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Diogo Figueiredo Pinho

Microbioma de *Quercus*: Decifrando as comunidades microbianas da rizosfera de sobreiro e carvalho

Quercus Microbiome: Deciphering the rhizosphere microbial communities of cork oak and pedunculate oak

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, especialização em Microbiologia, realizada sob a orientação científica da Doutora Maria Conceição Venâncio Egas, Diretora da Unidade de Sequenciação Avançada do Biocant, do Doutor Newton Carlos Marcial Gomes, Investigador Principal do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro, e do Doutor Daniel Francis Richard Cleary, Investigador Principal do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.

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Aos avós e pais

o júri

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palavras-chave

Declínio Agudo de Carvalho; Declínio Crónico do Sobreiro; Declínio da floresta; Microbioma; Propriedades do solo; Rizosfera; Saúde da árvore

resumo

As doenças-declínio da floresta são processos complexos, prolongados e generalizados, impulsionados por fatores bióticos e abióticos múltiplos, sequenciais e cumulativos. Embora as informações sobre as interações hospedeiro-microbioma-ambiente em sistemas agrícolas estejam a surgir rapidamente, estudos semelhantes sobre a saúde das árvores ainda estão na sua infância. Cada vez mais evidências têm destacado o papel dos microbiomas das árvores na saúde e resiliência do hospedeiro, particularmente na rizosfera. Usamos o declínio crónico do sobreiro (COD) e o declínio agudo do carvalho (AOD) como sistemas modelo para entender se as propriedades físico-químicas e o microbioma da rizosfera estão ligados à saúde das árvores. A rizosfera de árvores assintomáticas e afetadas por COD/AOD foram amostradas em locais e povoamentos que abrangeram diferentes estágios de declínio. As propriedades físico-químicas e microbioma da rizosfera foram caracterizados para estudar as relações entre essas propriedades, comunidades microbianas e o COD/AOD.

Os resultados revelaram que as propriedades físico-químicas da rizosfera e a composição do seu microbioma diferiram significativamente entre os locais e povoamentos tanto para o COD quanto para o AOD, mas apenas ao nível de saúde da árvore para o AOD. Ambas as espécies de *Quercus* despoletaram um processo de acidificação da rizosfera, o que aumentou os níveis de C e N e o rácio C:N. Isto sugere um mecanismo responsivo ao stress que leva ao aumento da absorção de nutrientes e à manutenção da resiliência sob pressão causada por fatores de declínio. Na verdade, o pH da rizosfera foi o principal condutor da composição microbiana. No sobreiro, vários microrganismos, incluindo membros dos géneros *Bacillus*, *Bryobacter*, *Cladophialophora* e *Phallus*, foram mais abundantes nos povoamentos saudáveis e parte do microbioma núcleo, o que os torna potenciais membros chave para a saúde e resiliência do sobreiro. No carvalho, as condições de solo menos extremas e uma elevada presença de microrganismos benéficos para o hospedeiro foram observados nas árvores saudáveis. Por outro lado, organismos patogénicos e oportunistas foram mais abundantes nos locais com níveis mais elevados de COD e AOD.

Além de informações básicas sobre as propriedades físico-químicas da rizosfera e microbioma em espécies de *Quercus*, esta tese reúne evidências da associação entre as condições de saúde do hospedeiro, propriedades e microbioma da rizosfera e liga os sintomas visíveis de declínio das árvores ao ambiente subterrâneo. Estas descobertas abrem caminhos estimulantes e exigem mais investigação para esclarecer as relações entre os microbiomas e *Quercus* ou outras espécies arbórias que sofrem eventos de declínio, assim como compreender os mecanismos subjacentes que melhoram a saúde, resistência e resiliência das árvores no contexto das alterações climáticas.

keywords

Acute Oak Decline; Chronic Cork oak Decline; Forest decline; Microbiome; Rhizosphere; Soil properties; Tree health

abstract

Forest decline-diseases are widespread, prolonged, complex processes driven by multiple, sequential, and cumulative biotic and abiotic factors. Although information about host-microbiome-environment interactions in agricultural systems is emerging rapidly, similar studies on tree health are still in their infancy. Increasing evidence has highlighted the role of tree microbiomes in host health and resilience, particularly in the rhizosphere. We used Chronic Cork oak Decline (COD) and Acute Oak Decline (AOD) as model systems to understand whether the rhizosphere physicochemical properties and microbiome are linked to tree health. We sampled the rhizosphere of asymptomatic and COD/AOD-affected trees across sites and stands that spanned different stages of decline. The rhizosphere physicochemical properties and microbiomes were characterised to study the relationships across these properties, microbial communities, and COD/AOD.

The results revealed that rhizosphere physicochemical properties and microbiome composition differed significantly across sites and stands for both COD and AOD, but only at tree health level for AOD. Both *Quercus* species undertook an acidification process in their rhizospheres, which boosted C and N levels and C:N ratio. This suggests a responsive stress mechanism for nutrient uptake enhancing and resilience maintenance under pressure caused by declining triggers. Indeed, rhizosphere pH was the main driver of microbial composition. In cork oak, several microorganisms, including members in the genera *Bacillus*, *Bryobacter*, *Cladophialophora*, and *Phallus*, were more abundant in the healthy stands and part of the core microbiome, which make them potential key members for cork oak health and resilience. In pedunculate oak, less extreme soil conditions and a high presence of host-beneficial microbiota were observed in the healthy trees. On the other hand, pathogenic and opportunistic organisms were more abundant in the sites experiencing higher levels of COD and AOD.

In addition to baseline information on the rhizosphere physicochemical properties and microbiome in *Quercus* species, this thesis gathers evidence of associations among host health conditions, rhizosphere properties, and microbiome and links aboveground tree decline symptoms to the belowground environment. These findings open exciting paths and call for further research to clarify the relationships between microbiomes and oaks or other tree species suffering decline-disease events and understand the underlying mechanisms that enhance tree health, resistance, and resilience in global change.

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ABBREVIATIONS

% – Percentage
˚C – Degree celsius
16S rDNA – 16S ribosomal RNA gene
ANCOM – Analysis of the Composition of Microbiomes
ANOSIM – Analysis of Similarities
ANOVA – Analysis of Variance
AOD – Acute Oak Decline
APFC – Association of Forestry Producers of Coruche
BC – Before Christ
bp – Base pair
C – Carbon
C:N ratio – Carbon-to-nitrogen ratio
CA – Correspondence Analysis
CCA – Canonical Correspondence Analysis
cm – Centimeter
COD – Chronic Oak Decline or Chronic Cork Oak Decline
CSS – Cumulative Sum Scaling
DBH – Diameter-at-Breast Height
dbRDA – Distance-based Redundancy Analysis
DNA – Deoxyribonucleic acid
DRA – DDJ's Sequence Read Archives
EcM – Ectomycorrhizal fungi
ENA – EBI's European Nucleotide Archive
FDR – False Discovery Rate
fitZIG – Zero-inflated Gaussian
g – Gram
H2O – Water
HSD – Honestly Significant Difference
HTS – High–Throughput Sequencing
INE – National Statistics Institute
INSDC – International Nucleotide Sequence Database Collaboration
ITS – Internal Transcribed Spacer
kg – Kilogram
log – Logarithm
logUQ – Log Upper Quartile
m – Meter
mg – Milligram
min – Minute
mm – Millimetre
N – Nitrogen
ng – Nanogram

NGS – Next Generation Sequencing
nM – Nanomolar
NMDS – Non–metric Multidimensional Scaling
OTU – Operational Taxonomic Unit
PCO – Principal Coordinates Analysis
PCR – Polymerase Chain Reaction
PERMANOVA – Permutational Analysis of Variance
pH – Potential of Hydrogen
PRIMER-E – Plymouth Routines In Multivariate Ecological Research
Q – Quality Score
QIIME – Quantitative Insights Into Microbial Ecology
qPCR – Quantitative Polymerase Chain Reaction
RDP – Ribosomal Database Project
RNA – Ribonucleic acid
s – Second
SOD – Sudden Oak Death
SRA – NCBI's Sequence Read Archive
TMM – trimmed Mean by M-Values
UK – United Kingdom
USA – United States of America
VS – Variance Stabilization
w/v – Weight per Volume
α – Alpha
μL – Microliter
μM – Micromolar

CHAPTER 1

GENERAL INTRODUCTION

General Introduction

Quercus suber and *Quercus robur*

Oak refers to trees of the genus *Quercus*, taxonomically located within the family *Fagaceae*, consisting of around 600 species worldwide (Zhang *et al.* 2015). Oaks are woody species widely distributed across the northern hemisphere, ranging from semi-arid Mediterranean shrublands to cool temperate and transitional boreal forests in Europe (Gil-Pelegrín *et al.* 2017). *Quercus suber* L (cork oak) and *Quercus robur* L (pedunculate oak) are foundation species and central components of the Southern European and Northern European national identities.

With a longstanding place in history and culture, oak trees were considered sacred trees by many different cultures. For example, in Greek mythology, it was believed that a nature spirit or nymph lived along with an oak tree in the form of a beautiful woman called Dryad (Britannica 2009). Moreover, oak trees were also associated with the god of thunder, the Greek Zeus, the Slavic Perun, the Germanic Thor, the Celtic Taranis, and the Lithuanian Pērkons (Britannica 2018). Indeed, it is believed that oaks are particularly susceptible to being struck by lightning. Even Shakespeare made a reference to "oak-cleaving thunderbolts" in King Lear. The cork oak, in particular, was described in detail by Theophrastus (372-285 BC) and Virgil (in *Aeneid* VII), where the head covering of the soldiers of ancient Latium was made by *stripped bark of the cork tree* (Aronson *et al.* 2009). Even more, in the best-known work of Spanish literature written by Miguel de Cervantes, Don Quijote de la Mancha, *Q. suber* is the second most frequently mentioned species, where Don Quixote often used these large trees as shelter (Pardo-de-Santayana *et al.* 2006; Aronson *et al.* 2009). The emblematic cultural and historical importance of oaks is likely related to their long lifespan with extraordinarily strong symbolisms of longevity, strength, stability, endurance, fertility, power, justice, and honesty (Plomion *et al.* 2018; Leroy *et al.* 2020).

In addition to their cultural and symbolic significance, both cork oak and pedunculate oak provide essential ecosystem services, including wood and non-wood products, climate regulation and mitigation, soil protection and formation,

nutrient cycling, biodiversity protection, water regulation and supply, cultural heritage and recreation (EEA 2016). These oak species provide a rich habitat for a diverse range of indigenous flora and fauna (Bugalho *et al.* 2011; Mitchell *et al.* 2019).

Cork oak is a typical western Mediterranean tree species (Figure 1.1A), occurring in areas below 800 meters in altitude, with average annual precipitation above 600 millimetres and mean temperature around 15 °C (Blanco *et al.* 1997). The cork oak forests, called *montado* in Portugal and *dehesa* in Spain, are mainly located in Portugal, which has a third of the total world cork oak area. Together with Spain, Morocco, and Algeria, they account for approximately 90% of over 2.2 million hectares worldwide (APCOR 2018). Cork oak is better able to thrive on moderately to slightly acidic soils, within a pH range from 4.7 to 6.5 on granite, schist, or sandy substrates (Serrasolses *et al.* 2009). Reaching up to 20 meters tall and living for centuries in suitable soils and rainfall (Pausas *et al.* 2009), the cork oak has a remarkable feature: it produces a corky bark that consists of continuous layers of suberized cells (Pereira 1988). Portugal is responsible for 50% of the world's cork production (100,000 tonnes), and therefore it is the largest producer of this unique, natural, renewable, sustainable outer bark (Silva *et al.* 2005; APCOR 2018). According to foreign trade data from the National Statistics Institute (INE), the Portuguese cork exports have been increasing over the last years (Figure 1.2), reaching 197 thousand tonnes of cork in 2017, which represented 986.3 million euros in terms of export value (APCOR 2018). This translated to approximately 2 % of Portuguese export goods and 1.2 % of total Portuguese exports (APCOR 2018). This highlights the extremely high value and importance of cork oak and the cork industry to the Portuguese economy and society.

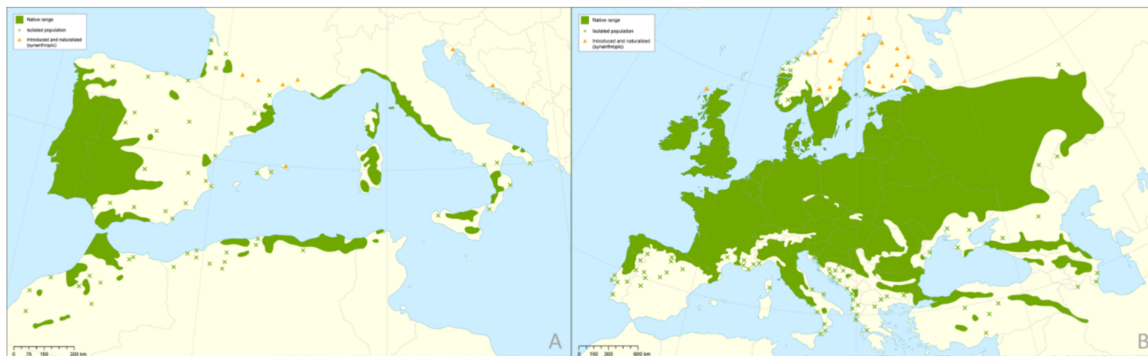

Figure 1.1 Cork oak (A) and pedunculate oak (B) map distribution compiled by members of the EUFORGEN Network. Source: Caudullo *et al.* (2017).

Figure 1.2 Portuguese cork exports between 2007 and 2017 in value (green bar) and mass (orange bar). Adapted from APCOR (2018).

Pedunculate oak is a native European species and occurs widely across most Europe (Figure 1.1B). Its large size characterizes this deciduous broadleaved tree, reaching over 40 metres in some cases and living for centuries, in rare cases more than a millennium (Eaton *et al.* 2016). Pedunculate oaks are vigorous trees with deep and penetrating roots, valuable for structural stability against windthrow, access to deeper water during drought periods, and drainage effects in heavy soils. Its growth is favoured in acidic, loamy nutrient soils, moist but well-drained,

yet also accepts most soil types, including sand and clay, ranging from neutral to alkaline (Praciak *et al.* 2013; Savill 2013). Oak wood is a hardwood valued for centuries due to its durable features. It was the primary timber source for shipbuilding and nowadays is mainly used for construction and wine barrels (Gil-Pelegrín *et al.* 2017). In particular, the wine barrel market represents an estimated value of 3,330 million dollars, in which the oak segment, with a nearly 50% revenue share, led the global wine barrel market in 2017 (FMI 2017).

Forest and tree decline

Trees have been able to adapt to disturbances, which are generally defined as "any relatively discrete event that disrupts the structure of an ecosystem, community, or population, and changes resource availability or the physical environment" (White and Pickett 1985). Disturbances are drivers of spatial and temporal heterogeneity and, therefore, crucial components of ecological system changes (Turner 2010). However, during the last decades, the incidence, occurrence, and magnitude of such disturbances have increased and had an adverse effect on trees' resilience and the onset of forest decline (Boyd *et al.* 2013; Trumbore *et al.* 2015; Pautasso *et al.* 2015). Despite the extensive and intense research, the factors responsible for many decline events remain elusive, likely due to the poorly understood aetiology (Ciesla and Donaubauer 1994) or the misusing of *decline* as a discrete disease class (Ostry *et al.* 2011). Whether decline and disease should be considered or not as two separate categories is still subject to discussion.

Nevertheless, decline is "an episodic event characterized by premature, progressive loss of tree and stand vigour and health over a given period without obvious evidence of a single clearly identifiable causal factor such as physical disturbance or attack by an aggressive disease or insect" (Ciesla and Donaubauer 1994). Several researchers suggested different models to explain tree decline and identify the main drivers. In 1991, Manion (1991) proposed the decline spiral model (Figure 1.3), which was first conceptualised by Sinclair (1965). The concept

behind this model assumes that decline is not attributable to a single causal agent but instead to multiple, sequential and cumulative abiotic and biotic factors organized in three distinct stages: 1) predisposing factors that slowly act in the long term, including climate, soil, air quality, genetic potential, and age; 2) inciting factors, which are of short duration, such as insect defoliation, frost, drought, and mechanical injury; and 3) contributing factors, which are long-term endemic factors, mainly of biological nature, such as bark beetles, nematodes, fungi, bacteria, and viruses, which can be very destructive to weakened trees and cause death (Ciesla and Donaubauer 1994). Another model, the host-stress-saprogen model, was suggested by Houston (1992) and assumes two groups of factors: environmental/stress factors (primary factors) that successively act on the trees, exposing them to pathogens (secondary factors), and often leading to tree death. Both models consider decline as a distinct class of disease. Alternative models based on the concept that decline is a part of forest dynamics have also been suggested. Muelier-Dombois (1992) proposed that, as part of forest dynamics and succession, declines are a natural process, and Auclair *et al.* (1992) noted that decline has climatic perturbations as a common inciting factor, triggering physical injuries with subsequent water stress and ending in the main decline characteristics.

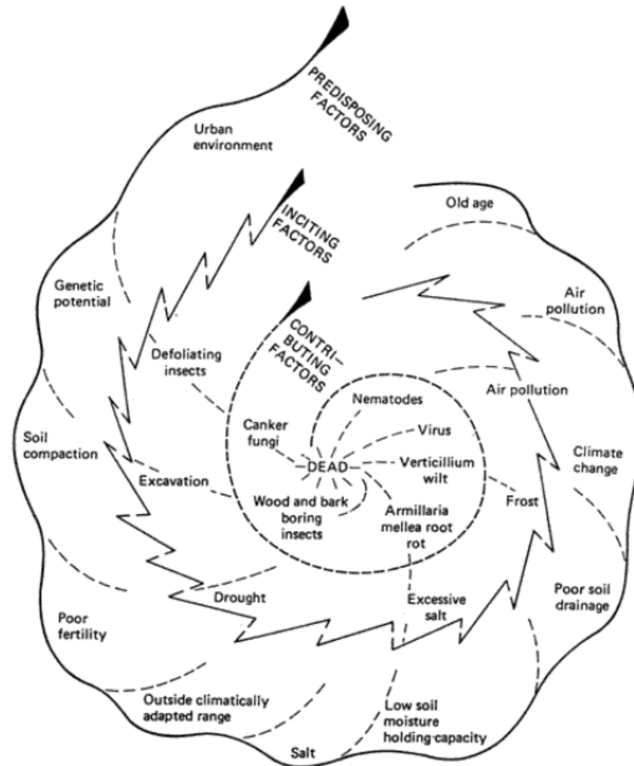

Figure 1.3 Forest decline spiral model proposed by Manion (1991). Three categories of multiple, sequential, and cumulative abiotic and biotic factors (predisposing, inciting, and contributing), shown as spiral rings, trigger tree decline towards death. The shorter the distance from the centre, the greater the factor's direct involvement in tree death and vice versa.

Like other declines, oak declines are difficult to define and quantify due to the complex interaction of the multitude of factors and the heterogeneous patterns that may vary between sites and differ within the decline (Denman *et al.* 2017; Gagen *et al.* 2019).

Oak declines

Forests across the world have always been shaped by climate (Bhatti *et al.* 2006; Neumann *et al.* 2017). However, nowadays, they have been challenged by the unprecedented increasing rate of temperature triggered by significant human-caused changes (IPCC 2007; Pachauri and Reisinger 2007). Predicted changes to

the global climate may be expected to form additional stress on forests. As suggested by the Auclair *et al.* (1992) model, climate change is likely to affect a broad range of tree species, including oaks. This phenomenon has already been reported to be responsible for more frequent and prolonged droughts (Allen *et al.* 2010), to have contributed to the establishment of exotic species (Dale *et al.* 2001), and to lead the spread and incidence of pests and diseases (Sturrock *et al.* 2011; Ennos 2015; Anderegg *et al.* 2015; Ramsfield *et al.* 2016).

Denman and Webber (2009) categorised oak declines into three classes: Chronic Oak Decline (COD), Acute Oak Decline (AOD), and Sudden Oak Death (SOD). The latter will not be further detailed as it is not part of the scope of this thesis.

Chronic Oak Decline

In Portugal and the UK, both cork oak and pedunculate oak are affected by COD. A considerable increase of severe episodes has occurred since the beginning of the twentieth century (Gibbs and Greig 1997; Thomas *et al.* 2002; Camilo-Alves *et al.* 2013). These syndromes are widespread in all their distribution, prolonged in time, and of complex nature. The main symptoms include early foliage deterioration, progressive death of branches, extensive dieback in the crown, and, in some cases, the action of secondary pests and diseases on impaired trees leads to mortality (Figure 1.4).

Figure 1.4 Cork oak trees without COD symptoms (A) and early foliage deterioration and extensive dieback in the crown (B).

Similar abiotic factors related to COD are often implicated for both *Quercus* species. These factors include air pollutants, climate perturbations, and local site conditions (Gibbs and Greig 1997; Thomas *et al.* 2002; Camilo-Alves *et al.* 2013). In addition, fires are also an important threat to cork oak in Portugal (Silva and Catry 2006), which is a doubtless worrying situation compared to other European countries (Silva and Catry 2006). Indeed, fires have changed the Portuguese land cover (Jones *et al.* 2011). The introduction of tree species with a high level of combustibility, such as maritime pine and eucalyptus trees, and their widespread, deliberated planting has triggered fire-prone areas that endanger indigenous forests like *montados*. In turn, indigenous species, such as cork oaks, are better at fighting climate changes, boosting biodiversity, and mitigating fire hazards (Fernandes *et al.* 2010; Alegria *et al.* 2019). Moreover, the chronic decline of cork oak is also associated with water stress. A study explored the physiological performance of declining trees during the summer drought and observed that

declining trees are less resistant to drought due to the lower water-use efficiency and non-photochemical quenching in summer (Camilo-Alves *et al.* 2017).

Dendrochronology provides a useful assessment to understand the relation between forest dynamics and environment as it surveys tree ring size and, therefore, the growth histories of oak trees (Haneca *et al.* 2009). Leal *et al.* (2008) analysed the variations in tree ring growth of cork oaks in Alentejo (Portugal) and revealed that the precipitation levels prior to the growing season have a primordial positive effect on the cork oak growth of the given season. Gagen *et al.* (2019) observed that pedunculate oak trees with COD symptoms located in the Forest of Dean (UK) had shown suppressed growth from early on in their lives, with an initial appearance of decline roughly 40 years after planting, in 1860. Other examples of dendrochronological works on *Quercus* species include a negative correlation between drought and Turkey oak (*Q. cerris* L.) growth in Central Italy (Di Filippo *et al.* 2010), or holm oak (*Q. ilex* L.) growth in Southwestern Spain (Natalini *et al.* 2016). Altogether, the cumulative effect of predisposing abiotic factors can influence oak growth and exacerbate the impact of the inciting and contributing biotic factors.

Harmful and destructive pests and diseases (biotic factors) have also been associated with cork oak and pedunculate oak declines. The brown-tail moth *Euproctis chysorrhoea* L. and chewer sawfly *Periclista andrei* Konow are two defoliating insects, incredibly destructive for cork oak in Portugal. Other harmful insects include the Oak Pinhole Borer *Platypus cylindrus*, the long-horned *beetles Cerambyx cerdo* L., *C. cerdo mirbeckii*, *C. welensii*, and the wood borer *Prinobius myardi* Mulsant (reviewed by Tiberi *et al.* 2016). A broad range of pathogens also infects cork oak trees from the aboveground to belowground parts. They comprise leaf (*Apiognomonia quercina*, *Cystodendron dryophilum*, *Dendrophoma myriadea*, and *Lembosia quercina*), stem, branch, and twig (*Biscogniauxia mediterranea*, *Diplodia corticola*, and *Neofusicoccum parvum*), and collar and root pathogens (*Armillaria mellea*, *Phytophthora cinnamomi*, and *Pythium spiculum*) (reviewed by Moricca *et al.* 2016). In particular, *Phytophthora cinnamomi* is an exotic invasive oomycete highlighted as the critical agent of evergreen oak decline (Brasier *et al.*

1993; Brasier 1996; Camilo-Alves *et al.* 2013). Likewise, weakened hosts are often attacked by destructive insects and pathogens in the UK. The range of damaging leaf-feeding pests of oak includes winter moth (*Operophtera brumata*), oak leaf roller moth (*Tortrix viridana*), and the recent arrival of oak processionary moth (*Thaumetopoea processionea*), which is currently restricted to London and surrounding counties and poses a hazard to human and animal health (Tomlinson *et al.* 2015). Additionally, the oak mildew (*Erysiphe alphitoides*, also known as *Microsphaera alphitoides*) is a fungal pathogen that usually acts after insect defoliation events (Thomas *et al.* 2002; Thomas 2008). The oak pinhole borer (*P. cylindrus*) is not itself responsible for killing oak trees. Instead, it infests and breeds in severely declined or already dead trees (Tilbury 2010). In the belowground environment, the main biotic factors are root disease fungi such as *Armillaria spp.*, *Collybia fusipes*, and *Phytophthora spp.* (Thomas *et al.* 2002; Thomas 2008).

Considerable knowledge has been accumulated about the many factors that adversely affect oak health. Still, progress has been slow to understand the origins and impact of the COD condition, which is probably related to the complex nature of the problem.

Acute Oak Decline

Acute Oak Decline (AOD) is an emerging threat affecting both native foundation oak species (*Q. robur* L and *Q. petraea*). Recently detailed by Denman and Webber (2009), AOD is spreading through Britain's woodlands, parklands, urban landscapes, and gardens. Affected trees occur in localised clusters (Brown *et al.* 2016) and can rapidly decline over 3-5 years (hence, the epidemiological title, acute). Due to the high number of symptomatic trees dying, much concern has been raised amongst landowners and managers (Denman and Webber 2009; Denman *et al.* 2014). Four key descriptors for AOD identification in the UK were provided by Denman *et al.* 2014: 1) dark exudates bleeding on the stems, 2) bark cracks from which the fluid seeps, 3) necrotic inner tissues, and 4) the presence of

larval galleries of the buprestid beetle *Agrilus biguttatus* in the phloem and outermost sapwood (Figure 1.5). Even more, the distribution of *A. biguttatus* and AOD spreading ranges similar coverage across the UK, in which 95% of AOD symptomatic trees have D-shape holes and larval galleries of the buprestid beetle (Brown *et al.* 2015, 2017).

Figure 1.5 Typical symptoms of AOD in pedunculate oak (Q. robur). Oak tree showing external active dark exudates bleeding on the stems (A); necrotic inner tissues (B); larval galleries of the buprestid beetle close to necrotic lesions (C); D-shape exit holes of the *A. biguttatus* (D); the buprestid beetle *A. biguttatus* as an adult (E) and larvae (F). Photos kindly provided by Sandra Denman.

Other European countries reported similar episodes, including Austria (Cech and Tomiczek 1986; Donaubauer 1987), Belgium (Vansteenkiste *et al.* 2004), France (Jacquiot 1949, 1950, 1976), Germany (Falck 1918; Krahl-Urban *et al.* 1944; Hartmann *et al.* 1989; Hartmann and Blank 1992; Kehr and Wulf 1993; Schlag 1994), Italy (Scortichini *et al.* 1993), the Netherlands (Oosterbaan 1990; Oosterbaan and Nabuurs 1991), Poland (Siwecki 1989; Kowalski 1996; Siwecki and Ufnalski 1998) and Spain (Soria *et al.* 1997; Biosca *et al.* 2003; Poza-Carrion *et al.* 2008). However, up to the present, there is a lack of detailed descriptions of these syndromes, and the exact causes remain elusive and unclear amongst European scientists (Denman *et al.* 2014). Nevertheless, certain bacterial species associated with AOD were not only detected in the UK but also in Spain (Biosca *et al.* 2003; Poza-Carrion *et al.* 2008; González and Ciordia 2020), more recently in Iran (Moradi-Amirabad *et al.* 2019) and Switzerland (Ruffner *et al.* 2020), which suggests a global widespread of AOD.

The bacterial species *Rahnella victoriana*, *Brenneria goodwinii*, and *Gibbsiella quercinecans* have been consistently isolated from AOD lesions across the UK forests (Denman *et al.* 2016; Brady *et al.* 2017). Modern molecular 'omics' analyses of AOD symptomatic field material provided the link between *B. goodwinii* and *G. quercinecans* and lesion formation. Using a contemporary approach for adapting and fulfilling Koch's postulates, Denman *et al.* (2018) demonstrated that *B. goodwinii* and *G. quercinecans* were capable of inducing oak tissue necrosis, and together with larvae of the buprestid beetle, formed the main symptoms of AOD. Broberg *et al.* (2018) took a step forward in understanding the role of these bacteria by profiling the metagenome, metatranscriptome, and metaproteome of inner bark tissue from AOD symptomatic and non-symptomatic trees. They provided evidence of the novel functional mechanisms behind the microbiota-host interactions of this complex arboreal decline-disease. *B. goodwinii* dominated the lesion microbiome, with significant expression of virulence factors, whereas *G. quercinecans* and *R. victoriana* contributed in a less extension to pathobiome microbiome activity.

In addition to knowledge of the AOD pathobiome (Denman *et al.* 2018; Broberg *et al.* 2018; Doonan *et al.* 2019), advances have been made in understanding the predisposing factors for AOD establishment in the UK. AOD tends to occur in warmer sites, with low rainfall, and at lower elevations, and significant importance was observed to the deposition of nitrogen, sulphur, and base cations (Brown *et al.* 2018). On the other hand, no significant association was found with soil type, but field investigations are underway to explore soil properties' impact on AOD (Brown *et al.* 2018). In a dendrochronological modelling study, Reed *et al.* (2020) observed that oak trees with long-term AOD had been predisposed many decades earlier before the onset of symptoms. The authors reported that, since the 1930s, diseased trees had reduced vigour growth, which conferred them poor growing conditions and predisposition to AOD compared to asymptomatic trees (Reed *et al.* 2020).

Tree microbiome

Trees are large, complex, long-living plants, which host microbial communities in above- and belowground niches. A better understanding of the role of microbiomes in host growth, health, and resilience has been emphasised by emergent and cumulative evidence showing that trees are expected to rely on their complex microbial communities to resist most biotic and abiotic pressures (Buonaurio *et al.* 2015; Hacquard and Schadt 2015; Koskella *et al.* 2017; Mercado-Blanco *et al.* 2018; Denman *et al.* 2018; Broberg *et al.* 2018; Terhonen *et al.* 2019; Bettenfeld *et al.* 2020). A range of factors, single or in combination, shapes tree microbiomes, involving tree-associated, environmental, physicochemical, and/or human-related factors (Table 1.1).

Table 1.1 Factors reported as shaping tree-associated microbiomes.

Factor group	Specific factor	References
Tree-associated	Species	Bonito *et al.* 2014; Müller *et al.* 2015; Urbanová *et al.* 2015; Fitzpatrick *et al.* 2018
	Genotype	Lamit *et al.* 2016; Gehring *et al.* 2017; Gallart *et al.* 2018; Gomes *et al.* 2019; Veach *et al.* 2019
	Plant compartment and niche	Uroz *et al.* 2010; Gottel *et al.* 2011; Coince *et al.* 2014; Beckers *et al.* 2017; Cregger *et al.* 2018
	Age	Meaden *et al.* 2016; Costa *et al.* 2018; McGee *et al.* 2020
Environmental	Season	Collignon *et al.* 2011; Shakya *et al.* 2013; Voříšková *et al.* 2014; López-Mondéjar *et al.* 2015; Žifčáková *et al.* 2016, 2017
	Climate change	Fransson *et al.* 2005; McHugh and Gehring 2006; Garcia *et al.* 2008; Gehring *et al.* 2017
Physicochemical	Soil	Shakya *et al.* 2013; Bonito *et al.* 2014; Colin *et al.* 2017; Plassart *et al.* 2019; Veach *et al.* 2019
Human-related	Forest management and land use	Hartmann *et al.* 2009, 2012, 2014; Creamer *et al.* 2016; Garau *et al.* 2019; Plassart *et al.* 2019; Goss-Souza *et al.* 2019, 2020

On the other hand, microbial assemblages can significantly affect their host tree. Beyond the negative impact of pathobiomes, they play positive roles, including disease control, induced systemic resistance, nutrient acquisition, abiotic stress resilience and environmental adaptation, and/or mycorrhizal establishment (Table 1.2).

Table 1.2 Microbial community effects on tree species.

Effect	Reference
Pathobiome	Buonaurio *et al.* 2015; Proença *et al.* 2017; Denman *et al.* 2018; Alves *et al.* 2018; Kovalchuk *et al.* 2018; Broberg *et al.* 2018; Gomes *et al.* 2019; Doonan *et al.* 2019
Disease control	Xue *et al.* 2015; Cazorla and Mercado-Blanco 2016; Koskella *et al.* 2017; Terhonen *et al.* 2019
Induced systemic resistance	Bonello *et al.* 2001; Arnold *et al.* 2003; Ganley *et al.* 2008; Wallis *et al.* 2008; Eyles *et al.* 2010
Nutrient acquisition	Grayston *et al.* 1997; Meier *et al.* 2013; Pena and Polle 2014
Abiotic stress resilience and environmental adaptation	Gehring *et al.* 2017; Timm *et al.* 2018
Mycorrhizal establishment	Garbaye 1994; Frey-Klett *et al.* 2007; Labbé *et al.* 2014

Within *Quercus* species, microbial communities have been characterized across the oak niches in an attempt to gain insight into the interactions and impacts on their host. On the aboveground parts of these trees, a unique and dynamic habitat termed the phyllosphere is colonized by microbial communities within (endophyte) and on the surface (epiphyte) of the leaves (Lindow and Brandl 2003). The phyllosphere of *Q. ilex* and *Q. macrocarpa* is colonized by diverse microbial communities influenced by season, land use, and drought (Jumpponen and Jones 2009, 2010; Peñuelas *et al.* 2012; Rico *et al.* 2014). Furthermore, associations between oak health and the phyllosphere microbiome have been reported. In a study of the endophytic fungal assemblages of three oak species (*Q. cerris*, *Q. pubescens,* and *Q. robur*) susceptible to decline, Ragazzi *et al.* (2003) reported a higher colonization frequency of pathogenic isolates (*Apiognomonia quercina*, *Colpoma quercinum*, *Diplodia mutila*, and *Phomopsis quercina*) in declining trees than in healthy trees. Another study reported that *Q. robur* trees showed foliar bacterial and fungal composition changes when infected by *Erysiphe alphitoides*, the causal agent of oak powdery mildew (Jakuschkin *et al.* 2016). Furthermore, the authors used network inference to unveil putative interactions between the pathogen and the foliar microbiome and found potential fungi and bacteria that likely interact with *E. alphitoides*, highlighting *Mycosphaerella punctiformis* and *Monochaetia kansensis* as potential antagonists (Jakuschkin *et al.* 2016).

In the stems of *Q. robur* afflicted with AOD, *R. victoriana*, *B. goodwinii*, and *G. quercinecans* were reported as crucial components of the AOD pathobiome and responsible for the necrotic lesions and dark exudate bleeds (Denman *et al.* 2018; Broberg *et al.* 2018; Doonan *et al.* 2019). Using high-throughput sequencing, Sapp *et al.* 2016 and Meaden *et al.* 2016 surveyed the stem bacterial community of the English oak tree (*Q. robur*) and found that they are greatly influenced by site location and spatial tree position. Moreover, Meaden *et al.* (2016) also found that the host age structures stem microbial composition, warning for the potential biases of this factor in disease diagnosing.

Recently, Costa *et al.* (2018) also reported differences in endophyte fungal communities in young and old twigs of cork oak (*Q. suber*). They observed low colonization rates and high diversity in the older twigs, whereas the younger twigs experienced high colonization rates and low endophyte diversity (Costa *et al.* 2018). Changes in the endophytic tree-associated communities and host health have also been reported in oak species under drought stress. Linaldeddu *et al.* (2011) isolated fungal endophytes from twigs, branches, and woody tissues of *Q. suber* and observed a decline in species diversity of trees under water stress. They observed a high frequency of *B. mediterranea* isolates and suggested that the proliferation of some potentially pathogenic endophytes could be a consequence of reduced fungal diversity triggered by drought conditions (Linaldeddu *et al.* 2011).

In a study of Persian oak trees (*Q. brantii*) located in healthy and declined stands, the high diversity of endophytic fungi isolated from twigs was complemented by shifts in microbiome composition, which may be correlated with tree decline, as suggested by Ghobad-Nejhad *et al.* (2018). Ecosystem-perturbing events also have adverse effects on the oak endophytic fungal microbiome. The abundance of a potential biocontrol endophyte observed in Persian oak branches was shown to decline due to dust storms, which suggested links between ecosystem-perturbing factors and oak decline induced by endophytic communities shifts (Hagh-Doust *et al.* 2017).

Although it is essential to understand the overall influence of the full-tree microbiome on tree growth, health, and resilience (Hacquard and Schadt 2015), until the present, most research has focused on belowground communities. This is partly due to the more massive biodiversity found in soil than the aboveground parts (Nielsen *et al.* 2015). Moreover, soil biodiversity is positively correlated with ecosystem services (Bakker *et al.* 2019), is the primary driver of ecosystem stability (Yang *et al.* 2018), and sustains services and preserves soil health under changing environmental conditions (Luyssaert *et al.* 2018; Dubey *et al.* 2019). Another important key point to explore these communities is that, through their roots, forest trees closely interact with a wide range of belowground biota, which includes a variety of life forms, such as earthworms, diplopoda, isopoda, mites, collembolans, tardigrades, enchytraeids, nematodes, protists, fungi, bacteria, and archaea (Bardgett and van der Putten 2014). However, the focus of this Ph.D. thesis is on bacterial and fungal communities.

Bacteria and fungi have coevolved with forest trees. They establish mutualistic relationships, including N-fixing bacteria and mycorrhizal associations, as well as complex interactions with a myriad of the tree- and soil-associated microbiome partners (Martin *et al.* 2017). Although the current knowledge of the rhizosphere microbiome is mostly focused on ectomycorrhizal fungi (EcM), it is known that these communities, together with bacteria, provide a multitude of vital services to their host. These services include nutrient acquisition, water supply, influence on seedling survival and establishment, promotion of plant growth, and tolerance increase to abiotic and biotic stresses (Smith and Read 2008; Van Der Heijden and Horton 2009; Bonfante and Genre 2010; Uroz *et al.* 2016; Baldrian 2017; Lladó *et al.* 2017; Mercado-Blanco *et al.* 2018; Terhonen *et al.* 2019). Moreover, EcM supports specific and diverse bacterial communities that help their symbiotic establishment (Garbaye 1994; Frey-Klett *et al.* 2007; Labbé *et al.* 2014).

Thanks to the advance of metabarcoding and metagenomics approaches, progress has been made in characterising the bacterial and fungal communities that inhabit roots, rhizospheres, and surrounding soils of *Quercus* species (Buée *et al.* 2009; Uroz *et al.* 2010; Jumpponen *et al.* 2010; Orgiazzi *et al.* 2012; Toju *et*

al. 2013b, a; Voříšková and Baldrian 2013; Voříšková *et al.* 2014; López-Mondéjar *et al.* 2015; Maghnia *et al.* 2017; Cobo-Díaz *et al.* 2017; Fernández-González *et al.* 2017; Reis *et al.* 2019; Ruiz Gómez *et al.* 2019; Lasa *et al.* 2019). Together with previous reports, several key factors, single or in combination, have been indicated as drivers of microbial communities. Several studies consistently reported land use and forest management, season and drought as primary factors that drive the bacterial and fungal assemblages of *Quercus* species (Azul *et al.* 2009, 2010; Dickie *et al.* 2009; Querejeta *et al.* 2009; Jumpponen and Jones 2009; Aponte *et al.* 2010; Pastorelli *et al.* 2011; Richard *et al.* 2011; Lagomarsino *et al.* 2011; Orgiazzi *et al.* 2012; Barrico *et al.* 2012; Voříšková *et al.* 2014; Franciolia *et al.* 2014; López-Mondéjar *et al.* 2015; Reis *et al.* 2018, 2019; Bastida *et al.* 2019). Other factors included soil compartment/niche (Uroz *et al.* 2010), *Quercus* species identity (Morris *et al.* 2008; Cavender-Bares *et al.* 2009; Toju *et al.* 2013a), vegetation and soil properties (Aponte *et al.* 2010), soil depth (Šnajdr *et al.* 2008), and ecosystem perturbations (Karpati *et al.* 2011; Fernández-González *et al.* 2017).

In the context of oak decline, information about the belowground microbial community is still scarce and mainly focuses on the fungal component (mostly EcM) and oomycete communities. Studies on EcM communities of *Q. ilex*, *Q. suber*, *Q. robur*, and *Q. petraea* reported changes according to tree health status, in which a greater EcM diversity was observed in trees with better crown health conditions (Kovacs *et al.* 2000; Montecchio *et al.* 2004; Lancellotti and Franceschini 2013; Corcobado *et al.* 2014, 2015; Bzdyk *et al.* 2019). Surprisingly, these studies also observed that root tips of declining oaks were still able to establish symbiotic mycorrhizal associations. However, as hypothesized by Montecchio *et al.* (2004), symptomatic individuals likely lose their ability to select the most efficient EcM, and less efficient symbionts can take their place. This is supported by a recent study, where the authors observed that AOD symptomatic trees had fewer fine root tips and less long-distance ectomycorrhizal exploration types compared to asymptomatic oaks (Barsoum *et al.* 2021). Moreover, even after *Q. robur* tree felling, EcM communities were capable of recovering, showing high resilience to disturbance events (Mosca *et al.* 2007). However, other

environmental disturbances, such as nitrogen pollution and deposition, have been reported as drivers of EcM communities, dramatically changing their diversity and composition (Lilleskov *et al.* 2002; Avis *et al.* 2003, 2008; Cox *et al.* 2010; Suz *et al.* 2014; van der Linde *et al.* 2018). Beyond the abiotic disturbances, biotic stressors also induce shifts in the EcM community. Two studies (Corcobado *et al.* 2014, 2015) observed that the presence of *P. cinnamomi* altered relationships between EcM abundance and *Q. ilex* trees, despite the seasonal and soil properties effects, and suggested that non-mycorrhizal root tips are vulnerable entry points for the pathogen (Corcobado *et al.* 2015). Recently, Ruiz Gómez *et al.* (2019) characterized the soil fungal and oomycete communities in holm oak (*Q. ilex*) stands across different decline levels and related their diversity and composition to the severity of the decay symptoms. Furthermore, they also observed an antagonistic association between the genus *Trichoderma* and *Phytophthora* spp. They suggested using this fungus as a potential biocontrol tool in the holm oak rot root management (Ruiz Gómez *et al.* 2019). Maghnia *et al.* (2017) studied the root-associated fungal community, including fungal endophytes, EcM fungi, and fungi in root-adhering soil. They reported that the three Moroccan cork oak forests subjected to increasing human disturbances exhibited distinct communities. The authors highlighted the need for joint initiatives on a Mediterranean scale for future conservation strategies (Maghnia *et al.* 2017) and suggested developing ecological management strategies based on rhizosphere microbiome-driven planting to improve cork oak forest sustainability (Maghnia *et al.* 2019). Finally, Scarlett *et al.* (2020) observed that asymptomatic oak trees had a higher abundance of ammonia-oxidising bacteria (AOB) driven positively by soil pH, which in turn was driven by C:N ratio. They suggested that soil acidification adjustment by increasing soil organic matter can mitigate the tree stress caused by oak decline (Scarlett *et al.* 2021).

Characterizing microbial communities through high-throughput sequencing and bioinformatics

In 2001, the first draft of the human genome was published (Lander *et al.* 2001; Venter *et al.* 2001). This milestone proved a breakthrough to the genomic era and was accomplished using Sanger DNA sequencing. However, due to the intrinsically limited throughput and high cost associated with this technology, the Human Genome Project took 13 years to complete and an estimated 300 million dollars worldwide (NIH 2019). A DNA sequencing technology initiative was launched to circumvent this problem, aiming to achieve a $1,000 human genome (Schloss 2008). Since then, there has been a rapid development of high-throughput sequencing (HTS) technologies and the emergence of the first sequences of environmental samples generated by HTS (Edwards *et al.* 2006; Poinar *et al.* 2006). The study of genetic material recovered directly from environmental samples is known as metagenomics (Handelsman *et al.* 1998). It is based on "the application of modern genomic techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species" (Chen and Pachter 2005). Researchers across the globe have been using this methodology to characterise the microbial communities of a wide range of environments (Medini *et al.* 2008). In particular, those inhabiting other 'host' organisms, such as plants, humans, and other animals, aiming to identify their role on host health through microbiome-wide association studies (Gilbert *et al.* 2016; Surana and Kasper 2017; Awany *et al.* 2019; Beilsmith *et al.* 2019).

Two main approaches can be used for this purpose: shotgun metagenomics or transcriptomics and metabarcoding. The present thesis focuses on metabarcoding data; thus, more attention will be given to this approach, with only a brief description of the shotgun metagenomics or transcriptomics approach. Nonetheless, the question formulation, study design (subject and control selection), sampling, storage, and extraction of total genetic material are the standard initial steps to both approaches (Figure 1.6).

Briefly, in shotgun metagenomics or transcriptomics, DNA or RNA libraries compatible with the sequencer to be used are constructed from the whole extracted genetic material. These libraries are used to generate gigabases or even terabases of genetic information. Next, this data is subjected to a pre-filtering step to remove redundant and low-quality sequences. The high-quality reads are assembled in contigs, and genes are identified and annotated for function. In parallel, reads are also mapped to reference genomes and marker genes to get read-based taxonomic profiling and mapped to annotated genes, proteins, or pathways to get read-based metabolic profiling (Quince *et al.* 2017). The result of this process provides information about 'who is there', 'what they are capable of doing' (if using DNA as starting material), and 'what they are actually doing' (if using RNA as starting material) (Quince *et al.* 2017).

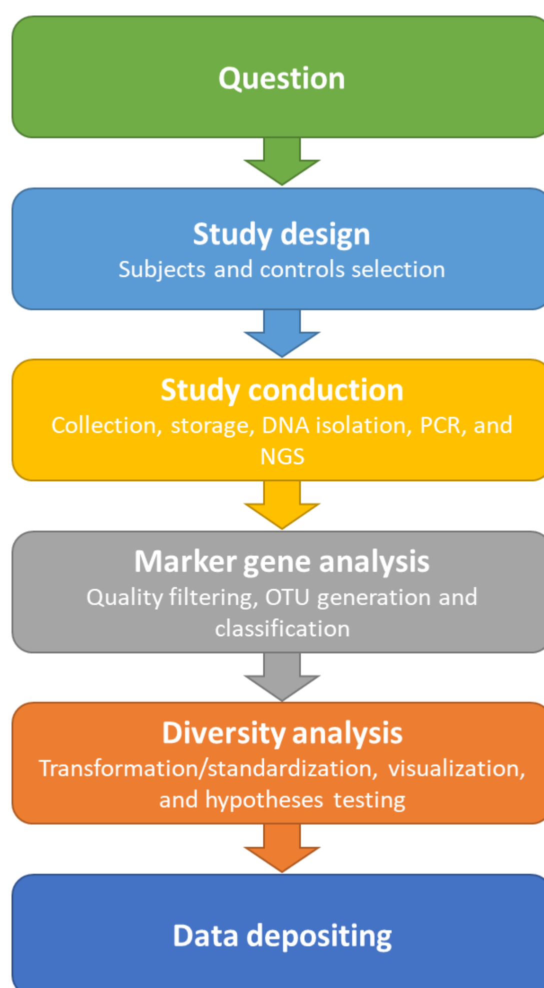

Figure 1.6 Main steps to conduct a microbiome study based on metabarcoding approach.

The metabarcoding approach is based on amplicon sequencing using the amplification of specific genomic regions in DNA (Figure 1.6 – study conduction). This targeting approach, commonly called DNA barcoding, allows taxonomic identification using any DNA fragment. The ideal DNA barcoding target should, however, be (1) nearly identical among individuals but variable enough for low-level taxonomic resolution (e.g., species-level), (2) the same standardized DNA region across distinct taxonomic groups, (3) a target region for easy assignment of new species into their phylogenetic group, (4) a genetic fragment that simultaneously contains extremely conserved priming regions with highly variable sequences between them, and (5) short enough to allow amplification of degraded DNA (Valentini *et al.* 2009). Unfortunately, such an ideal genetic marker does not exist. The most common metabarcoding markers used for profiling microbial communities are housekeeping genes, such as 16S rDNA for prokaryotic organisms (bacteria) and Internal Transcribed Spacer (ITS) for eukaryotes (fungi) (Herlemann *et al.* 2011; Klindworth *et al.* 2013; Tedersoo *et al.* 2014). These marker genes contain highly variable regions that allow detailed identification that, in turn, are flanked by highly conserved regions, which serve as binding sites for PCR primers. Essentially, "universal" primers are used in PCR to amplify particular targeting regions of these genes, allowing fast and cost-effective identification of a broad range of prokaryotic and eukaryotic organisms. After DNA extraction and quality assessment, the first step is library preparation. The indexes and sequencing adapters are attached to both ends of the enriched fragments, using a PCR approach. This enables massive parallel reading of multiple samples in sequencing platforms, such as MiSeq (Illumina).

Sequence data from complex microbial communities is challenging from the perspective of bioinformatics, biostatistics, and computing. The analysis of sequenced amplicon can be carried out by several software packages available, where the most commonly used are QIIME (Caporaso *et al.* 2010; Bolyen *et al.* 2019), mothur (Schloss *et al.* 2009; Schloss 2020), and Ribosomal Database Project (RDP) (Cole *et al.* 2014) (Figure 1.6 – marker gene analysis). Nonetheless, data processing generally begins with sequence read demultiplexing to its original sample based on the index sequence (barcode) added during library preparation.

At this stage, read quality filtering is usually carried out, allowing computational efficiency. According to the sequencing platform, read filtering thresholds are applied to remove redundant, low-quality, and short reads. Even though high-fidelity Taq polymerases are used to minimize biases and errors during PCR, recombinant and disparate templates, known as chimeras, are generated (Haas *et al.* 2011). These PCR products are created by incomplete extension, which is common and challenging to detect, resulting in inflated alpha diversity measures. Still, they have minimal effect on beta diversity measures (Ley *et al.* 2008). However, several software packages are available for chimera filtering, such as UCHIME (Edgar *et al.* 2011), ChimeraSlayer (Haas *et al.* 2011), Perseus (Quince *et al.* 2011), and DECIPHER (Wright *et al.* 2012). At the end of this initial process, high-quality reads are obtained and used in subsequent steps. After quality filtering, high-quality reads are clustered into operational taxonomic units (OTUs), which allows to classify groups of similar DNA sequences of a specific taxonomic marker gene (Blaxter *et al.* 2005). This makes OTUs pragmatic proxies for taxonomic classification and intends to represent a taxonomic unit depending on the sequence similarity threshold. An empirical study showed that most bacterial species had 97% similarity of 16S rDNA sequence; therefore, this threshold value is commonly used as a species-level proxy (Konstantinidis and Tiedje 2005). Three OTU-picking algorithms can be adopted to cluster sequences: *de novo*, closed reference, and open reference clustering (Rideout *et al.* 2014). In the first, OTUs are created by clustering reads against one another without any external reference sequence collection. In contrast, closed reference uses reference databases, in which reads cluster against a reference, and those not matching any reference sequence in the database are discarded. The third approach is a mixture of the previously described approaches. Essentially, open reference OTU picking is a two-step process comprising closed-reference OTU picking followed by *de novo* read clustering for sequences, which failed to match sequences in the reference database (Rideout *et al.* 2014). This is the best OTU picking algorithm in most microbiome studies as it retains all sequence data. The periodically updated databases used for the taxonomic assignment are chosen according to the selected microbial marker, and the most commonly used are Greengenes

(DeSantis *et al.* 2006), RDP (Cole *et al.* 2014), SILVA (Quast *et al.* 2013) for 16/18S rDNA, and UNITE (Nilsson *et al.* 2019) for ITS classification. The last step describes alpha (within-sample) and beta (between samples) diversities of microbial communities. This process provides information about the diversity and composition of microbial communities, answering the question 'who is there?'.

Beta diversity data is further explored and visualised (Figure 1.6 – diversity analysis). To achieve this, data are often transformed to correct the variable distribution shape so the data can meet the statistical assumptions and improve the data interpretability (Ramette 2007). Moreover, environmental data is standardized to remove the undue influence of different units and put different variables on the same scale (dimensionless variables) (Ramette 2007). Subsequently, transformed and standardized data can generate (dis)similarity or distance matrices. There are a vast number of distance/(dis)similarity) indices, which can be applied to generate the distance/(dis)similarity matrices. These include quantitative metrics, such as Bray–Curtis, Canberra, or weighted UniFrac, or qualitative (presence/absence) metrics, such as Jaccard and unweighted UniFrac (Knight *et al.* 2018). Variation in the (dis)similarity matrices can be visualised using, for example, cluster analysis, non-metric multidimensional scaling (NMDS), principal coordinates analysis (PCO), correspondence analysis (CA), distance-based redundancy analysis (dbRDA), and canonical correspondence analysis (CCA). These are data reduction techniques to decrease the number of dimensions (OTUs) and enable the plotting and visualization of microbiome data sets in 1D, 2D, or 3D scatterplots (Ramette 2007). Permutational analysis of variance (PERMANOVA), analysis of similarities (ANOSIM), and the Mantel test can be used to test a priori hypotheses based on treatments of discrete groupings of the sample data (Anderson and Walsh 2013). Differential abundance analysis is another common approach to test for significant differences in microorganisms/OTUs by comparing their abundances across treatments/groups (for example, healthy vs. diseased or control vs. treatment) (Lin and Peddada 2020). This is a challenging analysis due to the high dimensional, sparse (abundance of zeros), and compositional nature of the microbiome data sets. This has raised debate in the research community because there is a

panoply of normalization, (dis)similarity indices, and analytical strategies that can be used and have their pros and cons (Weiss *et al.* 2017; Hawinkel *et al.* 2019; Lin and Peddada 2020). Other normalization approaches include proportion, rarefaction, log upper quartile (logUQ), cumulative sum scaling (CSS), variance stabilization (VS), and trimmed Mean by M-Values (TMM). Differential abundance methods include the Mann-Whitney U test, nbinomWald test (DeSeq2), exact test (edgeR), variance modeling (Voom), metagenomeSeq (fitZIG – zero-inflated Gaussian), and analysis of the composition of microbiomes (ANCOM). However, the choice depends upon data characteristics (Weiss *et al.* 2017). Machine learning is a recent technique that enables sample clustering in groups based on metadata categories and has emerged as an exciting tool for predicting the future state of individuals (Qu *et al.* 2019).

With the exponential growth in studies producing sequencing data during the last decade, it has been standard practice and a prerequisite from peer-reviewed journals to deposit the sequencing data into public databases (Kodama *et al.* 2012) (Figure 1.6 – data depositing). Therefore, the last step conducted in a microbiome study is to archive the sequencing data and metadata in one of the public repositories of the International Nucleotide Sequence Database Collaboration (INSDC; EBI's European Nucleotide Archive (ENA), NCBI's Sequence Read Archive (SRA), and DDJ's Sequence Read Archives (DRA)) (Karsch-Mizrachi *et al.* 2012). These databases are routinely harmonized and support compatible data formats, creating an opportunity to increase the quality, accessibility and utility of information in microbiome research (Yilmaz *et al.* 2011).

Scope and objectives

Rhizosphere microbial members can either enhance or weaken the physiological performance of trees and are, therefore, seen as a critical component for their health, growth, and productivity (see Frey-Klett *et al.* 2011; Hacquard and Schadt 2015; Vandenkoornhuyse *et al.* 2015; Bettenfeld *et al.* 2020, and references therein). However, much remains to be learnt of the role that microbes play in oak tree health as well as in chronic and acute oak decline. Many ecological studies in oak species rely on traditional microbiological methodologies, which only detect the cultivable microorganisms and are mainly focused on EcM fungi and oomycete communities. Furthermore, most of the published studies to date focus on a single microbial domain, and those that assess multiple domains are rare. Given the above, using metagenomic approaches to study the microbial diversity and composition of cork oak and pedunculate oak-associated bacterial and fungal communities is a step forward. It will provide information and enable us to diagnose disease better and improve the health of oak trees.

The scope of this Ph.D. project is to characterize rhizosphere physicochemical properties and bacterial and fungal communities inhabiting *Quercus* species, using a metabarcoding approach, and test the hypothesis that the microbial community inhabiting the rhizosphere is linked to host health (Figure 1.7). Within this scope, the work developed in this thesis has the following specific objectives:

1) To characterize the rhizosphere physicochemical properties and microbial communities of cork oak (*Q. suber*) and English oak (*Q. robur*) (reported in chapters 2 and 3);
2) To discover associations between rhizosphere physicochemical properties, microbiome diversity and composition, and tree health across stands and sites in Portugal affected by COD disturbance (reported in Chapter 2);
3) To discover associations between rhizosphere physicochemical properties, microbiome diversity and composition, and tree health across sites in the UK affected by AOD disturbance (reported in Chapter 2);
4) To identify potential rhizosphere physicochemical and microbial markers of COD and AOD disturbances (reported in chapters 2 and 3);

5) To understand the relationships between COD or AOD-affected (cork) oak trees and rhizosphere physicochemical properties and microbiome diversity and composition (reported in Chapters 2 and 3).

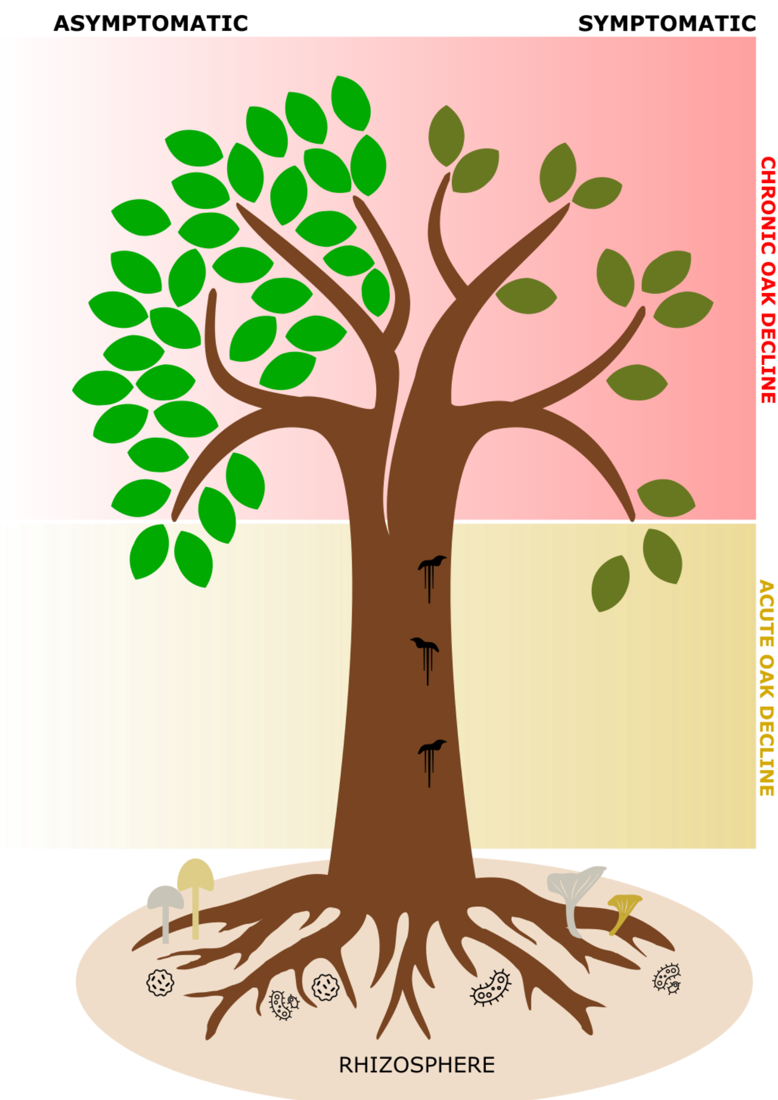

Figure 1.7 Rhizosphere microbiome and oak decline syndromes. The main symptoms of COD are located on the aboveground parts (red shadow) and include early foliage deterioration, progressive death of branches, and extensive dieback in the crown. In AOD, dark bleeds on the stems are a typical symptom (yellow shadow).

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CHAPTER 2

THE RHIZOSPHERE MICROBIOME OF CORK OAK UNDER CHRONIC DECLINE DISTURBANCE

The rhizosphere microbiome of cork oak under forest decline disturbance

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Abstract

In Southern Europe, the cork oak forest represents a unique traditional habitat under increasing pressure over the last decades. Chronic cork oak decline (COD) is a widespread, prolonged, complex disorder driven by multiple, sequential, and cumulative factors. Increasing evidence has highlighted the role of tree microbiomes in host health and resilience, particularly in the rhizosphere. Yet, little is known about how these microbiomes relate to cork oaks and COD. In this study, we sampled the rhizosphere of asymptomatic and COD-affected trees across sites and stands that spanned different stages of decline. The rhizosphere physicochemical properties and microbiomes were characterised to study the relationships across these properties, microbial communities, and COD.

The results revealed that rhizosphere physicochemical properties and microbiome composition differed significantly across sites and stands, but there was no significant association with tree health. The rhizosphere soils were more acidic, richer in nutrients (C and N) and higher C:N ratio in the site with better forest health. Rhizosphere pH and moisture were the main drivers of microbial composition. Several microorganisms, including members in the genera *Bacillus*, *Bryobacter*, *Cladophialophora*, and *Phallus,* were more abundant in the healthy stands and were also part of the core microbiome, suggesting potentially key functions for cork oak. In contrast, opportunistic organisms, including known plant pathogenic fungi and fungal parasites, were more abundant in the site experiencing a higher chronic decline.

This study provides baseline information on the rhizosphere physicochemical properties and microbiome in cork oak trees and reports on associations of soil conditions and rhizosphere communities with COD at site and stand levels. These findings open exciting routes for further research to clarify the relationships across microbiomes, soil, oaks, and chronic decline and understand the underlying mechanisms that enhance tree health, resistance, and resilience in global change.

Keywords: Chronic Cork oak Decline; Decline-disease; Microbiome; Rhizosphere; Soil chemistry; Tree health; Belowground

Introduction

Forest cover has declined across the globe (Boyd *et al.* 2013; Pautasso *et al.* 2015; Trumbore *et al.* 2015). Oaks (*Quercus* spp.), in particular, have suffered severe declines in many countries. In Europe, oak decline episodes have also increased in amplitude (Thomas *et al.* 2002; Denman *et al.* 2014; Haavik *et al.* 2015). Oak forests are among the most abundant of native European habitats. They provide several essential ecosystem services, including wood and non-wood products, climate regulation and mitigation, carbon sequestration, soil protection and formation, nutrient cycling, biodiversity protection, water regulation and essential components of cultural heritage and recreation (EEA 2016). In Southern Europe, cork oak (*Quercus suber* L.) forests are mainly located in Portugal, which has a third of the total area of cork oaks, and together with Spain, Morocco and Algeria account for approximately 90% of the estimated total area of over 2.2 million hectares (APCOR 2018). In Portugal, cork oak forests are known as *montado* (*dehesa* in Spain) and are classified as a multifunctional silvopastoral system from which cork is produced and extracted (Pereira 2007). They are also recognised as a global biodiversity hotspot and conservation priority (Myers *et al.* 2000; IPCC 2007). Portugal is the largest producer of this unique outer bark and is responsible for 50% of the world’s production. Therefore, these forests are of great socio-economic importance (APCOR 2018). However, *montados* are a human-managed ecosystem, which appears to be adversely impacted by the global climatic change (Acácio *et al.* 2017; Aubard *et al.* 2019).

Chronic cork oak decline (COD) is a widespread, prolonged, complex disorder characterised by gradual foliage deterioration, branch death, and crown dieback. Although *Phytophthora cinnamomi* has been described as a critical agent of evergreen oak decline (Brasier *et al.* 1993), this invasive oomycete is not, on its own, capable of driving such unprecedented decline (Brasier 1996; Moreira and Martins 2005; Camilo-Alves *et al.* 2013). Other factors associated with COD include drought (Camilo-Alves *et al.* 2017), soil type (Costa *et al.* 2008), forest management practices (Godinho *et al.* 2016; Arosa *et al.* 2017), pest attacks (Tiberi *et al.* 2016), and forest fires (Silva and Catry 2006). First conceptualised by

Sinclair (1965) and later proposed by Manion (1991), the decline spiral model suggests that forest decline-diseases are complex syndromes not attributable to single causal factors, but instead to multiple, sequential, and cumulative abiotic and biotic factors that predispose, incite, and contribute to tree deterioration, often ending in tree death (Ciesla and Donaubauer 1994).

Recent advances in the understanding of the drivers and mechanisms behind forest decline have emphasised the impact of microbial communities on tree health and resilience (Buonaurio *et al.* 2015; Koskella *et al.* 2017; Denman *et al.* 2018; Kovalchuk *et al.* 2018; Cregger *et al.* 2018; Broberg *et al.* 2018; Gomes *et al.* 2019; Pinho *et al.* 2020; Scarlett *et al.* 2021). Rhizosphere microbiomes are of particular importance to large, complex, and long-living hosts and are known to establish long-term interactions with trees (Hacquard and Schadt 2015; Mercado-Blanco *et al.* 2018; Terhonen *et al.* 2019). Bacterial and fungal communities are taxonomically and functionally diverse groups in the belowground environment. They influence nutrient and water acquisition in addition to curbing abiotic stress and disease (El Hassni *et al.* 2007; Uroz *et al.* 2016a; Baldrian 2017; Timm *et al.* 2018), and thereby impact host growth, health, and productivity (Bettenfeld *et al.* 2020). Moreover, soil microbial composition varies across space under the influence of several factors such as soil properties (Schreiter *et al.* 2014), environment and climate (Oliverio *et al.* 2017), anthropogenic and land-use practices (Lauber *et al.* 2013; Hartmann *et al.* 2014), and biotic stress (Berendsen *et al.* 2018). In the context of cork oak forests, advances have been made to gain insights into soil microbial communities, focusing on the influence of land-use and management practices, climate change, seasonal variation, and forest decline on bacterial (Costa *et al.* 2013; Bevivino *et al.* 2014; Franciolia *et al.* 2014; Reis *et al.* 2019) and fungal communities (Azul *et al.* 2009, 2010; Lancellotti and Franceschini 2013; Maghnia *et al.* 2017; Reis *et al.* 2018).

Soil acidity, carbon and nitrogen content, and C:N ratio are also critical determinants of various soil functions, such as plant growth, water supply regulation, raw materials recycling, and habitat for soil organisms (Bünemann *et al.* 2018). Changes in soil quality can impact soil properties and processes and,

consequently, the ability of soil to function effectively as a component of a healthy ecosystem (Schoenholtz *et al.* 2000). This can ultimately affect a wide range of soil ecosystem services (Bünemann *et al.* 2018), and therefore tracking changes in soil properties and functions represents a way to assess soil health (Seaton *et al.* 2021), even though their exact impacts are often difficult to evaluate (Gärdenäs *et al.* 2011).

In this study, we aimed to 1) characterise rhizosphere properties and microbiome composition; 2) test for a relationship between rhizosphere properties and microbiome diversity and composition; and 3) test for a relationship between the rhizosphere microbial community composition and chronic oak decline. We hypothesised that the rhizosphere properties and microbial communities differ between asymptomatic and COD-affected cork oaks due to the interdependency between the host and its microbiome under a specific soil context.

Materials and Methods

Site, stand, and tree selection and soil sampling

Two sites were selected based on the forest monitoring data of the Association of Forestry Producers of Coruche (APFC), the presence of cork oak trees with COD symptoms, and the willingness of landowners to allow access to the sites: Divor and Baixo Sorraia, both located in Coruche, Portugal (Supplementary Figure 2.1; APFC 2016a, b). Cork oak is the dominant tree species, occupying 78% and 77% of the forest area, respectively. Both sites are human-managed with similar forest practices. According to APFC data, since 1970, the cork oak area has been declining in both sites (APFC 2016a, b) and the phenotype data on crown condition showed that the overall deterioration in tree condition in Divor (APFC 2016a) is worse than in Baixo Sorraia (APFC 2016b). Therefore, these two sites were classified as sites with high (Divor) and low (Baixo Sorraia) levels of decline. Moreover, comparing crown phenotype data between 2010 and 2015 (Ribeiro and Surový 2010; APFC 2016a, b) allowed for selecting two cork oak stands within each site. These stands were designated as *vitality*, which showed low levels of

forest decline (cork oak mortality rate ranging between 0-1%), and *mortality*, which showed high levels of forest decline (cork oak mortality rate ranging between 9-22%).

Within each site and stand, 5 asymptomatic and 15 COD (visibly unhealthy) trees with a similar diameter-at-breast height (DBH, a proxy of tree age) were haphazardly selected, and a rhizosphere sample was collected (Supplementary Figure 2.1). At the *mortality* stand in Baixo Sorraia, only 5 asymptomatic and 5 COD trees were sampled due to the paucity of cork oak trees with similar DBH. The rhizosphere samples were collected after removing the top litter layer. At each tree, four cores were taken under the crown (2-4 m in approximate cardinal directions radially from the trunk), and the fine roots and attached rhizosphere were pooled and stored in a single sterile bag (composite sample). Samples were collected to a depth of 30 cm because the fine roots of trees are mainly distributed at this soil zone. Additionally, three single cores of bulk soil were randomly taken outside the vicinity of the tree root system (i.e., in surrounding open spaces within each site). The bulk soils were used as control samples to compare soil compartments and detect the cork oak-rhizosphere effect. In total, 70 rhizosphere and 12 bulk soil samples were collected in the *vitality* and *mortality* stands in Divor (high decline) and Baixo Sorraia (low decline) (Supplementary Table 2.1).

Physicochemical analysis

Rhizospheres were separated from the roots in the laboratory by gently shaking the roots and collecting the loosened soil. The rhizosphere and bulk soil samples were separately sieved through a 2-mm mesh, and c. 5 g of each soil sample was stored at -80 °C until DNA extraction. The remaining soil samples were analysed for moisture using the gravimetric method, pH (in water) using an automated Metrohm autosampler with a 719 S Titrino unit and a Sentek electrode, and total carbon (C) and total nitrogen (N) by dry combustion at 900 ˚C using a FlashEA1112 NC analyser (Thermo Fisher Scientific, USA). Soil C:N ratio was also calculated for each sample. The analyses were performed at the Chemical Analysis Laboratory of Forest Research, UK.

DNA extraction, PCR library preparation, high-throughput sequencing, and data processing

DNA was extracted from 500 mg of each rhizosphere and bulk soil sample using the NucleoSpin Soil kit (Macherey-Nagel, Germany) with buffer SL2 and Enhancer SX according to the manufacturer’s instructions. Soilless blank controls were included to ensure reagent sterility. Agarose gel electrophoresis, ND1000 spectrophotometer (NanoDrop Technologies, USA), and Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, USA) were used to check the DNA quality and yield. The DNA samples were stored at -20°C for further use.

The DNA extracted from the rhizosphere and bulk soil samples were used to amplify and sequence the bacterial V3-V4 variable regions of the 16S rDNA and the fungal Internal Transcribed Spacer 2 (ITS2). The bacterial primers used were: forward primer 341F 5′-CCTACGGGNGGCWGCAG-3′ and reverse primer 805R 5′-GACTACHVGGGTATCTAATCC-’3’ (Herlemann *et al.* 2011; Klindworth *et al.* 2013); and the fungal primers used were: forward primers pool ITS3NGS1_F 5’-CATCGATGAAGAACGCAG-3’3’, ITS3NGS2_F 5’-CAACGATGAAGAACGCAG-3’3’, ITS3NGS3_F 5’-CACCGATGAAGAACGCAG-3’3’, ITS3NGS4_F 5’-CATCGATGAAGAACGTAG-3’3’, ITS3NGS5_F 5’-CATCGATGAAGAACGTGG-3’3’, and ITS3NGS10_F 5’-CATCGATGAAGAACGCTG-3’3’, and reverse primer ITS3NGS001_R 5’-TCCTSCGCTTATTGATATGC-’3’ (Tedersoo *et al.* 2014).

The first PCR was performed using the KAPA HiFi HotStart ReadyMix PCR Kit (Roche, USA), 200 nM of each primer, and 12.5 ng of template environmental DNA in a total volume of 25 µL. The PCR conditions entailed a 3 min denaturation at 95 °C, followed by 25 cycles of 98 °C for 20 s, 55 °C (V3-V4) for 60 °C (ITS2) for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min in a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). Negative controls were included in each PCR batch. PCR products were run on an agarose gel (1% w/v) and purified using Agencourt AMPure XP (Beckman Coulter, USA) according to the manufacturer’s instructions.

Dual indexes and Illumina sequencing adapters from the Nextera XT Index kit (Illumina, USA) were attached to both ends of the amplicons by limited-cycle PCR. This second PCR used the KAPA HiFi HotStart ReadyMix PCR Kit (Roche, USA), 2.5 µL of each indexed adapter primer, and 2.5 µL of the first PCR product, in a total volume of 25 µL. PCR conditions included 3 min denaturation at 95 °C, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min in a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). PCR products were checked on an agarose gel (1% w/v) and one-step purified and normalised using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, USA). Amplicon libraries were pooled and pair-end sequenced using the MiSeq Reagent Kit v3 in the MiSeq platform (Illumina, USA) at Genoinseq (Cantanhede, Portugal). All sequence data described in this study are available in the European Nucleotide Archive (ENA) under accession number PRJEB33831.

Sequence reads were quality-filtered using PRINSEQ version 0.20.4 (Schmieder and Edwards 2011). Sequencing adapters and reads with less than 150 bp (bacteria) or 100 bp (fungi) were removed. Reads were trimmed where the average base quality was below Q25 in a window of 5 bp. The forward and reverse reads were merged by overlapping the paired-end reads with AdapterRemoval version 2.1.5 (Schubert *et al.* 2016) using default parameters. Chimeric reads were removed using USEARCH version 6.1 (Edgar 2010). The highly variable fungal ITS2 from the merged reads was extracted with ITSx version 1.0.11 (Bengtsson-Palme *et al.* 2013). The bacterial and fungal reads were clustered into Operational Taxonomic Units (OTUs) at 97% similarity using the open reference approach of the QIIME package version 1.8.0 (Caporaso *et al.* 2010). Taxonomy was annotated using UCLUST (Edgar 2010) with the databases SILVA 132 (Quast *et al.* 2013) for bacteria or UNITE version 7.1 (Kõljalg *et al.* 2013) for fungi. OTUs annotated as Archaea, “Chloroplast”, and “Mitochondria” were removed from the OTU tables.

Data Analysis

The OTU tables were rarefied for alpha diversity analyses to the lowest sequencing depth obtained from a sample (30,012 reads for bacteria and 26,863 for fungi). Species richness was determined using Chao1 (estimated number of OTUs) and the observed species (number of OTUs), and species diversity was calculated using Pielou's Evenness, Shannon's H', and Simpson's indices. These parameters were calculated using the script alpha_diversity.py of the QIIME package version 1.8.0 (Caporaso *et al.* 2010).

Relationships across soil properties, microbiome composition, soil compartment, site, stand, and tree health condition were investigated using PRIMER-E (Clarke and Gorley 2006). OTU tables were log(X+1) transformed, and similarity matrices were constructed using the Bray-Curtis similarity index. Distance-Based Redundancy Analysis (dbRDA) was then applied to explore the soil properties and microbial data. Permutational Multivariate Analysis of Variance (PERMANOVA) was used to test the differences according to soil compartment, site, stand, and tree health condition. Correlations between soil properties distance matrices and microbial similarity matrices were tested using a non-parametric Mantel test (RELATE function of PRIMER-E). The soil properties that best explained the bacterial and fungal community patterns were determined by BEST analysis (Clarke and Ainsworth 1993) using the same software.

Fungi were sorted into ecologically meaningful categories by assigning OTUs to ecological guilds, whenever possible, using FUNGuild (Nguyen *et al.* 2016).

DESeq 2.0 (Love *et al.* 2014) from the MicrobiomeAnalyst (Dhariwal *et al.* 2017) was used to perform the differential abundance analyses and determine which bacterial and fungal OTUs differed across the sites, stands, and tree health conditions. OTUs with less than two counts per sample and less than 20% prevalence across the samples were excluded. Moreover, OTUs with less than 10% of the variance were also removed based on the interquartile ranges. Variance stabilisation transformation was applied using weighted trimmer mean of M-values (TMM). The analysis outputs were used to create Volcano plots using Microsoft Excel.

Analysis of variance (ANOVA) or Kruskal-Wallis test were performed, where applicable, to assess the differences in soil properties and microbial data. Pairwise comparisons were assessed using Tukey's HSD post hoc or Mann–Whitney U test. All assumptions were checked before statistical analysis. IBM SPSS statistics 22 (IBM Corp. 2013) was used to carry out the statistical analysis.

Results

Soil compartments of cork oak

The physicochemical properties and microbiome composition differed significantly between rhizosphere and bulk soil of the cork oak trees ($P < 0.05$; Supplementary Figure 2.2; Supplementary Tables 2.2 and 2.3). In general, the rhizosphere was moister, more acidic, and had a higher content of C, N, and C:N ratio than bulk soil (Supplementary Table 2.2). While no clear trend was observed in alpha diversity, microbiome composition significantly differed between rhizosphere and bulk soil across all the sites and stands ($P<0.05$, Supplementary Figure 2.2, Supplementary Table 2.4). These results show that the physicochemical properties and microbial communities are specific to the soil compartment. Therefore, all further analyses were only based on the rhizosphere data to focus on the physicochemical properties and microbiome of this niche across sites, stands, and tree health conditions.

Rhizosphere physicochemical properties across sites, stands, and tree health conditions

The rhizosphere physicochemical properties differed significantly across the sites and stands (Table 2.1; $P < 0.05$) but not between the tree health categories (asymptomatic versus COD) (Supplementary Table 2.5; $P < 0.05$).

Table 2.1 Physicochemical properties of the asymptomatic and COD tree rhizospheres sampled at the *vitality* and *mortality* stands across Divor and Baixo Sorraia.

Site	Forest health condition	Moisture (%)	pH (H_2O)	Total C (mg/kg)	Total N (mg/kg)	C:N ratio
Baixo Sorraia	*Vitality*	9.13 ± 1.81^a	4.84 ± 0.24^a	$23,152 \pm 11,210^a$	$1,063 \pm 258^a$	21.30 ± 4.76^a
	Mortality	16.99 ± 5.17^b	4.82 ± 0.31^a	$63,994 \pm 42,607^b$	$2,821 \pm 1,759^b$	22.19 ± 2.02^a
Divor	*Vitality*	8.72 ± 2.00^a	5.51 ± 0.44^b	$13,279 \pm 4,176^c$	733 ± 254^c	18.35 ± 1.51^b
	Mortality	9.68 ± 2.44^a	6.05 ± 0.41^c	$14,427 \pm 4,376^c$	789 ± 261^c	18.50 ± 1.43^b

Mean ± standard deviation is shown. Superscript letters indicate significant differences ($P < 0.05$) as determined by the statistical analyses described in the methods section.

At the site level, rhizosphere pH was distinctly more acidic in Baixo Sorraia (4.83 on average) compared to Divor (5.78, $P < 0.05$, Table 2.1). Higher amounts of C and N and a higher C:N ratio were detected in Baixo Sorraia (Table 2.1, $P < 0.05$). At the stand level, moisture content was significantly higher in the *mortality* stand of Baixo Sorraia (16.99 % on average) than in the other three stands (9.18 %; $P < 0.05$; Table 2.1). Total C and N were approximately three times higher at the *mortality* stand (63,994 and 2,821 mg/ soil kg) than at the *vitality* stand (21,818 and 1,029 mg/ soil kg) in Baixo Sorraia. When compared to Divor, these parameters were four to five times higher than the *vitality* (13,720 and 760 mg/ soil kg) and *mortality* (14,202 and 775 mg/ soil kg) stands, respectively.

Rhizosphere microbial composition and core microbiome of cork oak

The rhizosphere microbiome in the present study included bacteria and fungi (Supplementary Table 2.6). The most abundant bacterial phyla were assigned to Proteobacteria (30%), Actinobacteria (21%), Acidobacteria (17%), Planctomycetes (11%), Verrucomicrobia (6%), Chloroflexi (4%), Bacteroidetes (3%), Firmicutes (2%), Patescibacteria (2%), and Gemmatimonadetes (1%), together comprising 98% of the sequences across the 43 phyla detected (Supplementary Figure 2.3A). A total of 9 fungal phyla were detected. Most of the sequences were assigned to the Ascomycota (47%), Basidiomycota (42%), Zygomycota (8%, currently circumscribed as Mucoromycota and Zoopagomycota (Spatafora *et al.* 2016)) and unidentified fungi (3%). The remaining phyla (less than 1% of total sequences)

accounted for 2 and 0.4% of the total bacterial and fungal communities, respectively (Supplementary Figure 2.3B).

The core microbiome was defined across all rhizospheres of cork oaks, regardless of site, stand and tree health, by setting a stringent criterion (threshold of 100%). Therefore, only OTUs present in all samples encompassed the core rhizosphere microbiome. Although all of the rhizosphere samples shared a tiny fraction of the total bacterial (0.5%) and fungal (0.3%) OTUs, this core microbiome represented a large fraction (31% and 22%, respectively) of all sequence reads. The core OTUs were assigned to 10 bacterial and 3 fungal phyla, namely Proteobacteria (38.1 %), Actinobacteria (28.5%), Acidobacteria (16.9%), Planctomycetes (9.7%), Verrucomicrobia (2.3%), Firmicutes (2.1%), Bacteroidetes (1.1%), Chloroflexi (0.8%), WPS-2 (0.2%), and Armatimonadetes (0.2%) for bacteria (Figure 2.1A, Supplementary Table 2.7), and Ascomycota (80%), Basidiomycota (16%), and Zygomycota (4%) for fungi (Figure 2.1B, Supplementary Table 2.7). The most abundant bacterial genera (> 1%) belonged to *Acidothermus* (11 OTUs, 9.25%), *Bradyrhizobium* (9 OTUs, 9.15%), *Bryobacter* (6 OTUs, 3.96%), *Conexibacter* (3 OTUs, 3.58%), Candidatus *Solibacter* (17 OTUs, 3.40%), *Sphingomonas* (5 OTUs, 3.12%), *Singulisphaera* (13 OTUs, 2.49%), *Bacillus* (3 OTUs, 1.98%), *Roseiarcus* (2 OTUs, 1.56%), *Mycobacterium* (5 OTUs, 1.53%), *Acidipila* (3 OTUs, 1.31%), *Haliangium* (8 OTUs, 1.21%), *Burkholderia-Caballeronia-Paraburkholderia* (5 OTUs, 1.14%), and *Acidibacter* (2 OTUs, 1.10%) (Supplementary Table 2.7). The most abundant fungal OTUs belonged to *Penicillium* (7 OTUs, 26.20%), *Cladophialophora* (3 OTUs, 4.40%), *Mortierella* (1 OTU, 3.00%), *Trichoderma* (1 OTU, 2.10%), *Sporothrix* (1 OTU, 1.60%), and *Phallus* (1 OTU, 1.50%) (Supplementary Table 2.7).

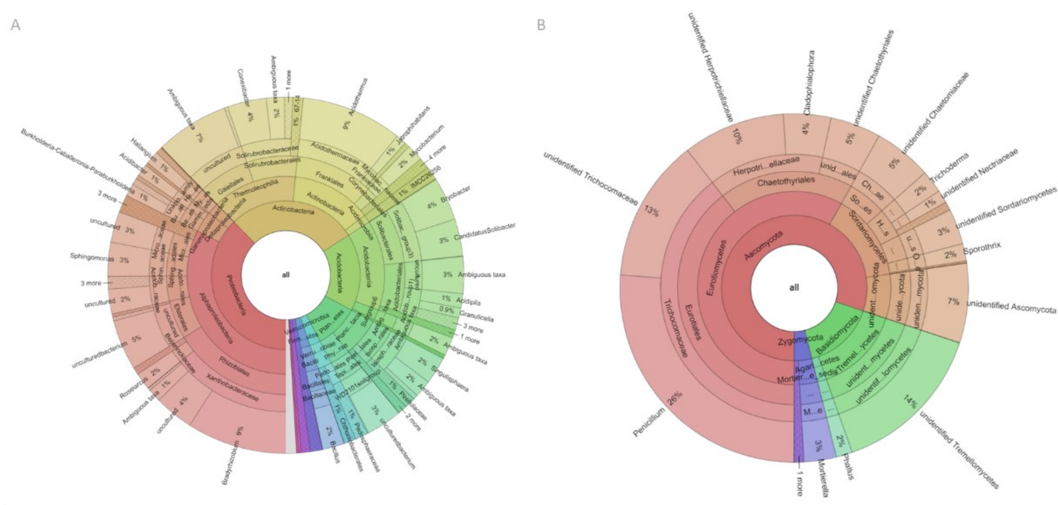

Figure 2.1 Rhizosphere core microbiome of the cork oak. Only the bacterial (A) and fungal (B) OTUs present in all rhizosphere samples were included. Krona charts are coloured at the phylum level for both communities.

Rhizosphere microbiome of cork oak across sites, stands, and tree health conditions

Bacterial α-diversity was significantly higher in Divor than in Baixo Sorraia ($P < 0.05$), but there were no significant differences according to stand and tree health conditions (Supplementary Table 2.8). There were no significant differences in fungal α-diversity between sites, stands, or tree health conditions (Supplementary Table 2.8).

However, distance-based Redundancy Analysis mainly separated Divor and Baixo Sorraia sites along the first axis, whereas the influence of the stand health condition was evident along the second axis (Figure 2.2). These were significant differences in composition (PERMANOVA: $P < 0.05$) for bacteria and fungi between sites and stand health conditions, but no significant difference between tree health conditions (Supplementary Table 2.9).

There were significant correlations between rhizosphere physicochemical properties and bacterial and fungal composition (Mantel $r = 0.655$, $p < 0.0001$; Mantel $r = 0.602$, $p < 0.0001$, respectively). Furthermore, BEST analysis revealed that moisture and pH were the best explanatory variables of variation in the

composition of bacteria ('Spearman's rs = 0.736, P < 0.0001) and fungi ('Spearman's rs = 0.640, P < 0.0001) (Supplementary Table 2.10), which suggests that they are the main drivers of rhizosphere microbial communities.

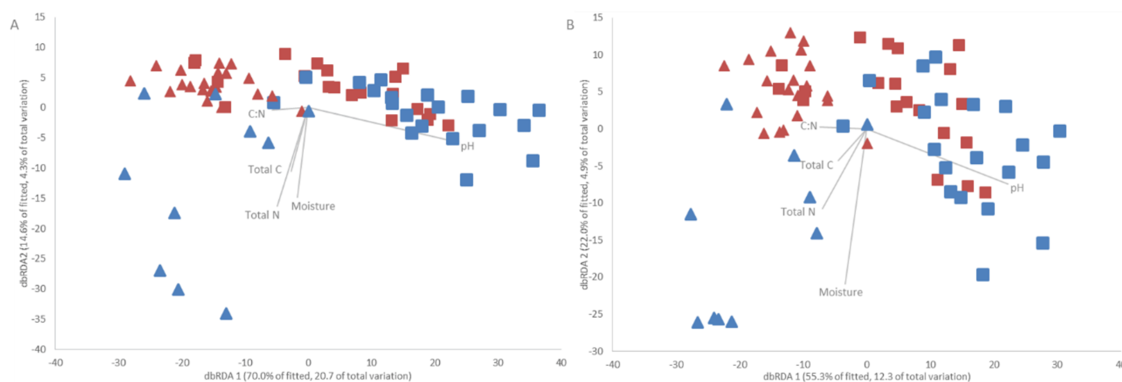

Figure 2.2 Distance-based Redundancy Analysis of bacterial (A) and fungal (B) composition and chemical properties of rhizospheres across sites and stand health conditions. All the rhizosphere chemical parameters are shown. The longer the line, the stronger the correlation. Each point represents the bacterial or fungal community of an individual soil sample. Baixo Sorraia – triangle; Divor – square; *Vitality* – red; *Mortality* – blue.

The DESeq 2.0 analysis revealed 1,612 bacterial and 231 fungal OTUs that differed significantly between Divor and Baixo Sorraia (Figure 2.3A and 2.3B, Supplementary Table 2.11, P < 0.05). Moreover, at the stand level, a total of 111 and 215 bacterial OTUs were significantly more abundant at the *vitality* stands of Divor and Baixo Sorraia, respectively (Figure 2.3C and 2.3E, Supplementary Table 2.11, P < 0.05). On the other hand, at the *mortality* stands of Divor and Baixo Sorraia, 169 and 196 bacterial OTUs were significantly more abundant, respectively (Figure 2.3C and 2.3E, Supplementary Table 2.11, P < 0.05). In the fungal community, 39 and 71 OTUs were more abundant at the *vitality* stands in Divor and Baixo Sorraia, respectively, and 42 and 35 OTUs at the *mortality* stands (Figure 2.3D and 2.3F, Supplementary Table 2.11, P < 0.05).

At the tree health level, only 1 bacterial and 7 fungal OTUs differed significantly between asymptomatic and COD trees, and they were specific to each site and stand. (Supplementary Figure 2.4; P < 0.05).

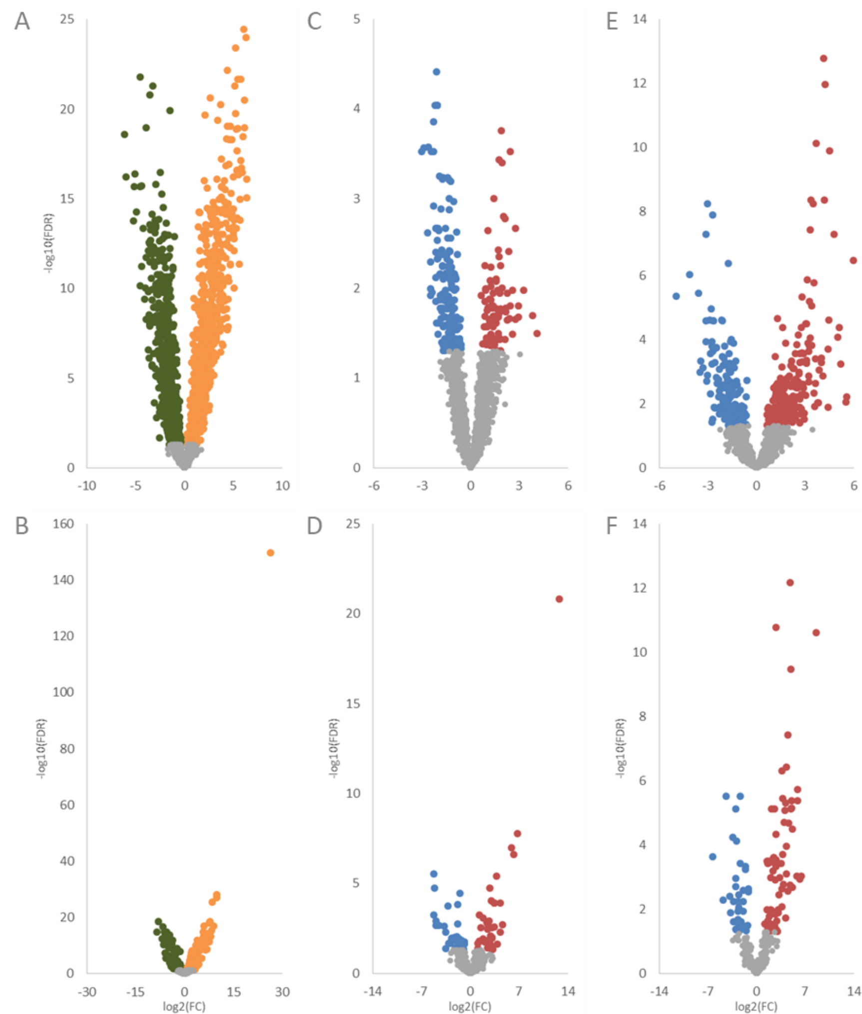

Figure 2.3 Volcano plots of differential bacterial (A, C, and E) and fungal (B, D, and F) OTU abundance analysis as calculated by DESeq 2.0 according to the site (A and B) and stand health condition (Divor: C and D; Baixo Sorraia E and F). Fold change FDR corrected p-values are plotted for each OTU. Significantly different taxa (FDR-corrected P < 0.05) are coloured according to site: Baixo Sorraia – green, Divor – orange; and stand health: *Mortality* – blue, *vitality* – red. No significant OTUs are shown as grey dots.

In the following, we focused only on OTUs that differed significantly and were commonly more abundant at both *vitality* or *mortality* stands in Divor and Baixo Sorraia. Nonetheless, given the main effects on the overall community structure (Figure 2.2), there were other OTUs that exclusively differed between the sites and stand health conditions (Supplementary Table 2.10).

A total of 30 bacterial and 11 fungal OTUs were commonly more abundant at the *vitality* stands. On the other hand, 22 bacteria and 5 fungi were commonly more abundant at the *mortality* stands (Tables 2.2-2.5). OTUs assigned to the phyla Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, WPS-2, and Basidiomycota were exclusively more abundant in the *vitality* stands (Table 2.2 and 2.3). At lower taxonomical levels, examples of OTUs included the bacterial genera *Bryobacter*, *Blastococcus*, *Crossiella*, *Saccharopolyspora*, *Flavisolibacter*, FCPS473, *Thermosporothrix*, *Tumebacillus*, *Bacillus*, *Acidicaldus*, and *Ramlibacter* (Table 2.2), and the fungal genera *Cladophialophora*, *Penicillium*, *Hydnobolites*, *Acremonium*, and *Phallus* (Table 2.3). On the other hand, OTUs of the class Subgroup 6 of the phylum Acidobacteria were more abundant at *mortality* stands (Table 2.4). The phylum Zygomycota was only detected in the mortality stands, namely *Mortierella* (Table 2.5). Further examples of OTUs, which were more abundant in the mortality stands, included the bacterial genera *Conexibacter*, *Phenylobacterium*, *Dongia*, *Bradyrhizobium*, *Sphingomonas*, *Burkholderia-Caballeronia-Paraburkholderia*, *Caenimonas*, and *Chthoniobacter* (Table 2.4) and the fungal genera *Penicillium* and *Acremonium* (Table 2.5).

Table 2.2 Bacterial OTUs more abundant in the *vitality* stands across Divor and Baixo Sorraia.

Site							Divor		Baixo Sorraia	
Phylum	Class	Order	Family	Genus	Species	OTU ID	*Vitality*	*Mortality*	*Vitality*	*Mortality*
Acidobacteria	Acidobacteriia	Acidobacteriales	unidentified	unidentified	unidentified	HQ118387.1.1335	0.03 ± 0.01	0.01 ± 0.00	0.03 ± 0.01	0.00 ± 0.00
						AB238777.1.1436	0.04 ± 0.02	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.00
						EU680464.1.1478	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
		Solibacterales	Solibacteraceae(Subgroup3)	Bryobacter	unidentified	FPLS01016962.1.1395	0.02 ± 0.01	0.00 ± 0.00	0.02 ± 0.00	0.00 ± 0.00
						KJ191777.1.1463	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.00 ± 0.00
Actinobacteria	Actinobacteria	Frankiales	Geodermatophilaceae	Blastococcus	unidentified	FOEE01000030.63.1587	0.02 ± 0.00	0.01 ± 0.00	0.04 ± 0.01	0.01 ± 0.00
		Pseudonocardiales	Pseudonocardiaceae	Crossiella	unidentified	HM445291.1.1353	0.08 ± 0.03	0.00 ± 0.00	0.14 ± 0.07	0.02 ± 0.02
				Saccharopolyspora	unidentified	New.CleanUp.ReferenceOTU467327	0.05 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.00 ± 0.00
Bacteroidetes	Bacteroidia	Chitinophagales	Chitinophagaceae	Flavisolibacter	unidentified	KF182253.1.1493	0.06 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.00 ± 0.00
				unidentified	unidentified	EF516100.1.1454	0.13 ± 0.02	0.07 ± 0.01	0.09 ± 0.03	0.03 ± 0.01
Chloroflexi	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae	FCPS473	unidentified	EU043754.1.1341	0.07 ± 0.01	0.02 ± 0.01	0.14 ± 0.03	0.02 ± 0.01
						HM275583.1.1311	0.26 ± 0.11	0.03 ± 0.01	0.55 ± 0.10	0.09 ± 0.03
				Thermosporothrix	unidentified	EF516303.1.1405	0.02 ± 0.00	0.01 ± 0.00	0.07 ± 0.02	0.02 ± 0.01
				unidentified	unidentified	New.ReferenceOTU622	0.04 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	Tumebacillus	unidentified	JX133323.1.1496	0.03 ± 0.00	0.01 ± 0.00	0.04 ± 0.01	0.00 ± 0.00
			Bacillaceae	Bacillus	unidentified	DQ129541.1.1486	0.41 ± 0.19	0.07 ± 0.02	0.75 ± 0.15	0.04 ± 0.01
						EU465896.1.1401	0.07 ± 0.04	0.01 ± 0.00	0.12 ± 0.04	0.01 ± 0.00
						JF210672.1.1372	0.32 ± 0.12	0.14 ± 0.02	0.99 ± 0.24	0.08 ± 0.02
						DQ298356.1.1410	0.03 ± 0.01	0.01 ± 0.00	0.04 ± 0.01	0.00 ± 0.00
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	unidentified	unidentified	EU445223.1.1517	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Planctomycetes	Planctomycetacia	Gemmatales	Gemmataceae	unidentified	unidentified	New.CleanUp.ReferenceOTU62885	0.02 ± 0.01	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
						New.ReferenceOTU80	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
		Isosphaerales	Isosphaeraceae	unidentified	unidentified	JQ311819.1.1446	0.03 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.00
Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	Acidicaldus	unidentified	EF019020.1.1348	0.04 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
				unidentified	unidentified	KM200466.1.1461	0.09 ± 0.02	0.04 ± 0.01	0.37 ± 0.05	0.14 ± 0.05
		Elsterales	unidentified	unidentified	unidentified	HQ119568.1.1355	0.21 ± 0.04	0.08 ± 0.02	0.16 ± 0.03	0.04 ± 0.03
		Rhizobiales	Xanthobacteraceae	unidentified	unidentified	FNLG01000001.4705154.4706639	0.03 ± 0.00	0.01 ± 0.00	0.09 ± 0.01	0.04 ± 0.01
	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	Ramlibacter	unidentified	KC358446.1.1261	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Verrucomicrobia	Verrucomicrobiae	Pedosphaerales	Pedosphaeraceae	unidentified	unidentified	EF516856.1.1486	0.06 ± 0.01	0.03 ± 0.00	0.08 ± 0.01	0.03 ± 0.01
WPS-2	unidentified	unidentified	unidentified	unidentified	unidentified	New.ReferenceOTU752	0.07 ± 0.02	0.02 ± 0.01	0.27 ± 0.03	0.11 ± 0.02

Each row represents a specific OTU and its relative abundance (%) across the stand health conditions and sites, based on the total community.

Table 2.3 Fungal OTUs more abundant in the *vitality* stands across Divor and Baixo Sorraia.

Site							Divor		Baixo Sorraia	
Phylum	Class	Order	Family	Genus	Species	OTU ID	*Vitality*	*Mortality*	*Vitality*	*Mortality*
Ascomycota	Dothideomycetes	Pleosporales	unidentified	unidentified	unidentified	SH008348.07FU_JX624287_reps	0.05 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.00 ± 0.00
	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora*	unidentified	New.ReferenceOTU183	0.04 ± 0.01	0.02 ± 0.02	0.06 ± 0.02	0.07 ± 0.06
				unidentified	unidentified	New.ReferenceOTU841	0.31 ± 0.12	0.06 ± 0.04	0.36 ± 0.16	0.10 ± 0.07
			unidentified	unidentified	unidentified	SH013078.07FU_EU888622_refs	0.14 ± 0.06	0.01 ± 0.00	0.28 ± 0.07	0.02 ± 0.01
		Eurotiales	Trichocomaceae	*Penicillium*	unidentified	New.ReferenceOTU1039	0.02 ± 0.01	0.00 ± 0.00	0.02 ± 0.01	0.00 ± 0.00
	Leotiomycetes	Helotiales	unidentified	unidentified	unidentified	SH018374.07FU_AF081443_refs	0.08 ± 0.05	0.01 ± 0.01	0.03 ± 0.01	0.00 ± 0.00
	Pezizomycetes	Pezizales	Pezizaceae	*Hydnobolites*	unidentified	New.ReferenceOTU62	2.15 ± 0.76	0.00 ± 0.00	0.73 ± 0.40	0.12 ± 0.11
	Sordariomycetes	Hypocreales	Hypocreales_fam_Incertae_sedis	*Acremonium*	unidentified	SH020352.07FU_GU187844_reps	0.10 ± 0.04	0.02 ± 0.00	0.04 ± 0.01	0.03 ± 0.03
Basidiomycota	Agaricomycetes	Phallales	Phallaceae	*Phallus*	*P. impudicus*	New.ReferenceOTU142	0.53 ± 0.17	0.15 ± 0.02	1.09 ± 0.16	0.14 ± 0.02
		Thelephorales	Thelephoraceae	unidentified	unidentified	SH009865.07FU_UDB000219_reps	0.46 ± 0.26	0.04 ± 0.02	0.11 ± 0.03	0.01 ± 0.00
Unidentified Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	SH479246.07FU_GU078635_reps_singleton	0.32 ± 0.13	0.04 ± 0.01	0.36 ± 0.08	0.01 ± 0.00

Each row represents a specific OTU and its relative abundance (%) across the stand health conditions and sites, based on the total community.

Table 2.4 Bacterial OTUs more abundant in the *mortality* stands across Divor and Baixo Sorraia.

Site							Divor		Baixo Sorraia	
Phylum	Class	Order	Family	Genus	Species	OTU ID	*Vitality*	*Mortality*	*Vitality*	*Mortality*
Acidobacteria	Subgroup6	unidentified	unidentified	unidentified	unidentified	EF018912.1.1402	0.03 ± 0.00	0.08 ± 0.01	0.03 ± 0.01	0.07 ± 0.02
						FJ004793.1.1373	0.15 ± 0.02	0.27 ± 0.03	0.11 ± 0.01	0.26 ± 0.05
						GU260708.8566.10112	0.02 ± 0.00	0.07 ± 0.01	0.00 ± 0.00	0.03 ± 0.01
						HM062099.1.1473	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.06 ± 0.01
						HQ598262.1.1507	0.02 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
						JX898169.1.1549	0.02 ± 0.00	0.05 ± 0.01	0.03 ± 0.00	0.08 ± 0.02
						JX898179.1.1548	0.13 ± 0.02	0.25 ± 0.03	0.06 ± 0.01	0.15 ± 0.03
Actinobacteria	Thermoleophilia	Solirubrobacterales	67-14	unidentified	unidentified	JX079307.1.1503	0.03 ± 0.00	0.05 ± 0.01	0.01 ± 0.00	0.04 ± 0.01
			Solirubrobacteraceae	Conexibacter	unidentified	FPLS01009980.35.1544	0.03 ± 0.00	0.05 ± 0.01	0.02 ± 0.00	0.13 ± 0.03
Planctomycetes	Phycisphaerae	Tepidisphaerales	WD2101soilgroup	unidentified	unidentified	JF185750.1.1331	0.01 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.02 ± 0.00
	Planctomycetacia	Gemmatales	Gemmataceae	unidentified	unidentified	KC541429.1.1521	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.01
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium	unidentified	AM116723.1.1227	0.05 ± 0.01	0.09 ± 0.02	0.03 ± 0.00	0.07 ± 0.02
		Dongiales	Dongiaceae	Dongia	unidentified	EU589252.1.1464	0.06 ± 0.01	0.14 ± 0.02	0.00 ± 0.00	0.04 ± 0.01
					unidentified	FPLL01000156.16.1491	0.01 ± 0.00	0.04 ± 0.01	0.00 ± 0.00	0.02 ± 0.01
		Reyranellales	Reyranellaceae	unidentified	unidentified	JQ923542.1.1456	0.01 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.02 ± 0.00
		Rhizobiales	Xanthobacteraceae	Bradyrhizobium	unidentified	HQ836181.1.1284	0.06 ± 0.01	0.10 ± 0.01	0.03 ± 0.01	0.07 ± 0.01
				unidentified	unidentified	FJ936955.1.1446	0.03 ± 0.00	0.11 ± 0.02	0.02 ± 0.01	0.08 ± 0.02
		Sphingomonadales	Sphingomonadaceae	Sphingomonas	unidentified	AB021492.1.1481	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.01
	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	Burkholderia-Caballeronia-Paraburkholderia	unidentified	AJ605717.1.1446	0.02 ± 0.00	0.06 ± 0.02	0.01 ± 0.00	0.08 ± 0.04
				Caenimonas	unidentified	FPLS01042243.15.1524	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.01
Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	Chthoniobacter	unidentified	EU135526.1.1360	0.02 ± 0.00	0.05 ± 0.01	0.01 ± 0.00	0.04 ± 0.00
		Pedosphaerales	Pedosphaeraceae	unidentified	unidentified	JN656844.1.1514	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.14 ± 0.04

Each row represents a specific OTU and its relative abundance (%) across the stand health conditions and sites, based on the total community.

Table 2.5 Fungal OTUs more abundant in the *mortality* stands across Divor and Baixo Sorraia.

Site							Divor		Baixo Sorraia	
Phylum	Class	Order	Family	Genus	Species	OTU ID	*Vitality*	*Mortality*	*Vitality*	*Mortality*
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium*	unidentified	SH013505.07FU_JN617674_refs	0.02 ± 0.01	0.07 ± 0.02	0.20 ± 0.05	0.52 ± 0.04
			unidentified	unidentified	unidentified	SH014171.07FU_KF767326_reps_singleton	0.00 ± 0.00	0.04 ± 0.03	0.01 ± 0.00	0.07 ± 0.03
	Sordariomycetes	Hypocreales	Hypocreales_fam_Incertae_sedis	*Acremonium*	*A. alternatum*	SH022859.07FU_HE608637_reps	0.02 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.11 ± 0.05
			unidentified	unidentified	unidentified	SH021118.07FU_EF619702_reps	0.00 ± 0.00	0.03 ± 0.01	0.12 ± 0.09	0.13 ± 0.05
Zygomycota	Mortierellomycotina_cls_Incertae sedis	Mortierellales	Mortierellaceae	*Mortierella*	unidentified	New.ReferenceOTU612	0.02 ± 0.01	0.27 ± 0.11	0.03 ± 0.01	0.20 ± 0.06

Each row represents a specific OTU and its relative abundance (%) across the stand health conditions and sites, based on the total community.

Focusing on the fungal ecological guilds, ectomycorrhiza (EcM) were the most abundant across the sites and stands (30.65 ± 0.99, Supplementary Table 2.12). A more detailed analysis, using DESeq 2.0, revealed that particular EcM OTUs were enriched in Divor: *Hydnobolites*, *Cortinarius*, and *Astraeus* were more abundant at the *vitality* stand, whereas *Amanita*, *Russula*, and *Tomentella* were more abundant at the *mortality* stand (Supplementary Table 2.11). In Baixo Sorraia, a higher abundance of *Hydnobolites*, *T. pseudoleptoderma*, *A. torrendii*, *Pisolithus*, *Russula vesca*, and *Tomentella* was detected at the *vitality* stand compared to the *mortality* stand, where a single OTU assigned to the genus *Russula* was more abundant (Supplementary Table 2.11).

Saprobes were the second most abundant guild (20.49 ± 0.54). Plant saprobes were more abundant at both *mortality* stands, whereas dung and soil saprobes' relative abundance were significantly higher in Divor ($P < 0.05$; Supplementary Table 2.12). Notably, plant pathogens and fungal parasites were significantly more abundant in Divor, particularly in the *mortality* stand ($P < 0.05$; Supplementary Table 2.12).

Discussion

Cork oaks have been suffering from chronic oak decline (COD), a complex disorder that jeopardises essential ecosystem services at socio-economic, environmental, and biodiversity levels. Increasing our knowledge of factors related to tree health, such as the rhizosphere microbiome, helps develop more efficient, affordable, and reliable forest management strategies, which may help predict and mitigate these events. In this study, we investigated the rhizosphere physicochemical properties and microbial communities of asymptomatic and COD-affected cork oak trees located across sites and stands that spanned different stages of decline.

Soil pH is critical in cork oak growth and development, with woodlands predominantly occurring in acidic soils (Serrasolses *et al.* 2009). At the soil-root

interface, the rhizosphere pH also influences the availability of many nutrients as a mechanism to compensate for unbalanced uptake (Hinsinger *et al.* 2003). Here, rhizosphere pH was less acidic and substantially poorer in nutrients in Divor, the site with serious concerns regarding forest health over the last decades (APFC 2016a). On the other hand, cork oak rhizospheres in Baixo Sorraia were more acidic, higher in C and N content, and C:N ratio. These results suggest that trees in Baixo Sorraia may be inducing an acidification process in their rhizosphere to enhance nutrient uptake. Using this pH-trigger mechanism, cork oak trees may undergo a set of physiological responses to maintain their resilience under the typical, harsh, and poor environmental conditions of the Mediterranean climate, where soils are usually shallow and nutrient-poor (Marañón 1988). Indeed, cork oak has a remarkable ability of rhizosphere acidification (Serrasolses *et al.* 2009), which also seems to be a universal stress mechanism among *Quercus* species (Pinho *et al.* 2020; Scarlett *et al.* 2021). This acidification process still requires experimental work to prove and understand the mechanisms adopted by trees to withstand abiotic and biotic disturbances. Moreover, increased C and N mobilisation also affect the rhizosphere's water content, as soils with a higher organic matter usually have higher water-holding capacities (Hudson 1994). Therefore, the higher amount of C and N in Baixo Sorraia, notably at the *mortality* stand, were complemented by increased water content in cork oak rhizospheres.

Our results showed that the rhizosphere microbial community differed significantly from the bulk soil. Proteobacteria, Acidobacteria, Actinobacteria, Ascomycota, and Basidiomycota dominated the rhizosphere niche, which lines up with previous findings in oak (Buée *et al.* 2009; Uroz *et al.* 2010; Fernández-González *et al.* 2017; Reis *et al.* 2018, 2019), beech, spruce (Uroz *et al.* 2016b), and poplar (Beckers *et al.* 2017; Cregger *et al.* 2018). An important part of this study aimed to identify the core rhizosphere microbiome of the cork oak. It is thought that the microbes consistently present across samples are likely to provide critical functions to their hosts (Shade and Handelsman 2012). Notably, our results showed that a negligible fraction of the total OTUs comprised the core microbiome (0.3-0.5 % of the total OTUs), but they represented a large proportion of the whole microbial community (22-31% of the total reads). Studies of the rhizosphere

microbiome of melojo oak (*Quercus pyrenaica*) and common bean (*Phaseolus vulgaris*) also reported a very low number of bacterial taxa, which were highly abundant in the core microbiome (Lasa *et al.* 2019; Pérez-Jaramillo *et al.* 2019). Overall, the core microbiome consisted of bacterial and fungal genera with potentially key functions for cork oak health and resilience, including carbon and nitrogen cycling (*Bradyrhizobium*, *Acidothermus*, *Conexibacter,* Candidatus *Solibacter*, *Bryobacter*, *Singulisphaera*, *Roseiarcus*, *Mycobacterium*, *Acidipila*, *Mortierella*, *Umbelopsis*, *Sporothrix*, *Phallus*, and *Purpureocillium*), antibiotic production (*Haliangium)*, plant growth-promoting (*Sphingomonas*), iron-reducing (*Acidibacter*), mineral-weathering (*Burkholderia*-*Caballeronia*-*Paraburkholderia*), and biological control (*Bacillus*, *Penicillium*, *Trichoderma*, and *Cladophialophora)*. Whether these taxa have positive roles in cork oak is unknown but merits further investigation.

Although we did not find a clear association between tree health and rhizosphere microbial composition, as initially hypothesised, there were marked differences between sites and stand health conditions. This suggests a scale effect whereby shifts in the rhizosphere microbiome of asymptomatic and decline conditions play out at the site and stand levels as opposed to the individual tree level. A more in-depth microbial community analysis allowed us to depict abundant bacterial and fungal taxa according to stand health. It is interesting to highlight that some of the genera that comprised the core microbiome of cork oak were also abundant components of the *vitality* stands, namely *Bacillus*, *Bryobacter*, *Cladophialophora*, and *Phallus*. Considering that they are part of the core microbiome and are significantly more abundant at the *vitality* stands, it is tempting to point them out as key providers of critical functions for cork oak trees. However, whether the association with these bacteria and fungi results in better tree health and resilience remains hypothetical and opens interesting routes for further research.

Moreover, differences in microbiome condition were significantly related to rhizosphere physicochemical properties across the sites and stand health, particularly soil pH and moisture. Indeed, within a wide range of soil properties, pH

has been described as one of the main contributors to community structure in *Quercus* species (Maghnia *et al.* 2017; Pinho *et al.* 2020; Scarlett *et al.* 2021).

EcM fungi, known to establish crucial mutualistic relationships with oak trees (Smith and Read 2008; Bonfante and Genre 2010), were abundant across the sites and stands but varied in their composition. In studies on *Quercus ilex*, *Q. suber*, *Q. robur*, and *Q. petraea*, EcM composition was shown to change with tree health status; root tips of declining oaks, however, were still able to establish symbiotic mycorrhizal associations (Kovacs *et al.* 2000; Montecchio *et al.* 2004; Lancellotti and Franceschini 2013; Corcobado *et al.* 2014, 2015; Bzdyk *et al.* 2019). As hypothesised by Montecchio *et al.* (2004), oaks may lose their ability to select the most efficient EcM and replace them with less efficient symbionts. Furthermore, we found plant pathogenic fungi and fungal parasites to be more abundant in Divor, particularly at the *mortality* stand. This finding suggests that trees located in this site, experiencing higher forest deterioration, are more prone to colonise opportunistic organisms.

Conclusions

The results presented in this study provide baseline information on the rhizosphere physicochemical properties and microbiome of cork oak trees and provide evidence that the rhizosphere communities, moisture, and pH are correlated to chronic cork oak decline at the site and stand levels. On the other hand, no clear association was found between the overall rhizosphere microbiome and COD (based on visual assessment of tree health). This may be due to the complex multifactorial nature and the poor aetiology of this syndrome combined with the heterogeneity of soil properties, leading to a great deal of variation in soil community composition (Fierer 2017; Veach *et al.* 2019). Nevertheless, enrichment of members of the core microbiome belonging to the genera *Bacillus*, *Bryobacter*, *Cladophialophora*, and *Phallus* at vitality stands provides an indication that these taxa could be used as potential microbial indicators for early detection of chronic cork oak decline. Future research should aim for more in-depth

knowledge of their role in the cork oak rhizosphere and COD and understand the underlying mechanisms. Translational applications could be developed using microbial and physicochemical markers to improve the prediction and detection of the emergent decline syndromes or, in the long-term, their use to efficiently enhance tree health, resistance, and resilience to curb biological threats and abiotic disturbances in the context of global change.

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Author Contributions

Conceptualisation, D.P., N.C.M.G., D.C., C.E.; methodology, D.P., C.B., H.F., N.C.M.G., D.C., C.S.S., C.E.; formal analysis, D.P., C.B., and H.F.; writing—original draft preparation, D.P.; writing—review and editing, D.P., N.C.M.G., D.C., C.E.; supervision, C.E.; project administration, C.E.; funding acquisition, C.S.S., C.E. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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CHAPTER 3

LINKING TREE HEALTH, RHIZOSPHERE PHYSICOCHEMICAL PROPERTIES, AND MICROBIOME IN ACUTE OAK DECLINE

Linking tree health, rhizosphere physicochemical properties, and microbiome in Acute Oak Decline

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Article

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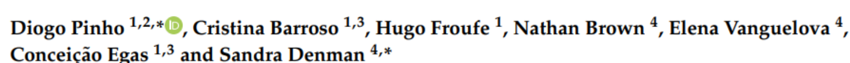
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Abstract

Forest decline-diseases are complex processes driven by biotic and abiotic factors. Although information about host-microbiome-environment interactions in agricultural systems is emerging rapidly, similar studies on tree health are still in their infancy. We used Acute Oak Decline (AOD) as a model system to understand whether the rhizosphere physicochemical properties and microbiome are linked to tree health by studying these two factors in healthy and diseased trees located in three sites in different AOD stages – low, mid and severe.

We found significant changes in the rhizosphere properties and microbiome composition across the different AOD sites and between the tree health conditions. Rhizosphere pH correlated with microbiome composition, with the microbial assemblages changing in more acidic soils. At the severe AOD site, the oak trees exhibited the lowest rhizosphere pH and distinct microbiome, regardless of their health condition, whereas, at the low and mid-stage AOD sites, only diseased trees showed lower pH and the microbial composition differed significantly from healthy trees. On these two sites, less extreme soil conditions and a high presence of host-beneficial microbiota were observed in the healthy oak trees.

For the first time, this study gathers evidence of associations among tree health conditions, rhizosphere properties, and microbiome and links aboveground tree decline symptoms to the belowground environment. It provides a baseline of rhizosphere community profiling of UK oak trees and paves the way for these associations to be investigated in other tree species suffering decline-disease events.

Keywords: Acute Oak Decline; Decline-disease; Microbiome; Rhizosphere; Soil chemistry; Tree health; Belowground; Aboveground

Introduction

Forests are globally important systems (Trumbore *et al.* 2015), often damaged by disturbances, which leads to significant loss of trees. This is of great concern as humans and environments rely on the functions, goods, and services provided by these essential ecosystems (Boyd *et al.* 2013; Pautasso *et al.* 2015; Trumbore *et al.* 2015). One of the far-reaching consequences of forest disturbance is tree decline and the onset of decline-diseases. Decline-diseases, defined by Manion and Lachance (1992) (Manion and Lachance 1992), are the result of the 'interaction of interchangeable, specifically ordered, abiotic and biotic factors that produce a general deterioration, often ending in the death of trees'. Three distinct phases in the decline process are recognised: predisposition, inciting, and contributing (Manion 1981). Predisposition factors are those related, in general, to site resilience and involve long term influences of the environmental and/or biological factors that weaken the host before the onset of decline. Inciting factors are short term and transient factors of biotic and/or abiotic origin, such as insect defoliating events, extreme weather conditions or human activities, amongst others. The contributing factors include secondary pests and diseases that can be very destructive to weakened trees and bring them to death. According to this concept, single or multiple factors within one category are not able, alone, to drive the decline (Ciesla and Donaubauer 1994). Recent evidence suggests that tree decline-disease interactions are more complex than previously envisaged, and occur at a range of scales spanning landscape to molecule (Brown *et al.* 2018; Broberg *et al.* 2018). In view of new research towards a deeper understanding of the mechanistic and functional components of plant-microbe interactions at the systems level (Levy *et al.* 2018), studies elucidating the drivers and mechanisms behind decline-diseases in the UK are underway using oak decline as a platform for investigation.

There are only two native oak species in the UK, *Quercus robur* L. (pedunculate oak) and *Quercus petraea* (Matt.) Liebl. (sessile oak). Both are foundation species and susceptible to Acute Oak Decline (AOD). This recent emerging threat is spreading through Britain's woodlands, parklands, urban landscapes, and

gardens, raising much concern amongst landowners and managers as affected trees can rapidly decline over 3-5 years with a high number of symptomatic trees dying (Denman and Webber 2009; Denman *et al.* 2014). Bark cracks, necrotic inner tissues and dark exudates bleeding on the stems, and larval galleries of the buprestid beetle *Agrilus biguttatus* are the distinctive set of visible symptoms characterising the disease (Denman *et al.* 2014). Similar symptoms have also been observed in continental European oaks (Denman *et al.* 2014). The bacterial species *Rahnella victoriana*, *Brenneria goodwinii*, and *Gibbsiella quercinecans* have been consistently isolated from AOD lesions (Denman *et al.* 2016; Brady *et al.* 2017). Using a contemporary approach for adapting and fulfilling Koch's postulates, *B. goodwinii* and *G. quercinecans* were proven capable of oak tissue necrosis, and together with larvae of the beetle, formed the main symptoms of AOD (Denman *et al.* 2018). Modern molecular ('omics') analyses of AOD symptomatic field material provided the link between *B. goodwinii* and *G. quercinecans* and lesion formation, where *B. goodwinii* has a dominant role (Broberg *et al.* 2018). Knowledge about the AOD pathobiome (Denman *et al.* 2018; Broberg *et al.* 2018; Doonan *et al.* 2019) and environmental factors that predispose oak trees to AOD (Brown *et al.* 2018) has led to remarkable advances in understanding and managing AOD in the UK. However, emerging evidence of 'the importance of the microbiome of the plant holobiont' (the holobiome) in host health emphasises the necessity for a better understanding of the microbiomes of the various niches of large, complex, long-lived plants like trees (Hacquard and Schadt 2015; Vandenkoornhuyse *et al.* 2015). A particularly important niche is the rhizosphere, as it is the link between nutrient and water supply and the host demand (Berendsen *et al.* 2012; Hacquard and Schadt 2015). The rhizosphere has a specific chemistry, is rich in microbial diversity, abundance and activity (Buée *et al.* 2009), and is distinct from the bulk soil (Uroz *et al.* 2010, 2016). It is thought that bacterial and fungal communities in this environment have a pivotal role in contributing to tree health and fitness (Hacquard and Schadt 2015), while simultaneously and responsively, the host shapes its microbial assemblage (Sasse *et al.* 2018). Although some research on the rhizosphere microbiomes of several tree species has been reported (Uroz *et al.* 2010, 2016; Gottel *et al.* 2011;

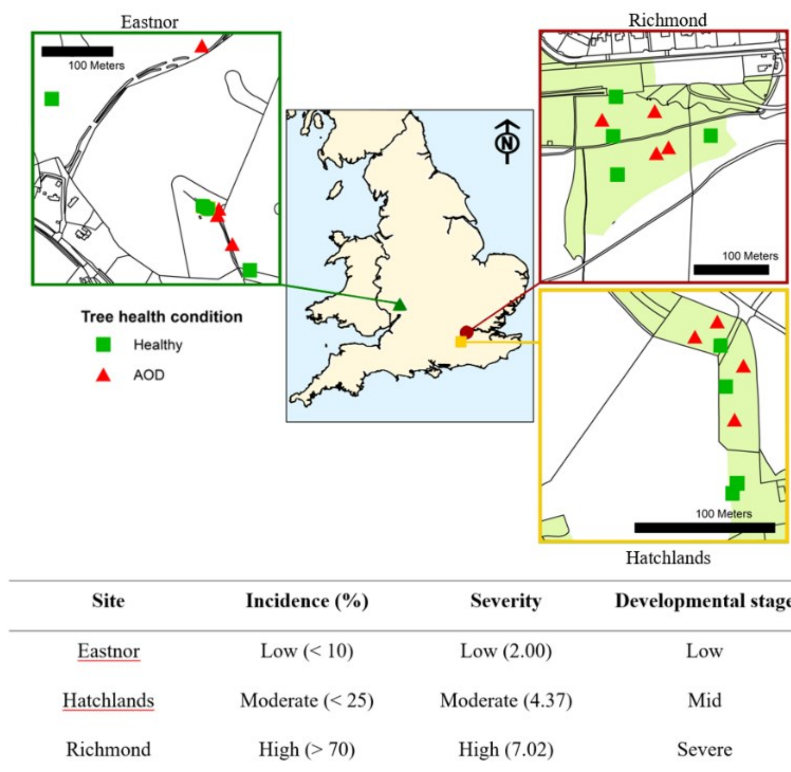
Cobo-Díaz *et al.* 2017; Beckers *et al.* 2017; Gallart *et al.* 2018), knowledge about the linkages among site factors, composition, and role of this specific niche microbiome concerning tree health condition is still lacking. Elucidating the relationships between microbial communities and forest hosts under decline-diseases disturbances is a complex process, which can be disentangled by gathering stepwise cumulative information.

As a starting point, this study describes and compares the physicochemical properties and microbiome of rhizosphere soils of healthy and AOD symptomatic pedunculate oak trees at three UK sites to determine whether rhizosphere physicochemical properties and microbial communities changed according to the tree health condition.

Materials and Methods

Site selection and soil sampling

Three parkland sites were selected based on the presence of pedunculate oak trees with AOD symptoms, the history of AOD monitoring and the willingness of landowners to allow access to the sites: Eastnor Castle Deer Park (Ledbury, SO743371), low-stage AOD; Hatchlands Park (Guildford, TQ067521), mid-stage AOD; and Richmond Park (London, TQ202743), severe-stage AOD (Figure 3.1). The three sites represent a range of AOD developmental stages, which were defined based on AOD incidence and severity monitoring data (Figure 3.1). At Eastnor, AOD symptomatic trees were first reported in 2016 with a small proportion of trees lightly affected, whereas trees at Hatchlands have been monitored for AOD since 2009 with a moderate proportion of moderately affected trees. Although Richmond has only been intensively monitored since 2010 (Brown *et al.* 2016), this site has a long history of severe AOD presence with a high number of severely affected trees and high levels of tree mortality (Gibbs and Greig 1997).



Site	Incidence (%)	Severity	Developmental stage
Eastnor	Low (< 10)	Low (2.00)	Low
Hatchlands	Moderate (< 25)	Moderate (4.37)	Mid
Richmond	High (> 70)	High (7.02)	Severe

Figure 3.1 Location of the UK sites and sampled healthy (green square) and AOD (red triangle) trees in Eastnor (low-stage AOD, green), Hatchlands (mid-stage AOD, yellow), and Richmond (severe-stage AOD, red). Each point represents the specific GPS location of each sampled tree. The overview of the AOD developmental stage is presented in the table below the maps. AOD incidence and severity classifications were defined based on the monitoring data of the number of trees affected (percentage) and bleeds per tree (average number of bleeds per stem), respectively. AOD developmental stage was set by combining disease incidence and severity.

At each site, four healthy and four AOD trees were randomly selected (Figure 3.1) based on the Forest Research monitoring records, following the visual tree health condition criteria as previously reported (Brown *et al.* 2016). The rhizosphere soils were collected after removing the top litter layer. At each tree, four cores were taken under the crown (2-4 m in approximate cardinal directions radially from the trunk), and the fine roots and attached rhizosphere soil were pooled in a single sterile bag. Samples were collected to a depth of 30 cm because the fine roots of trees are mainly distributed in this soil zone. Additionally, four single cores of bulk soil were randomly taken outside the vicinity of the tree root system (i.e. in

surrounding open spaces within each site). The bulk soils were used as control samples to enable comparisons between soil compartments and for the detection of oak rhizosphere effect. In total, 24 rhizosphere and 12 bulk soil samples were collected in November 2016 across Eastnor (low-stage AOD), Hatchlands (mid-stage AOD), and Richmond (severe-stage AOD), corresponding to 8 rhizosphere samples (4 healthy and 4 AOD trees) and 4 bulk soils per site.

Physicochemical analysis

Rhizosphere soils were separated from the roots in the laboratory by gently shaking the roots and collecting the loosened soil. The rhizosphere and bulk soil samples were separately sieved through a 2-mm mesh, and *c.* 5 g of each soil sample was stored at -80 °C until DNA extraction. The remaining soil samples were analysed for moisture using the gravimetric method, pH (in water) using an automated Metrohm autosampler with a 719 S Titrino unit and a Sentek electrode, and total carbon (C) and total nitrogen (N) by dry combustion at 900˚C using a FlashEA1112 NC analyser (Thermo Fisher Scientific, USA). Soil C:N ratio was also calculated for each sample. The analyses were performed at the Chemical Analysis Laboratory of Forest Research, UK.

DNA extraction, PCR library preparation, high-throughput sequencing and data processing

The DNA was extracted from 500 mg of each rhizosphere and bulk soil samples using the NucleoSpin Soil kit (Macherey-Nagel, Germany) with buffer SL2 and Enhancer SX according to the manufacturer’s instructions. Soilless blank controls were included to ensure reagent sterility. Agarose gel electrophoresis, ND1000 spectrophotometer (NanoDrop Technologies, USA) and Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, USA) were used to check the DNA quality and yield. The DNA samples were stored at -20°C for further use.

The DNA extracted from the rhizosphere and bulk soil samples were used for amplification and sequencing of the bacterial V3-V4 variable regions of the 16S rDNA and the fungal Internal Transcribed Spacer 2 (ITS2). The bacterial primers used were: forward primer 341F 5′-CCTACGGGNGGCWGCAG-3′ and reverse primer 805R 5′-GACTACHVGGGTATCTAATCC-3’ (Herlemann *et al.* 2011; Klindworth *et al.* 2013); and the fungal primers used were: forward primers pool ITS3NGS1_F 5’-CATCGATGAAGAACGCAG-3’, ITS3NGS2_F 5’-CAACGATGAAGAACGCAG-3’, ITS3NGS3_F 5’-CACCGATGAAGAACGCAG-3’, ITS3NGS4_F 5’-CATCGATGAAGAACGTAG-3’, ITS3NGS5_F 5’-CATCGATGAAGAACGTGG-3’, and ITS3NGS10_F 5’-CATCGATGAAGAACGCTG-3’, and reverse primer ITS3NGS001_R 5’-TCCTSCGCTTATTGATATGC-3’ (Tedersoo *et al.* 2014).

The first PCR was performed using KAPA HiFi HotStart ReadyMix PCR Kit (Roche, USA), 200 nM of each primer, and 12.5 ng of template environmental DNA in a total volume of 25 µL. The PCR conditions involved a 3 min denaturation at 95 °C, followed by 25 cycles of 98 °C for 20 s, 55 °C (V3-V4) or 60 °C (ITS2) for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min in a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). Negative controls were included. PCR products were run on an agarose gel (1% w/v) and purified using Agencourt AMPure XP (Beckman Coulter, USA) according to the manufacturer's instructions.

Dual indexes and Illumina sequencing adapters from the Nextera XT Index kit (Illumina, USA) were attached to both ends of the amplicons by limited-cycle PCR. This second PCR used KAPA HiFi HotStart ReadyMix PCR Kit (Roche, USA), 2.5 µL of each indexed adapter primer, and 2.5 µL of the first PCR product, in a total volume of 25 µL. PCR conditions included 3 min denaturation at 95 °C, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min in a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). PCR products were checked on an agarose gel (1% w/v) and one-step purified and normalized using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, USA). Amplicon libraries were pooled, and pair-end sequenced using the MiSeq Reagent Kit v3 in the MiSeq platform (Illumina, USA) at Genoinseq

(Cantanhede, Portugal). All sequence data described in this study are available in the European Nucleotide Archive (ENA) under accession number PRJEB29902.

Sequence reads were quality-filtered using PRINSEQ version 0.20.4 (Schmieder and Edwards 2011). Sequencing adapters and reads with less than 150 bp (bacteria) or 100 bp (fungi) were removed. Reads were trimmed where the average base quality was below Q25 in a window of 5 bp. The forward and reverse reads were merged by overlapping the paired-end reads with AdapterRemoval version 2.1.5 (Schubert *et al.* 2016) using default parameters. Chimeric reads were removed using USEARCH version 6.1 (Edgar 2010). The highly variable fungal ITS2 from the merged reads was extracted with ITSx version 1.0.11 (Bengtsson-Palme *et al.* 2013). The bacterial and fungal reads were clustered into Operational Taxonomic Units (OTUs) at 97% of similarity using the open reference approach of the QIIME package version 1.8.0 (Caporaso *et al.* 2010). Taxonomy was annotated using UCLUST (Edgar 2010) with the databases Greengenes version 13.8 (DeSantis *et al.* 2006) for bacteria, or UNITE version 7.1 (Kõljalg *et al.* 2013) for fungi. Singletons (OTUs represented by one single read) were removed from the OTU tables, as well as OTUs annotated as Archaea, "Chloroplast" and "Mitochondria".

Total abundance of soil bacterial and fungal communities

Total bacterial and fungal abundances were estimated by quantifying the copy number of the bacterial 16S rDNA and the fungal ITS2 using qPCR. Concentrated stock solutions (10^7 molecules μl^{-1}) of 16S and ITS amplicon standards were prepared by PCR amplification using the conditions described in PCR library preparation. An equimolar pool of all soil samples was used as template. For each DNA target, a set of calibration standards with known concentrations were generated by serial dilutions. Each sample and standard was quantified in triplicate using 2X Fast SYBR Green Master Mix (Thermo Fisher Scientific, USA), 0.5 µM of each PCR primer (the same used in library preparation) and 1 or 10 ng of template DNA for bacteria or fungi assay in a total volume of 10 µL. The qPCR

conditions involved a 20 s denaturation at 95 °C, followed by 40 cycles of 95 °C for 20 s, 58 °C for 15 s and 60 °C for 45 s for bacteria, and 20 s denaturation at 95 °C, followed by 40 cycles of 95 °C for 10 s, 60 °C for 45 s for fungi, in a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, USA). All qPCR assays included negative template controls using water instead of DNA.

Data Analysis

For alpha diversity analyses, the species richness was assessed using Chao1 (estimated number of OTUs) and the observed species (number of OTUs), and species diversity was calculated using Pielou's Evenness, Shannon, and Simpson indexes. These parameters were calculated using the script alpha_diversity.py of the QIIME package version 1.8.0 (Caporaso *et al.* 2010).

The relationships across soil properties, microbiome composition, soil compartment, site, and tree health condition were investigated using PRIMER-E (Clarke and Gorley 2006). OTU tables were log(X+1) transformed, and the Bray-Curtis similarity matrixes calculated. Distance-Based Redundancy Analysis (dbRDA) was then applied to explore the microbial and soil properties data according to soil compartment, site, and tree health condition. Permutational Multivariate Analysis of Variance (PERMANOVA) was used to test the differences according to the stated factors. Correlations between microbiome composition and soil properties were tested using a non-parametric Mantel test (RELATE function of PRIMER-E) and the soil properties that best explained the bacterial and fungal community patterns were determined by BEST analysis (Clarke and Ainsworth 1993) using the same software.

Fungi were sorted into ecologically meaningful categories by assigning ecological guilds to each community member, whenever possible, using FUNGuild (Nguyen *et al.* 2016).

Differential abundance analyses of each bacterial and fungal OTU detected in the rhizosphere soils across the sites and tree health conditions were performed using

DESeq 2.0 (Love *et al.* 2014) in the MicrobiomeAnalyst (Dhariwal *et al.* 2017). OTUs with less than two counts per sample and prevalence of less than 20% across the samples were excluded. Moreover, OTUs with less than 10 % of the variance, based on interquartile range, were also removed. Variance stabilization transformation was applied using weighted trimmer mean of M-values (TMM). The analysis outputs were used to create Volcano plots using Microsoft Excel. Relative abundances of the significantly abundant fungi (FDR corrected $P < 0.05$) were visually represented in bar plots using Excel or in Circle Packing charts built using the open-source framework RAW Graphs (Mauri *et al.* 2017).

Analysis of variance (ANOVA) or Kruskal-Wallis test were performed, where applicable, to assess the differences according to soil compartment, site, and tree health condition. Pairwise comparisons were assessed using Tukey's HSD post hoc or Mann–Whitney U test. All assumptions were checked before statistical analysis. IBM SPSS statistics 22 (IBM Corp. 2013) was used to carry out the statistical analysis

Results

Physicochemical properties and microbiome are different in the rhizosphere and bulk soil compartments

The physicochemical properties and microbiome differed between the oak rhizosphere and bulk soil compartments in all the three sites (Supplementary Table 3.1, Supplementary Table 3.2, and Supplementary Figure 3.1). At Richmond, the rhizosphere soil was significantly more acidic (pH 4.33 ± 0.14) compared to the bulk soil (5.12 ± 0.07; $P < 0.001$; Supplementary Table 3.1). Bulk soil and rhizosphere pH were similar at Eastnor and Hatchlands (average of 5.66 and 5.92, respectively). Total C and N, and C:N ratio were significantly higher in the rhizosphere than in the bulk soil compartment across all the sites ($P < 0.001$). In particular, the rhizosphere at Richmond showed 8 times more C (225,966 ± 55,586) and 6 times more N (12,921 ± 3,331) compared to the bulk soil

(Supplementary Table 3.1). In general, microbial diversity in rhizosphere soil was slightly higher compared to bulk soil (Supplementary Table 3.2). The soil compartments differed significantly in their microbiome composition, for both bacteria (Pseudo-F = 4.61, $P < 0.01$) and fungi (Pseudo-F = 8.04, $P < 1 \times 10^{-5}$) across the three sites (Supplementary Figure 3.1). The results show that the soil properties and microbial communities are specific to the soil compartment. To further characterize the rhizosphere properties and microbiome and their relation with site and tree health conditions, we excluded the bulk soil data because it is likely that this niche has different drivers from those found in the rhizosphere.

Rhizosphere physicochemical properties differ across sites and tree health conditions

The rhizosphere physicochemical properties varied significantly among the sites, in which Richmond showed the largest differences, and Eastnor and Hatchlands had identical rhizosphere properties (Table 3.1). The rhizosphere soil at Richmond was significantly more acidic (mean = 4.33; $P < 0.001$), the total C concentration was 3.6 times higher (225,966 mg/ soil kg on average; $P < 0.01$) and N concentration 2.8 times higher (12,922 mg/ soil kg, $P < 0.01$), than the other two sites (62,478 and 4,605 mg/ soil kg, respectively). (Table 3.1). Besides, C:N ratio increased significantly ($P < 0.01$) across the sites, in which Eastnor had the lowest values and Richmond the highest (Table 3.1).

Table 3.1 Physicochemical properties of the oak rhizosphere soils across the sites and tree health condition.

Site	AOD stage	Tree health condition	Moisture (%) *	pH (H_2O) ***	Total C (mg/kg) **	Total N (mg/kg) **	C:N ratio **
Eastnor	Low	Healthy	30 ± 1 ^{a}	6.09 ± 0.22 ^{ab}	61,810 ± 4,991 ^{a}	4,877 ± 439 ^{a}	12.69 ± 0.38 ^{a}
		AOD	19 ± 1 ^{c}	5.27 ± 0.31 ^{b}	59,670 ± 15,330 ^{a}	4,380 ± 729 ^{a}	13.40 ± 1.36 ^{ab}
Hatchlands	Mid	Healthy	24 ± 3 ^{bc}	6.43 ± 0.70 ^{a}	64,834 ± 13,077 ^{a}	4,739 ± 989 ^{a}	13.70 ± 0.56 ^{b}
		AOD	20 ± 1 ^{c}	5.42 ± 0.24 ^{b}	63,598 ± 3,852 ^{a}	4,423 ± 480 ^{a}	14.46 ± 0.83 ^{b}
Richmond	Severe	Healthy	27 ± 7 ^{ab}	4.35 ± 0.13 ^{c}	228,313 ± 53,690 ^{b}	12,905 ± 3,356 ^{b}	17.83 ± 0.58 ^{c}
		AOD	26 ± 6 ^{abc}	4.30 ± 0.14 ^{c}	223,618 ± 57,324 ^{b}	12,938 ± 3,305 ^{b}	17.28 ± 0.36 ^{c}

Mean values of soil physicochemical parameters ± standard deviation are shown (n = 24). Asterisks and letters indicate significant differences as determined by statistical analysis (* for $P < 0.05$; ** for $P < 0.01$; *** for $P < 0.001$).

When comparing the healthy and diseased trees within each site, there were significant differences ($P < 0.05$) in the rhizosphere soil pH (Table 3.1). At Eastnor and Hatchlands, the rhizosphere soil of AOD symptomatic trees was significantly ($P < 0.001$) more acidic (pH ranging from 4.84 to 5.74) than healthy trees (pH 5.59-7.52), but no differences were detected at Richmond, where, in both healthy and diseased trees, rhizosphere soils were very acidic (pH 4.14-4.49) (Table 3.1).

Taxonomic distribution of oak rhizosphere microbiome

Single gene community profiling took place on high quality isolated DNA. Yields of 1,661,144 and 1,647,971 16S rDNA and ITS2 high-quality reads were obtained, clustering into 36,841 and 6,519 bacterial and fungal OTUs at 97% distance sequence similarity, respectively (Supplementary Tables 3.3 and 3.4).

Regardless of the site and tree health condition, Proteobacteria (28.1 %), Actinobacteria (24.8 %), Acidobacteria (17.4 %), Verrucomicrobia (9.5 %), Planctomycetes (7.8 %), Firmicutes (3.2 %), Bacteroidetes (2.8 %), and Choroflexi (2.0 %) were the eight dominant phyla, totaling 95.5 % of the entire bacterial community Figure 3.2A). In the fungal community, 82.8 % of the identified OTUs were assigned to Basidiomycota (41.5 %), Ascomycota (32.1 %), and Zygomycota (15.1 %) phyla and the unidentified fungi represented 10.3 % of the total fungal community (Figure 3.2B). The remaining 37 bacterial and 5 fungal detected phyla, which individually represented less than 1% of the total community, were grouped in a group called “Others” and only accounted for 4.5 % and 1.0 % of all bacterial and fungal taxa, respectively (Figure 3.2).

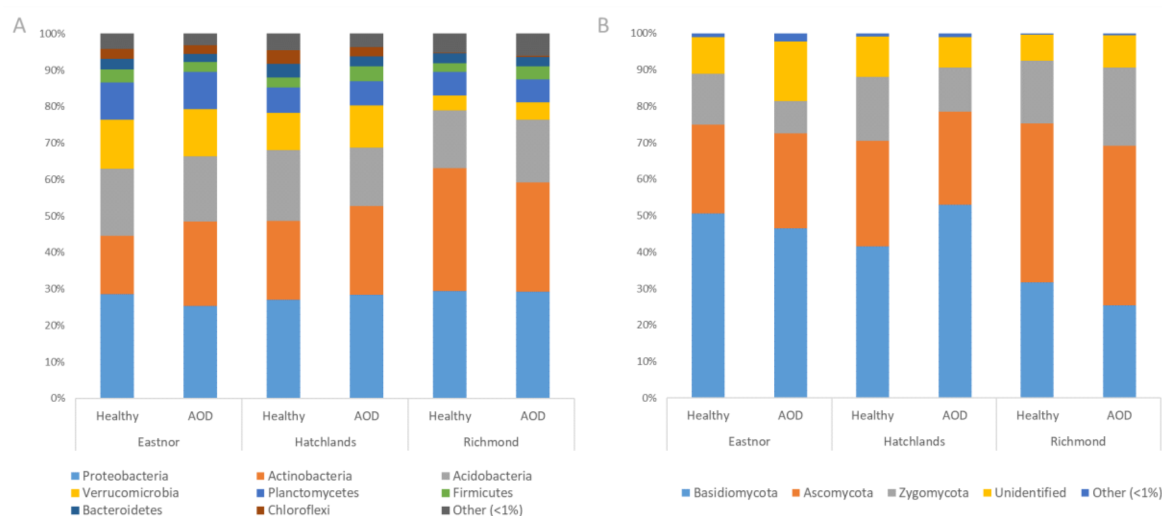


Figure 3.2 Relative abundance of bacterial (A) and fungal (B) phyla detected in the rhizosphere of the healthy and AOD trees in the Eastnor Park, Hatchlands Park, and Richmond Park (n = 24). The phyla that individually represented less than 1% of the total community were grouped in the artificial group “Others”. For bacteria: Gemmatimonadetes, Nitrospirae, WPS-2, WS3, TM7, unidentified Bacteria, Elusimicrobia, Chlamydiae, AD3, Armatimonadetes, Cyanobacteria, TM6, OD1, BRC1, Chlorobi, Tenericutes, Fibrobacteres, FBP, OP3, FCPU426, OP11, WS2, WS4, Spirochaetes, WS6, BHI80-139, GN02, NKB19, GAL15, NC10, [Thermi], Lentisphaerae, MVP-21, Aquificae, GN04, Kazan-3B-28, and WS5. For fungi: Rozellomycota, Chytridiomycota, Glomeromycota, Blastocladiomycota, and Microsporidia.

Oak rhizosphere microbiome composition differs across sites and tree health conditions

We sampled rhizosphere soils of 12 healthy and 12 AOD oak trees across three sites with different AOD developmental stages: Eastnor (low-stage AOD), Hatchlands (mid-stage AOD) and Richmond (severe-stage AOD). Bacterial diversity was more impacted than fungal diversity according to site (Supplementary Table 3.5). Richmond showed significantly lower bacterial diversity compared to Eastnor and Hatchlands ($P < 0.05$), which had similar diversity to each other. The total abundance of bacteria was similar across all three sites. According to the tree health condition, the bacterial diversity was similar between the healthy and AOD oaks within each site, and their total abundance varied significantly with no specific trend between the two health

conditions (Supplementary Table 3.5; P < 0.05). The fungal diversity and total abundance were similar across the sites and tree health conditions (Supplementary Table 3.5).

However, the rhizosphere microbiome composition varied significantly across the sites and tree health conditions, as indicated by dbRDA and PERMANOVA (Figure. 3.3 and Supplementary Table 3.6; P < 0.05). Richmond rhizospheres clustered separately from those of Eastnor and Hatchlands (Figure 3.3). PERMANOVA confirmed that the bacterial (Pseudo-F = 10.53, P < 1 x 10-5) and fungal (Pseudo-F = 6.43, P < 1 x 10-5) compositions changed significantly across the sites (Supplementary Table 3.6; P < 0.05). Pairwise comparisons revealed that Richmond differed significantly from Eastnor and Hatchlands, where bacterial community composition was similar (Supplementary Table 3.6; P < 0.05). The fungal composition varied significantly across all the sites (Supplementary Table 3.6; P < 0.05).

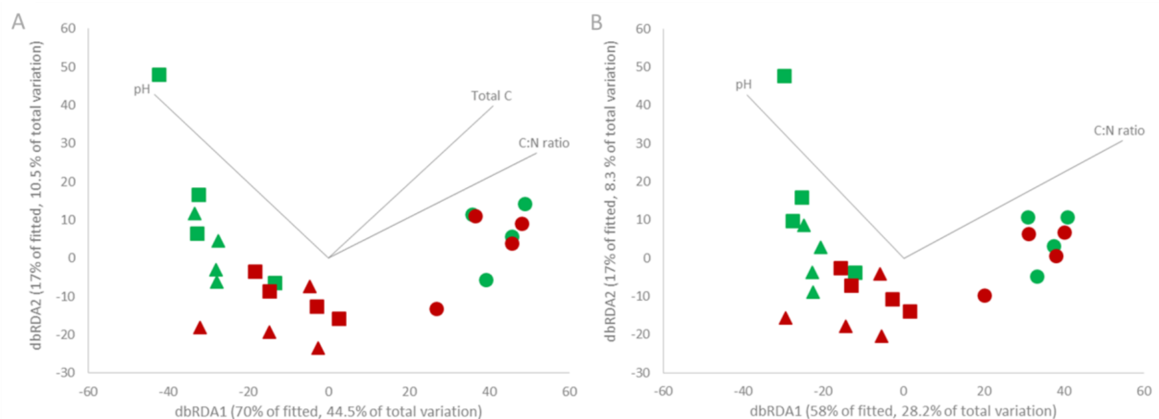


Figure 3.3 Distance-based redundancy analysis of the bacterial (A) and fungal (B) communities and rhizosphere soil chemical properties of the healthy and AOD trees across the sites (n=24). The soil parameters determined by BEST analysis are shown in each plot. The longer the line, the stronger the correlation between microbiome composition and soil chemistry. Eastnor – triangle; Hatchlands – square; Richmond – circle; healthy trees – green; AOD trees – red.

Within Eastnor and Hatchlands, healthy and AOD trees clustered into two distinct groups. The dbRDA and PERMANOVA results showed that the microbiome composition in the healthy oak trees was significantly different from the AOD trees for both bacterial (Pseudo-F = 3.37, $P < 0.05$) and fungal (Pseudo-F = 1.90, $P < 0.05$) communities (Figure 3.3, Supplementary Table 3.6). No differences were observed in Richmond.

Rhizosphere microbiome and physicochemical properties are correlated

The rhizosphere physicochemical properties and microbiomes showed strong correlation for both bacterial (Mantel $r = 0.81$, $P < 0.001$) and fungal (Mantel $r = 0.73$, $P < 0.001$) communities. Among the rhizosphere properties evaluated in this study, the BEST analysis revealed that rhizosphere pH was constantly present in all the combinations that best explained microbiome composition variance (Supplementary Table 3.7), together with total C, and C:N for bacteria (Spearman's $r_s = 0.896$, $P < 0.01$; Supplementary Table 3.7) and C:N ratio for fungi (Spearman's $r_s = 0.848$, $P < 0.01$; Supplementary Table 3.7).

Rhizosphere bacteria and fungi members differ across the sites and tree health conditions

According to the differential abundance analysis (DESeq 2.0), Eastnor and Hatchlands shared many common rhizosphere members (Figure 3.4A and 3.4D), only differing in 13 bacterial and 101 fungal OTUs ($P < 0.05$). However, a considerable number of rhizosphere bacterial and fungal members differed from Richmond: 2,237 bacterial and 409 fungal OTUs differed significantly ($P < 0.05$) between Eastnor and Richmond (Figure 3.4B and 3.4E), and 2,842 bacterial and 430 fungal OTUs between Hatchlands and Richmond (Figure 3.4C and 3.4F; $P < 0.05$).

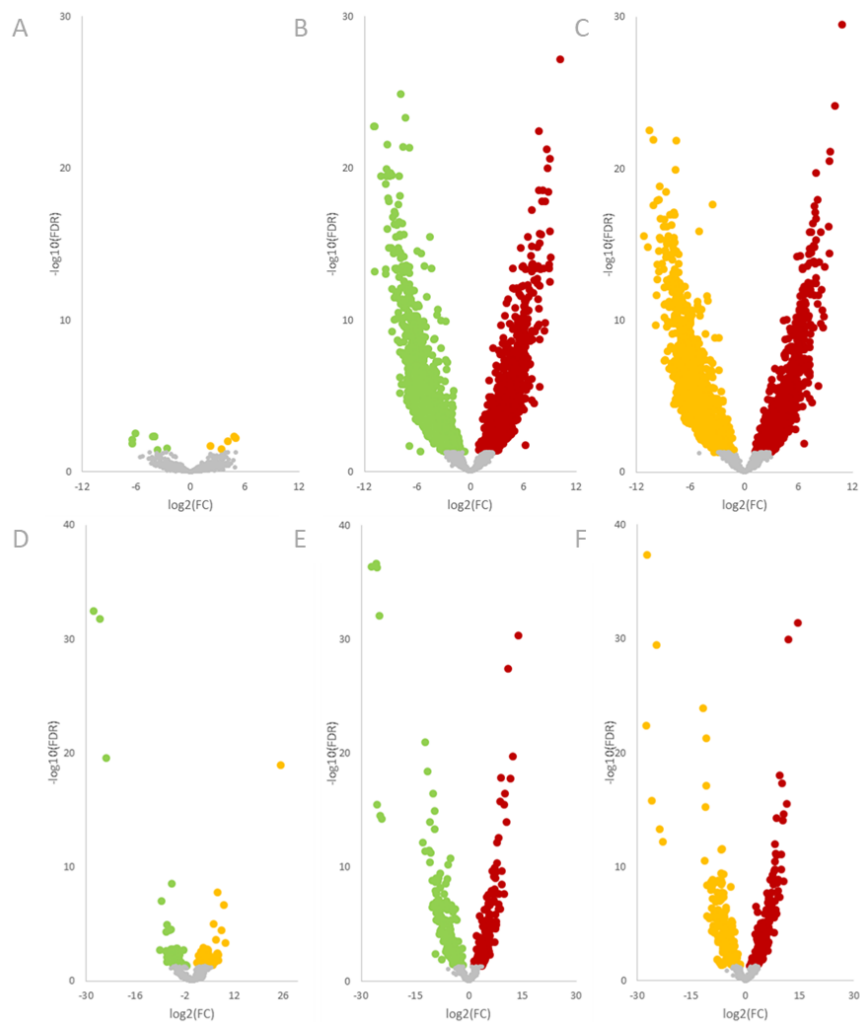


Figure 3.4 Volcano plots of differential bacterial (A, B and C) and fungal (D, E and F) OTU abundance analysis as calculated by DESeq 2.0 according to the site (A and D – Eastnor vs. Hatchlands; B and E – Eastnor vs. Richmond; C and F – Hatchlands vs. Richmond). Fold change FDR corrected p-values are plotted for each OTU. Significantly different taxa (FDR-corrected P < 0.05) are coloured according to site: Eastnor – green, Hatchlands – yellow, Richmond – red.

When looking at the top 20 bacterial OTUs showing the highest fold change (negative fold change in Eastnor or Hatchlands and positive fold change in Richmond, Figure 3.5), members from the phyla Verrucomicrobia and Actinobacteria were particularly enriched at Eastnor and Hatchlands sites. Richmond showed a higher abundance of the families *Acidobacteriaceae* (phylum Acidobacteria), *Acetobacteraceae*, *Rhodospirillaceae* (class Alphaproteobacteria), *Sinobacteraceae*, and *Xanthomonadaceae* (class Gammaproteobacteria).

Figure 3.5 Differentially abundant OTUs between Eastnor-Richmond (A) and Hathclands-Richmond (B). The top 20 (the highest log2 fold changes) significantly enriched OTUs determined by DESeq 2.0 are depicted (FDR-corrected P < 0.05). Negative log2 (FC): Eastnor (a) and Hatchlands (a); Positive log2 (FC): Richmond (a and b).

Moreover, members of the subdivision 6 Acidobacteria, the class Chloracidobacteria, and the phylum Nitrospirae were more abundant in Eastnor and Hatchlands (Supplementary Table 3.8). On the other hand, the families *Burkholderiaceae* (class Betaproteobacteria) and *Sphingobacteriaceae* (phylum Bacteroidetes) were particularly enriched in Richmond (Supplementary Table 3.8).

In the fungal community, DESeq 2.0 (Figure 3.6A) and FUNGuild (Figure 3.6B and 3.6C) analyses showed concordant results. The relative abundance of ectomycorrhizal fungal (EcM) was significantly higher ($P < 0.05$) at Eastnor (38 ± 11%) and Hatchlands (47 ± 12%) compared to Richmond (10 ± 3%; Figure 3.6B), namely *Cortinarius*, *Hebeloma*, *Humaria*, *Gymnomyces*, *Tomentella*, *Xerocomellus* and *Tuber* (Figure 3.6A and Supplementary Figure 3.2). *Russula* and *Cenococcum* were particularly enriched at Richmond (Figure 3.6A and Supplementary Figure 3.2). Oppositely, the percentage of saprobes in the rhizosphere was significantly lower ($P < 0.05$) in Eastnor (39 ± 10%) and Hatchlands (33 ± 9%) than in Richmond (64 ± 6%; Figure 3.6C), where *Aspergillus*, *Penicillium*, *Byssonectria*, *Nadsonia*, representatives of the phylum Zygomycota, and some plant pathogens, such as *Phaeoacremonium* and *Gibberella*, were particularly enriched (Figure 3.6A and Supplementary Figure 3.2).

As revealed above by dbRDA and PERMANOVA, healthy trees held different microbial compositions compared to AOD trees at Eastnor and Hatchlands. Aligned with that, the differential abundance analysis (DESeq 2.0) detected 848 bacterial and 70 fungal OTUs more abundant in healthy trees, and 436 bacterial and 58 fungal OTUs enriched in AOD trees (Supplementary Figure 3.3). No significant bacterial OTUs were detected at Richmond (Supplementary Figure 3.3).

Remarkably, similar taxa found in Eastnor (low-AOD stage) and Hatchlands (mid-AOD stage) were also enriched in the healthy trees. Among them, stood out the classes Chloracidobacteria (RB41), subdivision 6 Acidobacteria (iii1-15, mb2424, and RB40; phylum Acidobacteria), Nitrospira, and 0319-6A21 (phylum Nitrospirae), which were consistently more abundant (Figure 3.7). In the fungal

community, few common OTUs were detected across the tree health comparisons but instead, they were site-specific (Supplementary Figure 3.4).

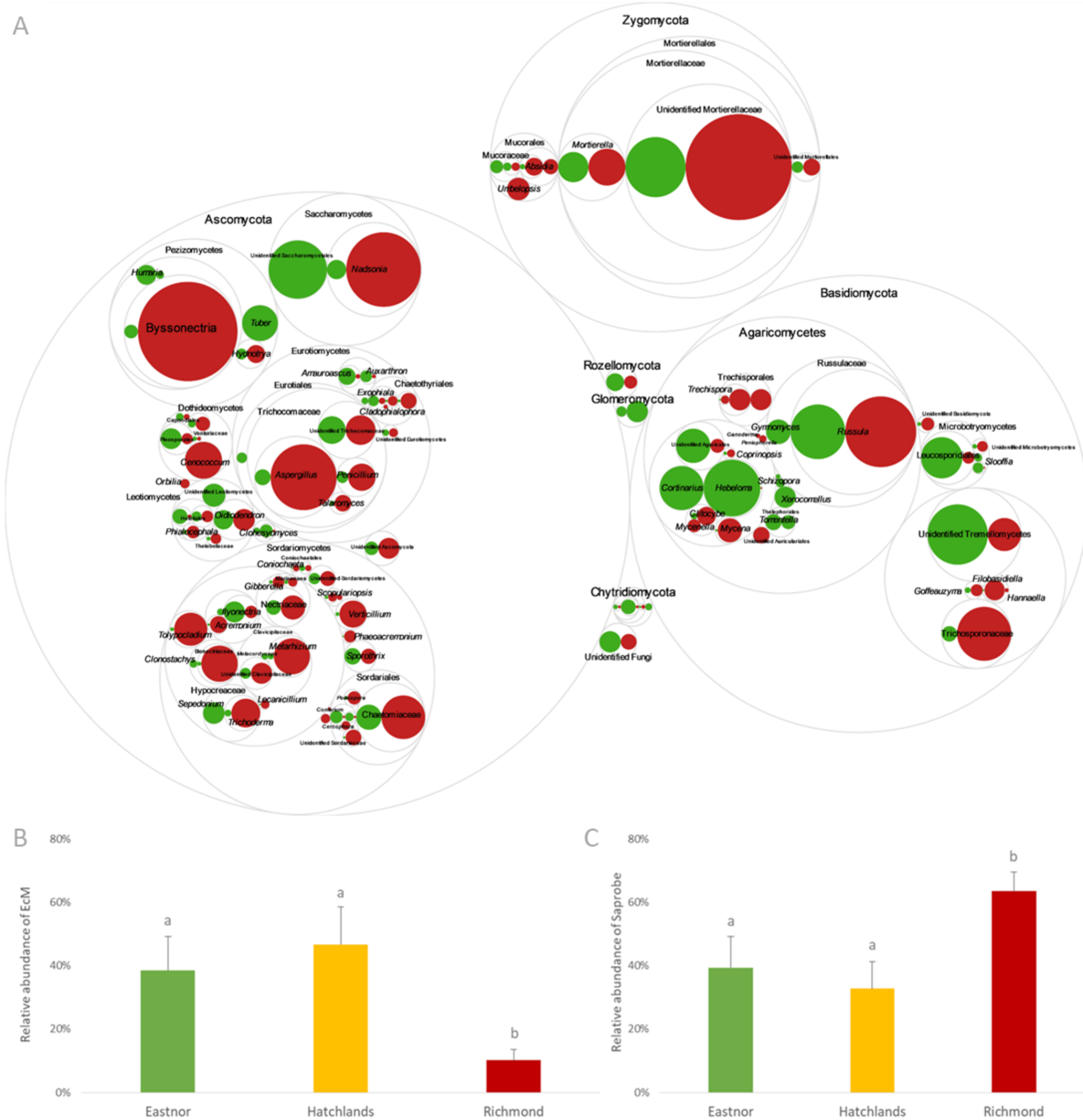


Figure 3.6 Fungi differentially abundant across the sites (A). Only significantly enriched OTUs determined by DESEq 2.0 are depicted (FDR-corrected P < 0.05). The size of the circles represents the relative abundance and the colour denotes the rhizosphere of oak trees located in Eastnor (green) and Richmond (red). Ecological guilds of fungal taxa detected in the rhizosphere soils in Eastnor, Hatchlands, and Richmond (B and C). Significant differences (P < 0.05) according to the site are indicated by different letters. EcM – Ectomycorrhizal fungi (B); Saprobe - saprophytic fungi (C).

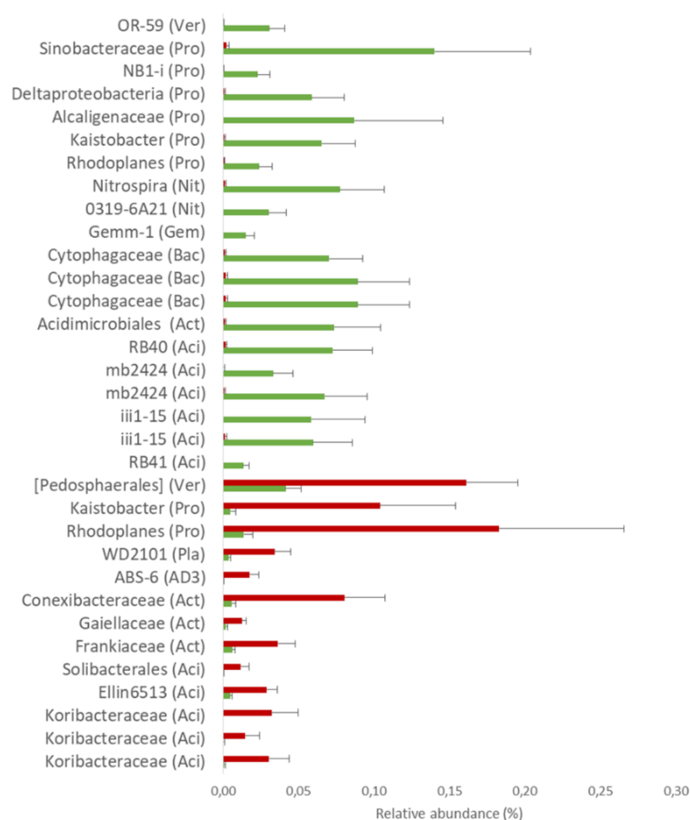


Figure 3.7 Relative abundance of the common bacterial OTUs differentially abundant between healthy and AOD oaks across the tree health comparisons. Phyla are indicated in brackets: Ver – Verrucomicrobia; Pro – Proteobacteria; Nit – Nitrospirae; Gem – Gemmatimonadetes; Bac – Bacteroidetes; Act – Actinobacteria; Aci – Acidobacteria; Pla – Planctomycetes. Error bars are shown as SEM. Green bar – healthy oaks; red bar – AOD oaks.

Discussion

In this work, we studied the rhizosphere physicochemical properties and microbiome (bacteria and fungi) of healthy and AOD symptomatic trees across three UK sites that ranged from different stages of the disease. We showed that host health, the rhizosphere physicochemical properties, and microbiome are linked at both site and tree conditions.

Across the three study sites (Figure 3.1), Richmond had suffered the longest and the most severe AOD symptoms (severe-AOD stage) but also had the most unfavourable soil environment. The soil was very acidic, dry, and compacted and

generally low in C and N content. Trees respond and adapt to unfavourable soil environments and this can take place through various mechanisms, including changes in root exudation and rhizodeposition, which consequently change the rhizosphere soil properties (Grayston *et al.* 1997; Karst *et al.* 2016; Gargallo-Garriga *et al.* 2018). In particular, plants can acidify their rhizosphere to facilitate nutrient uptake (Hinsinger *et al.* 2003). It is likely that all the oaks at Richmond have acidified their rhizosphere, regardless of tree health condition, in an effort to counter the poor soil environment and mobilize more nutrients for uptake to sustain growth and defence. At this site (severe-AOD stage), which showed the lowest rhizosphere pH, acidification dramatically boosted C and N levels in the rhizosphere compared to bulk soil, and these elements were higher than those measured at Eastnor and Hatchlands (low and mid-stage AOD sites, respectively). The same rhizosphere acidification effect was evident in the AOD affected trees at Eastnor and Hatchlands. Rhizosphere acidification seems to be an important oak response to withstand unfavourable environmental conditions and disease establishment, raising the hypothesis that oaks may trigger pH-related mechanisms to cause an increase in acidity levels when affected by or indeed leading to AOD. The understanding of oak stress reaction mechanisms is still lacking, and experimental work is required to detail and prove the mechanisms used by trees in responding and adapting to abiotic and biotic stress factors.

Rhizosphere acidification can also affect microbiome composition. Firstly, we found that the rhizosphere and bulk soil compartments differed in their microbial assemblies. This rhizosphere effect, triggered by root exudates and rhizodeposition, has been reported in many tree species, including oak (Uroz *et al.* 2010), beech, spruce (Uroz *et al.* 2016), poplar (Beckers *et al.* 2017; Cregger *et al.* 2018), wherein a diverse array of root exudates select inhabiting microorganisms (Paterson *et al.* 2007) with pivotal roles for host health, growth promotion, and productivity (Buée *et al.* 2009; Hacquard and Schadt 2015). Proteobacteria, Actinobacteria and Acidobacteria as well as Basidiomycota, Ascomycota, and Zygomycota were the main colonizers of the oak rhizosphere, as previously reported on *Quercus* species and other woody plants (Uroz *et al.* 2010, 2016; Beckers *et al.* 2017; Cregger *et al.* 2018).

In the second instance here, we show that the rhizosphere microbiome changed according to health conditions at both site and tree conditions. Oaks at Richmond (severe-AOD stage) showed the most dissimilar rhizosphere microbiome, whereas the oak trees at Eastnor and Hatchlands (low- and mid-stage AOD) presented a microbial composition dependent on the host health condition. Indeed, root exudates can change in quantity and quality in response to external stressors and affect the rhizosphere microbial community (Badri and Vivanco 2009; Timm *et al.* 2018). The rhizosphere pH strongly and consistently correlated with rhizosphere microbiome composition, with the bacterial and fungal assemblages changing in more acidic soils. It is well established that soil pH is one of the most important drivers in structuring soil bacterial and fungal communities and it has been reported as a predictor of diversity and composition of soil communities (Fierer 2017 and references within).

Acidobacteria of the class subdivision 6 and Chloracidobacteria, and members of the phylum Nitrospirae were consistently more abundant in healthy trees at Eastnor and Hatchlands, the low- and mid-stage AOD sites. Representatives of the phylum Acidobacteria are active members in the rhizosphere (Lee *et al.* 2008), capable of degrading complex plant polysaccharides (Ward *et al.* 2009), and harbouring putative extracellular peptidase genes for ammonium mobilization in N cycling (Eichorst *et al.* 2018). Moreover, they have recently been suggested as potential plant growth promoters (Kielak *et al.* 2016). The phylum Nitrospirae includes essential members for nitrogen cycling (Van Kessel *et al.* 2015). Whether the beneficial role of subdivision 6 Acidobacteria, Chloracidobacteria, and Nitrospirae on tree health remains speculative, it deserves further research to understand their increased abundance in healthier conditions and association with AOD.

In our study, ectomycorrhizal (EcM) fungi, which establish essential mutualistic relations with oak trees (Smith and Read 2008; Bonfante and Genre 2010), also varied in abundance and composition. At Eastnor and Hatchlands, EcM were more abundant and characterized by N-sensitive EcM fungi (e.g. *Cortinarius* and *Hebeloma*), whereas, at Richmond, this community was diminished and its profile

enriched in nitrophilic and acidophilous EcM fungi (e.g. *Russula*). Suz *et al.* (2014) (Suz *et al.* 2014) reported similar shifts of EcM abundance and composition across European temperate oak forests. These shifts were explained by the differences detected in rhizosphere soil pH and N content across the sites and were reported as drivers of changes in these communities (Suz *et al.* 2014; van der Linde *et al.* 2018). Other studies at local and continental scales also reported a reduction of EcM and changes in community profile with increasing N and decreasing pH (Avis *et al.* 2003, 2008; Cox *et al.* 2010; Lilleskov *et al.* 2011; Suz *et al.* 2014; van der Linde *et al.* 2018). However, our study is the first report of this occurrence linked to pH on oak in the UK and specifically associated with AOD symptomatic trees.

Interestingly, we found bacteria and fungi previously linked to plant diseases and that were more abundant in oak rhizospheres at Richmond (severe-AOD stage). Examples include members of the families *Burkholderiaceae* and *Xanthomonadaceae*, which were also isolated from tissues of oaks affected with AOD (Denman *et al.* 2016); representatives of the families *Sphingobacteriaceae* and *Burkholderiaceae*, which were reported in plant rhizospheres after pathogen infection (Chapelle *et al.* 2016); and plant pathogenic fungi such as *Gibberella* (Gordon *et al.* 2006; Aegerter and Gordon 2006) and *Phaeoacremonium* (Gramaje *et al.* 2015). Moreover, saprophytic fungi were also more abundant at Richmond as well as in the AOD trees. This suggests that declining trees have poor feeder root health and are undergoing partial root degradation or are too weak to fend off saprophytic attack. Thus, such organisms are able to take advantage of the situation, which is evidenced by the enrichment in bacteria and fungi with a saprophytic lifestyle and is a consequence of probable high decay material availability. Indeed, the results are supported by previous studies also reporting soil microbiome changes caused by biotic and abiotic forest disturbances, particularly the increase of saprophytic taxa over beneficial species (Hartmann *et al.* 2014; Štursová *et al.* 2014).

Conclusions

At first sight, our results appear to be a consequence of system perturbation that occurs in AOD establishment, in which the root exudation chemistry altered the rhizosphere soil pH and consequently changed the associated microbiome. However, the flip side of the results presented here raise the exciting hypothesis that the belowground rhizosphere communities can potentially protect oaks against the AOD, a decline-disease with aboveground symptoms. Understanding how trees and their associated microbiome respond to forest decline-disease episodes is vital for providing in-depth knowledge to develop more efficient, affordable, sustainable, and reliable forest management strategies for proactive prediction and mitigation of these events. In this sense, the present study gathers evidence of associations among tree health conditions, rhizosphere microbiome, and soil environment and paves the way for these associations to be investigated in other tree species suffering decline-disease events.

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Author Contributions

Conceptualization, D.P., C.E., E.V. and S.D.; methodology, D.P., C.B., H.F., N.B., E.V., C.E., and S.D; formal analysis, D.P., C.B. and H.F.; writing—original draft preparation, D.P.; writing—review and editing, D.P., E.V., C.E. and S.D.; supervision, C.E. and S.D.; project administration, S.D.; funding acquisition, S.D. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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CHAPTER 4

CONCLUDING REMARKS

Concluding remarks

Introduction

Cork oak (*Q. suber* L) and pedunculate oak (*Q. robur* L) forests are intrinsic components of the Portuguese and British national identities. In addition to the rich habitat provided to indigenous flora and fauna (Bugalho *et al.* 2011; Mitchell *et al.* 2019), the unique corky bark of cork oak and the obdurate structure of pedunculate oak make them extremely valuable and essential to local economies, cultures, and ecosystems (FMI 2017; APCOR 2018). However, the incidence, occurrence, and magnitude of perturbations have reduced the resilience of both species over the last decades (Thomas *et al.* 2002; Thomas 2008; Denman and Webber 2009; Camilo-Alves *et al.* 2013; Denman *et al.* 2014). Extensive knowledge of the factors that adversely affect oak health has increased our understanding of oak decline and its impact. However, progress towards resolving COD has been slow due to complex interactions of multiple factors decline (Denman *et al.* 2017; Gagen *et al.* 2019) along with the poorly understood aetiology (Ciesla and Donaubauer 1994) or the misuse of "decline" as a discrete disease class (Ostry *et al.* 2011). On the other hand, the aetiology of AOD follows the classic decline-disease spiral model (Figure 1.3) and the descriptors for AOD identification are clear and well established (Denman *et al.* 2014). Nevertheless, the predisposition factors that trigger these oak disorders are still unknown.

Missing pieces to these puzzles may include the microbial communities that inhabit the above- and belowground niches of oak trees. Previous studies have shown that woody, long-living plants rely on their complex microbiomes to resist biotic and abiotic perturbations (Buonaurio *et al.* 2015; Hacquard and Schadt 2015; Koskella *et al.* 2017; Mercado-Blanco *et al.* 2018; Denman *et al.* 2018; Broberg *et al.* 2018; Terhonen *et al.* 2019; Bettenfeld *et al.* 2020). Predicting and mitigating emergent oak syndromes under global change scenarios involves a better understanding of the role of microbiomes on oak tree growth, health, and resilience. Thanks to advances in metabarcoding and metagenomic approaches, progress has been made in characterising the bacterial and fungal communities inhabiting roots, rhizospheres and surrounding soils of *Quercus* species (Buée *et*

al. 2009; Uroz *et al.* 2010a; Jumpponen *et al.* 2010; Orgiazzi *et al.* 2012; Toju *et al.* 2013b, a; Voříšková and Baldrian 2013; Voříšková *et al.* 2014; López-Mondéjar *et al.* 2015; Maghnia *et al.* 2017; Cobo-Díaz *et al.* 2017; Fernández-González *et al.* 2017; Reis *et al.* 2019; Ruiz Gómez *et al.* 2019; Lasa *et al.* 2019). However, information on the belowground microbial community is still scarce in the context of oak decline. Therefore, exploring the rhizosphere microbiome contributes to a more thorough understanding of the impact of different rhizosphere members and their potential impact on oak health. This thesis assessed for the first time the associations between soil properties, rhizosphere microbiome and health status of oak trees.

General Discussion

Soil type and condition greatly influence water and nutrient availability to tree roots (Thomas 2008; Serrasolses *et al.* 2009). Oak species are thought to be drought-tolerant, especially *Q. suber* (Pereira *et al.* 2009), even though moist nutrient-rich conditions favour their growth (Lévy *et al.* 1992; Serrasolses *et al.* 2009; Lévesque *et al.* 2016). Looking at the physicochemical properties (Table 4.1), oak rhizospheres in the UK had more moisture and higher nutrient levels (24%, 116974 mg C/kg, 7377 mg N/kg on average) compared to cork oak in Portugal (11%, 28433 mg C/kg, 1346 mg N/kg). In fact, the rhizosphere samples were markedly different between the British and Portuguese sites during sampling. In the UK, soils mainly consisted of clay and were generally covered with plant litter and therefore had superior moisture-holding capacity and higher nutrient content. In Portugal, soils were shallow, sandy, with low or no plant litter, leading to limited moisture-holding capacity and low nutrient availability. These conditions may drive oak species to vulnerable situations due to shifts in rainfall distribution patterns (Knapp *et al.* 2008; Besson *et al.* 2014), water shortage during severe drought years (Gibbs and Greig 1997; Pereira *et al.* 2006; Avila *et al.* 2016), and nutrient deficits (Demchik and Sharpe 2000; Serrasolses *et al.* 2009; Kostić *et al.* 2021), resulting in frequent episodes of high tree mortality.

Table 4.1 Compilation of rhizosphere physicochemical properties described in chapters 2 and 3.

Country - Quercus species	Site	Forest Decline	Stand Health	Tree health condition	Moisture (%)	pH (H2O)	Total C (mg/kg)	Total N (mg/kg)	C:N ratio
UK - *Quercus robur*	Eastnor	Low	n.a.	Asymptomatic	30 ± 1	6.09 ± 0.22	61,810 ± 4,991	4,877 ± 439	12.69 ± 0.38
				AOD	19 ± 1	5.27 ± 0.31	59,670 ± 15,330	4,380 ± 729	13.40 ± 1.36
	Hatchlands	Mid	n.a.	Asymptomatic	24 ± 3	6.43 ± 0.70	64,834 ± 13,077	4,739 ± 989	13.70 ± 0.56
				AOD	20 ± 1	5.42 ± 0.24	63,598 ± 3,852	4,423 ± 480	14.46 ± 0.83
	Richmond	Severe	n.a.	Asymptomatic	27 ± 7	4.35 ± 0.13	228,313 ± 53,690	12,905 ± 3,356	17.83 ± 0.58
				AOD	26 ± 6	4.30 ± 0.14	223,618 ± 57,324	12,938 ± 3,305	17.28 ± 0.36
Portugal - *Quercus suber*	Baixo Sorraia	Low	*Vitality*	Asymptomatic	9 ± 2	4.74 ± 0.12	19,149 ± 1,543	959 ± 103	20.04 ± 0.91
				COD	9 ± 2	4.87 ± 0.26	24,486 ± 12,635	1,098 ± 284	21.72 ± 5.41
			Mortality	Asymptomatic	20 ± 5	4.79 ± 0.17	77,339 ± 50,422	3,275 ± 1,964	22.60 ± 2.13
				COD	15 ± 4	4.86 ± 0.40	50,649 ± 27,058	2,367 ± 1,386	21.78 ± 1.82
	Divor	High	*Vitality*	Asymptomatic	9 ± 2	5.45 ± 0.36	14,601 ± 2,034	814 ± 100	17.91 ± 0.76
				COD	9 ± 2	5.52 ± 0.46	12,838 ± 4,593	706 ± 283	18.50 ± 1.67
			Mortality	Asymptomatic	9 ± 3	5.87 ± 0.29	13,751 ± 3,031	745 ± 191	18.62 ± 1.34
				COD	10 ± 2	6.11 ± 0.42	14,652 ± 4,719	804 ± 279	18.46 ± 1.46

As described in Chapters 2 and 3, pH changed at site and tree levels, with both *Quercus* species undertaking an acidification process in their rhizospheres. This boosted C and N levels and C:N ratio and suggests rhizosphere acidification as a responsive stress mechanism among *Quercus* species in order to enhance nutrient uptake, which is significant for oak health and growth (Lévesque *et al.* 2016) and maintain their resilience under pressure caused by declining triggers. However, it is worth mentioning that rhizosphere acidification took place in different directions. While in Portugal the rhizospheres of cork oaks located in the site with low forest decline (Baixo Sorraia) had low pH (pH 4.82 on average), in the UK this was observed in the site with severe forest decline (Richmond, pH 4.33). Moreover, Divor (high forest decline, PT), Eastnor and Hatchlands (low and mid forest decline, UK) revealed similar rhizosphere pH values (5.74, 5.68 and 5.93, respectively), despite showing opposite forest decline levels. In Portugal, no pH differences were observed at the tree health level, in contrast to the evident rhizosphere acidification effect in the AOD affected trees at Eastnor and Hatchlands. The discrepancy between the low rhizosphere pH associated with better health of cork oak and the acidification linked to AOD symptoms in pedunculate oak suggests that different actions may be taking place in chronic and acute decline.

In cork oak, soil acidification may be a physiological mechanism for trees thriving under the Mediterranean climate's harsh and poor conditions, where soils are usually shallow and nutrient-poor (Marañón 1988). As mentioned in Chapter 2, the acidification process of cork oak trees boosted C and N levels and increased the C:N ratio in Baixo Sorraia, which contributes to nutrient uptake, enhances tree resilience and prevents the progression to more advanced chronic declining states. However, this acidification mechanism was not marked in Divor, which could explain the higher tree mortality rate in this location. Historical episodes of stress are known to predispose oaks many decades before revealing declining symptoms, which impacts their future resilience to disturbance and leaves them more susceptible to COD (Gagen *et al.* 2019).

On the other hand, an increase in rhizosphere acidity might be instigated by the pedunculate oaks themselves when under AOD-induced stress. This "cry for help" triggers acute metabolic changes (S. Denman and J. Draper unpublished information) that alter root exudation chemistry and bring about acidic conditions, which can be highly harmful to the health of the trees. However, it may also be true that this acidification process, triggered by unknown reasons, makes trees more susceptible and prone to AOD. Recently, Scarlett *et al.* (2020) reported that soil pH indirectly affected tree health via AOB-driven ammonia oxidation processes and suggested incrementing organic matter as a management practice to buffer acidification and reduce stress on declining oak trees.

Soil pH is well established as one of the most significant shapers and predictors of soil microbial communities (Fierer 2017, and references within). Here, the same was observed in both scenarios, in which rhizosphere pH correlated with the microbial communities, and rhizosphere acidification was associated with modified microbiome composition. First, rhizosphere and bulk soil compartments differed in their pH. The acidic conditions in the rhizosphere shaped the microbial assemblies, with Proteobacteria, Actinobacteria and Acidobacteria as well as Basidiomycota, Ascomycota, and Zygomycota dominating cork oak and pedunculate oak rhizospheres. This rhizosphere effect, triggered by root exudates and rhizodeposition, has been reported in previous studies on *Quercus* species

and other woody plants (Buée *et al.* 2009; Uroz *et al.* 2010b, 2016; Fernández-González *et al.* 2017; Beckers *et al.* 2017; Reis *et al.* 2018, 2019; Cregger *et al.* 2018). Second, rhizosphere acidification matched with health conditions at site and tree levels, which in turn had distinct microbiomes. Marked differences in rhizosphere pH and microbiome composition were evident between sites with opposite forest decline levels (Baixo Sorraia vs Divor and Eastnor/Hatchlands vs Richmond) for cork oak and pedunculate oak, respectively. However, there was no clear association between tree health and rhizosphere communities in chronic cork oak decline, as initially hypothesised, but instead at stand health level. As referred to in Chapter 2, *Bacillus*, *Bryobacter*, *Cladophialophora*, and *Phallus* (part of the core microbiome) were more abundant at the *vitality* stands, indicating that these bacterial groups may present beneficial traits for cork oak trees. On the other hand, tree health was associated with microbiome composition in AOD. Asymptomatic trees at Eastnor and Hatchlands (low- and mid-stage AOD sites) housed a distinct rhizosphere community compared to AOD-affected trees, with a consistently higher abundance of potential beneficial members, such as Acidobacteria of the class subdivision 6 and Chloracidobacteria, and the phylum Nitrospirae. In short, microbial community analyses allowed us to uncover bacterial and fungal taxa changes, where the better health scenarios had a plethora of bacteria and fungi with potential beneficial traits provided to their hosts. In contrast, plant pathogens and fungal parasites were more abundant in the rhizosphere of oaks located in the sites most affected by COD or AOD.

Altogether, these results support recent findings where the balance among the different rhizosphere organisms can determine host resilience to stress and play an important role in keeping them healthy or asymptomatic (Buonaurio *et al.* 2015; Hacquard and Schadt 2015; Koskella *et al.* 2017; Mercado-Blanco *et al.* 2018; Denman *et al.* 2018; Broberg *et al.* 2018; Terhonen *et al.* 2019; Bettenfeld *et al.* 2020). Here, the absence of visible decline symptoms (foliage deterioration, death of branches, and crown dieback in COD and dark exudates bleeding, bark cracks, necrotic tissues, and buprestid beetle larval galleries in AOD) is likely to indicate that trees are only experiencing background levels of stress and disturbance and are able to keep the right conditions to maintain a favourable microbiome in their

rhizosphere. In contrast, under specific contexts, shifts in pH may affect the rhizosphere environment. This may lead to an unbalanced microbiome unable to provide beneficial functions and curb opportunistic agents, which take advantage and push trees to evident, visible symptoms of health deterioration and decline. In this way, these favourable or unbalanced microbiomes can take effect to reduce or increase the predisposition of trees, respectively. Therefore, considering the pivotal role in promoting tree health and fitness (Hacquard and Schadt 2015), it is suggested that the rhizosphere microbiome may act as a predisposing factor in Manion's spiral model (Figure 4.1) for both chronic and acute decline in oak species.

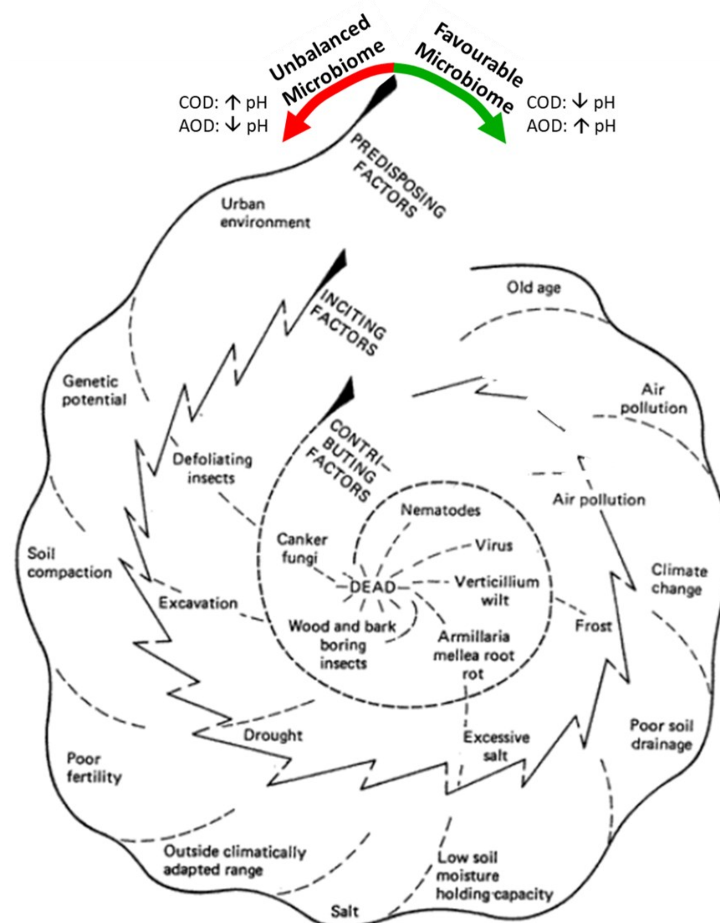

Figure 4.1 Adaptation of the forest decline spiral model proposed by Manion (1991) in the context of COD and AOD. Rhizosphere microbiome is indicated as a predisposing factor in the sense that trees can keep the right conditions to maintain a favourable microbiome, avoiding the further action of inciting and contributing factors. In contrast, an unbalanced microbiome can be unable to provide beneficial functions, enabling the action of inciting and contributing factors.

Final Considerations

The present thesis helped increase knowledge of oak rhizosphere microbiomes concerning chronic and acute decline syndromes. First, the studies in this thesis provide baseline information on rhizosphere physicochemical properties and microbiome for both cork oak and pedunculate oak trees in the context of COD and AOD, respectively. Furthermore, this thesis complements the knowledge reviewed in Chapter 1- Introduction and gathers evidence on the processes occurring in the belowground environment of *Quercus* species.

Although the information on rhizosphere physicochemical properties and microbiome revealed in this thesis is a step forward to understanding the complex multifactorial nature of COD and AOD syndromes, these findings also open new routes for further research. For example, the acidification process triggered in COD and AOD deserves controlled experiments to prove whether the acidification effect is a mechanism driving tree deterioration or a consequence of decline and to what extent it influences nutrient availability for tree health and resilience. Moreover, as demonstrated, the healthier sites, stands, and trees hold putative beneficial bacteria and fungi that merit additional research to test them as microbial indicators for early detection of COD and AOD by expanding and including more case studies from other sites. Also, unveiling their critical functions for the healthy growth and resilience of oak species and understanding their role on COD and AOD is of utmost importance in assessing their positive effect on Quercus species, including nutrient cycling and biological control, and plant growth. Altogether, understanding the underlying mechanisms that enhance tree health, growth, and resilience will help develop more efficient, affordable, and reliable forest management strategies, aiming to predict and mitigate these events in global change.

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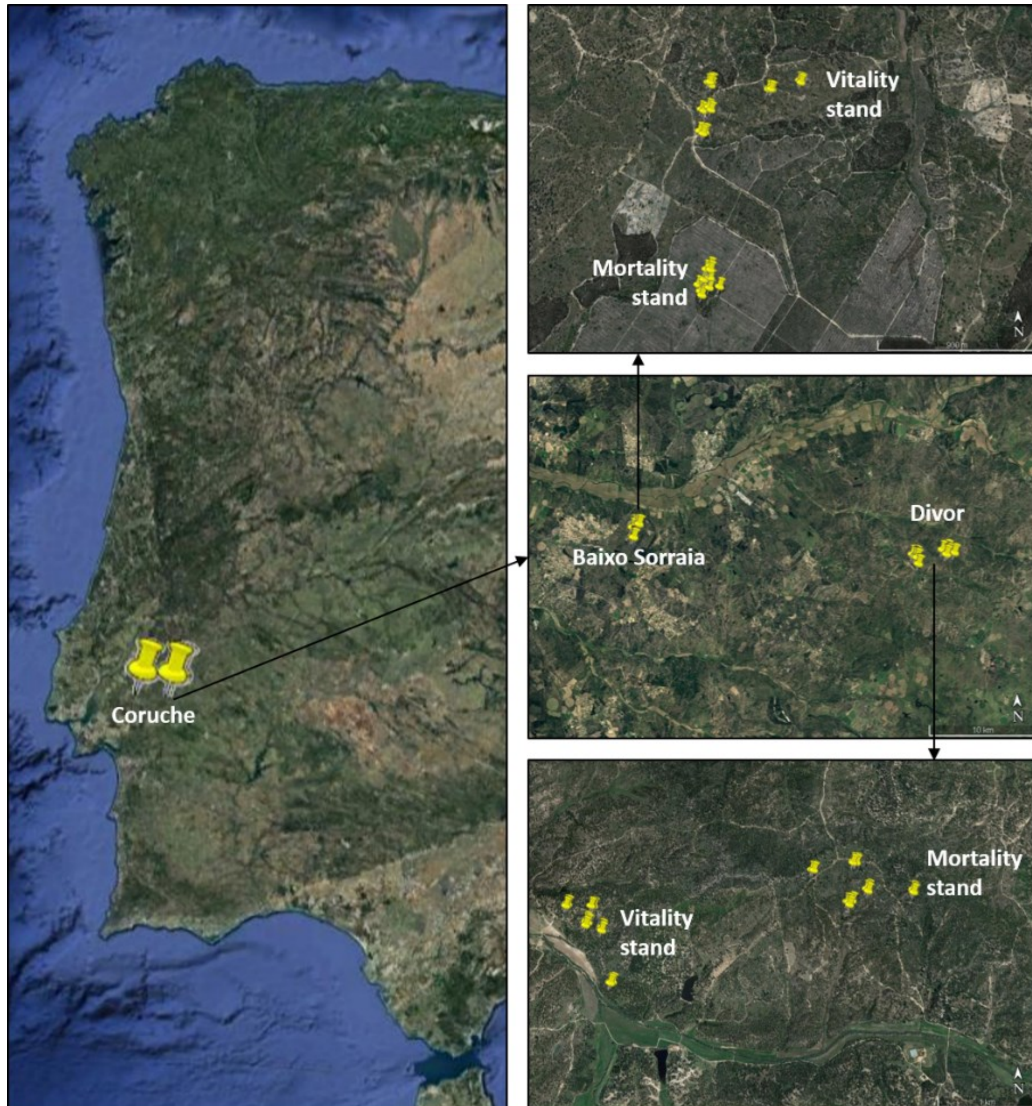
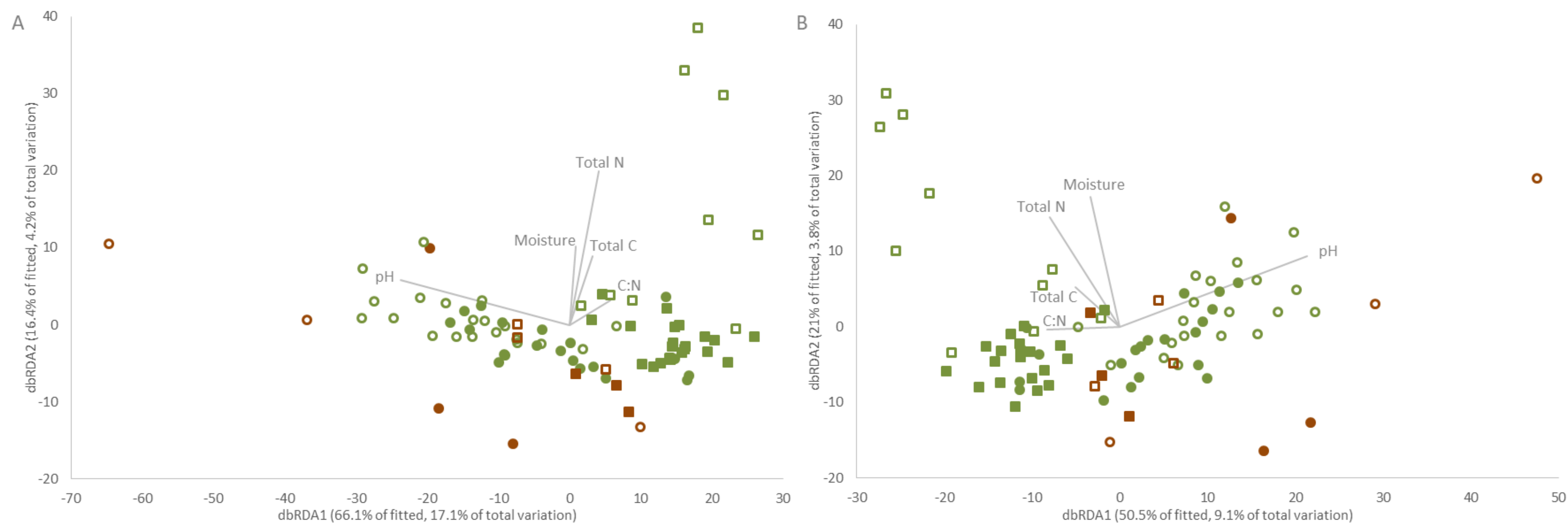
APPENDIX

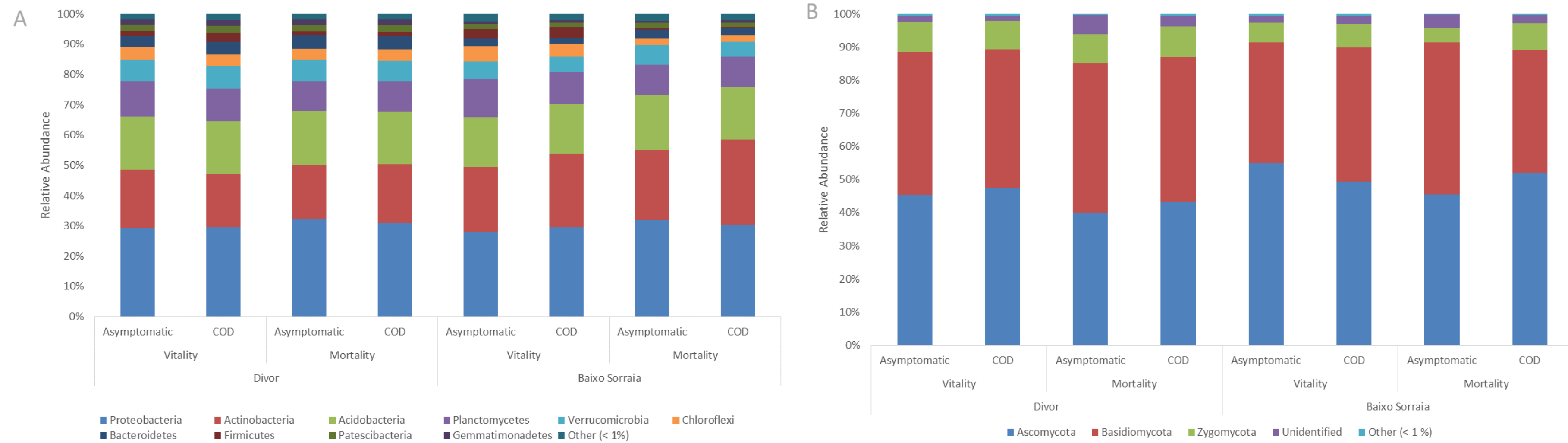
Appendix 1: Supplementary information of Chapter 2



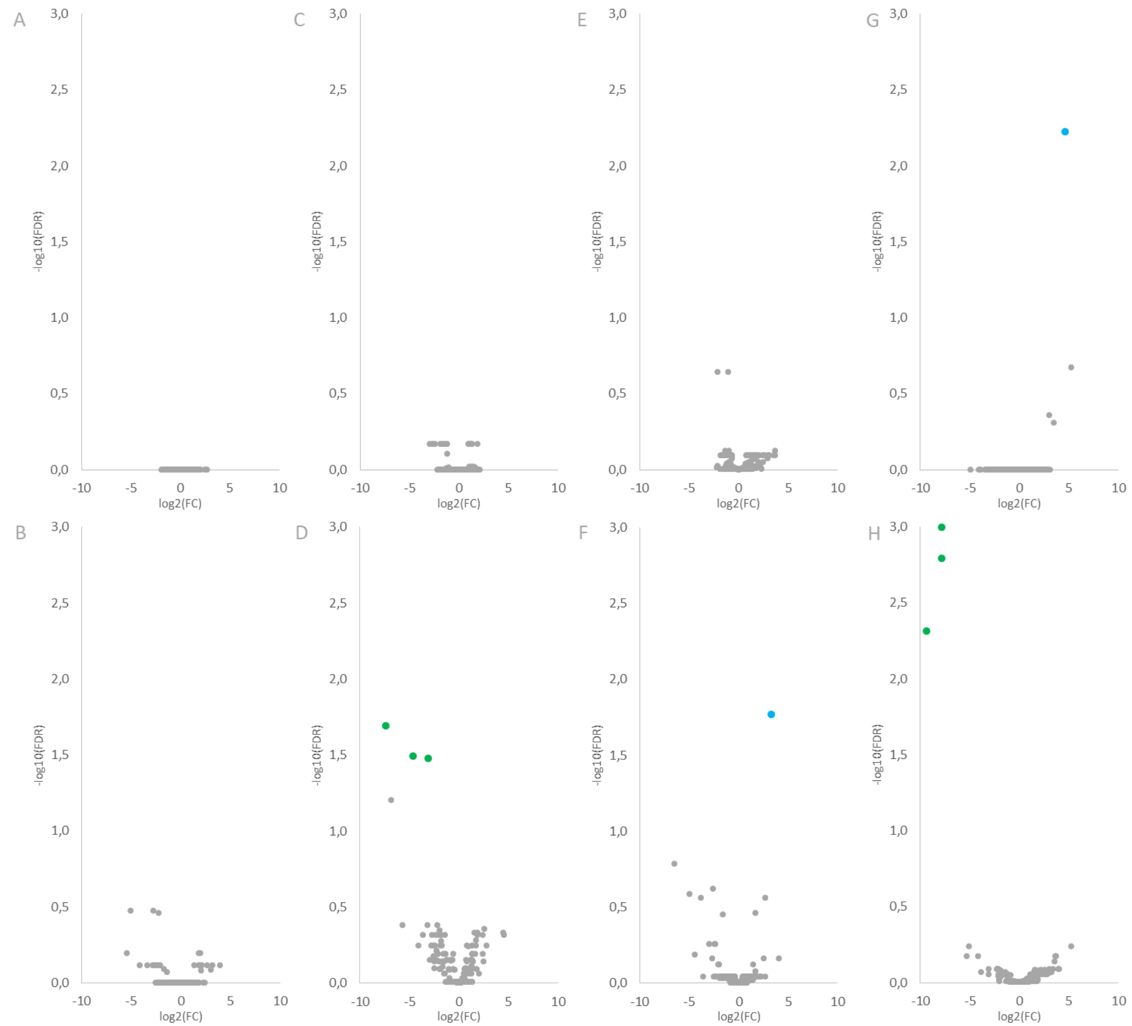
Supplementary Figure 2.1 Location of the cork oak trees sampled at the *vitality* and *mortality* stands in Divor and Baixo Sorraia sites. Each point represents the specific GPS location of each sampled tree.



Supplementary Figure 2.2 Distance-based Redundancy Analysis of bacterial (A) and fungal (B) composition in the rhizosphere and bulk soils of cork oak. All the soil physicochemical properties are shown. The longer the line, the stronger the correlation. Each point represents a bacterial or fungal community of an individual soil sample. Baixo Sorraia – square; Divor – circle; *Vitality* – closed symbol; *Mortality* – opened symbol; Rhizosphere – green; Bulk soil – brown.



Supplementary Figure 2.3 Relative abundance of (A) bacterial and (B) fungal phyla detected in the rhizosphere of the asymptomatic and COD trees within each site and stand. The phyla that individually represented less than 1% of the total community were grouped in the *Other* category. For bacteria, this consisted of WPS-2, Armatimonadetes, Elusimicrobia, Cyanobacteria, Chlamydiae, Dependentiae, Rokubacteria, Latescibacteria, Nitrospirae, Fibrobacteres, Omnitrophicaeota, BRC1, FBP, Entotheonellaeota, FCPU426, WS2, Unclassified, Hydrogenedentes, Tenericutes, Kiritimatiellaeota, Epsilonbacteraeota, Fusobacteria, Spirochaetes, Deinococcus-Thermus, Margulisbacteria, WS4, Aquificae, Dadabacteria, LCP-89, Caldiserica, Nitrospinae, and GAL15. For fungi, this consisted of Glomeromycota, Chytridiomycota, Rozellomycota, and Blastocladiomycota.



Supplementary Figure 2.4 Volcano plots of differential bacterial (A, C, E, and G) and fungal (B, D, F, and H) OTU abundance analysis as calculated by DESeq 2.0 within each site and stand health (Divor *vitality*: A and B; Divor *mortality*: C and D; Baixo Sorraia *vitality*: E and F; Baixo Sorraia *mortality*: G and H). Fold change FDR corrected p-values are plotted for each OTU. Significantly different taxa (FDR-corrected $P < 0.05$) are coloured according to tree health condition: asymptomatic – green; COD – blue. No significant OTUs are shown as grey dots.

Supplementary Table 2.1 Number of sampled asymptomatic trees, COD symptomatic trees, and bulk soils collected across the stands and sites.

Site	Stand health	Tree health	Number of trees	Number of bulk soils
Divor	*Vitality*	Asymptomatic	5	3
		COD	15	
	Mortality	Asymptomatic	5	3
		COD	15	
Baixo Sorraia	*Vitality*	Asymptomatic	5	3
		COD	15	
	Mortality	Asymptomatic	5	3
		COD	5	

Supplementary Table 2.2 Physicochemical properties of the rhizospheres and bulk soils sampled at the *vitality* and *mortality* stands across Baixo Sorraia and Divor.

Site	Stand health	Soil Compartment	Moisture (%)	pH (H_2O)	Total C (mg/kg)	Total N (mg/kg)	C:N ratio
Baixo Sorraia	*Vitality*	Rhizosphere	9.13 ± 1.81[a]	4.84 ± 0.24[a]	23,152 ± 11,210[a]	1,063 ± 258[a]	21.30 ± 4.76[ab]
		Bulk soil	9.87 ± 5.92[ab]	5.19 ± 0.17[bc]	6,066 ± 1,916[e]	316 ± 114[d]	19.50 ± 1.28[bc]
	Mortality	Rhizosphere	16.99 ± 5.17[b]	4.82 ± 0.31[ab]	63,994 ± 42,607[b]	2,821 ± 1,759[b]	22.19 ± 2.02[a]
		Bulk soil	8.00 ± 2.62[a]	5.59 ± 0.25[cde]	12,301 ± 3,158[cde]	591 ± 153[cd]	20.87 ± 0.79[ab]
Divor	*Vitality*	Rhizosphere	8.72 ± 2.00[a]	5.51 ± 0.44[cd]	13,279 ± 4,176[cd]	733 ± 254[c]	18.35 ± 1.51[c]
		Bulk soil	7.10 ± 4.87[a]	6.01 ± 0.28[de]	9,499 ± 9,559[acde]	691 ± 553[abcd]	11.30 ± 3.22[d]
	Mortality	Rhizosphere	9.68 ± 2.44[a]	6.05 ± 0.41[e]	14,427 ± 4,376[c]	789 ± 261[c]	18.50 ± 1.43[c]
		Bulk soil	8.65 ± 3.23[a]	6.70 ± 1.34[cde]	7,975 ± 2,872[de]	546 ± 230[cd]	14.94 ± 1.08[d]

Mean ± standard deviation is shown. Superscript letters indicate significant differences ($P < 0.05$) as determined by ANOVA or Kruskal-Wallis and their corresponding pairwise comparisons (Tukey's HSD post hoc or Mann–Whitney U test).

Supplementary Table 2.3 PERMANOVA results of soil compartment comparisons.

Microbial Group	Site	Stand Health	Soil compartment	t	p-value (perm)
Bacteria	Baixo Sorraia	*Vitality*	Rhizosphere – Bulk soil	1.3761	0.0072
		Mortality	Rhizosphere – Bulk soil	1.6177	0.0106
	Divor	*Vitality*	Rhizosphere – Bulk soil	1.6428	0.0007
		Mortality	Rhizosphere – Bulk soil	1.3452	0.0219
Fungi	Baixo Sorraia	*Vitality*	Rhizosphere – Bulk soil	1.6930	0.0010
		Mortality	Rhizosphere – Bulk soil	1.4950	0.0062
	Divor	*Vitality*	Rhizosphere – Bulk soil	1.6255	0.0003
		Mortality	Rhizosphere – Bulk soil	1.4735	0.0006

Supplementary Table 2.4 Alpha diversity of the rhizospheres and bulk soils sampled at the *vitality* and *mortality* stands across Divor and Baixo Sorraia.

Microbial Group	Site	Stand health	Soil Compartment	Chao1	Observed Species	Shannon	Pielou's Evenness	Simpson
Bacteria	Baixo Sorraia	*Vitality*	Rhizosphere	9278 ± 1286^{a}	5122 ± 646^{a}	10.42 ± 0.37^{a}	0.846 ± 0.018^{a}	0.9972 ± 0.0009^{a}
			Bulk Soil	8533 ± 1242^{a}	5002 ± 642^{a}	10.41 ± 0.41^{ab}	0.848 ± 0.021^{ab}	0.9971 ± 0.0012^{ab}
		Mortality	Rhizosphere	8753 ± 1126^{a}	4940 ± 582^{a}	10.37 ± 0.36^{a}	0.846 ± 0.018^{a}	0.9971 ± 0.0009^{a}
			Bulk Soil	9437 ± 520^{a}	5342 ± 257^{a}	10.63 ± 0.09^{ab}	0.859 ± 0.003^{ab}	0.9977 ± 0.0001^{ab}
	Divor	*Vitality*	Rhizosphere	12483 ± 1498^{b}	6499 ± 743^{bc}	11.04 ± 0.37^{bc}	0.872 ± 0.018^{bc}	0.9983 ± 0.0006^{bc}
			Bulk Soil	9991 ± 861^{a}	5690 ± 499^{ab}	10.78 ± 0.27^{abc}	0.865 ± 0.013^{abc}	0.9981 ± 0.0003^{abc}
		Mortality	Rhizosphere	12626 ± 918^{b}	6672 ± 430^{c}	11.15 ± 0.22^{c}	0.878 ± 0.012^{c}	0.9985 ± 0.0004^{c}
			Bulk Soil	9955 ± 1037^{a}	5756 ± 560^{ab}	10.81 ± 0.47^{abc}	0.865 ± 0.029^{abc}	0.9980 ± 0.0012^{abc}
Fungi	Baixo Sorraia	*Vitality*	Rhizosphere	1645 ± 187^{a}	1172 ± 136^{ab}	6.59 ± 0.64^{a}	0.646 ± 0.059^{a}	0.9594 ± 0.0469^{a}
			Bulk Soil	1051 ± 186^{b}	799 ± 102^{c}	6.23 ± 0.27^{ab}	0.647 ± 0.016^{a}	0.9619 ± 0.0056^{ab}
		Mortality	Rhizosphere	1459 ± 127^{c}	1038 ± 108de	6.32 ± 0.32^{b}	0.631 ± 0.032^{a}	0.9603 ± 0.0197^{b}
			Bulk Soil	1369 ± 111^{bc}	1121 ± 113^{abcde}	6.93 ± 0.51^{ab}	0.684 ± 0.040^{a}	0.9788 ± 0.0088^{ab}
	Divor	*Vitality*	Rhizosphere	1665 ± 235^{a}	1176 ± 164^{ad}	6.48 ± 0.42^{ab}	0.636 ± 0.033^{a}	0.9634 ± 0.0166^{ab}
			Bulk Soil	1203 ± 136^{b}	983 ± 110^{bce}	6.02 ± 0.67^{ab}	0.605 ± 0.059^{a}	0.9543 ± 0.0186^{ab}
		Mortality	Rhizosphere	1522 ± 171^{ac}	1151 ± 124^{ab}	6.50 ± 0.40^{ab}	0.640 ± 0.036^{a}	0.9640 ± 0.0192^{ab}
			Bulk Soil	1530 ± 623^{abc}	1261 ± 601^{abcde}	6.35 ± 0.96^{ab}	0.623 ± 0.067^{a}	0.9503 ± 0.0257^{ab}

Mean ± standard deviation is shown. Chao1 and Observed species (expressed as the number of OTUs) indicates species richness. Shannon, Pielou's Evenness, and Simpson indexes indicate species diversity. Superscript letters indicate significant differences ($P < 0.05$) ANOVA or Kruskal-Wallis and their corresponding pairwise comparisons (Tukey's HSD post hoc or Mann–Whitney U test). The statistical analyses were performed for bacterial and fungal communities separately.

Supplementary Table 2.5 Physicochemical properties of the asymptomatic and COD tree rhizospheres sampled at the *vitality* and *mortality* stands across Divor and Baixo Sorraia.

Site	Stand Health	Tree Health	Moisture (%)	pH (H_2O)	Total C (mg/kg)	Total N (mg/kg)	C:N ratio
Baixo Sorraia	*Vitality*	Asymptomatic	9.25 ± 1.62^{a}	4.74 ± 0.12^{a}	$19{,}149 \pm 1{,}543^{ab}$	959 ± 103^{ab}	20.04 ± 0.91^{abc}
		COD	9.09 ± 1.87^{a}	4.87 ± 0.26^{a}	$24{,}486 \pm 12{,}635^{a}$	$1{,}098 \pm 284^{a}$	21.72 ± 5.41^{ab}
	Mortality	Asymptomatic	19.53 ± 5.13^{b}	4.79 ± 0.17^{a}	$77{,}339 \pm 50{,}422^{bc}$	$3{,}275 \pm 1{,}964^{c}$	22.60 ± 2.13^{a}
		COD	14.45 ± 3.77^{bc}	4.86 ± 0.40^{a}	$50{,}649 \pm 27{,}058^{c}$	$2{,}367 \pm 1{,}386^{c}$	21.78 ± 1.82^{a}
Divor	*Vitality*	Asymptomatic	9.34 ± 1.86^{ac}	5.45 ± 0.36^{b}	$14{,}601 \pm 2{,}034^{d}$	814 ± 100^{bd}	17.91 ± 0.76^{d}
		COD	8.52 ± 2.00^{a}	5.52 ± 0.46^{b}	$12{,}838 \pm 4{,}593^{d}$	706 ± 283^{d}	18.50 ± 1.67^{cd}
	Mortality	Asymptomatic	9.07 ± 2.84^{ac}	5.87 ± 0.29^{bc}	$13{,}751 \pm 3{,}031^{d}$	745 ± 191^{bd}	18.62 ± 1.34^{bcd}
		COD	9.88 ± 2.26^{a}	6.11 ± 0.42^{c}	$14{,}652 \pm 4{,}719^{d}$	804 ± 279^{bd}	18.46 ± 1.46^{d}

Mean ± standard deviation is shown. Superscript letters indicate significant differences ($P < 0.05$) as determined by the statistical analyses described in the methods section.

Supplementary Table 2.6 Bacterial and Fungal OTU tables.

Due to the large OTU tables for the bacterial and fungal community, this link should be used to facilitate its consultation and view better.

Supplementary Table 2.7 Core microbiome of the cork oak.

Due to the large core microbiome tables for the bacterial and fungal community, this link should be used to facilitate its consultation and view.

Supplementary Table 2.8 Alpha diversity of the rhizospheres sampled in asymptomatic and COD cork oak trees across the *vitality* and *mortality* stands of Divor and Baixo Sorraia.

Microbial Group	Site	Stand Health	Tree Health	Chao1	Observed Species	Shannon	Pielou's Evenness	Simpson
Bacteria	Baixo Sorraia	*Vitality*	Asymptomatic	9174 ± 1042^{a}	5154 ± 579^{ab}	10.43 ± 0.33^{ab}	0.846 ± 0.016^{ab}	0.9973 ± 0.0007^{ab}
			COD	9312 ± 1355^{a}	5112 ± 666^{b}	10.41 ± 0.38^{b}	0.846 ± 0.019^{b}	0.9972 ± 0.0009^{a}
		Mortality	Asymptomatic	8790 ± 972^{a}	4984 ± 506a^{b}	10.44 ± 0.29^{ab}	0.851 ± 0.014^{ab}	0.9973 ± 0.0008^{ac}
			COD	8716 ± 1260^{a}	4896 ± 647^{b}	10.29 ± 0.40^{b}	0.840 ± 0.020^{b}	0.9969 ± 0.0009^{a}
	Divor	*Vitality*	Asymptomatic	12851 ± 1653^{b}	6524 ± 873^{ac}	11.07 ± 0.42^{ac}	0.874 ± 0.019^{ac}	0.9984 ± 0.0005^{cd}
			COD	12361 ± 1421^{b}	6490 ± 695^{c}	11.03 ± 0.35^{c}	0.872 ± 0.017^{c}	0.9982 ± 0.0006^{d}
		Mortality	Asymptomatic	12402 ± 489^{b}	6598 ± 335^{c}	11.09 ± 0.19^{c}	0.874 ± 0.010^{c}	0.9983 ± 0.0003^{bd}
			COD	12701 ± 1011^{b}	6696 ± 455^{c}	11.17 ± 0.23^{c}	0.879 ± 0.012^{c}	0.9985 ± 0.0004^{d}
Fungi	Baixo Sorraia	*Vitality*	Asymptomatic	1649 ± 134^{a}	1171 ± 108^{a}	6.76 ± 0.16^{a}	0.664 ± 0.017^{a}	0.9766 ± 0.0030^{a}
			COD	1643 ± 202^{a}	1173 ± 145^{a}	6.53 ± 0.73^{ab}	0.640 ± 0.067^{ab}	0.9537 ± 0.0529^{ab}
		Mortality	Asymptomatic	1368 ± 79^{b}	961 ± 83^{a}	6.20 ± 0.39^{b}	0.626 ± 0.041^{ab}	0.9537 ± 0.0243^{ab}
			COD	1549 ± 97^{a}	1114 ± 69^{a}	6.43 ± 0.15^{b}	0.636 ± 0.017^{b}	0.9669 ± 0.0097^{ab}
	Divor	*Vitality*	Asymptomatic	1622 ± 210^{ab}	1141 ± 139^{a}	6.51 ± 0.32^{ab}	0.641 ± 0.022^{ab}	0.9661 ± 0.0180^{ab}
			COD	1679 ± 242^{a}	1188 ± 170^{a}	6.48 ± 0.45^{ab}	0.635 ± 0.036^{ab}	0.9625 ± 0.0160^{b}
		Mortality	Asymptomatic	1505 ± 186^{ab}	1117 ± 143^{a}	6.34 ± 0.45^{ab}	0.627 ± 0.047^{ab}	0.9548 ± 0.0245^{ab}
			COD	1528 ± 165^{a}	1162 ± 114^{a}	6.55 ± 0.36^{ab}	0.644 ± 0.030^{ab}	0.9670 ± 0.0159^{ab}

Mean ± standard deviation is shown. Chao1 and Observed species (expressed as the number of OTUs) indicates species richness. Shannon, Pielou's Evenness, and Simpson indexes indicate species diversity. Superscript letters indicate significant differences ($P < 0.05$) based on ANOVA or Kruskal-Wallis and their corresponding pairwise comparisons (Tukey's HSD post hoc or Mann–Whitney U test). The statistical analyses were performed for bacteria and fungi separately.

Supplementary Table 2.9 PERMANOVA results of the site (si), stand (st), and tree health condition comparisons.

Microbial Group	Factor	Pseudo-F	p-value (perm)
Bacteria	Si	13.03	< 1.00E-05
	St	3.63	0.0003
	Si x St	2.26	0.0099
Fungi	Si	9.53	< 1.00E-05
	St	4.41	< 1.00E-05
	Si x St	3.58	< 1.00E-05

Microbial Group	Site	Stand health	Tree health	T	p-value (perm)
Bacteria	Baixo Sorraia	*Vitality*	Asymptomatic / COD	1.0094	0.3657
		Mortality	Asymptomatic / COD	0.8639	0.8735
	Divor	*Vitality*	Asymptomatic / COD	0.8805	0.9286
		Mortality	Asymptomatic / COD	0.9318	0.6844
Fungi	Baixo Sorraia	*Vitality*	Asymptomatic / COD	0.9287	0.7776
		Mortality	Asymptomatic / COD	1.0067	0.4230
	Divor	*Vitality*	Asymptomatic / COD	0.8358	0.9858
		Mortality	Asymptomatic / COD	0.9004	0.8237

Supplementary Table 2.10 Combinations of rhizosphere explanatory variables that best explain the variance in rhizosphere microbiome.

Microbial group	Environmental variable(s) (no. of variables)	Spearman's coefficient (r_s)*
Bacteria	pH, moisture (2)	0.736
	pH, total N (2)	0.728
	pH (1)	0.723
	pH, total C (2)	0.717
	pH, moisture, total N (2)	0.712
Fungi	pH, moisture (2)	0.640
	pH, moisture, total N (3)	0.628
	pH, moisture, total C (3)	0.627
	pH, moisture, C:N ratio (3)	0.620
	pH, moisture, total N, total C (4)	0.620

* Significant differences ($P < 0.05$) were obtained in all the combinations.

Supplementary Table 2.11 DESeq 2.0 analysis results.

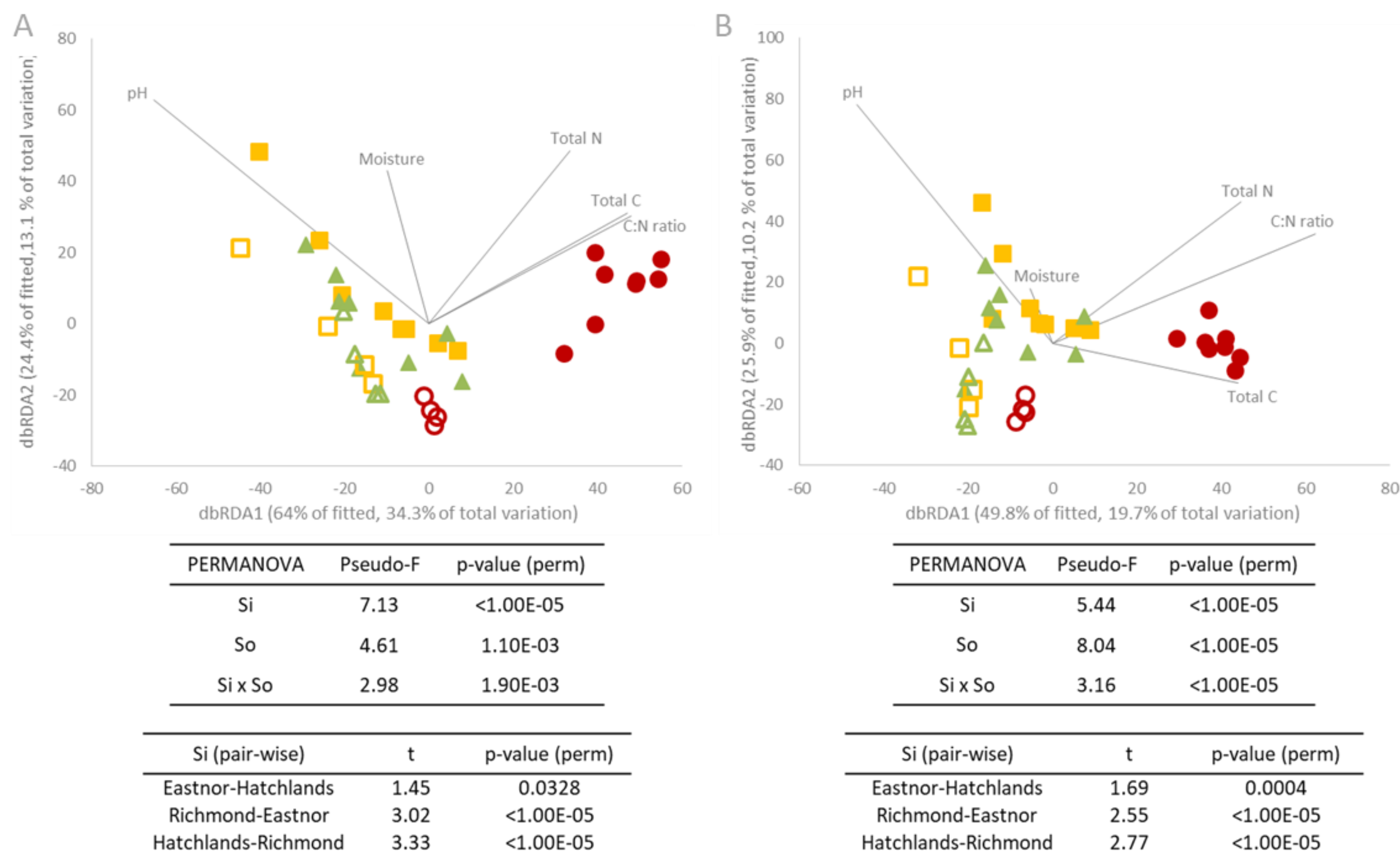
Due to the large DESeq 2.0 results tables for the bacterial and fungal community, this link should be used to facilitate its consultation and view.

Supplementary Table 2.12 Ecological guilds of fungal taxa detected in the rhizosphere of cork oaks at vitality and mortality stands across Divor and Baixo Sorraia.

Site	Divor		Baixo Sorraia	
Stand Health	***Vitality***	***Mortality***	***Vitality***	***Mortality***
Ectomycorrhizal	31.78 ± 2.22[a]	32.89 ± 2.52[a]	27.62 ± 3.07[a]	30.30 ± 3.22[a]
Saprotroph	20.87 ± 1.28[a]	19.93 ± 1.77[a]	19.12 ± 1.19[a]	22.05 ± 2.24[a]
Plant Saprotroph	0.00 ± 0.00[a]	0.01 ± 0.01[b]	0.00 ± 0.00[a]	0.01 ± 0.00[b]
Dung Saprotroph	0.05 ± 0.03[a]	0.04 ± 0.01[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]
Wood Saprotroph	0.42 ± 0.17[a]	0.20 ± 0.04[ab]	0.11 ± 0.03[b]	0.28 ± 0.07[a]
Soil Saprotroph	0.03 ± 0.01[ab]	0.05 ± 0.02[a]	0.01 ± 0.00[b]	0.02 ± 0.01[ab]
Plant Pathogen	0.54 ± 0.07[ab]	0.65 ± 0.15[a]	0.41 ± 0.06[bc]	0.32 ± 0.09[c]
Fungal Parasite	0.35 ± 0.06[a]	0.46 ± 0.05[b]	0.39 ± 0.06[ab]	0.21 ± 0.06[c]

Significant differences ($P < 0.05$) according to site and stand health are indicated by different letters.

Appendix 2: Supplementary information of Chapter 3



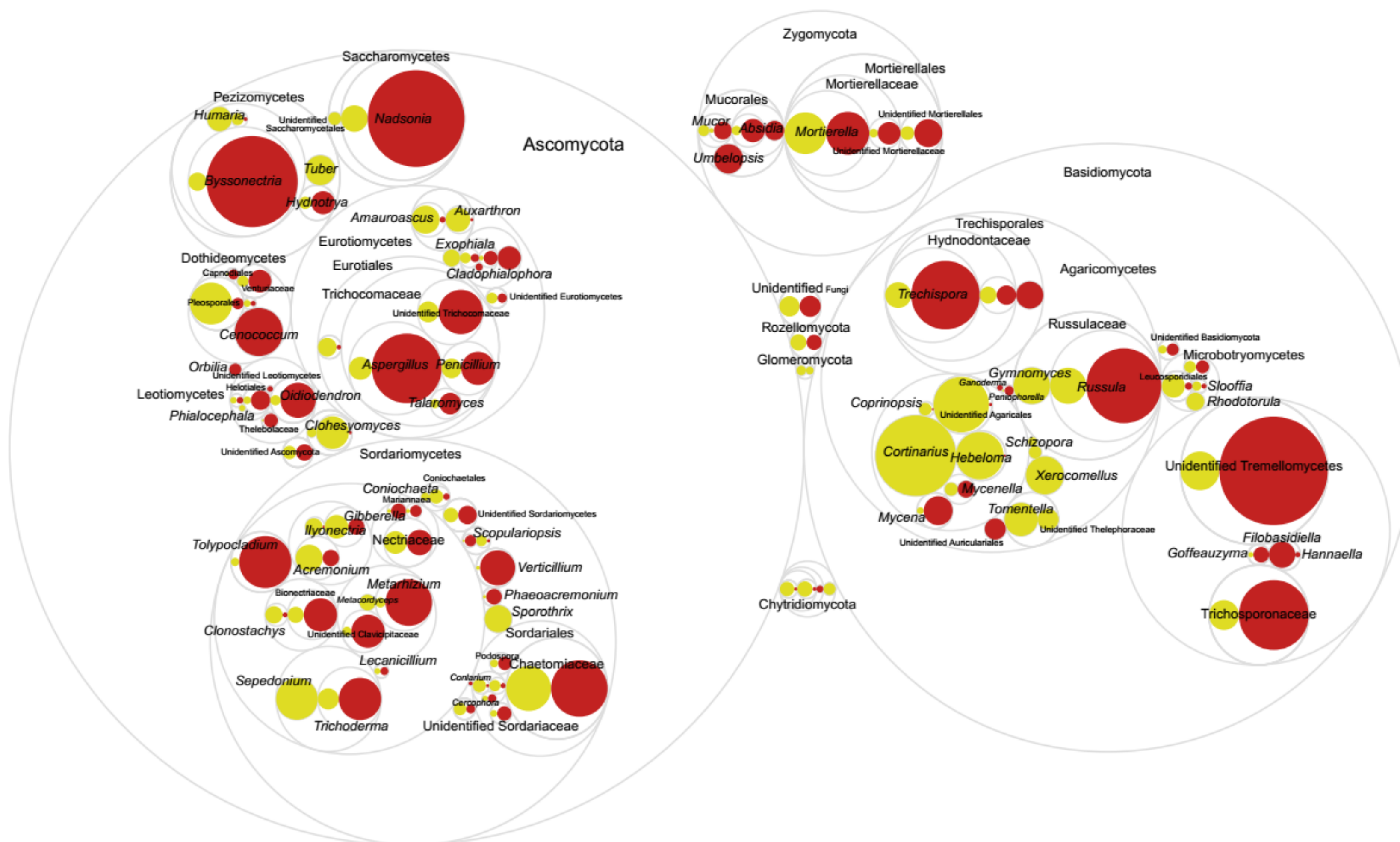
PERMANOVA	Pseudo-F	p-value (perm)
Si	7.13	<1.00E-05
So	4.61	1.10E-03
Si x So	2.98	1.90E-03

Si (pair-wise)	t	p-value (perm)
Eastnor-Hatchlands	1.45	0.0328
Richmond-Eastnor	3.02	<1.00E-05
Hatchlands-Richmond	3.33	<1.00E-05

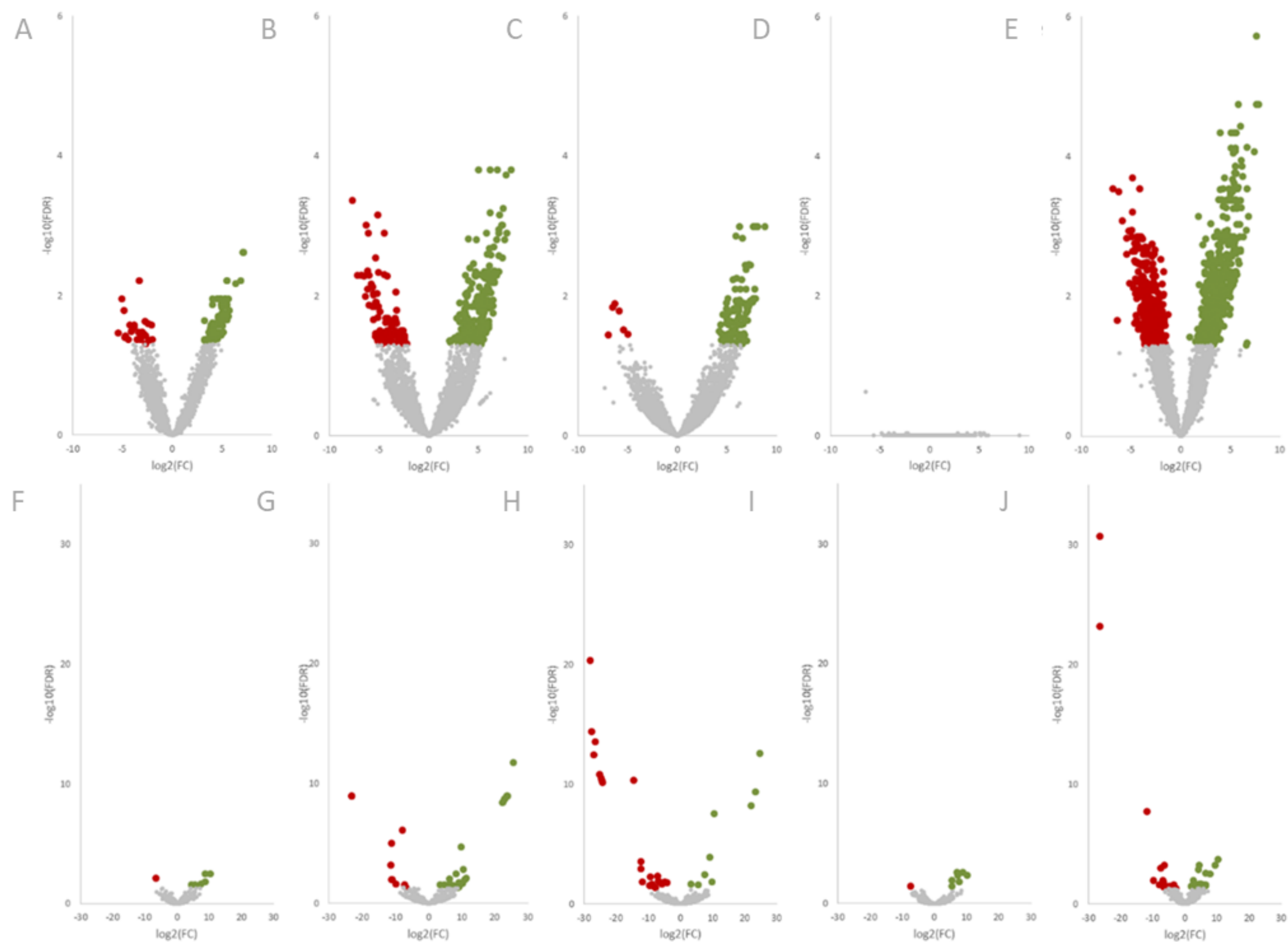
PERMANOVA	Pseudo-F	p-value (perm)
Si	5.44	<1.00E-05
So	8.04	<1.00E-05
Si x So	3.16	<1.00E-05

Si (pair-wise)	t	p-value (perm)
Eastnor-Hatchlands	1.69	0.0004
Richmond-Eastnor	2.55	<1.00E-05
Hatchlands-Richmond	2.77	<1.00E-05

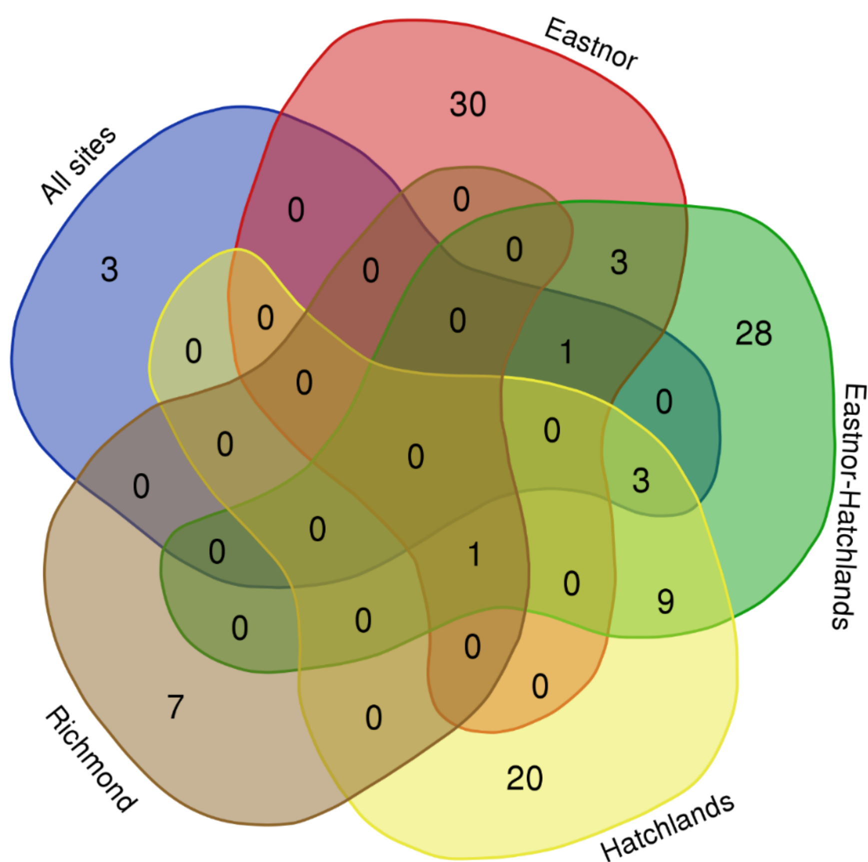
Supplementary Figure 3.1 Distance-based redundancy analysis of the (A) bacterial and (B) fungal communities and soil properties of the rhizosphere and bulk soil across the sites (n=36). The longer the line, the stronger the correlation between microbiome composition and soil chemistry. Eastnor – green triangle; Hatchlands – yellow square; Richmond – red circle; filled symbol – rhizosphere; open symbol – bulk soil. PERMANOVA results are shown in the below tables. Si – site; So – Soil compartment.



Supplementary Figure 3.2 Fungi differentially abundant across the sites. Only significantly enriched OTUs determined by DESEq 2.0 are depicted (FDR-corrected P < 0.05). The size of the circles represents the relative abundance and the colour denotes the rhizosphere of oak trees located in Hatchlands (yellow) and Richmond (red).



Supplementary Figure 3.3 Volcano plots of differential bacterial (A, B, C, D and E) and fungal (F, G, H, I and J) OTU abundance analysis as calculated by DESeq 2.0 according to tree health condition. Fold change as FDR corrected p-values are plotted for each OTU. Significantly different taxa (FDR-corrected P < 0.05) are coloured according to tree health condition: healthy – green (positive log2 (FC) axis), AOD – red (negative log2 (FC) axis). All sites (A, F) Eastnor (B, G); Hatchlands (C, H); Richmond (D, I); Eastnor and Hatchlands (E, J).



Supplementary Figure 3.4 Venn diagram of the intersection of the differentially abundant fungal OTUs between healthy and AOD trees detected across the tree health comparisons.

Supplementary Table 3.1 Physicochemical properties according to soil compartment.

Site	AOD stage	Soil Compartment	Moisture (%) *	pH (H2O) ***	Total C (mg/kg) ***	Total N (mg/kg) ***	C:N ratio ***
Eastnor	Low	Rhizosphere	25 ± 6 [ab]	5.68 ± 0.49 [ac]	60,740 ± 11,450 [a]	4,629 ± 651 [a]	13.04 ± 1.06 [ad]
		Bulk soil	28 ± 2 [b]	5.62 ± 0.26 [a]	34,401 ± 5,065 [bd]	2,908 ± 376 [b]	11.81 ± 0.64 [bd]
Hatchlands	Mid	Rhizosphere	22 ± 3 [a]	5.92 ± 0.73 [a]	64,216 ± 9,660 [a]	4,581 ± 793 [a]	14.08 ± 0.80 [a]
		Bulk soil	29 ± 4 [b]	5.93 ± 0.47 [a]	42,666 ± 8,150 [b]	3,860 ± 931 [ab]	11.17 ± 0.54 [b]
Richmond	Severe	Rhizosphere	27 ± 6 [ab]	4.33 ± 0.14 [b]	225,966 ± 55,586 [c]	12,921 ± 3,331 [c]	17.56 ± 0.55 [c]
		Bulk soil	15 ± 3 [c]	5.12 ± 0.07 [c]	27,328 ± 847 [d]	2,064 ± 102 [d]	13.25 ± 0.28 [ad]

Mean values of soil physicochemical parameters ± standard deviation are shown (n = 36). Asterisks and letters indicate significant differences as determined by statistical analysis (* for $P < 0.05$; ** for $P < 0.01$; *** for $P < 0.001$).

Supplementary Table 3.2 Microbial alpha diversity and total abundance according to soil compartment across the sites.

Microbial Group	Site	AOD stage	Soil Compartment	Chao 1	Observed Species	Shannon	Evenness	Simpson	Total abundance
Bacteria	Eastnor	Low	Rhizosphere	7,840 ± 804 [a]	4,234 ± 492 [a]	9.92 ± 0.37 [a]	0.82 ± 0.02 [a]	0.996 ± 0.001 [ab]	11.02 ± 0.11 [a]
			Bulk soil	7,506 ± 412 [ad]	4,143 ± 392 [a]	9.97 ± 0.31 [a]	0.83 ± 0.02 [a]	0.997 ± 0.001 [a]	10.92 ± 0.10 [a]
	Hatchlands	Mid	Rhizosphere	7,721 ± 682 [a]	4,326 ± 426 [a]	10.06 ± 0.31 [a]	0.83 ± 0.02 [a]	0.997 ± 0.001 [a]	11.08 ± 0.05 [a]
			Bulk soil	6,396 ± 229 [b]	3,519 ± 212 [ab]	9.51 ± 0.24 [ab]	0.81 ± 0.01 [ab]	0.995 ± 0.001 [b]	10.94 ± 0.14 [a]
	Richmond	Severe	Rhizosphere	5,478 ± 746 [c]	2,996 ± 441 [b]	8.77 ± 0.42 [c]	0.76 ± 0.02 [c]	0.988 ± 0.004 [c]	11.09 ± 0.05 [a]
			Bulk soil	6,618 ± 338 [bd]	3,598 ± 226 [ab]	9.17 ± 0.20 [bc]	0.78 ± 0.01 [bc]	0.990 ± 0.004 [c]	11.00 ± 0.05 [a]
Fungi	Eastnor	Low	Rhizosphere	1,187 ± 190 [a]	875 ± 152 [a]	6.77 ± 0.43 [ab]	0.69 ± 0.03 [ab]	0.974 ± 0.008 [ab]	9.08 ± 0.20 [bc]
			Bulk soil	1,062 ± 141 [ac]	807 ± 107 [ab]	6.46 ± 0.20 [ab]	0.67 ± 0.01 [ab]	0.966 ± 0.008 [ab]	8.87 ± 0.06 [ac]
	Hatchlands	Mid	Rhizosphere	1,125 ± 97 [a]	883 ± 70 [a]	6.94 ± 0.32 [a]	0.71 ± 0.03 [a]	0.976 ± 0.006 [a]	8.92 ± 0.26 [abc]
			Bulk soil	853 ± 130 [b]	710 ± 90 [ab]	6.44 ± 0.40 [ab]	0.68 ± 0.03 [ab]	0.959 ± 0.016 [ab]	8.64 ± 0.16 [a]
	Richmond	Severe	Rhizosphere	1,140 ± 102 [a]	767 ± 43 [ab]	6.27 ± 0.25 [b]	0.65 ± 0.02 [b]	0.961 ± 0.018 [b]	9.46 ± 0.15 [d]
			Bulk soil	896 ± 74 [bc]	674± 60 [b]	6.52 ± 0.29 [ab]	0.69 ± 0.02 [ab]	0.970 ± 0.011 [ab]	9.00 ± 0.07 [b]

Mean values ± standard deviation are shown (n = 36). Chao1 and Observed species (expressed as number of OTUs) indicate species richness. Pielou's Evenness, Shannon, and Simpson indexes indicate species diversity. Total abundance (qPCR) is expressed as log copy number of 16S or ITS genetic markers/g of soil. Significant differences ($P < 0.05$) are indicated by letters. The statistical analyses were performed for bacterial and fungal communities separately.

Supplementary Table 3.3 Bacterial OTU table.

Due to the large OTU table for the bacterial community, this link should be used to facilitate its consultation and view.

Supplementary Table 3.4 Fungal OTU table.

Due to the large OTU table for the fungal community, this link should be used to facilitate its consultation and view.

Supplementary Table 3.5 Microbial alpha diversity and total abundance of the oak rhizosphere soils across the sites and tree health conditions.

Microbial Group	Site	AOD stage	Tree health condition	Chao 1	Observed Species	Shannon	Evenness	Simpson	Total abundance
Bacteria	Eastnor	Low	Healthy	8,358 ± 804 ^{a}	4,566 ± 477 ^{a}	10.14 ± 0.39 ^{a}	0.83 ± 0.02 ^{a}	0.997 ± 0.001 ^{ab}	11.11 ± 0.05 ^{ab}
			AOD	7,323 ± 334 ^{ac}	3,903 ± 191 ^{ab}	9.70 ± 0.16 ^{a}	0.81 ± 0.01 ^{a}	0.996 ± 0.001 ^{b}	10.93 ± 0.07 ^{c}
	Hatchlands	Mid	Healthy	7,974 ± 466 ^{a}	4,570 ± 319 ^{a}	10.30 ± 0.25 ^{a}	0.85 ± 0.01 ^{a}	0.997 ± 0.001 ^{a}	11.11 ± 0.05 ^{a}
			AOD	7,469 ± 766 ^{a}	4,082 ± 377 ^{a}	9.82 ± 0.12 ^{a}	0.82 ± 0.00 ^{a}	0.996 ± 0.000 ^{b}	10.93 ± 0.07 ^{b}
	Richmond	Severe	Healthy	5,292 ± 765 ^{b}	2,950 ± 371 ^{b}	8.73 ± 0.23 ^{b}	0.76 ± 0.01 ^{b}	0.988 ± 0.002 ^{c}	11.04 ± 0.03 ^{b}
			AOD	5,664 ± 677 ^{bc}	3,041 ± 498 ^{b}	8.81 ± 0.54 ^{b}	0.76 ± 0.03 ^{b}	0.988 ± 0.005 ^{c}	11.13 ± 0.03 ^{a}
Fungi	Eastnor	Low	Healthy	1,181 ± 178 ^{a}	905 ± 128 ^{a}	6.84 ± 0.44 ^{ab}	0.70 ± 0.03 ^{ab}	0.974 ± 0.010 ^{a}	9.03 ± 0.14 ^{ab}
			AOD	1,193 ± 201 ^{a}	844 ± 167 ^{a}	6.69 ± 0.40 ^{abc}	0.69 ± 0.02 ^{ab}	0.975 ± 0.010 ^{a}	9.13 ± 0.24 ^{abc}
	Hatchlands	Mid	Healthy	1,101 ± 77 ^{a}	915 ± 66 ^{a}	7.15 ± 0.24 ^{a}	0.73 ± 0.02 ^{a}	0.980 ± 0.004 ^{a}	8.71 ± 0.13 ^{a}
			AOD	1,149 ± 109 ^{a}	851 ± 57 ^{a}	6.72 ± 0.23 ^{abc}	0.69 ± 0.02 ^{ab}	0.973 ± 0.006 ^{a}	9.13 ± 0.16 ^{abc}
	Richmond	Severe	Healthy	1,173 ± 78 ^{a}	777 ± 40 ^{a}	6.20 ± 0.33 ^{bc}	0.65 ± 0.03 ^{b}	0.954 ± 0.022 ^{a}	9.49 ± 0.20 ^{c}
			AOD	1,108 ± 113 ^{a}	757 ± 44 ^{a}	6.34 ± 0.07 ^{c}	0.66 ± 0.01 ^{b}	0.969 ± 0.002 ^{a}	9.42 ± 0.06 ^{bc}

Mean values ± standard deviation are shown (n = 24). Chao1 and Observed species (expressed as number of OTUs) indicate species richness. Pielou's Evenness, Shannon, and Simpson indexes indicate species diversity. Total abundance (qPCR) is expressed as log copy number of 16S or ITS genetic markers/g of soil. Significant differences ($P < 0.05$) are indicated by letters. The statistical analyses were performed for bacterial and fungal communities separately.

Supplementary Table 3.6 PERMANOVA and pairwise comparison results of the comparison of the bacterial and fungal community according to site and tree health condition (n = 24).

Microbial Group	**Factor**	**Pseudo-F**	**p-value (perm)**
Bacteria	Si	10.53	< 1.00E-05
	Tr	3.37	0.0181
	Si x Tr	1.51	0.1416
Fungi	Si	6.43	< 1.00E-05
	Tr	1.90	0.0426
	Si x Tr	1.29	0.1522
Microbial Group	**Pairwise (Si)**	**t**	**p-value (perm)**
Bacteria	Eastnor-Hatchlands	1.33	0.0791
	Eastnor-Richmond	3.77	2.00E-05
	Hatchlands-Richmond	4.01	2.00E-05
Fungi	Eastnor-Hatchlands	1.62	0.0003
	Eastnor-Richmond	2.85	< 1.00E-05
	Hatchlands-Richmond	3.06	3.00E-05

Si – Site; Tr – Tree health condition

Supplementary Table 3.7 Combinations of rhizosphere soil explanatory variables that best explain the variance in the rhizosphere microbiome (n = 24).

Microbial group	Environmental variable(s) (no. of variables)	Spearman's coefficient (rs)*
Bacteria	pH, C, C:N (3)	0.896
	pH (1)	0.895
	pH, C (2)	0.892
	pH, C:N (2)	0.892
	pH, N, C:N (3)	0.889
Fungi	pH, C:N (2)	0.848
	pH, C, C:N (3)	0.827
	pH (1)	0.825
	pH, N, C:N (3)	0.817
	pH, C (2)	0.799

* Significant differences (P < 0.01) were obtained in all the combinations

Supplementary Table 3.8 DESeq 2.0 analysis results at site level.

Due to the large DESeq 2.0 tables for the bacterial community, this link should be used to facilitate its consultation and view.