Universidade do Minho
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Effect of mycorrhization on *Quercus suber* L. tolerance to drought

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Effect of mycorrhization on *Quercus suber* L. tolerance to drought

Tese de Doutoramento
Programa Doutoral em Biologia de Plantas

Trabalho efetuado sob a orientação da
Profª Doutora Teresa Lino-Neto
da
Profª Doutora Paula Baptista
e do
Prof. Doutor Rui Tavares

janeiro de 2018

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Universidade do Minho, 26/01/2018

Francisca Rodrigues dos Reis

Francisca Reis

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"Em tudo obrigada!"

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Abstract

Mediterranean temperate forests have been identified as major biodiversity hotspots. These ecosystems are currently threatened by long term drought imposition, which will be further enhanced by the predicted climate changing. Cork oak (*Quercus suber* L.) is an evergreen species typically distributed within the Mediterranean Basin that has an important economic and social impact for the Iberian Peninsula. *Q. suber* forests can comprise different forest systems, ranging from forests with high tree density (400 trees/ha, *sobreirais*) to savannah-like landscapes with 60–100 trees/ha (*montados*). Although, well adapted to summer drought season, increasing of temperature and decreasing of precipitation is endangering the sustainability of cork oak forests. A key role for cork oak adaptation and tolerance to drought could be played by the microbial community, namely ectomycorrhizal fungi and bacteria.

In the present PhD project, seven cork oak forests from five different geographic locations, at different landscapes and gradient of water availability, were sampled. Ectomycorrhizal (ECM) community was assessed by barcoding of root tips present in soils samples, whereas the bacterial community of the same cork oak soils samples was assessed by metabarcoding using Illumina MiSeq sequencing. ECM community was predominantly dominated by Basidiomycota whereas bacterial community was highly enriched in *Proteobacteria*, *Actinobacteria* and *Acidobacteria*. A core microbial community was identified as belonging to all cork oak forests, namely *Tomentella* for ECM community and *Acidothermus*, *Afipia* and *Sphingomonas* for bacterial community. Both microbiomes clustered according to three bioclimate groups, humid, sub-humid and semi-arid/arid climates, although clustering was more evident for bacterial communities. When considering individual climate variables, bacterial and ECMF communities presented an opposite behavior. While ECMF occurrence was promoted by precipitation and impaired by temperature, bacteria presented the exact opposite trend. Correlations between ECM and mycorrhiza helper bacteria (MBH) communities revealed that *Russula/Bacillus* and *Russula/Streptomyces* interaction could play a potential role for cork oak drought stress acclimation.

To the best of our knowledge, this work comprises the most complete survey of cork oak microbiomes at different landscapes. A set of microbial interactions were suggested that could push forward future research on cork oak forests for preventing further drought stress consequences.

Keywords: cork oak, forest soil, ectomycorrhizal community, bacterial community, symbiotic interactions; Mediterranean bioclimates

Resumo

A floresta Mediterrânica está classificada como um dos principais pontos de conservação de biodiversidade. Estes ecossistemas estão atualmente ameaçados por períodos de seca prolongada, que serão intensificados com as alterações climáticas previstas. O sobreiro (*Quercus suber* L.) é uma espécie arbórea, de folhagem persistente, distribuída na bacia do Mediterrâneo, que tem um importante impacto socio-económico na Península Ibérica. As florestas de *Q. suber* estão distribuídas em diferentes sistemas florestais que variam, desde florestas com alta densidade de árvores (400 árvores/ha, sobreirais), até paisagens tipo-savana com 60-100 árvores/ha (montados). Apesar de bem adaptado à estação seca de Verão, o aumento da temperatura e a diminuição da precipitação registados estão a pôr em perigo a sustentabilidade das florestas de sobro. Neste contexto, a comunidade microbiana, nomeadamente a comunidade ectomicorrizica e bacteriana, podem desempenhar um papel essencial na adaptação e tolerância do sobreiro à secura.

Neste projecto de doutoramento, foram consideradas sete florestas de sobreiro, de cinco locais geográficos diferentes, em diferentes sistemas florestais e de acordo com um gradiente de disponibilidade de água. A comunidade ectomicorrízica (ECM) foi avaliada por *barcoding* enquanto a comunidade bacteriana foi analisada através de *metabarcoding* (sequenciação Illumina MiSeq). A comunidade ECM é predominantemente dominada por Basidiomycota, enquanto a comunidade bacteriana é altamente enriquecida em *Proteobacteria*, *Actinobacteria* e *Acidobacteria*. A comunidade microbiana comum a todas as florestas de sobreiro envolve *Tomentella* na comunidade ECM e *Acidothermus*, *Afipia* e *Sphingomonas* na comunidade bacteriana. Ambos os microbiomas agruparam de acordo com três grupos bioclimáticos, húmido, sub-húmido e semi-árido/árido, embora o agrupamento das comunidades bacterianas fosse mais evidente. Ao considerar variáveis climáticas individuais, as comunidades bacterianas e fungos ectomicorrízicos apresentaram um comportamento oposto. Embora a ocorrência de fungos ectomicorrízicos tenha sido promovida pela precipitação e prejudicada pela temperatura, as bactérias apresentam a tendência exactamente oposta. As correlações entre ECM e as bactérias auxiliares de micorrizas revelam que a interacção entre *Russula*/ *Bacillus* e *Russula*/ *Streptomyces* pode desempenhar um eventual papel na aclimatação do sobreiro ao stresse hidrico.

De acordo com o nosso conhecimento, este trabalho compreende a análise mais completa dos microbiomas de sobreiro em diversos regimes florestais. Um conjunto de interacções microbianas são sugeridas tendo em conta futuras linhas de investigação de forma a prevenir consequências negativas do stresse hídrico no normal desenvolvimento do sobreiro.

Palavras-chave: sobreiro, comunidade ectomicorrízica, comunidade bacteriana, interacções simbióticas, bioclimas Mediterrânicos

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List of Abbreviations

AL	Alcobaça
ANOSIM	Analysis of similarities
AM	Arbuscular Mycorrhizal
APCOR	Associação patronal do setor corticeiro
Aver.	Average
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
ºC	Celsius degrees
CHV1-4	*Cryphonectria parasitica* hypovirus 1
D	Simpson
DNA	Deoxyribonucleic Acid
ECM	Ectomycorrhizal
ECMF	Ectomycorrhizal fungi
EPPO	European Plant Protection Organization
EU	European Union
EUFORGEN	European Forest Genetic Resources Programme
EST	Expressed Sequence Tag
FAO	Food and Agriculture Organization
GPS	Global Positioning System
GR	Grândola
H´	Shannon
ha	Hectare
HC	Herdade da Contenda
HC-CT	Contenda
HC-MA	Monte Asparão
IPMA	Instituto Português do Mar e da Atmosfera
ITS	Internal Transcribed Spacer
L	Liter
LI	Limãos
M	Maximal precipitation
m	Minimal precipitation
Max	Maximal
MHB	Mycorrhizal Helper Bacteria
Min	Minimal
min	Minute
mm	Millimeters
mM	Millimolar
N	North
NCBI	National Center for Biotechnology Information
NCBI	National Center for
NGS	Next Generation Sequencing
NMDS	Non-metric multidimensional
OTU	Operational Taxonomic Unit
P	Annual precipitation
PCR	Polymerase Chain Reaction
PG	National Park of Peneda-Gerês
PG-ER	Ermida
PGPB	Plant Growth-Promoting Bacteria
PG-RC	Rio Cabril
P annual	Average of past 30 years of annual precipitation
P max	Precipitation of the months with highest precipitation levels
P min	Precipitation of the months with lowest precipitation levels
Q	Emberger index with lowest precipitation levels
qPCR	Quantitative Polymerase Chain Quantitative Polymerase Chain Reaction
rDNA	Ribossomal Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S	ECMF richness
s	Second
SIMPER	Similarity percentages
T annual	Average of past 30 years of annual temperature
T max	Temperature of hottest month
T min	Temperature of the coldest month
UK	United Kingdom
USA	United States of America
v/v	Volume *per* volume
W	West

Chapter 1

General introduction

Mycorrhization of Fagaceae forests within Mediterranean ecosystems

Mycorrhization of Fagaceae forests within Mediterranean ecosystems

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1.1. Abstract

Mediterranean Fagaceae forests are valuable due to their ecological and socio-economic aspects. Some profitable plant species, such as *Castanea* (timber and chestnut), *Quercus* (timber and cork) and *Fagus* (timber), encounter in this habitat the excellent edaphoclimatic conditions to develop. All Fagaceae plants are commonly associated to ECM fungal species, which are found in these forests in quite stable communities, mainly enriched in Russulaceae and Telephoraceae species. Currently, the Mediterranean Basin is considered as one of the global biodiversity hotspots, since many of their endemic plant species are not found elsewhere and are now under threat. Due to climate changing and introduction of disease agents, Fagaceae forests are facing an adaptation challenge to both biotic and abiotic threats. Although ECM communities are highly disturbed by climate factors and trees disease incidence, they could play an important role in increasing water availability to the plant and also improving plant tree defense against pathogens. Recent advances, namely on genomics and transcriptomics, are providing tools for increasing the understanding of Fagaceae mycorrhization process and stress responses to biotic and abiotic stresses. Such studies can provide new information for the implementation of the most adequate management policies for protecting threaten Mediterranean forests.

1.2. Fagaceae forests distribution

Plant nutrient acquisition is mainly performed by root symbionts in about 86% of land plant species (Brundrett, 2009). From the two most common mycorrhizal associations, arbuscular mycorrhizal (AM) fungi colonize a diverse spectrum of plant species, whereas ectomycorrhizal (ECM) fungi become specialized in trees and shrubs colonization playing an essential role in forest sustainability. The physiology of colonization is also different. AM hyphae are capable of enter inside the root cells forming arbuscules, whereas ECM hyphal growth takes place in intercellular spaces of root cells forming an Hartig net and the root tip is covered by a mantle (Bücking *et al.*, 2012). Boreal, temperate forests (Mediterranean, northern Hemisphere, South America), rainforests (Africa, India and Indo-Malay), and seasonal woodlands of Australia are the most important habitats for ECM communities (Tedersoo *et al.*, 2010). Both responsible for seedling establishment and tree growth, ECM are crucial for Pinaceae, Fagaceae, Betulaceae, Nothofagaceae, Leptospermoideae, Dipterocarpaceae and Amhersteae families in woodland and forest communities (Tedersoo *et al.*, 2010).

The Fagaceae family has a worldwide distribution and is well-recognized for comprising the largely widespread beeches (*Fagus*), chestnuts (*Castanea*) and oaks (*Quercus*) species. However, this family comprises a total of about 900 plant species, which are included in nine genera of both deciduous and evergreen trees and shrubs (Kremer *et al.*, 2012). Fagaceae family is currently divided into two subfamilies depending on their floral attributes, fruit morphology and germination: Castaneoideae (comprising *Chrysolepis*, *Castanea*, *Castanopsis*, and *Lithocarpus* genera) and the less consensual subfamily Fagoideae (Manos *et al.*, 2001). The placement of *Fagus* together with *Quercus* and *Trigonobalanoid* genera (*Trigonobalanus*, *Formanodendron* and *Colombobalanus*, which sometimes are collectively included under *Trigonobalanus*) in Fagoideae is still under debate (Nixon and Crepet, 1989, Manos *et al.*, 2001, Oh and Manos, 2008, Kremer *et al.*, 2012). Recently, a new genus, *Notholithocarpus*, has been isolated from *Lithocarpus*, since it is more closely related to *Quercus*, *Castanea*, and *Castanopsis* (Manos and Oh., 2008). Presenting a high economic value (mostly *Castanea*, *Quercus* and *Fagus* genera), due to their timber, fruits (chestnuts) and cork, the plantation areas of these plant species have been increasing in the past years (FAO, 2013).

Fagaceae forests are mainly distributed in the northern temperate hemisphere, presenting also a biodiversity hotspot in southeast Asia (reviewed by Kremer *et al.*, 2012). While the temperate, subtropical, and semiarid floras are particularly rich in *Quercus*, *Castanea* and *Fagus*, the warmer forests of southeast Asia are comparably diverse in the castaneoid *Lithocarpus* and *Castanopsis* genera (Fig. 1.1). Northern hemisphere temperate forests are all very similar, presenting high abundance of *Castanea*, *Fagus* and *Quercus* genera. These temperate forests are characterized by well-defined seasons and moderate climate, comprising at least 4-6 frost-free months with regular rates of precipitation (Manos and Oh, 2008). For this reason, European and North America ecosystems are the most closely related (Manos and Oh, 2008), being both currently affected by a decrease of native beech and oak forests and natural reforestation (Brunet *et al.*, 2010; Dulmer *et al.*, 2014). Anthropogenic influence and disease incidence are two major threats. The Fagaceae forest cut down and forest clearing for activities like agriculture or natural products extraction (*e.g.* coal mining) has been a major source of income but is degrading forest ecosystems (Bauman *et al.*, 2013). The population awareness for the need of appropriated reforestation programs is thus important to decrease forests erosion and desertification. The knowledge of ECM community of a particular geographic place could contribute for increasing

trees adaptation and reforestation survival rate (Ding *et al.*, 2011; Bauman *et al.*, 2013; Dulmer *et al.,* 2014).

Mediterranean climate features have provided unique conditions for the remarkable evolutionary adaptation and divergence of life. Mediterranean Basin only represents 1.5% of earth dry land but comprises about 10% of the total plant species identified (Blondel *et al.*, 2010). From 22,500 plant species found in this region, 11,700 (52%) are endemic to Mediterranean Basin and cannot be found anywhere else in the world (Valavanidis and Vlachogianni, 2011). However, the Mediterranean biodiversity has been currently threatened by the habitat loss and degradation, provided by the pollution levels, drought, alien invasive species spread and overexploitation, among others. For example, from the original Mediterranean forests and shrubs lands, 70% have been destroyed by 1990 (Acácio *et al.*, 2009). This resulted in the recognition of Mediterranean Basin as one of the first 25 Global Biodiversity Hotspots and a hyper-hot candidate for conservation due to the presence of exceptional totals of endemic plants (Myers *et al.*, 2000). For these reasons, the European Union (EU) has classified the Mediterranean Basin as an area of European Community importance and established the "EU Habitats Directive" for the conservation of wild animal and plant species and natural habitats. From the 37 world habitat types identified as priority, 26 occur only in the Mediterranean region (Condé *et al.*, 2005).

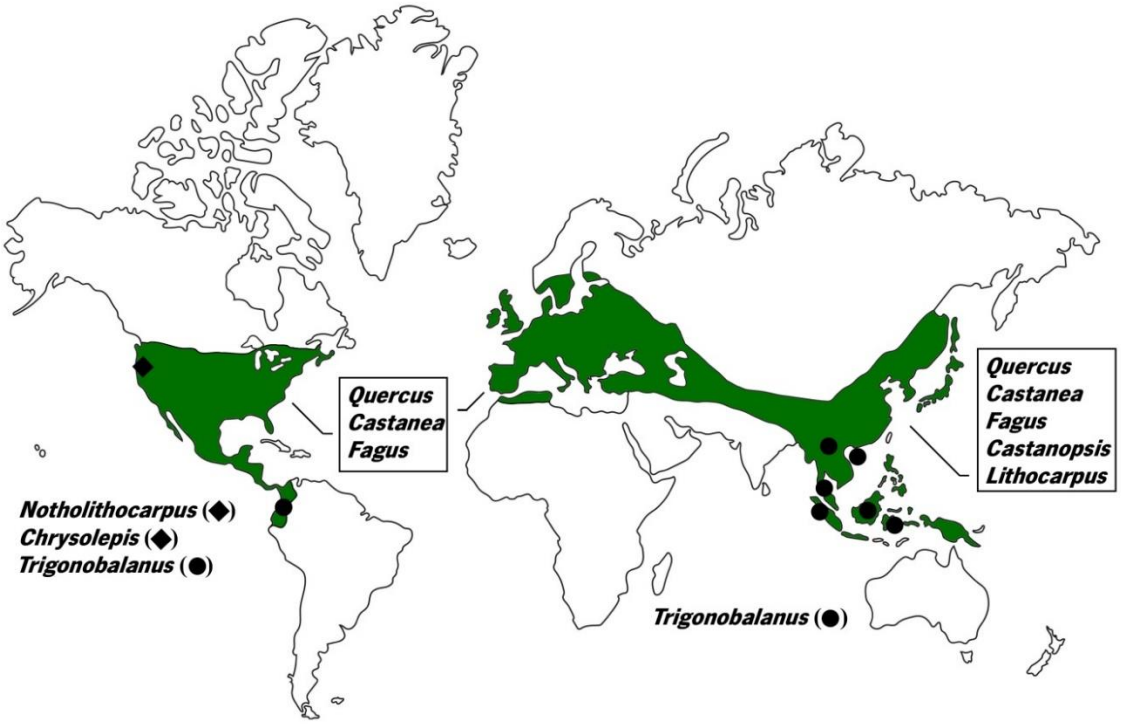

Figure 1.1 World distribution of Fagaceae genera (adapted from Kremer *et al.*, 2012). *Quercus, Castanea* and *Fagus* genera are the most widespread genera and dominate broadleaf deciduous Mediterranean forests.

Mediterranean natural forests contain about 100 different tree species, whereas only 30 are present in forests of central Europe (four times larger; Valavanidis and Vlachogianni, 2011). The Mediterranean forest is mainly composed by broadleaved evergreen tree species, such as oaks and mixed sclerophyllous trees, that alone present more than 20 species in the Mediterranean region (Valavanidis and Vlachogianni, 2011). Conifers are also frequently found (Aleppo pine – *Pinus halepensis*; stone pine - *P. pinea*), being the rare conifer species of *Abies*, *Juniperus* and *Taxus* commonly found in mountains. The most frequent oak species are the cork oak - *Quercus suber* – Fig. 1.2A; the holm oak – *Q. ilex* [considered as two subspecies: *Q. ilex* subsp. *ilex* and *Q. ilex* subsp. *rotundifolia* (Amaral-Franco, 1990) or as two different species: *Q. ilex* and *Q. rotundifolia* (Lumaret *et al.*, 2002)]; or the Turkey oak – *Q. cerris*. While some oak species, like holm oak and kermes oak (*Q. coccifera*), encircle whole the Mediterranean Sea, others like cork oak and Mediterranean oak (*Q. canariensis*) exhibit a denser distribution in the western region

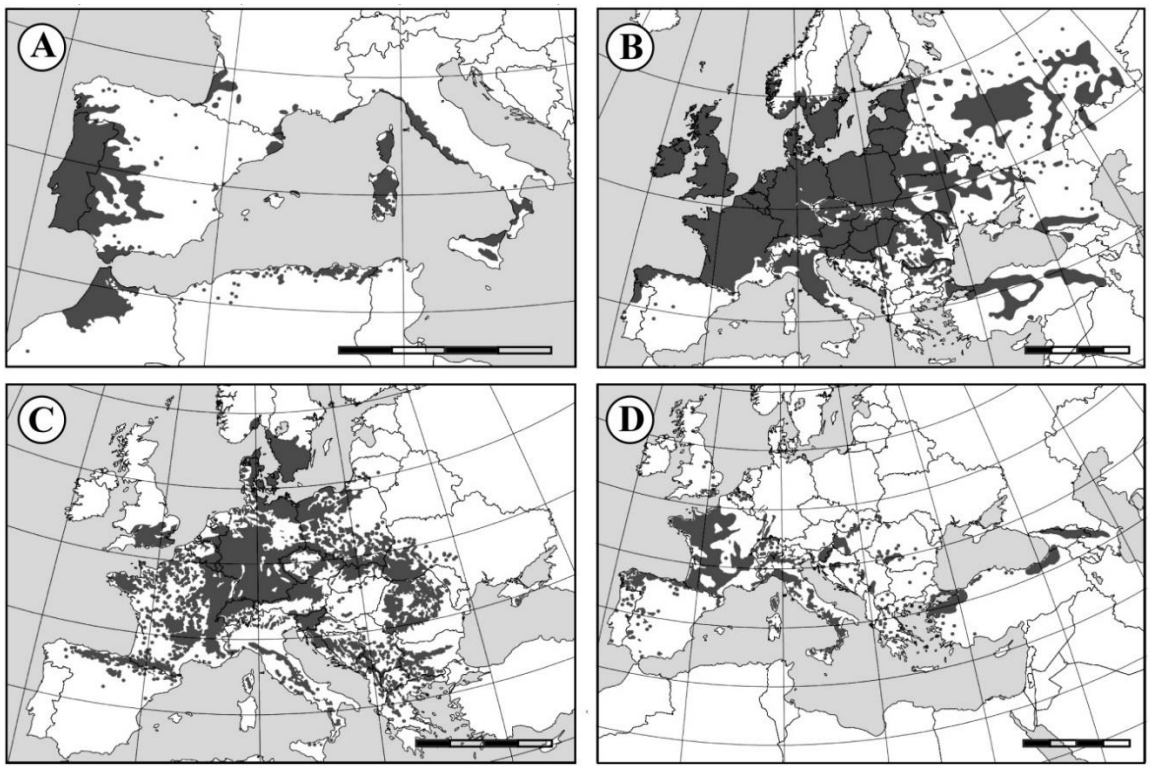

Figure 1.2. European distribution of the most important Fagaceae species for the economy of Mediterranean Basin countries. *Quercus suber* (A), *Fagus sylvatica* (B), *Quercus robur* (C) and *Castanea sativa* (D). (www.euforgen.org)

(Condé *et al.*, 2005). Although *Q. robur* is also found in Mediterranean countries, this tree species distribution is more evident in central and northern Europe (Fig. 1.2B), as also reported for *Fagus sylvatica* that has the preferable climate and soil properties in the central Europe

(Fig. 1.2C; EUFORGEN, 2016). *Castanea sativa* presents the smallest forest area in Europe (predominantly in north of Iberian Peninsula, France and west of Italy), mainly due to widespread diseases and cultural practices (Fig. 1.2D; Condé *et al.*, 2005). On the other hand, regions with increased water availability are more favorable for downy oak (*Q. pubescens*), Valonia oak (*Q. ithaburensis*) or golden oak (*Q. alnifolia* – Cyprus native) growth (Condé *et al.*, 2005). In all Mediterranean region, the dominant shrubs present in Fagaceae forests are highly aromatic, namely *Cistus*, *Genista*, *Calluna*, *Arbutus*, thyme and sage (Condé *et al.*, 2005).

1.3. Mycodiversity in Fagaceae forest ecosystems

The interaction between trees and ECM fungi are dependent on many factors, namely tree species, environmental conditions, belowground interactions, among others (Öpik *et al.*, 2006). Even season variations have an important role in ECM fungal dynamics in the soil. According to Voříšková *et al.* (2014), seasonal changes have a significant impact on fungal activity, biomass content and composition, as well as in the relative abundance of different fungal groups in temperate oak forests. A recent work performed in *Castanopsis fargesii, Lithocarpus harlandii, Pinus armandii*, and *Pinus massoniana* forests revealed that ECM community is much more dependent on the host plant species (33.3%) than soil origin (4.6%) (Ding *et al.*, 2011). This is an important result to take into consideration in reforestation programs, dictating that adequate tree species selection is essential due to ECM host preference.

Fagaceae forests present a quite stable ECM community, mainly consisting of Basidiomycota species, like Russulaceae (Russulales), Thelephoraceae (Thelephorales), *Boletus* (Boletales), Cortinariaceae, Inocybaceae and Amanitaceae (all Agaricales) species. When analyzing the ECM community diversity of Japanese and Chinese Fagaceae forests, the fungal families Russulaceae and Thelephoraceae were indeed the most abundant, being *Russula, Tomentella* and *Clavulina* the most common ECM fungi (Wang *et al.,* 2011; Toju *et al.*, 2014). Results also showed that these Fagaceae forests, comprising *Castanopsis sieboldii, Lithocarpus edulis,* and *Quercus salicina*, present 3-fold more abundant ECM fungi than non-Fagaceae forests (Lauraceae, *e.g. Machilus japonica* and *Neolitsea sericea*; Toju *et al.*, 2014). A North-American *C. dentata* forest also revealed the same trend as Asian Fagaceae forests, with Russulaceae as the major fungal family identified either by fruitbodies collection (aboveground analysis) or by morphotyping ECM root tips followed by direct sequencing of corresponding rDNA-ITS region (belowground

analysis) (Palmer *et al.*, 2008). Although highly abundant, the relative abundance of Boletales, Cortinariaceae, and Thelephoraceae was different in both fungal community views.

The temperate forests from the Mediterranean Basin uncover highly diverse ECM fungal communities, in which several hundreds of fungal species coexist (*e.g.* Richard *et al.*, 2005; Buée *et al.*, 2009). In a metanalysis study where fruitbodies surveys were compared in holm oak, cork oak and mixed forests from Andaluzia (Spain) region, a common dominance of Agaricomycetes species (*e.g.* Boletales and Russulales) was found (Ortega and Lorite., 2007). In this study, a higher diversity and number of exclusive species were reported for cork oak forests. The diversity and structure of other Mediterranean *Quercus*, *Fagus*, and *Castanea* ECM communities have also revealed a high dominance of Russulaceae, Cortinariaceae, Thelephoraceae, and Inocybaceae fruitbodies (Table 1.1).

DNA technologies have improved fungal ecology studies during the recent past years (Anderson *et al.*, 2007). Fruitbodies as well as root tip descriptions have been greatly enriched by soil-based metabarcoding DNA sequencing (Shokralla *et al.*, 2012). Even though this recent approach revealed a high potential for microbial diversity identification in every ecological guilds, there are still some issues remaining when applying Next Generation Sequencing (NGS) methods for assessing fungal diversity (Orgiazzi *et al.*, 2015). When studying Fagaceae ECM communities recurring to molecular methods, such as ITS barcoding of ECM tips (*e.g.* Richard *et al.*, 2005) or ITS metabarcoding of soil samples approaches (Buée *et al.*, 2009), which are methods not dependent on the ability of fungi to produce conspicuous fruitbodies, a different picture of ECM community is obtained. While ECM surveys based exclusively on fruitbodies identification (aboveground approaches) have been hyper-dominant in Basidiomycetes species (mainly Agaricomycetes), a high diversity of Ascomycetes has been detected using belowground approaches based on molecular methods (Peintner *et al.*, 2007; Orgiazzi *et al.*, 2012; Baptista *et al.*, 2015). In spite of that, a higher abundance of Basidiomycota OTUs (operational taxonomic units) has been consistently found. However, from 140 identified taxa among 558 ectomycorrhizal *Q. ilex* root tips, the Ascomycota *Cenococcum geophilum* dominated (35% of ECMs), together with Russulaceae (21.4%), Cortinariaceae (7.1%) and Thelephoraceae (25%) (Richard *et al.*, 2005). The same trend was detected by Azul *et al.* (2010) when studying the influence of managed oak woodlands dominated by *Q. suber*, under different land use practices, by using the same ECM root tips surveys complemented with ITS rDNA analysis. In this study, the Ascomycota *C. geophilum*, together with Russulaceae and Thelephoraceae, represented 56% of whole ECM fungal community.

A positive correlation between ECM fungal richness and silvo-pastoral exploitation regime and low mortality of cork was detected in this study (Azul *et al.*, 2010). In addition, the use of NGS DNA sequencing methods on *Fagus sylvatica* forest soils revealed that the most abundant fungal genera were *Russula*, *Boletus*, but also *C. geophilum* (Coince *et al.*, 2013). Moreover, *C. geophilum* was

Table 1.1. ECM communities present in Fagaceae forests in Mediterranean Basin ecosystems. Revision of published studies since 2000.

Fagaceae species	Ecosystem	ECM taxa	Approach	Reference
Q. ilex	Corsica Island, France	*Russula, Amanita, Tricholoma, Cortinarius*	Root tips	Richard *et al.*, 2004
Q. ilex	Mediterranean forests	*Cenococcum geophilum*	Root tips	De Román and De Miguel, 2005
Q. ilex	Mediterranean forests	*Cenococcum geophilum*, Russulaceae, Cortinariaceae Thelephoraceae	Root tips	Richard *et al.*, 2005
Q. ilex	Mediterranean forests	Thelephoraceae, Russulaceae, Cortinariaceae	Root tips	Richard *et al.*, 2011
Q. ilex	Southern France	Thelephoraceae, Pyrenomataceae	Root tips	Taschen *et al.*, 2015
Q. suber	Moroccan woodlands	*Pisolithus, Boletus aureus*	Fruitbodies survey	Yakhlef *et al.*, 2009
Q. suber	Portuguese montados (savanna-type forests)	*Cenococcum geophilum,* Russulaceae, Thelephoraceae	Root tips	Azul *et al.*, 2010
Q. suber	Declining forest in northwestern Sardinia, France	Pyronemataceae, Thelephoraceae, Russulaceae, Inocybaceae, Cortinariaceae	Root tips	Lancellotti and Franceschini, 2013
Q. suber	Portuguese forests and landscapes	*Russula*, *Tomentella*, *Cenoccoccum*	Root tips	Reis *et al.*, unpublished results
Q. suber and *Q. canariensis*	South of Spain	*Lactarius chrysorrheus, Cenococcum geophilum*	NGS	Aponte *et al.*, 2010
Q. petraea	Czech Republic	*Russula, Lactarius*	NGS	Voříšková *et al.*, 2014
Q. petraea and *Q. robur*	100-year-old forest in northeastern France	*Tomentella, Lactarius, Cenococcum*	Root tips	Courty *et al.*, 2008

Table 1.1. *continuation*

C. sativa	Greece	*Amanita caesaria, A. rubescens, Boletus edulis, B. aereus, Cantharellus cibarius, Craterellus cornucopioides, Hydnum repandum, H. rufescens*	Fruitbodies surveys	Diamandis and Perlerou, 2001
C. sativa	Italy	*Russula, Inocybe, Lactarius, Tricholoma, Cortinarius* and *Amanita*	Fruitbodies surveys	Laganà *et al.*, 2002
C. sativa	Italy	*Cenococcum geophilum, Boletus aestivalis, Lactarius chrysorrheus*	Root tips and fruitbodies survey	Peintner *et al.*, 2007
C. sativa	healthyand *Phytophthora*-infected forests in central Italy	*Cenococcum geophilum, Oidiodendron maius*	Root tips	Blom *et al.*, 2009
C. sativa	Portuguese orchards	*Russula, Inocybe, Lactarius, Tricholoma, Boletus, Cortinarius, Amanita*	Fruitbodies survey	Baptista *et al.*, 2010
C. sativa	Portuguese orchards	*Inocybe, Hypholoma, Amanita* (above) and *Inocybe, Amanita, Sistotrema* (below)	Fruitbodies survey and NGS	Baptista *et al.*, 2015

the main ECM fungus reported in root tips assessment in *Q. rubra* forests, although its abundance has oscillated significantly with tree age (Gebhardt *et al.*, 2007).

Although the ECM association is the dominant symbiotic relationship, Mediterranean Fagaceae species can also be simultaneously colonized by different mycorrhizal fungal types, such as AM and ericoid fungi, among others (Bergero *et al.*, 2000). Accordingly, in oak forests a higher number of AM fungal spores (mainly *Ambispora gerdemannii*) have been found when compared to other landscapes, such as pine forests, combined forests of pines and oaks, or in several agro-ecosystems (Chaturvedi *et al.*, 2012). In addition, the symbiotic relationship between plant and ECM fungi can be mediated by other microorganisms or plants (Herrmann, 2007; Toju *et al.*, 2014). For example, recent studies on red oak (*Q. rubra*) have showed that soil bacteria can help plants to establish ECM symbiosis by maintaining adequate plant signaling gene levels that will promote mycorrhization (Kurth *et al.*, 2015). Accordingly, as obligatory ECM hosts, *Quercus* are usually sensitive to shifts on microbial communities (Smith *et al.*, 2007).

To conclude, the enriched decaying litter soil from Fagaceae forests is an excellent habitat for fungal development and has been a natural source of many economically important

mushrooms (Boa, 2004). Those edible ECM fungi naturally associated with Fagaceae trees, mainly in *Castanea* or *Quercus* forests, comprise a main forest sub-product for population food supply, as well for the production of natural medicines (Boa, 2004; Savoie and Largeteau, 2011). However, ECM mushroom harvesting has been dramatically decreasing in the past century (Yun and Hall, 2004), mainly due to air pollution and litter accumulation in soil surface (Smit *et al.*, 2003). For all these reasons, the preservation of forests including Fagaceae forests has become, not only ecologically important, but also necessary for maintaining an ECM edible mushroom repository.

1.4. Disturbance and protection of Fagaceae forests from biotic threats

Beyond ecological and physiological importance to the forests, ECM community is essential for plant tree disease prevention and incidence (Smith and Read, 2008). The most devastating diseases of Fagaceae family are caused by *Phytophthora* spp. (ink disease and oaks decline) and *Cryphonectria parasitica* (blight disease). The sudden oak disease caused by *Phytophthora ramorum* has been responsible for the rapid mortality of native oak trees (*Quercus* spp. and *Lithocarpus densiflorus*) in central and northern California (USA) since its first observation in 1995 (DiLeo *et al.*, 2009). More recently, surveys revealed that *P. ramorum* was introduced into Pacific northwest nurseries (Hansen, 2003) and into at least eight European countries by movement of stock plants (Brasier *et al.*, 2004). Also, the introduction of the causal agent of chestnut blight disease (*C. parasitica*) by the importation of infected Asian chestnut trees to the USA east coast in the early 20th century almost led to the extinction of American chestnuts (*C. dentata*; Milgroom *et al.*, 1996). Indeed, this later epidemic has been considered as one of the greatest ecological disasters in USA history (Wheeler and Sederoff, 2009) and one of the most devastating plant disease epidemics caused by fungi or fungal-like oomycetes (Fisher *et al.*, 2012). Although pedunculate oaks (*Quercus petraea* and *Q. robur*), holm oak (*Q. ilex*) and *Castanopsis* have been also classified as *C. parasitica* host species by the European Plant Protection Organization (EPPO), corresponding plant damages are relatively less when compared with chestnut species. Although susceptible to this fungus, the relatively higher tolerance of European chestnut (*C. sativa*) in comparison to the American chestnut prevented the heavy mortality levels previously observed in USA (Heiniger and Rigling, 1994). However, when *C. parasitica* was first observed in Europe (Genova, Italy, in 1938; reviewed by Anagnostakis, 1987), the blight disease rapidly spread all over France, Spain and Portugal chestnut orchards (Robin and Heiniger, 2001).

Within the Mediterranean region, oomycetes from *Phytophtora* spp. are serious threats to Fagaceae forests. Between 1900 and 1950, the main *C. sativa* growing areas of southern Europe, especially Italy, France and Iberia, suffered heavy mortality due to the chestnut ink disease caused by *Phytophthora cambivora* and *P. cinnamomi* (reviewed by Brasier, 2000). After introduction in the late 18th century from a centre of origin in the Papua New Guinea-Celebes, this disease rapidly spread in France and in all chestnut growing areas (Vettraino *et al.*, 2002), being the main reason for abandonment of several chestnut orchards. In addition, *P. cinnamomi* has been reported as the agent responsible of ink disease of red oak (*Quercus rubra*; Robin *et al.*, 2012), and as the primary factor of root infection resulting in oaks decline and mortality in Mediterranean countries (Brasier *et al.*, 1993). Although cork and holm oaks decline have occurred in the Mediterranean Basin since the beginning of the 20th century, only in the early 1980s a severe oaks decline was reported across the Mediterranean region (Brasier, 1996). Oaks decline has been described as a complex disease triggered by several interacting environmental constraints, including pathogens (*P. cinnamomi*), as well as drought and other site factors (soil texture and fertility, slope) (Camilo-Alves *et al.*, 2013). The affected oak trees face a progressive defoliation that can go over 75% (Franceschini *et al.*, 2002). Typical symptoms of *Phytophthora* diseases have also been observed in *Fagus* stands of several European countries in the last two decades, which are caused by *P. citricola*, *P. cambivora* and *P. cactorum* (Schmitz *et al.*, 2006), and in Swedish *Q. robur* stands caused by *P. quercina* (Jönsson-Belyazio and Rosengren, 2006).

All *Phytophthora* diseases result in severe leaf loss, which would lead to the reduction of root sugar content and would alter the ECM community of diseased plants. Accordingly, tree crown defoliation has been shown to modify ECM community structure in Scots pine (Kuikka *et al.*, 2003) and increase the frequency of thin mantled ECM morphotypes (Saravesi *et al.*, 2008). Even artificial defoliation has been reported to negatively affect ECM symbionts by reducing the production of fungal biomass in interacting roots (Markkola *et al.*, 2004; Stark and Kytöviita, 2005). Comparing healthy and ink diseased chestnut stands, Blom *et al.* (2009) found differences in the richness of ECM communities and relative abundance of most important ECM fungi. *Cenococcum geophilum* was dominant on both stands, but its relative abundance was 1.5-fold higher in the infected orchard. Also, other Basidiomycota, such as Boletaceae, Paxillaceae, Sistotremataceae, Hydnaceae and Atheliaceae showed significantly higher values in infected soils, whereas Thelephoraceae, Cortinariaceae and Sebacinaceae showed an opposite trend (Blom *et al.*, 2009). As a result of oak decline disease, a reduction of ECM diversity and ECM root colonization

has been detected in *Q. ilex* trees (Causin, 1996; Montecchio *et al.*, 2004). In contrast, *Q. suber* declined trees do not present differences in ECM community when compared to healthy trees (Lancellotti and Franceschini, 2013). But, although no differences in ECM community have been detected in Spanish *Q. ilex* forest trees infected or not with *P. cinnamomi*, non-mycorrhizal root tips seem to be more susceptible to infection than mycorrhizal ones (Corcobado *et al.*, 2014). Although these results indicate that ECM communities are strongly affected in diseased Fagaceae plants, ECM fungal species could also contribute for disease protection. This feature could be provided by the formation of a mantle that serves as a physical barrier to the pathogen, by the production of antibiotics that inhibit pathogen growth and reproduction, by diverging plant exudates that could act as biochemical signals to the disease agent, by providing habitat for antagonistic rhizosphere microorganisms, or by improving plant vigor and protection potential (reviewed by Keen and Vancov, 2010). Accordingly, a number of ECM fungi have been already related to *P. cinnamomi* suppression in conifers and eucalyptus forests (Marx, 1972; Malajczuk, 1979; Malajczuk and McComb, 1979) and several ECM fungal isolates (mainly *Suillus brevipes*) have revealed high antagonistic potential against *Phytophtora* sp. (Mohan *et al.*, 2015). The direct protection of ECM fungi against both *P. cambivora* and *P. cinnamomi* infection was achieved after inoculation of *C. sativa* seedlings with *Laccaria laccata*, *Hebeloma crustuliniforme*, *H. sinapizans* and *Paxillus involutus* (Branzanti *et al.*, 1999). Biocontrol and bioprotection strategies by using ECM could then be the future key for Fagaceae diseases prevention and treatment. This kind of information would be important for advising tree nurseries involved in reforestation programs, even though artificial inoculation of *Q. garryana* and *F. sylvatica* seedlings has not been considered necessary in nursery practices (Southworth *et al.*, 2009; Pietras *et al.*, 2013). In any case, the inoculation of *Q. ilex* seedlings with *Hebeloma mesophaeum* revealed to increase the mycorrhizal colonisation and plant growth while reducing the need for fertilisers (Oliveira *et al.*, 2010). Also, *Q. ilex* and *Q. faginea* artificial mycorrhization with *Tuber melanosporum* improved seedling growth, water and phosphorous acquisition (Núñez *et al.*, 2006). Although the growth of cork oak nursery seedlings has not increased by artificial inoculation with *Pisolithus tinctorius*, several physiological parameters, such as higher photosynthetic capacity, water use efficiency, and N uptake capacity, benefit from mycorrhization (Sebastiana *et al.*, 2013).

In the recent past years, asymptomatic endophytic fungi have been also regarded as potential biocontrol agents for tree diseases (*e.g.* Arnold *et al.*, 2003; Blumenstein *et al.*, 2015). The oaks decline has been correlated with the diversity and amount of fungal endophytes present

on different tissues of *Quercus* spp., and many oak-specific endophytes are specifically described to accelerate the decline of oaks stand (Ragazzi *et al.*, 2001, 2003, 2004). *Q. cerris* exhibited a more diverse endophytic assemblage, but greater infection levels, than *Q. pubescens* suggesting a role of some pathogenic fungal endophytes in Mediterranean oak forests (Moricca *et al.*, 2012).

Other biocontrol agents against Fagaceae diseases are now arising. Strains of the chestnut blight fungus, *C. parasitica*, harboring asymptomatic mycoviruses (CHV1-4; reviewed by Xie and Jiang, 2014) are described to induce hypovirulence (virulence attenuation) (Dawe and Nuss, 2001). The use of the complex triple interaction (hypovirus, fungal pathogen, and chestnut tree) for controlling chestnut blight in orchards remains a possibility (Xie and Jiang, 2014). Antagonistic microbes or metabolites produced by them have been also studied as potential biocontrol agents against *Phytophthora* spp. causing chestnut ink disease (reviewed by Choupina *et al.*, 2014). Most promising results were obtained with *Trichoderma* sp., *Gliocladium* sp., and *Pseudomonas* sp. (Aryantha *et al.*, 2000).

1.5. Fagaceae mycorrhization in a Mediterranean changing climate

The sustainability of forests is extremely dependent on both biotic and abiotic factors and worldwide climate changes are affecting forests all over the world (Keenan, 2015). The effects of drought can be minimized by increasing water uptake through fine roots growth, deep taproots formation and by osmotic adjustment in water-stressed roots through the accumulation of osmolytes (reviewed in Brunner *et al.*, 2015). Due to their long-term evolutionary adaptation to long periods without rain and high temperatures, typical Mediterranean tree species, particularly evergreen oaks, are particularly adapted to cope with moderate drought without significant losses of production and survival (Ramirez-Valiente, 2009, 2011). For example, although not so drought tolerant as *Q. ilex* (described as one of the most drought-resistant oaks), cork oak presents rather drought tolerant traits such as deep roots (Kurz-Besson *et al.*, 2006). However, Mediterranean forests are now facing problems due to the rapid environmental changes (Lindner *et al.*, 2014). Forests become more likely to be exposed to extreme events, such as the increased risk of fire, extreme drought events or severe heat waves, which could even lead to the spread of pests and diseases (reviewed by Bussotti *et al.*, 2013; Moricca and Ragazzi, 2008; Moricca *et al.*, 2014). Recurrent episodes of extreme water stress can greatly increase the number of declined trees (also with the contribution of pathogens) and represent a major threat to the survival of Mediterranean plant species (Nardini *et al.*, 2014). Tree plasticity and adaptation to drought is now

slower than the increase of stress severity. In *Q. faginea*, a typical Mediterranean tree, the rate of plant adaptive response in xeric environment is significantly lower than drought increase occurring in Spain (Nuche *et al.*, 2014).

As individual plant responses to environmental changes are largely dependent on fungal symbionts (reviewed by Kivlin *et al.*, 2013), the microbial community present in the forest soil is suggested to play an essential role in plant drought stress resistance. The changing environmental conditions are likely to induce changes in plant physiology and root exudation, altering the composition of root exudates in chemoattractants or signal compounds (Kandeler *et al.*, 2006) and thus changing the structure of ECM communities associated with stressed plants (reviewed by Compant *et al.*, 2010). Accordingly, the increased drought imposed by reduction of rainfall induced significant shifts in *Q. ilex* ECM community composition (Richard *et al.*, 2011). The most common taxa identified in these forests are Thelephoraceae, Russulaceae and Cortinariaceae, but five consecutive years of increased drought have induced a positive response of Cortinariaceae species. In addition, when *F. sylvatica* plants were subjected to drought, no effect was detected in *Lactarius subdulcis* and *Byssocorticium atrovirens* mycorrhizae abundance, but *Xerocomus chysenteron* mycorrhizae occurrence increased almost two-fold (Shi *et al.*, 2002). Furthermore, beech plants mycorrhized with *X. chysenteron* and *L. subdulcis* were able to better cope with drought stress than others. These observations suggested that distinct ECM taxa differently respond to drought by specifically changing their occurrence/abundance in mycorrhized plants, and each plant could be differently affected by drought according to the associated-mycorrhizal community. Furthermore, the structure of *F. sylvatica* ECM communities and metabolic activity of each morphotype was reported to be dependent on the season, temperature and soil moisture, being certain morphotypes more abundant and active in winter than in summer (Bueé *et al.*, 2005). The same authors described *C. geophilum* morphotype as being more active during summer, when the increase in temperature and drought could influence its abundance and enzyme activity as reported in oak ecosystems (*Q. robur*, *Q. petraea* and *Q. pubescens*) (Herzog *et al.*, 2012). Therefore, the overall function of ECM community would result from the occurrence and functional feature of each morphotype. In a complex ecosystem as Fagaceae forests, more than one variable could be influencing ECM communities. European *Q. robur* and *Q. petrea* forests ECM community are influenced by precipitation, pH and N-deposition (Suz *et al.*, 2014).

Diverse drought tolerance levels exhibited by mycorrhized plants are most probably due to the well-recognized differences in drought resistance of specific ECM fungi. *Rhizopogon vinicolor* or

C. geophilum have been reported as drought-tolerant species, being *C. geophilum* also particularly efficient in protecting forest trees against drought damage, while *Laccaria laccata* is described as a drought-sensitive fungus unable to grow at very low water potentials (Coleman *et al.*, 1989; di Pietro *et al.*, 2007). Since the respiration activity of *C. geophilum* ectomycorrhizae has been reported to be significantly less altered than that of *Lactarius* sp., *C. geophilum* was suggested to better maintain the physiological integrity of beech roots facing drought stress (Jany *et al.*, 2002). In contrast, under high temperatures a decreased colonization with *C. geophilum* has been detected in *Quercus myrsinaefolia* (Kasai *et al.*, 2000), agreeing with the observation of its reduced respiration under increasing temperature (Malcolm *et al.*, 2008). In any case, *C. geophilum* being a hydrophilic and short-distance exploration fungus has been suggested as a potential indicator of environmental changes (reviewed by Lehto and Zwiazek, 2011). However, several problems have been discussed about its use in environmental assessments, including its resistance to other stress factors besides drought and its inability of forming fruitbodies.

The ability for water uptaking in a typical Mediterranean climate is essential for tree resistance to drought scenarios and ECMs have been recognized as crucial for drought resistance improvement (Kivlin *et al.*, 2013; Brunner *et al.*, 2015). The water status of drought-stressed trees is highly improved by the increased absorbing surface provided by the ECM fungi, through a higher efficient water conduction by mycelial strands, enhanced soil-root hydraulic conductivity, and other hormonal and nutritional effects that modify plant physiology (reviewed by Breda *et al.,* 2006). Moreover, ECM networks can redistribute water from deep soils to roots or move water among roots of drought-stressed plants (Egerton-Warburton *et al.*, 2007; Querejeta *et al.*, 2007). Accordingly, studies performed in *Q. alba* inoculated with *P. tinctorius* revealed higher water potentials and larger root systems than non-inoculated plants (Dixon *et al.*, 1980). Also, *Q. ilex* seedlings inoculated with *T. melanosporum* exhibited half of root hydraulic conductance than non-mycorrhized roots, but presented 2.5-fold more fine root surface area (Nardini *et al.*, 2000). The best ECM inoculum for improving drought tolerance is difficult to establish, but their choice should be based on fungal water uptake ability and exploration type. Hydrophilic fungi, such as *Russula*, *Hebeloma*, *Lactarius* and *Laccaria* are able to transport water in the apoplast, whereas hydrophobic fungi, like *Paxillus involutus* and *Suillus* spp., need to form mycelia cords to transport water in the symplast (reviewed in Lehto and Zwiazek, 2011). On the other hand, contact mycelia or short-distance exploration mycorrhizae are mainly hydrophilic, whereas long-distance exploration are hydrophobic fungal ECMs (Agerer, 2001). This particular information would be essential in

further research on ECM behavior in drought scenarios or on ECM fungal selection for *in vitro* and field assays.

Forest fires are common in Mediterranean region during summer period but fire risk is clearly increasing due to extreme environmental conditions. Indeed, during the last decade, Mediterranean forest fires (especially in Portugal and Greece) have been associated with extreme weather, in particular to extremely long dry periods with hot temperatures and high wind speeds (reviewed by Lindner *et al.*, 2014). Fire events could have significant effects on fungal communities of Mediterranean forests. After a fire event, the complexity of ECM communities tend to be reduced and replaced by a less diverse community, usually composed by resilient fungal species and previously rare species (Pezizales and *Rhizopogon* spp; reviewed by Buscardo *et al.*, 2010). Colonization by new fungal species can benefit from a competition decrease, being spores the main structures for post-fire natural recolonization. While *Telephora* spp. distribution was strongly affected by fire events in an oak forest, *Tomentella* spp. rapidly raised (Buscardo *et al.*, 2010). When studying the ECM root tips of a *Q. ilex* forest over a 3-year post-fire period, the richness of ECM community and the percentage of root tips were also significantly decreased (De Roman and De Miguel, 2005). *C. geophilum* was the most resilient ECM fungi and maintained its abundance all over the period.

1.6. Advances for Mediterranean Fagaceae-ECM studies

To better understand the symbiotic relationship that occurs between Fagaceae roots and ECM fungi, new molecular tools have been created. Several efforts have been made in order to know the genetic patrimony of several Fagaceae species. To the best of our knowledge, 18 Fagaceae genomes have already been sequenced: eight *Castanea* species and ten *Fagus* species, six of which considered as sub-species (http://www.fagaceae.org/). Other species, such as *Q. alba, Q. rubra* and *Q. suber*, have their genome sequencing ongoing (The Fagaceae Genome Web - http://www.fagaceae.org/home; Genosuber Project - http://www.genosuber.com/). Furthermore, several transcriptomic studies are now allowing the generation of a comprehensive catalog of transcripts from Fagaceae. Recently, a number of transcriptomic studies have been successful at generating expressed sequence tags (ESTs) libraries, mainly from oaks and chestnuts, recurring to NGS approaches (*e.g. Q. robur* and *Q. petraea*, Lesur *et al.*, 2015; *C. sativa* and *C. crenata*, Serrazina *et al.*, 2015). The use of a *Q. robur* gene catalog allowed the discovery of specific molecular mechanisms involved in the regulation of oak ECM symbiosis and the

identification of key molecular players involved in ECM formation (Tarkka *et al.*, 2013). Their main findings concern the plant defense genes attenuation and ethylene signaling enhancement during mycorrhization, cell wall remodeling mechanisms and alteration in several metabolic pathways (*e.g.* nitrogen, phosphorus and sugar transporters). Within a national initiative, a Portuguese consortium was created to study cork oak ESTs and thus develop a new genomic resource for studying *Q. suber* (Pereira-Leal *et al.*, 2014). This achievement has been used to better understand processes related with plant development (Rocheta *et al.*, 2014; Teixeira *et al.*, 2014) and adaptation responses to both biotic (Sebastiana *et al.*, 2014) and abiotic factors (Magalhães *et al.,* 2016). The global overview of up- and down-regulated genes in cork oak roots following inoculation with the *P. tinctorius* resulted in a better insight of those molecular events that control ECM symbiosis (Sebastiana *et al.*, 2014). ECM colonization resulted in extensive cell wall remodeling, activation of the secretory pathway, alterations in flavonoid biosynthesis, and expression of genes involved in the recognition of fungal effectors. Other identified genes could have putative roles in symbiotic processes such as nutrient exchange with the fungal partner, lateral root formation or root hair decay (Sebastiana *et al.*, 2014). The transcriptional response of *C. sativa* during the early contact with *P. tinctorius* revealed that gene expression alterations occur a few hours after contact, long before the development of a functional mycorrhiza (Sebastiana *et al.*, 2009). Host plant rapidly reacts by eliciting a defense program similar to that described for pathogenic interactions and represses genes normally implicated in water stress. All these identified processes are consistent with the idea that ECM fungi alter plant-specific cellular processes, such as development, metabolism or responses to abiotic and biotic stresses.

In addition to these plant-based tools, recent research has been made by the Mycorrhizal Genomics Initiative to sequence nuclear and mitochondrial genomes of 50 fungal species able to establish mycorrhizal symbiosis. Among them, 33 are already concluded, including 26 ECM, four ericoid, two orchidoid and one AM fungal species (reviewed by van der Heijden *et al.*, 2015). Genome sequencing of some ectomycorrhizal fungal species, such as *Laccaria bicolor*, *T. melanosporum* and *P. tinctorius*, opens a window to better understand these processes (Martin *et al.*, 2008, 2010).

Advances in Fagaceae genomics are providing new tools and methodologies for understanding the molecular processes of tree species adaptation to the main challenges (reviewed by Plomion *et al.*, 2015). The climate changes and associated threats, as well as the introduction and spread of new disease agents, could rapidly deteriorate Mediterranean Fagaceae forests.

The understanding of those mechanisms underlying tree adaptation to long term defense strategies, for both biotic and abiotic stresses, and processes leading to the association with beneficial organisms like ECM fungi, could have a major role in devising new strategies for forest sustainability. Innovative management practices and policy actions could be planned to preserve forest adaptation to a changing climate and new threats. Yet, the fundamental knowledge provided by all available genetic resources will not be sufficient for getting immediate effects on forest management. Re-forestation programs will be essential to forest sustainability maintenance, where natural ECM communities would play an important role.

1.7. Thesis main objectives and outline

Cork oak forest sustainability is a major concern for the upcoming years, due to the rising of climatic changes. Many difficulties in forest research, such as investigation timeline and environmental conditions control, have been the major causes of limited forest investigation. The present work pretends to establish cross-linked information between three research fields: plants, soil microbiology and climatic conditions. The main objective is to identify microbial taxa that can act as beneficial symbiotic partners for increasing cork oak tolerance to drought. To achieve this goal it is important to know the microbial communities residing in soils of cork oak stands that present distinct water availability levels, and determine those microorganisms that are more drought-sensitive or tolerant. These microbes could be used in the future as inocula for young cork oak plantlets at nurseries, or as forest fertilizers acting as plant growth promoter agents.

In **Chapter 1**, a general introduction was provided in order to review the main threats to Fagaceae forests, as well as the recent research advances conducted on ectomycorrhization, not only in Fagaceae species in general, but also in the particular case of cork oak. Description was made at worldwide perspective but then became more specific on Mediterranean ecosystems, the main focus of the present work. The following chapters (Chapter 2 to 4) are developed to answer the specific objectives, each including a brief introduction, material and methods as wells as results and discussion.

Environmental studies were conducted to get a global picture of soil microbiome residing on cork oak forest soils, as well to understand the main microbial player for cork oak forests tolerance to the uprising drought conditions. In **Chapter 2**, ectomycorrhizal community of Portuguese cork oak forests was evaluated by barcoding of ectomycorrhized root tips. As the seven sampled forests were located in different climatic regions, presenting distinct precipitation and

temperature parameters, a relation between climatic variables and ECM communities is analyzed. In **Chapter 3**, a metagenomic analysis was performed by high-throughput sequencing of bacterial ribosomal subunit 16S gene amplicons. Using the same cork oak soil samples, a general picture of bacterial microbiome associated with cork oak forests is obtained and related with climatic parameters as well. In **Chapter 4**, the combined analysis of symbiotic microorganisms identified in previous chapters is discussed, taking into account their potential biological role for drought resilience of cork oak forests under a climate change scenario. Finally, in **Chapter 5**, the concluding remarks and future perspectives of this thesis are described for disclosing the role of microbial communities on cork oak ectomycorrhizal formation under drought tolerance.

As a conclusion, this work proposes to cross-link the information regarding different research fields, in order to understand cork oak ectomycorrhization as a cooperative triangle that promotes cork oak forest sustainability. Because plant adaptation to new environmental conditions is not easy to access, this study pretends to give a step further on the clarification of the relationship between forest partners for an increasing resilience of forest to climatic change challenges.

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Chapter 2

Evaluation of ectomycorrhizal community

Ectomycorrhizal fungal diversity and community structure along a Mediterranean climate gradient in cork oak forests

Ectomycorrhizal fungal diversity and community structure along a Mediterranean climate gradient in cork oak forests

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Mycorrhiza (accepted under revisons)

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2.1. Abstract

Global warming is increasing temperature and causing deregulation of water cycle (precipitation storms and long dry seasons). One of the most affected ecosystems is located in the Mediterranean basin, where cork oak (*Quercus suber* L.) forests play an important ecological and economic role. Soil microbial communities, namely ectomycorrhizal fungi (ECMF), are largely responsible for this ecosystem sustainability. In this work, soil samples from cork oak forests residing in different Mediterranean climates occurring in Portugal (arid, semi-arid, sub-humid and humid) were collected and surveyed for ECM root tips. A global analysis performed on 3,565 ECM root tips, identified 161 OTUs from 32 different genera. *Russula, Tomentella* and *Cenoccocum* were the ECMF genera that contribute the most for community differences. ECMF communities from rainiest and drier cork oak forests displayed differences on their composition, being soils from rainiest climates more heterogeneous than driest soil samples. Climate variables are discussed as potential drivers able to shape ECM communities associated with cork oak. ECM community could be of utmost importance when considering the upcoming environmental changes (increased temperature and precipitation decline) that will further threat the sustainability of cork oak stands.

2.2.Introduction

Quercus suber L. is an evergreen oak tree species, typical from the Western Mediterranean region, presenting a significant ecologic and economic importance, especially for the Iberian Peninsula. Cork oak forest highest value lies in Portugal, which is the largest producer of cork (almost 50% of world production) and also one of the largest importers of cork for processing industries (APCOR, 2016). *Q. suber* grows in different forest systems - from the forest type under densities about 400 trees/ha (*sobreirais*) to low density stands (60–100 trees/ha; *montados*) of savannah-like landscape. *Montados* are typical from the southern region of Portugal (Alentejo), where an extensive agro-silvo-pastoral exploitation is frequently found due to the scattered cork oak tree cover, whereas *sobreirais* are typically found in central and northern Portugal.

Cork oak forests represent a very important ecosystem that is now facing increased threats from the predicted climate changes. The combination of increasing mean temperatures and drought, which could also result in increasing wildfires, is one of the main concerns of forest producers (Acácio *et al.,* 2009). Cork oak density and tree distribution are closely related to water availability (Joffre *et al.,* 1999). Climate models have recently been used for predicting the consequences of climatic changes that expect a shift of the dry limit of Mediterranean climate to

the north (Rego and Rocha, 2014). Adaptations to local environmental conditions could drive cork oak population genetic divergence (Ramírez-Valiente, 2009a) and preliminary results on still juvenile cork oak trees revealed that populations could possible cope with climate changes leading to drier and warmer conditions (Varela *et al.,* 2015). However, the moderate capacity of this species to cope with severe drought could lead to the disappearance/scarceness of actual populations (Ramírez-Valiente, 2009b). A severe decline of cork oaks has been reported across the Mediterranean region since early 1980s, although some regions (*e.g.* Tunisia and Sardinia) still present few decline signs (Ben Jamâa and Piazzetta, 2010; Lee *et al.,* 2011). Cork oak decline has been described to be primarily caused by drought stress, although other factors could also be important, as the presence of the root pathogen *Phytophtora cinnamomi* (Braisier, 1996). As a reduction in water availability is expected for the near future (IPPC, 2014), the long-term sustainability of these ecosystems may be further threatened, leading to a decrease of *Q. suber* growth and productivity (Moricca and Ragazzi, 2008; Moricca *et al.,* 2014). Regarding these environmental challenges, cork oak forest decline could implicate beyond economic losses, also microbial, plant and animal diversity losses (Hector *et al.,* 1999).

The microbial community present in the soil could establish different associations with plants, such as beneficial, neutral or harmful, playing an essential role in plant health and productivity (Rout, 2014). Mycorrhizal symbiosis is a well-known beneficial association. Indeed, through the establishment of ectomycorrhizae (ECM), ectomycorrhizal fungi (ECMF) are described as helpers in increasing plant survival and growth rate in forestry ecosystems (Selosse *et al.,* 2000; Menkis *et al.,* 2007), as well as improving host tree health (Hyder *et al.,* 2013). In the particular case of Mediterranean species, ECM are crucial for drought resistance improvement (Jany *et al.,* 2002). The diversity and structure of *Quercus* ECMF communities have already been studied recurring to ITS barcoding on ECM tips (Smith and Read 2008; Azul *et al.,* 2010; Shi *et al.,* 2011; Richard *et al.,* 2011; Lancellotti and Franceschini, 2013). The ECMF richness on cork oak *montados* is correlated with landscape and land use practices (Azul *et al.,* 2010) and an impact of cork oak decline on the diversity and composition of ECM, but not on ECM tip number, has been found (Lancellotti and Franceschini, 2013). In order to assess the ECMF community of *Q. suber* under different drought scenarios, we sampled cork oak forests with a gradient of water availability. Portugal displays diversified climate regions, ranging from humid to arid Mediterranean climate regions, also including sub-humid and semi-arid areas. With this work, we performed a comprehensive assessment of ECM fungal community associated with *Q. suber* root tips at

different landscapes. Differences on ECMF communities structure revealed that soils from humid Mediterranean forests present a more heterogeneous ECMF community when compared to more arid Mediterranean forests that display more similar communities. We suggest that one major drivers among climatic parameters for ECM root tips could be the minimal temperature/precipitation levels registered in locations.

2.3.Material and Methods

2.3.1.Selection of cork oak stands and sample collection

Sampling collection was made in five different geographic locations in Portugal (Figure 2.1). Cork oak stands selection was based on the previous information obtained from the stands (Varela and Eriksson, 1995), as well on the Mediterranean climate classification (Rego and Rocha, 2014) determined by the climatic parameter of Emberger (*Q*; Emberger, 1930). This parameter takes into account the annual precipitation (P), maximal (M) and minimal (m) temperatures of the hottest and coldest months during the sampling year,

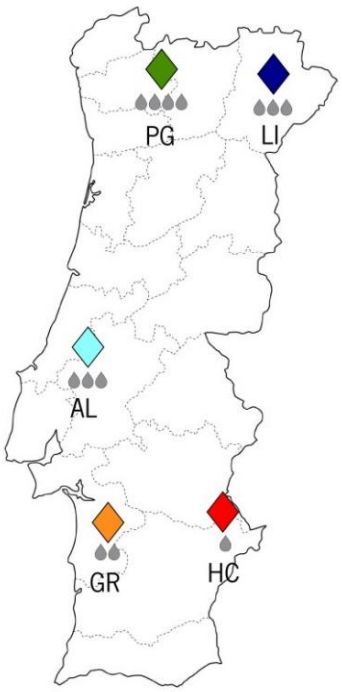

Figure 2.1. Distribution of studied Portuguese cork oak forests. Peneda Gerês (PG), Limãos (LI), Alcobaça (AL), Grândola (GR) and Herdade da Contenda (HC) were selected based on the climatic parameter of Emberger and water availability conditions (water gradient is represented by the number of drops).

Table 2.1. Characterization of cork oak sampling spots, including geographic and climatic conditions. Averages from the past 30 years (1986-2016) of annual precipitation (P annual), precipitation of the months with lowest (P min) and highest (P max) precipitation levels, annual temperature (T annual), temperature of the coldest (T min) and hottest months (T max), were used for determining the indexes of Emberger (*Q*). Soil tillage, forest system and vegetation cover were assessed to understand the agricultural exploitation.

Sampling spot	National Park of Peneda-Gerês (PG)		Limãos (LI)	Alcobaça (AL)	Grândola (GR)	Herdade da Contenda (HC)	
	Ermida (PG-ER)	Rio Cabril (PG-RC)				Contenda (HC-CT)	Monte Asparão (HC-MA)
GPS location	41°42´ 39.76"N 8°6´ 14.87"W	41°45' 43.05"N 8° 1' 39.09"W	41°31´ 51.54"N 6°49´ 56.56"W	39°27´ 41.13"N 9°2´ 42.52"W	38°11´ 32.37"N 8°37´ 11.41"W	38°1´ 45.25"N 7°0´ 27.26"W	38°2´ 24.78"N 7°1´ 55.59"W
Altitude	627	492	601	78	150	437	419
P annual (mm)	1448.4		772.8	651.6	735.6	558	
P min (mm)	22 (July)		15.4 (July)	4.2 (July)	3.7 (July/August)	2.7 (July)	
P max (mm)	220.2 (December)		121.6 (December)	106.8 (November)	124.7 (December)	97.7 (December)	
T annual (ºC)	12.7		15.0	17.0	16.6	16.9	
T min (m) (ºC)	9 (January)		4.5 (January)	10.4 (January)	10.1 (January)	9.7 (January)	
T max (M) (ºC)	21.4 (July/August)		21.7 (July/August)	23.8 (August)	23.2 (August)	24.8 (August)	
Q	186.6 (humid)		88.9 (sub-humid)	102.7 (sub-humid)	77.5 (semi-arid)	43.5 (arid)	
Soil tillage	non-tilled		tilled	non-tilled	tilled	non-tilled	
Forest system	*sobreiral*		*sobreiral*	*sobreiral*	*montado*	*sobreiral*	
Vegetation cover	*Genista* sp., *Cistus* sp., *Ulex* sp., *Erica* sp.		*Cistus* sp.	*Pistacia* sp., *Ulex* sp., *Rubus* sp., *Rosa* sp.	*Cistus* sp.	*Cistus* sp., *Lavandula* sp.	
Soil pH	4.97 (strongly acid)		5.10 (strongly acid)	5.49 (strongly acid)	6.01 (slightly acid)	5.51 (slightly acid)	
sand	94.48		90.57	~100	95.28	90.35	
silt	5.36		8.69	*~0*	4.12	8.44	
clay	0.16		0.73	~0	0.59	1.21	
Soil texture	Sand		Sand	Sand	Loamy sand	Sandy loam	

respectively [$Q = 100P/[(M^2 - m^2)]$; Tate and Gustard, 2000]. This information was obtained by the Portuguese Sea and Atmosphere Institute (IPMA; Table 2.1). Annual precipitation means

from the past 30 years (1986-2016) ranged between 1448.4 mm (National Park of Peneda-Gerês, PG) and 558 mm (Herdade da Contenda, HC), corresponding to the highest (186.6) and lowest (43.5) Emberger indexes obtained, respectively. From the locations presenting the extreme conditions, two independent forests were sampled [Ermida (PG-ER) and Rio Cabril (PG-RC) from PG location; Contenda (HC-CT) and Monte Asparão (HC-MA) from HC location]. Although presenting quite similar annual precipitation means from the past 30 years, the other locations [Alcobaça (AL), Limãos (LI), and Grândola (GR)] presented decreasing Emberger indexes from 102.7 to 77.5 (Table 2.1).

During the autumn season (November and December 2013) all seven cork oak stands were sampled. In each, five apparently healthy trees were selected at least 30 m apart from each other to avoid direct interlacing/connection of their roots. Soil cores were collected under the middle of tree canopy. After removing the uppermost layer of soil that consist in plant litter and other organic material, two independent soil cores (8 cm of diameter and 12 cm in depth) were collected in opposite directions from the cork oak trunk and kept at 4°C until processing. In total, 70 soil cores (7 forests x 5 plots x 2 cores) were collected.

In order to determine soil pH, samples were homogenized by mixture, dried at 40°C during 2 to 3 days and sieved through a 2 mm mesh. After being mixed with deionized water or 0.01 M CaCl2 (1:2.5) the supernatant pH was measured using a glass combination electrode. Soil granulometric analysis was performed using sieve analysis and SediGraph 5100 to determine grain size distribution in the different fractions. The percentage of sand, silt and clay was used for soil texture classification, using the soil texture triangle for Portugal (Gomes and Silva, 1962).

2.3.2. ECM root tips sorting

Each soil core was sieved twice. Particles retained by the 4 mm^2 sieve were discarded and residues with more 2 mm^2 were thoroughly washed. Root tips were isolated and grouped, according to their morphology, color and characteristic features under the dissecting microscope (Figure 2.2). Subsamples of each morphotype in every soil core were selected based on the strategy described by Richard *et al.* (2005; 2011) with some adaptations: (1) one ECM tip was sampled for each rare morphotype (*i.e.* represented by fewer than three ECM tips); (2) two ECM tips were sampled for all morphotypes represented by more than three and less than 10 mycorrhizae; and (3) three ECM tips were considered for all morphotypes represented by at least 10 ECM tips. These ECM subsamples were stored at -20°C until DNA extraction.

2.3.3. Molecular identification of ECMF

Molecular analysis was performed for each root tip. Total DNA was extracted using the Extract-N-Amp™ kit (Sigma-Aldrich, St. Louis, USA). Internal Transcriptional Spacer (ITS) PCR amplification was performed using 10 µl of the Extract-N-Amp™ PCR Reaction Mix (Sigma-Aldrich, St. Louis, USA), 4 µl of stored DNA, 6 µl of distilled water (sterilized) and 1 µl of each primer at 10 mM. All samples were amplified using ITS1F/ITS4 primers and negative amplifications were re-amplified using ITS1F/ITS2 primers (White *et al.*, 1990, Gardes and Bruns, 1993). In addition, DNA samples resulting in two different fungal ITS products were further re-amplified with ITS1F/ITS4B primers (White *et al.,* 1990; Gardes and Bruns, 1993). The thermocycling program included an initial denaturation step of 94°C for 3 min; 35 cycles of 94°C for 30 s, 53°C for 30 s,

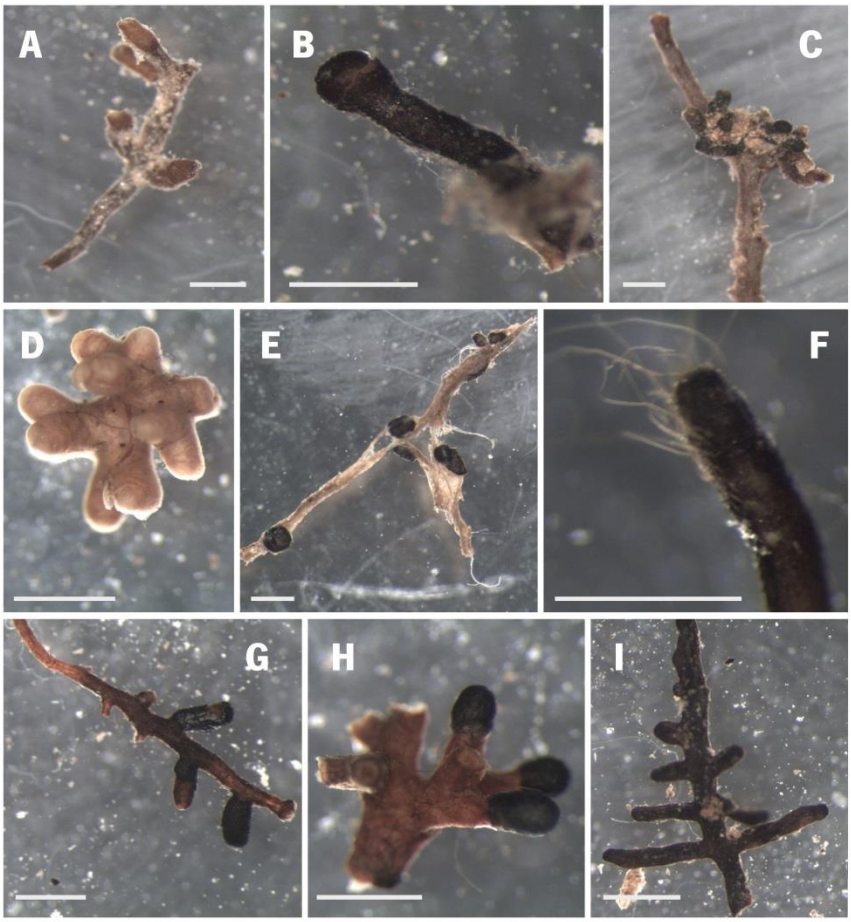

Figure 2.2. Cork oak ectomycorrhizal root tips found during the performed survey (scale = 1mm): *Russula delica* (A), *Tomentella* sp. (B and I), *Cantharellus tubaeformis* (C), *Lactarius camphoratus* (D), *Cennococcum geophilum* (E, F, G and H). Bar scale = 1 mm

Portugal) and visualized under UV light. Amplification products were sequenced by ITS1F primers at Macrogen (Amsterdam, The Netherlands).

Fungal sequences were blasted against UNITE (https://unite.ut.ee/) and NCBI (http://www.ncbi.nlm.nih.gov/) databases. The best BLAST hit was considered based on e-value, higher similarity identity and also on ecological considerations. UNITE identification was overestimated in relation to NCBI descriptions, except when morphological characterization indicated the opposite. Sequences that were identified up to the genus level were further edited with Seqman module of the program DNASTAR (DNASTAR 8, Madison, USA). Alignments of treated sequences were performed at MegAlign module of the same program and sequences presenting more than 97% of identity between them were classified as the same species.

2.3.4. Data and statistical analyses

ECMF community analysis was made based on genera-abundance data, taking into account their relative abundance on each soil sample. In order to simplify statistical analysis, replicates taken from the same tree were combined. Instead of 10 samples from each forest, five samples were considered (each tree considered as one sample). ECMF community's diversity was measured by computational indices that combine both relative abundance and diversity (Magurran, 2004). *Estimates S* - version 9 (RK Colwell, http://purl.oclc.org/estimates) was used to determine alpha [Simpson (D), Shannon (*H´*), Fisher´s alpha] and beta-diversity (Whittaker) indexes, as well as species richness estimators (first-order Jackknife), whereas *Species Diversity and Richness* - version 5 (Pisces Conservation Ltd. Lymington, UK; 2014) was used for rarefaction curves (Henderson and Seaby, 2007). Jaccard's and Bray-Curtis similarity indexes were calculated with the number of identified ECM root tips shared between samples using *Community Analysis Package Version 5* (Pisces Conservation Ltd. Lymington, UK; 2014). For evaluating microbial community changes, analysis of similarity (ANOSIM), similarity percentage analysis (SIMPER) and non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity were performed by *Community Analysis Package Version 5* software (Pisces Conservation Ltd. Lymington, UK; 2014). A Mantel test for correlating the community structure and geographic distances was determined using the Microsoft Excel add-in program XLSTAT (version 2017, Addinsoft, New York, USA). Other Excel tools were used for determining Pearson correlations between climatic conditions and fungal structure; one-way ANOVA followed by Tukey's multiple comparison test were performed using the analysis tools of *GraphPad Prism* software.

2.4. Results and discussion

2.4.1. General description of ECMF community

From the seven cork oak stands surveyed, 3,565 ECM root tips were isolated and 796 tips were used for molecular identification, 74% of which (587 sequences) allowed a successful identification. This analysis revealed 161 OTUs from 23 families and 32 different genera of ECMF (Table 2.2). Basidiomycota (seven orders) and Ascomycota (three orders) were the only phyla identified. From Basidiomycota, Agaricales was the richest order, comprising eight families and genera, being followed by Thelephorales with two families and six genera. Among Ascomycota, Pezizales order was the most diverse (three families and genera identified). Abundance analysis revealed Basidiomycota as the most abundant phylum (3,229 root tips – 82% of the collected root tips) followed by Ascomycota (651 root tips – 18%). Within Basidiomycota, the most abundant families (from 18 identified) were Russulaceae (43% of basidiomycete root tips) and Thelephoraceae (26%). More precisely, *Russula* (76% of Russulaceae root tips) and *Tomentella* (99% of Thelephoraceae root tips) were respectively the most dominant genera. Within Ascomycota, the most abundant root tips belonged to Mytilinidiales order (56% of ascomycete root tips) that only comprised *Cenococcum*.

2.4.2. How is the community structure affected in different Mediterranean climates?

Cork oak ECMF communities differed between sampling locations (Figure 2.3). Two biological replicas were conducted in those extreme water availability scenarios (PG- and HC-locations), corresponding to the most divergent bioclimate scenarios (humid and arid Mediterranean climates, respectively). Northern and rainiest forests (PG-ER, PG-RC and LI) presented higher ECMF abundance than southern and driest forests (HC-CT, HC-MA and GR) (significant difference at $p<0.001$; Figure 2.3A). When comparing northern and rainiest forests (PG-ER/PG-RC and LI) with southern and driest forests (HC-CT/HC-MA and GR), ECMF community differences were evident. This result is associated with a more heterogeneous picture of ECMF community found in those sites. Indeed, the northeast sampling forest (LI) presented the highest abundance of root tips among all sampled forests ($p<0.05$, except for PG-ER) and GR the lowest abundance (significantly different from PG-ER and LI; $p<0.05$). Although a higher number of genera

Table 2.2. Number of ECM root tips and their relative abundance (in brackets) from each genus, identified in each sampled cork oak forest. Each site is referred by their code, as used in table 2.1.

Phylum	Order	Family	Genus	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Ascomycota	Eurotiales	Elaphomycetaceae	*Elaphomyces*	108 (14.84)	14 (2.96)	8 (0.64)	0	0	0	0	130 (3.65)
	Mytilinidiales	Gloneaceae	*Cenococcum*	18 (2.47)	178 (37.63)	0	46 (9.77)	29 (26.61)	74 (23.49)	20 (8.97)	365 (10.24)
	Pezizales	Helvellaceae	*Helvella*	0	0	0	0	0	7 (2.22)	0	7 (0.20)
		Pyronemataceae	*Humaria*	0	0	89 (7.14)	0	0	0	0	89 (2.50)
		Tuberaceae	*Tuber*	0	0	0	0	53 (48.62)	0	7 (3.14)	60 (1.68)
Basidiomycota	Agaricales	Amanitaceae	*Amanita*	0	13 (2.75)	3 (0.24)	3 (0.64)	4 (3.67)	0	0	23 (0.65)
		Cortinariaceae	*Cortinarius*	26 (3.57)	3 (0.63)	19 (1.52)	48 (10.19)	0	22 (6.98)	39 (17.49)	157 (4.40)
		Entolomataceae	*Entoloma*	0	0	133 (10.67)	0	0	0	2 (0.90)	135 (3.79)
		Hymenogastraceae	*Hebeloma*	0	26 (5.50)	0	0	0	0	0	26 (0.73)
		Hydrophoraceae	*Hygrocybe*	0	6 (1.27)	44 (3.53)	9 (1.91)	0	0	0	59 (1.65)
			Hygrophorus	0	0	0	11 (2.34)	0	0	0	11 (0.31)
		Inocybaceae	*Inocybe*	0	4 (0.85)	8 (0.64)	12 (2.55)	2 (1.83)	22 (6.98)	0	48 (1.35)
		Hydnangiaceae	*Laccaria*	6 (0.82)	1 (0.21)	0	0	0	0	17 (7.62)	24 (0.67)
		Tricholomataceae	*Tricholoma*	0	0	35 (2.81)	0	0	1 (0.32)	3 (1.35)	39 (1.09)
	Atheliales	Atheliaceae	*Piloderma*	0	0	0	5 (1.06)	0	0	0	5 (0.14)
	Boletales	Boletaceae	*Boletus*	31 (4.26)	4 (0.85)	23 (1.85)	3 (0.64)	0	0	0	61 (1.71)
			Leccinum	0	1 (0.21)	0	0	0	0	0	1 (0.03)
			Xerocomus	7 (0.96)	2 (0.42)	0	0	0	0	0	9 (0.25)
		Sclerodermataceae	*Pisolithus*	0	0	0	0	0	1 (0.32)	0	1 (0.03)
	Cantharellales	Cantharellacae	*Cantharellus*	127 (17.45)	0	0	0	0	0	0	127 (3.56)
		Clavulinaceae	*Clavulina*	16 (2.20)	9 (1.90)	38 (3.05)	0	0	0	0	63 (1.77)
		Hydnaceae	*Sistotrema*	0	1 (0.21)	0	0	0	0	0	1 (0.03)
	Russulales	Russulaceae	*Lactarius*	160 (21.98)	11 (2.33)	2 (0.16)	64 (13.59)	0	5 (1.59)	1 (0.45)	243 (6.82)
			Russula	127 (17.45)	0	601 (48.23)	35 (7.43)	15 (13.76)	133 (42.22)	111 (49.78)	1022 (28.67)
	Sebacinales	Sebacinaceae	*Sebacina*	0	65 (13.74)	6 (0.48)	30 (6.37)	0	0	0	101 (2.83)
			Hydnellum	0	2 (0.42)	0	0	0	0	0	2 (0.06)
	Thelephorales	Bankeraceae	*Phellodon*	1 (0.14)	0	0	0	0	0	0	1 (0.03)
			Sarcodon	1 (0.14)	0	0	0	0	0	0	1 (0.03)
			Pseudotomentella	12 (1.65)	27 (5.71)	0	0	0	0	0	39 (1.09)
		Thelephoraceae	*Thelephora*	0	11 (2.33)	0	0	0	0	0	11 (0.31)
			Tomentella	88 (12.09)	95 (20.08)	237 (19.02)	205 (43.52)	6 (5.50)	50 (15.87)	23 (10.31)	704 (19.75)
			TOTAL	**728 (100)**	**473 (100)**	**1246 (100)**	**471 (100)**	**109 (100)**	**315 (100)**	**223 (100)**	**3565 (100)**

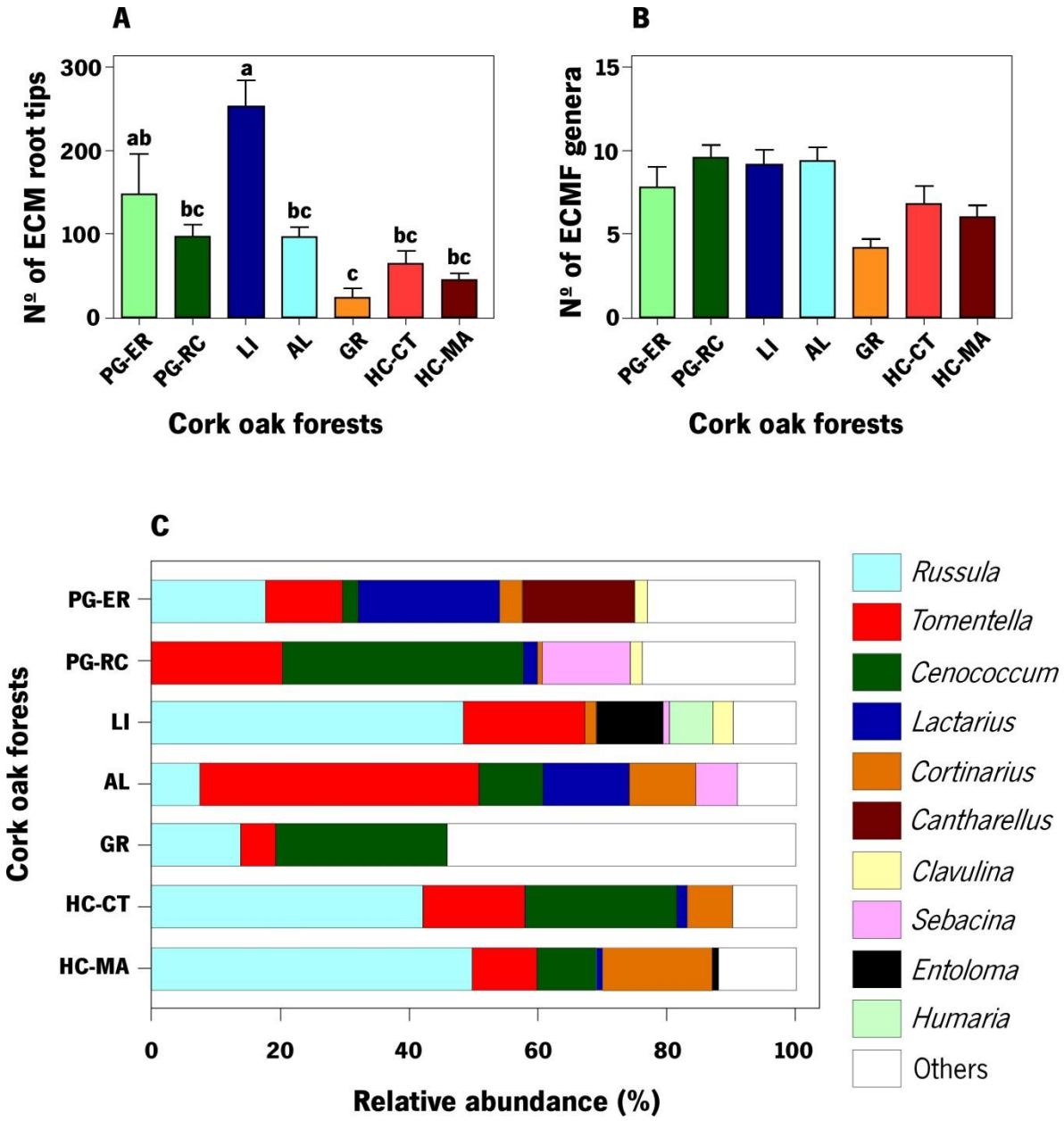

Figure 2.3. Abundance (A), richness (B) and most abundant ECM fungal genera (C) identified by root tips analysis from all sampled cork oak stands. Results are presented considering the statistical analysis of five replicates (cork oak trees) from each sampling site. Different letters denote statistically significant differences (at $p<0.05$; one-way ANOVA, Tukey test).

(14 and 19) were identified in the rainiest locations (PG-ER and PG-RC, respectively) and a fewer (9) in the driest (HC-CT and HC-MA each), no significant differences were revealed in genera richness among sampling sites (Figure 2.3B). In contrast, southern samples were more similar between each other and present a lower number of ECM root tips and ECMF genera. Even between forest replicas, a higher similarity was always found among sampled soils from southern than among northern soils.

ECM fungal communities were compared between forests/samples by computation of rarefaction curves (Figure 2.4) and diversity indices (Table 2.3; Table S2.1). Rarefaction curves suggested that ECM root tips from all forest sampling sites could give information about

Table 2.3. Diversity parameters for cork oak ECM fungal communities detected by root tip barcoding. Total number of ECMF root tips (N), ECMF number of identified genera (S), alpha diversity indexes [Simpson's index (D), Shannon index (*H'*), Fisher's alpha], richness estimator [1st order Jackknife] and beta diversity Whittaker index are represented. The lowest estimates are highlighted in bold, being the highest underlined. Each site is referred by their code, as used in table 2.1. Letters mean statistical differences for each parameter (p<0.05; one-way ANOVA, Tukey test), using the values determined for each replicate (5 replicates/site). All the replicate values are described in Table S1.

	N	S	D	H'	α Fisher	Jacknife index	Beta diversity (Whittaker)
PG-ER	728^{ab}	29^{ab}	3.67^{ab}	2.06^{ab}	5.35^{a}	50^{ab}	2.85
PG-RC	473^{bc}	36^{a}	3.43^{ab}	1.96^{a}	7.68^{a}	62.6^{a}	2.85
LI	1246^{a}	38^{a}	3.748^{ab}	2.19^{a}	6.57^{a}	67^{a}	3.24
AL	471^{bc}	36^{a}	3.83^{a}	2.32^{a}	7.69^{a}	61.8^{a}	2.94
GR	**109^{c}**	**12^{b}**	**3.06^{b}**	**1.46^{b}**	**3.03^{a}**	**21^{b}**	**2.10**
HC-CT	315^{bc}	22^{ab}	3.32^{ab}	1.73^{ab}	4.69^{a}	38.2^{ab}	2.38
HC-MA	223^{bc}	21^{ab}	3.68^{ab}	2.00^{ab}	4.86^{a}	35.6^{ab}	2.67

ECMFcommunity, although they did not reach a clear plateau (Figure 2.4). Northern forests presented a more diversified ECMF community, mainly LI and PG-RC, but also PG-ER. However, the central AL forest exhibited the most diversified ECMF community. Accordingly, AL forest competed with the most northeastern forest (LI) for higher alpha diversity indexes, while the southern forest GR consistently presented the lowest values (Table 2.3). Therefore, while AL and LI forests presented the most diverse ECMF communities, GR exhibited the poorest community of all sampled forests. Differences between the highest and lowest diversity indexes detected in these forests were always statistical significant (p<0.05), except for Fisher´s alpha (Table 2.3). A similar pattern was observed for beta diversity described by the Whittaker estimator, being LI forest the most diverse and GR the less diverse communities. At ecological level, both biological replicas (PG-ER/PG-RC and HC-CT/HC-MA) presented similar values for beta diversity index, which validated our sampling strategy-

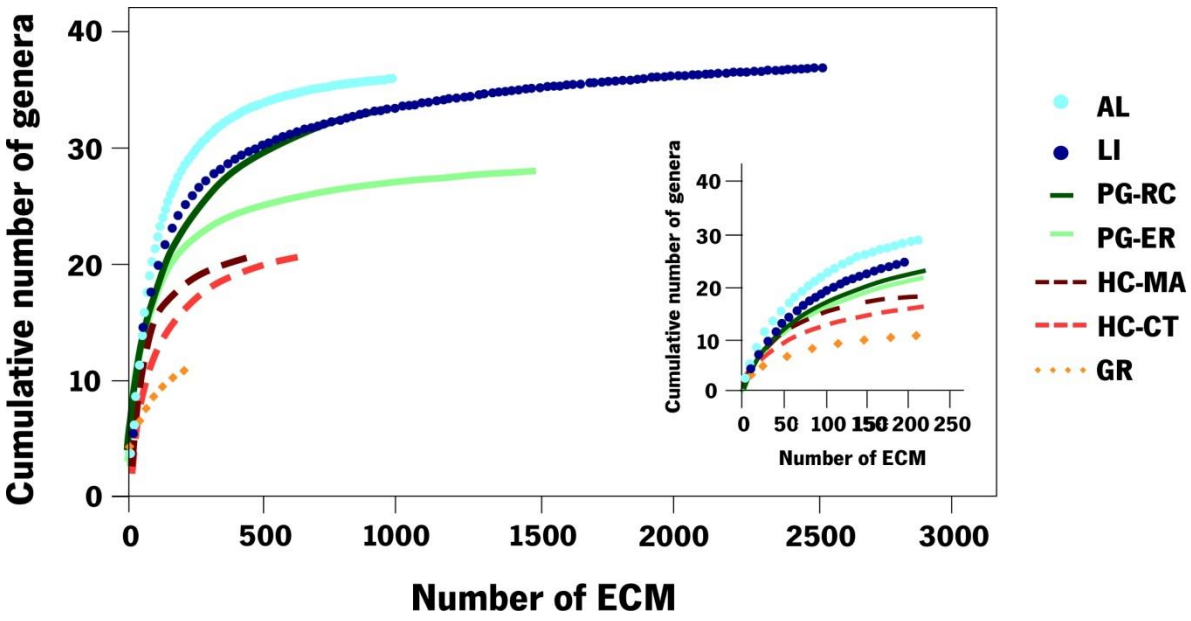

Figure 2.4. Rarefaction curves for ECM fungal communities present in the sampled cork oak forests. Inset: Detailed rarefaction curves normalized to the lowest number of ECM root tips found among forests (GR – 218 ECM root tips). Rarefaction curves were computed by *Species Diversity and Richness* software.

The similarity coefficient of Jaccard's based on presence-absence of species revealed a higher similarity of ECMF communities within southern forests when compared to similarities within northern forests (Figure 2.5). Even for those locations with extreme conditions (PG and HC), where two independent forests were sampled, the northern and wettest sites (PG-ER/PG-RC) presented less similar communities when compared to southern and driest samples (HC-CT/HC-MA). A similar picture was obtained when considering other similarity measures that take into account, not only the presence/absence of fungal taxa, but also their abundance (Bray-Curtis index; results not presented).

According to ANOSIM analysis (Table 2.4), ECMF intra-diversity among forests was statistical significant (p<0.05), being differences between AL/PG-ER, AL/PG-RC, HC-CT/HC-MA and HC-MA/GR forests the only ones that were not statistically significant (p<0.05). When considering distance between all sampling forests, where the most distant forests (HC-MA and PG-RC) were 430 km apart from each other, the ECMF community similarity significantly decreased as geographic distance increased (Mantel test for Jaccard's similarity index and geographic distance: r = −0.188, p<0.0001). This result was different at a regional scale. The most northern and rainiest forests (PG-ER, PG-RC and LI), where the most separated forests were

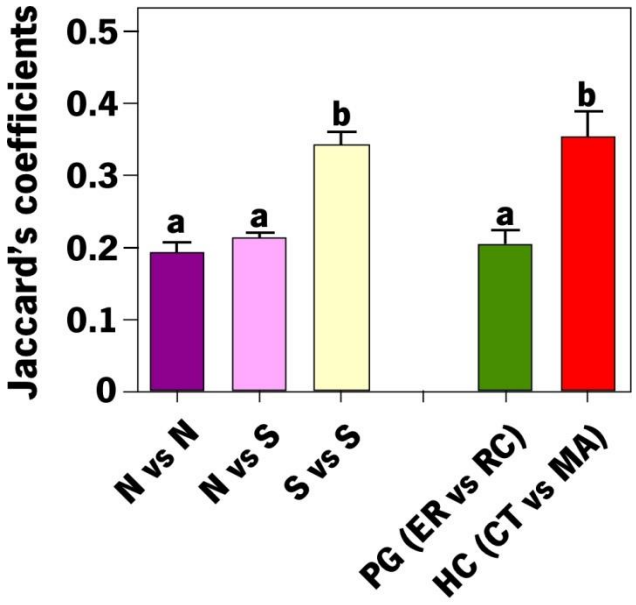

Figure 2.5. Pairwise Jaccard's similarity indices between fungal communities from different plots of northern (N: PG-ER, PG-RC and LI) and southern (S: GR, HC-CT and HC-RC) forests, as well among plots from locations presenting the same extreme conditions (PG and HC). Analyses were performed using the *Community Analysis Package* software. Different letters indicate a significant difference at $p < 0.001$ (one-way ANOVA, Tukey's test).

109 km apart, still presented a significant effect of geographic distance on ECMF community similarity (Mantel test: r = −0.258, $p = 0.008$). But, ECMF communities from the most southern and driest and driest forests (HC-CT, HC-MA and GR), where the most separated sampling sites were almost 150 km apart, did not reveal a significant similarity difference with geographic distance (Mantel test: r = −0.034, $p = 0.734$). These results suggest that distance is not the main driver for ECMF occurrence, especially for the driest regions, and agree with Miyamoto *et al.* (2015) work that described geographic distance as a minor driver of ECMF community structure at

Table 2.4. Analysis of similarity (ANOSIM) for Bray-Curtis among cork oak forests. Significant values mean that ECMF communities between both forests are different (italics at $p<0.05$; bold and italics at $p<0.01$).

	PG-ER	PG-RC	LI	AL	GR	HC-CT
PG-RC	*0.46*					
LI	*0.44*	***0.94***				
AL	0.13	0.28	***0.65***			
GR	***0.63***	***0.54***	***0.84***	***0.60***		
HC-CT	*0.30*	***0.50***	***0.84***	*0.24*	*0.29*	
HC-MA	*0.33*	***0.75***	***0.84***	***0.48***	0.23	-0.08

a regional scale. Drivers, such as forest topsoil's, vegetation composition and activity (Štursová *et al.,* 2016), as well environmental (Tedersoo *et al.,* 2012) or intrinsic fungal factors (Peay *et al.,* 2007) have been described as determinants of forest microbiomes.

Climatic parameters registered for all sampled sites did not affect the diversity parameters determined for cork oak ECMF community (results not shown). A positive correlation was detected between ECM occurrence and precipitation levels registered from the sampling sites, being fungal richness more influenced than abundance (Table 2.5). Indeed, richness was affected in all precipitation parameters analyzed (average from past 30 years, wettest and driest months; $p<0.01$), whereas abundance was only significantly correlated to the driest month ($p<0.01$, Table 2.5). Wide range of temperatures registered on sampling sites were negatively correlated with ECM root tips abundance and richness. This is particularly evident for ECM root tips abundance which was significantly reduced when considering forests facing higher temperatures during the coldest month ($p<0.001$). Higher average temperatures occurring during the last 30 years also significantly limited ECMF abundance ($p<0.05$) and richness ($p<0.01$). The combination of these climatic variables into the Emberger index revealed that ECMF richness was significantly increased in forests with wettest climates ($p<0.01$), but not ECMF abundance (Table 2.5).

The impact of several abiotic factors on ECMF communities have been reported, including the influence of atmospheric and soil temperature (*e.g.* Domisch *et al.,* 2002; Tibbett and Cairney, 2007) or drought (*e.g.* Shi *et al.,* 2002; reviewed by Reis *et al.,* 2017). Schmidt *et al.* (2017) also indicated that a season-long experimental drought alters fungal community composition. Climatic variables, such as precipitation levels and temperatures, occurring on sampled cork oak forests correlated with ECMF occurrence, although no causal effects between climate and community structure could be really anticipated. Using cork oak stands present in different climate regions, the present work reported a stronger correlation between rainiest climates and ECMF richness, when compared to ECMF abundance. Fungal abundance was also not significantly altered following treatments of extended precipitation (Jumpponen and Jones, 2014) and no evidences of increasing mycorrhizal hyphae, arbuscules, or vesicles by season water availability were detected in a four years' field work (Hawkes *et al.,* 2011). However, when studying an experimental hydrological gradient, an increase on ECMF abundance and presence/absence of certain ECMF species on oak species was detected (Cavander-Bares *et al.,* 2009). In the present work, we found that ECMF abundance is more influenced by temperature than precipitation levels, while richness was dependent on precipitation. Accordingly, long-term soil warming 5°C above the ambient

temperature significantly reduced the abundance of fungal biomarkers (Frey *et al.,* 2008) and Castro *et al.* (2010) described the precipitation as one of the main drivers on the community composition.

The most abundant genera differed among locations (Figure 2.3C). Detailed analysis of the ten most abundant genera in each cork oak stand revealed that *Tomentella* genus was common among all cork oak forests, followed by *Russula* that was only absent from the rainiest forest

Table 2.5. Correlations between total ECM fungal abundance and diversity with climatic conditions (precipitation and temperature: average from past 30 years, maximum and minimum of sampling year, Emberger index) in all seven sampled forests. Asterisks mean statistical significance at $p<0.05$ (*), at $p<0.01$ (**) or at $p<0.001$ (***).

	Climatic parameters		***F***
Abundance	Precipitation	Average	1.78
		Max	1.24
		Min	10.80**
	Temperature	Average	-5.52*
		Max	-12.91**
		Min	-41.97***
	Q		2,45
Richness	Precipitation	Average	10.93**
		Max	10.28**
		Min	11.86**
	Temperature	Average	-11.60**
		Max	-6.92*
		Min	-0.46
	Q		11.88**

(PG-RC), and *Cenococcum* only absent from LI forest. Southern forests presented more homogenous genera content, being always enriched in *Russula*, *Tomentella* and *Cenococcum* (46-82% of occurrence). Northern forests were more diverse and each presented somehow specific genera, such as *Cantharellus* (PG-ER), *Sebacina* (PG-RC), and *Humaria* (LI).

To the best of our knowledge this is the first report that compares ECMF structure on different landscapes and Mediterranean bioclimates. ECM fungal community associated with cork oaks was mainly represented by *Russula* (29%), *Tomentella* (20%) and *Cenoccoccum* (10%),

which is in accordance with other reports on *Q. suber* forests that also describe a highly enriched ECMF community on *C. geophilum*, Russulaceae and Thelephoraceae (Yakhlef *et al.,* 2009; Azul *et al.,* 2010; Lancellotti and Franceschini, 2013). This trend is commonly followed by other Fagaceae forests (reviewed by Reis *et al.,* 2017). While *C. geophilum* was the main ECM root tip identified in *Q. ilex* forests (Richard *et al.,* 2011), *Russula* has been the most abundant genus found in *Q. suber* landscapes (Azul *et al.,* 2010). However, there are also reports of *Russula* or *Cenococcum* absence in Fagaceae forests, such as in *Q. ilex* (De Román and De Miguel, 2005) or *Q. petrea* (Voríšková *et al.,* 2014) forests. In the present work, *Russula* comprised 50% (HC-CT) and 42% (HC-CT) of total ECM root tips identified in southern forests, but only 14% was found in the other southern forest (GR), whose samples were also singled out by an increased abundance of *Tuber* spp. (49%). Altogether, these results suggest a predominance of *Russula* in *Q. suber* stands, although displaying a scattered abundance. The heterogeneity of forest microbial diversity has been well-described and results from the dynamic ecosystem processes occurring at spatial and temporal scales (reviewed by Baldrian, 2017). The low tree density of GR southern forest, where an agro-silvo-pastoral management occurs, could partly explains the low diversity of ECMF community found in this forest, as reported elsewhere (Jones *et al.,* 2003; Smith *et al.,* 2005; Azul *et al.,* 2010).

The assemblage of fungal communities was also explored by non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis similarity index (Kruskal's stress=0.1917; Figure 2.6). Although distinct clusters were not well distinguished, southern forests seemed to be assembled together in the upper part of the graphic. The northern LI cork oak forest presented the less similar ECMF community of all sampled geographic places, almost describing a singular ECMF community. As most abundant genera, *Russula* (29% of total root tips), *Tomentella* (20%) and *Cenococcum* (10%), were the genera that contributed the most for samples divergence (*Russula* contributed up to 33.4% of total dissimilarity between samples, *Tomentella* up to 36.7%, and *Cenococcum* up to 33.4%; as detected by SIMPER dissimilarity analysis between groups; Figure 2.6). Due to their high abundance, the occurrence of ECM root tips from *Russula*, *Tomentella* and *Cenococcum* well discriminated all samples, but a clear scenario of taxa distribution among forests is missing, even between geographic replicas (PG-ER/PG-RC and HC-CT/HC-MA). In any case, genera such as *Elaphomyces* and *Lactarius* were more frequently found in humid than in arid climates, as well all identified genera from Thelephoraceae and

Sebacinaceae, or from Cantharellales order. Their abundance could be partly explained by ECMF differential tolerance/susceptibility to environmental conditions. Accordingly, temperature assays revealed that Thelephoraceae and Sebacinaceae species are susceptible to temperatures increases, while *Cenococcum geophilum* and *Rhizopogon* sp. are more tolerant (Kipfer *et al.,* 2010). In a rainfall exclusion experiment in *Q. ilex* forests, the global richness of the community was not affected, but significant shifts in the community composition were reported

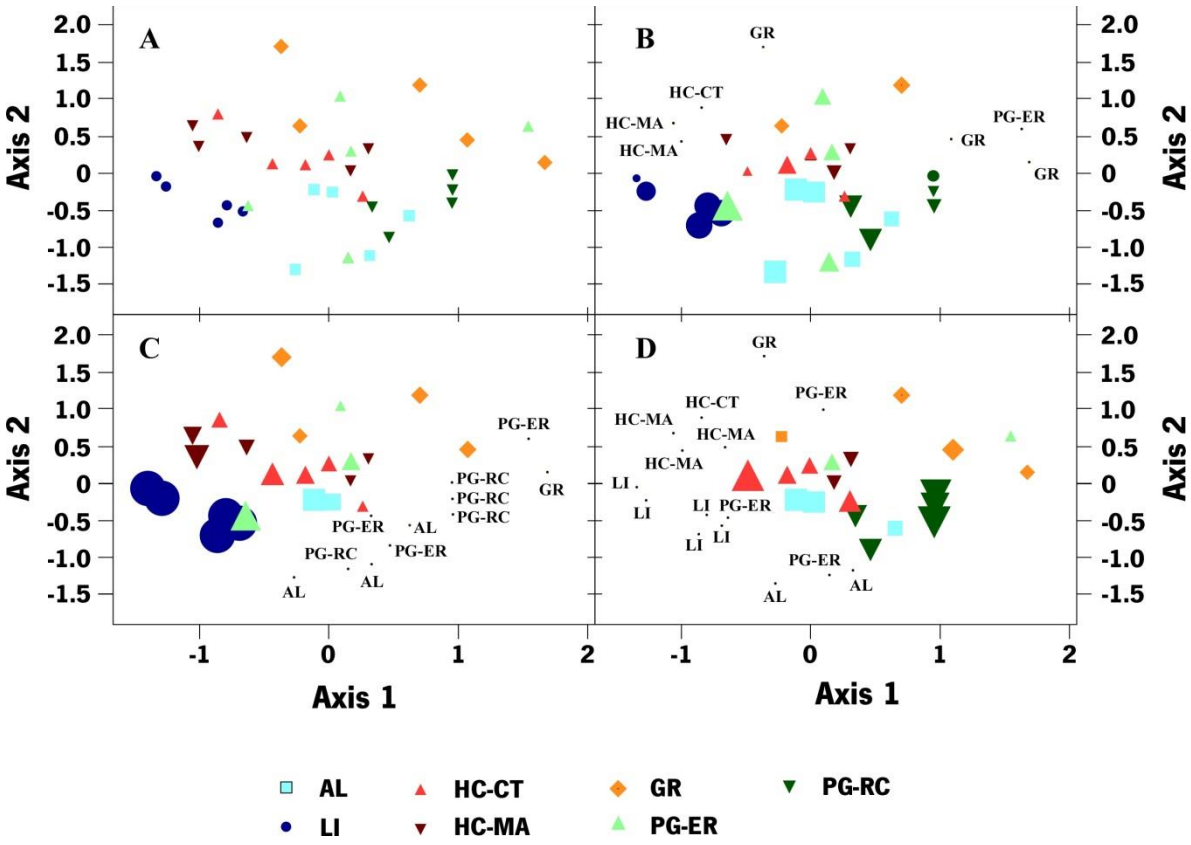

Figure 2.6. Nonmetric multidimensional scaling (NMDS) analysis of total root tips (A; Kruskal's stress = 0.1917) and abundance of the most discriminant genera – *Russula* (B), *Tomentella* (C) and *Cenococcum* (D) – in each sampling site. The two first dimensions are drawn. The circle size denotes the abundance of each genera. (PG: inverted triangles; LI: squares; AL: cruxes; GR: triangles; HC: circles)

(Richard *et al.,* 2011). Sebacinaceae were also less represented in a drought scenario, while members of Cortinariaceae species were significantly more abundant. Although the ECMF community composition of *Pinus edulis* have been recently considered as an extended phenotype of the host tree that could potentially promote the plant adaptation for facing the changing climate

(Gehring *et al.,* 2017), we have not found any taxa that presented an important increase under the most arid climates.

2.5. Conclusions

This study evaluated ECM fungal community of cork oak forests in different locations under distinct Mediterranean climates, ranging from humid to arid climates. To the best of our knowledge, the present work comprises the most complete assessment of ECMF communities associated to cork oak at different landscapes. This work reveals that the occurrence of ECM root tips from *Russula*, *Tomentella* and *Cenococcum* well discriminated cork oak forests. The most arid forests revealed to be less diverse and more homogenous among them than northern and humid forests. When relating ECMF community structure with each climatic parameter individually, temperature seems to negatively affect ECMF abundance, whereas richness appears to be positively affected by precipitation levels. Although further experimental support is needed for concluding about climate implication on occurrence of ECMF root tips, the results suggest their possible involvement on structuring ECMF communities and/or promote mycorrhization. As current climate models predict general temperature increase and precipitation decline for the near future, alterations on ECMF community could further compromise cork oak forest sustainability under climate changes.

Acknowledgments

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https://www.ipcc.ch/pdf/assessment-report/ar4/wg3/ar4-wg3-chapter9.pdf

2.7. Supplementary data

Table S1. Diversity parameters for ECM fungal communities from all replicates of studied cork oak plots, after identification by root tip barcoding. Species richness (S), alpha diversity indexes [Simpson's index (D), Shannon index (*H'*), Fisher's alpha], richness estimators [Chao, 1st order Jackknife] and beta diversity Whittaker index are represented. The lowest estimates are highlighted in bold, being the highest underlined. Each site is referred by their code, as used in table 2.1, and replicates referred from A to E.

	Sample	S	D	*H'*	αFisher	Chao	Jacknife
PG-ER	A	10	5.56	1.91	3.02	**<u>12.96</u>**	10
	B	4	3.07	1.17	**0.65**	4	4
	C	4	2.76	1.14	0.91	4	4
	D	7	3.86	1.57	2.01	7	7
	E	9	5.74	1.9	1.89	9	9
PG-RC	A	8	4.97	1.77	1.82	8	8
	B	9	3.28	1.51	2.83	10.48	9
	C	6	2.5	1.24	1.58	6	6
	D	10	2.54	1.43	2.74	10.99	10
	E	10	4.2	1.77	2.88	10.49	10
LI	A	8	4.55	1.69	1.47	8	8
	B	9	3.25	1.45	1.85	9.5	9
	C	6	5	1.68	1.24	6	6
	D	**<u>11</u>**	**<u>6.94</u>**	2.06	2.4	11.5	**<u>11</u>**
	E	7	2.52	1.16	1.3	7	7
AL	A	7	4.94	1.75	1.65	7	7
	B	10	8.3	**<u>2.19</u>**	2.6	10	10
	C	9	5.76	1.94	2.51	9	9
	D	10	4.87	1.81	2.82	10.33	10
	E	6	4.66	1.64	1.71	6	6
GR	A	4	2.14	1.01	1.65	4	4
	B	**2**	2	0.69	1.59	**2**	**2**
	C	4	1.94	0.94	1.97	4.46	4
	D	4	1.99	0.85	0.92	4	4
	E	**2**	**1.38**	**0.45**	1.05	**2**	**2**
HC-CT	A	4	2.42	1.06	1.02	4	4
	B	7	3.09	1.4	2.05	7.49	7
	C	9	5.08	1.84	**<u>3.24</u>**	11.94	9
	D	3	2.1	0.79	0.76	3	3
	E	6	2.64	1.23	1.34	6	6
HC-MA	A	3	2.28	0.94	0.84	3	3
	B	5	3.45	1.36	1.81	5	5
	C	6	3.97	1.51	1.81	6	6
	D	7	3.43	1.51	2.01	7	7
	E	4	2.76	1.13	0.99	4	4

Chapter 3

Evaluation of bacterial community

Cork oak soil bacterial communities at different landscapes

Cork oak soil bacterial communities are affected by climate variables

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*Francisca Reis contribution: all field and laboratory work, data analysis (helped by P. Castro on raw data filtering), discussion, and manuscript writin

3.1. Abstract

Cork oak (*Quercus suber* L.) forest is considered as one of the most important ecosystems from the Mediterranean Basin. Despite cork oak tolerance to drought, the increase of temperature and decrease of water availability is causing a serious decline of cork oak populations. This ecosystem sustainability is largely maintained by microbial communities present within soils. In the present work, the bacterial community of cork oak soils was assessed by metabarcoding using Illumina MiSeq. Soils from seven independent cork oak forests were collected from different geographic locations, chosen based on pluviometric indicators. Cork oak forest soils were highly enriched on *Proteobacteria*, *Actinobacteria* and *Acidobacteria*, but differences among forests were detected. Driest and warmer forests presented more homogeneous and diverse bacterial communities than wettest and coolest forests, resulting in a clear discrimination of microbiomes with bioclimate regions. Bacterial communities from humid, sub-humid and semi-arid/arid climates clustered into three distinct groups. As recent climate models forecast a general temperature increase and precipitation decline for the near future, the implication of climate variables on structuring bacterial microbiome is discussed. Cork oak forests sustainability could be compromised by alterations of bacterial community under the predicted upcoming climate changes.

3.2. Introduction

Mediterranean forests are one of the most important ecosystems on Earth and have been recognized as one of the global biodiversity hotspots, since many endemic species are now under threat (Médail and Quézel, 1999). Mediterranean forests are mainly composed by broadleaved evergreen tree species (holm – *Quercus ilex* and cork oak – *Quercus suber*; Valavanidis and Vlachogianni, 2011). Cork oak displays an important economic input for the Mediterranean countries, in particular for the Iberian Peninsula that presents the largest cork oak forest area and results in 80% of annual cork production (50% of which in Portugal). Currently, cork oak stands are facing environmental challenges due to the predicted temperature increasing, as well as the reduction of water availability (reviewed by Reis *et al.*, 2017). An adaptation of cork oak populations to drier and warmer conditions is expectable (Varela *et al.*, 2015), but the decline of existing populations have been described all over Mediterranean Basin (reviewed by Reis *et al.*, 2017). Indeed, the Mediterranean region has been considered as one of the most affected regions

worldwide by climate changes (Giorgi, 2006). For facing the upcoming changes, the microbial community present in the soil of cork oak forests has been recognized as one of the main drivers for forest sustainability (Reis *et al.*, 2017). From the huge diversity of microbes present in cork oak soils, fungal communities have been largely described, most of them related with the ability of ectomycorrhizal fungi to become associated to cork oak roots and thus promote water and nutrients transfer (Bevivino *et al.*, 2014). Bacterial communities are abundant in forests floor, soils and litter (Hardoim *et al.*, 2015), where they play important ecological roles, some of which in interaction with plants (Vandenkoornhuyse *et al.*, 2015). Among the important roles for the ecosystem, soil bacterial communities could significantly contribute for the decomposition process (Štursová *et al.*, 2012), N fixation or mineral weathering leading to the inorganic nutrients release (Reed *et al.*, 2011; Uroz *et al.*, 2011). The most abundant bacterial phyla present in most soils are *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* (Lauber *et al.*, 2009). Similar results were obtained for the bulk soil of *Q. petraea* forests in Czech Republic, accessed by 454-pyrosequencing, where *Acidobacteria* was the dominant phylum (40 to 50% of identified sequences by López-Mondéjar *et al.*, 2015). However, a strong domination by *Proteobacteria* was detected in cork oak forests from the Mediterranean region (Sardinia, Italy; Bevivino *et al.*, 2014). A different trend was registered for the rhizospheric bacterial community, where the high availability of C provided by tree roots and mycorrhizal hypha exudation, increased the microbial abundance and activity of extracellular enzymes (Collignon *et al.*, 2011; Drake *et al.*, 2013; Finzi *et al.*, 2015). In this scenario, copiotrophic bacteria community is enriched (Lladó and Baldrian, 2017).

Mediterranean plant species have been correlated with diverse climatic variables, such as precipitation, evaporation and temperature (Suc, 1984). In the particular case of cork oak, stands density are closely related to water availability (Joffre *et al.*, 1999). Portuguese *Q. suber* stands comprise two different forest systems depending on tree density. High density forests (about 400 trees/ha; *sobreirais*) are typically found in northern and central Portugal, whereas low density stands (60–100 trees/ha; *montados*) are more common in the southern and drier region.

Bacterial communities present in different soil layers – litter and deadwood, rhizosphere and bulk soil – bacteria are differently affected (Lladó *et al.*, 2017). In the present work, a global picture of the bacterial community associated with cork oak soils is described, taking into consideration cork oak forests from different locations of the greatest cork producer country (Portugal), which comprises different Mediterranean bioclimates. At the end, the contribution of climate variables for structuring bacterial communities is discussed.

3.3. Material and Methods

3.3.1. Cork oak stands and sample collection

Five independent geographic locations were selected based on local weather conditions and water availability levels (Portuguese Sea and Atmosphere Institute), previous information's of cork oak stands (Varela and Eriksson, 1995) and local Emberger indexes that define the corresponding Mediterranean bioclimates (Rego and Rocha, 2014; Chapter 2; Table 2.1). Based on annual precipitation means, *National Park of Peneda-Gerês* – PG (120.7 mm) and *Herdade da Contenda* – HC (46.5 mm) comprised the extreme conditions registered on the sampling year. At these geographic locations, two independent forests were sampled [Ermida (PG-ER) and Rio Cabril (PG-RC) from PG location; Contenda (HC-CT) and Monte Asparão (HC-MA) from HC location]. Other three locations displaying intermediate precipitation levels were also sampled [Limãos (LI, 772.8 mm), Alcobaça (AL, 651.6 mm), and Grândola (GR, 735.6 mm)]. Using climatic data during the sampling year [annual precipitation (P), maximal (M) and minimal (m) temperatures of the hottest and coldest months], the corresponding Emberger indexes (Q) were determined according to Tate and Gustard (2000), thus combining the sampled forests into four distinct Mediterranean climates: humid (PG, Q = 186.6), sub-humid (LI, Q = 88.9; AL, Q = 102.7), semi-arid (GR, Q = 77.5) and arid (HC, Q = 43.5). The same soil samples had been previously used for assessing ectomycorrhizal communities (through root tips barcoding) in these cork oak stands (Chapter 2).

Soils sampling was conducted on the seven cork oak forests during the autumn (November and December) of 2013, using the procedure described in Chapter 2. Five independent healthy trees, separated at least 30 m from each other, were selected. After removing the uppermost layer of soil that comprised plant litter (~0.5 to 1 cm depth) and other organic material (~1-3 cm depth), three soil cores (8 cm of diameter and 12 cm in depth) were collected under the middle of the cork oak canopy, in three tree trunk directions. Soils were stored at 4ºC until processing. In total, 105 soil cores (7 forests x 5 plots x 3 cores) were collected. Each soil core was sieved twice (4 mm and 2 mm meshes).

In order to determine soil pH, samples were homogenized by mixture, dried at 40°C during 2 to 3 days and sieved through a 2 mm mesh. After being mixed with deionized water or 0.01 M $CaCl_2$ (1:2.5), the supernatant pH was measured using a glass combination electrode. Soil granulometric analysis was performed using sieve analysis and *SediGraph 5100* to determine grain

size distribution in the different fractions. The percentage of sand, silt and clay was used for soil texture classification, using the soil texture triangle for Portugal (Gomes and Silva, 1962).

3.3.2. DNA extraction and Illumina sequencing of soil bacteria

Soil samples from three independent soil cores from each tree were mixed together using equal amounts and soil DNA was extracted from this single sample using *Power Soil DNA isolation kit* (MO BIO Laboratories), according to the instructions provided by the supplier with few modifications. The gene for the bacterial ribossomal subunit 16S was amplified using universal primers (16S amplicon PCR forward primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S amplicon PCR reverse primer = 5' CGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC), which targeted a segment comprising the variable regions V3 and V4. Sequencing of 16S rRNA was performed on the *Illumina MiSeq* platform recurring to the sequencing services of *Instituto Gulbenkian de Ciência* (Portugal).

3.3.3. Read processing and data analysis

Read pairs from each sample were trimmed with *Sickle* (Joshi and Fass, 2011), overlapped and prepared with strict quality and size filtering into uniform error-free reads with length of 400 bp. De-replication, removal of chimeric sequences and clustering with an identity threshold of 97% were performed using *Vsearch v2.3.2* (Rognes *et al.*, 2016). Taxonomic classification was assigned by using the ribosomal RNA gene reference database *SILVA, version 123* (Quast *et al.*, 2013). Unclassified sequences and low abundance taxa (less than 5 reads) were filtered from the operational taxonomic unit (OTU) tables before downstream analysis, as performed in previous work (Baptista *et al.*, 2015). For analysis of the microbial profile between samples, *QIIME 1.9.1* (Kuczynski *et al.*, 2011) was used to subsample the datasets for an even number of sequences, to mitigate biases due to differences in the sampling depth (Table S3.1).

3.3.4. Statistical and ecological data analysis

Bacterial community analysis was performed on family-abundance data, considering family OTUs with at least 5 reads for the total number of replicas from each forest. Bacterial community's diversity was evaluated using computational indices that combine both relative abundance and

diversity (Magurran, 2004). *Estimates S - version 9* (RK Colwell, http://purl.oclc.org/estimates) was used to determine alpha [Simpson (D), Shannon (*H'*), Fisher´s alpha] indexes, as well as species richness estimators (Chao), whereas *Species Diversity and Richness - version 5* (Pisces Conservation Ltd. Lymington, UK; 2014) was used for rarefaction curves (Henderson and Seaby, 2007). Bray-Curtis similarity index was calculated with the number of family bacterial OTUs shared between samples using *Community Analysis Package Version 5* (Pisces Conservation Ltd. Lymington, UK; 2014).

Changes in microbial community were analyzed by analysis of similarity (ANOSIM), similarity percentage analysis (SIMPER) and non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity, performed by *Community Analysis Package Version 5* software (Pisces Conservation Ltd. Lymington, UK; 2014). Total soil analysis comprised all five samples from each forest. Correlation between community structure and geographic distances was determined using the Mantle test of the *Microsoft Excel* add-in program *XLSTAT version 2017* (Addinsoft, New York, USA). Pearson correlations between climatic conditions and fungal structure were performed with *Excel* tools; one-way ANOVA followed by Tukey's multiple comparison test were performed using the analysis tools of *GraphPad Prism 7* (La Jolla California, USA).

The significance of OTUs abundance differences between samples was calculated with the F-test wrapper mt() from the *"R" phyloseq package version 1.16.1* (McMurdie and Holmes, 2013), with a Bonferroni correction for multiple pairwise comparisons. Taxonomic distribution of differentially abundant families between samples was represented in a heatmap created with the *"R" packages stats version 3.4.2* and *gplots version 3.0.1* (Warnes *et al.*, 2016).

3.4. Results and Discussion

3.4.1. General description of bacterial community associated to cork oak forest soils

A set of 3,967,918 paired-end reads were obtained from V3–V4 16S rDNA sequencing of different cork oak forest soil samples. Raw reads were filtered with stringent parameters of quality and size thresholds to exclude any error-containing sequences from following analysis. Forward and reverse reads within each pair were merged and truncated into single 400 bp reads, followed by removal of chimeric sequences. The high quality reads were clustered into OTUs based on sequence similarity considering a threshold of 97% (Stackebrandt and Goebel, 1994) and taxonomic classification assigned with the reference database *SILVA version 123* (Quast *et al.*,

2013). Low abundance (OTUs with less than 5 sequences) and unclassified taxa were removed from datasets. The filtered datasets ranged from a maximum of 74,917 to a minimum of 16,868 sequences *per* soil, whose differences might suggest significant variations in bacterial richness and abundance according to the sampled soil (Table S3.1).

In this study, a total of 1,116,477 high-quality reads resulted in the identification of 5,329 OTUs from 36 *Eubacteria* phyla, 109 classes and 442 families (Table S3.2; Figure 3.1A). *Proteobacteria* (126 families), *Actinobacteria* (54 families) and *Chloroflexi* (51 families) were the richest phyla, while 27 phyla individually registered less than 1% of total identified reads (74 families). Among the *Proteobacteria* phylum, *Alphaproteobacteria* and *Deltaproteobacteria* classes were the most diverse (comprising 36% and 29% of *Proteobacteria* families, respectively), followed by *Betaproteobacteria* and *Gammaproteobacteria* classes (17% and 14% of *Proteobacteria* families, respectively). Within the *Actinobacteria* phylum, which includes most of the known tree symbiotic bacteria, 56% of families belong to the *Actinobacteria* class, being *Frankiales* the most diverse order comprising seven families. *Ktedonobacteria* class (19 identified families) was the richest from the *Chloroflexi* phyla.

Considering the taxa abundance, *Proteobacteria* (31% of total reads), *Actinobacteria* (22%) and *Acidobacteria* (16%) were the most abundant phyla detected in all samples from the seven forest soils (Figure 3.1B). Similar profiles have been obtained from acidic soil of coniferous forests or temperate deciduous forests (Purahong *et al.*, 2014; Urbanová *et al.*, 2014; Lopéz-Mondéjar *et al.*, 2015). As copiotrophic bacterial phyla, *Proteobacteria* and *Actinobacteri*a are frequently found as highly abundant in rhizospheric environments where nutrients, namely C, are largely available (Lladó *et al.*, 2017). In cork oak soil samples, *Alphaproteobacteria* was the most conspicuous identified class, representing 20% of total reads (64% of *Proteobacteria* reads). Within this class, the *Rhizobiales* order (comprising 29% of *Proteobacteria* reads) mainly including reads assigned to the *Bradyrhizobiaceae* family (11%) were the most abundant taxa. Within *Actinobacteria* phylum, *Actinobacteria* was the most abundant class (13% of total reads, 60% of *Actinobacteria* phylum reads), while among *Acidobacteria* phylum, *Acidobacteria* class comprised more than 96% of *Acidobacteria* phylum reads (16% of total reads). The most conspicuous genera were *Afipia* (*Bradorhizobium*, *Proteobacteria*) with 0.08% to 0.23% of relative abundance in each forest, *Sphingomonas* (*Proteobacteria,* 0.01% to 0.19%, and *Acidothermus* (*Frankiales*, *Acidobacteria*, 0.06-0.43%). As these bacteria were found in all samples/forests, they are probably included in the

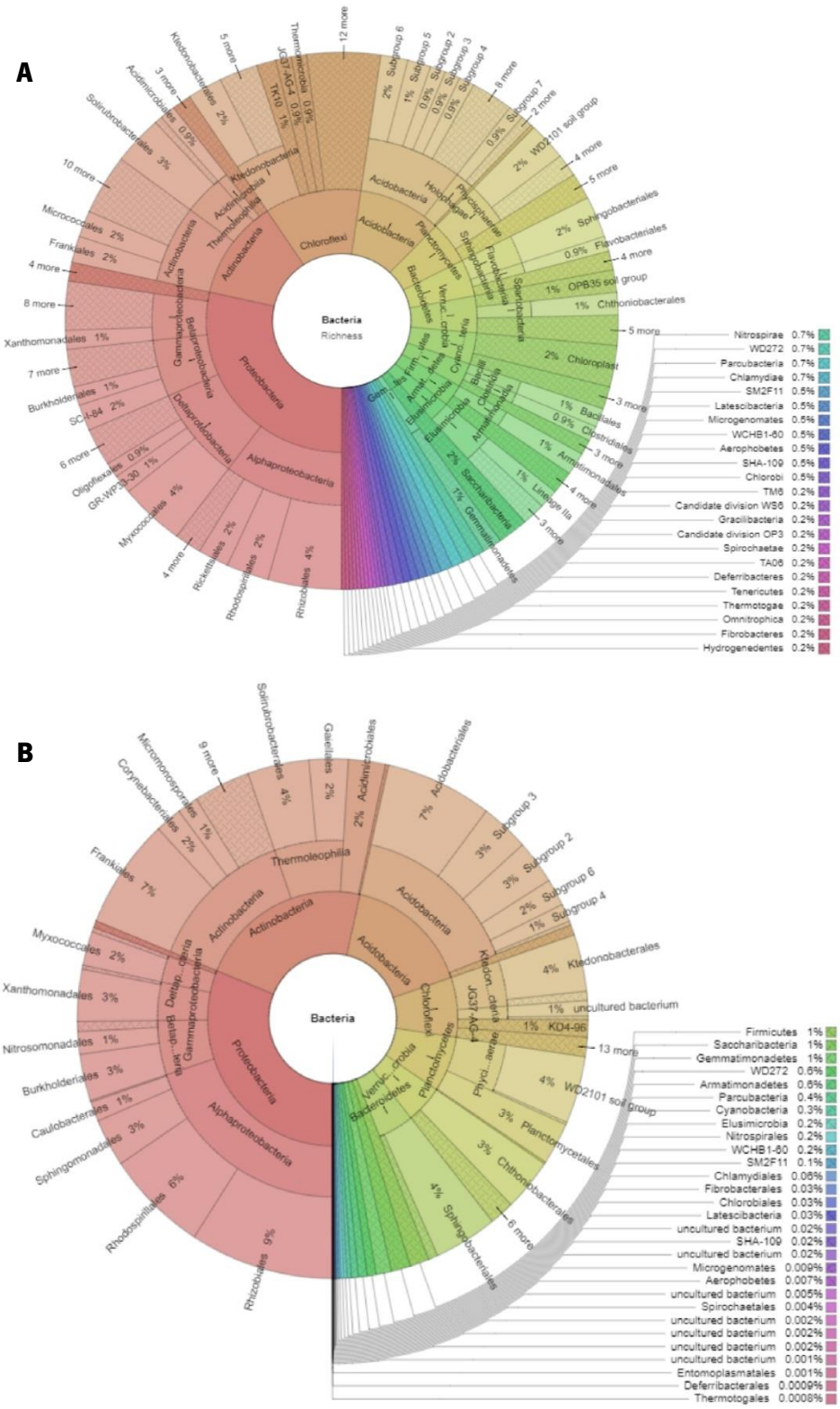

Figure 3.1. Representation of bacterial community richness (number of OTUs) (A) and abundance (number of reads) (B) present in all cork oak soils, identified up to class level. Graphics were generated using Krona tool (Ondov *et al.*, 2011). For more details click here for richness and here for abundance krona graphs.

core bacterial community associated with cork oak. The soil sampling procedure could have affected the described bacterial community. As the upper layer of decomposed litter was removed previously to soil sampling, the abundance of *Proteobacteria* taxa typically present in litter of temperate deciduous forests, such as *Bradyrhizobiaceae, Rhizobiaceae, Burkholderiaceae, Sphingomonadaceae* and *Pseudomonadaceae* (López-Mondéjar *et al.*, 2015), could have been underestimated. On the other hand, our sampling strategy favored the abundance of *Acidobacteriaceae* (*Granulicella* and *Edaphobacter* genera), which are more common in organic soil layers, and mainly *Acidoterrimonas* (*Actinobacteria*), *Acidobacterium* (*Acidobacteria*) and *Rhodoplanes* (*Alphaproteobacteria*) genera that are more abundant in mineral soil layers (López-Mondéjar *et al.*, 2015).

The bacterial communities of seven Portuguese cork oak stands were differently enriched. The number of identified high-quality reads in all forests was similar (137,468 – 162,577 reads), except for PG-ER that presented a non-significant higher number of reads (216,031; Table S3.1, Figure 3.2A). When considering the number of identified families, a significant trend was found as water availability decreased (Figure 3.2B). While wettest forests (PG-ER and PG-RC) presented 243

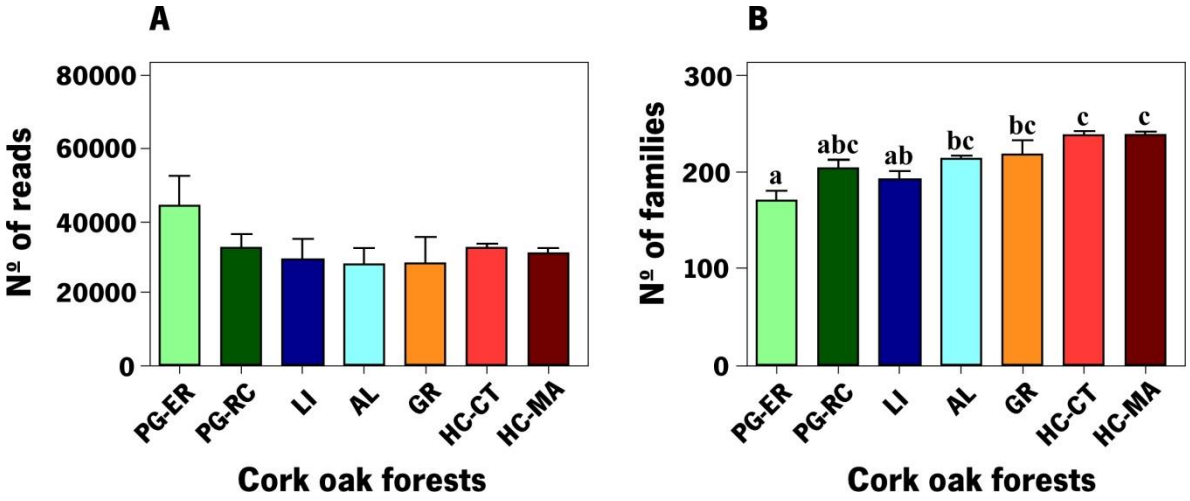

Figure 3.2. Abundance (A) and richness (B) identified by metabarcoding analysis of cork oak soils from all sampled cork oak stands. Richness results are presented considering only those OTUs comprising at least five reads in each sample. Statistical analysis was performed considering five replicates (cork oak trees) from each sampling site. Different letters denote statistically significant differences (at $p<0.05$; one-way ANOVA. Tukey test). Each site is referred by their code: PG - National Park of Peneda-Gerês (PG-ER - Ermida; PG – RC - Rio Cabril); HC - Herdade da Contenda (HC-CT – Contenda; HC-MA - Monte Asparão; LI – Limãos; AL – Alcobaça; GR - Grândola (see Table 2.1 for more details).

and 287 identified bacterial families, southern and driest forests (HC-CT and HC-MA) presented 323 and 308 families, respectively. Bacterial communities were compared between forests/samples by computation of rarefaction curves and diversity indexes (Figure 3.3). Rarefaction curves suggest that all forest soils were well-sampled and could give information about bacterial communities (Figure 3.3A). Accordingly, Chao richness estimator only revealed an overestimation of 0.43% of the identified family number for total bacterial community (data not shown).PG-ER soil was singled out from the others by presenting a lower family diversity. Accordingly, PG-ER forest systematically presented the lowest values for alpha diversity indexes (*H´* and D; Figure 3.3B), while the highest values were shared between southern forests (HC-MA,

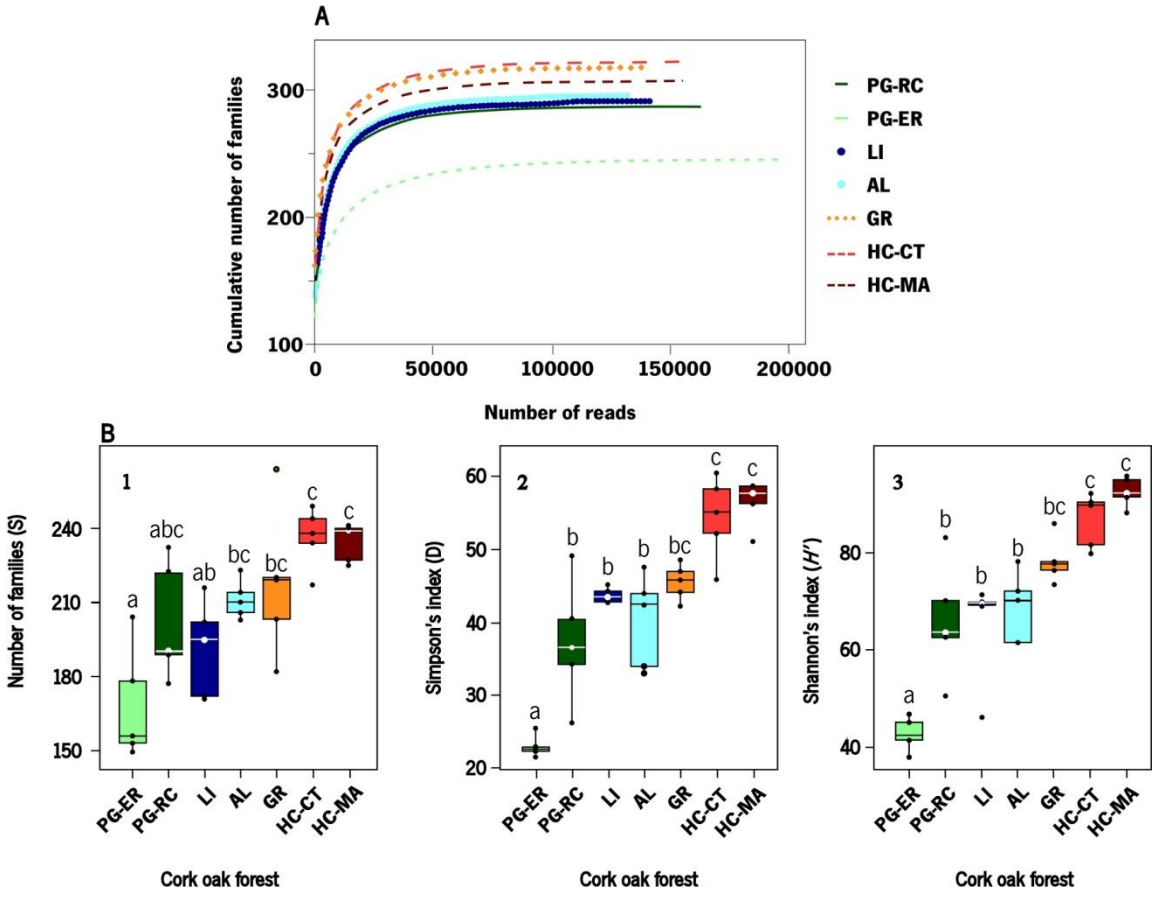

Figure 3.3. Diversity analysis of bacterial communities from the seven sampled cork oak forests by computation of rarefaction curves (A) and determination of diversity parameters (B). Rarefaction curves were computed by *Species Diversity and Richness* software, using data obtained from all samples of each forest and OTUs identified at 97% of identity. Families number (S), Simpson's (D) and Shannon's (*H'*) indexes are graphically represented by box plots, taking into consideration values from all samples of each stand. Each site is referred by their code, as used in Figure 3.2.

HC-CT and GR). Indeed, bacterial communities were significantly more diverse in southern forests (HC-MA, HC-CT, $p<0.001$). The most abundant bacterial taxa differed among locations. From all 36 identified bacterial phyla, 20 (55%) were shared between all sampled soils; however, when considering the identified families (442), only 33% were present in all 35 soil samples (Table S3.2). Northern and centra soils exhibited three specific phyla (*Tenericutes*, *Thermotogae* and *Spirochaetae*), but they only appeared on sporadic stands. Southern forests (HC-CT, HC-MA and GR) also exhibited two exclusive phyla (*Candidate division OP3* and *Hydrogenedentes*) that were present in several southern forests. Analysis of the ten most abundant taxa (phylum or class) in each cork oak forest revealed that *Proteobacteria* was the most abundant phylum (Figure 3.4). This result is corroborated by meta-analysis studies which attributed to *Proteobacteria* a central role on forest rhizosphere, as in 16 of 19 studies was the dominant phylum found in the rhizosphere (Hawkes *et al.*, 2007). The bacterial profiles exhibited by different soils are dependent on the distribution of certain nutrients and organic matter (Šnajdr *et al.*, 2008). The sampled cork oak stands comprised a mixture of forests (PG-ER, PG-RC, AL), but also agro-forest systems where landscape was used

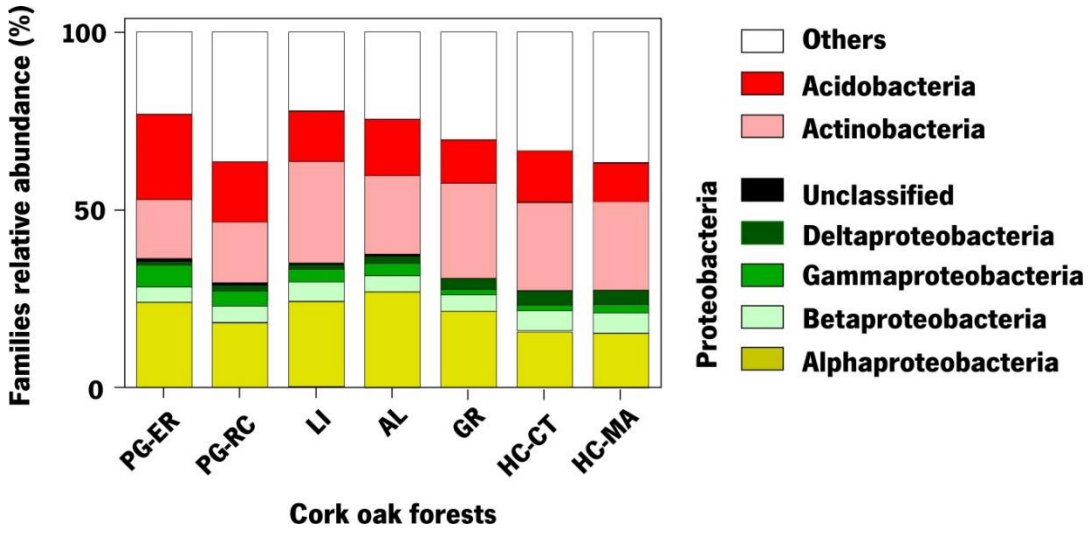

Figure 3.4. Most abundant bacterial phyla/classes (A) identified by metabarcoding analysis of cork oak soils from all sampled stands. Richness results are presented considering only those families comprising at least five reads in each sample. Statistical analysis was performed considering five replicates (cork oak trees) from each sampling site. Different letters denote statistically significant differences (at $p<0.05$; one-way ANOVA, Tukey test). Each site is referred by their code, as used in Figure 3.2.

for feeding animals (LI, GR, HC-CT, HC-MA). While forest soils are typically enriched in copiotrophic bacteria, agro-forest landscapes are enriched with oligotrophic bacterial taxa (Fierer *et al.*, 2007;

Peiffer *et al.*, 2013; Lladó *et al.*, 2017). The exclusive presence of *Candidate division OP3* and *Hydrogenedentes* on southern cork oak stands, where landscape was used for feeding animals, is in accordance with bacterial microbiomes typically found on grasslands/agricultural systems, as they have been described as being present in methanogenic environments, therefore being adapted to anaerobic conditions (Glockner *et al.*, 2010; Nobu *et al.*, 2015). In addition, as grasslands/agricultural systems present high nutrient content due to the intensive human exploitation, they comprise bacterial microbiomes enriched in *Proteobacteria*, *Actinobacteria* and *Bacteriodetes*; whereas. Forest soils being more undisturbed and presenting less nutrients are described to be enriched in *Acidobacteria*, *Actinobacteria, Proteobacteria* and *Bacteriodetes* (Bevivino *et al.*, 2014). Indeed, cork oak forests that present agricultural or pastoral exploration (LI, GR, HC-CT and HC-MA) presented lower abundance of *Acidobacteria* (11.07% to 14.51%), in contrast with typical cork oak forests (PG-ER, PG-RC and AL, 16.12% to 24.93%), but a higher abundance of *Actinobacteria* (24.65% to 29.45% was found in agro-pastoral stands in contrast to 15.91% to 21.70% found in typical forests. Within *Proteobacteria*, the *Alphaproteobacteria* and *Gammaproteobacteria* classes presented a significant different abundance among cork oak stands, displaying northern forests (PG-ER and LI) a higher abundance than southern ones (HC-CT and HC-MA, $p<0.001$; Figure 3.4). An opposite trend was found for *Deltaproteobacteria* that exhibited more abundance in southern samples when compared with northern ($p<0.05$).

3.4.2. Bacterial communities structure at different landscapes

Considering the 50 most abundant families that were differentially found in cork oak sites (at $p<0.05$), distinct bacterial communities could be perceived in different cork oak stands (Figure 3.5). Northern forests (PG-ER and PG-RC) presented the most distinctive communities, while the southern forests (HC-CT, HC-MA and GR) clustered together thus revealing a higher bacterial homogeneity. Furthermore, northern forests (PG-ER and PG-RC) presented higher dissimilarity between them than southern among them (HC-CT and HC-MA), as evaluated by the dissimilarity coefficient of Bray-Curtis that considers the presence-absence, as well as the abundance of families (data not shown). Similar results were obtained using Jaccard's index, which only takes into account the presence-absence of families (data not shown). Non-metric multidimensional scaling (NMDS), performed based on Bray-Curtis dissimilarity coefficient, assembled the samples from each cork oak stand (Kruskal's stress=0.1016; Figure 3.6), being

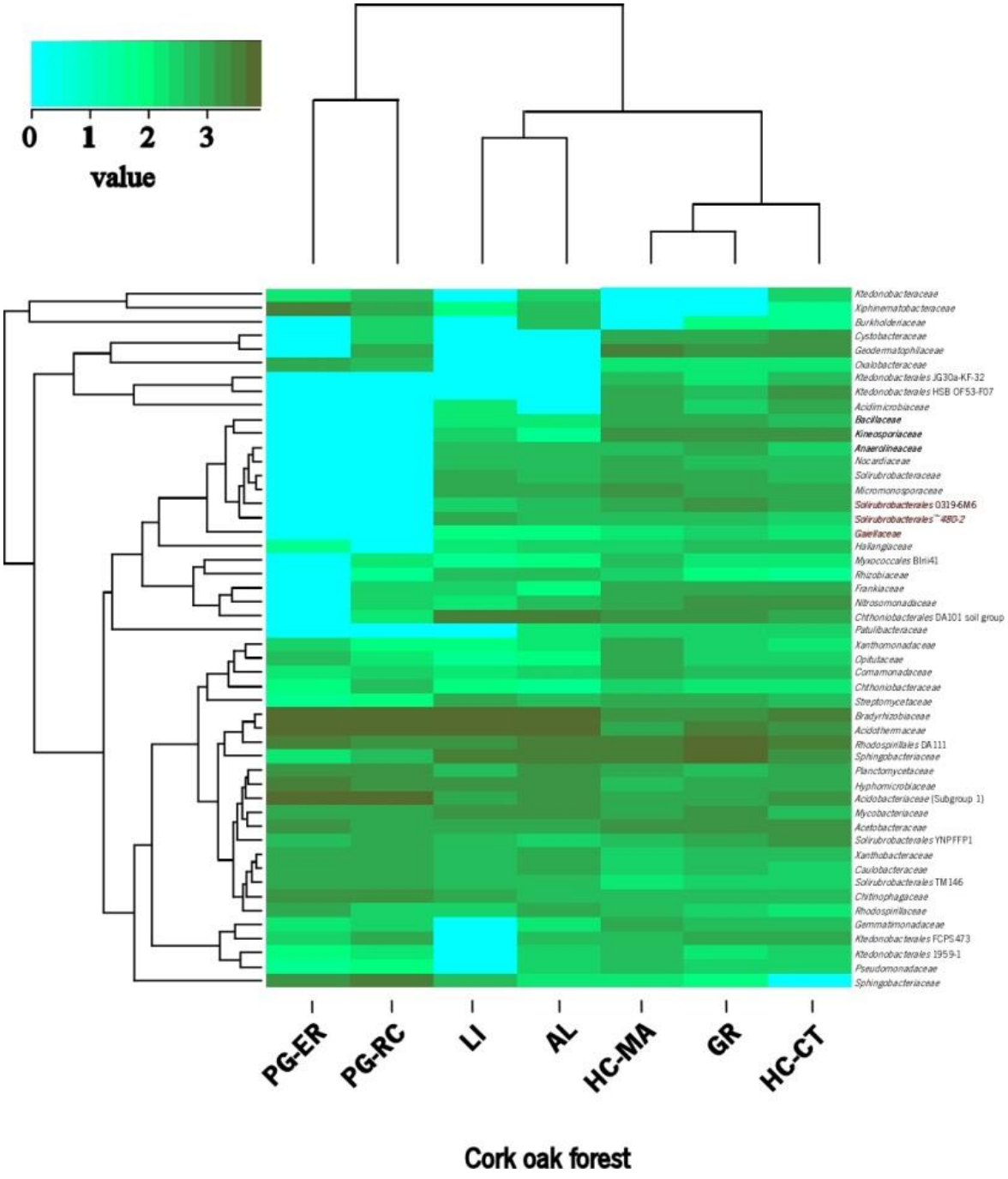

Figure 3.5. Taxonomic distribution of the 50 most abundant bacterial families within the different soils. The F-test with Bonferroni correction for multiple pairwise comparisons was used to select the classified OTUs whose differences in the relative abundance were significant between samples (p<0.05). Dendrograms were made through hierarchical clustering based on the calculated Canberra distance with UPGMA agglomeration method. Each site is referred by their code, as used in Figure 3.2.

axis 1 more discriminant than axis 2. According to the SIMPER analysis, *Acidobacteriaceae* (Subgroup 1; *Acidobacteria*) and *Acidothermaceae* (*Actinobacteria*) were the families that contributed the most for dissimilarity between groups, which could be related by the fact that both are the most abundant families in total soil analysis (Figure 3.1B; Table S3.2). Southern samples (HC-CT, HC-MA and GR) clustered better than northern samples (PG-ER and PG-RC) corroborating their higher homogeneity. According to this finding, within the forest biological replicates, southern forests (HC-CT and HC-MA) presented similar diversity indexes whereas northern forests (PG-ER and PG-RC) display significantly different D and *H'* indexes ($p<0.05$; Figure 3.3B). Bacterial intra-diversity among all cork oak stands is statistically significant, even when only considering southern or northern cork oak forests (ANOSIM analysis, $p<0.001$). However, while all cork stands were very well separated (R=0.814), northern forests (R=0.682) are better separated than southern forests (R=0.524). Similarity between the same northern and southern forests was also significantly low for ECM community assessment (Chapter 2).

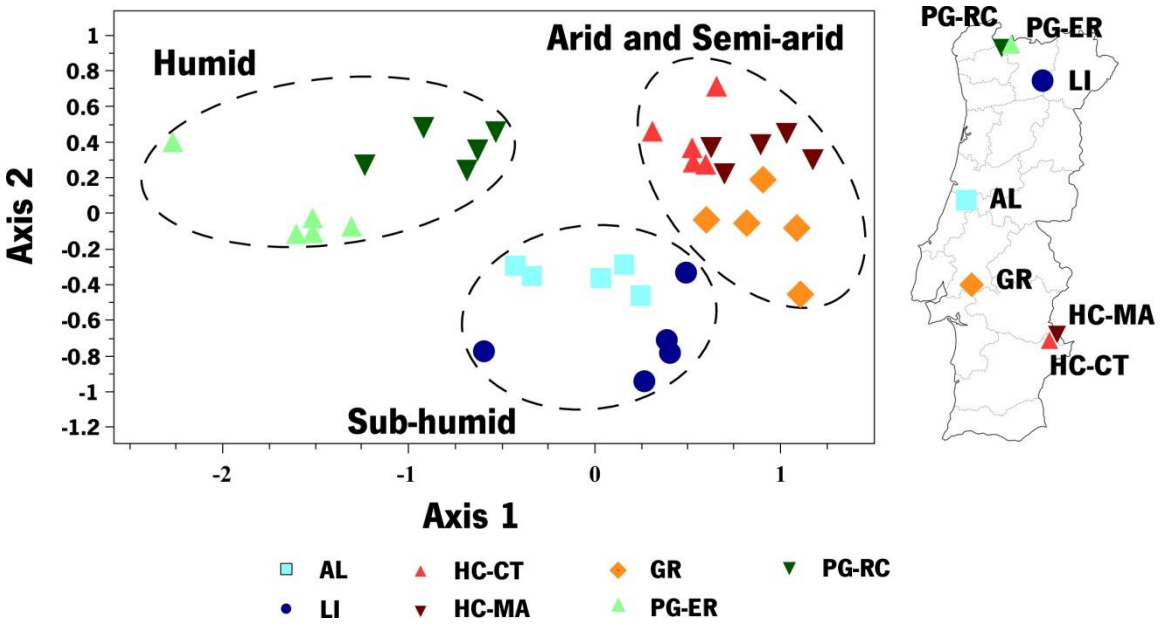

Figure 3.6. Nonmetric multidimensional scaling (NMDS) analysis of bacterial families represented by at least five reads in each sampling site (Kruskal's stress = 0.1016). Clustering analysis was performed with Bray-Curtis dissimilarity measure (A). The two first dimensions are drawn. For reference the geographic distribution of sampled forests is shown, displaying the symbols used for representing each forest stand. Each site is referred by their code, as used in Figure 3.2

Climatic variables (in particular precipitation, evaporation and temperature) are closely linked to Mediterranean vegetation (Suc, 1984). The combination of rainfall and temperature

variables for determination of indexes, such as the Emberger index (*Q*; Emberger, 1930), allows the quantitative expression of climatic conditions. Different Mediterranean climates can be defined based on a system of two axes: one represented by the average of the minimum temperatures of the coldest month and the other the *Q* index. The selection of cork oak stands for this study was mainly based on their different *Q* indexes that defined different Mediterranean climates: humid (PG-ER, PG-RC; *Q* = 186.6), sub-humid (LI and AL; *Q* = 88.9 and *Q* = 102.7), semi-arid (GR; *Q* = 77.5) and arid (HC-CT, HC-AS; *Q* = 43.5). When taking into consideration the families present in all soil samples, NMSD analysis of bacterial communities revealed that cork oak soils grouped differently according to the bioclimate where cork oaks reside (Figure 3.6). Three distinct clusters were formed, related with the humid, sub-humid and semi-arid/arid climates, which agree with the previously presented dendrogram (Figure 3.5). Both revealed a higher homogeneity of bacterial communities within arid/semi-arid samples than within humid/semi-humid bioclimates. For the most divergent Mediterranean bioclimate scenarios (PG and HC), two forest replicas were considered. Replicas displayed very similar bacterial communities, although arid (HC) replicas revealed to be more similar between each other than humid (PG) samples.

Detailed information of the 50 bacterial families which were more differentially present in the different sampled forests (p>0.05) revealed that *Bradyrhizobiaceae* (*Alphaproteobacteria*), *Acidothermaceae* (*Actinobacteria*) and *Acidobacteriaceae* (Subgroup 1; *Acidobacteria*) were the most positively influenced families by climatic condition (higher abundance in humid and sub-humid forests; Figure 3). Southern forests (GR, HC-MA and HC-CT) that clustered with high Canberra distance from northern/central forests were particularly enriched in *Geodermatophilaceae, Kineosporiaceae* and *Frankineae* (*Actinobacteria*), *Nitrosomonadaceae* (*Proteobacteria*), and *Chthoniobacterales* DA101 soil group bacteria (*Verrucomicrobia*). Furthermore, two families of *Ktedonobacteriales* (*Chloroflexi*) that are described to use more recalcitrant carbon substrates as well as inorganic nutrients (reviewed by Lladó *et al.*, 2017) were exclusively found in southern cork oak forests. The higher relative abundance of *Actinobacteria* community in driest soils could be related with the use of a mycelium-forming growth strategy in low hydraulic conductivity (Wolf *et al.*, 2013). Also, an increase of *Actinobacteria* and decrease of *Acidobacteria* communities was detected during summer drought, reflecting a bacterial lifestyle strategy in response to abiotic stress (Barnard *et al.*, 2013).

When disentangled the effect of each climatic parameter used for determining Emberger indexes on the bacterial community structure, a general picture of bacterial community adaptation

to cork oak forests could be suggested. The climatic parameters registered on all sampled sites presented a stronger relation with bacterial richness parameters, but only showed a weak relation with bacterial abundance (Table 3.1). Indeed, although bacteria abundance is positively affected by precipitation levels (at $p<0.05$), it was not really influenced by temperature. Interestingly, richness was strongly affected by both climatic variables. Precipitation negatively affected the bacterial composition of cork oak soils ($p<0.001$), being the levels of precipitation occurring during the driest month the most relevant. On the other hand, temperatures occurring on the hottest month presented exactly the opposite trend ($p<0.001$). Temperatures occurring during the coldest month,

Table 3.1. *Pearson* correlations between bacterial families' abundance and richness from all seven sampled forests with local climatic parameters (precipitation and temperature) and Emberger indexes (*Q*). Climate variables comprised the average precipitation and temperatures from past 30 years (average), from the wettest/hottest month of the sampling year (max) and from the driest/coldest month (min) of the sampling year. Asterisks mean statistical significance at $p<0.05$ (*), $p<0.01$ (**) or at $p<0.001$ (***).

	Climatic parameters		***F***
Abundance	Precipitation	average	5.945*
		max	6.036*
		min	4.721*
	Temperature	average	-5.895*
		max	-1.973
		min	0.005
	Q		4.389*
	pH		-6.376*
Richness	Precipitation	average	-19.190***
		max	-17.563***
		min	-27.116***
	Temperature	average	22.233***
		max	32.976***
		min	4.581*
	Q		-22.964***
	pH		8.958**

despite significant (p<0.05), were less important for bacterial composition. Shifts on bacterial communities of beech forest soils were also correlated with precipitation, as those plots with low precipitation levels presented more bacterial genera then control plots (Felsmann *et al.*, 2015). Interestingly, an opposite trend was found regarding the ectomycorrhizal community in the cork oak stands studied in the present work, where a significant increase of ECM root tips was detected with water availability increasing (Chapter 2). Our results revealed a clear discrimination of bacterial communities according to the bioclimate in which cork oaks reside. The combination of climatic variables into the Emberger index revealed that richness of bacterial communities is significantly decreased in forests with wettest climates (p<0.001) and abundance is stimulated in forests with higher Q values (p<0.05).

The bacterial taxa that are highly affected by climatic variables and Q index were *Chloroflexi, Firmicutes* and *Proteobacteria*, in particular *Gammaproteobacteria* and *Deltaproteobacteria* (Table 3.2). The previous finding that precipitation and temperatures display opposite effects on bacterial communities is maintained, but specific phyla or classes revealed inverse trends. Within phylum analysis, *Chloroflexi* (green non-sulfur bacteria; p<0.001) and *Firmicutes* (most Gram positive bacteria; p<0.01) are more abundant in wet forests and *Proteobacteria* (Gram negative bacteria; p<0.01) in drier forests. These results do not agree with the general idea that Gram negative bacteria present a higher sensitive behavior to environmental disturbances and drought stress than Gram positive bacteria due to their anatomical features (Uhlirova *et al.*, 2005; Schimel *et al.*, 2007; Barnard *et al.*, 2013). On the other hand, the abundance of *Chloroflexi* members presented a strong positive correlation with water availability, as previously reported by Chodak *et al.* (2015). The most abundant classes of *Proteobacteria* (*Alphaproteobacteria* and *Betaproteobacteria*) were not disturbed by the climatic conditions occurring in soil sampling sites, but *Gammaproteobacteria* and *Deltaproteobacteria* classes were positively and negatively correlated with precipitation, respectively. Studies in scots pine forest revealed similar features after drought and rewatering conditions (Chodak *et al.*, 2015). Also, Bouskill *et al.* (2013) observed increases of *Actinobacteria, Proteobacteria* and *Planctomycetes* in plots under rainfall exclusion, whereas decreases of *Acidobacteria, Bacteroidetes* and *Firmicutes* relative abundance were observed in the same conditions. Felsmann *et al.* (2015) described changes in *Actinobacteria, Proteobacteria, Acidobacteria* and *Firmicutes* communities. In general, they concluded that bacteria most well represented exhibit greater resilience and tolerance to

drought. Although less evident in most cases, an opposite trend to the observed with precipitation was verified for temperature averages from past 30 years and temperatures of the hottest months (Table 3.2). Interestingly, the abundance of *Actinobacteria* (p<0.01) and more significantly *Acidobacteria* (p<0.001) seem to be affected by low temperatures, but not by precipitation or high temperatures.

Although revealing high significant levels, previous considerations on the effect of climate on bacterial communities should be taken with care. Bacteria are highly dynamic within the ecosystem and many drivers are known to structure bacterial communities (Kaiser *et al.*, 2016). Bulk soil community is strongly influenced by organic matter content, soil moisture, cations, pH and nutrient content, while rhizosphere layer community is mainly regulated by tree species, litter quality, root and mycorrhiza exudation (Lladó *et al.*, 2017). In any case, climate variables, such as temperatures, precipitation, light and seasonality have been considered as major drivers of

Table 3.2. *Pearson* correlations between abundance of specific taxa and edaphoclimatic conditions in all seven sampled forests, including precipitation and temperature [average from past 30 years (aver.), from the wettest/hottest month (max) and from the driest/coldest month (min) of the sampling year], Emberger index (Q) and soil pH. Pooled of the 35 soil samples were used to analyses of total soil (TOTAL). Asterisks mean statistical significance at p<0.05 (*), p<0.01 (**) and p<0.001 (***).

Bacterial taxa	Precipitation			Temperature			Q	pH
	aver.	Max	min	aver.	Max	min		
Proteobacteria	-10.4**	-10.5**	-5.5*	7.2*	2.4	-1.7	-11.6**	8.9**
Alphaproteobacteria	0.8	0.5	1.6	-0.6	-5.8	-1.3	3.7	0.2
Betaproteobacteria	-2.9	-3.0	-1.1	1.4	1.4	-1.5	-4.0	0.7
Gammaproteobacteria	38.9***	35.1***	45.1***	-41.0***	-27.2***	-1.4	50.4***	-9.3**
Deltaproteobacteria	-22.3***	-19.5***	-38.3***	26.6***	56.8***	7.2*	-34.6***	6.4*
Actinobacteria	1.8	2.1	0.0	-0.1	-0.2	12.0**	3.1	0.1
Acidobacteria	-3.2	-3.7	0.0	0.4	-0.1	-34.2***	-4.3*	0.2
Chloroflexi	13.7***	14.0***	5.7*	-8.4**	-1.4	5.1*	15.5***	-6.5*
Planctomycetes	4.9*	5.0*	3.2	-4.9*	0.0	1.0	2.9	-6.6*
Verrucomicrobia	-4.5*	-4.7*	-1.5	2.6	0.3	-3.6	-4.5*	5.4*
Bacteroidetes	-2.5	-2.5	-0.8	1.1	0.3	-3.4	-4.8*	-1.6
Firmicutes	11.0**	10.9**	5.2*	-6.3*	-4.0	2.4	16.7***	-2.8
TOTAL	6.0*	6.0*	4.7*	-5.9*	-2.0	0.01	4.4*	-6.4*

bacterial communities present on temperate forests (Lladó *et al.*, 2017). While waiting for further experimental support, the implication of climate parameters structuring bacterial communities remains speculative. In the particular case of cork oak forests, other drivers could be additionally implied, like the economic exploration of forest (for agriculture, pasture or even for cork striping) or soil composition. Soil chemical properties, such as strong acidic environments (pH 3.5-4) may decrease *Proteobacteria*/*Acidobacteria* ratio by increasing *Acidobacteria* community (Fierer *et al.*, 2007; López-Mondéjar *et al.*, 2015). In cork oak forests, *Proteobacteria* presented a positive correlation with soil pH (p<0.01), though *Gammaproteobacteria* presented a strong negative correlation with pH (p<0.01). The decrease of *Gammaproteobacteria* community could lead to changes in other taxa community depending on biochemical sub-products (Padmanabhan *et al.*, 2003).

3.5. Conclusions

During the last decade, metagenomics approaches have been used as a major tool for the identification of bacterial communities in different ecosystems (Neelakanta and Sultana, 2013). In the particular case of forest soils, which are one of the richest microbial habitats on Earth, bacterial communities gave a significant contribution for the forest sustainability (Hardoim *et al.*, 2015). This work disclosed part of the bacterial biodiversity present in one of the major biodiversity hotspot - the Mediterranean forest. Part of the sustainability of this threaten ecosystem is largely maintained by microbial communities present within the soils, which are responsible for improving cork oak resistance to environmental changes (Bevivino *et al.*, 2014). In this work, we found that cork oak forest soils were highly enriched in *Proteobacteria*, *Actinobacteria* and *Acidobacteria*, although some differences were detected in seven Portuguese cork oak stands. Northern and more humid stands presented a heterogeneous and less diversified bacterial community than southern and arid forests, being detected a clear discrimination of bacterial communities with bioclimate. Indeed, when considering the most abundant families, the bacterial communities from humid, sub-humid and semi-arid/arid climates clustered into three distinct groups. An implication of climate on community structuring seems to be clear from the correlation analysis of climatic parameters and abundance/richness of bacteria taxa. However, it is still not possible to unequivocally attribute changes in bacterial communities to a climate effect and further experimentation is needed. As recent climate models forecast a general temperature increase and precipitation decline for the

near future, further experimentation on this topic is required, as alterations on bacterial community could affect cork oak forest sustainability under climate changes.

Data Accessibility

Sequence data have been deposited in the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number PRJNA428525.

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Supplementary data

Table S3.1. Number of reads obtained by Illumina MiSeq metabarcoding of 16S DNA samples taken from cork oak forest soils. Raw were processed as described in Materials and Methods section and clustered into OTUs. Taxonomic classification of OTUs represented by at least 5 reads is provided. Each site is referred by their code, as used in Table 2.1.

Cork oak stand/tree		Reads processing				OTUs	Singletons	OTUs (without singletons)	OTUs (≥5 reads)	Taxonomic classification of OTUs					
		Initial no.of reads pairs	Merged reads	Processed reads	Unclassified reads					Phylum	Class	Order	Family	Genera	Species
PG-ER	A	135,397	41,566	36,841	2,627	1,979	633	808	723	22	54	95	153	231	723
	B	122,571	39,493	34,962	2,535	1,871	614	746	686	22	52	91	156	227	686
	C	122,778	39,441	35,133	2,296	1,861	605	731	656	22	55	94	149	212	656
	D	271,293	81,651	74,917	4,410	2,550	620	1,295	1,182	25	63	114	204	310	1,182
	E	126,303	39,134	34,178	2,085	2,508	832	923	822	22	53	98	178	268	822
	Total	778,342	241,285	216,031	13,953	3,840	901	1,579	1,579	25	70	130	243	378	1,579
PG-RC	A	132,196	41,652	35,621	2,493	3,011	818	1,207	1,065	20	62	114	222	350	1,065
	B	126,003	40,576	34,071	2,858	3,131	823	1,298	1,143	23	71	124	232	368	1,143
	C	119,738	39,462	35,045	1,996	2,301	646	991	871	23	63	102	177	274	871
	D	108,895	31,300	26,628	2,038	2,446	716	1,029	885	19	57	100	189	302	885
	E	107,193	35,379	31,212	1,758	2,292	662	989	872	22	61	104	190	296	872
	Total	594,025	188,369	162,577	11,143	4,553	690	2,190	2,190	26	83	147	287	473	2,190
LI	A	68,813	23,326	19,184	1,372	2,347	819	798	695	18	50	90	171	274	695
	B	110,759	37,155	31,608	2,473	2,655	740	1,048	915	20	56	100	202	327	915
	C	119,580	47,123	35,404	8,591	2,796	801	1,141	1,000	24	66	114	216	357	1,000
	D	114,474	37,299	32,918	1,682	2,348	784	838	738	21	53	95	172	272	738
	E	95,941	31,794	26,525	1,846	2,892	969	1,020	884	19	58	100	195	314	884
	Total	509,567	176,697	145,639	15,964	4,784	885	2,034	2,034	26	79	146	292	490	2,034

Table S3.1. *continuation*

Cork oak stand/tree		Reads processing								Taxonomic classification of OTUs					
		Initial no.of reads pairs	Merged reads	Processed reads	Unclassified reads	OTUs	Singletons	OTUs (without singletons)	OTUs (≥5 reads)	Phylum	Class	Order	Family	Genera	Species
AL	A	99,671	34,317	28,806	2,051	2,928	877	1,109	957	20	53	97	203	337	957
	B	118,905	41,847	36,004	2,156	3,135	939	1,184	1,048	21	56	103	214	349	1,048
	C	81,758	29,069	23,464	2,016	2,931	879	1,056	913	22	62	112	223	347	913
	D	92,006	29,967	24,817	1,700	2,830	907	987	838	24	65	105	206	315	838
	E	90,369	31,227	25,75	1,779	3,027	898	1,096	935	22	63	108	210	323	935
	Total	482,709	166,427	138,841	9,702	5,064	861	2,037	2,036	30	83	151	295	479	2,036
GR	A	99,164	32,453	27,355	1,720	2,895	876	1,105	935	21	59	113	219	362	935
	B	106,653	33,980	28,636	1,769	3,109	984	1,173	1,006	21	58	110	220	363	1,006
	C	92,604	30,703	25,095	1,881	3,077	940	1,094	950	19	61	107	203	339	950
	D	70,382	21,911	16,868	1,638	2,720	981	839	712	20	53	95	182	281	712
	E	145,067	47,123	39,514	3,505	3,532	901	1,483	1,282	25	76	137	264	429	1,282
	Total	513,870	166,170	137,468	10,513	5,290	856	2,174	2,174	28	86	162	321	538	2,174
HC-CT	A	109,979	37,048	30,777	2,238	3,384	946	1,296	1,127	27	73	134	244	382	1,127
	B	108,734	38,478	32,382	2,255	3,279	939	1,270	1,108	23	64	114	234	376	1,108
	C	114,150	41,313	34,675	2,435	3,608	925	1,482	1,292	27	71	129	249	392	1,292
	D	111,442	37,223	31,527	2,223	2,973	863	1,157	1,014	22	62	115	217	343	1,014
	E	108,858	38,222	31,988	2,222	3,415	952	1,346	1,174	26	70	128	238	377	1,174
	Total	553,163	192,284	161,349	11,373	5,419	727	2,378	2,378	29	87	168	323	531	2,378
HC-MA	A	109,636	38,099	31,784	2,329	3,425	899	1,390	1,230	23	65	117	241	399	1,230
	B	102,089	35,395	29,391	1,907	3,518	1,060	1,344	1,169	22	65	122	239	381	1,169
	C	110,556	39,352	32,739	2,327	3,670	972	1,464	1,279	23	70	127	240	394	1,279
	D	103,063	34,736	28,466	2,123	3,440	1,021	1,265	1,098	22	68	121	225	378	1,098
	E	110,898	38,315	32,192	2,050	3,422	959	1,306	1,122	24	66	121	227	378	1,122
	Total	536,242	185,897	154,572	10,736	5,495	686	2,453	2,378	28	81	153	308	526	2,378
TOTAL		**3,189,576**	**1,317,129**	**1,116,477**	**83,384**	**7,429**	**809**	**5,329**	**5,329**	**36**	**109**	**260**	**442**	**943**	**5,329**

Table S3.2. Relative abundance of bacterial OTUs from each family, identified in each sampled cork oak forest. Each site is referred by their code, as used in Table 2.1.

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Acidobacteria	*Acidobacteria*	AT-s3-28	unc bacterium	0	0	0	<0.01	0	0	0	0
		Acidobacteriales	*Acidobacteriaceae* (Subgroup 1)	13.3	8.33	4.67	6.39	3.61	4.86	3.01	6.76
		Subgroup 12	unc bacterium	0.01	0.02	0.01	0	0	0	0	0.01
		Subgroup 13	unc *Acidobacteria* bacterium	0.01	0	0	0	0	0	0	<0.01
			unc bacterium	0.03	0	0	0	0.02	<0.01	0	0.01
		Subgroup 15	unc bacterium	0	0	0	0.01	0	<0.01	<0.01	<0.01
		Subgroup 17	unc *Acidobacteria* bacterium	0	0.01	0.08	0.05	0.07	0.1	0.04	0.05
			unc *Acidobacteriales* bacterium	0	0	0	0	0.01	0.02	<0.01	<0.01
			unc bacterium	0	0.02	0.02	0.05	0.02	0.08	0.07	0.04
		Subgroup 18	unc bacterium	0	0	0	0	<0.01	0	0	<0.01
		Subgroup 2	unc *Acidobacteria* bacterium	0.74	0.32	0.22	0.47	0.02	<0.01	0	0.28
			unc bacterium	4.48	2.3	1.27	3.44	1	0.7	0.66	2.11
			unc eubacterium WD2123	0.78	0.28	0	0.05	0	0	0	0.2
			unc forest soil bacterium	0.43	0.19	0.03	0.22	0.02	0.03	0.01	0.15
		Subgroup 25	unc bacterium	0	0	0	0	0.01	<0.01	0.01	<0.01
		Subgroup 3	Elev-16S-1166	0	0.01	0	0	0	<0.01	<0.01	<0.01
			SJA-149	0	0	0.01	0	0	0	0	<0.01
			Unknown Family	4.65	3.95	2.54	2.3	2.36	3.02	1.74	3.06
			unc bacterium	0.01	0	0	<0.01	0	0	0	<0.01
		Subgroup 4	45597	0	0	0.08	0.09	0.13	0.02	0.07	0.05
			DS-100	0	0	0.01	<0.01	0.02	0.03	0.05	0.02
			RB41	0.02	0.11	1.03	0.59	0.93	0.41	0.58	0.48
			Unknown Family	0.01	0.18	0.74	0.54	0.98	0.98	0.84	0.57
		Subgroup 5	unc *Acidobacteria* bacterium	0.02	0.04	0.07	0.07	0.08	0.09	0.11	0.07
			unc *Acidobacteriales* bacterium	0	0	0.02	0.02	0.01	0.02	0.02	0.01
			unc *Acidob*acterium sp.	0	0	0.01	0.01	0.01	0.01	0.03	0.01
			unc *Holophaga* sp.	0.01	0.02	0	<0.01	0	0	0	0.01
			unc bacterium	0.02	0.02	0.01	0.08	0.06	0.04	0.04	0.04
			unc *soil* bacterium	0	0	<0.01	0.01	0.01	0.02	0.02	0.01
		Subgroup 6	unc *Acidobacteria* bacterium	0.13	0.19	0.88	0.62	0.8	0.95	0.97	0.61
			unc *Acidobacteriaceae* bacterium	0	0	0.01	0.01	0	0	<0.01	<0.01
			unc *Acidobacteriales* bacterium	0.01	0.05	0.31	0.23	0.42	0.32	0.38	0.23
			unc bacterium	0.2	0.5	1.39	0.68	0.87	1.29	1.06	0.82
			unc bacterium 92	0	0	0.01	0	0	0.01	0.01	<0.01
			unc soil bacterium	0	0.02	0.02	0.01	0.04	0.05	0.04	0.02
			unidentified	0	0	<0.01	0	0	0	0	<0.01
	Holophagae	Elev-16S-816	unc bacterium	0	0	0.01	0	0	0	0	<0.01
		Subgroup 10	ABS-19	0.05	0.17	0.06	0.05	0.18	0.17	0.23	0.13
			Sva0725	0	0	0	0	0	0.01	0	<0.01
		Subgroup 7	unc *Acidobacteria* bacterium	0	<0.01	0	0	0.01	0.1	0.14	0.04
			unc bacterium	0.02	0.32	0.41	0.11	0.52	1.17	0.93	0.48

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Acidobacteria	*Holophagae*	Subgroup 7	unc *proteobacterium*	0	0	0	0.01	0	<0.01	0	<0.01
			unc soil bacterium	0	0	0	0	0	0	<0.01	<0.01
	Subgroup 26	unc bacterium	unc bacterium	0	0	0	0	0	0	<0.01	<0.01
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	*Acidimicrobiaceae*	0.01	0.1	0.48	0.31	0.51	0.94	0.77	0.42
			Acidimicrobiales Incertae Sedis	<0.01	0.01	0.02	0.02	0.03	0.02	0.04	0.02
			Iamiaceae	<0.01	0.05	0.05	0.05	0.03	0.04	0.05	0.04
			unc	1.87	2.03	2.11	1.53	2.1	1.85	1.49	1.86
	Actinobacteria	*Catenulisporales*	*Actinospicaceae*	0.09	0.04	0.02	<0.01	0.03	<0.01	0	0.03
			Catenulisporaceae	0.08	0.04	0.02	0.07	0.08	0.05	0.02	0.05
		Corynebacteriales	*Mycobacteriaceae*	2.48	1.05	1.59	2.49	1.52	0.69	1.01	1.58
			Nocardiaceae	0.02	0.06	0.09	0.01	0.04	0.03	0.05	0.04
			unc	0	<0.01	0.22	0.12	0.04	0.05	0.05	0.06
		Frankiales	*Acidothermaceae*	8.64	6.56	6.8	5.13	2.67	2.54	1.24	5.02
			Cryptosporangiaceae	<0.01	0.08	0.01	0	0.03	0.01	0.03	0.02
			Frankiaceae	0.14	0.54	0.83	0.46	0.91	0.92	0.82	0.63
			Geodermatophilaceae	0.01	0.81	0.17	0.04	1.14	1.8	2.55	0.9
			Nakamurellaceae	0.01	0.11	0.12	0.01	0.21	0.17	0.2	0.11
			Sporichthyaceae	<0.01	0.06	0.14	0.14	0.05	0.1	0.09	0.08
			unc	0	0.16	0.05	<0.01	0.13	0.28	0.34	0.13
		Kineosporiales	*Kineosporiaceae*	0.02	0.11	0.27	0.09	1.06	1.17	1.29	0.54
		Micrococcales	*Bogoriellaceae*	0	0	0	0	0.02	0.03	0.03	0.01
			Cellulomonadaceae	0	0	0.14	0.06	0.11	0.12	0.17	0.08
			Demequinaceae	0	0	0.01	0	0	0	0.01	<0.01
			Intrasporangiaceae	0	0.08	0.03	0	0.33	0.14	0.19	0.1
			Microbacteriaceae	0.05	0.3	0.48	0.33	0.17	0.14	0.22	0.23
			Micrococcaceae	<0.01	0.58	0.09	0	0.34	0.09	0.2	0.18
			Micrococcales Incertae Sedis	0	0	0	0.01	0.01	0.01	0.01	<0.01
		Micromonosporales	*Micromonosporaceae*	0.01	0.26	2.52	2.09	1.3	1.75	2.01	1.32
		PeM15	unc bacterium	0.01	0.01	0	0	0	0	0	<0.01
		Propionibacteriales	*Nocardioidaceae*	0.01	0.05	1.53	0.87	1.15	0.95	1.44	0.79
			Propionibacteriaceae	0	<0.01	0.02	0	0.11	0.07	0.17	0.05
		Pseudonocardiales	*Pseudonocardiaceae*	0.17	0.75	0.59	0.46	0.53	0.5	0.72	0.51
		SIFF498-N9D4	unc bacterium	0	0	0	0	0	0.01	0	<0.01
		Streptomycetales	*Streptomycetaceae*	0.2	0.31	1.1	0.9	1.44	0.66	1.02	0.75
		Streptosporangiales	*Streptosporangiaceae*	0	0	0.27	0.06	0.01	<0.01	0.04	0.05
			Streptosporangiales Incertae Sedis	0	0	0	0	0	0.02	0.01	<0.01
			Thermomonosporaceae	0.03	0.12	0.53	0.18	0.18	0.16	0.19	0.19
	MB-A2-108	unc *Micromonospora* sp.	unc *Micromonospora* sp.	0	0	<0.01	0.01	<0.01	0.02	0.01	0.01
		unc *actinobacterium*	unc *actinobacterium*	0	0	0.1	0	0	0	0	0.01
		unc bacterium	unc bacterium	0	0	0.17	0.02	0.25	0.27	0.26	0.13
	Rubrobacteria	*Rubrobacterales*	*Rubrobacteriaceae*	0	0	0.02	0	0.03	0.01	0.03	0.01
	TakashiAC-B11	unc bacterium	unc bacterium	0.05	0.08	0	0.01	0.05	0.05	0.07	0.04
	Thermoleophilia	*Gaiellales*	*Gaiellaceae*	<0.01	<0.01	0.12	0.09	0.33	0.15	0.2	0.12
			unc	0.17	0.57	3.81	2.7	4.24	3.26	2.94	2.35
		Solirubrobacterales	0319-6M6	0.01	0.04	0.47	0.57	1.48	0.84	1.06	0.59

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Actinobacteria	*Thermoleophilia*	*Solirubrobacterales*	288-2	0	<0.01	0.12	0.02	0.05	0	0.03	0.03
			480-2	0.04	0.05	1.63	0.9	0.98	0.6	0.67	0.64
			Conexibacteraceae	0.1	0.05	0.06	0.08	0.03	0.01	0.01	0.05
			Elev-16S-1332	0	0	0.13	0.05	0.06	0.09	0.09	0.06
			FCPU744	0	0	0.01	0	0.03	0	0.01	0.01
			FFCH12655	0	0	0.02	0.01	0.02	0	0	0.01
			Patulibacteraceae	0	0.01	0.55	0.4	0.51	0.45	0.62	0.34
			Solirubrobacteraceae	0	<0.01	0.8	0.59	0.67	0.69	0.96	0.49
			TM146	0.75	1.02	0.31	0.41	0.25	0.33	0.18	0.49
			YNPFFP1	0.83	1.04	0.75	0.38	1.14	2.02	0.8	1
			unc	0.11	0.14	0.04	0.02	0.2	0.61	0.27	0.2
			unc bacterium	0	0	0.03	0	<0.01	0	0.01	0.01
Aerophobetes	unc *Geobacter* sp.	unc *Geobacter* sp.	unc *Geobacter* sp.	0	0	0	<0.01	0	0	0.01	<0.01
	unc bacterium	unc bacterium	unc bacterium	0	0	0	0	0	0.03	0.01	0.01
Armatimonadetes	*Armatimonadia*	*Armatimonadales*	*Armatimonadaceae*	0	0	0	0	0	0	0.01	<0.01
			unc *Armatimonadetes* bacterium	0	0	0	0	0	0	0.01	<0.01
			unc *Chloroflexi* bacterium	0.05	0.11	0	0.02	0.02	0.04	0.05	0.04
			unc bacterium	0.18	0.37	0.01	0.05	0.01	0.02	0.11	0.12
			unc *eubacterium* WD294	<0.01	0.02	0	0	0	0	0	<0.01
			unc organism	0	0	0	0	<0.01	0	0	<0.01
	Chthonomonadetes	*Chthonomonadales*	*Chthonomonadaceae*	0.16	0.65	0.1	0.07	0.16	0.21	0.34	0.24
			unc *Armatimonadetes* bacterium	0	0	0	0	0.01	<0.01	0	<0.01
			unc bacterium	0	0.06	0.03	0.02	0.11	0.07	0.14	0.06
	unc *Armatimonadetes* bacterium	unc *Armatimonadetes* bacterium	unc *Armatimonadetes* bacterium	0.09	0.04	<0.01	0.01	0	0	0.01	0.03
	unc bacterium	unc bacterium	unc bacterium	0.08	0.1	0.02	0.02	0.05	0.03	0.08	0.06
	unc *soil* bacterium	unc soil bacterium	unc soil bacterium	0.03	0.01	0	0	0	<0.01	0	0.01
Bacteroidetes	*Bacteroidetes VC2.1 Bac22*	unc bacterium	unc bacterium	<0.01	0.01	0	0.01	0	<0.01	0	<0.01
	Bacteroidia	*Bacteroidales*	*Bacteroidaceae*	0.16	0.44	0	0	0.16	0	0	0.11
			Bacteroidales S24-7 group	0.02	0.04	0	0	0.01	0	0	0.01
	Cytophagia	*Cytophagales*	*Cytophagaceae*	0.14	0.21	0.15	0.21	0.19	0.24	0.33	0.21
	Flavobacteriia	*Flavobacteriales*	*Blattabacteriaceae*	0	0	0	0	0	0	0.09	0.01
			Cryomorphaceae	0	0	0.01	0	0	0	0.01	<0.01
			Flavobacteriaceae	0.01	0.01	0.76	0.36	0.13	0.15	0.42	0.24
			NS9 marine group	0.01	0.02	0.01	0.01	0.01	<0.01	0	0.01
	Sphingobacteriia	*Sphingobacteriales*	AKYH767	<0.01	0.03	0.05	0.03	0.01	0.05	0.03	0.03
			CWT CU03-E12	0.21	0.07	0	0	0	0	0.02	0.05
			Chitinophagaceae	2.24	2.43	2.23	1.34	1.28	1.52	1.72	1.86
			KD3-93	0.08	0.16	0.01	0.03	0.01	0.04	0.04	0.06
			NS11-12 marine group	0.01	0	0	0.01	<0.01	0	0.01	<0.01
			PHOS-HE51	0	0.01	<0.01	0	0.01	0.04	0.04	0.01
			S15-21	0	<0.01	0	0	0	0	0	<0.01
			Saprospiraceae	0	0	0.03	0.01	0	0	0	0.01
			Sphingobacteriaceae	3.19	3.18	2.07	0.79	0.73	0.35	1.14	1.75
			WCHB1-69	0	0	0	0	<0.01	0	0	<0.01
			env.OPS 17	0.18	0.32	0.05	0.27	0.15	0.41	0.36	0.25

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Bacteroidetes	WCHB1-32	unc bacterium	unc bacterium	0	0	0	0	0	<0.01	0	<0.01
Candidate division OP3	unc bacterium	unc bacterium	unc bacterium	0	0	0	0	<0.01	0.02	0.01	0.01
Candidate division WS6	unc bacterium	unc bacterium	unc bacterium	0	0	0.01	0	<0.01	0	0	<0.01
Chlamydiae	*Chlamydiae*	*Chlamydiales*	*Parachlamydiaceae*	<0.01	0.01	0	0	0	0	0	<0.01
			Simkaniaceae	0.11	0.01	0.02	0.02	0	<0.01	0	0.03
			cvE6	0.04	0.03	<0.01	0.09	0	0.01	0	0.03
Chlorobi	*Chlorobia*	*Chlorobiales*	OPB56	0.1	0.04	0.01	0.01	0.02	0.01	0	0.03
			SJA-28	0	0	0	0	0	0.01	0	<0.01
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	0	0.04	0.08	0.03	0.09	0.04	0.01	0.04
	Caldilineae	*Caldilineales*	*Caldilineaceae*	0	<0.01	0.04	0.04	0.15	0.1	0.27	0.08
	Chloroflexia	*Chloroflexales*	FFCH7168	0	0	0	0	<0.01	0	0	<0.01
			Roseiflexaceae	0	0.01	0.16	0.11	0.63	0.25	0.37	0.2
		Kallotenuales	AKIW781	0	0	0.01	<0.01	0.04	0.02	0.04	0.01
	Gitt-GS-136	unc *Caldilinea* sp.	unc *Caldilinea* sp.	0	<0.01	0.05	0	0.01	0	0	0.01
		unc *Chloroflexi* bacterium	unc *Chloroflexi* bacterium	0	0	0.02	0.02	0	0	0	0.01
	JG30-KF-CM66	unc *Caldilinea* sp.	unc *Caldilinea* sp.	0	0.01	0	<0.01	0.01	0.03	0.02	0.01
		unc bacterium	unc bacterium	0.03	0.04	0.02	0.05	0.06	0.06	0.05	0.04
	JG37-AG-4	unc *Chloroflexi* bacterium	unc *Chloroflexi* bacterium	0.01	0.06	0	0	0.06	0.67	0.12	0.13
		unc *Clostridium* sp.	unc *Clostridium* sp.	0.07	0.22	0.01	<0.01	0.02	0.02	0	0.05
		unc *Thermomicrobia* bacterium	unc *Thermomicrobia* bacterium	0.14	0.01	0.01	0	0	0.01	0	0.03
		unc bacterium	unc bacterium	0.76	1.99	0.44	0.08	0.92	1.96	0.51	0.97
	KD4-96	unc *Anaerolineaceae* bacterium	unc *Anaerolineaceae* bacterium	0.03	0.05	0.97	0.67	0.87	0.44	0.6	0.47
		unc *Anaerolineae* bacterium	unc *Anaerolineae* bacterium	0	0	0.05	0.02	0	0	0	0.01
		unc bacterium	unc bacterium	0.03	0.18	1.27	0.77	0.88	0.67	0.73	0.6
	Ktedonobacteria	B12-WMSP1	unc *Chloroflexi* bacterium	0	0	0	0	0	0.33	0.12	0.06
			unc bacterium	0	0.07	0	0	0.13	0.7	0.3	0.17
		C0119	unc *Chloroflexi* bacterium	0	<0.01	0	0	0.04	0.01	<0.01	0.01
			unc bacterium	0	0.06	0.02	0.01	0.23	0.18	0.14	0.09
			unc *soil* bacterium	0	0	0	0	0.03	0.03	0.02	0.01
		JG30-KF-AS9	*Chloroflexi* bacterium Ellin7237	0.01	0.01	0	0.26	<0.01	0.02	0.01	0.04
			unc bacterium	0.01	0.05	0	0.02	0.03	0.05	0.02	0.03
			unc *soil* bacterium	<0.01	0.01	0	0.07	0.01	0.03	<0.01	0.02
		Ktedonobacterales	1921-3	<0.01	0.13	0.02	0.01	0.23	1.08	1.19	0.37
			1959-1	0.1	0.14	0.01	0.27	0.14	0.26	0.51	0.2
			BacC-u-018	0	0	0	0	0	0.01	0	<0.01
			FCPS473	0.67	2.75	0.02	0.82	1.42	1.4	1.33	1.2
			G12-WMSP1	0	0.05	0	0	0	0.01	0	0.01
			HSB OF53-F07	0.05	0.93	0.06	0.21	0.67	1.57	1.04	0.63
			JG30a-KF-32	0.15	0.47	0.04	0.07	0.67	1.05	0.91	0.47
			Ktedonobacteraceae	0.31	0.74	0.27	0.79	0.42	0.72	0.17	0.48
			Thermosporotrichaceae	0.17	0.33	0	0.18	0.06	0.23	0.29	0.18
		Thermogemmatisporales	1921-2	<0.01	0.24	0	0	0	0.45	0.3	0.14
		unc bacterium	unc bacterium	0.01	0.04	0	0	0	0	0	0.01
	P2-11E	unc bacterium	unc bacterium	0	0.04	<0.01	0	<0.01	0.09	0.03	0.02

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Chloroflexi	S085	unc *Chloroflexi* bacterium	unc *Chloroflexi* bacterium	0	0	0.02	0.02	0.02	0.01	0.02	0.01
		unc bacterium	unc bacterium	0	0.01	0.03	0.02	0.11	0.06	0.06	0.04
	SAR202 clade	unc bacterium	unc bacterium	0	<0.01	0	0	0	0	0	<0.01
	SHA-26	unc bacterium	unc bacterium	<0.01	0	0	0	0.01	0	0.01	<0.01
	TK10	bacterium Ellin6519	bacterium Ellin6519	0	0	0	0	0	0.03	0.01	0.01
	TK10	bacterium Ellin6543	bacterium Ellin6543	0	0	<0.01	0	0.01	0	0	<0.01
		unc *Chloroflexi* bacterium	unc *Chloroflexi* bacterium	0.03	0.14	0.04	0.02	0.09	0.1	0.05	0.07
		unc *Dehalogenimonas* sp.	unc *Dehalogenimonas* sp.	0	0	<0.01	<0.01	0.02	<0.01	<0.01	<0.01
		unc bacterium	unc bacterium	0.07	0.5	0.41	0.23	0.89	0.99	0.78	0.53
	Thermomicrobia	AKYG1722	unc bacterium	0	0	0	0	0.02	0.01	<0.01	<0.01
		JG30-KF-CM45	unc *Chloroflexi* bacterium	0	0	0.01	0	0	0	0	<0.01
			unc *Sphaerobacter* sp.	0	0	0	0	0.01	0	0	<0.01
			unc bacterium	<0.01	0.08	0.14	0.03	0.2	0.13	0.18	0.1
	unc	unc bacterium	unc bacterium	0	0.01	0.01	0.02	0.03	0.01	0.02	0.02
	unc *Bellilinea* sp.	unc *Bellilinea* sp.	unc *Bellilinea* sp.	<0.01	0.05	0	0	0.01	0.01	0	0.01
Cyanobacteria	*Chloroplast*	*Chlorella* sp. CC-Bw-9	*Chlorella* sp. CC-Bw-9	0	0	0	0	0	<0.01	0	<0.01
		Ettlia pseudoalveolaris	*Ettlia pseudoalveolaris*	0	0	<0.01	0	0.03	0	0.01	0.01
		Grimmia sp. Qiu 01090	*Grimmia* sp. Qiu 01090	0	0.01	0.01	0.01	0.06	0.02	0.06	0.02
		Isoetes melanopoda	*Isoetes melanopoda*	0	0	0	0	0.01	0.06	0	0.01
		Neocystis brevis	*Neocystis brevis*	0	0	0	0	0.01	0	0.01	<0.01
		unc *Chlorella*	unc *Chlorella*	0	0	0	0	0	0.01	<0.01	<0.01
		unc *Chlorophyta*	unc *Chlorophyta*	<0.01	0	0	0.01	<0.01	0	0	<0.01
		unc bacterium	unc bacterium	0	0	0	0	0.05	0.01	0.01	0.01
		unc *cyanobacterium*	unc *cyanobacterium*	0	0	0	<0.01	0	0	0.01	<0.01
		unc eukaryote	unc eukaryote	0.01	0.01	0	0	0.08	0.01	0.02	0.02
		unc phototrophic eukaryote	unc phototrophic eukaryote	0.06	0.07	0	0	0.07	0	0	0.03
	Cyanobacteria	Subsection I	Family I	0.04	0.06	0	0	0.04	<0.01	0	0.02
		Subsection III	Family I	0.01	0.01	0.01	0	<0.01	0.01	0	0.01
		Subsection IV	Family I	0	0	0	0	0.02	0	0.03	0.01
	ML635J-21	unc bacterium	unc bacterium	0	0.04	0	0.01	0.01	0.01	<0.01	0.01
	Melainabacteria	*Obscuribacterales*	unc bacterium	0.25	0.07	0.06	0.09	0.05	0.19	0.16	0.13
			unc *cyano*bacterium	0.01	0.01	0	0	0	0	0	<0.01
Deferribacteres	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	0	<0.01	0	0	<0.01	0	0	<0.01
Elusimicrobia	*Elusimicrobia*	FCPU453	unc bacterium	0	<0.01	0	0	0	0.01	0	<0.01
		Lineage IIa	*actinobacterium* YJF1-30	0.05	0.01	0.04	0.02	0.04	0.02	0	0.03
			unc *Elusimicrobia* bacterium	0	0	0	0	0.02	0	0	<0.01
			unc *Termite* group 1 bacterium	0	0	<0.01	0	0	0	0	<0.01
			unc bacterium	0.06	0.03	0.06	0.04	0.15	0.05	0.06	0.06
			unc soil bacterium	0.01	0.01	0.02	0.01	0.01	<0.01	0	0.01
		Lineage IIb	unc bacterium	<0.01	0.01	0.01	0	0.02	0.01	0.01	0.01
			unc soil bacterium	0	0	0	0	0.01	0	0	<0.01

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Elusimicrobia	*Elusimicrobia*	Lineage IV	unc *Termite* group 1 bacterium	0.01	0.01	0	0	0	<0.01	<0.01	<0.01
			unc bacterium	0.16	0.16	0.01	0.09	0.02	0.05	0.06	0.08
			unc soil bacterium	<0.01	0	0	0.01	0	0	0	<0.01
Fibrobacteres	*Fibrobacteria*	*Fibrobacterales*	*Fibrobacteraceae*	0.01	<0.01	0.01	0.01	0.07	0.09	0.06	0.03
Firmicutes	*Bacilli*	*Bacillales*	*Alicyclobacillaceae*	0	0.02	0.01	0	0.07	0	0	0.01
			Bacillaceae	0.06	0.04	0.68	0.19	1.66	0.98	3.06	0.9
			Paenibacillaceae	0	0.05	0.07	0.11	0.1	0.06	0.04	0.06
			Planococcaceae	0.01	0.24	0.02	<0.01	0.09	0.04	0.14	0.08
			Sporolactobacillaceae	0	<0.01	0	0	0.01	0	0	<0.01
	Clostridia	*Clostridiales*	*Clostridiaceae* 1	0.01	0	0.06	0.01	0.18	0.07	0.06	0.05
	Clostridia	*Clostridiales*	*Lachnospiraceae*	0.07	0.15	0	0	0.07	0	0	0.05
			Peptostreptococcaceae	0	0	0	<0.01	0.03	0.06	0.23	0.04
			Ruminococcaceae	0	0	0.03	0	0.03	0.04	0.01	0.01
	Erysipelotrichia	*Erysipelotrichales*	*Erysipelotrichaceae*	0	0	<0.01	0.01	0.04	0	0.02	0.01
	Negativicutes	*Selenomonadales*	*Veillonellaceae*	0.01	0	0.01	0	0.01	0.01	0.01	0.01
			unc	0	0	<0.01	0	0.01	0	0	<0.01
	OPB54	unc bacterium	unc bacterium	0	0	0	0	0	0.01	0	<0.01
Gemmatimonadetes	*Gemmatimonadetes*	AT425-EubC11 terrestrial group	unc bacterium	0	0	0.01	0.02	0.09	0.04	0.07	0.03
		BD2-11 terrestrial group	unc bacterium	0	0	0	<0.01	0	0.01	<0.01	<0.01
		Gemmatimonadales	*Gemmatimonadaceae*	0.26	0.85	0.92	0.57	1.52	1.33	1.34	0.93
		S0134 terrestrial group	unc *Gemmatimonas* sp.	0	0	0.01	0	<0.01	0	0	<0.01
			unc bacterium	0	0	<0.01	0	0	0.01	0.06	0.01
Gracilibacteria	unc bacterium	unc bacterium	unc bacterium	0	0	0	0.01	0	<0.01	<0.01	<0.01
Hydrogenedentes	unc bacterium	unc bacterium	unc bacterium	0	0	0	0	0	0.01	0.01	<0.01
Latescibacteria	unc *Latescibacteria* bacterium	unc *Latescibacteria* bacterium	unc *Latescibacteria* bacterium	0	0	0.01	0.01	0.01	<0.01	0	<0.01
	unc bacterium	unc bacterium	unc bacterium	0	<0.01	0.06	0.02	0.05	0.03	0.01	0.02
	unc *Microgenomates* bacterium	unc *Microgenomates* bacterium	unc *Microgenomates* bacterium	<0.01	0	0	<0.01	0	0	0	<0.01
	unc bacterium	unc bacterium	unc bacterium	0	0	0	0	0	0.04	0.02	0.01
	Nitrospira	*Nitrospirales*	*0319-6A21*	0.01	0.21	0.22	0.18	0.05	0.04	0.03	0.1
			47209	0.19	0.12	0.01	<0.01	0	0.03	0	0.06
			Nitrospiraceae	<0.01	0.04	<0.01	0.06	0.07	<0.01	0.04	0.03
	NPL-UPA2	unc bacterium	unc bacterium	0	0	0	0.01	<0.01	0	<0.01	<0.01
	unc *Parcubacteria* bacterium	unc *Parcubacteria* bacterium	unc *Parcubacteria* bacterium	0	0.02	0.01	<0.01	0.02	0.04	0.03	0.02
	unc bacterium	unc bacterium	unc bacterium	0.16	0.77	0.11	0.11	0.18	0.18	0.4	0.27
	unc *soil* bacterium	unc soil bacterium	unc soil bacterium	0.08	0.45	0.03	0.06	0.1	0.08	0.15	0.14
	BD7-11	unc bacterium	unc bacterium	0.01	0.04	0	0.02	0.02	0.1	0.02	0.03
	OM190	unc bacterium	unc bacterium	0	<0.01	0.02	0.05	0.02	0.09	0.06	0.03
	Phycisphaerae	*CPla-3 termite group*	unc bacterium	0.01	0.03	0.02	0.05	0.03	0.09	0.12	0.05
			unc *planctomycete*	0.03	0.04	0.02	0.07	0.04	0.03	0.04	0.04
			unc soil bacterium *PBS-22*	0	<0.01	0.01	0.03	0.01	0.02	0.03	0.01
		Phycisphaerales	*Phycisphaeraceae*	0.02	0.06	0.02	0.14	0.03	0.05	0.07	0.05
		Pla1 lineage	unc bacterium	0	0	<0.01	0.02	0	<0.01	0	<0.01
		S-70	unc bacterium	0	<0.01	0	0.01	0.02	<0.01	0.01	0.01
		WD2101 soil group	*Planctomycetales* bacterium *Ellin6207*	0	0.04	0.01	<0.01	0.03	0.04	0.06	0.03

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Latescibacteria	*Phycisphaerae*	WD2101 soil group	*planctomycete LX80*	0	0.02	0	<0.01	0.02	0.03	0.01	0.01
			planctomycete WWH14	0	0	0	0.01	0	0.02	0.01	0.01
			unc *Planctomycetaceae* bacterium	0.28	0.58	0.08	0.14	0.05	0.05	0.08	0.19
			unc *Planctomycetales* bacterium	0.05	0.2	0.14	0.32	0.16	0.3	0.37	0.21
			unc bacterium	1.66	3.05	1.61	2.43	3.18	4.2	5.89	3.09
			unc eubacterium *WD2101*	0.02	0.18	0.02	0.05	0.03	0	0.04	0.05
			unc eubacterium *WD283*	0.12	0.48	0	0.01	<0.01	0.01	0	0.1
			unc *planctomycete*	0.02	0.2	0.11	0.16	0.68	0.61	0.87	0.36
			unc soil bacterium	0	0.04	0.09	0.09	0.2	0.34	0.29	0.14
	Pla4 lineage	unc bacterium	unc bacterium	0	0.01	0	<0.01	0	0.04	0.01	0.01
	Pla4 lineage	unc deep-sea bacterium	unc deep-sea bacterium	0	0	0	0.01	0	0.01	0	<0.01
	Planctomycetacia	*Planctomycetales*	*Planctomycetaceae*	3.03	4.16	1.26	4.01	1.35	2.34	1.9	2.62
	vadinHA49	unc bacterium	unc bacterium	0.01	0.12	0.01	0.02	0.02	0.08	0.06	0.05
		unc *planctomycete*	unc *planctomycete*	0	0.01	0	0	0	0	0	<0.01
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	1.49	1.23	1.38	1.72	0.79	0.61	0.74	1.15
			Hyphomonadaceae	0	<0.01	0.2	0.32	0.03	0.06	0.05	0.09
		DB1-14	unc bacterium	<0.01	0.01	0	0.01	0	<0.01	0.01	0.01
		Rhizobiales	1174-901-12	<0.01	0.19	0	0	0.01	0	0.01	0.03
		Rhizobiales	A0839	0	0.06	0.09	0.11	0.03	0.04	0.01	0.04
			Beijerinckiaceae	0.02	0.15	0.11	0.03	0.2	0.13	0.15	0.11
			Bradyrhizobiaceae	4.48	3.94	5.18	4.07	1.57	2.25	1.89	3.4
			DUNssu044	0	0	0.01	0	0	0	0	<0.01
			DUNssu371	0.06	0.11	0.04	0.26	0.04	0.02	0.01	0.08
			Hyphomicrobiaceae	2.8	2.28	2.61	2.31	1.06	1.41	0.83	1.95
			JG34-KF-361	0	0	0.2	0.08	0.01	0.01	0.04	0.04
			KF-JG30-B3	0.06	0.18	0.19	0.2	0.18	0.19	0.11	0.15
			MNG7	0	0.05	0.11	0.06	0.01	0.01	0.02	0.03
			Methylobacteriaceae	0.06	0.07	0.09	0.05	0.24	0.23	0.42	0.16
			Methylocystaceae	<0.01	0.01	0	0	0	0	0	<0.01
			Phyllobacteriaceae	<0.01	0.05	0.42	0.36	0.1	0.05	0.1	0.14
			Rhizobiaceae	0.01	0.03	0.4	0.39	0.19	0.05	0.2	0.16
			Rhizobiales Incertae Sedis	1.52	1.58	1.35	1.38	0.61	0.7	0.44	1.11
			Rhodobiaceae	0	0	0.06	0.1	<0.01	0.01	0.01	0.02
			Xanthobacteraceae	1.03	1.31	1.27	1.25	0.69	0.73	0.57	0.98
			alphal cluster	1.97	0.69	0.25	0.54	0.2	0.19	0.09	0.65
			unc bacterium	0.08	0.07	0.05	0.02	0.03	0.04	0.09	0.06
		Rhodobacterales	*Rhodobacteraceae*	0	0	0.01	0	0.01	0	0	<0.01
		Rhodospirillales	*Acetobacteraceae*	2.14	1.27	1.06	1.15	1.66	1.59	1.31	1.5
			DA111	4.42	2.09	1.75	4.73	5.19	3.4	2.3	3.42
			I-10	0.01	0.03	<0.01	0.04	<0.01	0.01	<0.01	0.01
			JG37-AG-20	0.06	0.04	0.17	0.34	0.04	0.01	0.01	0.09
			KCM-B-15	<0.01	0.01	0.09	0.19	0.02	0.01	0.01	0.04
			ML80	0	0	<0.01	0.01	0	0	0	<0.01
			MNC12	0	<0.01	<0.01	<0.01	<0.01	0	0	<0.01
			MND8	0	0	0.02	0.07	0	0	0	0.01

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	MSB-1E8	0	0	0	0.02	0	0	0	<0.01
			Rhodospirillaceae	1.42	0.79	0.87	1.71	0.49	0.54	0.72	0.96
			Rhodospirillales Incertae Sedis	0.37	0.3	0.67	0.55	0.43	0.36	0.37	0.43
		Rickettsiales	AKIW1012	<0.01	0	0	0	<0.01	0	0	<0.01
			Anaplasmataceae	<0.01	0	0	0	0	0	0	<0.01
			EF100-94H03	0.09	0.02	0.01	<0.01	<0.01	0	0	0.02
			Holosporaceae	0.01	0	0.01	0	0	0	0	<0.01
			LWSR-14	0	0	0	0	0	0	<0.01	<0.01
			Mitochondria	0	0	0.01	0	0	0.01	<0.01	<0.01
			Rickettsiales Incertae Sedis	0.01	0.01	0.01	0.01	<0.01	0	0	<0.01
			SM2D12	0.13	0.08	0.03	0.04	0.01	0.03	0.05	0.06
		Sphingomonadales	*Erythrobacteraceae*	0	0.01	0.03	0.06	0.17	0.03	0.06	0.05
			Sphingomonadaceae	0.26	0.82	3.95	4.09	6.47	2.55	4.02	2.91
			unc	0	0	<0.01	0.02	0.08	0.01	0.03	0.02
	Betaproteobacteria	B1-7BS	unc bacterium	0.01	0	0	0	0	0	0	<0.01
		Burkholderiales	*Alcaligenaceae*	0.33	0.14	0.1	0.1	0.03	0.03	0.05	0.13
			Burkholderiaceae	1.44	1.01	2.1	1.78	0.42	0.21	0.35	1.05
			Comamonadaceae	0.42	1.16	0.91	0.75	1.27	1.22	1.58	1.01
			Oxalobacteraceae	1.37	0.88	0.28	0.26	0.5	0.57	0.79	0.71
			unc	0	0.01	0	0	0	0	0	<0.01
		Methylophilales	*Methylophilaceae*	0	0	0	0	<0.01	0	0.01	<0.01
		Neisseriales	*Neisseriaceae*	0.01	0.01	0	0	0	0	0	<0.01
		Nitrosomonadales	*Gallionellaceae*	0	0.01	0	0	0	0.01	0	<0.01
			Nitrosomonadaceae	0.51	1.09	1.25	1.09	2.03	2.43	1.84	1.41
		Rhodocyclales	*Rhodocyclaceae*	0.01	0.09	0.11	0.02	0.07	0.09	0.11	0.07
		SC-I-84	unc *Burkholderiaceae* bacterium	0	0.01	0.01	0	0	0	0	<0.01
			unc *Nitrosomonadaceae* bacterium	0	0	0.01	0	0	0	0	<0.01
			unc *Rhodocyclaceae* bacterium	0.02	0.02	0.1	0.12	0.05	0.13	0.02	0.06
			unc bacterium	0	0.07	0.45	0.2	0.12	0.47	0.19	0.2
			unc *beta proteobacterium*	0.01	0.02	0.12	0.01	0.01	0.01	0.01	0.02
			unc *proteobacterium*	0	0	0.02	<0.01	0.01	0.03	0.02	0.01
	Betaproteobacteria	SC-I-84	unc soil bacterium	0	0.02	0.02	0.01	0	0.01	0.01	0.01
		TRA3-20	unc *Burkholderiales* bacterium	0	0	0.01	0	0.01	0.01	0.02	0.01
			unc bacterium	0.04	0.07	0.07	0.07	0.13	0.14	0.32	0.12
			unc *beta proteobacterium*	0	0.07	0.15	0.07	0.05	0.12	0.22	0.09
		UCT N117	unc *beta proteobacterium*	0	0	0.01	0.01	0.01	0.05	0.06	0.02
	Deltaproteobacteria	*Bdellovibrionales*	*Bacteriovoracaceae*	0	<0.01	0	0	0	0	<0.01	<0.01
			Bdellovibrionaceae	0.17	0.14	0.05	0.1	0.01	0.05	0.12	0.1
		Deltaproteobacteria Incertae Sedis	*Syntrophorhabdaceae*	0	0	0.01	<0.01	0	0	0	<0.01
		Desulfobacterales	*Nitrospinaceae*	0	0	0.01	0	<0.01	0.01	0.02	0.01
		Desulfurellales	*Desulfurellaceae*	0	0	0.01	0	0.01	0	0	<0.01
		Desulfuromonadales	AKYG597	0	0	0.02	0.03	0.01	0.01	<0.01	0.01
			Desulfuromonadaceae	0	0	0	0	<0.01	0.01	0	<0.01

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Proteobacteria	*Deltaproteobacteria*	GR-WP33-30	unc *Gemmatimonadetes* bacterium	0	0	0.01	0.02	<0.01	<0.01	0	0.01
			unc bacterium	0.13	0.01	0.1	0.19	0.03	0.04	0.04	0.08
			unc *delta proteobacterium*	0	0	<0.01	0.03	0.01	0	0	0.01
			unc *proteobacterium*	0	0	0.01	0.04	0.03	0.01	0.01	0.02
			unc soil bacterium	<0.01	0	0	0.01	0	0	0	<0.01
		Myxococcales	27F-1492R	0	<0.01	0	0	<0.01	0.03	0.03	0.01
			Amb-16S-1034	0	<0.01	0	0.01	0	<0.01	<0.01	<0.01
			Blrii41	0.02	0.12	0.19	0.19	0.25	0.42	0.66	0.25
			Blfdi19	0	0.02	0.02	0.05	0.03	0.05	0.06	0.03
			Cystobacteraceae	0.09	0.48	0.09	0.27	1.3	1.66	1.36	0.72
			Elev-16S-1158	0	0	<0.01	0.01	0.04	0.02	0.03	0.01
			Haliangiaceae	0.11	0.36	0.48	0.59	0.83	0.88	0.61	0.52
			KD3-10	0.02	0.02	0.01	0.02	0.09	0.04	0.06	0.04
			MSB-4B10	0	0	0.01	0	0.01	0.04	0.05	0.02
			Myxococcaceae	0	0	0	0	0.03	0	<0.01	<0.01
		Myxococcales	P3OB-42	0.02	0.02	0.07	0.09	0.01	0.01	0.02	0.03
			Phaselicystidaceae	0	0.01	0.08	0.12	0.1	0.1	0.16	0.07
			Polyangiaceae	0.37	0.38	0.05	0.14	0.04	0.14	0.18	0.2
			Sandaracinaceae	0	0.02	0.06	0.1	0.2	0.23	0.32	0.13
			Vulgatibacteraceae	0	0	0	0	<0.01	0.03	0.01	0.01
			mle1-27	<0.01	0	0.01	0	0	<0.01	<0.01	<0.01
			unc	0.05	0.03	0	0.08	0.12	0.15	0.2	0.09
		Oligoflexales	*Oligoflexaceae*	0.01	<0.01	0	<0.01	0	0	0	<0.01
			bacterium enrichment culture clone auto112_4W	<0.01	0	0	0	0	0	0	<0.01
			unc bacterium	0.09	0.04	0	0.03	<0.01	0.01	0.01	0.03
			unc soil bacterium	<0.01	<0.01	0	0	0	0	0	<0.01
		Sh765B-TzT-29	unc bacterium	0	0	0.01	0.01	0	<0.01	0.01	<0.01
			unc soil bacterium	0	0	0	0.01	0	0	0	<0.01
	Elev-16S-509	unc bacterium	unc bacterium	0.12	0.06	0	0.03	0	0	0	0.04
	Epsilonproteobacteria	*Campylobacterales*	*Helicobacteraceae*	0	<0.01	0	0	<0.01	0	0	<0.01
	Gammaproteobacteria	*Aeromonadales*	*Aeromonadaceae*	<0.01	<0.01	0	0	0	0	0	<0.01
		Cellvibrionales	*Cellvibrionaceae*	0	0.02	0	0	<0.01	0.07	0.1	0.03
		Chromatiales	*Chromatiaceae*	0	0.01	0.01	0	0	0	0	<0.01
			Ectothiorhodospiraceae	0	0	0	0	0.01	0.02	0.02	0.01
		Enterobacteriales	*Enterobacteriaceae*	0	0	0.12	0.02	0.06	0.01	0	0.03
		KI89A clade	unc bacterium	0	0	<0.01	0.03	0	0	0	<0.01
		Legionellales	*Coxiellaceae*	0.03	0.01	0.05	0	0.01	<0.01	0	0.02
			Legionellaceae	0.01	0	0	0	0	<0.01	0	<0.01
		NKB5	unc bacterium	0.01	0	0	<0.01	0	0	0	<0.01
			unc *gamma proteobacterium*	0.01	0	0	0	0	0	0	<0.01
			unc organism	<0.01	0	0	0	0	0	0	<0.01
		Pseudomonadales	*Moraxellaceae*	0.02	0.06	0	0	0.15	0.01	0.01	0.03
			Pseudomonadaceae	0.09	0.08	0.26	0.23	0.04	0.04	0.12	0.12
		Xanthomonadales	*Nevskiaceae*	0.09	0.1	0	0.04	0	0	0.01	0.04
	Gammaproteobacteria	*Xanthomonadales*	*Solimonadaceae*	0	0.02	0	0	0.07	0.02	0.02	0.02

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	0.53	0.27	1.08	1.03	0.54	0.66	0.98	0.71
			Xanthomonadales Incertae Sedis	1.95	1.14	0.84	1.19	0.55	0.57	0.92	1.08
			unc	3.38	2.61	1	1.04	0.2	0.25	0.29	1.39
	SK259	unc bacterium	unc bacterium	0.3	0.22	0.07	0.07	0.03	0.07	0.04	0.13
	TA18	unc *Acidobacteria* bacterium	unc *Acidobacteria* bacterium	0.1	0.16	0.22	0.13	0.03	0.02	0.03	0.1
		unc bacterium	unc bacterium	0.3	0.22	0.13	0.22	0.1	0.09	0.05	0.17
SHA-109	unc bacterium	unc bacterium	unc bacterium	0.01	0.01	0	<0.01	0	0.01	0	0.01
	unc soil bacterium	unc soil bacterium	unc soil bacterium	0.01	0.02	0.03	0.01	0.01	0.01	0.01	0.01
SM2F11	unc *Parcubacteria* bacterium	unc *Parcubacteria* bacterium	unc *Parcubacteria* bacterium	0	0.01	<0.01	<0.01	0.03	0.04	0.02	0.01
	unc bacterium	unc bacterium	unc bacterium	0.05	0.08	0.07	0.03	0.18	0.21	0.13	0.11
Saccharibacteria	candidate division TM7 bacterium JGI 0001002-L20	candidate division TM7 bacterium JGI 0001002-L20	candidate division TM7 bacterium JGI 0001002-L20	0	0	0.01	0	0.01	0.01	0	<0.01
	metal-contaminated soil clone K20-27	metal-contaminated soil clone K20-27	metal-contaminated soil clone K20-27	0	0	<0.01	0.01	0	0	0	<0.01
	soil bacterium WF55	soil bacterium WF55	soil bacterium WF55	0.04	0.02	0.04	0.07	0.06	0.01	0.06	0.04
	unc *Candidatus Saccharibacteria* bacterium	unc *Candidatus Saccharibacteria* bacterium	unc *Candidatus Saccharibacteria* bacterium	0.03	0.04	0.1	0.08	0.25	0.09	0.11	0.09
Saccharibacteria	unc bacterium	unc bacterium	unc bacterium	0.52	0.59	1.37	1.13	2.03	0.78	1.14	1.03
	unc bacterium *SBR2096*	unc bacterium *SBR2096*	unc bacterium *SBR2096*	0.01	0.04	0.05	0.09	0.02	0.01	0.06	0.04
	unc soil bacterium	unc soil bacterium	unc soil bacterium	0	0.01	0.04	0.02	0.03	0.01	0	0.01
	unidentified	unidentified	unidentified	0	0	0	0	0.02	0	0	<0.01
Spirochaetae	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	0.02	0	0	<0.01	0	0	0	<0.01
TA06	unc bacterium	unc bacterium	unc bacterium	0	0	0.02	0	0.03	0.06	0.02	0.02
TM6	unc bacterium	unc bacterium	unc bacterium	0.05	0.03	<0.01	0.06	0.01	0	0	0.02
Tenericutes	*Mollicutes*	*Entomoplasmatales*	*Entomoplasmataceae*	0	0	0	0.01	0	0	0	<0.01
Thermotogae	*Thermotogae*	*Thermotogales*	*Thermotogaceae*	0	0.01	0	0	0	0	0	<0.01
Verrucomicrobia	OPB35 soil group	Unknown Order	Unknown Family	0.08	0.08	0.02	0.12	0.02	0.01	0.04	0.05
		bacterium enrichment culture clone auto10_4W	bacterium enrichment culture clone auto10_4W	0.24	0.06	0	<0.01	0	0	0	0.05
		bacterium enrichment culture clone auto67_4W	bacterium enrichment culture clone auto67_4W	0.23	0.11	0.01	0.01	0	<0.01	0	0.06
		unc *Verrucomicrobia* bacterium	unc *Verrucomicrobia* bacterium	0.35	0.2	0.08	0.15	0.06	0.07	0.18	0.17
		unc bacterium	unc bacterium	0.71	0.34	0.15	0.5	0.71	0.3	0.65	0.49
		unc soil bacterium	unc soil bacterium	0.04	0.01	0	0.06	0.03	0.02	0.04	0.03
	Opitutae	*Opitutae* vadinHA64	unc bacterium	0	0	0.01	0.01	0	<0.01	0.02	<0.01
		Opitutales	*Opitutaceae*	0.54	0.55	0.18	0.27	0.42	0.37	0.93	0.48
		Puniceicoccales	*Puniceicoccaceae*	<0.01	0	0	0	0	0	0	<0.01
	S-BQ2-57 soil group	unc *Verrucomicrobia* bacterium	unc *Verrucomicrobia* bacterium	<0.01	0	0	<0.01	0	0	0	<0.01
		unc bacterium	unc bacterium	0.05	0.01	0.04	0.02	0.01	0	0	0.02
	Spartobacteria	*Chthoniobacterales*	01D2Z36	0.01	0.01	0.13	0.08	0	0.01	<0.01	0.03
			Chthoniobacteraceae	0.43	0.3	0.14	0.04	0.12	0.11	0.3	0.22
			DA101 soil group	0.38	0.96	4.27	4.08	3.22	2.25	2.01	2.28
			FukuN18 freshwater group	<0.01	0.01	0	0.03	0.01	0.01	0.01	0.01

Table S3.2. *continuation*

Phylum	Class	Order	Family	PG-ER	PG-RC	LI	AL	GR	HC-CT	HC-MA	TOTAL
Verrucomicrobia	*Spartobacteria*	*Chthoniobacterales*	LD29	0.01	<0.01	0	0	0.01	0.06	0.02	0.01
			Xiphinematobacteraceae	2.51	0.76	0.09	0.58	0.12	0.08	0.01	0.71
	UA11	unc *soil* bacterium	unc *soil* bacterium	0	0	0.01	0	0.02	0.03	0.05	0.01
	Verrucomicrobia Incertae Sedis	Unknown Order	Unknown Family	0.46	0.16	0.14	0.06	0.02	<0.01	0.03	0.15
	Verrucomicrobiae	*Verrucomicrobiales*	*Verrucomicrobiaceae*	0.04	0.03	0.03	0.01	0.01	0.01	0.06	0.03
WCHB1-60	unc bacterium	unc bacterium	unc bacterium	0.13	0.16	0.33	0.13	0.12	0.08	0.09	0.15
	unc soil bacterium	unc soil bacterium	unc soil bacterium	0	0.01	0.02	0.02	0.01	0.01	0.02	0.01
WD272	unc *Chitinophagaceae* bacterium	unc *Chitinophagaceae* bacterium	unc *Chitinophagaceae* bacterium	0.01	0	<0.01	0	0	0	0	<0.01
	unc *Firmicutes* bacterium	unc *Firmicutes* bacterium	unc *Firmicutes* bacterium	0.01	0.08	0	0	0	<0.01	0	0.01
	unc bacterium	unc bacterium	unc bacterium	1.05	1.72	0.1	0.11	0.1	0.34	0.09	0.56
				100	100	100	100	100	100	100	100

Chapter 4

Beneficial symbiotic communities

Bacteria could help the ectomycorrhizae establishment under climate variations

Bacteria could help the ectomycorrhizae establishment under climate variations

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*Francisca Reis contribution: data analysis, discussion, and manuscript writing

4.1. Abstract

Rhizosphere microbiome is one of the main sources of plant protection against drought. Beneficial symbiotic microorganisms, such as ectomycorrhizal (ECM) fungi and mycorrhiza helper bacteria (MHB), interact with each other in order to achieve the common goal of increasing or maintaining host plant fitness. This mutual aid benefits all three partners and comprises a natural system for drought acclimation in plants. Cork oak tolerance to drought scenarios is widely known, but adaptation to more extreme situations is not occurring as fast as needed for facing the eminent climate changes and protecting forest sustainability. In the present work, the relative abundance of ECM and MHB described to participate in ECM-MBH interactions were evaluated and cross-linked with cork oak forest communities under drought stressed gradient. While *Cenococcum* and *Russula* were the most abundant MHB-interacting ECM genera in cork oak stands, *Bacillus*, *Streptomyces*, and *Burkholderia* were the most conspicuous helper bacteria. Specific interactions among both communities revealed *Russula/Bacillus* and *Russula/Streptomyces* as the major potential interactions that could play a role in cork oak drought stress acclimation. The higher abundance of these microorganisms on drier climates could represent an advantage to cork oak forest resilience to upcoming climatic changes.

4.2. Introduction

The prediction of a global climate change for the next century is one of the main threats for forest ecosystems sustainability, particularly within Mediterranean basin region. Cork oak (*Quercus suber* L.) is one predominant broadleaf species of this region and gives an important economic input to the Iberian economy, where the most extensive cork oak forests are located (Reis *et al.,* 2017). As many other oak species, cork oaks are able to establish many symbiotic relationships with microbes, resulting in negative, neutral or beneficial interactions to the host (Reis *et al.,* 2017). Ectomycorrhizal fungi play an essential role for plant drought stress resistance, being strongly associated with forest tree sustainability, namely within temperate Fagaceae forests (Reis *et al.,* 2017). Among beneficial microorganisms, plant growth-promoting bacteria (PGPB) are well-known mycorrhizal fungi, stimulating their mycelia extension, increasing the colonization and contact of host roots and reducing environmental changes for attaining optimal conditions for mycelium growth (Figure 4.1; Frey-Klett *et al.,* 2007). One of the most studied ecological advantages of MHB is the bacteria able to stimulate plant growth, either directly by providing

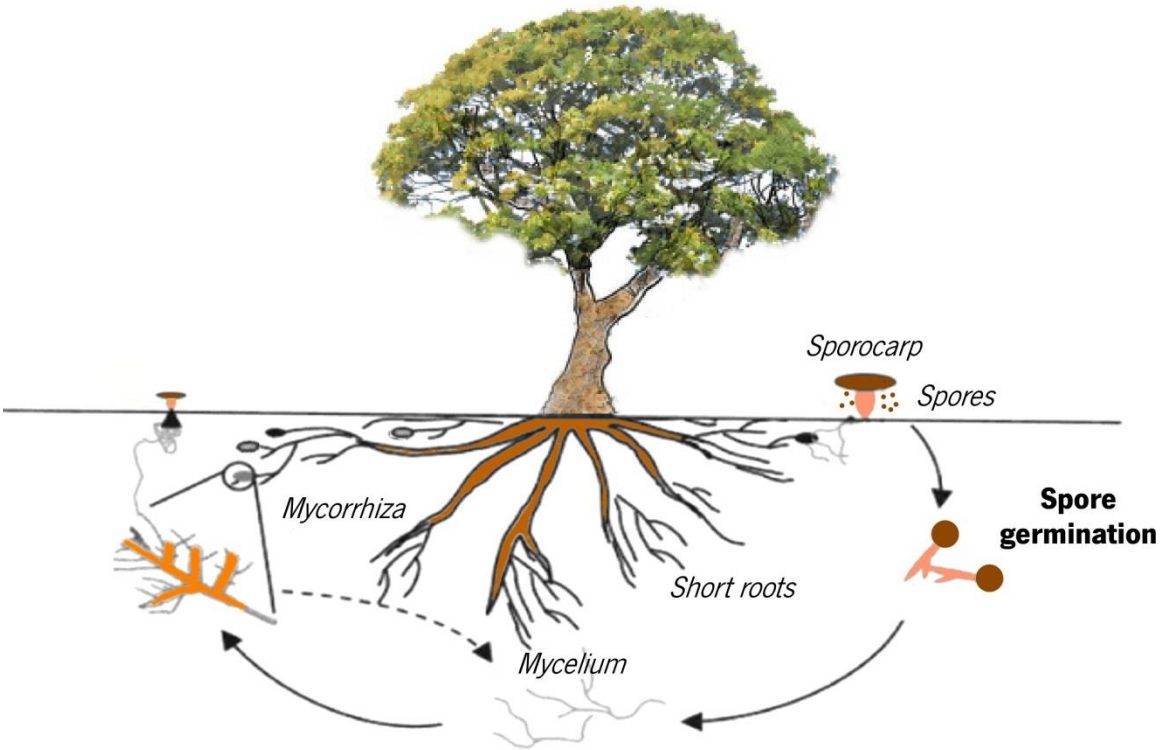

Figure 4.1. Potential mechanisms of helper bacteria during the process of ectomycorrhization, including ECM spore germination, pre-symbiotic fungal growth and ectomycorrhiza formation (adapted from Deveau and Labbé, 2016).

hormones or indirectly by promoting the acquisition of nutritional resources and preventing damage of phytopathogens (Siddiqui, 2005). Within PGPB group, mycorrhiza helper bacteria (MHB) specifically interact with improvement of drought tolerance they provide to host plant (Creus *et al.,* 2004; Forchetti *et al.,* 2007). In this work, the key ectomycorrhizal fungi identified by a previous root tips survey in cork oak stands under a drought gradient will be compared with bacterial communities identified in the same soil samples by high-throughput sequencing. A better understanding of MHB community and mycorrhizal root tips occurrence could provide clues for a better forest management in a drier climate.

4.3. Results and Discussion

4.3.1. General analysis of ECM fungi and MHB of cork oak stands

Cork oak ectomycorrhizal community has been correlated with several climatic parameters, such as precipitation and temperature, as well as land use practices (Azul *et al.,* 2010; Chapter 2). In a previous study, where 32 ECM fungal genera were identified as being associated with cork oak root tips, *Russula*, *Tomentella* and *Cenococcum* genera were found to

contribute the most for ECM community divergence in Portuguese cork oak stands (Chapter 2). In this study, forests were located on different Mediterranean climate regions, as evaluated by their precipitation and temperature levels, expressed by Emberger indexes (*Q*; Emberger, 1930): humid (PG-ER and PG-RC forests; $Q = 186.6$), sub-humid (LI and AL forests; $Q = 88.9$ and $Q = 102.7$), semi-arid (GR forest; $Q = 77.5$) and arid (HC-CT and HC-MA forests; $Q = 43.5$). From the identified genera, eleven included members potentially suitable of interacting with MHB (Table 4.1; Frey-Klett *et al., .* 2007; Kataoka *et al.,* 2009; Rigamonte *et al.,* 2010; Kurth *et al.,* 2013; Egamberdieva *et al.,* 2017) and were selected for further analysis. Although Frey-Klett *et al.* (2007) referred Basidiomycetes as the only ECM fungi capable of interacting with MHB, more recent studies revealed that Ascomycetes, such as *Cenococcum geophilum*, are also able to cooperate with *Bacillus subtilis* (Kataoka *et al.,* 2009). *Cenococcum* and *Russula* were the most conspicuous MHB-interacting genera in cork oak stands, whereas *Pisolithus* and *Piloderma* presented an erratic and low abundance.

Using the same cork oak stands/samples, a bacterial community survey was performed by metabarcoding using an Illumina platform, being found a large abundance of symbiotic bacteria (Chapter 3). From the 812 identified bacterial genera, which presented at least 5 reads in all analysed soil samples, nine have been described as acting as MHB, the most conspicuous of which were *Bacillus*, *Streptomyces* and *Burkholderia* (Table 4.1). The first two bacterial genera, as well as *Pseudomonas*, are the most studied MHB and promote plant growth by producing phytohormones (indole acetic acid – IAA; Egamberdieva *et al.,* 2017) and siderophores (Pii *et al.,* 2015). Within MHB, functional groups can be distinguished based on the bacterial ability to interact with ECM fungi, arbuscular mycorrhizal (AR) fungi, or both (Frey-Klett *et al.,* 2007). *Arthrobacter* is the only identified genus capable of only interacting with ECM species, whereas *Bradyrhizobium, Burkholderia, Rhizobium* and *Rhodococcus* have been described to be only associated to AR fungi (*Glomus* sp.; Frey-Klett *et al.,* 2007; Rigamonte *et al.,* 2010; Egamberdieva *et al.,* 2017). Altogether, the bacterial genera that are able to interact with ECM were 1.9-fold more abundant than AR specific genera. This result agrees with the study of cork oak forest soils, since Fagaceae only forms ectomycorrhizae (Reis *et al.,* 2017). Semi-arid and arid cork oak stands present the higher relative abundance of ECM genera described as capable of interact with MHB, 92.66% and 68.59% of total identified root tips, respectively (Table 4.1). Among bacteria genera, humid forests presented 0.5-fold less MHB than other sampled stands (Table 4.1).

Table 4.1. Relative abundance of ECM and MHB genera identified in cork oak stands and able to be in an ECM-MHB interaction. Cork oak forests were classified according to Emberger classification, humid (two forests, 5 soil samples/each), sub-humid (two forests, 5 soil samples/each), semi-arid (one forest, 5 soil samples), and arid (one forest, 5 soil samples). MHB genera that are able to only interact with ectomycorrhizal fungi [ECM], arbuscular fungi [AR], or able to interact with both [ECM/AR] are indicated. TOTAL comprises pooled ECM root tips or MHB identified reads. For detailed information consult Chapter 2 and 3.

Microbial taxa	Humid (%)	Sub-humid (%)	Semi-arid (%)	Arid (%)
ECM				
Amanita	1.08	0.35	3.67	0.00
Boletus	2.91	1.51	0.00	0.00
Cantharellus	10.57	0.00	0.00	0.00
Cenococcum	16.32	2.68	26.61	17.47
Hebeloma	2.16	0.00	0.00	0.00
Laccaria	0.58	0.00	0.00	3.16
Lactarius	14.24	3.84	0.00	1.12
Piloderma	0.00	0.29	0.00	0.00
Pisolithus	0.00	0.00	0.00	0.19
Russula	10.57	37.04	13.76	45.35
Tuber	0.00	0.00	48.62	1.30
TOTAL	**58.45**	**45.72**	**92.66**	**68.59**
MHB				
Arthrobacter [ECM]	0.25	0.04	0.34	0.14
Bacillus [ECM/AR]	0.05	0.44	1.66	2.00
Bradyrhizobium [AR]	0.05	0.05	0.03	0.07
Burkholderia [AR]	1.23	1.91	0.42	0.27
Paenibacillus[ECM/AR]	0.01	0.08	0.08	0.05
Pseudomonas [ECM/AR]	0.09	0.24	0.04	0.08
Rhizobium [AR]	0.02	0.39	0.19	0.12
Rhodococcus [AR]	0.02	0.01	0.03	0.02
Streptomyces [ECM/AR]	0.25	0.99	1.44	0.84
TOTAL	**1.96**	**4.17**	**4.22**	**3.59**

4.3.2. Interaction between ECM and MHB genera

Plants are not only colonized by fungi but also by symbiotic bacteria, such as mycorrhizal helpers that confer beneficial effects to their hosts (Compant *et al.,* 2010). These interactions are very specific and MHB could inhibit the plant symbiosis with certain fungi to enhance the mycorrhizae formation with other fungal species (reviewed by Frey-Klett *et al.,* 2007). Indeed, distinct ECM fungal isolates respond differentially to the same MHB (Duponnois and Garbaye, 1991), even when using different strains of the same ECM fungal species (Dunstan *et al.,* 1998). For example, a *Streptomyces* sp. promotes mycelial growth of *Amanita muscaria* and *Suillus*

bovinus, while inhibiting the *Hebeloma cylindrosporum* growth, due to the production of an antibiotic to which *A. muscaria* is tolerant but *H. cylindrosporum* not (Keller *et al.,* 2006). This called "fungal isolate specificity" may reflect the environmental and genetic co-evolution occurring in the same geographical location (reviewed by Frey-Klett *et al.,* 2007). In the present work, different ECM/MHB genera were identified in different locations, which have been selected by their climatic parameters. Humid and sub-humid forests were dominated by *Burkholderia* bacteria, whereas driest forests (semi-arid and arid) were richer in *Bacillus* species (Table 4.1). Also *Streptomyces* was present in drier forests (sub-humid, semi-arid and arid). On the other hand, ECM fungal genera, such as *Boletus*, *Cantharellus*, *Hebeloma* and *Lactarius* were mainly identified in wettest forests, being *Pisolithus*, *Russula* and *Tuber* mainly found in driest cork oak stands (Table 4.1).

As far as we know, there are no studies on cork oak regarding MHB interaction with mycorrhizal fungi. To better understand the possible interaction between MHB and ECM genera identified in this work, correlations between both taxonomic groups were performed (Table 4.2). Two significant and positive correlations are particularly singled out, due to the high abundance of corresponding interacting partners and statistical significance (*Russula*/ *Bacillus*, at $p<0.01$; *Russula*/ *Streptomyces*, at $p<0.05$). *Bacillus* has been described to increase the ectomycorrhizal infection of *Laccaria* and *Suillus* spp., in *Pseudostuga menziesii*, *Eucalyptus diversicolor* and *Pinus sylvestries* (reviewed by Frey-Klett *et al.,* 2007). The abundance of *Bacillus* genera in driest cork oak forests could thus enhance the ectomycorrhization with *Russula* in such environmental conditions. However, different *Bacillus* species were found to have opposite ecological behaviours, playing either positive or negative roles, as described by Marulanda *et al.,* (2006, 2009). In addition, although *Bacillus* spp. were able to act as MHB in citric orchards (Freitas and Vildoso, 2004), in *Pinus pinea* host there was not a synergistic effect with *Pisolithus* species for enhancing mycorrhizal infection (Probanza *et al.,* 2001). Also *Streptomyces* have been described to promote fungal extension and mycorrhiza formation of *A. muscaria* on Norway spruce (Maier *et al.,* 2004), and significantly increase the mycorrhizal colonization of *Sorghum* roots (Abdel-Fattah and Mohamedin, 2000). While waiting for experimental support, the hypothesis of *Bacillus* or *Streptomyces* role on cork oak ectomycorrhization in drought environments remains speculative.

Humid cork oak forests present a diversified ECM distribution (Chapter 2). The most abundant MHB genus in these regions is *Burkholderia*, which has been described to increase 1.9 - 2.4-fold the ectomycorrhizal formation in the *Pinus sylvestris-Lactarius rufus* system

(Poole *et al.,* 2001). Interestingly, *Lactarius* is also highly abundant on cork oak forests where *Burkholderia* is dominant (humid forests), as revealed by the positive (but non-significant) correlation (Table 4.2). Although the effect of *Burkholderia* on cork oak ectomycorrhization still needs experimental support, the beneficial effect of this MHB in plants under stress has been reported. Potato and Cucurbitaceae plants take advantage from the infection with a *Burkholderia* sp. under drought stress conditions (Nowak *et al.,* 1995). Cadmium stressed plants of *Solanum lycopersicum* were also protected by a *Burkholderia* sp. (Dourado *et al.,* 2013).

Table 4.2. *Pearson* correlations between ECM and MHB genera relative abundance in all forests/samples (a total of seven forests x five samples; n=35). *Pearson* correlations were performed with Excel tools. Statistical significant correlations are highlighted in bold, where asterisks mean statistical significance at $p<0.05$ (*) or at $p<0.01$ (**).

	Arthrobacter	*Bacillus*	*Bradyrhizobium*	*Burkholderia*	*Paenibacillus*	*Pseudomonas*	*Rhizobium*	*Rhodococcus*	*Streptomyces*
Amanita	0,03	-0,48	-0,92	-0,70	-0,04	-1,02	-0,25	3,49	-0,12
Boletus	-0,84	-1,00	-2,93	2,73	-0,99	-0,01	-1,02	0,18	-1,94
Cantharellus	-1,13	-1,19	-4,08	0,27	-4,69	-1,69	-2,73	-0,10	-2,80
Cenococcum	3,41	-0,40	0,28	-2,08	0,10	-2,37	-3,97	3,42	-1,68
Hebeloma	0,09	-0,49	**5,63***	-0,12	-1,69	-0,15	-0,41	-0,63	-0,78
Laccaria	-0,30	0,07	**4,50***	-0,35	0,00	-0,40	-0,13	0,00	-0,47
Lactarius	-1,85	-2,03	-0,82	4,00	0,08	0,02	1,71	-0,93	-1,48
Piloderma	-0,43	-0,32	0,10	3,06	**4,26***	0,18	**8,46****	-0,63	0,00
Pisolithus	-0,43	-0,06	-0,59	-0,68	1,31	-0,51	-0,79	-0,63	0,08
Russula	-0,24	**7,85****	0,24	-0,48	0,03	0,04	1,62	-0,01	**6,35***
Tuber	0,43	2,19	-2,19	-0,49	1,89	-0,97	-0,20	0,83	0,00

4.3.3. MHB abundance for a higher drought stress resilience

Climatic changes, such as increasing of temperature and CO_2 concentrations, as well as the reduction of water availability, will be the most important challenges for cork oak forests in a nearby future (reviewed by Reis *et al.,* 2017). In this work, we found that MHB community is more affected by climate variables than ECM community (Table 4.3). In general, precipitation and temperature affect each ECM/MHB genus abundance in opposite ways. Precipitation was previously described to be more determinant for microbial abundance than temperature (Chapter 2 and 3). Although most ECM/MHB genera show a similar trend, specific MHB genera (*Burkholderia* and *Pseudomonas*) are more affected by temperatures than precipitation. Within the eleven ECM

genera, *Cantharellus* and *Russula* are the most affected by climatic parameters, where precipitation was clearly more important for their distribution than temperature, although in different ways. Indeed, *Russula* distribution is highly dependent (at $p<0.001$) on the *Q* parameter, which differs from the few descriptions of *Russula* spp. under abiotic stress conditions intolerance of some species to drought stress (Smith and Read, 2008).

Most MHB genera are negatively correlated with precipitation and *Q*, except *Arthrobacter*, *Burkholderia* and *Rhodococcus* that present positive but non-significant correlations (Table 4.3).

Table 4.3. *Pearson* correlations between ECM/MHB relative abundance and climate parameters [average precipitation and temperatures from the past 30 years (1986-2016; aver.), from the wettest/hottest month (Max) and from the driest/coldest month (min) of the sampling year], as well with indexes of Emberger (*Q*). TOTAL comprises pooled ECM root tips or MHB identified reads. *Pearson* correlations were performed with Excel tools. Statistical significant correlations are highlighted in bold, where asterisks mean statistical significance at p<0.05 (*), at p<0.01 (**) or at p<0.001 (***).

Microbial taxa	Precipitation			Temperature			*Q*
	aver.	max	min	aver.	max	min	
ECM							
Amanita	0.57	0.67	0.08	-0.20	-0.58	0.38	0.50
Boletus	3.53	3.45	3.49	-3.61	-2.69	-0.18	3.37
Cantharellus	**7.21***	**7.26***	**5.49***	-6.49	-3.58	0.01	**6.41***
Cenococcum	0.83	1.04	0.01	-0.25	0.00	2.79	0.41
Hebeloma	2.50	2.52	1.96	-2.28	-1.32	0.00	2.25
Laccaria	-0.16	-0.14	-0.23	0.13	0.80	0.18	-0.38
Lactarius	0.80	0.71	0.32	-0.27	-0.20	1.23	2.45
Piloderma	-0.39	-0.45	-0.53	0.77	0.30	0.63	0.00
Pisolithus	-0.78	-0.73	-0.83	0.67	1.63	0.17	-1.18
Russula	**-8.76****	**-8.77****	-3.71	**4.52***	4.02	-2.61	**-13.93*****
Tuber	-0.51	-0.36	-1.70	1.17	0.15	0.96	-0.74
TOTAL	*-0.40*	*-0.23*	*-1.41*	*0.60*	*1.17*	*0.99*	*-1.78*
MHB							
Arthrobacter	2.28	2.55	1.05	-1.75	-1.10	0.17	1.24
Bacillus	**-17.42*****	**-9.71****	**-12.26****	**10.10****	**12.80****	0.78	**-17.42*****
Bradyrhizobium	-0.02	-0.03	0.00	0.00	0.24	0.00	-0.06
Burkholderia	1.21	0.87	3.81	-1.93	**-5.98***	**-5.02***	0.00
Paenibacillus	**-12.24****	**-12.54****	**-9.85****	**12.83****	3.78	0.01	**-7.79****
Pseudomonas	-0.45	-0.71	0.10	0.08	-0.41	**-4.55***	0.00
Rhizobium	**-9.86****	**-11.47****	-3.00	6.90·	0.41	**-4.37***	**-4.18***
Rhodococcus	0.85	0.94	0.80	-1.06	-0.95	-0.34	0.24
Streptomyces	**-16.03*****	**-15.68*****	**-12.88****	**15.38*****	**4.24***	-0.05	**-14.47*****
TOTAL	***-7.41****	***-7.58*****	***-4.25****	***5.71****	*1.76*	*-0.87*	***-6.85****

The higher abundance of *Burkholderia* in humid/sub-humid forests could be indeed more related with the preference for low temperatures occurring in those forests than with higher precipitation levels. *Bacillus*, *Paenibacillus* and *Streptomyces* were the most negatively affected genera by precipitation levels. This result agrees with the higher abundance of *Bacillus* and *Streptomyces* in drought stressed forests (33-fold and 6-fold in semi-arid, 40-fold and 3-fold in arid forests, in relation with humid forests, respectively). Both genera are described as increasing first and second order root mycorrhization rate, respectively (Bending *et al.,* 2002; Schrey *et al.,* 2005). *Bacillus* is described as playing an essential role during ectomycorrhizal infection (1.8 – 3.9 fold) of *Eucalyptus diversicolor* (Dunstan *et al.,* 1998), and different *Bacillus* spp. have been implicated in the hormonal production of IAA, cytokinines, gibberellins and abscisic acid in a wide range of host plants colonized by AM fungi (reviewed by Egamberdieva *et al.,* 2017). Beyond antibiotic production, *Streptomyces* is also able to produce a fungal growth-promoting substance, auxofuran, which enhanced fungal growth (Riedlinger *et al.,* 2006). *Streptomyces* sp. has been associated to the osmotic pressure increase of cells, accelerated callose accumulation, and lignification of sieve cell walls, which together provide a positive effect on drought tolerance (Hasegawa *et al.,* 2005).

4.4. Conclusions

Cork oak forest sustainability is promoted by diverse interactions occurring between plant host, microbial community and environmental conditions (Reis *et al.,* 2017). The climatic changes that are emerging are enforcing the scientific community to take a closer look on the different partners of forest ecosystems. Mycorrhizal fungi and bacteria that help them to infect plants and form mycorrhizae (MHB) have been used in agricultural systems to improve crop production and increase tolerance to both biotic and abiotic stresses (Compant *et al.,* 2010). The ability of forming ectomycorrhizae is a natural strategy for forest trees to cope with environmental changes. Keeping this strategy in mind, the information obtained from ECM and bacterial communities residing on cork oak forest soils was combined and potential microbial partners of ECM-MHB interaction were further studied. Although cork oak ECM community has been reported to be positively affected by precipitation levels (Chapter 2), only *Cantharellus* and *Russula* revealed to be affected by precipitation among all the identified ECM genera able to interact with MHB. In contrast, MHB community was strongly affected by different climate variables (precipitation and temperature), each genera exhibiting a preference for a cork oak forest. The ectomycorrhizal *Russula* and helper bacteria *Bacillus* and *Streptomyces* were highly affected by climatic drivers, presenting a higher

abundance on drier climates. In addition, two interactions, *Russula/Bacillus* and *Russula/Streptomyces* were also described to play potential roles on cork oak drought stress acclimation. For these reasons, we speculate that these microorganisms/interactions could have an important role for cork oak forest sustainability in drought environments.

Few works have been conducted in order to better understand interactions between microbial communities within forests soils. Future research is need in this field to help preventing drought stress consequences on the forest, particularly in Mediterranean ecosystems. We hope that this work will help to create new research lines for improving forests sustainability by using MHB and ECM fungi.

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Chapter 5

Concluding remarks and future perpectives

5.1. Concluding remarks and future perspectives

Forests ecosystems sustainability is in danger due imminent climatic changes, such as the rapidly increasing of temperature and decreasing of precipitation rates. In particular, Mediterranean forests represent one of the most threatened ecosystems and have been classified as a major biodiversity hotspot since they include many endemic species. For this reason, new effective approaches to help forest resilience and tolerance to drought stress are urgent.

Microbial communities are a main element for sustaining forests ecosystems, due to the many beneficial symbiotic partners that help plant adaptation and resistance to both biotic and abiotic stresses. Indeed, mycorrhizal and PGPB communities are the most common and well-known helpers for promoting plant adaptation to climates. A deeper knowledge on forest microbial communities can give a new input about their role for drought management and would provide comprehensive overviews of the microbiome diversity at large scale. As plant-microbe interactions are the major natural source of environmental adaptation, the study of such interactions need to be more explored. Among plant-interacting microbes, ECMF communities have a significant impact for forest ecosystems. ECMF contributes for the host defense against phytopathogens, as well as for the nutrient and water uptake in exchange of carbohydrates from photosynthesis. Therefore, well-nourished plants can provide more and better nutrients for ECM fungal partners. Improved plant tolerance responses are particularly important when plant hosts are under stress, such as during long term drought that frequently occurs in the regions where cork oak is widely distributed. In **Chapter 2**, the ECMF community of cork oak forests in regions presenting a water availability gradient, from humid to arid climates, is described. An overall analysis of all sampled forest soils allowed to infer that *Russula*, *Tomentella* and *Cenoccoccum* were responsible for the most abundant ECM root tips and were determinant for discriminating all sampling sites. While ECMF abundance was influenced by the drought gradient studied, richness was not. The community structure, mainly richness, was directly affected by precipitation but inversely by temperature. To the best of our knowledge, this was the most complete assessment of cork oak ECMF communities regarding climatic variables influence.

Together with ECMF, bacterial communities are one important element for the forest sustainability triangle – plant/soil/microbe – and comprise the most prevalent microbes in forest soils. In contrast with ECMF communities, cork oak bacterial community still remains poorly studied. The global picture of the bacterial community associated with cork oak soils is described in **Chapter 3**. Using the same soil samples as Chapter 2, cork oak forests were highly enriched in

Proteobacteria, *Actinobacteria* and *Acidobacteria*. As core bacterial microbiome, *Acidothermus*, *Afipia* and *Sphingomonas* were the most abundant genera identified. Driest forests presented significantly more bacterial families than wettest forests, being bacterial communities clearly discriminated with climate. The relation of bacterial communities with climate variables and bioclimates revealed that bacterial community composition seems to be affected by climatic parameters as found for ECMF community. Interestingly, an opposite trend was observed for both microbial communities studied. Precipitation promoted and temperature reduced the ECMF community abundance, whereas bacterial community presented exactly the contrasting behavior. In contrast with our findings, bacterial community was found to be widely more influenced by season drivers in temperate oak forests than fungi (Voříšková *et al.*, 2014; López-Mendéjar *et al.*, 2015). In cork oak soils, the most affected bacterial taxa by climate variables were *Chloroflexi, Firmicutes* and *Proteobacteria*, in particular *Gammaproteobacteria* and *Deltaproteobacteria*. The identified taxa could play an important role in the acclimation of cork oak to an eventual climate changing scenario.

Plants are known to interact with many symbiotic microbial species. Ectomycorrhizal fungi (ECM) and mycorrhiza helper bacteria (MHB) interact with each other, in order to achieve the common goal of increasing or maintaining host plant fitness. Taking into consideration the information obtained on previous chapters, **Chapter 4** evaluated the relation of MHB on ectomycorrhizal establishment on cork oak forests under a water availability gradient. Among both communities analyzed, MHB revealed to be more sensitive to drought stress than ECMF community. Humid forests were richer in *Burkholderia* bacterial genus, whereas *Bacillus* was predominant in arid forests. Moreover, specific interactions between *Russula/Bacillus* and *Russula/Streptomyces* were recognised as major putative interactions for cork oak drought stress acclimation. These microbial combinations could represent an advantage for cork oak forest resilience to climatic changes, but other beneficial bacterial taxa were also identified in the present study, such as nitrogen fixation species (*Rhizobium* spp. and *Bradyrhizobium* spp.). On the other hand, PGPB other than MHB were also very common among soil samples and could help cork oak to increase growth rates as well as its plant defences.

The main focus of this thesis was the assessment of microbial communities associated to cork oak forest. For covering regions with different climates, in which the most separated cork oak forests were 430 km apart, only a single time point was used for collecting soils. In order to have a better picture of climate influence on microbial community's dynamics, a regular biomonitorization

of sampled cork oak forests during all year's seasons would be interesting to perform, taking into consideration the climatic data from previous days of sampling. Fungal community evaluation by high throughput sequencing could also bring an important input about other fungal trophic levels and non-ECM fungal cork oak community. Since microbial assemblages are quite important for forest ecology, *in vitro* and *in vivo* studies for studying microbial interactions with cork oak plants should be performed. Fungal and bacterial interactions of most susceptible or resilient organisms with cork oak plants could be conducted for analysing the direct effects of co-habitation, including fungal/bacterial growth and ability to form (or promote) ectomycorrhization. On the other hand, *in vivo* co-inoculation assays with suggested microbial interactors (*Russula/Bacillus* and *Russula/Streptomyces*) can be performed on non-stressed, short-term and long-term stressed cork oak plantlets for studying the effect of these microorganisms on plant tolerance to drought. Such assays should be firstly performed in a greenhouse but, if justified, could be repeated on experimental field stands. These tasks should be followed by physiological evaluation of plants (root osmolite contents and photosynthesis), as well as plant development features (biometric data) and mycorrhizae formation (Brzostek *et al.*, 2015).

Environmental and genetic factors are nown to contribute for cork oak adaptation to drought. To be adapted to long drought seasons during summer, cork oak developed several physiological mechanisms to tolerate drought stress and growth under adverse climatic conditions. However, due to the rapid environmental changes cork oak forests are now facing, tree plasticity and adaptation to drought are slower than the increase of stress severity (Nuche *et al.*, 2014). To better tolerate such environmental challenges, plants developed sophisticated molecular mechanisms involving gene regulation, changes in metabolic processes and organ morphology adaptation.

New technology is revolutionizing diverse research fields, including RNA sequencing and expression levels quantification, which have been increasing the amount of generated information. Several Portuguese institutions have recently associated into a national consortium (COEC – Cork oak ESTs Consortium) for *de novo* sequencing the *Q. suber* transcriptome under different physiological conditions and plant tissues (Pereira-Leal *et al.*, 2014). In this project, the University of Minho team contributed by studying the transcriptome of cork oak drought stress responses (Magalhães *et al.,* 2016), as well as the ectomycorrhizal formation with *P. tinctorius* (Sebastiana *et al.*, 2014). Related with this previous work, much research was performed within the scope of this PhD thesis that was not integrated in this thesis. For the genetic assessment of cork oak drought

pathway, two-months-old *Q. suber* plantlets were subjected for one month to specific five watering regimens for imposition of moderate to severe drought. Morphological observations and physiological assays revealed that plants were indeed suffering from drought imposition gradient and transcriptome was validated by qPCR analysis of ten drought responsive genes (Magalhães *et al.,* 2016). Specific genes that could be involved in both processes were selected by cross-linking both transcriptomes and will be further studied in the future, since it is well known the relation between both genetic pathways in other species (Marjanović *et al.*, 2005; Dietz *et al.*, 2011; Xu *et al.*, 2015).

Within the scope of a funded project (SuberStress, PTDC/AGR-AAM/099556/2008), and based on cork oak transcriptomic data in different stressful conditions (biotic – infection by *Phytophthora cinnamomi* and abiotic – drought, heat, cold and salt stress), several cork oak genes (seven) were selected for further analysis on drought stressed cork oaks. Gene expression patterns were followed by RT-PCR and in those situations where a differential expression was detected (four genes) further validation was achieved by using qPCR. Genes displaying a highly differential expression under drought stress conditions, mainly transcription factors (but also unknown genes) whose function was not related yet with drought responses, were selected for further functional characterization using *Arabidopsis thaliana* as model (two genes). After selecting *Arabidopsis* homologue genes of cork oak, insertional *Arabidopsis* mutants and overexpression lines of *Q. suber* genes were obtained. Morphological and physiological assays in standard and drought imposition conditions are being performed in these lines to better understand the role of these *Q. suber* genes on drought tolerance.

The results presented in this thesis provide a deeper understanding of microbial communities in cork oak forests under different landscapes and climatic scenarios and fulfill the major aim of this thesis. Several research suggestions came out that could be useful for helping to mitigate drought consequences on cork oak forests in the future. Moreover, the undergoing work will increase the understanding of drought plant responses. Therefore, the results presented in this thesis do not intent to be the end of a story but the beginning of many more.

5.2. References

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“Quem aos vinte não é e aos trinta não tem,

aos quarenta não será ninguém”

Amílcar Reis