metabolic profile), but only the best fitted ones were presented (quantitative functional Shannon and metabolic profile). Both community assemblage and metabolic profile were included in the models as the distance to a unique centroid in the plotted NMDSs. Our models considered a complete set of hypotheses showed in Figure 3.3a and 3.3b for bacterial and fungal communities, respectively. These hypotheses were based on literature, previous exploratory analyses (ANOVA, correlations), and our own previous experience (Flores-Rentería et al., 2015; Chapter 2). First, we hypothesized that microbial functioning will depend on microbial community structure (Giller et al., 1997; McGuire and Treseder, 2010; Ushio et al., 2013; Wagg et al., 2014; Flores-Rentería et al., 2015), and both would be dependent on abiotic and biotic conditions, such as pH, (Hamman et al., 2007; Fierer et al., 2009), SOM (Curiel Yuste et al., 2007; Franklin and Mills, 2009), soil moisture (Curiel Yuste et al., 2007; Saul-Tcherkas et al., 2012), nutrients (O'Donnell et al., 2001; Franklin and Mills, 2009; Laughlin et al., 2014; Legay et al., 2014), and that all these variables would be on their turn, under the tree influence (Classen et al., 2003; Pugnaire et al.,

2004; Legay et al., 2014). Additionally, we included in our model causal relations among abiotic variables, i.e. SOM influence over soil moisture, pH and C:N (Abu-Hamdeh, 2001; Boix-Fayos et al., 2001; Pugnaire et al., 2004). Standardized path coefficients were estimated by using the maximum likelihood algorithm (Shipley, 2002).

To determine the possible links between microbial assemblage and metabolic profile (for both bacterial and fungal communities) independent Mantel Tests of correlation (*mantel* function on vegan package in R) were performed between the Bray–Curtis dissimilarity indices of each bacterial and fungal DGGE matrix and the corresponding Bray–Curtis dissimilarity indices of the bacterial and fungal EcoPlates. The Mantel Test uses the similarity of two dissimilarity matrices by permuting each of the elements in the dissimilarity matrix 999 times to derive a distribution of correlation values (Franklin and Mills, 2009). The resulting R-statistic is similar to the Pearson's Product Moment Correlation Coefficient; with increasingly similar dissimilarity matrices, the Mantel R-statistic will approach 1. Abundance proxies of microbial assemblage and metabolic profile matrixes were not transformed.

Prior to analyses, all variables were tested for normality, and log transformations were applied to meet variance homoscedasticity when required, except abundance matrices of microbial assemblage and metabolic profiles. SEMs were performed by using IBM®, SPSS® (IBM Corporation Software Group, Somers, NY) and IBM®, SPSS® AMOS 20.0 software (IBM Corporation Software Group, Somers, NY), the rest of analyses were performed using R 3.1.0 (The R Foundation for Statistical Computing, (2014).

## Results

*Cover and forest fragmentation effect on soil microbial communities*

As expected, soils in the holm oak forest fragments studied were strongly influenced by the canopy cover. Under canopy, significantly higher values of nutrients, SOM, soil moisture, and lower Ca and pH values were found compared with open areas (Fig. 2.3 Chapter 2; Table S3.1).

Structural alpha-diversity of the fungal community was neither affected by coverage nor agricultural matrix, only fungal community evenness *($E_S$)* was sensitive to the influence of the agricultural matrix (Table 3.1); whereas bacterial community structure was mainly influenced by the agricultural matrix, with higher values of bacterial richness *(S*) and Shannon (*H'*) at small fragments and lower values at forest interior (Table 3.1). On the contrary, the metabolism of both bacterial and fungal communities was strongly influenced by the coverage, showing higher functional alpha-diversity under the influence of the tree canopy in all measured parameters: functional richness *(SS)*, Shannon *(SH')*, and evenness *($SE_S$)* (Table 3.1). Additionally, the agricultural matrix positively influenced, although to a lesser extent, the functional alpha-diversity of both bacterial and fungal communities. Specifically, bacterial functional Shannon *(SH')*, and richness (*SS*) were higher at forest edge and small fragments, and those of fungi in small fragments (Table 3.1). An interactive effect between coverage and matrix influence was found for bacterial *SH'* and *$SE_S$*, which were higher in soils from small fragments and under the tree canopy (Table 3.1). Substrate consumption in both bacterial (Table S3.2) and fungal (Table S3.3) communities was mainly dependent on the tree canopy, affecting 24 and 19 substrates, respectively. The agricultural matrix also affected the consumption of some substrates, more evidently in the case of bacteria (15) than fungi (4; Tables S3.2 and S3.3).

**Table 3.1** Structural and functional alpha-diversity of soil microbial communities in holm oak forest fragments in Spain. Data are mean ± standard error. Two-way ANOVA results are presented (left columns), for factors C = coverage and MI = Matrix influence.

| | Under canopy | | | Open areas | | | Factorial ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|
| **Structural alpha-diversity** | **Forest interior** | **Forest edge** | **Small fragments** | **Forest interior** | **Forest edge** | **Small fragments** | ***C*** | ***MI*** | ***C x MI*** |
| ***Bacterial community*** | | | | | | | | | |
| Richness *(S)* | 34±0.52 | 36.93±0.38 | 37.53±0.52 | 32.6±0.6 | 35.27±0.5 | 36.8±0.47 | n.s. | $F_{2,84}$=8.1 $p$<0.001 | n.s. |
| Shannon diversity *(H)* | 3.3±0.09 | 3.38±0.08 | 3.39±0.09 | 3.25±0.11 | 3.32±0.10 | 3.35±0.07 | n.s. | $F_{2,84}$=3.8 $p$=0.027 | n.s. |
| Evenness *($E_S$)* | 0.94±0.03 | 0.94±0.03 | 0.94±0.04 | 0.94±0.03 | 0.93±0.04 | 0.93±0.04 | n.s. | n.s. | n.s. |
| ***Fungal community*** | | | | | | | | | |
| Richness *(S)* | 29.4±0.47 | 28.8±0.32 | 27.93±0.37 | 29.73±0.45 | 29.27±0.37 | 28.73±0.31 | n.s. | n.s. | n.s. |
| Shannon diversity *(H)* | 3.06±0.10 | 3.10±0.09 | 3.14±0.09 | 3.12±0.09 | 3.14±0.09 | 3.17±0.08 | n.s. | n.s. | n.s. |
| Evenness *($E_S$)* | 0.91±0.04 | 0.92±0.04 | 0.94±0.04 | 0.92±0.03 | 0.93±0.04 | 0.94±0.03 | n.s. | $F_{2,84}$=12.9 $p$<0.001 | n.s. |
| **Functional alpha-diversity** | | | | | | | | | |
| ***Bacterial community*** | | | | | | | | | |
| Functional richness *(SS)* | 28.53±0.37 | 29.07±0.26 | 29.07±0.27 | 27±0.35 | 28.8±0.28 | 27.07±0.37 | $F_{1,84}$=*14.11* $p$<0.001 | $F_{2,84}$=4.30 $p$=0.017 | n.s. |
| Functional Shannon diversity *(SH)* | 3.05±0.08 | 3.07±0.11 | 3.13±0.07 | 2.73±0.09 | 2.99±0.08 | 2.81±0.1 | $F_{1,84}$=85.09 $p$<0.001 | $F_{2,84}$=9.87 $p$<0.001 | $F_{2,84}$=10.04 $p$<0.001 |
| Functional Evenness *($SE_S$)* | 0.91±0.04 | 0.91±0.06 | 0.93±0.03 | 0.83±0.05 | 0.89±0.04 | 0.85±0.06 | $F_{1,84}$=62.65 $p$<0.001 | $F_{2,84}$=5.93 $p$=0.002 | $F_{2,84}$=7.17 $p$<0.001 |
| ***Fungal community*** | | | | | | | | | |
| Functional richness *(SS)* | 19.33±0.52 | 22±0.43 | 23.2±0.39 | 12±0.52 | 14.87±0.45 | 19.07±0.50 | $F_{1,84}$=74.41 $p$<0.001 | $F_{2,84}$=19.28 $p$<0.001 | n.s. |
| Functional Shannon diversity *(SH)* | 2.64±0.13 | 2.79±0.11 | 2.85±0.1 | 2.1±0.17 | 2.27±0.13 | 2.56±0.14 | $F_{1,84}$=58.25 $p$<0.001 | $F_{2,84}$=10.78 $p$<0.001 | n.s. |
| Functional Evenness *($SE_S$)* | 0.90±0.04 | 0.90±0.05 | 0.91±0.05 | 0.88±0.06 | 0.85±0.07 | 0.87±0.05 | $F_{1,84}$=16.28 $p$<0.001 | n.s. | n.s. |

Structural beta-diversity of microbial communities based in both quantitative (abundance; Fig. 3.1a-b) and qualitative data (presence/absence; Fig. 3.1c-d) pointed out to a negative influence of the agricultural matrix in small fragments and edges, in comparison to the forest interior, which generally showed higher beta-diversity. When qualitative data were analyzed (Fig. 3.1c-d), only the bacterial community was also influenced by coverage, with higher beta-diversity observed in open areas (Fig. 3.1d). In the case of quantitative data, the higher beta-diversity of the fungal community was observed in the forest interior in comparison with forest edges and small fragments (Fig. 3.1b); whereas qualitative data analysis revealed an interaction between coverage and matrix influence, with the highest beta-diversity of fungal communities under canopy and open areas in forest interior (Fig. 3.1d).

On the contrary, when functional beta-diversity was analyzed (quantitative data Fig. 3.2a-b), both bacterial and fungal communities were significantly influenced by coverage and not by the matrix influence, showing in both cases higher values in open areas than under canopy (Fig. 3.2a-b). By contrast, for both bacteria and fungi, functional beta-diversity (qualitative data Fig. 3.2c-d) was affected by the interaction between coverage and matrix influence with the highest beta-diversity usually observed for forest interiors, in both open areas and under canopy (Fig. 3.2c-d).

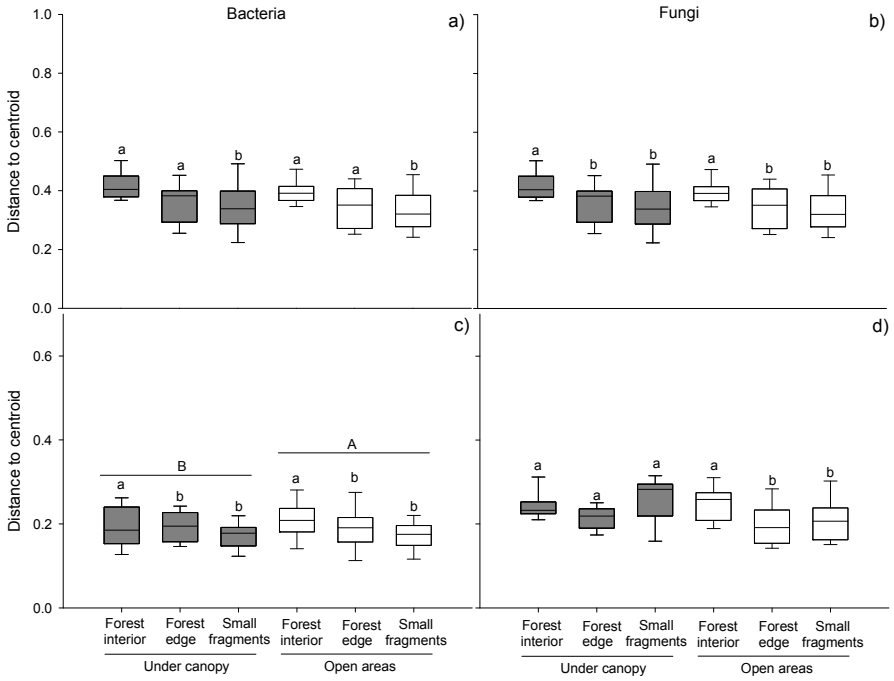


**Figure 3.1** Structural $\beta$-diversity of bacterial (a,c) and fungal (b,d) communities, determined by the distance to the centroid of multivariate dispersion using quantitative (abundance) (a-b) or qualitative (presence/absence) (c-d) datasets, of soils collected at three agricultural matrix influence levels, under canopy or in open areas, in fragmented holm oak forests in Spain. Note that higher distance to centroid (over-dispersion) means higher $\beta$-diversity. Different capital letters represent differences among coverage treatments, while different lowercase letters represent differences among matrix influence levels; Tukey HSD multiple comparison of the mean ($p$<0.05). The line within the box is the median value indicating the interquartile range (25th to 75th percentiles), and the whiskers extend to the most extreme value within the 1.5 interquartile range.

*Controlling factors of structure and metabolism of soil microbial communities*

Bacterial and fungal communities were significantly correlated with some environmental variables (Table S3.4): e.g. PC1 of nutrients-PCA was strongly correlated with functional alpha-diversity in all cases (Table S3.4). Bacterial and fungal metabolism was strongly correlated with all the environmental variables measured, e.g. SOM and soil moisture were positive correlated with functional alpha-diversity of both bacterial and fungal communities (Table S3.4).

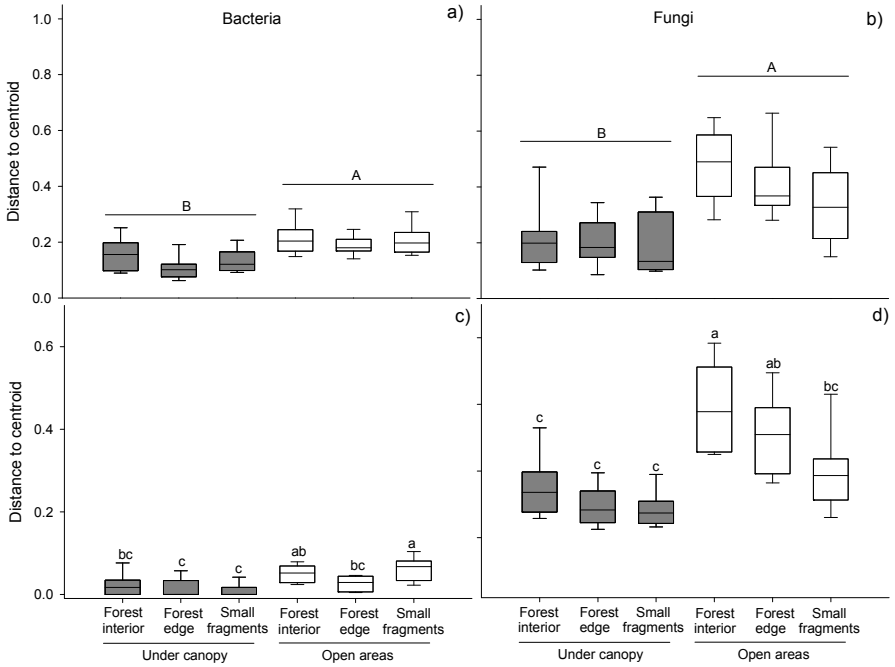


**Figure 3.2** Functional $\beta$-diversity of bacterial (a,c) and fungal (b,d) communities, determined by the distance to the centroid of multivariate dispersion using quantitative (abundance) (a-b) or qualitative (presence/absence) (c-d) dataset, of soils collected at three agricultural matrix influence levels, under canopy or in open areas, in fragmented holm oak forests in Spain. Note that higher distance to centroid (over-dispersion) means higher $\beta$-diversity. Different capital letters represent differences among coverage treatments, while different lowercase letters represent differences among matrix influence levels; Tukey HSD multiple comparison of the mean ($p$<0.05). The line within the box is the median value indicating the interquartile range (25th to 75th percentiles), and the whiskers extend to the most extreme value within the 1.5 interquartile range.

The assemblage of bacterial and fungal communities, based on quantitative data, was strongly influenced by tree coverage and agricultural matrix (Fig. S3.1), although the NPMANOVA indicated that the matrix influence exerted the strongest effect, in both cases (Table S3.5). The respective assemblage of OTUs within bacterial and fungal communities (NMDS) showed a good fit (stress value of bacteria=19.98, Fig. S3.1a, and fungi=20.76, Fig. S3.1b). Similar results were obtained when qualitative (presence/absence) matrices were analyzed, with no substantial changes concerning the factors controlling the grouping of OTUs with respect to

results obtained with quantitative (abundance) matrices (Fig. S3.1c-d; Table S3.5). According to the *envfit* permutation test, soil physicochemical properties were highly correlated suggesting them as contributing factors influencing the grouping of bacteria and fungi (Fig. S3.1). Specifically, all nutrients, except organic carbon and Mo in the case of fungi, affected the assemblage of both bacterial and fungal communities (Fig. S3.1a-b; Table S3.6). Tree influence index (*Tii*), soil organic matter (SOM), soil moisture and pH also influenced the grouping of bacteria (Fig. S3.1a-b; Table S3.6).

Regarding the metabolic profile of both bacteria and fungi (quantitative data; Fig. S3.2a-b) the NMDSs analysis showed very good fit (stress values of 10.04 and 16.74, respectively), and both microbial communities were affected by the tree influence (i.e. coverage in Table S3.5). Additionally, the metabolic profile of bacterial community in open areas was significantly influenced by the agricultural matrix (Table S3.5). Contrary to the assemblage of the bacterial community, its metabolic profile was only affected by total and organic C, N, P, SOM, soil moisture, tree influence and pH (Fig. S3.2a; Table S3.7), being, in consequence strongly segregated by the influence of the tree canopy. By contrast, the fungal metabolic profile was not significantly influenced by any variable (Fig. S3.2b; Table S3.7). Unlike results obtained from quantitative data analyses of metabolic profile, qualitative analyses (i.e. just testing the capability of substrate utilization, not its relative use) showed that the bacterial and fungal metabolic profiles were clustered with almost no influence of the environmental variables (Fig. S3.2c-d, Table S3.5). Most variables had not longer influence in the ordination of qualitative (presence/absence) bacterial metabolic profile, being influenced only by tree influence, soil moisture, SOM, organic and total C, N, P (Fig. S3.2c); whereas fungal metabolic profile by tree influence and total carbon (Fig. S3.2d).

The structural-equation models (SEM) proposed for bacterial and fungal communities (Fig. 3a-b) and based on the correlations observed above, provided a good general fit, as indicated by the non-significant *f* value and by the goodness-of-fit indices (RMSEA, NFI and GFI). Squared multiple correlations for SEMs showed that the variance of the bacterial functional Shannon (*SH'*) was highly explained ($R^2$= 0.60) in comparison with the community assemblage variance ($R^2$= 0.42; Table S3.8). When the effect of the bacterial community structure (i.e. the effect of Shannon *H'* over functional Shannon *SH'*; or assemblage over metabolic profile) over its functioning was removed (data not show), the explained variance dropped almost 8% in the case of Shannon (*SH'*) model ($R^2$= 0.55; Fig 3.3c), and a 12% in the case of the metabolic profile model ($R^2$= 0.37; Fig 3.3e; Table S3.8). Both bacterial functional Shannon (*SH'*) and metabolic profile were affected by soil moisture and bacterial structure (Shannon *H'* and assemblage, respectively). Agricultural matrix indirectly affected bacterial Shannon (*H'*) and assemblage, mainly through its effect over the size of the trees (tree influence), which on the other hand, exerted a strong positive effect over pH and SOM quantity (Fig. 3c,e). Additionally, the agricultural matrix influenced the bacterial community assemblage and the quantity of nutrients (i.e. PC1; Fig. 3.3c), which in turn influenced bacterial structural Shannon (*H'*) (Fig. 3.3e). Soil pH affected both bacterial Shannon (*H'*) and community assemblage, but with opposite influence, negatively to the Shannon (*H'*; Fig. 3.3c) and positively to the community assemblage (Fig. 3.3e).

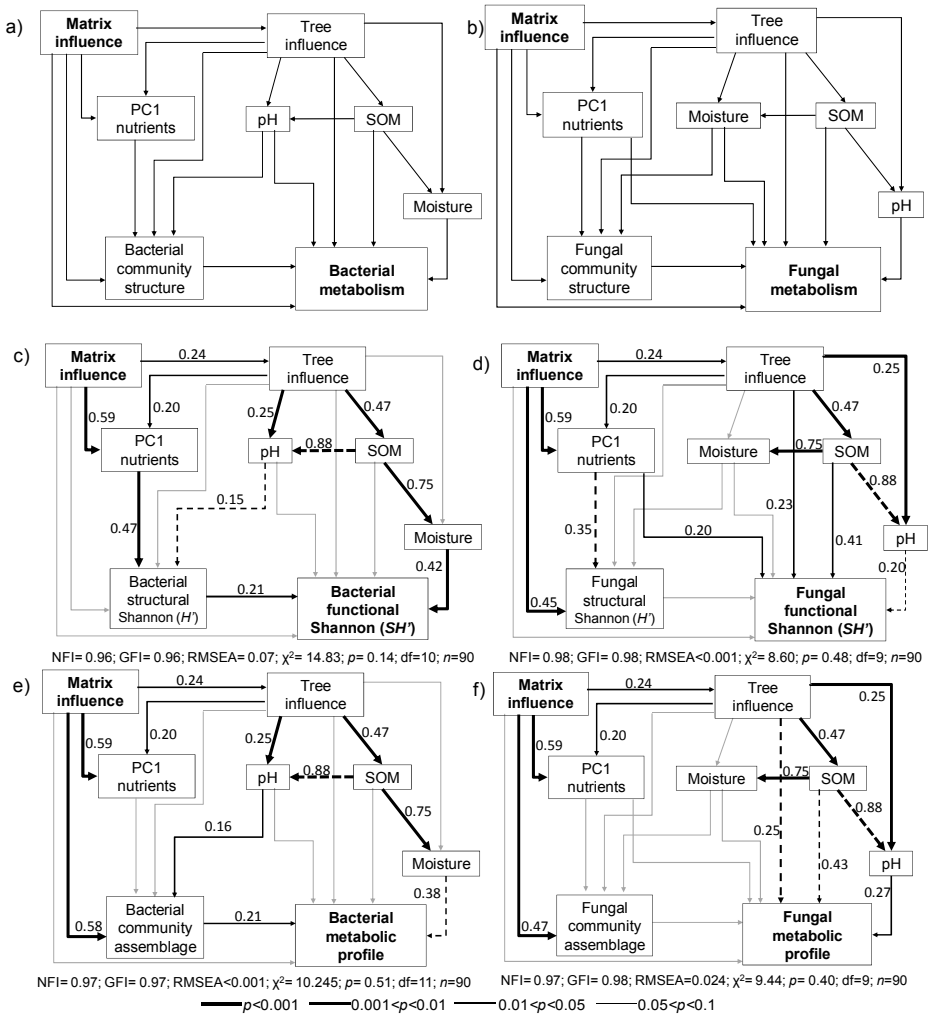


**Figure 3.3** Structural equation models: general (a-b), and fitted to bacterial (c) and fungal (d) Shannon diversity (*H'*) (c-d, respectively), and bacterial (e) and fungal (f) community assemblage (e-f, respectively), representing hypothesized causal relationships among matrix influence, biotic and abiotic predictors. Arrows depict casual relationships: positive effects are indicated by solid lines, and negative effects by dashed lines, with standardized estimated regression weights (SRW) indicated. SRW of each microbial diversity indicator are showed in Table 3.2, and squared multiple correlations for the structural equation models in Table S3.8. Arrow widths are proportional to *p* values. Paths with coefficients non-significant different from 0 ($p > 0.1$) are presented on gray.

The agricultural matrix exerted both a direct and indirect (through its effect on nutrients) influence over fungal Shannon (*H'*) and community assemblage, as well as over tree influence (Fig. 3.3d,f). Fungal functional Shannon (*SH'*) and community assemblage were drove by SOM, tree influence and soil pH, showing opposite patterns to those observed in each the functional Shannon (*SH'*) or community assemblage models (Fig. 3.3d,f; Table 3.2).

**Table 3.2** Microbial community metabolism and direct (D), indirect (I) and total (T) effects of tested variables, based on standardized regression weights (SRW), for each structural equation model. Significant direct effects are noted in bold. Tii= Tree influence index; PC1= Principal component 1 of the nutrients PCA.

| *Bacterial functional alpha-diversity* | | | | *Fungal functional alpha-diversity* | | | |
|---|---|---|---|---|---|---|---|
| | **D** | **I** | **T** | | **D** | **I** | **T** |
| Matrix influence | -0.02 | 0.16 | 0.13 | Matrix influence | 0.09 | 0.23 | 0.32 |
| *Tii* | 0.13 | 0.34 | 0.47 | *Tii* | **0.23** | 0.21 | 0.45 |
| SOM | 0.23 | 0.36 | 0.60 | SOM | **0.41** | 0.09 | 0.50 |
| Soil moisture | **0.42** | 0.00 | 0.42 | Soil moisture | -0.12 | 0.00 | -0.11 |
| pH | -0.02 | -0.03 | -0.05 | pH | **-0.20** | 0.00 | -0.20 |
| PC1 nutrients | 0.00 | 0.10 | 0.10 | PC1 nutrients | **0.20** | 0.00 | 0.20 |
| Bacterial alpha-diversity | **0.21** | 0.00 | 0.21 | Fungal alpha-diversity | 0.01 | 0.00 | 0.01 |
| *Bacterial metabolic profile* | | | | *Fungal metabolic profile* | | | |
| | **D** | **I** | **T** | | **D** | **I** | **T** |
| Matrix influence | 0.00 | 0.00 | 0.00 | Matrix influence | -0.08 | -0.12 | -0.20 |
| *Tii* | -0.15 | -0.25 | -0.40 | *Tii* | **-0.25** | -0.22 | -0.47 |
| SOM | -0.13 | -0.36 | -0.49 | SOM | **-0.43** | -0.15 | -0.57 |
| Soil moisture | **-0.38** | 0.00 | -0.38 | Soil moisture | 0.10 | 0.02 | 0.12 |
| pH | 0.05 | 0.03 | 0.08 | pH | **0.27** | 0.00 | 0.27 |
| PC1 nutrients | 0.00 | -0.04 | -0.04 | PC1 nutrients | -0.08 | -0.01 | -0.10 |
| Bacterial assemblage | 0.21 | 0.00 | 0.21 | Fungal assemblage | -0.10 | 0.00 | -0.10 |

*Relations between assemblage and function in soil microbial communities*

Relationships among indicators of structural and functional alpha-diversity (richness, diversity and evenness) showed significant but weak correlations; i.e. bacterial richness (*S*) and bacterial functional richness (*SS'*) showed a $R^2$=0.26 (Table S3.4), whereas fungal community structure and function (richness, diversity and evenness) were uncorrelated (Table S3.4).

In the case of the bacterial community, SEMs revealed a direct effect of structure (Shannon and assemblage) over functioning (functional Shannon and metabolic profile; Fig. 3.3c,e; Table 3.2). In the case of fungal communities, SEMs revealed that neither fungal Shannon (*H'*) nor community assemblage exerted a significant effect on fungal functioning, neither on Shannon *SH'* nor on metabolic profile (Fig. 3.3d, f; Table 3.2). Indeed, Mantel test showed that the dissimilarity matrices of fungal community assemblage and metabolic profile were not significantly correlated (*R*=0.04; *p*=0.26); while, dissimilarity matrices of bacterial assemblage and metabolic profile exhibit a significant correlation (*R*=0.12; *p*=0.006).

## Discusion

*Tree coverage and forest fragmentation effect on soil microbial community*

Our results indicate that the assemblage and diversity of microbial communities, as well as their capacity to metabolize different substrates, exhibited dissimilar susceptibility to the agricultural matrix influence (used as proxy of forest fragmentation), in many cases strongly mediated by its influence over tree growth and plant-soil interactions (i.e. increasing plant productivity and, in turn, the amount of soil organic matter). This different response to the agricultural matrix and the tree influence of the studied microbial community structure (DGGE) and functional (EcoPlates) indicators suggests a partial decoupling between the factors controlling the composition/assemblage of species and the functioning of microbial communities. Indeed, bacterial richness (S) and Shannon (H') were positively affected by the agricultural matrix influence, whereas the functional alpha-diversity of bacterial and fungal communities was positively influenced by both factors (matrix influence and coverage), and particularly by the tree (canopy cover). The presence of the tree, therefore, exerted a strong positive influence over the relative amount of consumed substrates, corroborating the findings of studies that have previously showed a higher microbial functional diversity (Classen et al., 2003), as well as enzymatic activity (Chapter 2), under canopy in comparison with open areas. The generally higher bacterial alpha-diversity (i.e. richness and Shannon) in areas more influenced by the agricultural matrix (i.e. small forest fragments), can be also explained by the formation of new available niches after disturbance (Curiel Yuste et al., 2012). The partial decoupling of the factors controlling the structure and functioning of microbial communities might be an indicative of the strong differences in the composition of the active microbial community from the total community

at local scales (O'Donnell et al., 2001; Jones and Lennon, 2010). Indeed, it might be that the unfavorable environmental conditions of the open areas can be associated with dormancy of most bacterial and fungal lineages present in the community, as it has been previously observed (Jones and Lennon, 2010). However, we cannot discard a bias due to the used techniques, since DGGE explores the most abundant, still representative OTUs of the microbial community (Vaz-Moreira et al., 2013), whereas EcoPlates represent the cultivable ones (Classen et al., 2003).

This general tendency of positive influence of both tree coverage and agricultural matrix over local diversity of the microbial communities (i.e. structural richness and Shannon and all functional indicators) contrasted, moreover, with the results obtained when analyzing their beta-diversity, as a measure of the spatial structural and functional heterogeneity of these communities at the landscape scale. Indeed, areas highly influenced by the agricultural matrix (i.e. small fragments and forest edges), or by the tree, showed more spatially homogeneous (less beta-diverse) microbial communities suggesting a clear scale-dependent response of microbial communities to environmental perturbations. Hence, forest fragmentation with high agricultural matrix and tree influences would enhance bacterial alpha-diversity, both structural (*S* and *H*') and functional (*SS, SH* and $SE_s$), while decreasing both the structural and functional spatial heterogeneity (less beta-diversity) of these communities. This landscape convergence of microbial communities under the tree canopies and at highly influenced areas by the agricultural matrix (small fragments and forest edges) could be attributable to the environmental filtering of these communities by more uniform soil properties in these areas, i.e. abiotic homogenization, in comparison with open areas and forest interiors, since a beta-diversity decrease indicates community similarity increase over space, i.e. biotic homogenization (Olden et al.,

2004). These results would finally indicate that despite the fact that forest fragmentation might be associated with species enrichment at the local scale, it might cause a general species and functional impoverishment of soils at the landscape scale, with likely negative consequences for the capacity of these soils to respond to engines of climate change (Curiel Yuste et al., 2011; Flores-Rentería et al., 2015).

*Controlling factors of soil microbial communities*

Our results showed, in general, the strong effect of the agricultural matrix over the proxies for microbial community structure (i.e. alpha-diversity and assemblage), that could be mainly attributable to the soil nutrient enrichment associated with fragmentation. Variability in soil nutrient contents was mainly explained by the matrix influence (44%) even more than by the tree cover (26%). Indeed, as it has been previously reported, a big portion of the variability in microbial structure was explained by nutrient availability (Wardle, 1998; O'Donnell et al., 2001; Fierer and Jackson, 2006; Franklin and Mills, 2009; Bowen et al., 2011; Ramirez et al., 2012; Tardy et al., 2014). While almost all nutrients influenced bacterial and fungal assemblages, only macronutrients (C, N, P) influenced their metabolism. On the other hand, the use of both quantitative and qualitative analyses allowed us to unveil the strong influence of the canopy cover over the microbial metabolic profile (influencing the amount of substrate metabolized, but not the capability for substrate utilization), highlighting the importance of using both approaches to explore, for example, functional redundancy and complementarity within these microbial communities (Lozupone and Knight, 2008; Miki et al., 2010). According to our results, microbial communities with different structure (i.e. under canopy vs. open areas) exhibited the same capability for substrate utilization, given that the tree canopy cover only influenced the amount of substrate metabolized, suggesting a functional redundancy of these

microbial communities (i.e. the ability of one microbial taxon to carry out a process as another; Allison and Martiny, 2008).

SEMs allowed us to disentangle the complexity of the direct and indirect effects of the agricultural matrix over the microbial ecology and potential roles of these communities within the plant-soil-microbial system. The magnitude of the agricultural matrix effect, and hence the effect of forest fragmentation, over both functional Shannon and metabolic profile, is the result of a complex cascade of causal-effect relations involving changes in plant performance and modifications of nutrient quantity (and probably quality and/or availability). Similarly to the findings in Chapter 2, the agricultural matrix influences the tree size and modified the micro-environmental conditions (nutrients, SOM, pH and moisture), which in turn, strongly influenced the relative amount of consumed substrates and the ability of microbial communities to metabolize different substrates. In a causal-effect cascade, trees exert a strong direct effect over pH, nutrients and SOM, which, in turn influencing variables directly related to microbial functioning. In particular, and according to SEMs and Chapter 2 results, the quantity of SOM, which in our study was strongly influenced by the tree and is usually strongly correlated with higher microbial metabolism (Gomez et al., 2004; Frąc et al., 2012), appeared to be indirectly related with bacterial metabolism through increasing moisture availability, among other variables.

On the other hand, the direct effect of agricultural matrix over the soil microbial communities drawn by SEMs (i.e. fungal structural Shannon and both bacterial and fungal assemblages) suggests that there are other factors, besides soil nutrients, not measured in this study, influencing the species composition of soil microbial communities. One possibility is that the agricultural matrix is influencing the quality of soil substrates (e.g. configuration of humic molecules, presence of secondary metabolites)

inducing changes in the microbial community (Asensio et al., 2012), as suggested in our study by the influence of the agricultural matrix on the microbial preference for the consumption of determinate substrates.

SEMs also showed that in this cascade of causal-effect relations the paths controlling the capacity of bacterial and fungal communities to metabolize different substrates markedly differed. For instance, the capacity of fungi to metabolize different substrates was strongly and directly influenced by the tree, with no apparent influence of the structure of the fungal community. These results point out to the strong link of fungi with trees, as organisms that have co-evolved with plants in the colonization of terrestrial ecosystems (Boer et al., 2005) and have developed the enzymatic machinery able to degrade the complex vegetal molecules (Kohler et al., 2015). However, it is important to consider limitation issues when using EcoPlates, particularly in the case of fungi, although it is still a largely accepted and useful tool to explore the potential activity of microbial communities (Gomez et al., 2004).

Regarding bacterial community ecology, SEMs and Mantel test clearly showed how bacterial functioning was related to their community structure (alpha-diversity and community assemblage). The relationship between microbial structure and their metabolism is leastways highly complex (O'Donnell et al., 2001; Mendes et al., 2015), hence it has so far presented conflicting results (Griffiths et al., 2000; O'Donnell et al., 2001; Bell et al., 2005; Langenheder et al., 2010; Levine et al., 2011; Curiel Yuste et al., 2014; Tardy et al., 2014; Mendes et al., 2015). However, the relationship between bacterial structure and their metabolism found here and in others studies (Bell et al., 2005; Tardy et al., 2014), suggest that environmental alterations, such as those derived from forest fragmentation, which produces changes on the microbial structure could lead to decreases on their overall functioning.

## Conclusions

Our study reinforces our knowledge about the important alterations that forest fragmentation exerts on tree-soil-microbial interactions, as presented in Chapter 2. Forest fragmentation positively affects soil fertility and tree growth and productivity (SOM accumulation), inducing changes in both abiotic (moisture and pH) and biotic (higher microbial alpha-diversity) factors that ultimately improve the conditions for microbial metabolism at local scale. However, forest fragmentation and tree cover tend to homogenize the microbial community structure and their metabolism (lower microbial beta-diversity) at landscape scale with potential negative consequences on the capacity of these soils to respond to the climate change. Collectively, our results suggest that forest fragmentation has a deep effect on microbial diversity and function through direct and indirect ways, affecting the functioning of the plant-soil-microbial system and the cycling of nutrients.

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**Table S3.1** Characteristics of soils collected in holm oak fragmented forests in Spain, included in the structural equation models. Data are mean ± standard error. The factor "canopy cover" strongly affected all variables with significant highest values under canopy except in the case of pH. One-way ANOVA testing the "matrix influence" factor within each "canopy cover" level is also presented (extracted from Chapter 2).

| | **Under canopy** | | | | **Open areas** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Forest interior** | **Forest edge** | **Small fragments** | **ANOVA** | **Forest interior** | **Forest edge** | **Small fragments** | **ANOVA** |
| Tree influence index (*Tii*) | 318.1 ± 3.1 | 603.3 ± 4.2 | 572.6 ± 4.9 | **$F_{2,42}$=5.08 $p$=0.01** | 109.1 ± 1.8 | 239.2 ± 2.8 | 219.0 ± 3.1 | **$F_{2,42}$=5.80 $p$=0.01** |
| Soil organic matter (%) | 7.7 ± 0.5 | 9.9 ± 0.5 | 16.1 ± 1.0 | $F_{2,42}$=1.81 $p$=0.18 | 3.5 ± 0.3 | 4.4 ± 0.4 | 4.7 ± 0.5 | $F_{2,42}$=0.89 $p$=0.42 |
| Soil moisture (%) | 13.4 ± 0.5 | 15.5 ± 0.7 | 19.0 ± 0.9 | $F_{2,42}$=1.27 $p$=0.29 | 7.2 ± 0.3 | 10.3 ± 0.5 | 8.4 ± 0.4 | **$F_{2,42}$=4.93 $p$=0.01** |
| pH | 8.0 ± 0.1 | 8.0 ± 0.1 | 7.9 ± 0.1 | $F_{2,42}$=0.75 $p$=0.48 | 8.2 ± 0.1 | 8.2 ± 0.1 | 8.2 ± 0.1 | $F_{2,42}$=1.19 $p$=0.31 |
| C:N | 10.4 ± 0.5 | 9.0 ± 0.3 | 9.5 ± 0.4 | $F_{2,42}$=0.98 $p$=0.38 | 9.9 ± 0.5 | 9.3 ± 0.4 | 9.7 ± 0.5 | $F_{2,42}$=0.15 $p$=0.86 |

**Table S3.2** Response of bacterial consumption of individual substrates to tree canopy coverage (*C*), agricultural matrix influence (MI), and its interaction (C x MI), in soil from holm oak forest fragments in Spain. Significant effects are noted in bold. The effect of factors is summarized on the left of the table.

| | | Factorial ANOVA | | | | | |
|---|---|---|---|---|---|---|---|
| | | *C* | | *MI* | | *C x MI* | |
| | | *F* | *P* | *F* | *P* | *F* | *P* |
| | 2-Hydroxy benzoic acid | 2.34 | 0.130 | 0.44 | 0.648 | 0.40 | 0.675 |
| | Gamma-hydroxybutyric acid | 1.27 | 0.264 | 0.91 | 0.408 | 2.24 | 0.112 |
| | Alpha-ketobutyric acid | 2.62 | 0.109 | 1.14 | 0.325 | 0.87 | 0.422 |
| | D-malic acid | 0.80 | 0.374 | 0.09 | 0.915 | 1.23 | 0.296 |
| | L-phenylalanine | 2.26 | 0.137 | 0.14 | 0.869 | 0.38 | 0.683 |
| Coverage | Alpha-cyclodextrin | **11.65** | **<0.001** | 2.79 | 0.067 | 2.25 | 0.112 |
| Coverage | Glycogen | **11.83** | **<0.001** | 1.21 | 0.305 | 0.58 | 0.561 |
| Coverage | Alpha-D-lactose | **6.28** | **0.014** | 2.10 | 0.129 | 2.12 | 0.127 |
| Coverage | D-xylose | **10.06** | **0.002** | 2.80 | 0.066 | 2.06 | 0.134 |
| Coverage | D-mannitol | **10.98** | **<0.001** | 1.79 | 0.173 | 0.77 | 0.466 |
| Coverage | D-galactonic acid-gamma-lactone | **13.53** | **<0.001** | 1.14 | 0.325 | 0.05 | 0.955 |
| Coverage | Itaconic acid | **74.92** | **<0.001** | 1.62 | 0.204 | 1.62 | 0.205 |
| Coverage | L-threonine | **6.31** | **0.014** | 0.82 | 0.444 | 0.56 | 0.574 |
| Coverage | Phenylethylamine | **61.21** | **<0.001** | 0.56 | 0.571 | 0.51 | 0.601 |
| Coverage + Matrix influence | Tween 40 | **33.54** | **<0.001** | **3.66** | **0.030** | 1.89 | 0.158 |
| Coverage + Matrix influence | Tween 80 | **59.11** | **<0.001** | **6.15** | **0.003** | 0.76 | 0.471 |
| Coverage + Matrix influence | D-cellobiose | **29.95** | **<0.001** | **4.76** | **0.011** | 0.30 | 0.744 |
| Coverage + Matrix influence | Beta-methyl-D-glucoside | **32.39** | **<0.001** | **5.91** | **0.004** | 0.47 | 0.629 |
| Coverage + Matrix influence | i-erythritol | **27.73** | **<0.001** | **7.55** | **<0.001** | 1.63 | 0.201 |
| Coverage + Matrix influence | N-acetyl-D-glucosamine | **25.07** | **<0.001** | **4.60** | **0.013** | 0.18 | 0.837 |
| Coverage + Matrix influence | D-glucosaminic acid | **22.45** | **<0.001** | **3.27** | **0.043** | 0.10 | 0.906 |
| Coverage + Matrix influence | Glucose-1-phosphate | **23.35** | **<0.001** | **6.11** | **0.003** | 0.16 | 0.849 |
| Coverage + Matrix influence | D,L-alpha-glycerol phosphate | **7.92** | **0.006** | **5.72** | **0.005** | 0.27 | 0.765 |
| Coverage + Matrix influence | D-galacturonic acid | **44.92** | **<0.001** | **6.33** | **0.003** | 2.64 | 0.077 |
| Coverage + Matrix influence | 4-Hydroxy benzoic acid | **46.01** | **<0.001** | **3.96** | **0.023** | 1.51 | 0.227 |
| Coverage + Matrix influence | L-arginine | **46.72** | **<0.001** | **3.80** | **0.026** | 1.19 | 0.310 |
| Coverage + Matrix influence | L-asparagine | **45.91** | **<0.001** | **6.63** | **0.002** | 0.24 | 0.785 |
| Coverage + Matrix influence | L-serine | **58.23** | **<0.001** | **6.18** | **0.003** | 0.24 | 0.786 |
| Coverage + Matrix influence | Putrescine | **40.46** | **<0.001** | **4.92** | **0.010** | 0.11 | 0.896 |
| **C x MI** | Pyruvic acid methyl ester | **8.57** | **0.004** | **5.13** | **0.008** | **3.32** | **0.041** |
| **C x MI** | Glycyl-L-glutamic acid | **36.73** | **<0.001** | **10.21** | **<0.001** | **6.36** | **0.003** |

**Table S3.3** Response of fungal consumption of individual substrates to tree canopy coverage (*C*), agricultural matrix influence (MI), and its interaction (C x MI), in soil from holm oak forest fragments in Spain. Significant effects are noted in bold. The effect of factors is summarized on the left of the table.

| | | Factorial ANOVA | | | | | |
|---|---|---|---|---|---|---|---|
| | | *C* | | *MI* | | *C x MI* | |
| | | *F* | *P* | *F* | *P* | *F* | *P* |
| | Pyruvic acid methyl ester | 2.78 | 0.099 | 0.84 | 0.436 | 0.29 | 0.751 |
| | Alpha-cyclodextrin | 1.8 | 0.184 | 1.14 | 0.325 | 0.11 | 0.896 |
| | D-glucosaminic acid | 0.3 | 0.585 | 1.71 | 0.188 | 1.11 | 0.335 |
| | D,L-alpha-glycerol phosphate | 0.16 | 0.691 | 1.02 | 0.365 | 1.08 | 0.345 |
| | D-galactonic acid-gamma-lactone | 1.45 | 0.232 | 0.33 | 0.720 | 0.54 | 0.585 |
| | 4-Hydroxy benzoic acid | 2.08 | 0.153 | 0.34 | 0.713 | 0.06 | 0.942 |
| | Itaconic acid | 0.29 | 0.591 | 2.32 | 0.104 | 0.52 | 0.599 |
| | Alpha-ketobutyric acid | 3.02 | 0.086 | 0.72 | 0.492 | 1.43 | 0.245 |
| | L-phenylalanine | 1.54 | 0.217 | 0.22 | 0.802 | 1.24 | 0.295 |
| | Phenylethylamine | 0.57 | 0.453 | 2.40 | 0.097 | 0.68 | 0.509 |
| Coverage | Tween 40 | **24.8** | **<0.001** | 0.56 | 0.575 | 0.78 | 0.461 |
| Coverage | Tween 80 | **25.4** | **<0.001** | 0.78 | 0.460 | 0.47 | 0.628 |
| Coverage | Glycogen | **26.7** | **<0.001** | 1.72 | 0.186 | 0.56 | 0.573 |
| Coverage | D-cellobiose | **39.7** | **<0.001** | 2.92 | 0.059 | 0.55 | 0.576 |
| Coverage | Beta-methyl-D-glucoside | **98.1** | **<0.001** | 1.37 | 0.259 | 1.12 | 0.331 |
| Coverage | D-xylose | **44.8** | **<0.001** | 1.18 | 0.311 | 2.66 | 0.076 |
| Coverage | D-mannitol | **19.8** | **<0.001** | 0.54 | 0.584 | 0.73 | 0.485 |
| Coverage | N-acetyl-D-glucosamine | **35.1** | **<0.001** | 1.18 | 0.312 | 0.22 | 0.807 |
| Coverage | Glucose-1-phosphate | **5.14** | **0.03** | 2.14 | 0.124 | 0.80 | 0.454 |
| Coverage | D-galacturonic acid | **27.4** | **<0.001** | 1.07 | 0.349 | 1.14 | 0.326 |
| Coverage | 2-Hydroxy benzoic acid | **17.9** | **<0.001** | 1.79 | 0.173 | 0.52 | 0.597 |
| Coverage | D-malic acid | **9.82** | **<0.001** | 0.72 | 0.491 | 0.12 | 0.890 |
| Coverage | L-arginine | **14.8** | **<0.001** | 1.00 | 0.374 | 0.15 | 0.864 |
| Coverage | L-asparagine | **19.3** | **<0.001** | 0.36 | 0.701 | 0.26 | 0.771 |
| Coverage | L-serine | **36.1** | **<0.001** | 0.15 | 0.859 | 0.79 | 0.457 |
| Coverage | Putrescine | **4.08** | **0.047** | 0.05 | 0.954 | 0.18 | 0.834 |
| Matrix influence | Alpha-D-lactose | **13.4** | **<0.001** | **3.40** | **0.038** | 1.95 | 0.149 |
| Matrix influence | i-erythritol | **73.2** | **<0.001** | **4.33** | **0.016** | 0.68 | 0.507 |
| Matrix influence | Glycyl-L-glutamic acid | **21.7** | **<0.001** | **4.14** | **0.019** | 1.50 | 0.228 |
| Matrix influence | L-threonine | 3.45 | 0.067 | **3.21** | **0.045** | 0.00 | 0.997 |
| C x MI | Gamma-hydroxybutyric acid | **11.1** | **<0.001** | **4.53** | **0.013** | **3.17** | **0.047** |

**Table S3.4** Pearson's correlations of structural and functional components of bacterial and fungal communities and characteristics of soil collected in open areas or under canopy of fragmented holm oak forests in Spain. Significant effects are noted in bold.

| | Bacterial richness (*S*) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) | (13) | (14) | (15) | (16) | (17) | (18) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (2) Bacterial structural Shannon (*H*) | **0.84** | | | | | | | | | | | | | | | | | |
| (3) Bacterial structural evenness ($E_S$) | 0.02 | **0.55** | | | | | | | | | | | | | | | | |
| (4) Fungal structural richness (*S*) | 0.001 | 0.01 | 0.001 | | | | | | | | | | | | | | | |
| (5) Fungal structural Shannon (*H*) | 0.13 | 0.07 | -0.08 | **0.72** | | | | | | | | | | | | | | |
| (6) Fungal structural evenness ($E_S$) | 0.18 | 0.09 | -0.12 | 0.20 | **0.82** | | | | | | | | | | | | | |
| (7) Bacterial functional richness (*SS*) | 0.20 | 0.15 | -0.04 | -0.03 | 0.01 | 0.04 | | | | | | | | | | | | |
| (8) Bacterial functional Shannon (*SH*) | **0.23** | **0.26** | 0.14 | -0.12 | -0.01 | 0.09 | **0.52** | | | | | | | | | | | |
| (9) Bacterial functional evenness ($SE_S$) | 0.19 | **0.24** | 0.17 | -0.11 | -0.01 | 0.08 | **0.25** | **0.95** | | | | | | | | | | |
| (10) Fungal functional richness (*SS*) | **0.49** | **0.40** | -0.03 | -0.03 | 0.05 | 0.09 | **0.34** | **0.57** | **0.52** | | | | | | | | | |
| (11) Fungal functional Shannon (*SH*) | **0.48** | **0.41** | 0.01 | -0.02 | 0.01 | 0.03 | **0.29** | **0.56** | **0.53** | **0.95** | | | | | | | | |
| (12) Fungal functional evenness ($SE_S$) | 0.18 | **0.21** | 0.13 | -0.04 | -0.14 | -0.16 | -0.01 | **0.25** | **0.27** | **0.26** | **0.5** | | | | | | | |
| (13) Tree influence index (*Tii*) | **0.30** | **0.25** | 0.00 | -0.19 | -0.05 | 0.09 | **0.45** | **0.48** | **0.39** | **0.47** | **0.49** | **0.34** | | | | | | |
| (14) Soil organic matter (%) | **0.22** | 0.06 | -0.19 | 0.00 | 0.02 | 0.04 | **0.46** | **0.62** | **0.55** | **0.54** | **0.57** | **0.41** | **0.43** | | | | | |
| (15) Soil moisture (%) | 0.07 | -0.03 | -0.12 | -0.07 | 0.07 | 0.16 | **0.42** | **0.66** | **0.6** | **0.41** | **0.43** | **0.33** | **0.43** | **0.83** | | | | |
| (16) Soil pH | **-0.26** | -0.20 | 0.01 | -0.13 | -0.05 | 0.03 | **-0.28** | **-0.5** | **-0.46** | **-0.47** | **-0.5** | **-0.37** | -0.16 | **-0.74** | **-0.56** | | | |
| (17) C:N ratio | -0.09 | -0.16 | -0.16 | **0.21** | 0.18 | 0.09 | -0.02 | -0.03 | -0.03 | -0.08 | -0.08 | 0.01 | 0.02 | **0.25** | 0.15 | -0.06 | | |
| (18) PC1 of nutrients PCA | **0.54** | **0.44** | -0.01 | **-0.34** | -0.07 | 0.18 | **0.25** | 0.19 | 0.11 | **0.45** | **0.42** | 0.13 | **0.37** | **0.23** | 0.07 | -0.10 | -0.18 | |
| PC2 of nutrients PCA | 0.11 | 0.01 | -0.12 | 0.13 | 0.08 | 0.01 | **0.43** | **0.64** | **0.58** | **0.49** | **0.51** | **0.34** | **0.32** | **0.90** | **0.76** | **-0.71** | 0.10 | -0.07 |

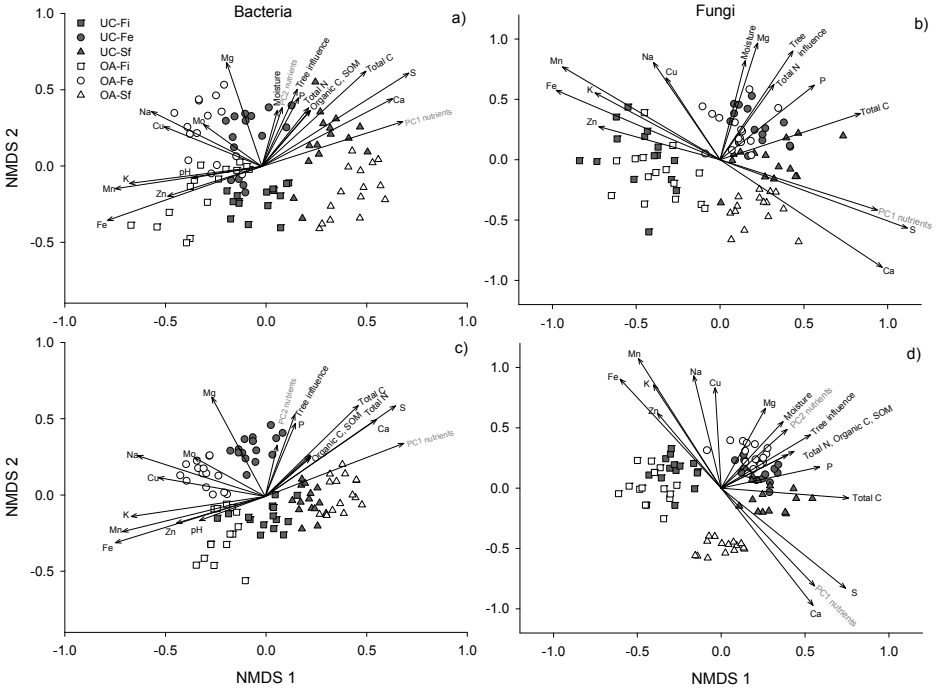



**Figure S3.1** Assemblage of bacterial (a,c) and fungal (b,d) communities, plotted by Non-metric multidimensional scaling (NMDS) using quantitative (abundance) (a-b) or qualitative (presence/absence) (c-d) datasets, of soils collected at three agricultural matrix influence levels, under canopy or in open areas, in fragmented holm oak forests in Spain. Vector length and direction respectively represent the relative magnitude of explained variation and that of a positive increase. See probability values in Table S3.6. Only significant vectors are showed, $p < 0.05$. Coverage is represented by different colors: gray = under canopy (UC); white = open areas (OA). Soil provenances are represented by different symbols: squares = forest interior of large fragments (Fi); circles = forest edge of large fragments (Fe); triangles = small fragments (Sf).

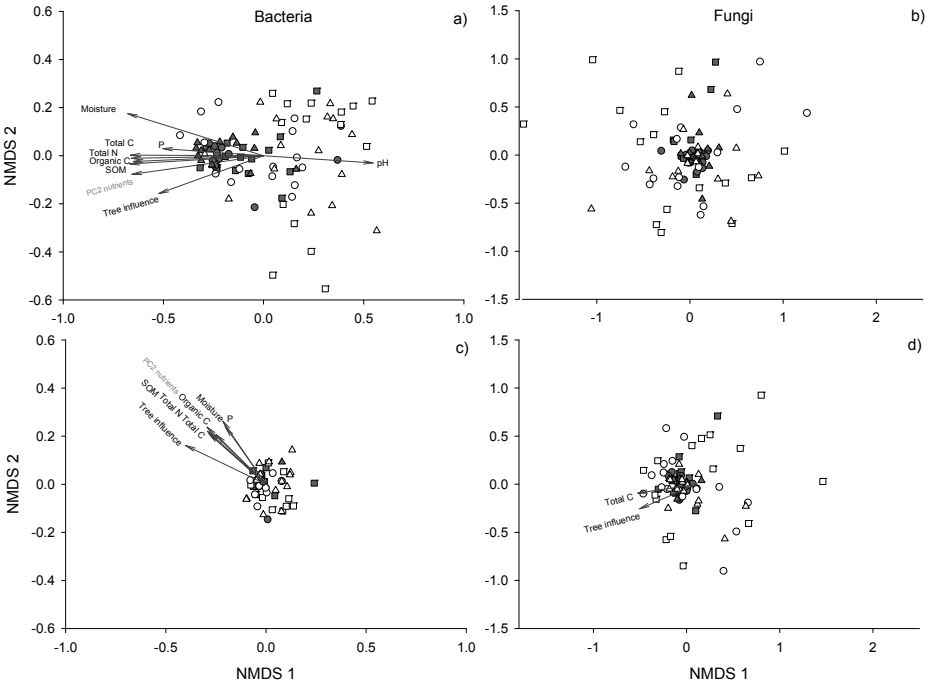



**Figure S3.2** Metabolic profile of bacterial (a,c) and fungal (b,d) communities, plotted by Non-metric multidimensional scaling (NMDS) using quantitative (abundance) (a-b) or qualitative (presence/absence) (c-d) datasets, of soils collected at three agricultural matrix influence levels, under canopy or in open areas, in fragmented holm oak forests in Spain. Vector length and direction, respectively, represent the relative magnitude of explained variation and that of a positive increase. See probability values in Table S3.7. Only significant vectors are showed, $p < 0.05$. Coverage is represented by different colors: gray = under canopy (UC); white = open areas (OA). Soil provenances are represented by different symbols: squares = forest interior of large fragments (Fi); circles = forest edge of large fragments (Fe); triangles = small fragments (Sf).

**Table S3.5** Non-parametric multivariate analysis of variance using both quantitative (abundance) and qualitative (presence/absence) datasets, of soil microbial community assemblage and metabolic profile of holm oak forest fragments in Spain. Significant effects are noted in bold. MI = Matrix influence (forest interior, forest edge, small fragments); C = coverage

| | | ***Quantitative data set*** | | | | | | | | ***Qualitative data set*** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Two-way** | | | **One-way** | | | | | **Two-way** | | | **One-way** | | | | |
| | | | | | **Under canopy** | **Open areas** | **Forest interior** | **Forest edge** | **Small** | | | | **Under canopy** | **Open areas** | **Forest interior** | **Forest edge** | **Small** |
| | | **C** | **MI** | **C x MI** | **MI** | **MI** | **C** | **C** | **C** | **C** | **MI** | **C x MI** | **MI** | **MI** | **C** | **C** | **C** |
| | | *n*=90 | *n*=90 | *n*=90 | *n*=45 | *n*=45 | *n*=30 | *n*=30 | *n*=30 | *n*=90 | *n*=90 | *n*=90 | *n*=45 | *n*=45 | *n*=30 | *n*=30 | *n*=30 |
| Bacterial assemblage | *F* | **4.62** | **14.21** | **5.11** | **8.36** | **11** | **6.37** | **4.25** | **4.38** | **11.05** | **130.49** | **15.03** | **20.64** | **24.77** | **17.68** | **9.53** | **13.49** |
| | *P* | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | $R^2$ | **0.04** | **0.22** | **0.08** | **0.29** | **0.34** | **0.19** | **0.13** | **0.14** | **0.06** | **0.33** | **0.16** | **0.50** | **0.54** | **0.39** | **0.25** | **0.33** |
| Fungal assemblage | *F* | **6.02** | **9.31** | **3.82** | **5.69** | **7.52** | **3.87** | **3.52** | **6.59** | **14.93** | **29.24** | **9.81** | **14.29** | **25.69** | **10.12** | **7.73** | **15.98** |
| | *P* | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | $R^2$ | **0.05** | **0.16** | **0.07** | **0.21** | **0.26** | **0.12** | **0.11** | **0.19** | **0.08** | **0.33** | **0.11** | **0.41** | **0.55** | **0.27** | **0.22** | **0.36** |
| Bacterial metabolic profile | *F* | **15.89** | **2.66** | **2.20** | 1.77 | **2.79** | **7.42** | 1.87 | **10.71** | 1.49 | 1.95 | 1.95 | 0.84 | **2.35** | **3.01** | -0.745 | 1.41 |
| | *P* | **<0.001** | **0.006** | **0.033** | 0.068 | **<0.001** | **<0.001** | 0.101 | **<0.001** | 0.24 | 0.10 | 0.08 | 0.57 | **0.028** | **0.02** | 0.99 | 0.245 |
| | $R^2$ | **0.15** | **0.05** | **0.04** | 0.08 | **0.12** | **0.21** | 0.06 | **0.28** | 0.02 | 0.04 | 0.04 | 0.04 | **0.10** | **0.10** | -0.03 | 0.05 |
| Fungal metabolic profile | *F* | **5.64** | 1.16 | 0.699 | 1.022 | 0.904 | **2.309** | **2.744** | **1.884** | **5.69** | 1.42 | 0.97 | 1.27 | 1.18 | **2.84** | 1.79 | **3.0** |
| | *P* | **<0.001** | 0.293 | 0.862 | 0.436 | 0.616 | **0.002** | **0.003** | **0.019** | **<0.001** | 0.185 | 0.489 | 0.253 | 0.29 | **0.005** | 0.115 | **<0.001** |
| | $R^2$ | **0.06** | 0.03 | 0.001 | 0.05 | 0.04 | **0.08** | **0.09** | **0.06** | **0.06** | 0.03 | 0.02 | 0.06 | 0.05 | **0.09** | 0.06 | **0.10** |

**Table S3.6** Probability values from permutation test (envfit in vegan R-package) fitted on microbial assemblage (see Fig. S3.1). Values indicate results from 10,000 permutations.

| | ***Quantitative*** | | | | ***Qualitative*** | | | |
|---|---|---|---|---|---|---|---|---|
| | Bacterial assemblage | | Fungal assemblage | | Bacterial assemblage | | Fungal assemblage | |
| | $R^2$ | *p*-value | $R^2$ | *p*-value | $R^2$ | *p*-value | $R^2$ | *p*-value |
| Tree influence index | **0.18** | **<0.001** | **0.15** | **<0.001** | **0.194** | **<0.001** | **0.241** | **<0.001** |
| Soil organic matter (%) | **0.12** | **0.00** | 0.06 | 0.05 | **0.078** | **0.026** | **0.132** | **<0.001** |
| Soil moisture (%) | **0.09** | **0.02** | **0.08** | **0.02** | 0.032 | 0.252 | **0.163** | **<0.001** |
| pH | **0.08** | **0.03** | 0.02 | 0.38 | **0.084** | **0.021** | 0.060 | 0.069 |
| Ratio C:N | 0.00 | 0.94 | 0.01 | 0.61 | 0.008 | 0.708 | 0.011 | 0.607 |
| PC1 nutrients | **0.37** | **<0.001** | **0.32** | **<0.001** | **0.379** | **<0.001** | **0.365** | **<0.001** |
| PC2 nutrients | **0.10** | **0.01** | 0.06 | 0.05 | **0.073** | **0.038** | **0.161** | **<0.001** |
| Organic C | **0.12** | **0.00** | 0.06 | 0.06 | **0.072** | **0.036** | **0.127** | **0.002** |
| Total C | **0.42** | **<0.001** | **0.26** | **<0.001** | **0.359** | **<0.001** | **0.402** | **<0.001** |
| Total N | **0.13** | **0.00** | **0.08** | **0.03** | **0.076** | **0.031** | **0.152** | **<0.001** |
| P | **0.15** | **<0.001** | **0.15** | **<0.001** | **0.158** | **<0.001** | **0.246** | **<0.001** |
| K | **0.29** | **<0.001** | **0.22** | **<0.001** | **0.294** | **<0.001** | **0.279** | **<0.001** |
| Ca | **0.39** | **<0.001** | **0.41** | **<0.001** | **0.354** | **<0.001** | **0.422** | **<0.001** |
| Mg | **0.31** | **<0.001** | **0.12** | **0.00** | **0.308** | **<0.001** | **0.148** | **<0.001** |
| Cu | **0.19** | **<0.001** | **0.09** | **0.02** | **0.190** | **<0.001** | **0.158** | **<0.001** |
| Fe | **0.45** | **<0.001** | **0.36** | **<0.001** | **0.412** | **<0.001** | **0.437** | **<0.001** |
| Mn | **0.35** | **<0.001** | **0.37** | **<0.001** | **0.355** | **<0.001** | **0.431** | **<0.001** |
| Na | **0.27** | **<0.001** | **0.13** | **0.00** | **0.302** | **<0.001** | **0.215** | **<0.001** |
| S | **0.58** | **<0.001** | **0.46** | **<0.001** | **0.489** | **<0.001** | **0.538** | **<0.001** |
| Mo | **0.10** | **0.01** | 0.02 | 0.49 | **0.121** | **0.004** | 0.057 | 0.079 |
| Zn | **0.16** | **<0.001** | **0.19** | **<0.001** | **0.144** | **<0.001** | **0.188** | **<0.001** |

**Table S3.7** Probability values from permutation test (envfit in vegan R-package) fitted on metabolic profile (see Fig. S3.2). Values indicate results from 10,000 permutations.

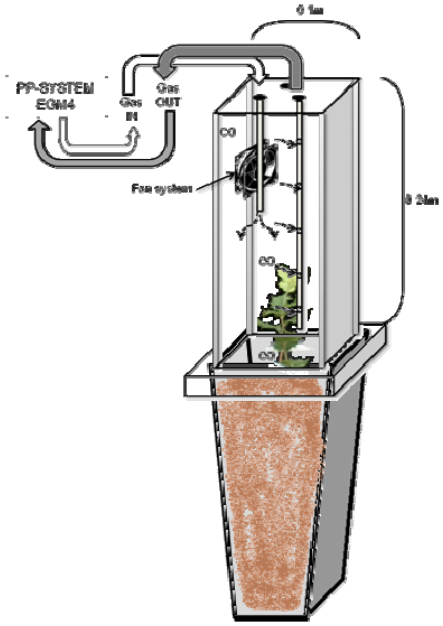
| | Quantitative | | | | Qualitative | | | |
|---|---|---|---|---|---|---|---|---|
| | Bacterial metabolic profile | | Fungal metabolic profile | | Bacterial metabolic profile | | Fungal metabolic profile | |
| | $R^2$ | *p*-value | $R^2$ | *p*-value | $R^2$ | *p*-value | $R^2$ | *p*-value |
| Tree influence index | **0.20** | **<0.001** | 0.03 | 0.22 | **0.129** | **0.002** | **0.073** | **0.036** |
| Soil organic matter (%) | **0.29** | **<0.001** | 0.02 | 0.41 | **0.104** | **0.015** | 0.044 | 0.143 |
| Soil moisture (%) | **0.33** | **<0.001** | 0.01 | 0.56 | **0.099** | **0.014** | 0.022 | 0.390 |
| pH | **0.19** | **<0.001** | 0.01 | 0.75 | 0.063 | 0.059 | 0.026 | 0.333 |
| Ratio C:N | 0.01 | 0.80 | 0.00 | 0.94 | 0.008 | 0.707 | 0.047 | 0.122 |
| PC1 nutrients | 0.03 | 0.23 | 0.06 | 0.08 | **0.060** | **0.073** | 0.054 | 0.089 |
| PC2 nutrients | **0.28** | **<0.001** | 0.02 | 0.49 | **0.082** | **0.025** | 0.053 | 0.100 |
| Organic C | **0.27** | **<0.001** | 0.02 | 0.43 | **0.096** | **0.019** | 0.040 | 0.175 |
| Total C | **0.28** | **<0.001** | 0.04 | 0.15 | **0.112** | **0.006** | **0.072** | **0.040** |
| Total N | **0.28** | **<0.001** | 0.02 | 0.45 | **0.098** | **0.017** | 0.042 | 0.158 |
| P | **0.16** | **<0.001** | 0.03 | 0.26 | **0.083** | **0.026** | 0.042 | 0.152 |
| K | 0.00 | 0.89 | 0.06 | 0.06 | 0.010 | 0.644 | 0.038 | 0.184 |
| Ca | 0.01 | 0.77 | 0.02 | 0.37 | 0.000 | 0.979 | 0.018 | 0.460 |
| Mg | 0.02 | 0.46 | 0.00 | 0.94 | 0.005 | 0.804 | 0.004 | 0.842 |
| Cu | 0.03 | 0.25 | 0.01 | 0.82 | 0.007 | 0.745 | 0.027 | 0.299 |
| Fe | 0.02 | 0.45 | 0.04 | 0.15 | 0.016 | 0.514 | 0.031 | 0.257 |
| Mn | 0.00 | 0.92 | 0.05 | 0.10 | 0.002 | 0.933 | 0.029 | 0.278 |
| Na | 0.00 | 0.94 | 0.02 | 0.45 | 0.011 | 0.634 | 0.014 | 0.553 |
| S | 0.03 | 0.28 | 0.04 | 0.17 | 0.021 | 0.414 | 0.041 | 0.159 |
| Mo | 0.00 | 0.83 | 0.01 | 0.64 | 0.008 | 0.716 | 0.006 | 0.774 |
| Zn | 0.02 | 0.45 | 0.02 | 0.53 | 0.023 | 0.354 | 0.017 | 0.462 |

**Table S3.8** Squared multiple correlations for the structural equation models.

| | **Estimate** |
|---|---|
| Tree influence index (*Tii*) | 0.06 |
| Soil organic matter (%) | 0.22 |
| Soil moisture (%) | 0.64 |
| pH | 0.62 |
| PC1 nutrients of the PCA | 0.45 |
| Bacterial alpha-diversity | 0.25 |
| Bacterial assemblage | 0.26 |
| Fungal alpha-diversity | 0.13 |
| Fungal assemblage | 0.17 |
| *Bacterial metabolic alpha-diversity* | 0.60 |
| *Bacterial metabolic profile* | 0.42 |
| *Fungal metabolic alpha-diversity* | 0.52 |
| *Fungal metabolic profile* | 0.55 |

# Capítulo 4

## Habitat fragmentation can modulate drought effects on the plant-soil-microbial system in Mediterranean holm oak (*Quercus ilex*) forests

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Microbial Ecology 69(4): 798-812 (2015)

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The chapter IV of the thesis: FLORES-RENTERÍA, Dulce. Effects of forest fragmentation on the plant-soil-microbial interactions = Efectos de la fragmentación del encinar en las interacciones planta-suelo-microorganismos (2016), is published in: Flores-Rentería, D., J. Curiel Yuste, A. Rincón, F. Brearley, J. García-Gil and F. Valladares (2015). Habitat Fragmentation can Modulate Drought Effects on the Plant-soil-microbial System in Mediterranean Holm Oak (*Quercus ilex*) Forests. Microbial Ecology 69(4): 798-812 (2015). doi: 10.1007/s00248-015-0584-9. http://hdl.handle.net/10261/128289

El capítulo IV de la tesis doctoral: FLORES-RENTERÍA, Dulce. Efectos de la fragmentación del encinar en las interacciones planta-suelo-microorganismos = Effects of forest fragmentation on the plant-soil-microbial interactions (2016, está publicado en: Flores-Rentería, D., J. Curiel Yuste, A. Rincón, F. Brearley, J. García-Gil and F. Valladares (2015). Habitat Fragmentation can Modulate Drought Effects on the Plant-soil-microbial System in Mediterranean Holm Oak (*Quercus ilex*) Forests. Microbial Ecology 69(4): 798-812 (2015). doi: 10.1007/s00248-015-0584-9. http://hdl.handle.net/10261/128289

## Abstract

Ecological transformations derived from habitat fragmentation have led to increased threats to above-ground biodiversity. However, the impacts of forest fragmentation on soils and their microbial communities are not well understood. We examined the effects of contrasting fragment sizes on the structure and functioning of soil microbial communities from holm oak forest patches in two bio-climatically different regions of Spain. We used a microcosm approach to simulate the annual summer drought cycle and first autumn rainfall (rewetting), evaluating the functional response of a plant-soil-microbial system. Forest fragment size had a significant effect on physicochemical characteristics and microbial functioning of soils, although the diversity and structure of microbial communities were not affected. The response of our plant-soil-microbial systems to drought was strongly modulated by the bioclimatic conditions and the fragment size from where the soils were obtained. Decreasing fragment size modulated the effects of drought by improving local environmental conditions with higher water and nutrient availability. However this modulation was stronger for plant-soil-microbial systems built with soils from the northern region (colder and wetter) than for those built with soils from the southern region (warmer and drier) suggesting that the responsiveness of the soil-plant-microbial system to habitat fragmentation was strongly dependent on both the physicochemical characteristics of soils and the historical adaptation of soil microbial communities to specific bioclimatic conditions. This interaction challenges our understanding of future global change scenarios in Mediterranean ecosystems involving drier conditions and increased frequency of forest fragmentation.

## Introduction

The impacts of global change disturbances are directly responsible for increased rates of biodiversity loss, which are altering the functioning of ecosystems and affecting important ecosystem services (IPCC, 2007). In the Mediterranean basin, forest fragmentation, resource overexploitation and poor management are the main drivers of forest degradation, and their impacts are expected to be aggravated by climate change (Valladares et al., 2014). Habitat fragmentation negatively affects population size and/or diversity of organisms in large habitats (reviewed by Didham et al. 1996, Fischer and Lindenmayer 2007), although it is still not clear if it has a consistent effect on biodiversity loss (Fahrig, 2003), since neutral (e.g. microorganisms; Rantalainen et al. 2008) or even positive effects (Dooley and Bowers, 1998; Díaz et al., 1999) have also been reported. It is well known that consequences of habitat fragmentation are strongly dependent upon the size of the remaining area (Fernández et al., 2002; Lindenmayer and Fischer, 2006), because of the complex processes related to edge effects (Fernández et al., 2002), and resource constraints in smaller fragments (Zanette et al., 2000). Most Mediterranean ecosystems have historically suffered major transitions involving fragmentation due to agricultural practices that transform the landscape dominated by forest and shrublands into isolated patches (Valladares et al., 2004), in a process that is expected to become more frequent over the next century (Millennium-Ecosystem-Assessment, 2005). Evidence of fragmentation effects in Mediterranean ecosystems has been reported for a range of different organisms such as birds, butterflies, plants and microorganisms, and includes the disruption of biotic interactions such as pollination, seed dispersal and herbivory (Santos and Tellería, 1998; Díaz et al., 1999; Díaz and Alonso, 2003; Lázaro-Nogal et al., 2012; Valladares et al., 2014).

The global climate is expected to change rapidly and deeply over the next century (IPCC, 2007). Particularly in the Mediterranean basin, an increase in temperature of 1.8 °C is predicted for the next 40 years, coupled

with a reduction in rainfall frequency of 5-10% with more intense droughts (IPCC, 2007). The functioning of Mediterranean ecosystems is largely governed by the soil water regime (Rambal et al., 2003; Barba et al., 2013) and reductions in soil water are very likely to cause a concomitant reduction in carbon and water fluxes (Orchard and Cook, 1983; Reichstein et al., 2002; Barba et al., 2013). The increased intensity of droughts in recent decades has led to the reduction of tree productivity in the Mediterranean Basin (Ogaya and Peñuelas, 2004; Barba et al., 2013), and to forest decline in some areas (Hereş et al., 2012). These global changes directly affect plant communities, but also simultaneously and interactively affect the associated belowground microorganisms (Castro et al., 2010). The changes in precipitation patterns can also affect soil nutrients and carbon cycling by impacting upon the activity of microbial communities (Barnard et al., 2013), although a considerable debate about how water stress affects soil microbial communities and their overall activity still exists (Williams, 2007; Castro et al., 2010; Treseder et al., 2012). Relative shifts in soil microbial communities depends on their different inherent resistances to drought (Schimel et al., 2007), with soil bacterial community generally considered more sensitive than the fungal community (Drenovsky et al., 2004; Williams, 2007; Castro et al., 2010; Curiel Yuste et al., 2011; Barnard et al., 2013).

Soils and their microorganisms are essential for the performance and regulation of global biogeochemical cycles (Wardle et al., 2004; de Vries et al., 2012; Bahn et al., 2013); their activity is controlled by both biotic and abiotic factors such as quantity and quality of litter inputs, temperature, and moisture (Drenovsky et al., 2004; Castro et al., 2010; Curiel Yuste et al., 2011; Evans et al., 2013; Göransson et al., 2013; Fuchslueger et al., 2014). Changes in soil communities and the loss of soil biodiversity threaten the multifunctionality and sustainability of ecosystems, with negative impact on plant diversity and nutrient cycling and retention (Wagg et al., 2014), whereas a more diverse microbial

community could be associated with higher resistance to disturbance (Allison and Martiny, 2008). In parallel, microbial processes have been related to variations in ecosystem properties, such as tree productivity (Wardle et al., 2004; Allison and Martiny, 2008; Grigulis et al., 2013).

Most studies of habitat fragmentation have focused on above-ground organisms, while only a few have addressed effects of fragmentation on soil physicochemical characteristics, functioning (Lázaro-Nogal et al., 2012), or microbial structure (Rantalainen et al., 2008). In Mediterranean gypsic soils, Lázaro-Nogal et al. (2012) found that the synergistic interaction between habitat fragmentation and habitat quality was negatively correlated with soil nutrients and enzymatic activity. Results of simulated fragmentation from Rantalainen et al. (2008) suggested that habitat fragmentation did not have a direct effect on soil microorganisms. However, the effects of habitat fragmentation on the plant-soil-microorganism system remain largely unknown and especially their interaction with drought.

Our general objective was to study the impact of drought and rewetting on the structure and functioning of microbial communities in soils from two climatically different regions and from forest fragments of contrasting sizes in a microcosm experiment. To assess potential interactions between fragmentation and climate, we explored the physiological responses to drought of oak seedlings grown in soils from contrasting fragment sizes. First, we hypothesized that physicochemical characteristics of soils and their microbial biomass would differ in forest fragments of contrasting sizes. Second, we hypothesized that the functional response of the plant-soil-microbial system to climatic simulations (drought and rewetting) would be determined by the particular initial microbial communities and biogeochemical properties associated with the size of the fragment.

## Material and methods

*Soil provenance*

We selected three large (> 10 ha) and three small (< 0.5 ha) holm-oak (*Quercus ilex* L. ssp. ballota (Desf.) Samp; Fagaceae) forest fragments, resulting from the conversion to an agricultural landscape, located in two climatically different regions of central Spain (12 forest fragments in total). In the northern region (Lerma; 41°58'-42°02'N, 03°45'-03°52'W; 930m asl) the studied fragments were in an area of 1500 ha, they showed similar characteristics in spatial structure and vegetation, and were separated by at least 50 m to a maximum of 11 km (Fig. 1.5a in Chapter 1). This region is characterized by 554 mm mean annual precipitation and 11 °C mean annual temperature (Ninyerola et al., 2005). The dominant tree species is also holm oak, with isolated Lusitanian oak *Quercus faginea* and Spanish juniper *Juniperus thurifera* L. and understory shrubs typical of wetter and cooler supramediterranean localities (e.g. *Cistus laurifolius* L.*, Genista scorpius* (L.) DC*, Thymus zygis* Loefl. ex L.; see Santos and Tellería, 1998; Díaz et al., 1999, for further details). In the southern region (Quintanar de la Orden; 39°30'-39°35'N, 02°47'-02°59'W; 870 m asl), the studied fragments were in an area of 1000 ha, they showed similar characteristics in spatial structure and vegetation, and were separated by at least 50 m to a maximum of 8 km (Fig. 1.5b in Chapter 1). This region is characterized by 434 mm mean annual precipitation and 14 °C mean annual temperature (Ninyerola et al., 2005). The dominant tree is the holm oak *Q. ilex* with the understory composed of shrubby kermes oak *Quercus coccifera* L. and shrub species typical of xeric mesomediterranean localities (e.g. *Asparagus acutifolius* L.*, Cistus ladanifer* L.*, Rhamnus alaternus* L.*, Rhamnus lycioides* Brot.; see Santos and Tellería, 1998; Díaz and Alonso, 2003 for further details). Both sites are characterized by a pronounced summer drought period, usually lasting from July to September (Fig. 1.7 in Chapter 1). The

climatic characteristics of the two study regions are representative of the mesomediterranean and supramediterranean bioclimatic zones of the Iberian Peninsula, respectively (Rivas-Martínez, 1981). The dominant soils are classified as Cambisols (calcics) (WRB, 2007), sandy loam texture, with 17% sand, 39% silt and 44% clay for the southern region and, 11% sand, 42% silt and 47% clay for the northern region. In both regions, the original forest is now highly fragmented due to land conversion for intensive cultivation of cereal crops, legumes, and some grapes (Santos and Tellería, 1998; Díaz and Alonso, 2003). Remnants of forests were left between crops and now are imbibed in the agricultural matrix, yet clearly differentiated. Trees within large fragments were homogeneously distributed with low tree density, while the small fragments consist of at least four trees and with a high tree density (authors' personal observations).

*Soil sampling*

We selected four trees with basal area of ~500-600 $cm^2$ within each fragment and region with six fragments in each region, making 48 trees in total. Four soil samples were taken in four orientations under the canopy of each tree to a depth of 0-15 cm, and combined to a single sample per tree. Soils were collected at the end of the dry season. Once in the laboratory, soils were sieved (< 2 mm), and air-dried for two weeks. Acorns of *Q. ilex* were collected from the southern region.

Soil physicochemical characteristics and microbial biomass were measured in all replicates of each fragment size. Since the level of spatial heterogeneity of soils at very small spatial scales would have blurred functional differences among fragments of contrasting size, and due to practical limitations of the experiment, conditions were standardised by combining soils into composite samples of small and large fragment types from each bioclimatic region; thus we finally obtained a total of four

treatments: two factors (region and fragment size) with two levels each (region: northern and southern; fragment size: large and small).

*Soil abiotic characteristics and microbial biomass*

Physicochemical soil characteristics and microbial biomass were determined on air-dried soils, for all 48 soil samples. Water holding capacity of soils was determined by soaking the samples in water for 2 h and then draining for 24 h in a humid environment. Soil aggregate stability was determined on 2 mm aggregates by a water-drop test (Imeson and Vis, 1984) using at least 20 aggregates per replicate. Soil pH was determined in a water slurry (1:5 w/v in $H_2O$); soil organic matter (SOM) was assessed by loss on ignition at 400 °C for 4 hours. Organic N was determined by the Kjeldahl method (Bremner, 1960). Available phosphorus was determined by the Burriel-Hernando extraction method (Burriel and Hernando, 1950), and $K^+$, $Ca^{2+}$, $Na^+$ and $Mg^{2+}$ were extracted with ammonium acetate (1M, pH 7) and subsequently determined by inductively coupled plasma spectrometry (Optima 4300DV, Perkin-Elmer, Waltham, USA). Microbial biomass carbon was determined by the chloroform fumigation-extraction method modified by Gregorich et al.(1990).

*Experimental design*

Two-litre pots were filled with a mixture of 5:1 soil:perlite (v:v). Randomly selected holm oak acorns from the southern region were soaked in water for 24 h before planting. Pots were randomly arranged in the greenhouse (25 °C and 40 % air humidity), and regularly watered to field capacity based on weight loss (between 35-45% soil water content). Other seedlings that eventually germinated were carefully removed from the pots. We established three experimental periods during the dry-rewetting simulation: 1) pre-drought with seedlings growing in well-watered pots for 80 days; 2) drought for 55 days with no water supply (the drought was

terminated when seedlings started to die); and 3) two days after rewetting, aboveground biomass was harvested and pots were watered to field capacity. We then measured the water potential of the harvested seedlings. Intermediate non-intrusive measures were carried out as indicated below.

*Seedling growth and physiological variables*

Seedling height and diameter were recorded every fifteen days (seven seedlings for southern large and northern small fragments, and eight seedlings for southern small and northern large fragments; 30 in total). Physiological variables were recorded at the same time interval: stomatal conductance (*gs*) was measured with a leaf porometer SC-I (Decagon, Pullman, USA), during the period of maximal conductance (10:00-12:00 h). Predawn and midday maximum photochemical efficiency of photosystem II ($F_v/F_m$) were measured with a portable pulse-modulated fluorometer FMS2 (Hansatech, Norfolk, UK), for which the leaves were previously held in the leaf clip holder for 30 min. Final Specific Leaf Area (SLA) was determined as leaf area (determined with an optical scanner) per gram dry mass. Final predawn stem water potentials ($\Psi_{PD}$) were determined with a Scholander pressure chamber (Scholander et al., 1965).

*Soil microbial community activity and fingerprinting profiles*

Three pots per treatment were selected for collection of soil samples at pre-drought, drought and after rewetting (12 soil samples per condition; 36 in total), which were stored at 4 °C for a maximum of two weeks or -20 °C for subsequent enzymatic and molecular analyses, respectively. The acid phosphatase activity (phosphoric monoester hydrolases, EC 3.1.3.2) assay was based on the detection of *p*-nitrophenol (PNP) released after 0.5 g of soil was incubated in 0.1 M maleate buffer at pH 6.5 (37 °C, 90 min) with p-nitrophenyl phosphate disodium as substrate (Tabatabai, 1994). The urease activity (amidohydrolase, EC 3.5.1.5) assay was based on the

detection of $NH^{4+}$ released after 0.5 g of soil was incubated in 1M phosphate buffer at pH 7 (30 °C, 90 min) with 1M urea as a substrate (Nannipieri et al., 1980).

Community structure of soil fungal and bacterial communities was assessed by the DNA community fingerprinting technique of denaturing gradient gel electrophoresis (DGGE). Soil DNA was extracted with the MoBio Powersoil DNA isolation kit (Solana Beach, USA), and yields assessed by electrophoresis at 80 V on a 1.2 % agarose gel. For fungi, the internal transcribed spacer nrDNA region ITS-1 was PCR-amplified using the primer pair ITS1-F/ITS2 (Gardes and Bruns, 1993). The universal primers 338F/518R were used for amplification of the bacterial 16S rRNA gene (Muyzer et al., 1993). A GC clamp was added to the 5' end of forward fungal (ITS1-F) and bacterial (338F) primers to stabilize the melting behaviour of the DNA fragments (Muyzer et al., 1993). PCRs were carried out on a PTC-200 Thermocycler (MJ Research, Massachusetts, USA), with 50 µl of reaction mixture containing 10x $NH_4$ reaction buffer, 2 and 1.5 mM $MgCl_2$ (for fungi and bacteria, respectively), 0.2 mM total dNTPs, 2.5 U Taq (Bioline, London, UK), 1 µM of each primer, 0.5 µl of 10 mg $ml^{-1}$ bovine serum albumin (BSA) and 50 ng of template DNA, determined using a Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, USA). PCR cycling parameters were: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 or 45 s, and 72 °C for 30 or 45 s, with a final extension at 72 °C for 5 or 10 min (for fungi and bacteria, respectively). Negative controls (with ultrapure water instead of DNA) were included in each PCR. DGGE was carried out on a DCode universal mutation detection system (Bio-Rad, Hemel Hempstead, UK); using 10 % polyacrilamide gels, with denaturant urea-formamide gradients of 10-50 % for fungi (Anderson et al., 2003) and 40-55 % for bacteria (Grossman et al., 2010), with the concentrations of 7 M urea and 40 % formamide (v/v) for the 100 % denaturant.

Electrophoreses were run at 60 °C 75 V for 16 h and 14 h, for fungi and bacteria respectively, loading equal volumes of amplified DNA. Gels were stained with SYBR Gold nucleic acid stain (Molecular Probes, Leiden, The Netherlands) and digitized using an InGenius3 Imaging System and Genesnap 6.08 (Syngene, Cambridge, UK). DGGE fingerprint profiles were analyzed with a KODAK 1D Image Analysis software (Eastman Kodak Co. 2000; Rochester, NY, USA). Bands were adjusted with a Gaussian model with a profile width of 80%. Noise was eliminated by removing bands below a 10% band peak intensity threshold. Species delimitation can be contended, especially taking into account the inherent limitations of the different analytical methods, such as in the case of DGGE where a unique band does not necessarily represent a unique species and one species can be represented by multiple bands on the gel (Cleary et al., 2012; Vaz-Moreira et al., 2013). Consequently, each band of the DGGE profile is hereafter referred to as an operational taxonomic unit (OTU) rather than a species. Although this is a simplification of the real taxonomic diversity of soil microbes, it allowed us to comparatively investigate differences and changes in the microbial community structure with respect to the studied factors. Gel bands were analyzed by using internal reference bands (bands present in all lanes), and known reference markers loaded in lanes at either side of the gel. The number and pixel intensity of bands in a particular sample were considered comparative proxies of richness and proportional abundance of fungal or bacterial OTUs, respectively (Cleary et al., 2012). Similar analysis of DGGE banding patterns have been previously used in other studies (Anderson et al., 2003; Farnleitner et al., 2004; Gafan et al., 2005; Cleary et al., 2012; Suzuki et al., 2012; Vaz-Moreira et al., 2013).

*Plant-soil system $CO_2$ exchange*

Net ecosystem exchange (NEE), defined as the net balance between Gross Primary Productivity (GPP) and Ecosystem Respiration ($R_{eco}$), was

measured for each individual plant-soil microcosm using a non-steady-state dynamic (closed dynamic) approach. For that purpose, a rectangular plexiglas chamber with a base of 0.01 $m^2$ and a volume of 0.0024 $m^3$ with a small fan to mix the air internally was built to fit over the microcosms (Fig. S4.1). The change in $CO_2$ concentrations was measured with a $CO_2$ infrared gas analyzer (EGM-4, PP-systems, MA, USA). The chamber was covered with aluminium foil to determine ecosystem respiration ($R_{eco}$) including both autotrophic (plants) and heterotrophic (microorganisms) components. Respiration rates were measured in both the transparent and the aluminium chamber for 52 s before the simulated drought and then every 15 days. During the drought simulation, diurnal $CO_2$ exchange measurements were taken in all microcosms four times per day (7:00-9:00 h, 11:00-13:00 h; 14:00-16:00 h; 18:00-20:00 h). Soil respiration was determined 3, 24 and 48 h after rewetting, only at 14:00-16:00 h, which was the period where the maximum activity was previously observed.

NEE (transparent chamber) and $R_{eco}$ (opaque chamber) were determined by calculating the $CO_2$ increase in the closed loop (see Fig. S4.1) in the respective measurements according to the formula (Street et al., 2007): $I_c = \frac{\rho * V * \left(\frac{dC}{dt}\right)}{A}$, where $I_c$ is the net $CO_2$ increase (μmol $m^{-2}$ $s^{-1}$), ρ is air density (mol $m^{-3}$), $V$ the chamber volume ($m^3$), $dC/dt$ represents the slope of $CO_2$ concentration increase in the chamber over time (μmol $mol^{-1}$ $s^{-1}$), and $A$ is the chamber surface area ($m^2$). Gross primary productivity (GPP) was calculated by subtracting $R_{eco}$ from NEE. The maximum difference between atmospheric ($C_a$) and internal $CO_2$ ($C_i$) was used to correct for chamber leaks using a linear equation (Pérez-Priego et al., 2010).

*Data analysis*

The effects of fragment size (large or small), region (north or south) and condition (pre-drought, drought or rewetting) and their interactions were

analyzed by three-way Analysis of Variance (ANOVA), with pot as a random effect. The effect of fragment size and region on soil abiotic characteristics, microbial biomass and final seedling measurements (height, diameter, biomass, root/shoot ratio, SLA, water potential) were analyzed by two-way ANOVAs. Repeated-measures ANOVAs were used to test the effect of the same factors during the drought in continuously measured variables (plant physiology and soil moisture). The analysis of survival percentages was carried out with a Peto and Peto test using $X^2$ (Pyke and Thompson, 1986).

The diversity of both bacterial and fungal communities was estimated from the number and intensity of bands (OTUs). Richness (S), Shannon ($H'$) and evenness ($E_H$) diversity indexes were calculated as follows: Shannon $(H') = -\sum_{i=1}^{S}\left({}^{n_i}/_{N}\right)\cdot ln\cdot\left({}^{n_i}/_{N}\right)$; Evenness $(E_H) = \frac{H'}{ln\,S}$ where $n_i$ is the band intensity, $N$ is the sum of all intensities of a sample and $S$ is the number of bands of a sample (richness). Principal component analysis (PCA) was conducted to reduce the $n$-dimensional DGGE data obtained for each sample into linear axes explaining the maximum amount of variance, using the relative intensity of the bands obtained from the DGGE. We used the first two principal components of the PCA to define the structure of bacterial and fungal communities (Suzuki et al., 2012). With the scores of the principal components of the PCA we explored the effect of soil moisture over the structure of soil microbial communities using three-way ANOVAs with the same factors as above, except the condition (soil moisture) which was tested by pairs (pre-drought vs drought and drought vs rewetting). In all cases, pot was used as a random effect and Tukey's test was used for post-hoc multiple comparisons to determine significant differences.

We performed two correlation analyses: 1) Pearson's correlations (calculated to investigate the univariate relations between variables); and 2) stepwise multiple regressions to study which measured variables best

explained the microbial diversity and variations in community structure. To perform stepwise multiple regressions first we reduced the number of explanatory variables to four, eliminating redundant variables (correlation among explanatory variables; see Table S4.1), and by choosing those variables that generated the highest Pearson's coefficients. A sequential Bonferroni correction was used to account for multiple comparisons (Hill and Lewicki, 2005). Simple linear regressions were run to analyze moisture sensitivity of carbon fluxes. All variables were tested for normality, and log transformations were applied when required (GPP, $R_{eco}$ and soil moisture), prior to analyses. STATISTICA 8.0 (StatSoft, Inc. 2007; Tulsa OK, USA) was used for performing all analyses.

## Results

*Soil characteristics*

In general, the physicochemical characteristics of soils were highly affected by fragment size and less by bioclimatic region (i.e. northern or southern). Soils from smaller fragments showed higher water holding and cation exchange capacities, as well as higher $Mg^{2+}$, $Ca^{2+}$ and $Na^{+}$ concentrations compared with soils from larger fragments (Table 4.1). Concentration of SOM, nitrogen and phosphorus were higher in soils from small than from large fragments, and also higher in the northern region compared to the southern one; whereas $K^{+}$ was higher in small fragments and in the southern region (Table 4.1). Soils from the northern region presented a significantly less alkaline pH and more stable aggregates in comparison with those from the southern region. A significant interaction between fragment size and region on soil microbial biomass was also detected (Table 4.1), showing that microbial biomass was highest in soil from large fragments from the northern region. SOM was strongly correlated with cation exchange capacity, organic nitrogen and $Ca^{2+}$ (Table S4.1), as well as with water holding capacity ($R^2$=0.89 p<0.001).

During the drought simulation, soils from small fragments of both regions showed higher soil moisture ($F_{10,\ 260}$= 3.7; $p$<0.001), compared to soils from large fragments, particularly those from the northern region (Fig. S4.2 and Table S4.2).

*Soil microbial diversity*

Significant interactions among condition, region and fragment size was observed in both fungal richness and diversity (Table S4.3), mainly driven by large fragments from the southern region with the highest values of both variables at pre-drought and after rewetting (Fig. 4.1a and b).

**Table 4.1**. Physicochemical characteristics of soils from two climatically different regions and two fragment sizes of holm oak forest in Spain. Data = mean (SE) ($n$=12). The effect of factors is summarized on the left of the table. SOM = soil organic matter; CEC=cation exchange capacity. Significant differences ($P$<0.05) between main effects are indicated with capital letters (among region) and lower case letters (among fragment size).

| | | Northern region | | Southern region | |
|---|---|---|---|---|---|
| | | Large fragment | Small fragment | Large fragment | Small fragment |
| Fragment effect | Water holding capacity (%) | 40.1A,b (2.4) | 49.8A,a (2.2) | 38.9A,b (0.9) | 45.2A,a (1.6) |
| | CEC ($cmol_c Kg^{-1}$) | 26.5A,b (4.3) | 39.5A,a (3.2) | 27.6A,b (1.5) | 35.7A,a (2.3) |
| | $Mg^{2+}$ ($cmol_c Kg^{-1}$) | 1.4A,b (0.1) | 2.5A,a (0.2) | 1.6A,b (0.1) | 3.1A,a (0.5) |
| | $Ca^{2+}$ ($cmol_c Kg^{-1}$) | 24.2A,b (4.1) | 35.6A,a (2.9) | 24.5A,b (1.4) | 30.5A,a (1.8) |
| | $Na^{+}$ ($cmol_c Kg^{-1}$) | 0.04A,b (0.004) | 0.07A,a (0.01) | 0.05A,b (0.003) | 0.07A,a (0.01) |
| Region effect | SOM (%) | 12.1A,b (1.9) | 19.8A,a (2.4) | 10.3B,b (0.7) | 14.1B,a (1.8) |
| | N organic (%) | 0.4A,b (0.1) | 0.8A,a (0.1) | 0.3B,b (0.02) | 0.5B,a (0.1) |
| | $P_2O_5$ ($mg\ Kg^{-1}$) | 13.8A,b (1.0) | 34.4A,a (4.2) | 2.5B,b (0.4) | 16.5B,a (1.7) |
| | $K^{+}$ ($cmol_c Kg^{-1}$) | 0.9B,b (0.14) | 1.7B,a (0.1) | 1.5A,b (0.1) | 2.0A,a (0.1) |
| | pH | 7.1B,a (0.3) | 7.3B,a (0.1) | 7.9A,a (0.04) | 7.9A,a (0.03) |
| | Aggregate stability (%) | 56.9A,a (3.1) | 57.1A,a (4.0) | 47.8B,a (3.7) | 49.1B,a (2.9) |
| | Microbial biomass ($mg\ C\ kg^{-1}$) | 1013.4a (6 6.9) | 625.1b (96.7) | 621.6b (29.6) | 725.4ab (41.3) |

Similarly, significant interactions between condition, region and fragment size were observed for bacterial richness and between condition and region for bacterial diversity (Table S4.3). Both interactions showed very similar patterns: a significant decrease of bacterial richness and diversity during drought, and a partial recovery after rewetting (Fig. 4.1d and e), been more evident in soils from the northern region.

Fungal evenness increased significantly with drought (Fig. 4.1c), while bacterial evenness during drought showed an increase only in the northern region (Fig. 4.1f).

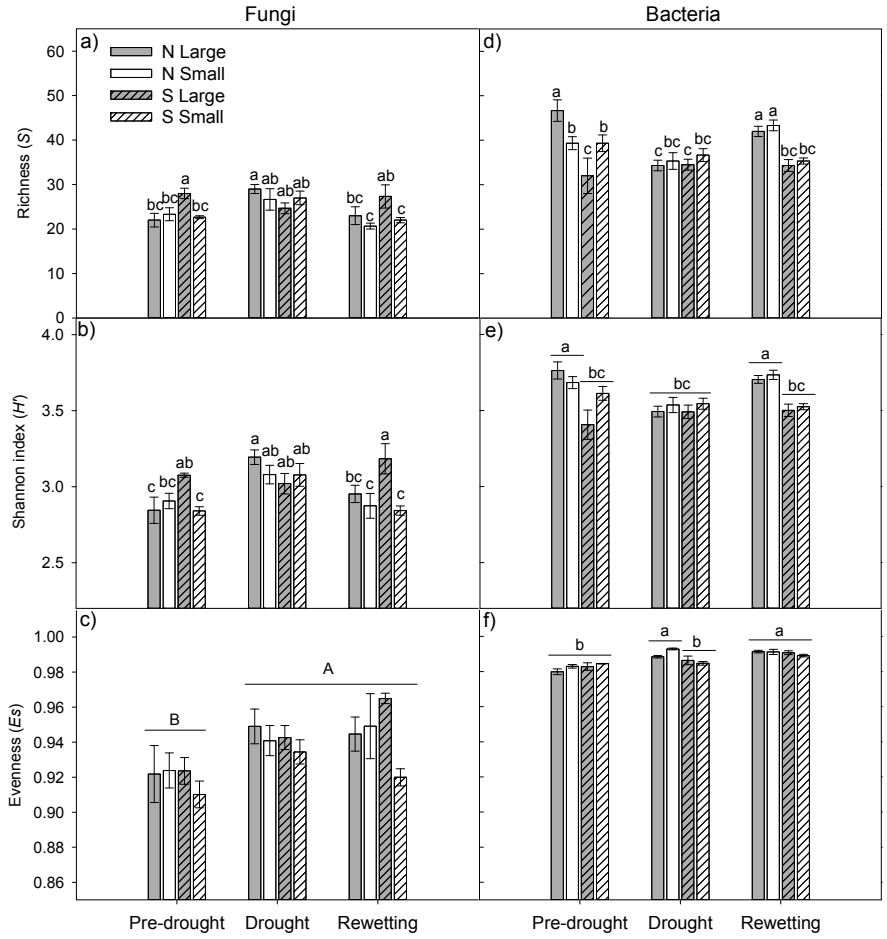


**Figure 4.1** Response of richness, Shannon diversity and evenness of fungal (*a, b, c*) and bacterial (*d, e, f*) communities of soils from two climatically different regions and two fragment sizes of Holm oak forests in Spain, to experimental simulation of drought and rewetting. *Grey/white* bars represent large/small fragments, respectively. *Open/filled* bars represent the northern or the southern region respectively. Data = mean ± SE. Significant differences given by post-hoc multiple comparisons by Tukey's test ($P<0.05$) of the three-way ANOVA are indicated: main effects by capital letters (among condition) and significant interaction between factors by lower case letters (triple in the case of *a*, *b* and *d*; condition x region in the case of *e* and *f*; see Table S4.3).

The first two PCA components explained 20.5 % of the total variance in the fungal community composition (Fig. 4.2a). The pre-drought fungal community was strongly influenced by region, but not by fragment size (Table 4.2). A high variability was observed in the fungal community from the northern region (Fig. 4.2a). Under drought, an interaction between drought and region was observed (Table 4.2, Fig. 4.2a). Once soils were rewetted, the fungal community structure was significantly affected by region and fragment size (Table 4.2, Fig. 4.2a). In the case of bacterial community composition, the first two PCA components explained 33.2 % of the total variance (Fig. 4.2b). Neither region nor fragment size affected the structure of the pre-drought bacterial community (Table 4.2, Fig. 4.2b). Both drought and rewetting led to a significant separation of the structure of bacterial communities from different regions, according to the principal component 1 of the PCA (Table 4.2, Fig. 4.2b). Fragment size and its interaction with drought and rewetting had no significant effect over the structure of microbial communities.

*Enzymatic activity*

Significant interaction between all factors (condition x region x fragment size) was observed for urease activity, with higher activity in soils from small fragments of the southern region at pre-drought (Fig. 4.3a), but higher activity in soil from small fragments of the northern region during simulated drought and rewetting. For phosphatase activity, fragment size showed a significant main effect ($F_{1, 1}$= 9.53; $p$=0.005) with higher activity in soils from small fragments (Table S4.3). Additionally, a significant interaction between condition and region was observed, where activity in soils from the northern region decreased significantly with drought (Fig. 4.3b); whereas activity in soils from the southern region remained very similar during the experiment.

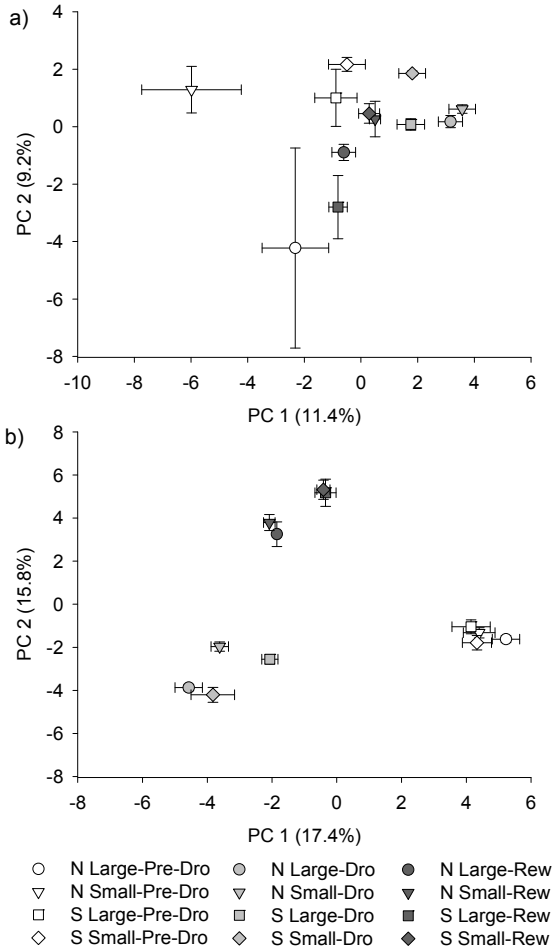


**Figure 4.2** Principal component analyses (PCA) for soil fungal (*a*) and bacterial (*b*) communities of soils from two climatically different regions and two fragment sizes of holm oak forests in Spain, and exposed to experimental drought and rewetting simulations. Soil treatments are represented by different symbols: circles = large fragments from the northern region; inverted triangles = small fragments from the northern region; squares = large fragments from the southern region; diamonds = small fragments from the southern region. Simulated experimental conditions are represented by different colours: white = pre-drought (*Pre-Dro*); grey = drought (*Dro*); black = rewetting (*Rew*). Error bars = SE.

**Table 4.2.** Response of fungal and bacterial community composition (first two components of PCA analyses) to the factors region, fragment size and drought at different experimental phases: pre-drought, drought and rewetting simulations, in soils from two climatically different regions and two fragment sizes of holm oak forest in Spain. Data were analysed by three way ANOVA ($n$=6), and significant effects are noted in bold (*<0.05; **< 0.01; ***<0.001). PC1= first component and PC2= second component of Principal Component Analysis (PCA).

| | Fungal (20.5%) | | | | Bacterial (33.2%) | | | |
|---|---|---|---|---|---|---|---|---|
| | PC1 (11.4%) | | PC2 (9.2%) | | PC1 (17.4%) | | PC2 (15.8%) | |
| | *P* | *F* | *P* | *F* | *P* | *F* | *P* | *F* |
| **Drought (D)** | **<0.001***** | 63.22 | 0.515 | 0.44 | **<0.001***** | 539.49 | **<0.001***** | 81.852 |
| **Region (R)** | 0.155 | 2.23 | 0.070 | 3.77 | 0.421 | 0.685 | 0.296 | 1.173 |
| **Fragment size (F)** | 0.280 | 1.25 | **0.030*** | 5.67 | 0.311 | 1.097 | 0.807 | 0.062 |
| **D x R** | **0.001**** | 16.09 | 0.203 | 1.76 | **0.025*** | 6.178 | 0.188 | 1.902 |
| **D x F** | 0.156 | 2.21 | 0.250 | 1.42 | 0.911 | 0.013 | 0.369 | 0.855 |
| **R x F** | 0.161 | 2.16 | 0.433 | 0.65 | 0.237 | 1.521 | **<0.001***** | 37.134 |
| **D x R x F** | 0.098 | 3.09 | 0.147 | 2.32 | **0.017*** | 7.214 | **0.004**** | 11.155 |
| **Rewetting (Rw)** | **<0.001***** | 90.87 | **<0.001***** | 17.05 | **<0.001***** | 85.59 | **<0.001***** | 629.044 |
| **Region (R)** | **0.007**** | 9.73 | 0.686 | 0.17 | **<0.001***** | 29.08 | 0.053 | 4.405 |
| **Fragment size (F)** | **0.034*** | 5.39 | **<0.001***** | 23.20 | 0.295 | 1.175 | 0.449 | 0.602 |
| **Rw x R** | **0.029*** | 5.78 | 0.054 | 4.32 | 0.398 | 0.758 | **0.002**** | 13.269 |
| **Rw x F** | 0.150 | 2.29 | 0.129 | 2.55 | 0.625 | 0.249 | 0.729 | 0.123 |
| **R x F** | 0.750 | 0.10 | **0.024*** | 6.24 | **0.024*** | 6.287 | **0.005**** | 10.833 |
| **Rw x R x F** | 0.761 | 0.09 | 0.589 | 0.30 | **0.012*** | 8.068 | **0.019*** | 6.885 |

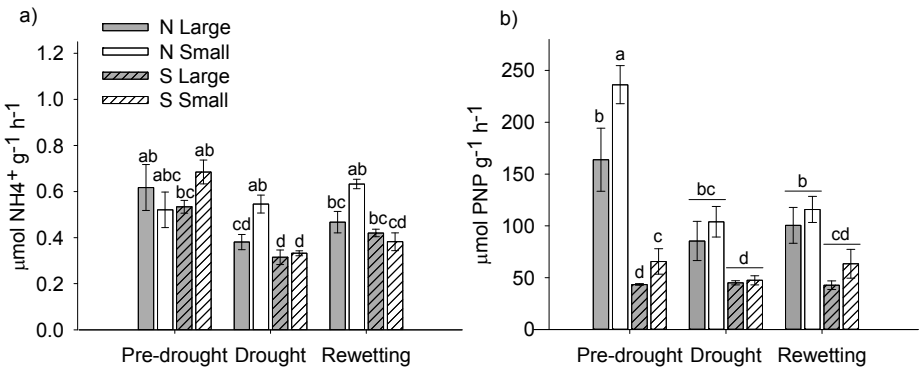


**Figure 4.3** Enzymatic activities: urease (*a*) and phosphatase (*b*) of soils from two climatically different regions and two fragment sizes of holm oak forests in Spain, and exposed to experimental drought and rewetting simulations. *Grey/white* bars represent large/small fragments respectively. *Open/filled* bars represent the northern or the southern region, respectively. Data = means ± SE. Significant differences given by post-hoc multiple comparisons by Tukey's test ($P$<0.05) of the three-way ANOVA are indicated: main effect of fragment size in phosphatase activity (not represented), and significant interaction between factors by lower case letters (triple in the case of urease; condition x region in the case of phosphatase; see Table S4.3).

*Net ecosystem exchange in plant-soil-microbial system*

In the plant-soil-microbial system, the main component of net ecosystem exchange (NEE) was ecosystem respiration ($R_{eco}$), with a lower contribution of gross primary productivity (GPP; Fig. 4.4). Drought caused an overall significant decrease in both $R_{eco}$ and GPP (Fig. 4.4a and b, respectively; Table S4.4). A consistent significant interaction between region and fragment size was detected, showing that the soils from small fragments of the northern region had higher $R_{eco}$, during the drought simulation (Fig. 4.4a; Table S4.4). A significant interaction between time, region and fragment size was observed in the pulse of $CO_2$ after rewetting (Table S4.5), showing higher $R_{eco}$ in soils from the northern region especially at 3 and 48 hrs (Fig. 4.4c).

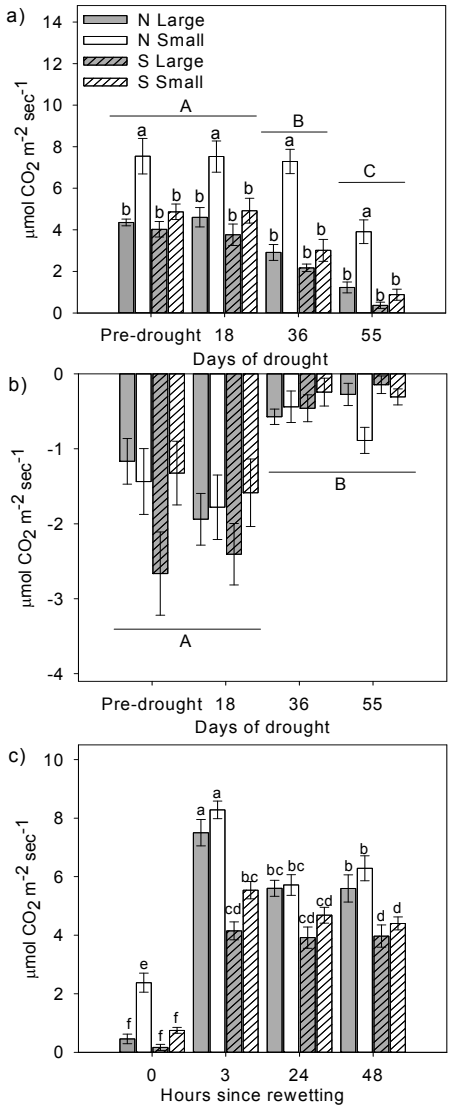


**Figure 4.4** $CO_2$ fluxes of the plant-soil-microbial system of soils from two climatically different regions and two fragment sizes of Holm oak forests in Spain. Ecosystem respiration (*a*) and Gross primary productivity (*b*) both in drought simulation, and ecosystem respiration in rewetting simulation (*c*), where 0 means immediately before rewetting. *Grey/white* bars represent large/small fragments respectively. *Open/filled* bars represent the northern or the southern region, respectively. Data = means ± SE. Significant differences given by post-hoc multiple comparisons by Tukey's test ($P<0.05$) of the three-way ANOVA are indicated: main effects by capital letters (among condition) and significant triple interaction between factors by lower case letters (condition x region x fragment size, see Table S4.4 and S4.5).

*Plant growth and physiology*

Growth and physiological activity of seedlings were not affected by region or fragment size (Table 4.3, Table S4.2), despite the differences in moisture and nutrient status observed between soils (Table 4.1). However, the physiological activity of seedlings was significantly affected by drought (Table S4.2). Seedlings grown in soils from the southern region showed the lowest SLA (Table 4.3). Seedling survival varied significantly depending upon treatments, from no mortality in soils of small fragments from the northern region, to almost 50% seedling mortality in soils of large fragments from the southern region (Table 4.3). We did not find any consistent relationships between seedling growth (height, diameter, biomass, SLA), physiology (GPP, photochemical efficiency, stomatal conductance, water potential), and soil microbial communities (bacterial and fungal diversity and structure), soil biogeochemical properties (nutrients, soil moisture, stability), or soil functioning (enzymatic activity, $R_{eco}$; Table S4.6).

*Influence of soil moisture on the plant-soil-microbial system*

Linear regression analyses showed that $R_{eco}$ was highly sensitive to soil moisture ($R^2$=0.499, $p$<0.001) (Fig. 4.5a), whereas GPP was not (Fig. 4.5b). As expected, correlations showed that soil functioning ($R_{eco}$ and enzymatic activities) were strongly related to soil moisture (Table 4.4). Fungal community structure (i.e. PC1 of fungal PCA; Fig. 4.2a) was negatively correlated with $R_{eco}$ and phosphatase activity (Table 4.4). Fungal diversity was negatively correlated with $R_{eco}$ and both enzyme activities (Table 4.4). Bacterial community structure (i.e. PC1 of bacterial PCA; Fig. 4.2b) was positively correlated with $R_{eco}$ and urease activity (Table 4.4). Bacterial richness and diversity were also correlated with $R_{eco}$ and phosphatase activity (Table 4.4).

**Table 4.3.** Seedling growth and physiology during drought simulation in soils from two climatically different regions and two fragment sizes of holm oak forest in Spain. Data = mean (SE) ($n$= 7 for southern large and northern small fragments, and $n$= 8 for southern small and northern large fragments). Significant differences ($P$<0.05) are indicated with capital letters (among region for a given fragment size) and lower case letters (among fragment size for a given region).

| | Northern region | | Southern region | |
|---|---|---|---|---|
| | **Large fragment** | **Small fragment** | **Large fragment** | **Small fragment** |
| Germination (days) | 64.1 (8.2)A,a | 45.0 (3.8)A,a | 60.6 (6.4)A,a | 53.6 (5.9)A,a |
| Height (cm) | 6.64 (0.9)A,a | 8.6 (1.2)A,a | 8.6 (1.9)A,a | 6.1 (0.9)A,a |
| Diameter (mm) | 2.12 (0.2)A,a | 2.44 (0.3)A,a | 2.3 (0.3)A,a | 2.3 (0.4)A,a |
| Biomass (g) | 1.3 (0.3)A,a | 1.7 (0.3)A,a | 1.4 (0.3)A,a | 1.2 (0.4)A,a |
| Root/shoot ratio | 1.2 (0.1)A,a | 0.9 (0.1)A,a | 1.0 (0.2)A,a | 1.0 (0.1)A,a |
| SLA ($cm^2\ g^{-1}$) | 81.2 (3.4) B,a | 71.7 (2.3)B,a | 84.7 (3.8)A,a | 86.2 (3.5)A,a |
| $F_v/F_m$ predawn | 0.66 (0.09)A,a | 0.59 (0.10)A,a | 0.63 (0.16)A,a | 0.78 (0.04)A,a |
| $F_v/F_m$ midday | 0.60 (0.05)A,a | 0.51 (0.07)A,a | 0.61 (0.03)A,a | 0.67 (0.03)A,a |
| Stomatal conductance ($mmol\ s^{-1}\ m^{-2}$) | 68.3 (15.2)A,a | 74.7 (17.8)A,a | 52 (11.2)A,a | 59.8 (13.7)A,a |
| Water potential (MPa) | -1.5 (0.4)A,a | -1.7 (0.2)A,a | -1.3 (0.4)A,a | -2.2 (1.7)A,a |
| Survival (%) | 87.5 ab | 100.0a | 57.1b | 87.5ab |

Results of the stepwise multiple regressions showed a strong relationship between soil moisture and the diversity and structure of microbial communities (Table 4.5). However, an opposite relationship with soil moisture was detected for the two microbial communities, with soil moisture negatively correlated with fungal structure and diversity and positively correlated with bacteria structure (Table 4.5). Furthermore, fungal community evenness was significantly affected by pH, aggregate stability and SOM, whereas fungal diversity was also affected by SOM (Table 4.5).

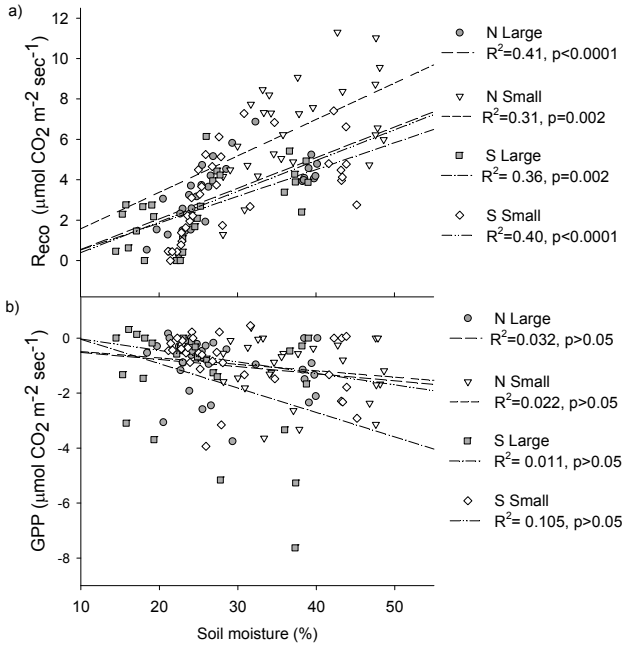


**Figure 4.5** Relationships between Ecosystem Respiration (*a*), Gross Primary Productivity (*b*) and soil moisture from two climatically different regions and two fragment sizes of holm oak forests in Spain exposed to experimental drought and rewetting simulations. Soil treatments are represented by different symbols: grey circles = large fragments from the northern region; white inverted triangles = small fragments from the northern region; grey squares = large fragments from the southern region; white diamonds = small fragments from the southern region. $R^2$ and *P* values of simple linear regressions are presented.

**Table 4.4.** Correlations between soil functioning and soil moisture, fungal and bacterial diversity in soils from two climatically different regions and two fragment sizes of holm oak forest in Spain. Data were analysed by Pearson's correlation ($n$=36) and significant effects are noted in bold (*<0.05; **< 0.01; ***<0.001). $R_{eco}$= ecosystem respiration; PC1= first component and PC2= second component of Principal Component Analysis (PCA). For the full correlation Table see Table S4.7.

| | $R_{eco}$ | Phosphatase | Urease |
|---|---|---|---|
| Soil moisture | **0.68*** ** | **0.45** ** | **0.58*** ** |
| Fungal richness | **-0.38*** | -0.33 | -0.34 |
| Fungal Shannon index | **-0.37*** | **-0.38*** | **-0.43*** |
| PC1 Fungal | **-0.58*** ** | **-0.49** ** | -0.30 |
| Bacterial richness | **0.41*** | **0.42*** | 0.29 |
| Bacterial Shannon index | **0.41*** | **0.40*** | 0.25 |
| PC1 Bacterial | **0.45** ** | 0.34 | **0.51** ** |
| PC2 Bacterial | **0.38*** | -0.10 | 0.06 |

**Table 4.5.** Relationships between fungal and bacterial community structure and explanatory physicochemical soil variables in soils from two climatically different regions and two fragment sizes of holm oak forest in Spain. Data were analysed by stepwise multiple regression ($t$-test) and significant effects using corrected $P$-value (sequential Bonferroni method) are noted in bold ($n$=36); n.s. not significant. $\beta$ = standardized coefficient. SOM = soil organic matter.

| | Fungi | | | | Bacteria | | | |
|---|---|---|---|---|---|---|---|---|
| | PC1 ($R^2$= 0.55) | Richness ($R^2$= 0.23) | Shannon ($R^2$= 0.41) | Evenness ($R^2$= 0.39) | PC1 ($R^2$= 0.84) | Richness ($R^2$= 0.37) | Shannon ($R^2$= 0.34) | Evenness ($R^2$= 0.30) |
| **Soil moisture** | | | | | | | | |
| $\beta$ | **-0.79** | -0.35 | **-0.52** | **-0.51** | **0.98** | 0.33 | 0.23 | **-0.52** |
| Pearson correlation | **-0.74** | -0.35 | **-0.53** | **-0.52** | **0.91** | 0.36 | 0.25 | **-0.50** |
| $P$ | * | n.s. | * | * | * | n.s. | n.s. | * |
| **pH** | | | | | | | | |
| $\beta$ | 4.78 | -8.62 | -13.36 | **-15.05** | -1.95 | 9.30 | 9.82 | -5.62 |
| Pearson correlation | 0.17 | -0.22 | -0.38 | **-0.41** | -0.11 | 0.26 | 0.27 | -0.15 |
| $P$ | n.s. | n.s. | n.s. | * | n.s. | n.s. | n.s. | n.s. |
| **Aggregates (%)** | | | | | | | | |
| $\beta$ | 5.35 | -9.88 | -15.21 | **-16.95** | -2.26 | 11.11 | 11.68 | -6.37 |
| Pearson correlation | 0.16 | -0.23 | -0.38 | **-0.41** | -0.11 | 0.28 | 0.28 | -0.15 |
| $P$ | n.s. | n.s. | n.s. | * | n.s. | n.s. | n.s. | n.s. |
| **SOM (%)** | | | | | | | | |
| $\beta$ | -1.14 | 2.40 | **3.80** | **4.39** | 0.15 | -2.89 | -2.93 | 2.04 |
| Pearson correlation | -0.14 | 0.21 | **0.37** | **0.41** | 0.03 | -0.28 | -0.28 | 0.19 |
| $P$ | n.s. | n.s. | * | * | n.s. | n.s. | n.s. | n.s. |

## Discusion

*Effects of the fragment size and the bioclimatic region over the plant-soil-microbial system*

In our study the physicochemical characteristics of soils were highly affected by fragment size and less by the bioclimatic region (i.e. northern vs. southern region). Soils from small fragments of both regions showed higher concentrations of essential ions, partially confirming our initial hypothesis that physicochemical characteristics of soils would differ in forest fragments of contrasting sizes. In a fragmented forest, the influence of the agricultural matrix tends to increase as the fragments become smaller due to an amplified relative importance of edge effects (Fernández et al., 2002). This influence over the physicochemical characteristic of small fragments and of the edge of large fragments tend to be higher when the matrix is agricultural (Boutin and Jobin, 1998). Among other factors (lower tree competition and higher light availability), this increased fertility mediated by the matrix could also explain the presence of larger and more productive trees growing in the smaller fragments at both sites (authors personal observations), which result in higher soil organic matter (SOM) content. Soil water holding capacity was also higher in soils from small fragments, probably due to their higher SOM content, which typically increases the capacity of a soil to retain water (Boix-Fayos et al., 2001; Franzluebbers, 2002). This increase in resources under small fragments was also reflected in an increase in the functionality of the plant-soil-microbial system; both $R_{eco}$ and phosphatase activities were higher in soils from small fragments sizes in both regions.

Unlike functional indicators, our results indicate that the microbial communities were more sensitive to the particular bioclimatic conditions of the two regions studied than to the size of the fragment. Under a low influence of the matrix (large fragments), soils from the colder and wetter

region (northern) had higher microbial biomass and a more bacterial-rich community, while soils from the warmer and drier southern region had the lowest values of microbial biomass but the most diverse fungal communities. However, fragment size did not significantly affect the diversity and structure of the microbial communities, suggesting a strong resistance (Allison and Martiny, 2008) of these communities to the potential changes in soil physicochemical properties associated with fragment size.

*Effects of climatic simulations over the functioning of the plant-soil-microbial system and its interactions with fragment size and bioclimatic origin*

Drought negatively affected the plant-soil-microbial system due to the strong effect of soil moisture over different functional indicators of the plant-soil-microbial system, such as autotrophic and heterotrophic respiration as well as plant productivity (Orchard and Cook, 1983; Reichstein et al., 2002; Barba et al., 2013). Decreases in metabolic activity related to drought have been found in other studies for both soil (Rey and Jarvis, 2006; Curiel Yuste et al., 2007), and enzymatic activities (Sardans and Peñuelas, 2005; Zornoza et al., 2006; Hueso et al., 2011). A synchrony in the reduction of $R_{eco}$ and enzymatic activity was expected when the microcosms were water-limited since both processes involve microbial aerobic activity, largely depend upon adequate water conditions (Orchard and Cook, 1983; Reichstein et al., 2002; Rambal et al., 2003; Sardans and Peñuelas, 2005; Zornoza et al., 2006; Curiel Yuste et al., 2007; Schimel et al., 2010; Hueso et al., 2011; Barba et al., 2013). On the contrary, the observed lower effect of drought over GPP (only significant by the end of the drought) evidenced a higher resistance to drought of these seedlings with respect to microbes. This higher resistance to drought could be due to a higher capacity of plants to explore the water resources of soil than microorganisms, which are more static and dependant on water micro-

conditions (Rambal et al., 2003; Ogaya and Peñuelas, 2004; Castro et al., 2010; Curiel Yuste et al., 2011; de Vries et al., 2012).

Regarding microbial communities, the forced climatic simulations (drought and rewetting) were associated with strong and opposed fluctuations in the diversity of both bacteria and fungi. The observed increase in fungal richness and diversity together with the decrease in bacterial diversity during the drought reinforces the idea that fungi overcome water limitations better than bacteria (Drenovsky et al., 2004; Schimel et al., 2007; Curiel Yuste et al., 2011; Barnard et al., 2013; Göransson et al., 2013; Grigulis et al., 2013). This can be explained because bacteria are organisms evolved in aqueous environment, and are more diverse under optimal water conditions, whereas fungi are organisms evolved in terrestrial environments, tending to be more diverse under water limitations (Curiel Yuste et al., 2011; Göransson et al., 2013; Grigulis et al., 2013). This was in agreement with the fact that soil rewetting produced an opposite effect with respect to that observed for drought on the microbial communities, decreasing fungal diversity and increasing bacterial diversity, respectively.

Collectively, our results suggest that the bioclimatic origin of the soil microbial communities strongly determined both the modulation by fragmentation of the effect of drought and the capacity of the microbial community to respond to the simulated climatic fluctuations. Our results only partially support our second hypothesis i.e. that the functional response of the plant-soil-microbial system to climatic simulations (drought and rewetting) would be determined by the particular initial microbial communities and biogeochemical soil properties associated with fragment size. Only in the small fragments of the northern and wetter region did the initial biological and physicochemical soil properties (e.g. higher bacterial richness and SOM content) appear to have a strong

positive effect over the functioning of the plant-soil-microbial system during drought (increasing $R_{eco}$, GPP, urease activity under dry condition in small with respect to large fragments), whereas fragment size had less of an effect (significant only for GPP) in the southern and drier region. The sensitivity of the microbial communities (diversity and evenness) to the climatic simulations was also strongly dependent on the initial bioclimatic origin of the soils. Indeed, the stronger sensitivity of bacterial diversity and evenness from the northern (colder and wetter) with respect to the southern (warmer and drier) region indicates a different degree of historical adaptation to dry conditions from these two communities. It is therefore likely that the harsher historical climatic conditions in the southern region may have acted as a strong habitat filter by selecting drought tolerant microbial species, more resistant to the simulated dry conditions of the experiment (Curiel Yuste et al., 2014; Evans and Wallenstein, 2014). In turn, the fast and significant increase in bacterial richness and diversity together with the strong pulse in $CO_2$ after rewetting again suggest that soil microbial communities grown in soils from the northern region, richer in organic matter, were more resilient to changes in water availability. Therefore, whereas reducing the size of the fragment increases the fertility and water availability for both microbes and plants (hence ameliorating the effect of drought over the functioning of the plant-soil-microbial system), the lack of sensitivity to this increase in resources of the plant-soil-microbial systems with soils from the southern region could only indicate a lack of responsiveness of the soil microbial communities from this drier site, which were unable to recover the function.

## Conclusions

A schematic overview of the main findings from this study is shown in Figure 4.6, ilustrating a continuum of drier to wetter conditions of soil and the interplay between the two climatically different regions and the fragment sizes. We observed here that, under optimal conditions (no water limitations) and in soils from the wetter region, the highest metabolic rates ($R_{eco}$ and enzyme activities) were generally associated with microbial communities dominated by rich bacterial communities, whereas under drought and in the southern and drier region, the relatively lower metabolic rates were associated with microbial communities dominated by rich fungal communities. Under drought stress, fragmentation modulates the functional response of both plants and microbes, especially in the relatively richer soils from the northern region, whereas fragment size did not substantially modulate the functional response of the microcosms with soils from the southern and drier region.

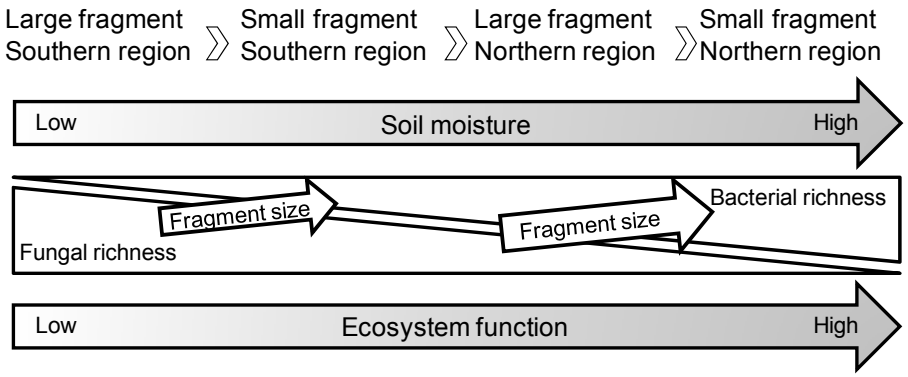


**Figure 4.6** Schematic overview of simultaneous variations in fungal and bacterial richness, soil moisture, ecosystem functioning and their modulation though fragment size, as suggested by the results of the present study.

Our results, therefore, suggest that the drier conditions expected in the future for the already water limited Mediterranean basin will favour fungal-dominated soil microbial communities, leading to a deceleration of processes associated with the plant-soil-microbial system. Moreover, the interaction found here between drought and fragment size suggests that depending on the local bioclimatic conditions and soil physicochemical characteristics, habitat fragmentation could ameliorate to some extent the negative effect of increasing droughts by increasing the fertility and water holding capacity of soils.

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## Supplementary material

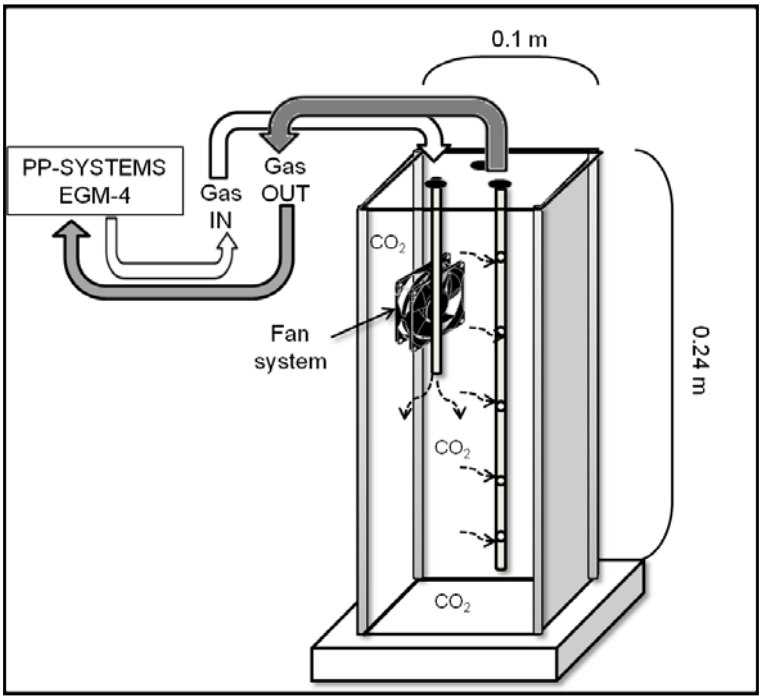


**Figure S4.1** Schema of the chamber used for measuring $CO_2$ fluxes of the plant-soil-system in Mediterranean holm oak forests fragments, showing the connections with the EGM-4 of PP-systems®. The frame of the bottom and top of the chamber were made of acrylic, and connected with aluminium rods. Walls were made of "NRS90 clear" polyester film of 75 µm thickness (Llumar®, Martinsville, USA). Arrows indicate the airflow inside the chamber.

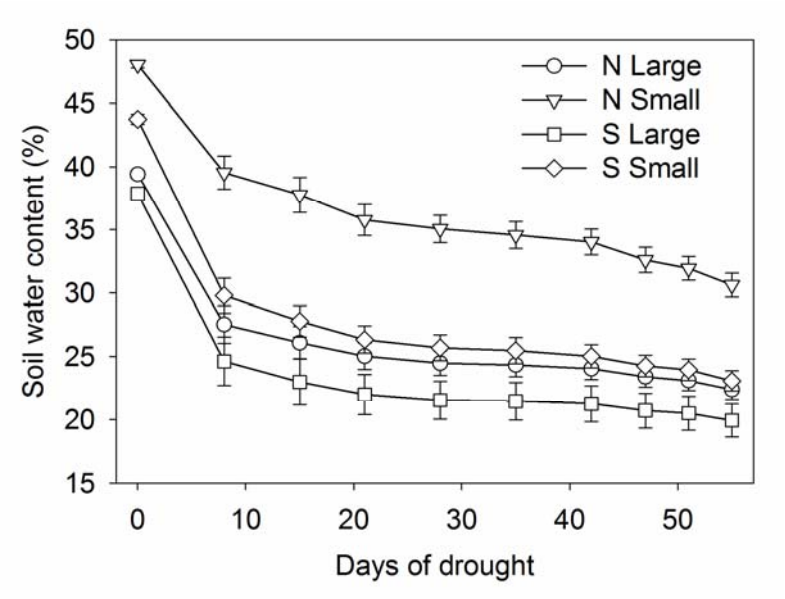


**Figure S4.2** Water content in the plant-soil-system during the experimental drought from two climatically different regions and two fragment sizes of holm oak forest in Spain. Different symbols represent distinct soil treatment: circles = large fragments from northern region; inverted triangle = small fragments from northern region; squares = large fragments from southern region; diamonds = small fragments from southern region. Error bars = SE ($n$= 7 for southern large and northern small fragments, and 8 for southern small and northern large fragments).

**Table S4.1** Correlation between physicochemical characteristics of soils (total values) (*n*=48) from two climatically different regions and two fragment sizes of holm oak forest in Spain. Significant correlation are in bold (*<0.05; **< 0.01; ***<0.001). CEC=cation exchange capacity; SOM=Soil organic matter. † Variables latter included in stepwise multiple regressions (see Table 5).

| | CEC | $Mg^{2+}$ | $Ca^{2+}$ | $Na^{+}$ | SOM | N organic | $P_2O_5$ | $K^{+}$ | pH | Aggregate stability | Microbial biomass (mg C $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Water holding capacity (%) | **0.91**$^{***}$ | **0.65**$^{***}$ | **0.90**$^{***}$ | **0.66**$^{***}$ | **0.89**$^{***}$ | **0.85**$^{***}$ | **0.67**$^{***}$ | **0.42**$^{**}$ | 0.21 | 0.20 | -0.23 |
| CEC ($cmol_c Kg^{-1}$) | | **0.69**$^{***}$ | **0.99**$^{***}$ | **0.66**$^{***}$ | **0.90**$^{***}$ | **0.83**$^{***}$ | **0.54**$^{***}$ | **0.51**$^{***}$ | **0.42**$^{**}$ | 0.11 | -0.23 |
| $Mg^{2+}$ ($cmol_c Kg^{-1}$) | | | **0.61**$^{***}$ | **0.48**$^{***}$ | **0.64**$^{***}$ | **0.63**$^{***}$ | **0.49**$^{***}$ | **0.65**$^{***}$ | 0.17 | -0.15 | -0.26 |
| $Ca^{2+}$ ($cmol_c Kg^{-1}$) | | | | **0.64**$^{***}$ | **0.90**$^{***}$ | **0.83**$^{***}$ | **0.53**$^{***}$ | **0.43**$^{**}$ | **0.41**$^{**}$ | 0.14 | -0.21 |
| $Na^{+}$ ($cmol_c Kg^{-1}$) | | | | | **0.64**$^{***}$ | **0.51**$^{***}$ | **0.59**$^{***}$ | **0.42**$^{**}$ | 0.13 | 0.17 | **-0.40**$^{*}$ |
| SOM (%) † | | | | | | **0.94**$^{***}$ | **0.69**$^{***}$ | **0.29**$^{*}$ | 0.06 | 0.08 | -0.14 |
| N organic (%) | | | | | | | **0.74**$^{***}$ | 0.20 | -0.05 | 0.001 | -0.05 |
| $P_2O_5$ ($mg Kg^{-1}$) | | | | | | | | 0.13 | **-0.33**$^{*}$ | 0.08 | -0.19 |
| $K^{+}$ ($cmol_c Kg^{-1}$) | | | | | | | | | **0.53**$^{***}$ | -0.10 | **-0.35**$^{*}$ |
| pH † | | | | | | | | | | 0.12 | -0.28 |
| Aggregate stability (%) † | | | | | | | | | | | 0.13 |

**Table S4.2** Soil moisture and seedling response during drought simulation to the factors region and fragment size in soils from two climatically different regions and two fragment sizes of holm oak forest in Spain. Data were analysed by repeated measures analysis of variance (*n*= 7 for southern large and northern small fragments, and *n*= 8 for southern small and northern large fragments) and significant effects are noted in bold (*<0.05; **< 0.01; ***<0.001).

| | | **Region(R)** | **Fragment size (F)** | **R x S** | **Drought (D)** | **D x R** | **D x F** | **DxRxF** |
|---|---|---|---|---|---|---|---|---|
| Soil moisture | F | **24.21** | **45.71** | 3.7 | **291.95** | **8.11** | **3.73** | 0.94 |
| | P | **<0.001**$^{***}$ | **<0.001**$^{***}$ | 0.065 | **<0.001**$^{***}$ | **<0.001**$^{***}$ | **<0.001**$^{***}$ | 0.49 |
| $F_v/F_m$ predawn | F | 0.553 | 0.556 | 1.586 | **12.880** | 0.394 | 0.581 | 1.663 |
| | P | 0.464 | 0.463 | 0.219 | **<0.001**$^{***}$ | 0.758 | 0.629 | 0.182 |
| $F_v/F_m$ midday | F | 0.794 | 0.366 | 1.737 | **23.342** | 0.386 | 1.825 | 1.877 |
| | P | 0.381 | 0.550 | 0.199 | **<0.001**$^{***}$ | 0.763 | 0.149 | 0.140 |
| Stomatal conductance (mmol $s^{-1}$ $m^{-2}$) | F | 2.36 | 0.909 | 1.362 | **22.356** | 0.607 | 1.384 | 0.125 |
| | P | 0.128 | 0.349 | 0.254 | **<0.001**$^{***}$ | 0.613 | 0.254 | 0.945 |

**Table S4.3** Soil microbial communities and enzymatic activities responses to condition (C=drought simulation and rewetting), region (northern and southern) and fragment size (large and small) in soils from two climatically different regions and two fragment sizes of holm oak forest in Spain. Significant effects of the three-way ANOVA ($n$=9) are noted in bold (*<0.05; **< 0.01; ***<0.001).

| | | Fungal Richness | Fungal Shannon | Fungal evenness | Bacterial Richness | Bacterial Shannon | Bacterial evenness | Phosphatase | Urease |
|---|---|---|---|---|---|---|---|---|---|
| **Condition (C)** | P | **0.007**$^{**}$ | **0.002**$^{**}$ | **0.003**$^{**}$ | **0.026**$^{*}$ | 0.058 | **<0.001**$^{***}$ | **<0.001**$^{***}$ | **<0.001**$^{***}$ |
| | F | **6.12** | **8.34** | **7.24** | **4.24** | 3.21 | **43.58** | **16.19** | **18.31** |
| **Region (R)** | P | 0.198 | 0.398 | 0.348 | **0.001**$^{**}$ | **0.001**$^{**}$ | **0.034**$^{*}$ | **<0.001**$^{***}$ | **0.005**$^{**}$ |
| | F | 1.75 | 0.74 | 0.91 | **14.74** | **14.8** | **5.04** | **90.57** | **9.43** |
| **Fragment size (F)** | P | **0.037**$^{*}$ | **0.007**$^{**}$ | 0.063 | 0.471 | 0.245 | 0.132 | **0.005**$^{**}$ | **0.030**$^{*}$ |
| | F | **4.86** | **8.83** | 3.81 | 0.54 | 1.42 | 2.44 | **9.53** | **5.32** |
| **C x R** | P | 0.057 | 0.087 | 0.989 | **0.016**$^{*}$ | **0.049**$^{*}$ | **0.001**$^{**}$ | **<0.001**$^{***}$ | **0.013**$^{**}$ |
| | F | 3.23 | 2.7 | 0.01 | **4.94** | **3.41** | **9.94** | **13.54** | **5.26** |
| **C x F** | P | 0.228 | 0.141 | 0.559 | 0.867 | 0.941 | 0.151 | 0.231 | 0.601 |
| | F | 1.58 | 2.13 | 0.6 | 0.14 | 0.06 | 2.05 | 1.56 | 0.52 |
| **R x F** | P | 0.354 | 0.091 | 0.075 | 0.05 | 0.06 | 0.075 | 0.318 | 0.504 |
| | F | 0.89 | 3.11 | 3.47 | 4.26 | 3.89 | 3.46 | 1.04 | 0.46 |
| **C x R x F** | P | **0.043**$^{*}$ | **0.026**$^{*}$ | 0.227 | **0.042**$^{*}$ | 0.053 | 0.485 | 0.347 | **0.004**$^{**}$ |
| | F | **3.58** | **4.27** | 1.58 | **3.63** | 3.32 | 0.75 | 1.11 | **7.12** |

**Table S4.4** Ecosystem respiration and Gross Primary productivity responses to condition (C=drought simulation and rewetting), region (northern and southern) and fragment size (large and small), in soils from two climatically different regions and two fragment sizes of holm oak forest in Spain. Significant effects of the three-way ANOVA ($n$=120) are noted in bold (*<0.05; **< 0.01; ***<0.001). $R_{eco}$= ecosystem respiration; GPP= Gross Primary Productivity.

| | | $R_{eco}$ | GPP |
|---|---|---|---|
| **Drought time (Dt)** | P | **<0.001*** ** | **<0.001*** ** |
| | F | **48.86** | 14.19 |
| **Region (R)** | P | **<0.001*** ** | 0.76 |
| | F | **62.12** | 0.09 |
| **Fragment size (F)** | P | **<0.001*** ** | 0.739 |
| | F | **71.88** | 0.11 |
| **Dt x R** | P | 0.505 | 0.172 |
| | F | 0.79 | 1.69 |
| **Dt x F** | P | 0.537 | 0.319 |
| | F | 0.73 | 1.18 |
| **R x F** | P | **<0.001*** ** | 0.287 |
| | F | **25.43** | 1.15 |
| **Dt x R x F** | P | 0.609 | 0.399 |
| | F | 0.611 | 0.99 |

**Table S4.5** Ecosystem respiration responses to time (T=3, 24 and 48 hrs after rewetting), region (northern and southern) and fragment size (large and small), in soils from two climatically different regions and two fragment sizes of holm oak forest in Spain. Significant effects of the three-way ANOVA ($n$=90) are noted in bold (*<0.05; **< 0.01; ***<0.001). $R_{eco}$ = ecosystem respiration.

| | | $R_{eco}$ rewetting |
|---|---|---|
| **Time (T)** | P | **<0.001*** ** |
| | F | 200.55 |
| **Region (R)** | P | **<0.001*** ** |
| | F | 104.82 |
| **Fragment size (F)** | P | **<0.001*** ** |
| | F | 25.43 |
| **T x R** | P | **<0.001*** ** |
| | F | 8.45 |
| **T x F** | P | 0.258 |
| | F | 1.37 |
| **R x F** | P | 0.821 |
| | F | 0.05 |
| **T x R x F** | P | **0.038*** |
| | F | 2.39 |

**Table S4.6** Correlation of physicochemical characteristics of soils with soil microbial communities and soil functioning in soils from two climatically different regions and two fragment sizes of holm oak forest in Spain, subjected to drought-rewetting simulation. Significant Pearson's correlation ($P$<0.05) are in bold. Underlined variables used mean values of the initial physicochemical characteristics of soils. SOM=Soil Organic Matter; PC1= first and PC2= second component of PCA; $R_{eco}$= ecosystem respiration; GPP= Gross Primary Productivity; gS=stomatal conductance; SLA=Specific leaf area. Numbers 18, 36 and 55 represent days since last watering.

| | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) | (13) | (14) | (15) | (16) | (17) | (18) | (19) | Root/Shoot ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Water holding capacity | 0.18 | 0.30 | 0.15 | -0.38 | 0.25 | -0.35 | -0.07 | 0.34 | 0.03 | -0.33 | 0.02 | **0.62** | 0.14 | **-0.80** | 0.01 | 0.41 | 0.001 | -0.31 | 0.30 | 0.02 |
| $Mg^{2+}$ | 0.25 | 0.44 | 0.17 | -0.15 | 0.36 | -0.11 | 0.38 | 0.25 | -0.24 | -0.14 | 0.20 | **0.50** | -0.07 | **-0.71** | 0.24 | 0.24 | -0.19 | 0.11 | -0.15 | 0.27 |
| SOM | 0.15 | 0.21 | 0.12 | -0.41 | 0.17 | -0.38 | -0.22 | 0.31 | 0.11 | -0.35 | -0.04 | **0.56** | 0.19 | **-0.71** | -0.08 | 0.41 | 0.06 | -0.41 | 0.41 | -0.05 |
| $P_2O_5$ | 0.25 | 0.20 | 0.13 | -0.41 | 0.09 | -0.40 | -0.28 | 0.23 | 0.09 | -0.40 | -0.02 | **0.48** | 0.22 | **-0.61** | -0.10 | 0.40 | 0.08 | -0.43 | 0.42 | 0.05 |
| $K^+$ | 0.03 | 0.36 | 0.10 | 0.02 | 0.41 | 0.07 | **0.55** | 0.25 | -0.23 | 0.08 | 0.17 | 0.40 | -0.19 | **-0.56** | 0.31 | 0.08 | -0.24 | 0.29 | -0.32 | 0.10 |
| Aggregate stability | 0.27 | -0.10 | 0.03 | -0.26 | -0.30 | -0.31 | **-0.57** | -0.12 | 0.15 | -0.36 | -0.08 | -0.06 | 0.29 | 0.09 | -0.28 | 0.19 | 0.22 | **-0.46** | **0.46** | 0.12 |
| Microbial biomass | 0.43 | -0.11 | -0.01 | 0.17 | **-0.48** | 0.08 | -0.18 | **-0.55** | -0.19 | -0.11 | 0.13 | **-0.67** | 0.08 | **0.78** | -0.10 | -0.19 | 0.07 | 0.09 | -0.13 | **0.57** |
| Soil moisture | 0.14 | 0.16 | -0.03 | -0.09 | -0.03 | -0.10 | -0.09 | 0.03 | -0.06 | -0.13 | -0.13 | 0.19 | -0.08 | -0.22 | 0.001 | 0.23 | 0.01 | -0.01 | -0.02 | 0.01 |
| Richness Fungi | -0.18 | 0.07 | -0.15 | 0.12 | -0.13 | -0.16 | 0.06 | 0.28 | -0.04 | -0.14 | -0.07 | 0.11 | -0.17 | 0.13 | 0.32 | -0.25 | -0.04 | 0.21 | -0.21 | -0.27 |
| Shannon index Fungi | -0.24 | -0.03 | -0.09 | 0.10 | -0.13 | -0.16 | -0.03 | 0.19 | 0.02 | -0.12 | -0.10 | 0.10 | -0.18 | 0.12 | 0.21 | -0.19 | -0.08 | 0.16 | -0.13 | -0.27 |
| Evenness Fungi | -0.21 | -0.21 | 0.07 | 0.01 | -0.06 | -0.05 | -0.22 | -0.14 | 0.14 | 0.001 | -0.09 | -0.01 | -0.13 | 0.07 | -0.13 | 0.06 | -0.11 | -0.02 | 0.09 | -0.14 |
| PC1 Fungi | -0.08 | -0.19 | -0.30 | 0.01 | -0.16 | 0.07 | -0.08 | 0.17 | -0.20 | -0.03 | 0.17 | 0.00 | 0.10 | 0.20 | 0.10 | -0.25 | -0.01 | 0.23 | 0.07 | 0.13 |
| PC2 Fungi | -0.14 | 0.21 | 0.07 | -0.05 | 0.06 | -0.31 | 0.16 | 0.28 | -0.26 | -0.22 | -0.25 | **0.58** | -0.32 | **-0.55** | 0.17 | 0.27 | -0.21 | 0.27 | -0.25 | -0.08 |
| Richness Bacteria | 0.05 | -0.23 | 0.001 | 0.03 | -0.33 | -0.08 | -0.30 | -0.32 | -0.20 | -0.12 | -0.11 | -0.09 | -0.13 | 0.22 | -0.18 | 0.19 | -0.07 | 0.18 | -0.02 | 0.29 |
| Shannon index Bacteria | 0.06 | -0.24 | 0.01 | 0.00 | -0.33 | -0.12 | -0.34 | -0.29 | -0.22 | -0.16 | -0.09 | -0.02 | -0.12 | 0.15 | -0.19 | 0.22 | -0.08 | 0.17 | 0.03 | 0.31 |
| Evenness Bacteria | 0.06 | 0.06 | 0.02 | -0.12 | -0.03 | -0.23 | -0.19 | 0.21 | -0.10 | -0.23 | 0.04 | 0.35 | 0.07 | -0.34 | -0.07 | 0.18 | 0.03 | -0.10 | 0.21 | 0.00 |
| PC1 Bacteria | -0.04 | -0.03 | -0.02 | 0.02 | -0.01 | 0.06 | -0.01 | -0.11 | 0.06 | 0.09 | -0.13 | -0.06 | -0.11 | 0.07 | 0.001 | 0.08 | 0.01 | 0.04 | -0.09 | -0.06 |
| PC2 Bacteria | -0.14 | -0.01 | 0.04 | -0.01 | 0.13 | -0.01 | -0.13 | 0.07 | 0.26 | 0.09 | -0.13 | 0.13 | -0.05 | -0.17 | -0.08 | 0.17 | 0.11 | -0.21 | 0.13 | -0.40 |
| Phospohatase activity | 0.13 | -0.17 | -0.05 | -0.38 | -0.13 | -0.24 | -0.42 | 0.03 | 0.20 | -0.23 | -0.12 | 0.01 | 0.40 | -0.01 | -0.21 | 0.20 | 0.27 | **-0.47** | **0.52** | 0.09 |
| Urease activity | 0.31 | 0.26 | 0.23 | 0.02 | 0.19 | 0.08 | 0.16 | -0.09 | 0.10 | 0.05 | 0.20 | -0.14 | 0.20 | -0.11 | -0.09 | 0.01 | 0.12 | -0.30 | 0.06 | 0.09 |
| $R_{eco}$ pre-drought | 0.17 | **0.50** | 0.25 | 0.17 | 0.23 | -0.24 | 0.00 | 0.38 | -0.01 | -0.22 | 0.21 | **0.59** | -0.16 | **-0.71** | 0.06 | 0.23 | 0.04 | -0.19 | 0.02 | -0.43 |
| $R_{eco}$ 18-drought | 0.12 | 0.37 | 0.15 | 0.18 | 0.17 | -0.19 | -0.14 | 0.34 | -0.02 | -0.19 | 0.23 | **0.60** | -0.23 | **-0.62** | 0.08 | 0.26 | 0.03 | -0.09 | 0.05 | -0.42 |
| $R_{eco}$ 36-drought | 0.10 | 0.27 | 0.11 | -0.10 | 0.09 | -0.42 | -0.18 | 0.44 | 0.00 | -0.32 | -0.07 | **0.68** | -0.03 | **-0.68** | 0.05 | 0.44 | 0.20 | -0.25 | 0.19 | -0.32 |
| $R_{eco}$ 55-drought | 0.13 | 0.28 | 0.28 | -0.23 | 0.24 | -0.41 | -0.10 | 0.24 | 0.28 | -0.26 | -0.14 | **0.47** | 0.05 | **-0.61** | 0.03 | **0.47** | 0.20 | **-0.48** | 0.23 | -0.29 |
| GPP pre-drought (1) | | **0.67** | 0.21 | 0.10 | 0.01 | -0.01 | 0.31 | -0.23 | -0.32 | -0.19 | 0.29 | -0.15 | 0.04 | -0.06 | 0.25 | 0.27 | 0.24 | -0.04 | -0.35 | **0.64** |
| GPP 18-drought (2) | | | **0.56** | 0.33 | 0.30 | -0.11 | **0.60** | -0.05 | -0.18 | -0.17 | 0.25 | 0.13 | -0.28 | **-0.52** | 0.22 | 0.12 | -0.11 | -0.06 | **-0.58** | 0.06 |
| GPP 36-drought (3) | | | | 0.36 | **0.56** | 0.05 | **0.54** | -0.44 | 0.33 | 0.18 | 0.24 | -0.20 | -0.17 | -0.40 | -0.26 | 0.06 | -0.23 | -0.44 | -0.27 | -0.18 |
| GPP 55-drought (4) | | | | | 0.15 | 0.40 | 0.37 | -0.14 | -0.26 | 0.38 | **0.58** | -0.10 | **-0.51** | 0.06 | 0.05 | -0.20 | -0.03 | 0.37 | **-0.57** | -0.34 |
| $F_v/F_m$ predawn pre-drought (5) | | | | | | **0.54** | **0.65** | -0.18 | **0.61** | **0.71** | 0.36 | -0.11 | -0.01 | **-0.47** | 0.02 | 0.19 | 0.06 | -0.43 | -0.04 | -0.36 |
| $F_v/F_m$ predawn18-drought (6) | | | | | | | 0.20 | **-0.46** | 0.38 | **0.89** | **0.63** | **-0.57** | -0.03 | 0.25 | -0.18 | -0.23 | -0.03 | -0.05 | 0.03 | -0.18 |
| $F_v/F_m$ predawn 36-drought (7) | | | | | | | | 0.01 | 0.01 | 0.39 | 0.22 | -0.04 | -0.11 | -0.32 | 0.36 | 0.15 | 0.22 | -0.04 | **-0.57** | -0.02 |
| $F_v/F_m$ predawn 55-drought (8) | | | | | | | | | -0.42 | -0.32 | -0.19 | **0.75** | 0.25 | -0.37 | 0.31 | 0.03 | 0.41 | 0.16 | 0.15 | -0.21 |
| $F_v/F_m$ midday pre-drought (9) | | | | | | | | | | **0.49** | 0.001 | -0.44 | 0.14 | 0.01 | -0.27 | -0.05 | -0.16 | **-0.66** | 0.39 | -0.43 |
| $F_v/F_m$ midday 18-drought (10) | | | | | | | | | | | 0.40 | -0.42 | -0.02 | 0.10 | -0.11 | 0.00 | 0.20 | -0.15 | 0.01 | -0.37 |
| $F_v/F_m$ midday 36-drought (11) | | | | | | | | | | | | -0.30 | 0.01 | 0.03 | -0.11 | -0.43 | -0.12 | -0.01 | -0.02 | -0.02 |
| $F_v/F_m$ midday 55-drought (12) | | | | | | | | | | | | | -0.25 | **-0.67** | 0.38 | **0.45** | 0.13 | 0.34 | -0.11 | -0.20 |
| $g_S$ pre-drought (13) | | | | | | | | | | | | | | 0.07 | -0.42 | -0.24 | 0.43 | **-0.66** | **0.76** | 0.25 |
| $g_S$ 18-drought (14) | | | | | | | | | | | | | | | 0.02 | -0.39 | 0.04 | 0.24 | 0.03 | 0.28 |
| $g_S$ 36-drought (15) | | | | | | | | | | | | | | | | 0.41 | 0.28 | **0.57** | **-0.60** | 0.18 |
| $g_S$ 55-drought (16) | | | | | | | | | | | | | | | | | **0.45** | 0.06 | -0.23 | 0.15 |
| Final Water Potential (17) | | | | | | | | | | | | | | | | | | -0.14 | 0.11 | 0.10 |
| Final SLA (18) | | | | | | | | | | | | | | | | | | | **-0.62** | 0.15 |
| Final seedling biomass (19) | | | | | | | | | | | | | | | | | | | | -0.07 |

**Table S4.7** Correlation between initial physicochemical characteristics of soils with soil microbial communities and soil functioning, in soils from two climatically different regions and two fragment sizes of holm oak fragments forest in Spain, subjected to drought-rewetting simulations ($n$=36). Significant Pearson's correlation (p<0.05) are in bold (*<0.05; **< 0.01; ***<0.001). Underlined variables used mean values of the initial physicochemical characteristics of soils. CEC=cation exchange capacity, SOM=Soil Organic Matter. PC1= first and PC2= second component of PCA; $R_{eco}$= ecosystem respiration. † Variables latter included in stepwise multiple regressions (see Table 5)

| | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) | (13) | Urease |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Water holding capacity | 0.30 | -0.31 | -0.32 | -0.15 | -0.03 | **0.37*** | 0.18 | 0.25 | 0.23 | -0.14 | -0.01 | **0.48**** | **0.43*** | 0.29 |
| CEC | 0.28 | -0.27 | -0.30 | -0.18 | 0.001 | **0.44**** | 0.08 | 0.15 | 0.22 | -0.13 | 0.01 | **0.43*** | 0.28 | 0.23 |
| $Mg^{2+}$ | 0.16 | -0.23 | -0.33 | -0.34 | 0.10 | **0.50**** | -0.03 | 0.03 | 0.07 | -0.09 | -0.01 | 0.15 | -0.07 | 0.06 |
| $Ca^{2+}$ | 0.3 | -0.28 | -0.29 | -0.14 | -0.02 | **0.40*** | 0.12 | 0.19 | 0.24 | -0.13 | 0.01 | **0.48**** | **0.37*** | 0.27 |
| $Na^{+}$ | 0.26 | -0.27 | -0.32 | -0.23 | 0.02 | **0.47**** | 0.06 | 0.12 | 0.19 | -0.12 | 0.01 | **0.37*** | 0.21 | 0.19 |
| SOM † | 0.32 | -0.30 | -0.29 | -0.07 | -0.06 | 0.28 | 0.24 | 0.30 | 0.25 | -0.13 | -0.02 | **0.53**** | **0.55**** | 0.33 |
| N organic | 0.32 | -0.30 | -0.28 | -0.05 | -0.08 | 0.22 | 0.29 | **0.35*** | 0.25 | -0.13 | -0.03 | **0.54**** | **0.61***** | **0.35*** |
| $P_2O_5$ | 0.32 | -0.33 | -0.32 | -0.08 | -0.07 | 0.23 | 0.32 | **0.37*** | 0.23 | -0.14 | -0.05 | **0.52**** | **0.61***** | **0.35*** |
| $K^{+}$ | 0.01 | -0.03 | -0.13 | -0.29 | 0.14 | **0.46**** | -0.29 | -0.26 | -0.02 | -0.02 | 0.05 | -0.09 | **-0.46**** | -0.14 |
| pH † | -0.15 | 0.19 | 0.13 | -0.13 | 0.13 | 0.27 | **-0.48**** | **-0.48**** | -0.08 | 0.05 | 0.11 | -0.29 | **-0.70***** | -0.29 |
| Aggregate stability † | 0.21 | -0.25 | -0.19 | 0.08 | -0.13 | -0.16 | **0.48*** | **0.5*** | 0.13 | -0.08 | -0.10 | **0.39*** | **0.75***** | 0.34 |
| Microbial biomass | -0.09 | -0.10 | -0.12 | -0.05 | 0.001 | -0.34 | **0.38*** | 0.34 | -0.20 | 0.02 | -0.16 | -0.19 | 0.21 | 0.02 |
| Soil moisture (1) † | | **-0.37*** | **-0.48**** | **-0.41*** | **-0.70***** | 0.21 | **0.4*** | 0.33 | **-0.36*** | **0.78***** | 0.04 | **0.68***** | **0.45**** | **0.58***** |
| Richness Fungi (2) | | | **0.91***** | 0.16 | 0.32 | 0.05 | **-0.5**** | **-0.49**** | 0.02 | -0.23 | -0.33 | **-0.38*** | -0.33 | -0.34 |
| Shannon index Fungi (3) | | | | **0.55**** | **0.35*** | 0.05 | **-0.44*** | **-0.42*** | 0.20 | **-0.38*** | -0.17 | **-0.37*** | **-0.38*** | **-0.43*** |
| Evenness Fungi (4) | | | | | 0.18 | -0.01 | -0.03 | 0.02 | **0.46**** | **-0.47**** | 0.25 | -0.10 | -0.24 | -0.33 |
| PC1 Fungi (5) | | | | | | 0.00 | -0.33 | -0.26 | **0.47*** | **-0.72***** | -0.16 | **-0.58***** | **-0.49**** | -0.30 |
| PC2 Fungi (6) | | | | | | | -0.09 | -0.08 | 0.12 | -0.11 | -0.26 | 0.001 | -0.26 | -0.19 |
| Richness Bacteria (7) | | | | | | | | **0.98***** | -0.20 | 0.29 | 0.03 | **0.41*** | **0.42*** | 0.29 |
| Shannon index Bacteria (8) | | | | | | | | | -0.06 | 0.17 | 0.09 | **0.41*** | **0.40*** | 0.25 |
| Evenness Bacteria (9) | | | | | | | | | | **-0.70***** | **0.45**** | -0.04 | -0.27 | -0.20 |
| PC1 Bacteria (10) | | | | | | | | | | | -0.02 | **0.45**** | 0.34 | **0.51**** |
| PC2 Bacteria (11) | | | | | | | | | | | | **0.38*** | -0.10 | 0.06 |
| $R_{eco}$ (12) | | | | | | | | | | | | | **0.50**** | **0.50**** |
| Phosphatase (13) | | | | | | | | | | | | | | **0.37*** |

# Capítulo 5

## The influence of forest fragmentation and bioclimatic region over the bio-physicochemical soil characteristics overshadows potential seedling provenance effects in the resistance of the plant-soil-microbial system to drought

Manuscript in preparation

## Abstract

Besides the strong dependence on the water regimen that characterizes the functioning of Mediterranean ecosystems, forest fragmentation might be considered one of the most important human-related threats for these ecosystems. Predictions, moreover, suggest that the impacts of those two engines of global change, climate (drought) and forest fragmentation will be progressively aggravated in the Mediterranean Basin. In this regard, it is expectable that these environmental perturbations will have less severe consequences for organisms historically grown under more extreme climatic conditions (drought) and/or historically more exposed to fragmentation. Here, we studied the interactive effect of climate and fragmentation (influence of the surrounding agricultural matrix) to evaluate how and in which strength both seedling provenances and soil origin could affect the functional response of the plant-soil system. We used a microcosm approach with holm oaks from three different provenances grown in soils from two different origins (bio-climatic regions) collected from stands under three different scenarios of fragmentation influence: no influence (inside large fragments), mild influence (edges of large fragments) and strong influence (small fragments). We used indicators of whole system functioning (ecosystem respiration), of plant physiological performance (gross primary productivity, stomatal conductance, photochemical efficiency), plant biomass allocation patterns (height, diameter, biomass, root:shoot ratio, specific leaf area), and degree and diversity of mycorrhization (percentage of ectomycorrhizal fungal, morphotypes number). The response of the plant-soil system to drought was strongly dependent on the effect of local bioclimatic conditions and the influence of the agricultural matrix over soil: microcosm were functionally more resistant to drought if soils were collected from small fragments and forest edges and from a region historically more adapted to drought (i.e.

southern and drier region). On the contrary, seedling provenance barely conditioned the response of the microcosms to drought. Our results further showed that the higher percentage of ectomycorrhizal fungi found in microcosms with soils from the northern, wetter and colder region, negatively affected the response of the plant-soil system to drought, suggesting that microbial diversity might not always have a positive effect over the functioning and resistance of ecosystems to environmental perturbations. Together, our results suggest that the bio-geochemical properties of soils, resulting from the historical conditions at which they have been developed, and not the historical adaptation of plant to climate, determined the response of the plant-soil system to drought.

## Introduction

The functioning of Mediterranean ecosystems is habitually constrained by water availability, given that both plant productivity (Larcher, 2000; Sanchez-Gomez et al., 2006) and soil activity (Lavorel et al., 1998; Reichstein et al., 2002; Rambal et al., 2003; Almagro et al., 2009; Barba et al., 2013) are strongly subjected to seasonal and spatial variations of this resource. In this regard, the expected further reduction in soil water availability, due to forecasted temperature increase and rainfall reduction for this region (IPCC, 2007) is likely to be associated with strong and irreversible modifications of carbon and water cycles (Reichstein et al., 2002; Cook and Orchard, 2008; Barba et al., 2013). Actually, the increased intensity of drought in recent decades has led to a decrease in tree productivity (Ogaya and Peñuelas, 2004; Barba et al., 2013), and even to forest decline (Hereş et al., 2012) in some areas of the Mediterranean Basin, which has been already related to changes in soil microbial communities (Curiel Yuste et al., 2012) and $CO_2$ fluxes (Barba et al., 2013).

Climate change is not the only threat affecting the ecology and functioning of ecosystems in the Mediterranean Basin. Other perturbations, such as habitat fragmentation, can be considered among the most impacting human-related activities that have contributed to the historical transformation of this area of the world (Alados et al., 2004; Valladares et al., 2004; Blondel, 2010), although its effects on the ecosystem functioning have been less studied than climate related ones (i.e. drought). Besides, most studies on how forest fragmentation affects the ecology of terrestrial ecosystems have mainly focused on above-ground organisms, and only a few have paid attention to its effects over the soil system (Zheng et al., 2005; Malmivaara-Lämsä et al., 2008; Riutta et al., 2012) and its interactive effect with drought, a very important factor in Mediterranean ecosystems (Lázaro-Nogal et al., 2012; Flores-Rentería et al., 2015). These previous

studies have shown that decreasing the size of the forest fragment, as a proxy of fragmentation extent, can modulate the effects of drought by improving local environmental conditions, increasing the availability of water and nutrients for both plants and microorganisms (Flores-Rentería et al., 2015). The increased soil fertility and higher availability of water of fragmented forests has been further associated with higher rates of soil microbial activity (Chapters 2 and 3), which highlights the important role that fragmentation may have on microbial communities and their role in the ecosystem functioning (Chapters 3 and 4).

Historical precipitation regimes can influence the response to drought of plants (and even of particular provenances in the case of Q. ilex; e.g. (Gratani, 1995; Gratani et al., 2003; Pesoli et al., 2003; Gimeno et al., 2009; Andivia et al., 2012), and microbial communities (Evans and Wallenstein, 2012; Curiel Yuste et al., 2014; Evans and Wallenstein, 2014), although the simultaneous effect of drought over the response of plants, microbes and the strong interactions between plant and microbes has been poorly explored (Richard et al., 2009; Gehring et al., 2014; Flores-Rentería et al., 2015). Moreover, there is a lack of studies designed to understand how the interaction of those two important engines of global change, drought and fragmentation, may affect the ecology of Mediterranean ecosystems. The interaction of different drivers frequently generates non-additive effects that, in turn, can either attenuate or exacerbate the ecosystem responses to individual drivers (Matesanz et al., 2009; Lázaro-Nogal et al., 2012; Flores-Rentería et al., 2015).

The general objective of this study was to advance our understanding on the interactive effect of drought and forest fragmentation over the plant-soil system, and to evaluate how the provenance of plants and soils, as a proxy for historical adaptation to different environmental conditions, could affect its functioning. For this, we used a microcosm

approach with different Q. ilex provenances grown in soils from fragments with different influence of the agricultural matrix, collected in holm-oak forest located in two climatically distinct regions of the Iberian Peninsula. As there are evidences of differential functional response of different Q. ilex populations to drought (Gratani et al., 2003; Andivia et al., 2012), first, we hypothesized that seedling provenance will determine their response to drought, i.e. those from drier sites would have higher resistance to drought. Second, we postulated that the native microbial community of soils associated with seedlings will buffer the effect of drought stress on the plant-soil system, i.e. soils with original microbial propagules would have higher resistance to drought. Third, we hypothesized that the functional response of the plant-soil system to drought will be determined by the specific biogeochemical characteristics of soils derived from the bioclimatic region and the agricultural matrix influence, i.e. soils with a higher influence of the agricultural matrix, more fertile and with higher water holding capacity (Flores-Rentería et al., 2015), and historically adapted to drought (e.g. from the drier region), would resist better the simulated drought.

## Material and methods

*Soil origin*

We selected three large (> 10 ha) and five small (< 0.5 ha) forest fragments, resulting from the conversion of a continuous holm-oak (*Quercus ilex* L. ssp. ballota (Desf.) Samp) forest into a patchy agricultural landscape, in two climatically different bioclimatic regions of Spain (Fig. 1.4 in Chapter 1). The large fragments were subdivided into forest interior (>30 m from the edge) and forest edge. In the northern region (Lerma; 41°58'-42°02'N, 03°45'-03°52'W; 930 m asl), the studied fragments were within an area of 1500 ha, showed similar spatial structure and vegetation characteristics, and were separated of at least 50 m to a maximum of 11 km (Fig. 1.5a in Chapter 1). This region is characterized by 554 mm mean annual precipitation and 11 °C mean annual temperature (Ninyerola et al., 2005). The dominant tree species is the holm oak, with isolated Lusitanian oak *Q. faginea* Lam. and Spanish juniper *Juniperus thurifera* L. and understory shrubs typical of wetter and cooler supramediterranean localities (e.g. *Cistus laurifolius* L., *Genista scorpius* (L.) DC, *Thymus zygis* Loefl. ex L.; see (Santos and Tellería, 1998; Díaz et al., 1999) for further details). In the southern region (Quintanar de la Orden; 39°30'-39°35'N, 02°47'-02°59'W; 870 m asl), the studied fragments were within an area of 1000 ha, showed similar spatial structure and vegetation characteristics, and were separated of at least 50 m to a maximum of 8 km (Fig. 1.5b in Chapter 1). This region is characterized by 434 mm mean annual precipitation and 14 °C mean annual temperature (Ninyerola et al., 2005). The dominant tree is the holm oak *Q. ilex* with the understory composed of shrubby Kermes oak *Quercus coccifera* L. and shrub species typical of xeric mesomediterranean localities (e.g. *Asparagus acutifolius* L., *Cistus ladanifer* L., *Rhamnus alaternus* L., *Rhamnus lycioides* Brot.; see (Santos and Tellería, 1998; Díaz and Alonso,

2003) for further details). Both sites are characterized by a pronounced summer drought period, usually lasting from July to September (Fig. 1.7 in Chapter 1), and their climatic characteristics are representative of the mesomediterranean and supramediterranean bioclimatic zones of the Iberian Peninsula, respectively (Rivas-Martínez, 1981). The dominant soils are classified as Cambisols (calcics) (WRB, 2007), of sandy loam texture, with 17% sand, 39% silt and 44% clay for the southern region and, 11% sand, 42% silt and 47% clay for the northern region. In both regions, the original forest is now highly fragmented due to the land conversion for intensive cultivation of cereal crops, legumes, and some grapes (Santos and Tellería, 1998; Díaz et al., 1999; Díaz and Alonso, 2003). Remnants of forests have been left between crops and they are now imbibed within the active agricultural matrix, yet clearly differentiated. Main physicochemical characteristics of soils at each level of agricultural matrix influence are presented in Table S5.1.

*Soil sampling and preparation*

Soils were collected within each fragment and region at the end of the dry season from a depth of 0-15 cm, sieved (< 2 mm) *in situ*, and dried at room temperature. In each respective case, half of soil was autoclaved at 120 °C for 1h, to eliminate native resistant soil microbial propagules. We considered three factors and a total of 12 soil treatments: 1) the factor agricultural matrix influence consisted of three levels: forest interior (Fi), forest edge (Fe) and small fragment (Sf), 2) the region of two: northern (N) and southern (S), and 3) the factor native soil microbial community (coded as "native or no native microbial community") of two: presence (non-autoclaved soil) and absence (autoclaved soil) of native microbial community. Both soils were finally expected to harbor microorganisms, but our interest was put on the origin of this microbial community (see Fig. S5.1).

*Seedling provenance*

Randomly selected holm oak acorns from three different bioclimatic regions (Fig. 1.4 in Chapter 1): Galaico-Leonesa (41°56'N, 06°15'W; 730 m asl; coded as "*Gal*"), La Mancha (39°51'N, 02°58'W; 760 m asl; coded as "*Lam*"), and Sistema Iberico (39°88'N, 01°38'W; 1042 m asl; coded as "*Sib*") were planted in 300 cc cells filled with peat (45 cells per tray), in an open-air nursery at the Centro Nacional de Mejora Forestal "El Serranillo" (Guadalajara, central Spain, 40°39'N, 3°19'W), for seventeen months. These seedling provenances were selected because they were close to the regions where the studied soils were collected and because their provenance region presented a precipitation and temperature gradient. The Galaico-Leonesa zone (supramediterranean) is characterized by 756 mm mean annual precipitation and 10 °C mean annual temperature, and has predominantly cambisols humic soils; the Sistema Iberico zone (also supramediterranean) is characterized by 608 mm mean annual precipitation and 12 °C mean annual temperature, with cambisols calcic soils; La Mancha (mesomediterranean) is characterized by 446 mm mean annual precipitation and 14 °C mean annual temperature, with cambisols calcic soils (Rivas-Martínez, 1981; Jiménez et al., 1996; Ninyerola et al., 2005). A total of 120 seedlings of each provenance were grown in the respective soil origin and treatment (i.e. full factorial), with ten replicates per treatment, and kept growing in a greenhouse under controlled conditions for a year (see details below; design in Table S5.2). Seedling survival, height and diameter were recorded every two months during this acclimatization period, after which, the initial number of replicates was reduced by mortality to 3-10 depending on the treatment (Table S5.2).

*Experimental design*

The experimental design encompassed treatments seedlings and soil microbial communities historically adapted to the wettest and coldest (Galaico-Leonesa and northern region, respectively), or to the driest and warmer conditions (La Mancha and southern region). A total of 360 seedlings were transferred to individual 1600 cc microcosms (in trays with six), and each one was filled with a mixture of 1:1 (v:v) soil:substrate (2:1:1, v:v:v, peat:sand:vermiculite; autoclaved), each tray containing the three seedling provenances and one of the 12 soil treatments: northern/southern region, forest interior/edge/small fragment and presence/absence of native microbial community. Trays were randomly arranged in the greenhouse (25 °C and 40 % air humidity), regularly moved to avoid micro-site effects, and watered to field capacity based on weight loss (between 30-35% soil water content, SWC). Just after transplanting, 3 g of slow-released fertilizer were applied (NUTRICOTE, Projar, Madrid, Spain) to each microcosm. One year after the experiment set up, we applied a drought simulation defined by three experimental periods: 1) pre-drought with seedlings in well-watered pots (28-32% SWC); 2) mild-drought after 20 days at ~12-18% SWC; and 3) severe-drought after 40 days at ~4-6% SWC. For each microcosm, we determined, within three consecutive days (randomizing the treatments within each day and along the day), the variables: $CO_2$ exchange, stomatal conductance, and soil temperature, as well as photochemical efficiency that was determined at pre-drought and severe-drought. Additionally, seedling water potential and growth variables (height, diameter and biomass) were measured after severe-drought, together with mycorrhizal percentages and the number of fungal morphotypes per seedling. Soil water content was determined based on weight loss per tray, every three days.

*$CO_2$ exchange in the plant-soil system*

Each individual plant-soil microcosm was measured for net ecosystem exchange (NEE), defined as the net balance between Gross Primary Productivity (GPP) and Ecosystem Respiration ($R_{eco}$), by using a non-steady-state dynamic (closed dynamic) approach. For that purpose, a rectangular plexiglas chamber with a base of 0.01 $m^2$ and a volume of 0.004 $m^3$ with a small fan to mix the air internally was built to fit over the microcosms (see Figure S4.1 in Chapter 4). The change of $CO_2$ concentrations was measured with a $CO_2$ infrared gas analyzer (EGM-4, PP-systems, MA, USA). The chamber was covered with opaque aluminum foil to determine ecosystem respiration ($R_{eco}$) including both autotrophic (plants and mycorrizas) and heterotrophic (microorganisms) components. Respiration rates were measured in each the transparent and the opaque chamber, for 52 s. Diurnal $CO_2$ exchange measurements were taken randomly in all microcosms (11:00-17:00). NEE (transparent chamber) and $R_{eco}$ (opaque chamber) were determined by calculating the $CO_2$ increase in the closed loop in the respective measurements according to the formula (Street et al., 2007): $I_c = \frac{\rho * V * \left(\frac{dC}{dt}\right)}{A}$, where $I_c$ is the net $CO_2$ increase (μmol $m^{-2}$ $s^{-1}$), ρ is air density (mol $m^{-3}$), $V$ the chamber volume ($m^3$), $dC/dt$ represents the slope of $CO_2$ concentration increase in the chamber over time (μmol $mol^{-1}$ $s^{-1}$), and $A$ is the chamber surface area ($m^2$). Gross primary productivity (GPP) was calculated by subtracting $R_{eco}$ from NEE. The maximum difference between atmospheric ($C_a$) and internal $CO_2$ ($C_i$) was used to correct for chamber leaks using a linear equation (Pérez-Priego et al., 2010). Carbon fluxes ($R_{eco}$ and GPP) were additionally corrected for seedling size using total and foliar biomass, respectively. Additionally, soil temperature was recorded immediately after $CO_2$

exchange measurement at 10 cm depth in each microcosm by using a wireless multilogger thermometer (Omega, Connecticut, USA).

*Seedling growth and physiological variables*

Stomatal conductance (*gs*) was measured with a leaf porometer SC-I (Decagon, Pullman, USA), during the period of maximal conductance (10:00-12:00 h). Predawn and midday maximum photochemical efficiency of photosystem II ($F_v/F_m$) were measured with a portable pulse-modulated fluorometer FMS2 (Hansatech, Norfolk, UK), with leaves previously held in the leaf clip holder for 30 min. Seedling biomass (shoot, foliar, and root), height and diameter, as well as Specific Leaf Area (SLA) determined as leaf area (obtained by measuring scanned leaves) per gram dry mass, were recorded at the end of the assay.

*Mycorrhizal assessment and morphological identification*

For each seedling, the entire root was separated from shoot and divided in *old root* (within the initial peat root ball) and *new root* (newly developed in the microcosm), washed free of substrate and cut into 2-cm segments. The ectomycorrhizal (EM) percentage of each seeding was assessed on new roots by counting all single root tips under the stereo-microscope, that were classified as mycorrhizal or non-mycorrhizal according to the presence or absence of fungal mantle and mycelium and to the lack or presence of root hairs, respectively. Ectomycorrhizal percentages were obtained by dividing the number of mycorrhizal root tips by the total number of root tips. EM root tips were classified by morphotypes based on the characteristics of their mantle and extra-matrical mycelium (branching, surface color, texture, emanating hyphae and rhizomorphs; Agerer, 1997). Relative frequency (expressed as percentage) of each EM morphotype was calculated as its absolute frequency (number of seedlings in which a morphotype occurred divided by the total number of seedlings) divided by

the sum of the absolute frequencies of all morphotypes (Rincón et al., 2007). The relative abundance of an EM morphotype (expressed as percentage) was estimated by dividing the number of tips of this morphotype by the total number of tips of the morphotypes found in all seedlings (Rincón et al., 2007). Relative frequency and abundance measured respectively the presence and quantity of a given morphotype relative to all morphotypes, in the roots of seedlings of a given sub-population of the experiment.

*Data analysis*

All statistical analyses were performed with the Statistica v.10.0 Software Package (StatSoft, Inc. 2007; Tulsa OK, USA). Prior to analyses, all variables were tested for normality, and log transformations were applied to meet variance homoscedasticity when required; mycorrhizal percentages were arc-sin transformed.

The effect of the factors: "region of soils" (soils from the northern or southern regions), "matrix influence over soils" (soils with low influence from forest interior, soils with mid-influence collected from forest edges and soils with high influence collected from small fragments), "soil microbial communities" (native or no-native soil microbial communities), "seedling provenance" (provenance from *Gal, Sib* or *Lam*), and their interactions were tested on final seedling measurements (height, diameter, biomass, foliar biomass, root/shoot ratio, SLA, mycorrhizal percentages, number of morphotypes), by Analysis of Variance (ANOVA). Repeated-measures ANOVAs were used to test the effects of all factor and their interactions along the drought period, on continuously measured variables ($R_{eco}$, GPP, $g_S$, SWC, $F_v/F_m$). Subsequent repeated-measures ANOVAs were applied to explore the response within each seedling provenance or native microbial community to the factors region and matrix influence. Finally, repeated-measures ANOVAs were performed without factors that were not

significant within the overall factorial analysis (i.e. native microbial community and seedling provenance). *T*-tests were performed to analyze the differences among relative frequency of EM morphotypes. Survival was analyzed by a Peto and Peto test using $X^2$ (Pyke and Thompson, 1986). Correlations among all variables were determined by Pearson's correlation analysis. Simple linear regressions were run to analyze soil moisture sensitivity of carbon fluxes and stomatal conductance.

## Results

*Impact of drought over the plant-soil system*

As expected, drought negatively affected the functioning of the plant-soil system (i.e. $R_{eco}$, GPP, $g_S$, predawn and midday $F_v/F_m$, and SWC; Fig. 5.1; Table 5.1). The response to drought of the whole system was strongly dependent on the region of origin of soils, the degree of matrix influence over soils, and their interaction (the last except in the case of $F_v/F_m$). Contrarily, neither the seedling provenance nor the soil microbial community (i.e. native or not), or their interaction with any other factor, affected the system along the drought simulation (Table 5.1). Only in the case of SWC, the soil microbial communities had an interactive effect with drought and region, revealed as an initial (pre-drought) negative effect of native microbial communities in the southern region over this variable. Regarding the significant interactive effect of region and matrix over SWC, at pre-drought, the highest SWC values were observed in soils from the northern region in forest interior and edges that latter, at mild and severe-drought, showed the lowest values (Fig. S5.2).

Since neither seedling provenance nor soil microbial community (native or not) had significant effects on the functional response of the plant-soil system to drought, we subsequently performed the analysis only considering the factors “region of soil origin” and "soil with different influence of the agricultural matrix” (Table S5.3).

**Table 5.1** Impact of drought over the whole plant-soil system in holm oak forest soils from two climatically different regions of Spain (R), collected in fragments with different agricultural matrix influence (MI), with native or no-native microbial communities (MC), and holding different seedling provenances (Se). Data were analysed by repeated measures ANOVA with all studied factors and their interactions. Significant effects are noted in bold. $R_{eco}$= Ecosystem respiration; GPP= gross primary productivity; $g_S$= stomatal conductance; $F_v/F_m$ =photochemical efficiency (measured at pre- and final-drought); SWC= Soil water content.

| | $R_{eco}$ | | GPP | | $g_S$ | | $F_v/F_m$ Predawn | | $F_v/F_m$ Midday | | SWC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* |
| *Region (R)* | **10.40** | **<0.001** | 1.27 | 0.262 | 3.43 | 0.066 | **11.18** | **<0.001** | **6.95** | **0.009** | **34.78** | **<0.001** |
| *Matrix influence (MI)* | **8.74** | **<0.001** | 1.79 | 0.170 | **3.95** | **0.021** | **12.44** | **<0.001** | **11.67** | **<0.001** | **17.71** | **<0.001** |
| *Microbial communities (MC)* | 0.61 | 0.437 | 3.20 | 0.075 | 1.83 | 0.177 | 1.75 | 0.187 | 2.22 | 0.137 | 3.01 | 0.084 |
| *Seedling (Se)* | 0.05 | 0.952 | 1.14 | 0.322 | 0.16 | 0.851 | 1.29 | 0.276 | 0.84 | 0.432 | 0.16 | 0.852 |
| R x MI | 2.85 | 0.060 | 0.03 | 0.969 | 0.39 | 0.678 | 2.10 | 0.125 | 1.64 | 0.197 | **7.07** | **<0.001** |
| R x MC | 0.41 | 0.522 | 0.19 | 0.659 | 0.03 | 0.862 | 0.75 | 0.387 | 0.55 | 0.458 | 2.93 | 0.088 |
| MI x MC | 1.10 | 0.333 | 0.10 | 0.906 | 1.20 | 0.302 | 2.41 | 0.093 | 2.51 | 0.084 | 0.39 | 0.680 |
| R x Se | 0.19 | 0.827 | 1.15 | 0.318 | 2.02 | 0.135 | 2.34 | 0.099 | 2.36 | 0.097 | 1.69 | 0.188 |
| MI x Se | 0.15 | 0.963 | 0.19 | 0.942 | 0.63 | 0.638 | 0.12 | 0.977 | 0.11 | 0.978 | 0.85 | 0.496 |
| MC x Se | 0.08 | 0.920 | 1.17 | 0.314 | 1.84 | 0.161 | 2.71 | 0.069 | 3.53 | 0.051 | 0.84 | 0.431 |
| R x MI x MC | 0.87 | 0.419 | 2.61 | 0.076 | 2.20 | 0.113 | 1.13 | 0.327 | 1.43 | 0.242 | 1.47 | 0.232 |
| R x MI x Se | 0.12 | 0.977 | 0.41 | 0.802 | 0.39 | 0.815 | 1.51 | 0.202 | 1.28 | 0.281 | 1.03 | 0.392 |
| R x MC x Se | 0.44 | 0.646 | 1.08 | 0.340 | 0.18 | 0.831 | 1.96 | 0.143 | 1.28 | 0.281 | 0.96 | 0.384 |
| MI x MC x Se | 0.62 | 0.646 | 1.18 | 0.319 | 0.57 | 0.687 | 0.52 | 0.723 | 0.45 | 0.773 | 0.65 | 0.631 |
| R x MI x MC x Se | 0.86 | 0.491 | 0.20 | 0.939 | 1.26 | 0.286 | 1.78 | 0.134 | 2.01 | 0.095 | 0.22 | 0.929 |
| *Drought (D)* | **861.40** | **<0.001** | **372.21** | **<0.001** | **697.33** | **<0.001** | **758.62** | **<0.001** | **879.58** | **<0.001** | **10491.31** | **<0.001** |
| D x R | **5.11** | **0.006** | **9.46** | **<0.001** | **21.03** | **<0.001** | **23.08** | **<0.001** | **21.38** | **<0.001** | **85.45** | **<0.001** |
| D x MI | **3.41** | **0.009** | **5.79** | **<0.001** | **8.06** | **<0.001** | **9.68** | **<0.001** | **9.44** | **<0.001** | **27.56** | **<0.001** |
| D x MC | 1.16 | 0.315 | 1.29 | 0.276 | 0.32 | 0.729 | 0.32 | 0.573 | 0.05 | 0.827 | **4.77** | **0.009** |
| D x Se | 0.37 | 0.831 | 0.54 | 0.707 | 1.26 | 0.283 | 0.89 | 0.414 | 0.51 | 0.600 | 0.05 | 0.994 |
| D x R x MI | **2.42** | **0.048** | **4.92** | **<0.001** | **2.43** | **0.047** | 1.89 | 0.153 | 1.88 | 0.156 | **9.56** | **<0.001** |
| D x R x MC | 1.03 | 0.359 | 0.36 | 0.699 | 0.67 | 0.514 | 0.28 | 0.597 | 0.34 | 0.561 | **4.31** | **0.014** |
| D x MI x MC | 1.07 | 0.373 | 0.55 | 0.699 | 1.56 | 0.185 | 1.35 | 0.262 | 1.11 | 0.332 | 0.27 | 0.898 |
| D x R x Se | 0.31 | 0.872 | 0.74 | 0.562 | 0.14 | 0.965 | 1.85 | 0.160 | 0.65 | 0.521 | 0.89 | 0.471 |
| D x MI x Se | 0.82 | 0.587 | 0.86 | 0.547 | 1.43 | 0.184 | 0.38 | 0.826 | 0.37 | 0.832 | 0.63 | 0.749 |
| D x MC x Se | 1.92 | 0.106 | 1.08 | 0.364 | 0.21 | 0.935 | 2.43 | 0.091 | 2.29 | 0.103 | 1.68 | 0.153 |

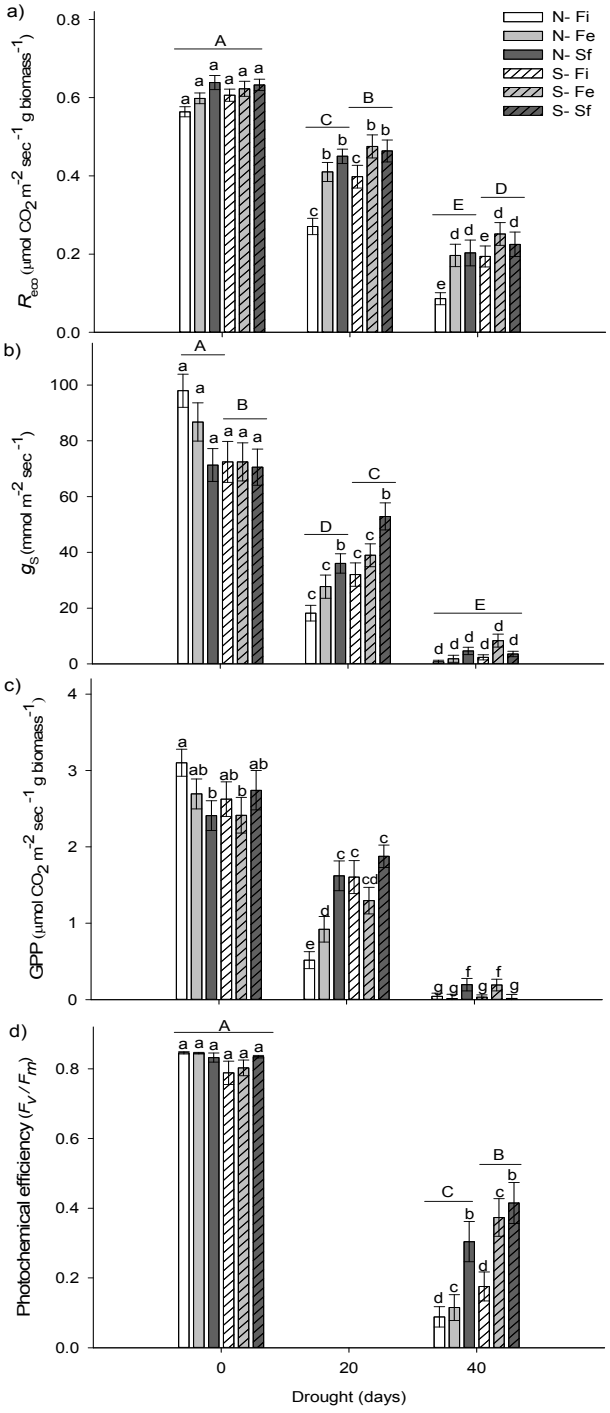


**Figure 5.1** a) Ecosystem respiration ($R_{eco}$), b) stomatal conductance ($g_S$), c) gross primary productivity (GPP), and d) photochemical efficiency ($F_v/F$m) along drought simulation in soils from fragmented holm oak forests and two climatically different regions of Spain. *Open/filled* bars are northern (N) or southern (S) region, respectively. The agricultural matrix influence is represented by different colors: white = forest interior (*Fi*; low influence), grey = forest edge (*Fe*; mid influence), dark grey = small fragments (*Sf*; high influence). Data = means ± SE. Significant differences by Tukey's test ($p$<0.05) of repeated measures ANOVA analyses are indicated: capital letters represent the interactive effect between drought x region; lower case letters represent the interactive effect between drought x matrix influence (graphics a, b, and d) and between drought x region x matrix influence (graphic c). See supplementary material Table S5.3.

The impact of both factors, soil region and matrix influence, over the plant-soil system along drought was revealed by ecosystem respiration ($R_{eco}$; Fig. 5.1a), stomatal conductance ($g_S$; Fig. 5.1b), and gross primary productivity (GPP; Fig. 5.1c), which showed a clear common pattern: at pre-drought no ($R_{eco}$ and $g_S$) or few (GPP) variation among treatments; at mild-drought higher activity in soils from the southern region ($R_{eco}$, $gs$), and especially in those from small fragments and forest edges in both regions; and at severe-drought higher activity in soils from the southern region ($R_{eco}$) and in small fragments ($R_{eco}$ and GPP) and forest edges ($R_{eco}$) in comparison with forest interiors (Fig. 5.1). These results were reinforced by those observed at severe-drought on the photochemical efficiency of plants in the system that was higher in soils from the southern region, especially in small fragments and forest edges (Fig. 5.1d; Table S5.3).

Physiological variables ($R_{eco}$, GPP, $g_S$, $F_v/F_m$) of the plant-soil system were positively correlated among them all along the drought period (Table 5.2). Significant correlations were also found among overall seedling size variables (i.e. height, diameter, SLA, biomass, root:shoot; Table 5.2); microcosm systems holding bigger seedlings (i.e. height, diameter and biomass) with lower root:shoot ratios, exhibited lower activity in plant (GPP, $g_S$, $F_v/F_m$) and soil ($R_{eco}$) components, especially at mild- and severe-drought (Table 5.2). These bigger seedlings also presented a positive relation with EM percentages and hence, EM was negatively correlated with the plant-soil system activity ($R_{eco}$, GPP, $g_S$, $F_v/F_m$; Table 5.2).

Regression analyses grouped by main factors (region, matrix influence, soil microbial community and seedling provenance), indicated that variables indicative of the system activity e.g. $R_{eco}$, GPP, $g_S$, were highly sensitive to SWC, showing global $R^2$ values of 0.6, 0.53 and 0.55, respectively, and independently of the grouping factor (i.e. region, matrix influence, soil microorganism, seedling provenance; Table S5.4).

**Table 5.2** Correlations between indicative variables of the plant-soil system performance along drought, and structural traits of seedlings grown in soils from two climatically different regions of holm oak fragmented forest in Spain. Significant Pearson's correlation ($p$<0.05) are highlighted in bold. $R_{eco}$= ecosystem respiration; GPP= Gross Primary Productivity; $g_S$=stomatal conductance; Pre/Mid $F_v/F_m$ = predawn and midday photochemical efficiency, respectively; SWC= soil water content; SLA=Specific leaf area; EM= Ectomycorrhizal. 0= pre-drought; 20= mild-drought; 40= severe-drought.

| | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) | (13) | (14) | (15) | (16) | (17) | (18) | (19) | (20) | (21) | (22) | No. EM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $R_{eco}$- 0 | **0.29** | **-0.17** | -0.05 | -0.07 | **-0.21** | **0.68** | **0.36** | **0.22** | **0.29** | **0.53** | **0.28** | **0.19** | **0.45** | **0.44** | **0.41** | **-0.45** | **-0.20** | **0.26** | **-0.71** | **-0.50** | **0.47** | **-0.33** | **-0.22** |
| (1) GPP -0 | | **0.52** | **0.24** | **0.29** | **0.24** | **-0.50** | **0.18** | **-0.19** | **-0.34** | **-0.36** | **0.17** | **-0.26** | **-0.47** | **-0.47** | **-0.49** | **0.33** | **0.16** | **-0.16** | **0.29** | **0.44** | **-0.17** | 0.06 | **-0.14** |
| (2) $g_S$ -0 | | | **0.30** | **0.37** | **0.20** | **-0.29** | -0.01 | 0.08 | **-0.28** | **-0.31** | -0.05 | **-0.14** | **-0.32** | **-0.33** | **-0.42** | **0.32** | **0.20** | -0.10 | **0.26** | **0.39** | **-0.19** | -0.02 | 0.11 |
| (3) Pre $F_v/F_m$ -0 | | | | **0.92** | **0.18** | -0.07 | 0.12 | **0.20** | -0.04 | **-0.15** | 0.04 | 0.02 | 0.03 | 0.03 | **-0.24** | 0.09 | 0.06 | 0.04 | **0.15** | **0.15** | -0.05 | 0.06 | 0.07 |
| (4) Mid $F_v/F_m$ -0 | | | | | **0.19** | **-0.14** | 0.09 | **0.20** | -0.08 | **-0.22** | 0.03 | 0.01 | -0.02 | -0.03 | **-0.31** | **0.15** | 0.08 | 0.03 | **0.20** | **0.23** | -0.07 | 0.05 | 0.11 |
| (5) SWC -0 | | | | | | **-0.34** | **-0.31** | **-0.39** | **-0.18** | **-0.19** | -0.04 | -0.11 | **-0.22** | **-0.23** | **-0.52** | 0.06 | 0.09 | -0.06 | **0.10** | **0.15** | -0.03 | 0.02 | 0.05 |
| (6) $R_{eco}$- 20 | | | | | | | **0.65** | **0.41** | **0.52** | **0.59** | **0.30** | **0.41** | **0.68** | **0.65** | **0.61** | **-0.51** | **-0.21** | **0.25** | **-0.69** | **-0.62** | **0.41** | **-0.28** | **-0.21** |
| (7) GPP -20 | | | | | | | | **0.65** | **0.35** | **0.23** | **-0.20** | **0.33** | **0.52** | **0.51** | **0.30** | **-0.27** | -0.08 | 0.10 | **-0.45** | **-0.39** | 0.08 | **-0.20** | **0.14** |
| (8) $g_S$ -20 | | | | | | | | | **0.19** | 0.12 | 0.12 | **0.29** | **0.42** | **0.42** | **0.26** | **-0.23** | -0.11 | 0.02 | **-0.27** | **-0.31** | 0.01 | **-0.14** | -0.11 |
| (9) SWC -20 | | | | | | | | | | **0.39** | **0.22** | **0.44** | **0.57** | **0.57** | **0.69** | **-0.18** | **-0.23** | 0.08 | **-0.33** | **-0.36** | **0.22** | -0.07 | **-0.21** |
| (10) $R_{eco}$- 40 | | | | | | | | | | | **0.29** | **0.22** | **0.41** | **0.39** | **0.51** | **-0.41** | **-0.23** | **0.21** | **-0.54** | **-0.45** | **0.48** | **-0.20** | **-0.14** |
| (11) GPP -40 | | | | | | | | | | | | **0.35** | **0.34** | **0.34** | **0.18** | **-0.23** | -0.12 | **0.17** | **-0.25** | **-0.20** | **0.42** | 0.01 | -0.03 |
| (12) $g_S$ -40 | | | | | | | | | | | | | **0.67** | **0.68** | **0.39** | **-0.24** | **-0.15** | 0.09 | **-0.33** | **-0.37** | **0.15** | -0.04 | **-0.14** |
| (13) Pre $F_v/F_m$ -40 | | | | | | | | | | | | | | **0.97** | **0.57** | **-0.43** | **-0.21** | **0.23** | **-0.58** | **-0.56** | **0.31** | **-0.19** | **-0.18** |
| (14) Mid $F_v/F_m$ -40 | | | | | | | | | | | | | | | **0.59** | **-0.42** | **-0.20** | **0.21** | **-0.56** | **-0.55** | **0.28** | **-0.15** | **-0.17** |
| (15) SWC -40 | | | | | | | | | | | | | | | | **-0.31** | **-0.18** | **0.13** | **-0.40** | **-0.46** | **0.26** | **-0.14** | **-0.25** |
| (16) Height | | | | | | | | | | | | | | | | | **0.22** | **-0.25** | **0.58** | **0.61** | **-0.44** | **0.27** | 0.07 |
| (17) Diameter | | | | | | | | | | | | | | | | | | **-0.17** | **0.24** | **0.43** | **-0.44** | 0.06 | 0.09 |
| (18) SLA | | | | | | | | | | | | | | | | | | | **-0.26** | **-0.15** | **0.30** | -0.03 | -0.07 |
| (19) Foliar biomass | | | | | | | | | | | | | | | | | | | | **0.76** | **-0.43** | **0.33** | **0.31** |
| (20) Biomass | | | | | | | | | | | | | | | | | | | | | **-0.27** | **0.25** | **0.24** |
| (21) Root:shoot | | | | | | | | | | | | | | | | | | | | | | **-0.15** | -0.09 |
| (22) EM percentage | | | | | | | | | | | | | | | | | | | | | | | **0.15** |

*Response of microbial communities to drought*

Separated analyses for soils with native and no-native microbial communities, revealed that the interactive effect of the influencing factors soil region and matrix influence along drought differed according to the microbial communities (Table 5.3). The influence of the agricultural matrix affected the response of the system on $R_{eco}$ (both native and no- native microbial communities), GPP (native microbial communities), $g_S$ (both native and no-native microbial communities) and $F_v/F_m$ (no-native microbial communities; Table 5.3), with higher values in soils from the southern region and in small fragments and forest edges. The region of origin of soils affected the response to drought of both systems with or without native microbial communities, measured as GPP, $g_S$ and $F_v/F_m$ (Table 5.3). Only in the case of $R_{eco}$ the influence of soils region was just evident in microcosms with native microbial communities (Table 5.3), i.e. higher values of $R_{eco}$ in soils from the southern region; whereas systems with no-native microbial communities had not significant influence of the soil region.

**Table 5.3** Impact of drought simulation on the plant-soil system, separately analysed for each native or no-native microbial communities by repeated measures ANOVA. Soils were collected in holm oak forests from two climatically different regions of Spain (R), in fragments with different agricultural matrix influence (MI). Significant effects are noted in bold. $R_{eco}$= Ecosystem respiration; GPP= gross primary productivity; $g_S$= stomatal conductance; $F_v/F_m$ =photochemical efficiency (measured at pre- and final-drought); SWC= Soil water content.

| | $R_{eco}$ | | GPP | | $g_S$ | | $F_v/F_m$ Predawn | | $F_v/F_m$ Midday | | SWC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* |
| **Native microbial communities** | | | | | | | | | | | | |
| *Region(R)* | **11.54** | **<0.001** | 0.11 | 0.738 | 1.46 | 0.230 | 2.29 | 0.133 | 1.25 | 0.266 | **9.20** | **0.003** |
| *Matrix influence (MI)* | **5.54** | **0.005** | 0.56 | 0.571 | **4.83** | **0.010** | **5.80** | **0.004** | **6.07** | **0.003** | **10.68** | **<0.001** |
| R x MI | 2.52 | 0.085 | 2.36 | 0.099 | 2.06 | 0.132 | **3.26** | **0.042** | 2.92 | 0.058 | **9.43** | **<0.001** |
| *Drought (D)* | **546.00** | **<0.001** | **263.55** | **<0.001** | **435.59** | **<0.001** | **456.61** | **<0.001** | **510.37** | **<0.001** | **5957.99** | **<0.001** |
| D x R | **5.75** | **0.004** | **5.40** | **0.005** | **9.16** | **<0.001** | **8.19** | **0.005** | **8.17** | **0.005** | **58.27** | **<0.001** |
| D x MI | **2.97** | **0.020** | **7.70** | **<0.001** | **5.90** | **<0.001** | 2.45 | 0.091 | 2.46 | 0.089 | **16.20** | **<0.001** |
| D x R x MI | 1.39 | 0.240 | **6.94** | **<0.001** | 2.16 | 0.074 | 1.55 | 0.217 | 1.23 | 0.297 | **8.32** | **<0.001** |
| **No-native microbial communities** | | | | | | | | | | | | |
| *Region(R)* | 2.67 | 0.105 | 1.36 | 0.246 | 2.33 | 0.130 | **7.06** | **0.009** | **4.90** | **0.029** | **23.45** | **<0.001** |
| *Matrix influence (MI)* | **5.48** | **0.005** | 1.85 | 0.163 | 0.48 | 0.623 | **8.53** | **<0.001** | **8.45** | **<0.001** | **12.83** | **<0.001** |
| R x MI | 1.95 | 0.148 | 1.15 | 0.322 | 1.03 | 0.362 | 0.07 | 0.934 | 0.13 | 0.875 | 0.93 | 0.396 |
| *Drought (D)* | **410.11** | **<0.001** | **160.38** | **<0.001** | **338.32** | **<0.001** | **359.36** | **<0.001** | **452.85** | **<0.001** | **5780.60** | **<0.001** |
| D x R | 1.56 | 0.212 | **4.96** | **0.008** | **9.92** | **<0.001** | **12.90** | **<0.001** | **13.38** | **<0.001** | **36.54** | **<0.001** |
| D x MI | **2.53** | **0.041** | 1.70 | 0.152 | **5.79** | **<0.001** | **8.39** | **<0.001** | **9.24** | **<0.001** | **17.27** | **<0.001** |
| D x R x MI | 1.18 | 0.322 | 1.73 | 0.146 | 1.26 | 0.285 | 0.62 | 0.539 | 0.86 | 0.426 | **4.24** | **0.003** |

The ectomycorrhizal percentage of seedlings was unaffected by either the region or the matrix influence, but it was tightly dependent on seedling provenance and soil microbial communities (Fig. 5.2; Table S5.5). Higher mycorrhizal percentages were found in *Gal* than in *Sib* seedlings, and when they grew in soils with native microbial communities compared with those without native microorganisms (Fig. 5.2b; Table S5.5). A total of ten EM fungal morphotypes were described according to their morphological characteristics (Figs. 5.2c and 2d, S5.3; Table S5.6). The mean number of fungal morphotypes per seedling was affected by the interaction between region and matrix influence (Figure 5.2c; Table S5.5), with general higher values found in soils from the northern region compared with the southern one, and particularly in forest interiors in the northern region compared with forest edges and small fragments (Fig. 5.2c). Contrarily, the mean number of fungal morphotypes was unaffected either by the presence of native microbial communities or by the provenance of seedlings (Fig. 5.2d; Table S5.5).

When analyzed per treatment, the frequency/abundance morphotype profiles showed a dominant morphotype M01 across all treatments, followed by fungi M02 and M05 (Fig. 5.3; Fig. S5.3). Also, morphotypes M03, 06 and 09 are present only in soils with native microbial communities. When comparing between regions (Fig. 5.3a,c), the morphotype profiles revealed that soils from the northern region were more diverse than those from the southern one, with 7 and 4 fungal morphotypes in average, respectively (*t*-value= 5.19; Fig. 5.3a,c). Similarly, the soils with native microbial communities were more diverse than those with no-native microbial communities, with 7.3 and 4.6 morphotypes in average, respectively (*t*-value= 2.83; Fig. 5.3b,d).

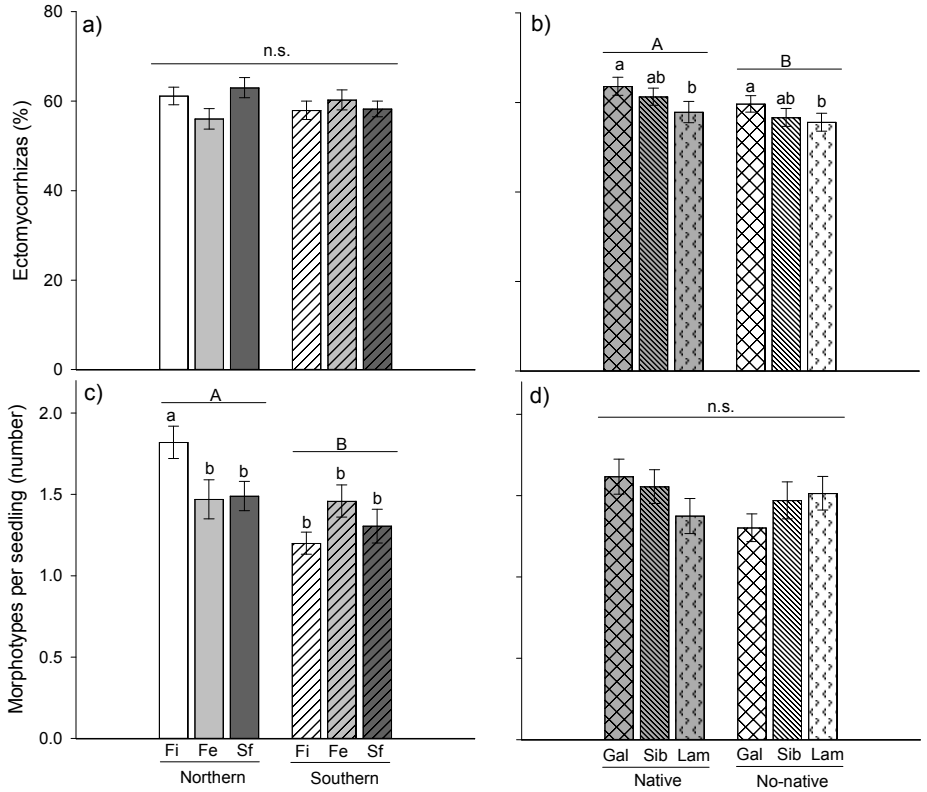


**Figure 5.2** Ectomycorrhizal percentages (a,b) and number of morphotypes (c,d) on seedlings grown in soils from fragmented holm oak forests and two climatically different regions of Spain, under drought. Effects of factors: region and matrix influence (a,c), where open/filled bars are northern or southern region, respectively; agricultural matrix influence is represented by different colors: white = forest interior (*Fi*; low influence), grey = forest edge (*Fe*; mid influence), dark grey = small fragments (*Sf*; high influence). Effects of factors: microbial communities and seedling provenance (b,d), where grey/white bars represent native or no native microbial communities, respectively; seedling provenance is represented by different fill patterns, Gal= Galaico-Leonesa, Sib = Sistema Iberico Lam = La Mancha. Data = means ± SE. ANOVA analyses and significant differences by Tukey's test ($p$<0.05), different lower case letters indicate differences among agricultural matrix index (a,c) or seedling provenance (b,c), while different capital letters indicate differences between region (a, c) or microbial communities (b, d). ns = not significant. See supplementary material Table S5.5.

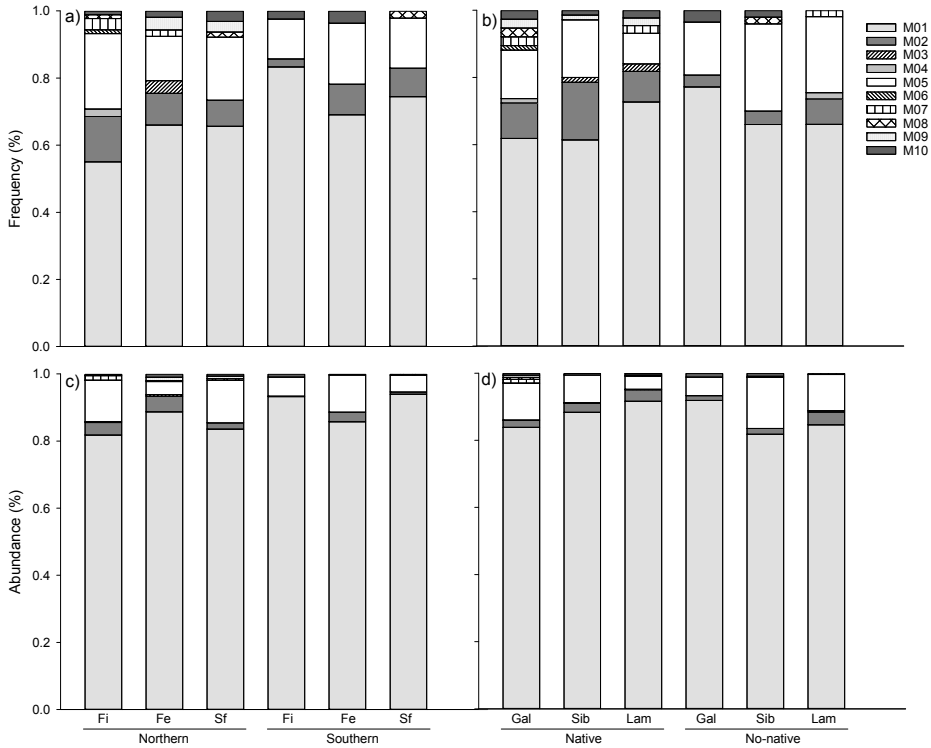


**Figure 5.3** Relative frequency (a,b) and abundance (c,d) profiles of fungal morphotypes found forming ectomycorrhizas with seedlings grown in soils of fragmented holm oak forests from two climatically different regions of Spain, under drought; profiles grouped by region and matrix influence (a,c) or by microbial communities and seedling provenances (b,d). Agricultural matrix influence: Fi= forest interior, Fe= forest edge, Sf= small fragment; seedling provenance: Gal= Galaico-Leonesa, Sib= Sistema Iberico, Lam= La Mancha.

*Response of holm oak seedlings to drought*

Seedling survival was highly affected by the region of the soils and the influence of the agricultural matrix, with general higher survival rates of seedlings in soils from the southern region than the northern one, and in microcosms with soils from areas with higher agricultural matrix influence (i.e. higher survival at small fragments, then forest edges, and lower at forest interiors; Fig. 5.4; Table S5.7).

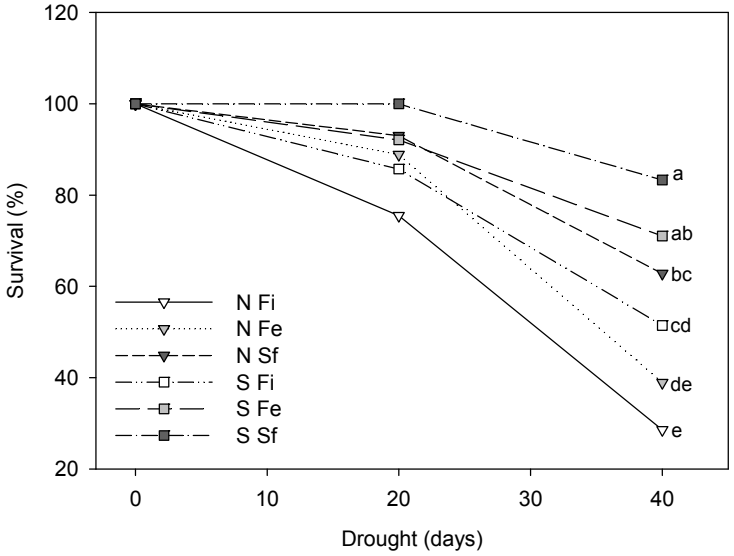


**Figure 5.4** Survival, along drought simulation, of seedlings grown in soils of fragmented holm oak forests from two climatically different regions of Spain. Inverted triangles and squares are northern (N) and southern (S) region, respectively. The agricultural matrix influence is represented by different colors: white = forest interior (*Fi*; low influence), grey = forest edge (*Fe*; mid influence), dark grey = small fragments (*Sf*; high influence). Significant differences among treatments, indicated by different letters, were separated by Peto and Peto $X^2$ analyses, see supplementary material Table S5.7.

Contrary to the studied functional traits ($R_{eco}$, GPP, $g_S$, $F_v/F_m$), results from factorial ANOVA indicates that plant morphological traits were barely affected by the soil region or the matrix influence, but mostly by the provenance of the seedling and/or the microbial community (Table S5.5). Only the Specific Leaf Area (SLA) was influenced by the interaction of soil region x microbial community (native vs non-native), and matrix influence x microbial community x seedling provenance (Table S5.5), although the Tukey's test did not separate significant differences among groups. The root:shoot ratio was dependent on seedling provenance with higher values observed for *Gal* and *Lam* compared with *Sib* seedlings (Fig. S5.4; Table S5.5). Biomass was affected by the interaction of seedling provenance and microbial communities (Table S5.5), with the lowest biomass observed for

Sib seedlings grown in soils with native microbial communities. Seedling height, diameter, and foliar biomass were not affected by any factor (Table S5.5). Seedling morphological traits displayed a high variation, reflected in great standard error values (Fig. S5.4; Table S5.8).

Additionally, for each seedling provenance, the functional traits of the plant-soil system along drought were dissimilarly affected by the influencing factors (i.e. soil region and matrix influence) (Table 5.4); systems with *Gal* and *Lam* seedlings were consistently affected by region and matrix influence along drought in all (*Gal*) or most (*Lam*, except for $R_{eco}$, and GPP affected only by matrix influence) functional traits (Table 5.4), with higher values of $R_{eco}$, GPP, $g_S$ and $F_v/F_m$ observed in small fragments and forest edges and in the southern region along drought; whereas the response to drought with *Sib* seedlings was only conditioned by region (Table 5.4), with higher values of GPP, $g_S$ and $F_v/F_m$ in the southern region along drought, while $R_{eco}$ was unaffected, and $g_S$ was also affected by the matrix influence (Table 5.4), exhibiting the highest values in small fragments and under mild-drought conditions.

**Table 5.4** Impact of drought simulation on the plant-soil system, separately analysed for each seedling provenance by repeated measures ANOVA. Soils were collected in holm oak forests from two climatically different regions of Spain (R), in fragments with different agricultural matrix influence (MI). Significant effects are noted in bold. $R_{eco}$= Ecosystem respiration; GPP= gross primary productivity; $g_S$= stomatal conductance; $F_v/F_m$ =photochemical efficiency (measured at pre- and final-drought); SWC= Soil water content.

| | $R_{eco}$ | | GPP | | $g_S$ | | $F_v/F_m$ Predawn | | $F_v/F_m$ Midday | | SWC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* |
| **Galaico-Leonesa** | | | | | | | | | | | | |
| *Region(R)* | 3.47 | 0.066 | 0.75 | 0.389 | 1.65 | 0.202 | 2.37 | 0.127 | 1.33 | 0.253 | 3.30 | 0.073 |
| *Matrix influence (MI)* | 2.79 | 0.067 | 0.81 | 0.448 | 0.43 | 0.651 | **4.91** | **0.010** | **4.68** | **0.012** | **10.36** | **<0.001** |
| R x MI | 2.39 | 0.098 | 0.67 | 0.516 | 0.13 | 0.882 | 0.60 | 0.551 | 0.49 | 0.612 | **5.05** | **0.008** |
| *Drought (D)* | **329.38** | **<0.001** | **154.08** | **<0.001** | **320.68** | **<0.001** | **319.87** | **<0.001** | **323.31** | **<0.001** | **3708.5** | **<0.001** |
| D x R | **3.43** | **0.035** | **6.80** | **<0.001** | **10.29** | **<0.001** | **4.17** | **0.044** | **5.32** | **0.024** | **26.17** | **<0.001** |
| D x MI | **3.03** | **0.019** | **4.10** | **0.003** | **3.39** | **0.011** | **5.57** | **0.005** | **6.16** | **0.003** | **11.89** | **<0.001** |
| D x R x MI | 1.94 | 0.106 | **6.56** | **<0.001** | 0.82 | 0.517 | 0.72 | 0.491 | 0.68 | 0.511 | **5.91** | **<0.001** |
| **Sistema Iberico** | | | | | | | | | | | | |
| *Region(R)* | 3.98 | 0.050 | 0.73 | 0.395 | 0.27 | 0.605 | 0.11 | 0.740 | 0.02 | 0.881 | **15.02** | **<0.001** |
| *Matrix influence (MI)* | 2.68 | 0.075 | 1.41 | 0.250 | 2.90 | 0.061 | **4.51** | **0.014** | **5.16** | **0.008** | **3.86** | **0.026** |
| R x MI | 0.84 | 0.438 | 0.01 | 0.986 | 0.43 | 0.655 | 2.51 | 0.088 | 2.32 | 0.105 | 2.24 | 0.114 |
| *Drought (D)* | **308.84** | **<0.001** | **138.06** | **<0.001** | **217.54** | **<0.001** | **278.75** | **<0.001** | **322.30** | **<0.001** | **4010.4** | **<0.001** |
| D x R | 1.79 | 0.171 | **4.53** | **0.012** | **3.65** | **0.028** | **4.44** | **0.039** | **5.33** | **0.024** | **36.73** | **<0.001** |
| D x MI | 1.54 | 0.193 | 1.62 | 0.172 | **3.36** | **0.012** | 1.65 | 0.200 | 1.84 | 0.166 | **10.33** | **<0.001** |
| D x R x MI | 1.40 | 0.237 | 1.68 | 0.159 | 1.19 | 0.318 | 1.90 | 0.158 | 0.91 | 0.407 | **3.11** | **0.017** |
| **La Mancha** | | | | | | | | | | | | |
| *Region(R)* | 3.80 | 0.056 | 3.15 | 0.081 | **7.50** | **0.008** | **10.08** | **0.002** | **8.15** | **0.006** | **24.48** | **<0.001** |
| *Matrix influence (MI)* | **3.83** | **0.027** | 0.31 | 0.731 | 2.45 | 0.095 | **4.25** | **0.019** | **3.31** | **0.043** | **9.88** | **<0.001** |
| R x MI | 0.42 | 0.660 | 0.09 | 0.911 | 1.15 | 0.324 | 2.19 | 0.120 | 1.81 | 0.172 | 2.84 | 0.066 |
| *Drought (D)* | **268.25** | **<0.001** | **104.16** | **<0.001** | **202.19** | **<0.001** | **194.46** | **<0.001** | **283.32** | **<0.001** | **3294.2** | **<0.001** |
| D x R | 0.94 | 0.392 | 1.24 | 0.294 | **5.49** | **0.005** | **16.01** | **<0.001** | **12.53** | **<0.001** | **27.10** | **<0.001** |
| D x MI | 0.85 | 0.494 | **2.72** | **0.033** | **5.86** | **<0.001** | **4.07** | **0.022** | **3.30** | **0.043** | **9.83** | **<0.001** |
| D x R x MI | 0.75 | 0.563 | 0.62 | 0.651 | 1.06 | 0.382 | 0.85 | 0.434 | 1.07 | 0.350 | **2.52** | **0.045** |

## Discusion

*Impact of drought over the whole plant-soil system*

All functional indicators of the entire plant-soil system (i.e. $R_{eco}$, GPP and $g_S$) were strongly affected by drought, regardless of the studied factors (i.e. region, matrix influence, microbial communities, and seedling provenance), which might be explained by the strong relationship between the functioning of all different ecological compartments under study (plants and soil microbial communities) and soil water availability (Orchard and Cook, 1983; Reichstein et al., 2002; Barba et al., 2013). The responsiveness of the plant-soil system to drought was different depending on the original bioclimatic conditions of soils and on the influence of the agricultural matrix on them, with a better resistance to drought of plant-soil systems with soils from small fragments and forest edges (i.e. $R_{eco}$, GPP, photochemical efficiency at mild/severe drought, and $g_S$ at mild drought) and from the southern warmer and drier region (i.e. $R_{eco}$, photochemical efficiency at mild/severe drought, and $g_S$ at mild drought). Both, the degree of fragmentation and the soil origin had also a profound repercussion on the survival of seedlings (i.e. higher survival in small and forest edges, especially in soils from the southern region), being less dependent on the seedling provenance or the presence of the native microbial community. These results suggest that the modulation of drought effects over the plant-soil system was mainly determined by the particular biogeochemical characteristics of soils associated with the bioclimatic region and the agricultural matrix, supporting our third hypothesis. As we have previously found in these study systems, the physicochemical characteristics of soils largely depend on the influence of the surrounding agricultural matrix, with wetter, more organic and fertile soils found in small fragments and forest edges (Flores-Rentería et al., 2015) (Chapter 2 and 3), whereas the

bioclimatic region of soils, additionally to its effect over certain biophysical characteristics (i.e. basic pH and higher $K^+$ on southern region) also influenced the microbial tolerance to drought (Flores-Rentería et al., 2015). In fact, the GPP exhibited a different response to the agricultural matrix influence depending on the particular bioclimatic conditions (e.g. the matrix only influenced GPP in soils from the northern region at mild-drought).

*Response of microbial communities to drought and its effect over the ecosystem functioning*

The lack of significant effect of the presence of native soil microbial communities on the plant-soil functioning could be due to the overriding effect of the soil physicochemical characteristics, although we cannot discard other factors such as an insufficient amount of soil inoculum in the microcosm.

When we focused in particular microbial communities (i.e. ectomycorrhizal association, EM), EM seedlings percentages were influenced by both factors native microbial communities and seedling provenance, with higher values in soils with native microbial communities, and on *Gal* provenance compared with *Lam* seedlings. The increased EM percentages associated with the native microbial communities, suggest an stimulation of lateral root tip formation (e.g. via hormones, (Rincón et al., 2003) making more sites available for fungal colonization. On the other hand, the different EM percentages depending on seedling provenance could be related with host compatibility issues, or with the different carbon economy of seedlings, that destined their carbon gain differentially to survival, growth or/and the maintenance of EM symbiosis. The increased EM fungal diversity, in soil with native microorganisms of the northern region points out to these particular EM fungi as competitive enough to

surpass priority effects of fungi already established in the nursery (Rincón et al., 2014).

However, despite these differences, only the differences in EM diversity (i.e. morphotypes per seedling) and morphotype frequency, both associated with the region of soil origin (north and south) appeared to determine the response of the plant-soil-microbial system to drought.

Although seedling growth was positively correlated with EM percentages (height, foliar and total biomass) and diversity (foliar and total biomass), plant-soil microcosms with more diverse EM fungal profiles (i.e. northern region) showed the lowest physiological performance under drought ($R_{eco}$, $F_v/F_m$), which together with negative correlations between physiological traits ($R_{eco}$, GPP, $g_S$, $F_v/F_m$) and EM percentages or diversity, suggests a rather negative impact of EM abundance and diversity on the functioning of the whole system. There has been proposed a mutualism-parasitism continuum considering parasitic mycorrhizal fungi on plants when net cost of the symbiosis exceeds net benefit (Johnson et al., 1997). This continuum can be affected by both biotic and abiotic environmental factors (Johnson et al., 1997), but also by the fungal genotype and ecotype (Mandyam and Jumpponen, 2015), our results suggesting a shift in the mutualistic relation explained by both the dominance of an opportunistic nursery EM fungal morphotype, and/or the stressful drought conditions of the system.

*Response of holm oak seedlings to drought*

Our results showed that, despite the significant reduction in photosynthetic efficiency and gross primary productivity, *Q. ilex* seedlings, regardless of their provenance, were able to maintain carbon gain under low water availability (mild drought, ~12-18% SWC), a stress-tolerance strategy previously reported for this species (Corcuera et al., 2005; Valladares et al.,

2008; Gimeno et al., 2009), which can be a particularly important trait to cope with unpredictable heat waves (Ghouil et al., 2003; Gimeno et al., 2009). We found not enough support for our initial hypothesis that seedling provenance would influence the response to drought of the plant-soil system, since only two morphological traits (i.e. biomass, root:shoot ratio) were dependent on this factor, whereas their physiology or survival rates were unaffected. However, our experimental design was likely limiting in capturing differences attributable to adaptive features of seedlings driven by the climate of the locality from which they originated, as it has been repeatedly observed for *Q. ilex* in other studies (Gratani, 1995; Gratani et al., 2003; Pesoli et al., 2003; Andivia et al., 2012), but instead revealed an overriding effect of soil properties, dependent on both the region (i.e. bioclimatic conditions) and the agricultural matrix influence (i.e. fragmentation).

## Conclusions

The influence of the agricultural matrix over the soil physicochemical characteristics, depending on the local bioclimatic conditions, could modulate to some extend the functional response of the plant-soil system to drought stress. Besides, the local bioclimatic conditions of soils could control the adaptation to drought of the native microbial communities, which in turn influenced the functional response of the soil system to drought. Despite of the positive effect of native soil microbial communities on ectomycorrhizal percentages and fungal diversity, both negatively impacted the response of the plant-soil system to drought. Expected seedling provenance effects were probably overridden by the strong influence of the factors agricultural matrix influence and region, suggesting that the soil physicochemical characteristics were more influent on the response of the plant-soil system to drought.

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## Supplementary material

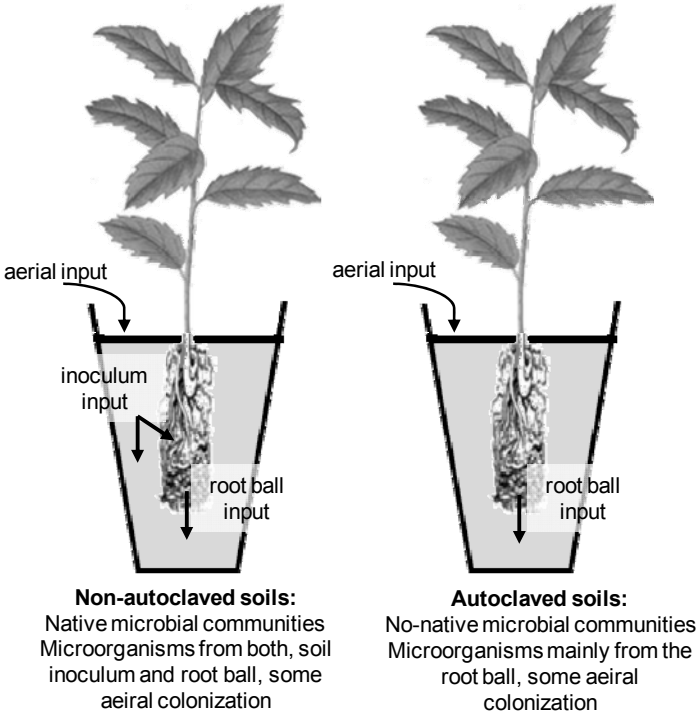


**Figure S5.1** Contribution to the total microbial community established by the autoclaving of soils. Non-autoclaved soils would have a high contribution of the microbial community from the external inoculum, denominated *native microbial community*, with scarce aerial colonization; whereas the autoclaved soils would have a microbial community predominantly spread from the seedling root ball and with more probable aerial colonization called *no-native microbial community*.

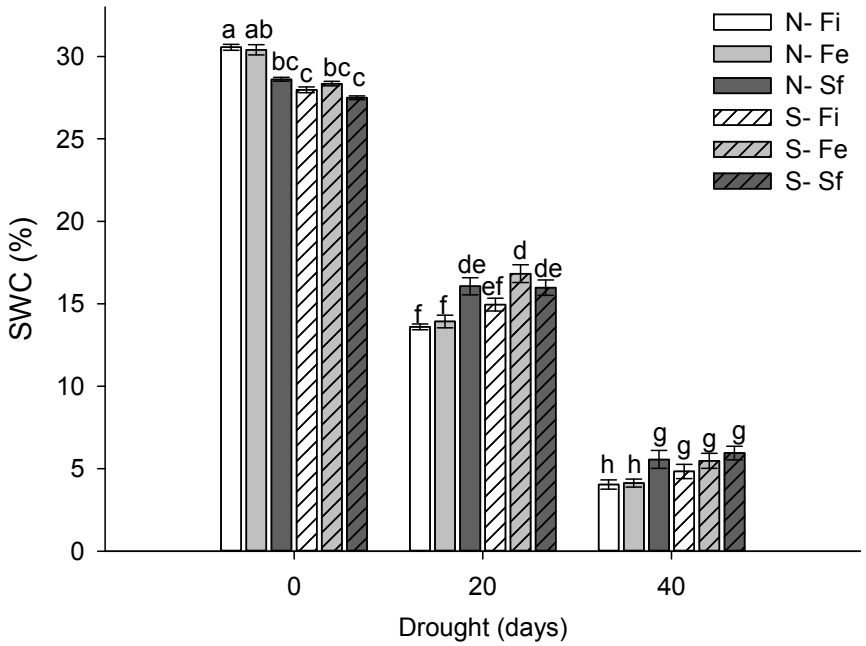


**Figure S5.2** Soil water content (SWC) along drought simulation in soils of fragmented holm oak forests from two climatically different regions of Spain. *Open/filled* bars are northern (N) and the southern (S) region, respectively. Agricultural matrix influence is represented by different colors: white = forest interior (*Fi*; low influence), grey = forest edge (*Fe*; mid influence), dark grey = small fragments (*Sf*; high influence). Data = means ± SE. Repeated measures ANOVA analyses and significant differences separated by Tukey's test ($p$<0.05): different letters indicate a different interactive effect between drought x matrix influence x region. See supplementary material Table S5.1.


**Figure S5.3** Most representative ectomycorrhizal fungal morphotypes a) not mycorrhizal root tip, b) M01, c) M02 and d) M05, of seedlings grown in holm oak forest soils from two climatically different regions of Spain, under drought. See Table S5.6 for a full description.

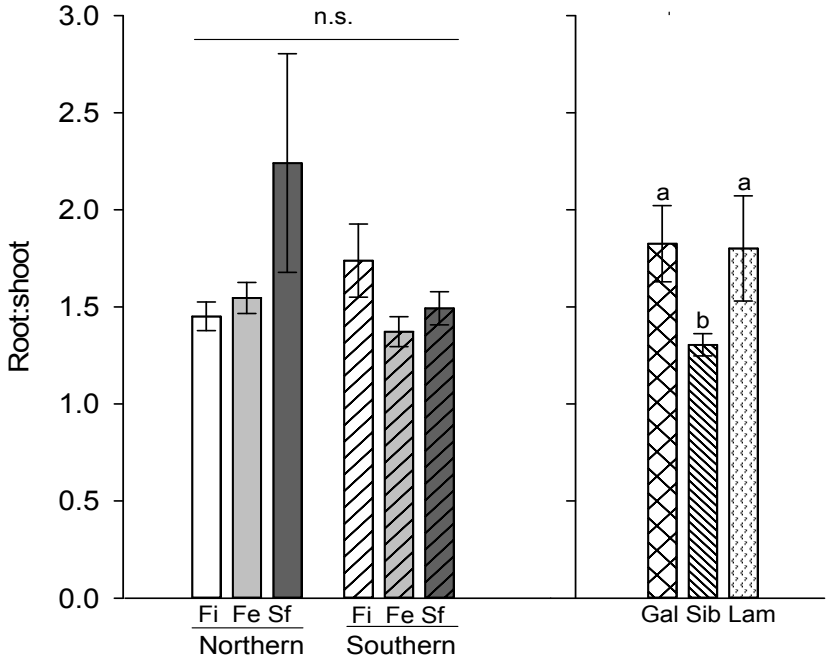


**Figure S5.4** Final root:shoot ratio of seedlings grown in soils of fragmented holm oak forests from two climatically different regions of Spain, under drought. Left: open/filled bars represent northern (N) or southern (S) region, respectively. The agricultural matrix influence is represented by different colors: white = forest interior (*Fi*; low influence), grey = forest edge (*Fe*; mid influence), dark grey = small fragments (*Sf*; high influence). Right: seedling provenance is represented by different fill patterns (*Gal* = Galaico-Leonesa; *Sib* = Sistema Iberico; *Lam* = La Mancha). Data = means ± SE. ANOVA analysis and significant differences by Tukey's test ($p$<0.05), significant differences among seedling provenances are represented by different letters. See supplementary material Table S5.5.

**Table S5.1** Summary of the physicochemical characteristics of soils from fragmented holm oak forests. Data = mean±SE. Elaborated from Flores-Rentería et al. (2015).

| | Northern | | | Southern | | |
|---|---|---|---|---|---|---|
| | **Forest interior** | **Forest edge** | **Small fragments** | **Forest interior** | **Forest edge** | **Small fragments** |
| **SWC (%)** | 31.33 ± 1.22 | 46.41 ± 1.4 | 49.84 ± 2.17 | 37.86 ± 0.85 | 39.65 ± 1.34 | 45.17 ± 1.63 |
| **SOM (%)** | 5.5 ± 0.47 | 16.84 ± 1.88 | 19.84 ± 2.39 | 9.32 ± 1.12 | 11.00 ± 1.12 | 14.09 ± 1.75 |
| **pH** | 6.36 ± 0.41 | 7.69 ± 0.06 | 7.32 ± 0.11 | 7.90 ± 0.04 | 7.83 ± 0.05 | 7.87 ± 0.04 |
| **Organic N (%)** | 0.23 ± 0.01 | 0.58 ± 0.07 | 0.78 ± 0.12 | 0.29 ± 0.01 | 0.29 ± 0.03 | 0.46 ± 0.07 |
| **$P_2O_5$ (mg $Kg^{-1}$)** | 12.32 ± 1.75 | 14.91 ± 1.22 | 34.39 ± 4.21 | 2.93 ± 0.57 | 7.79 ± 1.25 | 16.47 ± 1.68 |
| **$K^+$ (mg·$Kg^{-1}$)** | 170.8 ± 32.8 | 462.8 ± 55.2 | 529.9 ± 38.9 | 532.9 ± 69.3 | 556.24 ± 67.6 | 791.48 ± 55.8 |

**Table S5.2** Number of plant-soil microcosms with soils collected at two locations (Northern/Southern), in fragmented forests (Fi= forest interior, Fe= forest edge, Sf= small fragment), with native or no-native microbial communities (MC), and with different seedling provenances (Gal= Galaico Leonesa; Sib= Sistema Iberico; Lam= La Mancha), one year after their establishment in the greenhouse, under controlled conditions.

| | Northern | | | | | | Southern | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Native MC** | | | **No-native MC** | | | **Native MC** | | | **No-native MC** | | |
| | **Fi** | **Fe** | **Sf** | **Fi** | **Fe** | **Sf** | **Fi** | **Fe** | **Sf** | **Fi** | **Fe** | **Sf** |
| **Gal** | 10 | 6 | 10 | 8 | 8 | 6 | 7 | 7 | 7 | 8 | 7 | 7 |
| **Sib** | 9 | 7 | 7 | 8 | 6 | 5 | 6 | 8 | 8 | 3 | 7 | 6 |
| **Lam** | 6 | 5 | 7 | 8 | 4 | 8 | 6 | 4 | 4 | 6 | 5 | 4 |

**Table S5.3** Response of the plant-soil system to drought simulation (D) in holm oak forest soils from two climatically different regions of Spain (R), collected in fragments with different agricultural matrix influence (MI). Data were analysed by repeated measures ANOVA. Significant effects are noted in bold. $R_{eco}$= Ecosystem respiration; GPP= gross primary productivity; $g_S$= stomatal conductance; $F_v/F_m$ =photochemical efficiency (measured at pre- and final-drought); SWC= Soil water content.

| | $R_{eco}$ | | GPP | | $g_S$ | | $F_v/F_m$ Predawn | | $F_v/F_m$ Midday | | SWC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* |
| *Region(R)* | **12.04** | **<0.001** | 1.19 | 0.277 | 3.53 | 0.061 | **8.39** | **0.004** | **5.15** | **0.024** | **30.13** | **<0.001** |
| *Matrix influence (MI)* | **9.52** | **<0.001** | 2.05 | 0.131 | **3.91** | **0.021** | **11.68** | **<0.001** | **11.43** | **<0.001** | **22.67** | **<0.001** |
| R x MI | **3.53** | **0.031** | 0.11 | 0.896 | 0.27 | 0.764 | 1.65 | 0.194 | 1.28 | 0.281 | **7.75** | **<0.001** |
| *Drought (D)* | **945.14** | **<0.001** | **396.66** | **<0.001** | **767.92** | **<0.001** | **824.35** | **<0.001** | **978.40** | **<0.001** | **11383.67** | **<0.001** |
| D x R | **5.90** | **0.003** | **9.75** | **<0.001** | **18.89** | **<0.001** | **21.11** | **<0.001** | **21.64** | **<0.001** | **87.88** | **<0.001** |
| D x MI | **4.06** | **0.003** | **7.14** | **<0.001** | **10.34** | **<0.001** | **9.43** | **<0.001** | **10.42** | **<0.001** | **32.23** | **<0.001** |
| D x R x MI | 2.37 | 0.052 | **5.73** | **<0.001** | 2.17 | 0.072 | 2.07 | 0.128 | 2.07 | 0.129 | **10.47** | **<0.001** |

**Table S5.4** Regressions for ecosystem respiration ($R_{eco}$), gross primary productivity (GPP) and stomatal conductance ($g_S$), with soil water content (SWC), in soils of fragmented holm oak forests from two climatically different regions of Spain. Significant effects are noted in bold. Agricultural matrix: Fi= forest interior, Fe= forest edge, Sf= small fragment; Seedling provenance: Gal= Galaico Leonesa; Sib= Sistema Iberico; Lam= La Mancha. $R_{eco}$= Ecosystem respiration; GPP= gross primary productivity; $g_S$= stomatal conductance.

| | | Region | | Matrix influence | | | Microbial communities | | Seedling provenance | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Northern | Southern | Fi | Fe | Sf | Native | No-native | Gal | Sib | Lam |
| | ***n*** | 384 | 327 | 252 | 222 | 237 | 372 | 339 | 273 | 237 | 201 |
| $R_{eco}$ | **SWC (%)** | | | | | | | | | | |
| | $R^2$ | 0.62 | 0.59 | 0.67 | 0.57 | 0.58 | 0.63 | 0.58 | 0.62 | 0.55 | 0.63 |
| | *p* | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| **GPP** | **SWC (%)** | | | | | | | | | | |
| | $R^2$ | 0.59 | 0.46 | 0.63 | 0.51 | 0.44 | 0.57 | 0.51 | 0.52 | 0.53 | 0.57 |
| | *p* | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| $g_S$ | **SWC (%)** | | | | | | | | | | |
| | $R^2$ | 0.61 | 0.46 | 0.61 | 0.53 | 0.46 | 0.55 | 0.55 | 0.56 | 0.51 | 0.57 |
| | *p* | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |

**Table S5.5** Response to all factors studied: region, matrix influence, microbial communities and seedling provenance of seedlings grown in soils of fragmented holm oak forests from two climatically different regions of Spain, subjected to drought simulation. Data analysed by factorial ANOVA ($p$<0.05) and significant effects are noted in bold. EM = Ectomycorrhizal.

| | Height | | Diameter | | SLA | | Biomass | | Foliar biomass | | Root: shoot ratio | | EM percentages | | EM morphotypes | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* | F | *p* |
| *Region(R)* | 0.14 | 0.710 | 0.86 | 0.354 | 1.29 | 0.257 | 2.31 | 0.130 | 0.46 | 0.498 | 0.76 | 0.386 | 0.83 | 0.362 | 8.90 | 0.003 |
| *Matrix influence (MI)* | 0.54 | 0.582 | 0.76 | 0.468 | 1.03 | 0.359 | 2.62 | 0.075 | 1.16 | 0.317 | 1.10 | 0.334 | 0.84 | 0.435 | 1.05 | 0.354 |
| *Microbial communities (MC)* | 0.85 | 0.358 | 0.00 | 0.979 | 0.19 | 0.666 | 1.63 | 0.203 | 0.24 | 0.626 | 3.25 | 0.073 | **3.98** | **0.047** | 1.53 | 0.218 |
| *Seedling (Se)* | 0.26 | 0.771 | 2.24 | 0.109 | 2.09 | 0.126 | 2.31 | 0.102 | 0.44 | 0.645 | **3.11** | **0.047** | **3.58** | **0.030** | 0.44 | 0.645 |
| R x MI | 1.22 | 0.297 | 0.76 | 0.470 | 2.16 | 0.118 | 1.84 | 0.162 | 0.22 | 0.805 | 1.29 | 0.279 | 2.40 | 0.093 | **3.31** | **0.039** |
| R x MC | 0.04 | 0.838 | 0.02 | 0.899 | **3.02** | **0.030** | 0.41 | 0.525 | 0.49 | 0.487 | 1.20 | 0.275 | 0.29 | 0.594 | 0.19 | 0.668 |
| MI x MC | 2.67 | 0.072 | 0.46 | 0.631 | 2.25 | 0.108 | 0.77 | 0.466 | 2.09 | 0.127 | 2.71 | 0.069 | 1.06 | 0.347 | 0.74 | 0.481 |
| R x Se | 0.31 | 0.732 | 0.08 | 0.925 | 0.37 | 0.690 | 0.60 | 0.548 | 0.05 | 0.950 | 0.93 | 0.396 | 0.01 | 0.985 | 2.48 | 0.087 |
| MI x Se | 0.67 | 0.614 | 0.10 | 0.981 | 1.11 | 0.355 | 0.18 | 0.951 | 0.36 | 0.834 | 0.35 | 0.842 | 0.58 | 0.677 | 0.98 | 0.420 |
| MC x Se | 2.42 | 0.092 | 0.99 | 0.372 | 1.43 | 0.241 | **3.68** | **0.027** | 1.32 | 0.268 | 1.06 | 0.349 | 0.63 | 0.536 | 1.19 | 0.305 |
| R x MI x MC | 0.07 | 0.933 | 0.40 | 0.672 | 0.11 | 0.892 | 0.12 | 0.888 | 0.52 | 0.595 | 1.83 | 0.162 | 1.49 | 0.228 | 0.87 | 0.421 |
| R x MI x Se | 0.61 | 0.652 | 0.87 | 0.482 | 1.01 | 0.404 | 0.97 | 0.424 | 0.32 | 0.862 | 0.84 | 0.501 | 0.39 | 0.818 | 0.26 | 0.901 |
| R x MC x Se | 0.71 | 0.493 | 1.78 | 0.171 | 2.72 | 0.068 | 1.99 | 0.140 | 1.19 | 0.305 | 0.49 | 0.616 | 0.84 | 0.433 | 1.70 | 0.185 |
| MI x MC x Se | 0.77 | 0.545 | 1.18 | 0.319 | **2.46** | **0.047** | 0.65 | 0.627 | 0.81 | 0.522 | 0.70 | 0.592 | 0.11 | 0.980 | 1.94 | 0.105 |
| R x MI x MC x Se | 0.21 | 0.930 | 0.86 | 0.490 | 1.35 | 0.252 | 0.77 | 0.545 | 0.53 | 0.712 | 0.56 | 0.693 | 0.88 | 0.477 | 2.65 | 0.054 |

**Table S5.6** Description of the ectomycorrhizal fungal morphotypes of seedlings grown in holm oak forest soils from two climatically different regions of Spain, under drought. See Fig. S5.3

| Morphotype | Macroscopic description | Rhizomorphs |
|---|---|---|
| M01 | Dark brown/reddish mantle, felty mantle surface, un-branched, straight to slightly bent tips. See Fig. S5.3b. | Present |
| M02 | Dark brown/reddish mantle, smooth mantle surface, un-branched, straight tips. See Fig. S5.3c. | Present |
| **M03** | Black/reddish mantle, smooth mantle surface, un-branched, straight tips | Absent |
| **M04** | Black mantle, smooth mantle surface, un-branched, slightly bent tips | Present |
| M05 | White/yellow mantle, smooth mantle surface with loose hyphae, dichotomous and straight tips. See Fig. S5.3d. | Present |
| M06 | Black/reddish mantle, grainy mantle surface with loose hyphae, dichotomous/coralloid, slightly bent tips | Absent |
| M07 | Yellowish mantle, smooth mantle surface with loose hyphae, pinnate, slightly bent tips | Absent |
| M08 | White mantle, un-branched, loose hyphae, slightly bent tips | Absent |
| M09 | Black mantle, grainy mantle surface with loose black hyphae, un-branched, slightly bent tips | Present |
| M10 | Black mantle, grainy mantle surface with loose black hyphae, tuberoid | Present |

**Table S5.7** Effect of all the studied factors: region, matrix influence, microbial communities and seedling provenance, on the survival of *Quercus ilex* seedlings subjected to drought and grown in soils of fragmented holm oak forests from two climatically different regions of Spain. Significant effects (Peto and Peto analyses) are noted in bold ($p$<0.05). Gal= Galaico Leonesa; Sib= Sistema Iberico; Lam= La Mancha.

| Factor | Pair of factors | $X^2$ |
|---|---|---|
| **Region** | Northern vs. Southern | **23.89**** |
| **Matrix influence** | Forest interior vs. forest edge | **8.34**** |
| | Forest interior vs. small fragments | **30.64**** |
| | Forest edge vs. small fragments | **6.69**** |
| **Microbial communities** | Native vs. No-native | 1.63 n.s. |
| **Seedling provenance** | Gal vs. Sib | 0.10 n.s. |
| | Gal vs. Lam | 0.08 n.s. |
| | Sib vs. Lam | 0.33 n.s. |

**Table S5.8** Growth of different provenances of holm oak seedling in soils collected from fragmented forests in two climatically different regions of Spain, with native or no-native microbial communities and subjected to drought simulation. Data = means ± SE. Agricultural matrix influence: Fi= forest interior, Fe= forest edge, Sf= small fragment; Seedling provenance: Gal= Galaico-Leonesa; Sib= Sistema Iberico; Lam= La Mancha; SLA= Specific Leaf Area.

| | | | | *n* | Height (cm) | Diameter ($mm^2$) | SLA ($cm^2\ g^{-1}$) | Biomass (g) | Foliar biomass (g) | Root:shoot ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| Northern | Fi | Native | Gal | *10* | 20.1±2.4 | 33.8±6.0 | 22.2±0.9 | 12.5±1.3 | 2.1±0.2 | 1.7±0.1 |
| | | | Sib | *9* | 21.6±3.7 | 38.5±4.0 | 23.1±0.8 | 10.6±1.2 | 2.4±0.5 | 1.3±0.2 |
| | | | Lam | *6* | 25.8±4.0 | 50.8±8.1 | 20.7±1.1 | 13.7±1.2 | 2.4±0.3 | 1.4±0.1 |
| | | No-native | Gal | *8* | 20.3±2.4 | 41.7±5.8 | 23.3±0.9 | 13.4±0.5 | 2.4±0.3 | 1.7±0.2 |
| | | | Sib | *8* | 22.9±3.2 | 41.7±2.6 | 21.7±1.0 | 11.5±1.1 | 2.4±0.3 | 1.3±0.1 |
| | | | Lam | *8* | 24.1±2.8 | 53.8±13.4 | 22.8±1.3 | 13.2±1.4 | 2.7±0.5 | 1.2±0.2 |
| | Fe | Native | Gal | *6* | 20.2±2.6 | 32.7±6.9 | 24.9±1.0 | 12.2±1.3 | 2.1±0.4 | 1.7±0.3 |
| | | | Sib | *7* | 18.1±1.5 | 39.3±5.2 | 21.7±1.2 | 9.7±0.7 | 2.1±0.3 | 1.3±0.2 |
| | | | Lam | *5* | 21.4±2.2 | 44.9±8.4 | 20.0±1.5 | 12.1±1.4 | 1.9±0.3 | 1.6±0.2 |
| | | No-native | Gal | *8* | 25.2±1.5 | 37.4±6.9 | 25.0±1.9 | 12.3±1.1 | 2.0±0.3 | 1.7±0.2 |
| | | | Sib | *6* | 22.9±3.0 | 38.7±3.9 | 22.9±1.4 | 11.9±1.1 | 2.4±0.4 | 1.3±0.1 |
| | | | Lam | *4* | 17.9±4.5 | 34.9±3.5 | 34.1±1.5 | 11.8±2.4 | 1.9±0.7 | 1.8±0.2 |
| | Sf | Native | Gal | *10* | 20.2±2.4 | 39.4±6.5 | 23.1±1.0 | 11.2±1.1 | 2.3±0.4 | 1.6±0.5 |
| | | | Sib | *7* | 22.3±3.7 | 33.3±4.3 | 22.6±1.1 | 9.4±0.9 | 2.3±0.4 | 1.1±0.1 |
| | | | Lam | *7* | 22.3±2.3 | 36.9±4.8 | 21.9±1.0 | 12.2±1.0 | 2.9±0.5 | 1.1±0.1 |
| | | No-native | Gal | *6* | 21.6±4.7 | 35.2±8.4 | 23.3±3.0 | 11.7±1.7 | 1.6±0.5 | 4.5±2.6 |
| | | | Sib | *5* | 19.0±4.5 | 44.7±6.8 | 24.7±3.4 | 11.7±1.3 | 2.1±0.5 | 1.3±0.2 |
| | | | Lam | *8* | 13.8±2.9 | 31.1±7.7 | 25.5±1.8 | 8.8±1.2 | 1.5±0.4 | 3.9±2.2 |
| Southern | Fi | Native | Gal | *7* | 22.1±1.8 | 38.3±6.8 | 27.2±4.3 | 12.5±1.1 | 2.5±0.4 | 1.5±0.2 |
| | | | Sib | *6* | 17.2±2.6 | 38.1±7.5 | 24.1±1.6 | 9.2±1.2 | 2.0±0.5 | 1.3±0.4 |
| | | | Lam | *6* | 21.7±3.5 | 34.1±5.1 | 22.1±1.6 | 10.6±0.8 | 1.6±0.4 | 2.1±0.4 |
| | | No-native | Gal | *8* | 19.4±3.2 | 30.0±7.6 | 23.4±1.1 | 10.0±1.0 | 1.6±0.2 | 2.4±0.6 |
| | | | Sib | *3* | 21.9±3.1 | 44.8±8.4 | 18.4±0.4 | 13.8±1.0 | 2.6±0.1 | 1.2±0.3 |
| | | | Lam | *6* | 21.7±2.6 | 35.6±7.4 | 21.1±0.6 | 13.5±1.9 | 2.9±0.7 | 1.3±0.1 |
| | Fe | Native | Gal | *7* | 18.1±2.8 | 38.7±6.8 | 23.5±0.4 | 9.8±1.2 | 1.9±0.5 | 1.2±0.2 |
| | | | Sib | *8* | 18.1±1.4 | 42.1±5.2 | 21.5±1.3 | 9.4±1.2 | 2.1±0.4 | 1.2±0.2 |
| | | | Lam | *4* | 21.4±2.8 | 30.1±13.5 | 20.5±1.6 | 9.6±1.6 | 2.0±0.6 | 1.6±0.3 |
| | | No-native | Gal | *7* | 21.4±2.4 | 30.0±2.6 | 21.5±1.5 | 10.2±1.0 | 1.7±0.3 | 1.6±0.2 |
| | | | Sib | *7* | 19.8±1.4 | 36.5±2.5 | 21.3±1.2 | 10.8±1.1 | 2.4±0.4 | 1.3±0.1 |
| | | | Lam | *5* | 16.9±2.6 | 52.7±11.1 | 22.0±1.5 | 10.9±2.2 | 1.8±0.7 | 1.6±0.2 |
| | Sf | Native | Gal | *7* | 24.9±3.0 | 33.7±2.1 | 21.5±1.1 | 12.0±1.2 | 2.6±0.3 | 1.3±0.1 |
| | | | Sib | *8* | 20.0±1.9 | 33.0±5.0 | 22.7±2.7 | 8.8±0.9 | 1.9±0.3 | 1.4±0.2 |
| | | | Lam | *4* | 25.4±2.3 | 46.5±7.3 | 30.0±5.4 | 13.2±0.4 | 2.1±0.5 | 1.6±0.3 |
| | | No-native | Gal | *7* | 19.4±2.9 | 29.1±5.9 | 25.1±1.4 | 10.6±1.3 | 2.0±0.3 | 1.5±0.1 |
| | | | Sib | *6* | 18.9±3.9 | 39.0±3.7 | 22.0±2.1 | 10.8±1.6 | 2.1±0.5 | 1.5±0.3 |
| | | | Lam | *4* | 19.3±2.0 | 34.2±9.6 | 21.9±1.3 | 12.2±0.5 | 2.0±0.2 | 2.0±0.3 |

# Capítulo 6

Discusión general y conclusiones

# Discusión general

## *6.1 Respuesta del sistema planta-suelo-microorganismo a la fragmentación*

### *6.1.a Efecto de la fragmentación sobre el crecimiento de las plantas*

Nuestros resultados demuestran que la fragmentación de los encinares ejerce una influencia positiva sobre el tamaño de las encinas, con árboles más grandes en los bordes del encinar y en los fragmentos pequeños. Este incremento puede deberse, entre otros factores, a la mayor disponibilidad de recursos (agua, nutrientes o luz) como consecuencia de la disminución de competencia con otros árboles, aunque no podemos descartar otros factores como el manejo, o la historia de uso de estos encinares. Cabe destacar que este efecto positivo es un patrón consistente en ambas regiones de estudio, así como en otros encinares fragmentados en la Península Ibérica (Santos y Tellería, 1998), lo que sugiere que la relación entre el tamaño del arbolado y la influencia de la fragmentación esconde un fuerte componente causal. Asimismo, este aumento de tamaño puede deberse a una retroalimentación positiva, como se ha estudiado para otras especies del género *Quercus* en los ecosistemas Mediterráneos (ver Aponte et al., 2011), donde la mayor disponibilidad de recursos tendría un efecto positivo sobre tamaño de las encinas, las cuales a su vez, proporcionarían mayor cantidad de recursos para las comunidades subterráneas, lo que a su vez estimula la actividad y reciclado de nutrientes para la planta.

### *6.1.b Repercusión del aumento de las encinas sobre las condiciones ambientales y funcionales del suelo*

El aumento del tamaño de los árboles asociado a la fragmentación repercute positivamente en el funcionamiento del suelo, principalmente modificando las condiciones micro-ambientales del mismo, tanto abióticas (mayor fertilidad y humedad, menor pH y temperatura) como bióticas (más

materia orgánica y biomasa microbiana). Este incremento en la capacidad funcional de los suelos se vio reflejado en la mayor respiración del ecosistema (incluyendo planta y suelo, $R_{eco;}$ capítulos 4 y 5) y la mayor actividad enzimática (capítulo 2) y consumo de fuentes de carbono (capítulo 3), efecto que en algunos casos perduró al aplicarse una sequía crónica, p. ej. mayor actividad enzimática (capítulo 4). Si bien otros estudios han descrito esta influencia de la cobertura vegetal sobre la actividad del suelo (Lázaro-Nogal et al., 2012; Riutta et al., 2012), el trabajo desarrollado en esta tesis añade una dimensión mecanicista, mostrando la interacción de los diferentes factores responsables de este incremento del funcionamiento microbiano como consecuencia de la fragmentación del hábitat.

Los modelos de ecuaciones estructurales mostraron que el efecto neto de la fragmentación en el funcionamiento del suelo en un encinar meso-mediterráneo se explica a través de una compleja cascada de relaciones causa-efecto interconectadas, tanto directas como indirectas, entre los diferentes factores (abióticos y bióticos) y compartimentos ecológicos (planta y microorganismos) del ecosistema. Las encinas más grandes, con mayor influencia de la matriz agrícola, presentaron mayor productividad, lo que se refleja tanto en el mayor contenido de materia orgánica y fertilidad (p. ej. proporción C:N y PC1 de nutrientes), como en un incremento en su capacidad de retención de agua (humedad del suelo), muy asociado a ese mayor contenido en materia orgánica (Boix-Fayos et al., 2001; Franzluebbers, 2002). Las encinas, directamente y a través de su influencia sobre la materia orgánica, amortiguaron la temperatura del suelo, posiblemente mediante la disminución de la tasa de evaporación (Belsky y Canham, 1994; Hastwell y Morris, 2013). El tamaño y cercanía de las encinas, además, se vio asociado con una acidificación neta del suelo probablemente debida a la acumulación de materia orgánica y de ácidos

húmicos, y al efecto acidificante del entorno rizosférico relacionado con la absorción de nutrientes y la liberación de metabolitos secundarios (Dakora y Phillips, 2002; Faget et al., 2013).

Aunque la cercanía al árbol explica gran parte de la variabilidad espacial de la respiración del suelo, como se ha estudiado en ecosistemas similares (Matías et al., 2012; Barba et al., 2013), observamos también una relación muy significativa con la temperatura y humedad del mismo, independientemente de la cercanía a la planta, lo que indica la enorme dependencia que la actividad metabólica del suelo tiene del clima. Asimismo, las actividades enzimáticas derivadas de la actividad microbiana fueron sensibles a pequeñas variaciones en el pH, un factor que ha sido descrito con anterioridad como extremadamente importante para determinar la actividad metabólica de los microorganismos del suelo (Fierer y Jackson, 2006; Sinsabaugh et al., 2008; Legay et al., 2014). En este respecto, los modelos de ecuaciones estructurales nos permitieron discernir que el efecto del incremento de la materia orgánica sobre las actividades enzimáticas no es directo, tal como sugieren otros estudios (Sinsabaugh et al., 2008; Burns et al., 2013; Baldrian, 2014), si no que se produce indirectamente a través de su influencia sobre otros factores, como la biomasa microbiana, el pH, la humedad o la relación C:N, que sí ejercen un control directo sobre la actividad metabólica de las comunidades microbianas. Por otra parte, el observado efecto directo del tamaño de los árboles y su cercanía sobre la actividad enzimática podría reflejar la estimulación de la capacidad descomponedora de materia orgánica, estímulo en parte atribuible a los exudados radicales (Dakora y Phillips, 2002; Kuzyakov, 2010).

Por tanto, podemos concluir que el tamaño de las encinas (fuertemente dependiente de la influencia de la matriz) y los cambios micro-ambientales inducidos por las mismas controlan fuertemente el

funcionamiento del suelo, y que este control responde a complejas relaciones causa-efecto entre la planta y las comunidades microbianas.

### *6.1.c Efecto de la fragmentación del encinar sobre la fertilidad y las comunidades microbianas del suelo*

Además del efecto sobre las encinas, la matriz agrícola circundante favoreció directamente la acumulación de nutrientes en suelos de fragmentos pequeños y en los bordes de los fragmentos grandes en comparación con los interiores (capítulo 2-4). Este efecto de fertilización podría ser el resultado del arrastre por escorrentía de los nutrientes de dicha matriz hacia los suelos más influenciados (Boutin y Jobin, 1998).

Al igual que la fertilidad del suelo, la fragmentación del hábitat en los encinares mediterráneos fragmentados afectó directamente a la diversidad genética y a la estructura de las comunidades microbianas, efecto que fue aún mayor que el indirecto a través de las encinas (capítulo 3). De hecho, como se ha señalado previamente, gran parte de la variabilidad de las comunidades microbianas fue explicada por la disponibilidad de nutrientes (Wardle, 1998; O'Donnell et al., 2001; Fierer y Jackson, 2006; Bowen et al., 2011; Ramirez et al., 2012; Tardy et al., 2014), fuertemente influenciadas por la fragmentación del encinar.

Al contrario que la estructura y diversidad genética de las comunidades microbianas, su funcionamiento (actividad enzimática y diversidad de substratos metabolizados) dependió principalmente de las modificaciones micro-ambientales del suelo causadas por las encinas. Esto sugiere un desacoplamiento parcial entre los factores que controlan la estructura de las comunidades microbianas y su funcionamiento: el ensamblaje y diversidad de las comunidades no están totalmente ligados a su capacidad de metabolizar diferentes sustratos y de mineralizar diferentes nutrientes. Estos resultados podrían indicar diferencias en la composición de la comunidad activa y la comunidad microbiana total

(O'Donnell et al., 2001; Jones y Lennon, 2010; Placella et al., 2012; Barnard et al., 2013), dado que las condiciones ambientales desfavorables (p. ej. áreas abiertas) pueden asociarse con inactividad de parte de las comunidades de bacterias y hongos (Jones y Lennon, 2010).

A pesar de esta diferente sensibilidad de la estructura y de la funcionalidad de las comunidades microbianas, tanto los modelos de ecuaciones estructurales como la comparación de matrices de disimilitudes mostraron una relación entre la diversidad bacteriana y su capacidad de metabolizar diferentes sustratos. La existencia de esta compleja relación ha sido frecuentemente analizada, a veces con resultados contradictorios (Griffiths et al., 2000; O'Donnell et al., 2001; Bell et al., 2005; Langenheder et al., 2010; Levine et al., 2011; Curiel Yuste et al., 2014; Tardy et al., 2014; Mendes et al., 2015), aunque la relación encontrada aquí y en estudios previos (Bell et al., 2005; Tardy et al., 2014), sugiere que las alteraciones ambientales, como las derivadas de la fragmentación del hábitat, con la capacidad de producir cambios en la estructura y diversidad de las comunidades microbianas, podrían ejercer un efecto negativo sobre su funcionamiento.

### *6.1.d Dependencia espacial de la respuesta de las comunidades microbianas a la fragmentación*

La influencia positiva tanto de la cercanía y tamaño de las encinas como de la influencia de la matriz sobre el funcionamiento y diversidad local (alfa-diversidad) de las comunidades microbianas contrasta con la heterogeneidad espacial (beta-diversidad) tanto estructural como funcional, observada (capítulo 3). Precisamente, las áreas con más influencia de la matriz agrícola (fragmentos pequeños y bordes) o de las encinas (con cobertura), mostraron comunidades microbianas más homogéneas (menos beta-diversas), en contraste con la mayor heterogeneidad espacial de las mismas en áreas no perturbadas del interior

de fragmentos grandes o zonas no cubiertas por encinas. En primer lugar, esto sugiriere que la respuesta de las comunidades microbianas a las perturbaciones es dependiente de la escala espacial a la que se determina, lo cual debería ser tenido en cuenta si queremos entender y predecir las respuestas de estas comunidades ante perturbaciones ambientales. Por otro lado, ya que la disminución de beta-diversidad puede ser utilizada como un indicador de homogenización del hábitat (Olden et al., 2004), la convergencia observada podría atribuirse a filtros medioambientales causados por la homogenización de las condiciones abióticas y/o bióticas en las áreas menos beta-diversas (fragmentos pequeños, bordes y con cobertura), con respecto a las áreas más beta-diversas (interiores del encinar y áreas abiertas). La mayor homogenización de las comunidades en escenarios de fragmentación, especialmente bajo vegetación, indican que, a pesar del enriquecimiento de las comunidades microbianas y su funcionalidad a escala micro-local (alfa-diversidad), a escala de paisaje la fragmentación está asociada con un empobrecimiento estructural y funcional de las mismas, probablemente con consecuencias negativas sobre la capacidad de estos suelos para responder a futuras alteraciones climáticas (Curiel Yuste et al., 2011; Barnard et al., 2013).

### *6.1.e Dependencia de la región de origen del suelo sobre la respuesta del sistema planta-suelo-microorganismo a la fragmentación*

Los resultados obtenidos para los microcosmos con suelos procedentes de la región del norte (supra-mediterránea) confirmaron los resultados observados en los microcosmos con suelos de la región sur y los estudios realizados en campo en el encinar de la región sur (meso-mediterráneo). Los resultados confirman la influencia positiva de la fragmentación sobre el tamaño de las encinas y las características físico-químicas del suelo, siendo las diferencias ocasionadas por la influencia de la matriz agrícola más determinantes para la planta y el suelo, que las diferencias entre las

regiones de estudio (capítulo 4 y 5). Por el contrario, las condiciones bioclimáticas particulares de cada región ejercieron un control diferente sobre la biomasa y la diversidad de las comunidades microbianas. Por una parte, los suelos de la región del norte, más húmeda y fría, mostraron mayor biomasa microbiana asociada a mas cantidad de materia orgánica, y comunidades con mayor diversidad bacteriana, especialmente en suelos con poca influencia de la matriz agrícola (fragmentos grandes), en contraste con la región del sur, en general con menor biomasa microbiana y una comunidad fúngica más diversa que la de la región norte (capítulo 4). Esto sugiere una dominancia fúngica en la región más seca y cálida, probablemente relacionada con la mayor adaptación de los hongos a estos ambientes (Curiel Yuste et al., 2011; Grigulis et al., 2013; Fuchslueger et al., 2014). Asimismo, todos los rasgos fisiológicos funcionales medidos en las plántulas de encina (GPP, conductividad estomática, eficiencia fotoquímica) respondieron positivamente a la fragmentación, manteniendo tasas metabólicas más altas las plántulas crecidas en suelos con una mayor influencia de la matriz agrícola (fragmentos pequeños y bordes), que finalmente se reflejaba en su mayor supervivencia (capítulo 4 y 5).

Nuestros resultados sugieren que la fragmentación del encinar tiene un fuerte impacto en el sistema planta-suelo-microorganismo independientemente de la región de estudio, lo cual sugiere que puede extrapolarse también a otras regiones de encinares fragmentados con influencia agrícola.

## *6.2 Respuesta del sistema planta-suelo-microorganismo a la sequía en ambientes fragmentados*

### *6.2.a Efectos de la sequía crónica sobre la capacidad funcional del sistema planta-suelo-microorganismo*

En general, la sequía crónica aplicada a los experimentos controlados en microcosmos (capítulos 4 y 5) afectó sustancialmente al funcionamiento del sistema planta-suelo-microorganismo, altamente dependiente de la disponibilidad de agua, lo que se reflejó en los diversos indicadores funcionales medidos (enzimas, respiración y productividad del ecosistema y diversidad funcional). Esta disminución de la actividad metabólica ha sido descrita en estudios previos tanto para la respiración del suelo (Rey y Jarvis, 2006; Curiel Yuste et al., 2007), y sus actividades enzimáticas (Sardans y Peñuelas, 2005; Zornoza et al., 2006), como para la productividad y crecimiento de *Q. ilex* (Pesoli et al., 2003; Ogaya y Peñuelas, 2004). Por otro lado, el efecto de la sequía sobre el funcionamiento del componente autotrófico del sistema (GPP de microcosmos de *Q. ilex*) fue menos acentuado que sobre los indicadores que integraban el funcionamiento microbiano ($R_{eco}$, actividades enzimáticas). Esta diferencia se vio enfatizada a medida que las condiciones de sequía se agudizaron, evidenciando una menor disminución del metabolismo bajo sequía en comparación con los microorganismos dentro del sistema.

### *6.2.b Efectos de la sequía sobre la diversidad de bacterias y hongos*

Uno de los resultados más concluyentes obtenidos durante la simulación climática (sequía y rehidratación; capítulo 4) fue la respuesta antagónica de las comunidades bacteriana y fúngica a lo largo del gradiente de disponibilidad hídrica; las comunidades bacterianas aumentaron su diversidad en condiciones de mayor disponibilidad agua (antes de la sequía y en rehidratación) y lo contrario pasó con los hongos, más diversos ante

condiciones de limitación hídrica. Estos resultados refuerzan la idea anteriormente comentada de que los hongos tienen mejores estrategias para evitar las limitaciones hídricas en comparación a las bacterias (Drenovsky et al., 2004; Curiel Yuste et al., 2011; Barnard et al., 2013). Mientras que las bacterias, organismos que han evolucionado en el medio acuoso (Boer et al., 2005) encuentran un entorno óptimo en condiciones de buena disponibilidad hídrica, los hongos, organismos mayoritariamente evolucionados en el medio terrestre, son más abundantes, diversos y capaces de mantener su metabolismo en ambientes hídricos limitantes (Curiel Yuste et al., 2011; Grigulis et al., 2013). La capacidad de los hongos de evitar las zonas secas y orientarse mediante el sistema de hifas hacia fuentes de agua más eficientemente que las bacterias podría estar detrás de esta mayor ventaja en condiciones de sequía (Curiel Yuste et al., 2011; Barnard et al., 2015).

#### *6.2.c Dependencia de la región de origen sobre la respuesta de las comunidades microbianas a la sequía y la fragmentación*

La respuesta de las comunidades microbianas (diversidad y ensamblaje) en el sistema planta-suelo-microorganismos ante la sequía y rehidratación simuladas dependieron en gran medida de la región bioclimática de origen (capítulo 4). Así, las comunidades microbianas de la región norte, más húmeda y fría, mostraron una mayor sensibilidad a la sequía que las de la región sur, lo cual sugiere que las condiciones históricas (más limitación hídrica en la región del sur), pudieron actuar como filtro ambiental seleccionando especies microbianas tolerantes a la sequía, que resistieron mejor a las condiciones simuladas (Cruz-Martinez et al., 2009; Curiel Yuste et al., 2014; Evans y Wallenstein, 2014).

Además, la región de estudio afectó la diversidad de morfotipos de hongos ectomicorrícicos asociados a las plántulas, que fue mayor para los suelos más sensibles a la sequía de la región del norte, en comparación con

los del sur (capítulo 5). Además de esta correlación negativa entre diversidad ectomicorrízica y resistencia a la sequía, encontramos que la respuesta a la sequía del sistema planta-suelo se correlacionó negativamente con un mayor porcentaje de micorrización. Estos resultados sugieren el aumento en la diversidad y tasa de micorrización puede haber afectado positivamente a la presencia de morfotipos posiblemente oportunistas, que bajo condiciones sub-optimas de humedad pudieron tener un efecto negativo en la respuesta del sistema (capítulo 5; ver Rincón et al., 2007; Smith y Read, 2010). También observamos que la presencia de microorganismos nativos del suelo afectó positivamente a la diversidad y al porcentaje de micorrización de las plántulas, lo que sugiere que los propágulos de hongos ectomicorrícicos provenientes de suelos de encinares fragmentados presentaron compatibilidad con las plántulas de *Q. ilex* y fueron suficientemente competitivos para establecerse a pesar de los posibles efectos de prioridad, generados por las micorrizas ya establecidas (Rincón et al., 2014).

Al contrario que los indicadores de diversidad genética y composición (estructura) de las comunidades microbianas, la respuesta funcional de estas comunidades a la sequía estuvo principal y fuertemente determinada por los efectos de la fragmentación del encinar, siendo los suelos con mayor influencia de la matriz agrícola (fragmentos pequeños y bordes), con una mayor cantidad de materia orgánica y por lo tanto con mayor capacidad de retención de agua, los que mantuvieron tasas metabólicas mayores durante la sequía simulada (actividad ureasa, $R_{eco}$; capítulos 4 y 5, respectivamente). Nuestros resultados, por tanto, indican que la fuerte influencia de la fragmentación sobre las características fisicoquímicas del suelo y sobre el funcionamiento del sistema planta-suelo-microorganismo oscureció cualquier posible efecto de la adaptación diferencial de las plántulas fruto de las condiciones bioclimáticas históricas

asociadas con su procedencia (capítulo 5), como ha sido observado en otros estudios (Gratani, 1995; Gratani et al., 2003; Pesoli et al., 2003; Andivia et al., 2012). Este resultado muestra que para entender la respuesta de los ecosistemas al cambio climático en escenarios de cambios ambientales más generales puede ser más importante entender los efectos de la fragmentación sobre las condiciones abióticas (físico-químicas) y bióticas (mas biomasa asociada a menos competencia) del ecosistema que entender la capacidad de adaptación de los organismos a las condiciones concretas de sequía.

### *6.3 Consideraciones finales*

Colectivamente, nuestros resultados sugieren que los efectos positivos de la fragmentación del encinar sobre las características abióticas del suelo (fisicoquímicas) y las condiciones bióticas (menos competencia por recursos y mayor biomasa vegetal), podrían aminorar en cierta medida los efectos negativos de la sequía sobre el sistema planta-suelo-microorganismo en los encinares mediterráneos rodeados de una matriz agrícola. Además, nuestros resultados señalan que la estructura actual de las comunidades microbianas, fruto de la selección histórica a la que los microorganismos han sido sometidos, determina el funcionamiento de estas comunidades así como el funcionamiento ecosistémico y su respuesta a la sequía. Sin embargo, queda por entender cómo el empobrecimiento estructural y funcional neto de las comunidades microbianas asociado con la fragmentación observada a escala de paisaje (beta-diversidad) afecta al funcionamiento del sistema planta-suelo-microorganismo y a su respuesta ante el cambio climático, concretamente a la sequía.

## Conclusiones

1. La fragmentación del encinar Mediterráneo, medida como el grado de influencia de la matriz agrícola, produjo importantes alteraciones en el sistema planta-suelo-microorganismo, que fueron tanto directas como indirectas.

2. Encinas más grandes en las zonas con una influencia media (borde) y alta (fragmentos pequeños) de la matriz agrícola, fueron también más productivas, observándose mayor cantidad de materia orgánica en el suelo. Esta acumulación de materia orgánica indujo cambios en las condiciones abióticas (microclima y pH) y bióticas (biomasa microbiana) del suelo, impactando positivamente la actividad microbiana y por lo tanto la funcionalidad del sistema.

3. La fertilidad del suelo se vio directamente favorecida por la influencia de la matriz agrícola, afectando a su vez la composición y diversidad local (alfa diversidad) de las comunidades microbianas. La estructura de las comunidades bacterianas se relacionó positivamente con su capacidad para metabolizar diferentes fuentes de carbono.

4. La fragmentación del encinar afectó negativamente a la estructura de las comunidades microbianas y su funcionalidad a escala de paisaje (beta-diversidad), con posibles consecuencias negativas sobre la capacidad de respuesta de estos suelos al cambio global.

5. El futuro incremento de la sequía previsto para la cuenca Mediterránea podría tener repercusiones en la proporción de las comunidades microbianas favoreciendo a las comunidades fúngicas sobre las bacterianas, y ralentizando las tasas metabólicas del suelo.

6. La adaptación histórica de las comunidades microbianas a la sequía depende de la región bioclimática de origen del suelo, y puede tener un efecto importante sobre la capacidad de resistencia/resiliencia de las comunidades microbianas ante el aumento de la sequía.

7. La micorrización de las plantas se asoció a una respuesta negativa del sistema a la sequía, probablemente en relación con el balance coste-beneficio (en términos de carbono para la planta) del mantenimiento de la simbiosis.

8. El efecto tanto de la región bioclimática de origen del suelo, como de la matriz agrícola, predominó sobre las posibles diferencias derivadas de la procedencia de las encinas esto indica que para comprender la respuesta de los ecosistemas al cambio climático posiblemente sea más importante analizar la influencia de estos factores que la capacidad de adaptación y diferenciación poblacional de ciertas especies de plantas a las condiciones de sequía.

9. La fragmentación del encinar podría modular la respuesta funcional de los microorganismos a la sequía como resultado de su efecto sobre el incremento de la capacidad de retención de agua y de la fertilidad de los suelos, incrementando a su vez la diversidad local de las comunidades microbianas. Sin embargo, queda por entender cómo el empobrecimiento de las comunidades microbianas a escala de paisaje y asociado con la fragmentación, afecta al funcionamiento del sistema planta-suelo-microorganismo y a su respuesta a la sequía.

## Conclusions

1. Mediterranean holm oak forest fragmentation, measured as the degree of influence of the agricultural matrix, directly and indirectly impacted the plant-soil-microbial system causing significant alterations.
2. Trees grew bigger in medium and high agricultural matrix influence (i.e. forest edge and small fragments) resulting in the accumulation of more organic matter in soils. This higher levels of organic matter induced changes in the abiotic (microclimate and pH) and biotic (microbial biomass) edaphic conditions, stimulating the microbial activity and consequently the functioning of the soil-system.
3. Soil fertility was directly enhanced by the influence of the agricultural matrix, which in turn affected the composition and local diversity (alpha-diversity) of microbial communities; at the same time, the structure of these communities was positively related with their ability to metabolize different carbon sources.
4. Holm oak forest fragmentation negatively affected the structure and metabolism of microbial communities at landscape scale (beta-diversity), with potential negative consequences on the response of these soils to climate change related constraints.
5. The forecasted drought increase in the Mediterranean basin could have an impact on the relative proportions of soil microbial communities, favoring fungal-dominated soil communities, probably leading to a deceleration of metabolic soil rates.
6. Historic adaptation of microbial communities to drought was related with the bioclimatic region of soils, which could have major effects on the resistence/resilience abilities of microbial communities to increased drought.

7. Micorrization improved seeding development, but it was associated with a negative response of the whole plant-soil system to drought, probably related with the high cost-benefit carbon balance for the plant to maintain the symbiosis.

8. The effect of both the bioclimatic region of soils and the agricultural matrix influence overshadowed possible differences arising from the provenance of seedlings, indicating the importance of analyzing these factors over those related with adaptation capacity and population differentiation of plants to drought for understanding the response to climate change at the ecosystem level.

9. Holm oak forest fragmentation could modulate the functional response of microorganisms to drought, as a result of the induced increment in the water holding capacity and fertility of soils, and the enhancement of local diversity of microbial communities. However, it is still unknown how the impoverishment of microbial communities at the landscape scale associated with forest fragmentation, will affect the functioning of the plant-soil-microbial system and its response to drought.

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## Synopsis

The ecological consequences of habitat fragmentation on soil functioning largely depend on the influence of the plants that triggers a cascade of causal-effect relations that stimulates soil microbial activity. The negative effects of drought on the fragmented holm oaks forest could be ameliorated by the increased fertility and water holding capacity of soils on the small fragments, especially in soils with historic adaptation to drought.

## Sinopsis

Las consecuencias ecológicas de la fragmentación del hábitat sobre el funcionamiento del suelo dependen en gran medida de la influencia de las plantas que desencadenan una cascada de relaciones causa-efecto que estimula la actividad microbiana del suelo. Los efectos negativos de la sequía en los encinares fragmentados podrían ser modulados por el aumento de la capacidad de retención de agua y la fertilidad de los suelos en los fragmentos pequeños, especialmente en suelos con adaptación histórica a la sequía.