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Role of olive tree phyllosphere microorganisms in the biological control of olive leaf spot and olive knot

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Trabalho efetuado sob a orientação da
Profª Doutora Paula Baptista
da
Profª Doutora Teresa Lino-Neto
e do
Prof. Doutor José Alberto Pereira

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DECLARAÇÃO

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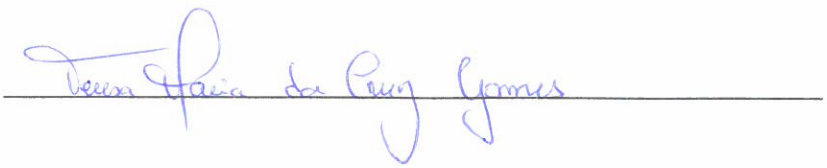

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Abstract

The olive leaf spot (OLS) and the olive knot (OK) diseases are key constraints to olive production, due to their high incidence and related losses. However, none of the available control measures are effective against both diseases. This work aims to characterize the phyllosphere fungal communities, which reside in and on leaf/twig tissues of olive tree, and to understand their role in conferring host protection against these two diseases. Fungal communities of cultivars displaying differences on disease susceptibility were assess by culture-dependent approach and compared either among asymptomatic and symptomatic plant tissues or among different levels of disease incidence. The isolation of fungal communities was performed in autumn and spring. The relationship between foliar composition on fungi, secondary metabolites and host susceptibility was also evaluated.

Phyllosphere fungal community revealed to be rich and abundant, comprising species belonging mainly to Ascomycota phyla and Cladosporiaceae family. Endophytic and epiphytic communities were distinct and affected primarily by season. In addition, climatic factors and the presence of disease were important in shaping epiphytes, whereas plant organ and genotype (at cultivar level) were the major drivers of endophytes. The interplay between the pathogen, the plant and its indigenous microbiota, also seemed to be critical for the establishment of fungal communities in the olive phyllosphere. The level of disease incidence was linked to host cultivar and to fungal and metabolite (phenolic and volatile compounds) composition of their leaves. Thus, it is possible that cultivar susceptibility might be in part related with the composition of fungal and metabolites. Some key fungal taxa and metabolites were identified to play an important role in conferring cultivar susceptibility/tolerance to OLS disease. Similarly, several fungal taxa were found to be specific to either asymptomatic or symptomatic plant tissues, suggesting their competitive or cooperative activity with the pathogen. Further investigations are still required to identify the functional role of these fungi and metabolites in conferring host plant protection to OLS and OK diseases.

Keywords: endophytes; epiphytes; disease susceptibility; metabolites; microbe-microbe interaction; microbe-host interaction

Resumo

O olho-de-pavão e a tuberculose são importantes ameaças à produção olivícola, devido à sua incidência e perdas relacionadas. Não existe nenhum método de luta que se tenha mostrado eficaz contra estas duas doenças. Este trabalho tem como objetivo caracterizar a comunidade fúngica da filosfera da oliveira, que reside interna e externamente nas suas folhas/ramos, de forma a compreender o seu papel na proteção da planta contra estas duas doenças. A comunidade fúngica foi avaliada em cultivares que apresentam diferenças de suscetibilidade às doenças, recorrendo a métodos culturais, e comparada entre material assintomático e sintomático ou entre diferentes níveis de incidência de doença. O isolamento de fungos foi realizado durante o outono e a primavera. Foi ainda avaliada a relação entre a composição foliar de fungos e de metabolitos secundários, e a suscetibilidade da planta às referidas doenças.

A comunidade fúngica da filosfera mostrou ser rica e abundante, incluindo espécies pertencentes maioritariamente ao filo Ascomycota e à família Cladosporiaceae. A composição da comunidade endofítica foi distinta da epifítica, e mostrou ser fortemente influenciada pela estação do ano. Vários fatores climáticos e a presença de doença foram ainda cruciais na estruturação dos epifíticos, enquanto o órgão e o genótipo da planta (cultivar) influenciaram também a composição de endófitos. A interação entre o patogénico, a planta e a sua flora microbiana nativa, também revelou ser crítica para o estabelecimento das comunidades fúngicas na filosfera da oliveira. O nível de incidência de doença mostrou estar relacionado com a cultivar, e com a composição de fungos e metabolitos (fenóis e voláteis) das suas folhas. Este resultado sugere que a suscetibilidade da cultivar possa estar relacionada com a sua composição em fungos e metabolitos, tendo, alguns deles, mostrado ter um papel importante na suscetibilidade/ tolerância da cultivar ao olho-de-pavão. Algumas espécies fúngicas mostraram também estar fortemente associados quer a material sintomático ou assintomático, sugerindo que possam estabelecer relações de competição ou cooperação com o patogénico. Estudos adicionais são ainda necessárias para identificar a função destes fungos e metabolitos na proteção da oliveira contra o olho-de-pavão e a tuberculose da oliveira.

Palavras-chave: endofíticos; epifíticos; suscetibilidade à doença; metabolitos; interação microrganismo-microrganismo; interação microrganismo-planta

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List of Abbreviations

AIC	Akaike's information criterion
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
BCA	Biological control agent
BLAST	Basic Local Alignment Search Tool
°C	Celsius degrees
CIA	Co-inertia analysis
CFU	Colony forming unit
CCA	Canonical correlation analysis
DAD	Diode Array Detection
DistLM	Distance based linear models
DNA	Deoxyribonucleic Acid
DW	Dry weight
EU	European Union
FAO	Food and Agriculture Organization
FW	Fresh weight
GC	Gas chromatography
GC-MS	Gas chromatography- mass spectrometry
H´	Shannon index
ha	Hectare
HPLC	High performance liquid chromatography
HS-SPME	Headspace solid-phase microextraction
IAA	Indol-3-acetic acid
ITS	Internal transcribed spacer
IndVal	The indicator value
NCBI	National Center for Biotechnology Information
NMDS	Non-metric multidimensional analysis
OK	Olive Knot
OLS	Olive leaf spot
OOB	Out-of-bag error
OTU	Operational Taxonomic Unit
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PCA	Plate count Agar
Psv	*Pseudomona savastanoi* pv s*avastanoi*
RDA	Redundancy analysis
SPSS	Statistical Package for the Social Sciences
rDNA	Ribossomal Deoxyribonucleic acid
SE	Standard error
UV	Ultraviolet light
1/D	Simpson's Reciprocal Index

Chapter 1

General Introduction

1.1 Major diseases of olive tree

Olive tree (*Olea europaea* L.) is one of the most economically important oil-producing crops in many countries of the world, particularly in the Mediterranean basin countries, including Portugal (Loumou and Giourga, 2003). However, its production and productivity is seriously constrained by various diseases, being the olive leaf spot (OLS) and olive knot (OK) considered to be the major causes of olive-crop damage worldwide (Obanor et al., 2008; Quesada et al., 2010; Salman et al., 2011; Marchi et al., 2005).

The OLS, caused by the mitosporic fungus *Venturia oleaginea* (Castagne) Rossman & Crous (syn. *Fusicladium oleagineum, Spilocaea oleaginea*), is the most important foliar disease of olive (Viruega et al., 2013). To a lesser extent the fungus can also cause lesions on fruits (Viruega et al., 2013) and peduncle (Trapero-Casas et al., 2009). This specific biotrophic pathogen from Venturiaceae family shows a typical leaf subcuticular development with the production of asexual spores, resulting in black circular spots as symptoms of the disease (Fig. 1.1; Rongai el al., 2012). Up to date, there is no evidence of sexual spore's production by *V. oleaginea,* and therefore, the infection spread is dependent on the number of available spores (Viruega et al., 2013). The establishment of infection requires high humidity and temperatures around 8-25°C, with optimal temperature of 15°C, occurring mostly during autumn and earlier spring (Trapero-Casas et al., 2009). The time interval between infection and the appearance of the first symptom, which is known as the incubation time of the pathogen, could vary between 1 and 10 months, depending on the climatic conditions (Trapero-Casas et al., 2009). This latent phase of the pathogen have great importance, since during extreme abiotic conditions, the pathogen can persist in infected leaves for a long period in the tree canopy (Viruega et al., 2013). When new favorable conditions are met, the pathogen resume growth and produce new conidia, which are dispersed by water splashing or wind (Viruega et al., 2013; Trapero-Casas et al., 2009).

Crop losses caused by OLS mostly result from the severe defoliation of infected trees, leading sometimes to the death of defoliated branches (Gonzalez-Lamothe et al., 2002). As most infected leaves fall prematurely, olive trees become weakened and flower formation is inhibited affecting largely the production (Belfiore et al., 2014). In several countries, such as Palestine and California, the OLS was reported to cause 20-70% of yield losses (cited by Rongai et al., 2012; Hajjeh et al., 2014).

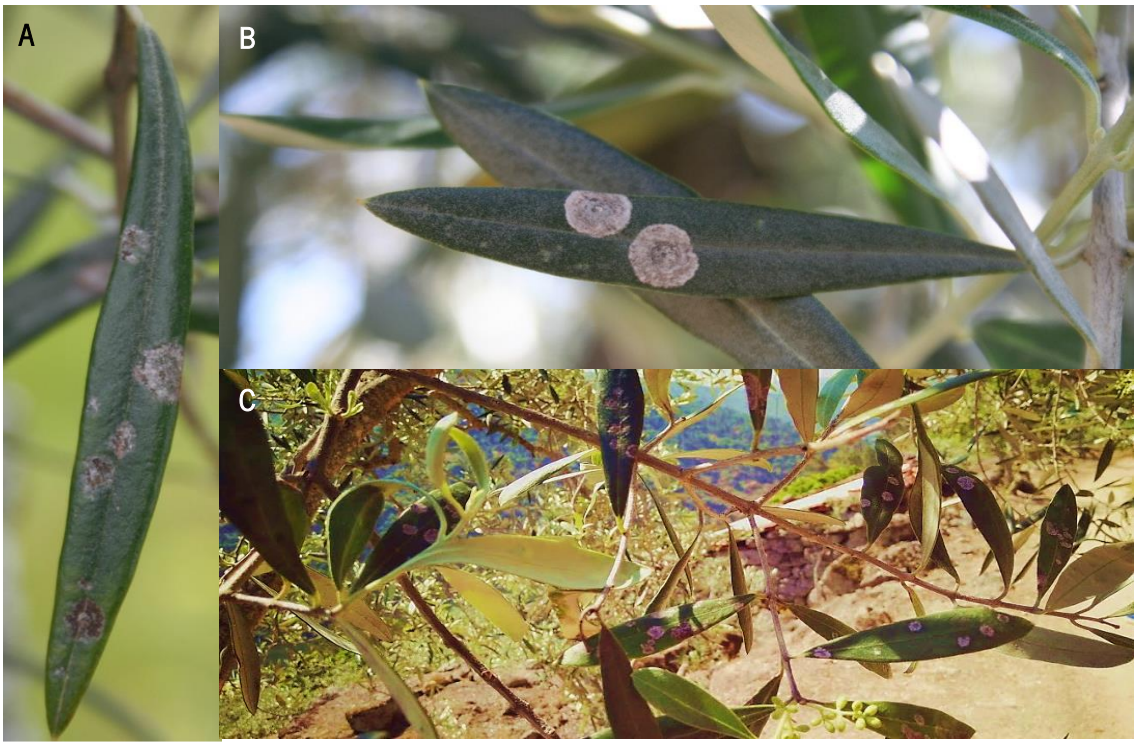

Fig. 1.1. Olive tree leaves exhibiting symptoms of olive leaf spot (OLS) disease. (**A**) Circular grizzly to almost black lesions on the adaxial surface of the leaf are the typical symptoms of the disease during the winter–spring. (**B** and **C**) These lesions change to a whitish scab during the summer when the cuticle is separated from epidermal cells on infected leaf. Credits: Teresa Gomes, IPB-ESA.

The OK disease, caused by the gram-negative, aerobic bacterium *Pseudomonas savastanoi* pv. *savastanoi* (Psv), is characterized by the formation of tumorous galls (knots) in the branches of olive tree (Fig. 1.2). Leaves and fruits may occasionally be also infected by Psv (Trapero-Casas and Ramos, 2015). Psv is usually found in olive tree phyllosphere as an epiphyte (Quesada et al., 2010) and/or endophyte (Marchi et al., 2009), associated with other bacterial species (Buonaurio et al., 2015). These associated bacteria, belonging mainly to *Pantoea*, *Pectobacterium*, *Erwinia* and *Curtobacterium,* have been shown to increased Psv bacterial populations and disease symptoms in knots (Buonaurio et al., 2015). The infection of olive tree is believed to be caused by the epiphytic Psv (Quesada et al., 2010), through wounds and fissures on twigs, particularly cracks caused by late spring frost, hail storms and agricultural practices (*e.g.* pruning and crop harvesting) (Lavermicocca et al., 2002; Trapero-Casas et al., 2009). Upon infection, the pathogen starts to colonize the tissues around the fresh wounds, producing cavities that are filled with the bacterium or invade directly the xylem vessels (Rodríguez-Moreno et al.,

2009; Maldonado-González et al., 2013). The formation of knots result mainly from the bacterial synthesis of indol-3-acetic acid (IAA) and cytokinins, that induce the increase in plant cell size (hypertrophy) followed by an abnormal cell division (hyperplasia) (Rodríguez-Moreno et al., 2008; Quesada et al., 2012).

Infection normally occurs during the wet seasons, *i.e.* spring and autumn, during which climatic conditions are optimal for disease development (Lavermicocca et al., 2002). In particular, rainfall, temperature and relative humidity, were found to be correlated with Psv population size (Quesada et al., 2007). The range of temperatures can vary widely (4-30°C), but the optimal temperature for growth and dispersal of Psv is at 23-24°C (Trapero-Casas et al., 2009). Furthermore, the precipitation is also an important factor for the Psv-bacteria dispersion, via water splash from galls (Quesada et al., 2010).

In several Mediterranean countries, such as Italy or Spain, OK disease infection can reach up to 70%, depending on the cultivar susceptibility (Godena et al., 2009; Trapero-Casas et al., 2009). Severe infections can lead to the death of the branches and progressively to a greater weakness resulting in loss of tree vigor (Tjamos et al., 1993). The effect of the disease on the olive crop is not well studied but reductions in yield can be expected (Quesada et al., 2010). There are also some reports indicating that heavy infection diminishing the quality of olive oil (Tjamos et al., 1993).

Fig. 1.2. Olive knot disease symptoms in olive tree caused by the *Pseudomonas savastanoi* pv. *savastanoi.* Knots on branches (**A**) and on young stem (**B**). At the onset of infection, the knots are pale-green, and gradually turn greenish-brown or brown and hard with a cracked surface. Credits: Teresa Gomes, IPB-ESA.

1.2. Disease management

Disease management of olive leaf spot and olive knot diseases in the field are mainly based on preventive measures (Quesada et al., 2010; Trapero-Casas et al., 2009). This is of particularly importance for OK disease, because it is extremely difficult to eradicate the pathogen after their establishment in a tree or orchard (Quesada et al., 2010). These preventive measures include mostly the use of resistant cultivars, pruning practices and chemical treatments (Viruega et al., 2013; Mundt, 2002; Trapero-Casas et al., 2009).

The use of resistant cultivars could be an approach to suppress OLS and OK diseases, but still few explored (Cacciola et al., 2012). Indeed, information available about cultivar susceptibility to OLS and OK diseases is scarce and mainly comes from field observations and from different methodologies, which data could not be compared (Quesada et al., 2010; Salman et al., 2013). The few studies performed on cultivar susceptibility to these two diseases suggest that true resistance is uncommon among cultivated olive cultivars (Quesada et al., 2010; Salman et al., 2013). Although no completely resistant cultivars are yet available, there are several reports indicating differences in the degree of susceptibility to OLS and OK among cultivars (Quesada et al., 2010; Trapero-Casas and Ramos, 2015). For instances, by using artificial inoculations with Psv, Marcelo et al. (1999) showed that the Portuguese cultivars *Cobrançosa* was relatively resistant, *Branquita* and *Santulhana* expressed some symptoms, and *Cordovil de Serpa* and *Galega Vulgar*, were most susceptible to the OK disease. Field observations in Trás-os-Montes region (Northeast of Portugal) indicated that some Portuguese cultivars are less susceptible to OLS and OK diseases (*i.e.* cv. *Cobrançosa*) than others (*i.e.* cvs. *Madural* and *Verdeal Transmontana*) (Fig. 1.3; Teresa Gomes, Pers. Comm.). However, the replace of established crops by these cultivars less susceptible to OLS and OK diseases can add high costs, making it a less appealing short-term solution (Landum et al., 2016).

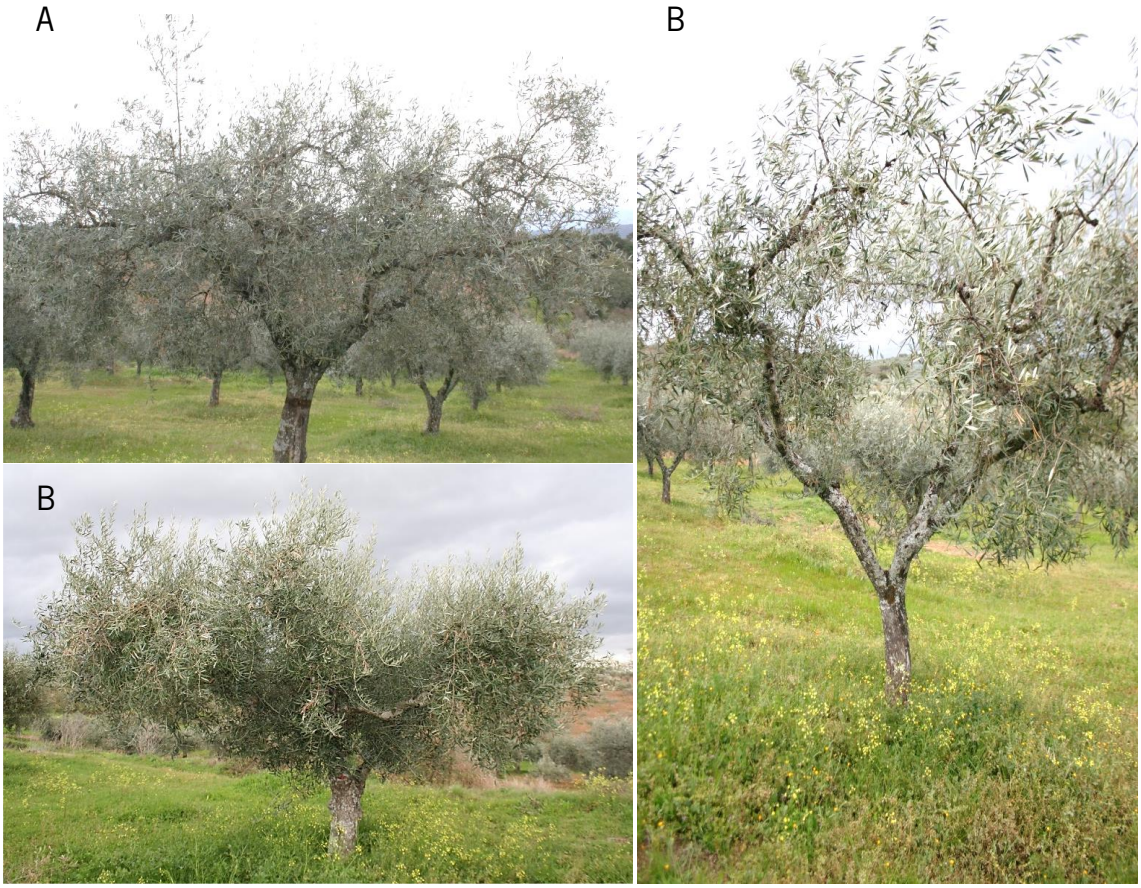

Fig. 1.3 Portuguese olive cultivars with different susceptibilities to OLS and OK-disease: A) Cobrançosa the most tolerant, whereas B) Madural and C) Verdeal Transmontana are the most susceptible. Credits: Teresa Gomes, IPB-ESA.

The pruning practices can also be implemented as a measure to OLS and OK disease control. The higher canopy density can affect the pathogen development, since the increase of humidity and tree shadow favors the infection spread (Trapero-Casas et al., 2009).

Chemical treatments by fungicides are commonly applied to prevent infections of both OLS and OK diseases (Trapero-Casas et al., 2009). They are mainly based on the use of copper-based compounds (*e.g.* copper hydroxide) (Trapero-Casas et al., 2009). However, these treatments are not always successful, and the cost-effective benefit is highly doubtful (Quesada et al., 2010; Obanor et al., 2008). Besides becoming less effective, fungicides are additionally harmful for human health and environment. Thus, strategies for sustainable prevention and management of these two diseases are one of the major challenges within the scientific community (Al-Khatib et al., 2010; Krid et al., 2012). Biological control of plant pathogens using antagonists or natural enemies inhabiting vegetative tissues with potential to eliminate or control their population has gained importance as a sustainable alternative to crop production (Cook, 1993; Di Francesco et

al., 2016). The use of these agents meets many of the European policy aims of moving towards more sustainable crop production systems (Directive 2009/128/EC) and the "Guidelines for integrated production of olives" published by IOBC/WPRS (Malavolta and Perdikis, 2012). Despite no effective biological control are still available against both OLS and OK diseases, some authors have already reported encouraging results in this field. Indeed, the antagonistic activity of *Pseudomonas fluorescens* and *Bacillus subtilis* isolates, from knots and leaves of olive trees, were reported to be effective against Psv under *in vitro* conditions (Krid et al., 2010). Similarly, Al-Khatib et al. (2010) showed that different bacterial (from air and water) isolates (*B. subtilis, B. megaterium, B. cereus*) displayed antifungal activity against the *V. oleaginea.* In line with this, challenges for the upcoming years will be to elucidate the role of the microbial community present in olive tree phyllosphere in the control of OLS and OK diseases.

1.3. The phyllosphere and their role in plant protection against diseases

The phyllosphere, here used to refer to the total above-ground portions of plants (Vacher et al., 2016), is a habitat for a variety of microorganisms (Newton et al., 2010), including bacteria, fungi, yeasts and oomycetes (Lindow & Brandl, 2003; Vorholt, 2012). These microorganisms can live epiphytically, at the surface of the tissues, or endophytically, at the inner tissues (Newton et al., 2010). Endophytes and epiphytes have been described as non-pathogenic components of plant microbiome (Bacon and White Jr., 2015).

There is growing evidence that these microbial communities inhabiting the phyllosphere play an important role in determining the fitness, health and productivity of their hosts (Rastogi et al., 2013). Such features are more commonly described to endophytes than to epiphytes. Endophytes can, for instances, promote plant nutrition and protection against abiotic (*e.g.* drought and extreme temperatures) and biotic stresses, such as plant pathogens and insects (Bacon and White Jr., 2015). The protection conferred by endophytes against pathogens is largely attributed to endophytic production of secondary metabolites in colonized plants. These compounds may suppress pathogen infections either directly, by antibiosis, mycoparasitism and competition, or indirectly by induction of plant defense system (Lacava and Azevedo, 2014). These mechanisms frequently operated simultaneously. Some of these compounds include antibiotics (*e.g.* terpenoids, alkaloids and polypeptides), volatile organic compounds (*e.g.* acids, alcohols, alkyl pyrones, ammonia, esters, hydrogen cyanide, ketones, and lipids) and enzymes (Gao et al., 2010; Ownley et al., 2010). Compared with endophytes, relatively little is known about host plant protection

conferred by epiphytes against pathogens. However, the biocontrol of diseases affecting several crops by epiphytes have been already described (*e.g.* Völksch and May, 2001; Lanna Filho et al, 2010). This functional aspect of phyllosphere microorganisms raises the potential to design and managed these microbial populations in order to enhance crop productivity with reduced agrochemical inputs and at lower cost (Newton et al., 2010). To accomplish this, we must first understand the biotic and abiotic factors driving microbial colonization patterns and the interactions that could take place in the phyllosphere either between microorganism–microorganism or microorganism–plant (Rastogi et al., 2013).

1.4. Major drivers of phyllosphere microbiota composition

The composition and diversity of phyllosphere microorganisms can be affected by several factors, being climatic conditions, host genetics, geographic location, and microbial interactions, considered the most determinant (Varanda et al., 2016; Liu et al., 2017).

Several studies have been showed that the microbial composition of the phyllosphere is greatly influence by **climatic factors** (*e.g.* temperature, humidity, precipitation), due to their rapid fluctuation (Whipps et al., 2007; Laforest-Lapoint et al., 2016). This is particularly important within epiphytes due to the greater fluctuation of temperature and relative humidity in the surface of leaves than in the inner plant tissues (Whipps et al., 2007). Temperature and humidity seems to have higher impact on the establishment of the microorganisms on the phyllosphere (Bernard et al., 2013), while both wind and precipitation are more important as dispersal factors (Kinkel, 1997; Zak, 2002).

Specific studies have also showed that the **host plant** drives the microbial community composition in the phyllosphere (Whipps et al., 2007; Müller et al. 2015). Indeed, different cultivars of the same plant species, growing in the same location, have been showed to displayed distinct phyllosphere microbial community (Whipps et al., 2008; Lemanceau et al., 2017). Although plant genotype seems to be an important factor determining the structure of phyllosphere microbial communities, the mechanisms responsible for such effect remain to be elucidated (Whipps et al., 2008). There is reason to believe that specific tissue features of each genotype, could be one possible explanation for such effect, by leading the establishment of particular microbial species (Lemanceau et al., 2017). Indeed, physical factors related with cell wall, cuticle and trichomes, have been showed to influenced microbial growth and survival in the phyllosphere (Whipps et al., 2008). Similarly, chemical composition of leaves is also major determinants of microbial

colonization (Lindow and Brandl, 2003; Whipps et al., 2008). It was suggested that plants produce compounds by which they actively regulate the communities of microbes that enter into their tissues (Bacon and White Jr., 2015). These compounds, which often reduce microbial growth rates, include phenolic compounds, and other secondary metabolites, such as volatile organic compounds (VOC's) (Farré-Armengol et al., 2016; Lattanzio et al., 2006). Phenolic compounds are one of the most widely distributed groups of secondary metabolites throughout the plant kingdom (Bhattacharya et al., 2010). Some of these compounds, such as phenols, phenolic acids, flavonoids and dihydrochalcones, showed antifungal activity against pathogens (Lattanzio et al., 2006). Also, phytoalexins (belonging to flavonoids) have been showed to either, exhibit bacteriostatic properties, similar to antibiotics, or to limit sporulation, spore germination and hyphal growth of phytopathogenic fungi (Kulbat, 2016). Plants can also release a wide range of VOC's that play a relevant role in determining the microbial communities that inhabit phyllosphere, through their antimicrobial effects and their role as carbon sources for some microorganisms (Farré-Armengol et al., 2016). Terpenoids, phenylpropanoids, benzenoids, aldehydes, alcohols and sesquiterpenoids are major constituents of plant VOC's emissions that have antimicrobial activity and strongly influence phyllospheric microbial colonization (Whipps et al., 2008; Farré-Armengol et al., 2016). Further studies should address the relationship between plant genetic control of phenotypic characteristics and their effects on phyllosphere microbial composition (Whipps et al., 2008).

The **spatial** proximity between plants can also contribute to the composition of phyllospheric microbial communities (Finkel et al., 2012; Rastogi et al., 2012). In general, microbial communities inhabit the phyllosphere of trees become more dissimilar as the geographic distance between the trees increases (Redford et al., 2011). Recent studies suggest that **microbe-microbe** interactions also play an important role in microbial assemblages in the phyllosphere (Hassani et al., 2018). Although the contribution of such interaction to the overall community structure remains poorly described, it is likely that both competitive and cooperative interaction within and between microbial kingdoms would be the main mechanisms employed by microbiota members to persist within the phyllosphere (Hassani et al., 2018). Cooperative interactions could refer, for instances, to the reciprocal exchange of metabolites, enhanced dispersal and molecular communication (quorum sensing) between microorganisms (Hassani et al., 2018). Direct or indirect competition between phyllosphere microbiota members occurs through several mechanisms, including competition for resources and antagonism (Hassani et al., 2018). In the antagonism can participate several

chemical compounds (such as volatiles and antibiotics) that directly suppress the growth of microbial opponents (Hassani et al., 2018). For instances, previous studies have already showed that some microorganisms produce VOC's that inhibit the growth of other microorganisms either of the same or of different kingdom (Schmidt et al., 2015; Quintana-Rodriguez et al., 2015). The extent of inhibition depends on the bacteria-fungus or fungus-fungus interactions, being the sensitivity to volatiles distinct according the microbial species (Kai et al., 2007, 2009; Vespermann et al., 2007; Garbeva et al., 2014).

Despite all these studies, we still have an incomplete understanding of how phyllosphere microorganisms interact among themselves and with their plant hosts. Similarly, phyllosphere microbiota functions and their implications for plants and agricultural crops are mostly unknown.

1.5 Thesis main objectives and outlines

The phyllosphere is a complex environment where numerous and diverse microorganisms live and interact with each other and with plant. The main aim of this work was to characterize fungal communities that inhabit the olive tree phyllosphere and elucidate their potential role in conferring host protection to olive leaf spot (OLS) and olive knot (OK) diseases. The identification of autochthonous microorganisms that could be used in the future as biological control agents against both diseases was also aimed. The obtained information may be useful for development of novel sustainable agricultural practices.

In **chapter 1,** a general introduction was provided in order to review two of the most important diseases affecting olive tree (OLS and OK). Specifically, pathogens and plant symptomatology were described. Emphasis was also given to the exploitation of biological control to manage both diseases. Description of phyllosphere and drivers shaping microbial communities were provided. The following chapters (chapters 2 to 5) are used to address the main objectives of the work, each one including a brief introduction, material and methods, results and discussion.

In **chapter 2**, the way environmental factors (season and climatic conditions), as well as plant organs (leaves and twigs), could drive both endo- and epiphytic fungal assemblages on olive tree phyllosphere is evaluated. In **chapter 3**, the influence of fungal OLS pathogen *Venturia oleaginea* on the resident fungal communities of olive leaves is highlighted. The effect of host plant on the outcome of competitive and cooperative microbial interactions is discussed and related with OLS disease susceptibility. In **chapter 4**, the impact of the OK pathogen *Pseudomonas savastanoi* pv. *savastanoi* on fungal communities of olive tree twigs is studied. The effect of host plant on the

final fungal assemblage is discussed and related with OK disease susceptibility. Key-species associated to asymptomatic and OK-symptomatic twigs and to a particular host cultivar are also identified. In **chapter 5**, the relationship between olive tree cultivars (and their underlying susceptibility to OLS) with the level of OLS disease incidence is evaluated. Specifically, this chapter aimed to determine whether differences in susceptibility of different olive tree cultivars to OLS disease is linked to both fungal communities and metabolites (volatile and phenolic compounds) of host plant leaves. Finally, in **chapter 6**, the concluding remarks and future perspectives of this thesis are presented for disclosing the role of fungal communities associated to olive tree phyllosphere in conferring host protection to OLS and OK diseases.

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Chapter 2

Endophytic and epiphytic phyllosphere fungal communities are shaped by different environmental factors in a Mediterranean ecosystem

Endophytic and epiphytic phyllosphere fungal communities are shaped by different environmental factors in a Mediterranean ecosystem

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2.1 Abstract

The diversity and factors influencing fungal assemblages in phyllosphere of Mediterranean tree species have been barely studied, especially when endophytic and epiphytic communities are simultaneously considered. In this work, the endophytic and epiphytic fungal communities from olive tree phyllosphere were studied. This tree species is natural from the Mediterranean region and adapted to grow under adverse climatic conditions. The main objective was to determine whether there are differences between both fungal communities, and to examine whether different abiotic (climate-related) and biotic (plant organs) factors play a pivotal role in structuring these communities. Both communities differed in size and composition, with epiphytic community being richer and more abundant, displaying also a dominance of melanized fungi. Season was the major driver of community composition, especially of epiphytes. Other drivers shaping epiphytes were wind speed and temperature; while plant organ, rainfall, and temperature were the major drivers for endophytic composition. In contrast, canopy orientation caused slight variations in community composition of fungi, but with distinct effects in spring and autumn seasons. In conclusion, epiphytic and endophytic communities are not driven by the same factors. Several sources of variation undergo complex interactions to form and maintain phyllosphere fungal community in Mediterranean climates. Climatic parameters have influence on these fungal communities, suggesting that they are likely to be affected by climate changes in a near future.

2.2 Introduction

Phyllosphere is the above ground component of plants, supporting a diverse community of microorganisms that live both within (as endophytes) and/or on the surface (as epiphytes) of plant tissues (Lindow and Brandl, 2003). Phyllosphere fungi have been considered as determinant factors for plant health and productivity (Berg et al., 2014), but different drivers that shape these fungal communities on woody plants remain unclear, especially in Mediterranean region (Moricca and Ragazzi, 2008). Reports are only limited to Mediterranean oak forests (*e.g.* Moricca and Ragazzi, 2008; Collado et al., 1999; Linaldeddu et al., 2011; Martinez-Alvarez et al., 2012; Moricca et al., 2012) pine stands (*e.g.* Martinez-Alvarez et al., 2012; Botella et al., 2010) and poplar plantations (*e.g.* Martín-García et al., 2011). Some of these studies tried to determine the factors that affect community composition of endophytic fungi, such as climatic variables (Martinez-Alvarez et al., 2012, Botella et al., 2010), type of plant organs (Moricca et al., 2012; Botella et al., 2010;

Martín-García et al., 2011) and seasonality (Collado et al., 1999). However, the results of these studies were often contradictory. Climatic variables such as average temperature, water availability and solar radiation, were reported as drivers of endophytic fungal community composition inhabiting twigs and needles of *Pinus halepensis* (Botella et al., 2010). In other pine plantations (P. *sylvestris*, P. *nigra*, and P. *pinaster*) and native oak forests (*Quercus pyrenaica*), the influence of climatic variables was not clearly understood (Martinez-Alvarez et al., 2012). Previous studies have reported that the composition of fungal endophytes was different in leaves and twigs of Q. *cerris* and Q. *pubescens* (Moricca et al., 2012); while in other perennial plants, there were no obvious evidences to prove tissue/organ specificity of fungal endophytes (Martín-García et al.,.2011). Furthermore, most studies have focused exclusively on endophytic fungi and have not considered epiphytic community, even though epiphytes may live less than a millimeter apart and distinction between both life forms may not be clear. In woody plants, fungal endophytes are transmitted horizontally (Rodriguez et al., 2009), so presumably most of them grow on leaf surface before penetrating into plant tissues (Porras-Alfaro and Bayman, 2011). However, the surface and internal tissues of these above-ground organs represent two different habitats. Epiphytes are exposed to numerous external environmental stress factors, such as temperature, humidity and solar radiation. On the other hand, endophytes have a more sheltered habitat but are challenged by plant defense reactions (Rastogi et al., 2013). Different stress factors faced by epiphytes and endophytes might have important effects on their community composition. The only studies that compared epiphytic and endophytic fungal communities on leaves of deciduous shrubs (Osono, 2007) or woody plants, such as *Coffea arabica* (Santamaria and Bayman, 2005) and *Camellia japonica* (Osono, 2008), reported that they have distinct communities.

Mediterranean-type ecosystems, like those found in the Iberian Peninsula, are frequently challenged by several stress factors, such as temperature, rainfall, and UV radiation. Therefore, a better knowledge of epi- and endophytic fungal communities that thrive in phyllosphere of Mediterranean environments is needed. This would be useful for getting a better understanding of factors that influence fungal composition and structure, in order to predict their response to climate change. In this study, we determined the influence of some climatic variables (i.e. rainfall, temperature, relative humidity and wind speed), seasons (spring vs. autumn), cardinal direction (north vs. south) and plant organ type (leaf vs. twig) on the composition of both epi- and endophytic fungi present on phyllosphere of olive trees. *Olea europaea* L. is an ancient drought-tolerant crop, which has significant ecological and socioeconomic importance for Mediterranean countries

(Loumou and Giourga, 2003). By identifying factors that play a dominant role in the structure of phyllospheric fungal communities in Mediterranean ecosystems, novel strategies could be developed for mitigating the impacts of climate change and for improving agriculture.

2.3 Material and methods

2.3.1 Study site and sample collection

Samples were collected from three olive orchards located in Mirandela, Northeast of Portugal [N 41° 32.593'; W 07° 07.445' (orchard 1), N 41° 32.756'; W 07° 07.590' (orchard 2) and N 41° 29.490'; W 07° 15.413' (orchard 3)] (Fig.2.2). This is a mountainous region, with altitudes ranging between 300 and 500 m, displaying a Mediterranean climate, with cold and rainy winters and long, hot and dry summers. The average annual rainfall in Mirandela ranged from 500 to 700 mm, mainly occurring between October and February, and annual average temperature ranged from 3 to 26°C (SNIRH 2016). The selected orchards were managed by following integrated production guidelines (Malavolta and Perdikis, 2012). Each orchard included Portuguese olive cultivars, wherein trees were planted at 7 × 7 m spacing. In each orchard, olive branches were collected from 21 randomly chosen trees, in two cardinal directions (north and south) at the operator's height (one sample from north and other from south × 21 trees × 3 orchards = 126 branches). The same trees were sampled at two different periods, *i.e.*, in autumn 2013 (from October to November) and spring 2014 (from March to May) (Fig. 2.1).

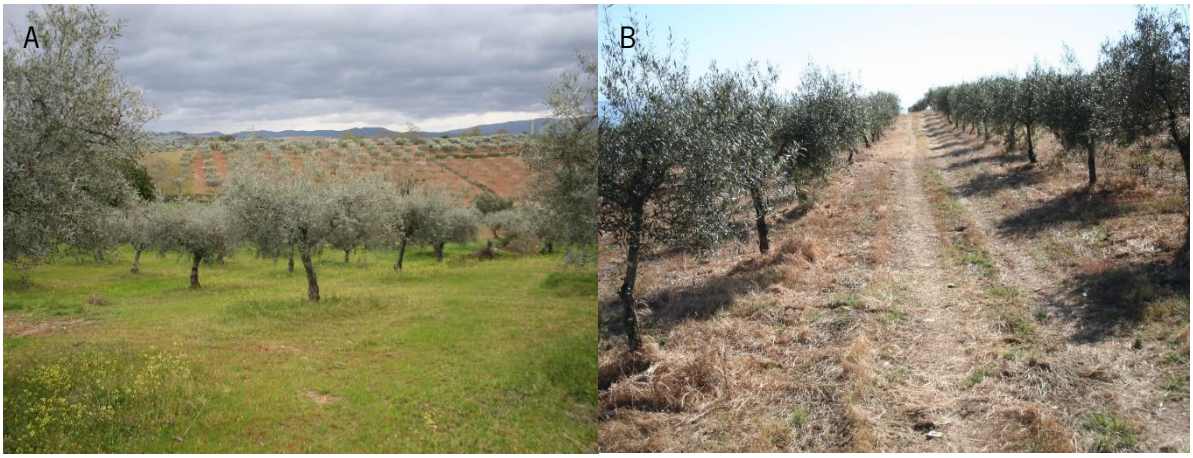

Fig. 2.1 Olive orchards during the seasonal sample collection: A) Autumn and B) Spring. Credits: Teresa Gomes, IPB-ESA.

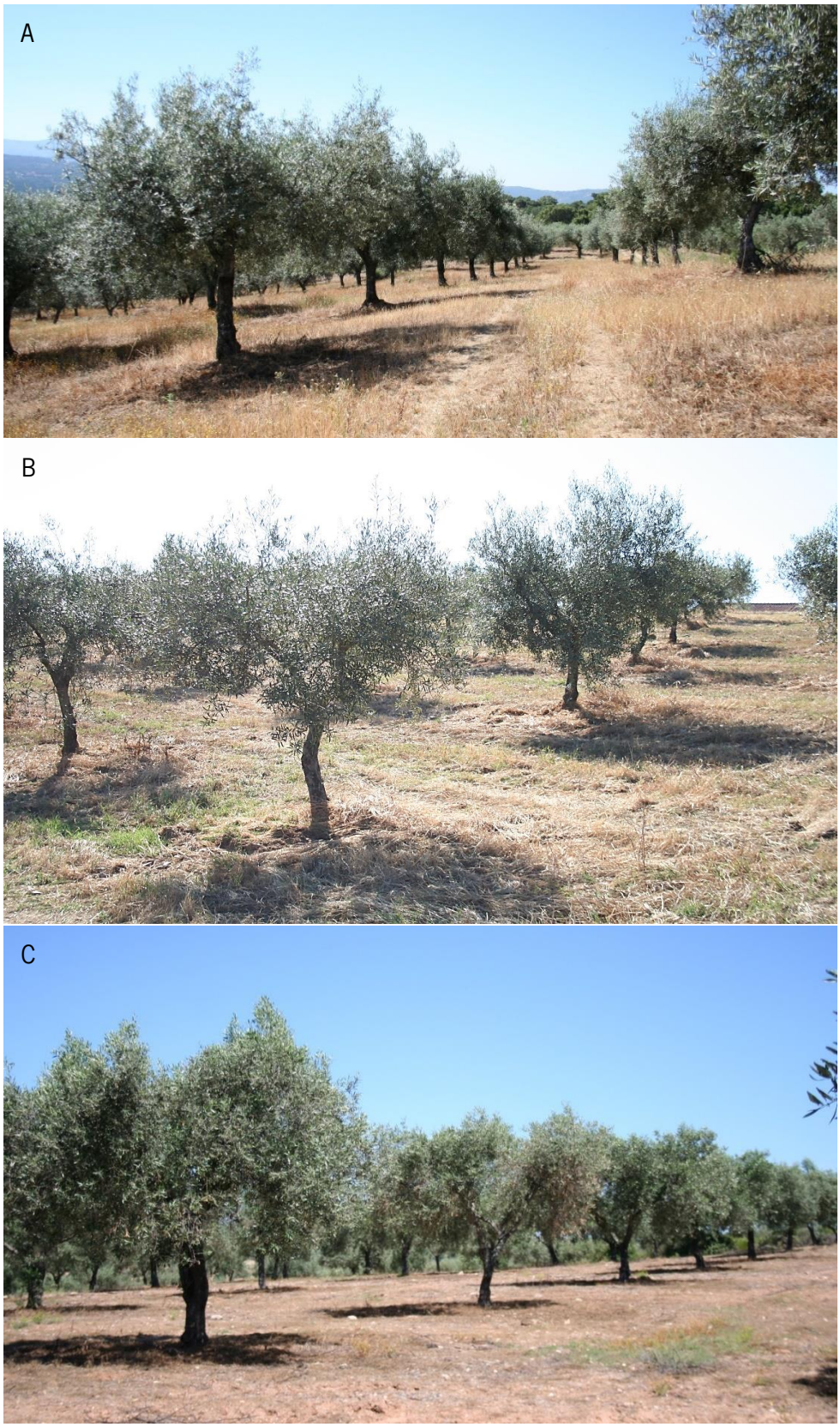

Fig. 2.2 Olive orchards used for sample collection: A) olive orchard 1 (Paradela1); B) olive orchard 2 (Sucçães) and C) olive orchard 3 (Paradela 2).

2.3.2 Climatic data

During the surveyed period, meteorological data was collected at a weather station located near olive orchards at Mirandela (N 41° 43.333'; W 07° 21.944, elevation: 357 m). Cumulative rainfall, average daily mean temperature, relative humidity (maximum and minimum) and wind speed were determined at 3, 5, 10, and 20 days before sampling dates.

2.3.3 Fungal isolation and identification

From each branch, five segments of twigs and leaves were randomly selected and used to isolate epiphytes and endophytes. For isolating fungal epiphytes, around 1 g of leaves and twigs were separately added to 9 mL of sterile potassium phosphate buffer pH 7.0 (8 g/L NaCl; 0.20 g/L KCl; 1.4 g/L Na2HPO4; 0.24 g/L KH2PO4). This suspension was placed on a rotary shaker (200 rpm) for 60 min., at room temperature, to dislodge microorganisms from the plant surface. Aliquots of 1 mL of the resulting microbial suspensions were separately plated in triplicate on Potato Dextrose Agar (PDA, Difco) and Plate Count Agar (PCA, Himedia) media, supplemented with 0.01% (w/v) chloramphenicol (Oxoid). Plates were incubated at 25 ± 2°C in the dark and were observed daily for microbial growth and colony counting. Results of epiphytes were expressed as log CFU/cm^{2}, i.e. the number of individual colonies of fungi adhered to the surface of leaf/twig. An ellipse equation ($A = \pi ab \times 2$) was used for estimating leaf surface. A cylinder equation ($A = 2\pi rh + 2\pi r^2$) was used for determining twig surface. In these equations, A is the area, *a* and *b* are the longitudinal and transverse axes of leaf, respectively, and r and h are radius and height of twig segments, respectively. The average area of leaf and twig was 13.5 ± 1.3 cm^2 and 11.4 ± 0.1 cm^2, respectively.

Endophytic fungi were isolated from the same plant fragments used to isolate epiphytes. After removing epiphytes by surface disinfection through a procedure described by Martins et al. (2016), each leaf or twig was cut into segments (4–5 mm for twigs and ca. 5 × 5 mm for leaves), which were transferred into the same culture media used to isolate epiphytes. In total, 7,440 plant tissue segments were inoculated for each season (spring and autumn). To validate surface sterilization procedure, the surface of sterilized plant tissues was imprinted onto PDA and PCA media.

Fungal colonies were subcultured on fresh medium until pure epi/endophytic cultures were obtained. Each fungal colony was further identified by morphological and molecular approaches. Isolates were first grouped, according to their morphological similarity (colony

morphology, hyphae, spores and reproductive structures). One representative isolate of each morphotype was selected for molecular identification, using internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). Total genomic DNA was extracted from harvested mycelia/spores using REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK). The ITS region (ITS1, 5.8S, ITS2) was amplified using ITS1/ITS4 or ITS5/ITS4 primers sets (White el al., 1990) according to a PCR protocol previously described by Oliveira et al. (2012). The amplified products (≈650 bp) were purified and sequenced using Macrogen Inc. (Seoul, South Korea) services. The obtained DNA sequences were analysed with DNASTAR v.2.58 software and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST algorithm. Blast results were sorted according to higher identity score and lowest E-value. For sequence identities >98%, the genus and species were accepted; for sequence identities between 95% and 97%, only the genus was accepted; and for sequence identities <95%, isolates were labelled as 'unknown' fungi. The sequences obtained are available at GenBank with the following accession numbers: KU324941-KU325040; KU325041-KU325240; KU325241-KU325457; KT804020-KT804039; KT804040-KT804069; KT804070-KT804119; KT804120-KT804149. Pure cultures of each identified isolate were preserved and deposited in the culture collection of the Polytechnic Institute of Bragança (School of Agriculture).

2.3.4 Diversity of fungal endophytes and epiphytes

For the following analysis, only those OTUs identified up to the genus were considered, being excluded the "unknown fungi". Diversity of fungal epiphytes and endophytes was determined in tissue samples (leaf, twig and total), oriented in a north-south position for each season (spring and autumn). Diversity was assessed by evaluating the abundance (average number of isolates per tree) and richness (average number of operational taxonomic units per tree), and by computing Simpson's Reciprocal Index (1/D) and Shannon–Wiener (H') diversity indices with Species Diversity and Richness v. 4.0 (Seaby and Henderson, 2006). All diversity indices and estimators are presented as the mean of replicates (= tree), displaying respective SE values or total number (the values of all samples lumped together). To determine differences among means, an analysis of variance (ANOVA) with *SPSS* v.18 was performed. Averages were then compared using Tukey's test (P <0.05). Species-richness within tissue samples was also estimated by using species accumulation curves, which were calculated using sample-based rarefaction index (Mao Tau) and EstimateS v. 9.1.0 (Colwell, 2013), using 1000 runs of bootstrapping with replacement.

2.3.5 Data analysis

Both univariate and multivariate methods were used to identify which climatic or biological factors may drive the structure and composition of epiphytic and endophytic fungal community. For each fungal community (endophytic or epiphytic), mean fungal abundance, richness and Simpson's Reciprocal Index diversity were modelled separately by using *R* software (R Core Team, 2014). Organ (leaf vs. twig), cardinal direction (north vs. south) and season (spring vs. autumn) were considered as explanatory variables. Thus, the full linear model for each independent variable was as follows:

$$Y_i = \alpha + \beta_1 \times Organ_i + \beta_2 \times Cardinal\ direction_i + \beta_3 \times Season_i + \varepsilon_i \quad \text{where } \varepsilon_i \sim N(0, \sigma^2)$$

For each response, model selection followed Akaike's Information Criterion (AIC), according to Zuur et al. (2009). Then, model was validated by plotting the fitted values vs. residuals, checking for non-linear patterns. For the endophyte community, an interaction term had to be included between organ and season in the diversity model. The optimal model for each response and community remained as follows:

Endophytic community:

$$Abundance_i = \alpha + \beta_1 \times Organ_i + \varepsilon_i$$
$$Richness_i = \alpha + \beta_1 \times Organ_i + \beta_2 \times Season_i + \varepsilon_i$$
$$Diversity_i = \alpha + \beta_1 \times Organ_i + \beta_2 \times Season_i + \beta_3 \times Organ_i{:}Season_i + \varepsilon_i$$

Epiphytic community:

$$Abundance_i = \alpha + \beta_1 \times Season_i + \varepsilon_i$$
$$Richness_i = \alpha + \beta_1 \times Season_i + \varepsilon_i$$
$$Diversity_i = \alpha + \beta_1 \times Cardinal\ direction_i + \beta_2 \times Season_i + \varepsilon_i$$

Secondly, non-metric multidimensional scaling (NMDS) was carried out with two similarity indexes, *i.e.* Jaccard's and Bray-Curtis, in order to assess variability of endophytic and epiphytic assemblages with respect to season (autumn vs. spring), cardinal direction (north vs. south) and plant organ (leaf vs. twig). Both similarity indexes provided different types of information. Jaccard's similarity index only compares the presence or absence of fungal taxa among samples, without considering species abundance (Magurran, 2004). On the other hand, Bray-Curtis's similarity index compares the presence or absence of fungal taxa as well as species abundance among samples.

This coefficient ignores cases in which species are absent in both community samples and is strongly influenced by abundant species (Bray and Curtis, 1957). Kruskal's stress was used to estimate model's goodness of fit (commonly acceptable when is <0.2). A one-way analysis of similarity (ANOSIM) was used to determine significant differences between fungal community groupings obtained in NMDS ordination, using Bray–Curtis distance matrices. ANOSIM generates a *P*-value (significant level below to 0.05) and R-value (gives the degree of discrimination between groups), which ranges from 0 (indistinguishable) to 1 (completely dissimilar) (Clarke and Gorley, 2015). All these analyses were performed using *Community Analysis Package* v.4.0 (Henderson and Seaby, 2007).

To corroborate the obtained results, a "cross-table" multivariate analysis (co-inertia analysis, CIA) was performed using R software. This analysis aims to find a co-structure between two sets of variables that are linked by the same individuals, where the resulting sample scores the most covariant (Dolédec, 1994). The co-inertia function from the *ade4* package in R was used to perform the analyses and the *table.value* function from the same package was used to visualize the results.

Causes of variation in endophytic and epiphytic fungal assemblages due to climatic factors (*i.e.* mean temperature; maximum and minimum relative humidity; total rainfall and wind speed) were also investigated using distance-based linear models (DistLM) (Mcardle and Anderson, 2001). This analysis was performed on climatic data, obtained on 3, 5, 10, and 20 days before fungal sampling, which were individually used or clustered together (for each climatic variable, days were lumped together). Similarity matrices were generated from square root transformed data of fungal abundance using the Bray–Curtis method and climatic data were log transformed [log (x + 1)] to normalize their distribution. A marginal test was firstly performed, where the individual climatic variables were separately fitted to assess their relationship with fungal data. Using step-wise selection procedure and AIC, a sequential test was performed to identify the subset of climatic variables that best predicts the observed structural pattern of fungal community. Both marginal and sequential tests were undertaken with 999 permutations using *PRIMER 7.0* (Clarke and Gorley, 2015).

2.4 Results

2.4.1 Endophytic and epiphytic fungal communities differ in diversity and composition

A total of 290 fungal operational taxonomic units (OTUs) were isolated from all plant tissues surveyed in both seasons (autumn and spring). Compared to endophytes (125 OTUs), epiphytes had greater diversity (242 OTUs; Table S2.1). Despite having high diversity, species accumulation curves did not reach an asymptote (Fig. 2.3A), indicating that more species would be isolated with additional sampling. For endophytes 135 OTUs would be expected (instead of the observed 125) and for epiphytes 254 OTUs were expected instead of the observed 242. Both Simpson and Shannon-Winer indices were similar between endophytic and epiphytic communities (3.5 vs. 3.6 and 0.7 vs. 1.1, respectively), despite the average number of epiphytic fungal OTUs per tree being significantly higher ($P < 0.001$) than endophytic (5.4 vs. 2.9) (Table S2.1). Moreover, epiphytes were found to be more abundant than endophytes. Overall, the average number of epiphytic isolates per tree was up to 2.4-fold significantly higher ($P<0.001$) than that of endophytes (Table S2.1).

Endophytic and epiphytic fungal communities were significantly different in terms of species composition (ANOSIM, R = 0.465, $P = 0.001$) and were distinguished from each other by their most common families (Fig. 2.4). Within epiphytic communities, most abundant families were Davidiellaceae, Psathyrellaceae, Pleosporaceae, Nectriaceae and Trichocomaceae. Within endophytic communities, the most frequently isolated fungal OTUs belonged to following families Leptosphaeriaceae, Pleosporaceae, Pyronemataceae, Trichocomaceae, and Pezizaceae. Most OTUs identified in this study were either exclusively endophytic (48) or epiphytic (165), and only 77 OTUs were found in both fungal communities (Fig. S2.1A). ANOSIM analysis indicated that dissimilarity between the composition of endophytic and epiphytic fungal communities was greater in spring (R = 0.976; $P = 0.001$) than in autumn (R = 0.539; $P = 0.001$), as well as in olive tree twigs (R=0.542; $P=0.001$) than in leaves (R=0.303; $P = 0.001$). In contrast, dissimilarity between these two fungal communities was almost the same in south (R = 0.404; $P = 0.001$) and in north directions (R = 0.388; $P = 0.001$). These results were corroborated by NMDS ordination, in which a clear differentiation of endophytic from epiphytic fungal communities was evident for different seasons and olive tree organs, but not for cardinal direction (Fig. 2.5).

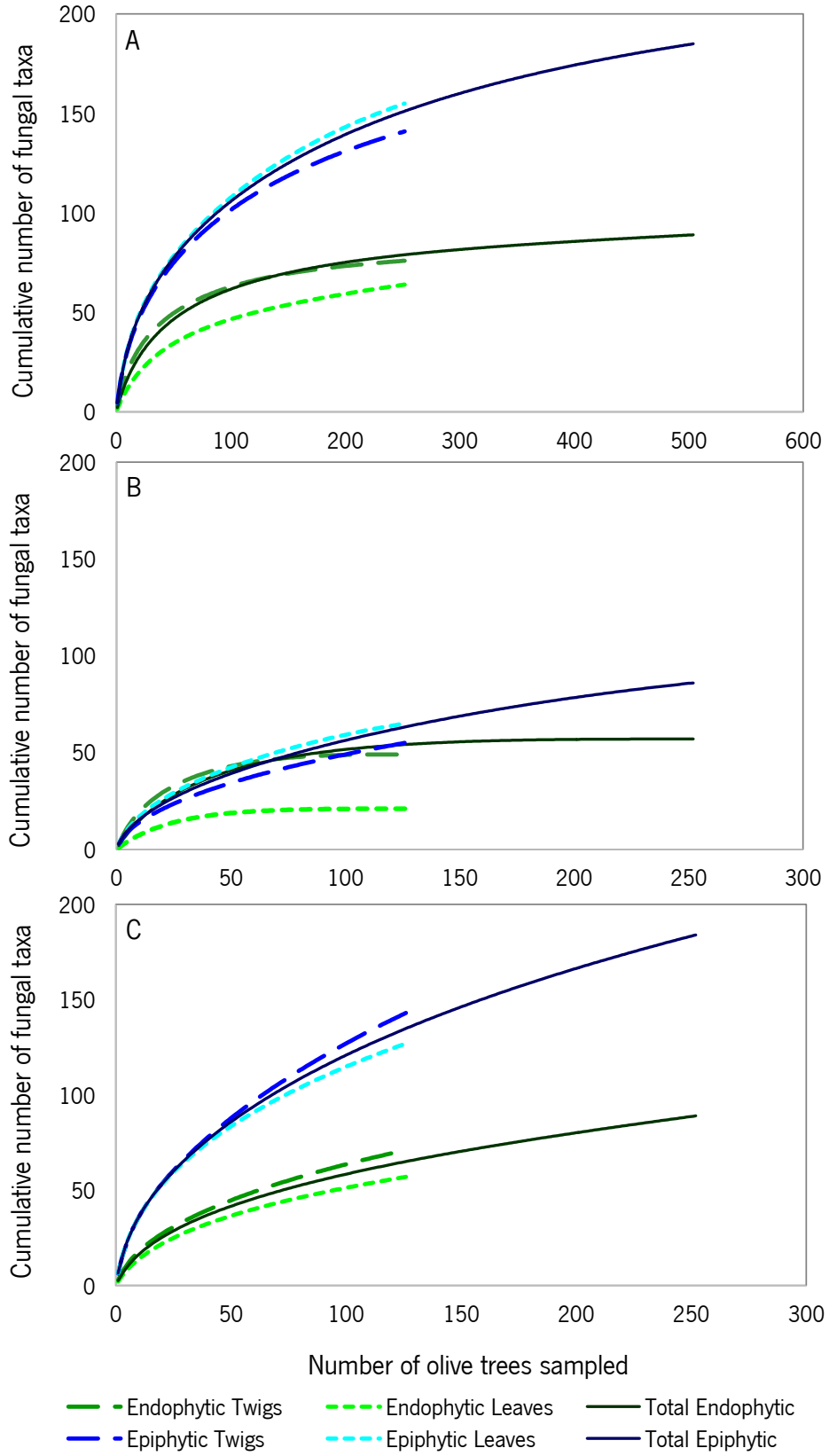

Fig. 2.3 Species accumulation curves for all fungal endophytes and epiphytes isolated from each plant tissue (twigs and leaves) and from whole plant (total), collected from 63 olive trees (*Olea europaea* L.), in both seasons (**A**), autumn (**B**) and spring (**C**).

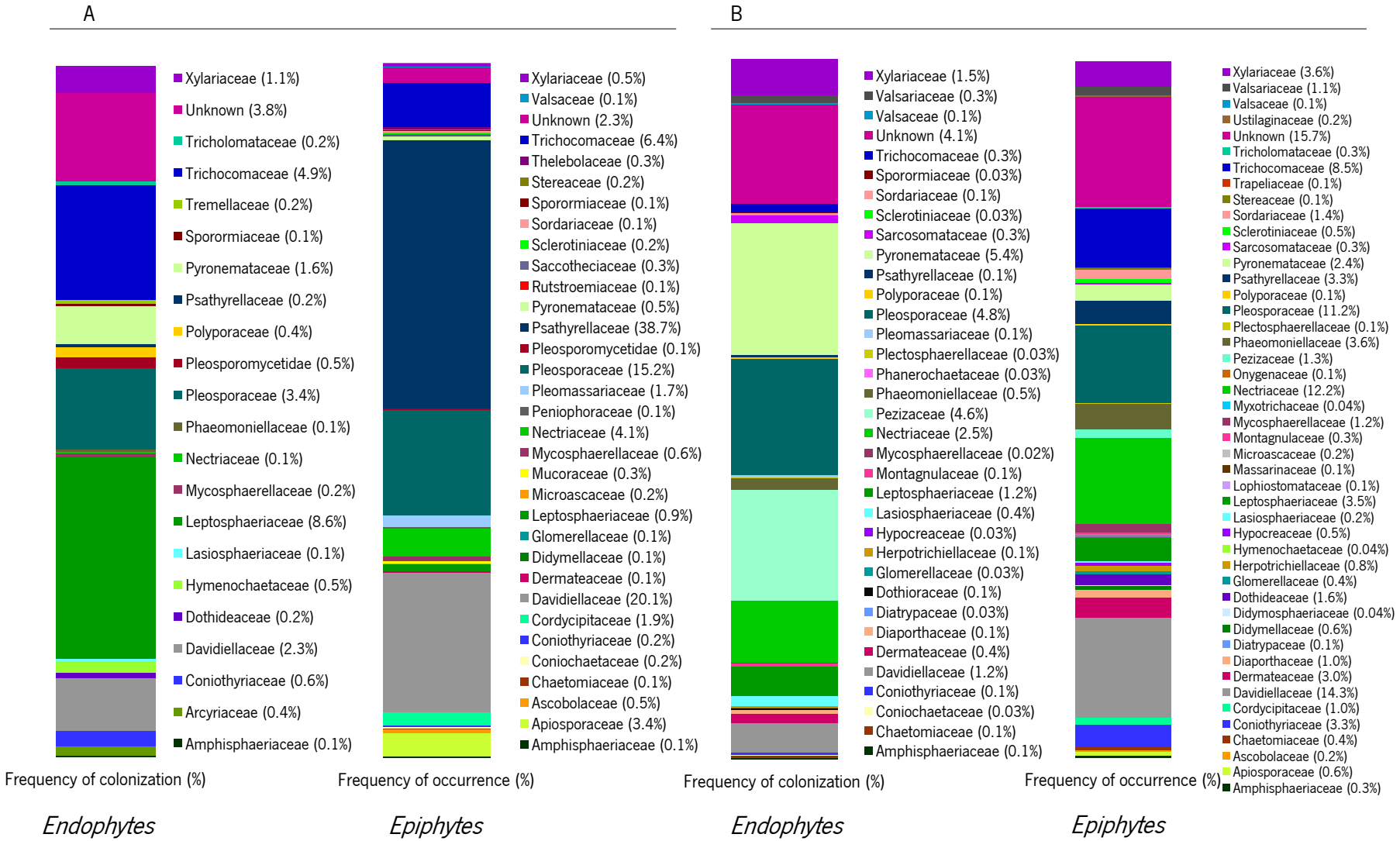

Fig. 2.4 Frequency of fungal endophytes and epiphytes families present in *Olea europaea* L. tissues (both on twigs and leaves) surveyed either in autumn (**A**) or spring (**B**).

2.4.2 Diversity and composition of phyllosphere fungal community depends on the season

Fungal diversity (*i.e.* species richness and 1/D) of endophytes and epiphytes was significantly (P<0.001) higher in spring than in autumn (Fig. 2.6). Since higher diversity was found in spring season, fungal surveys should to be carried out with greater sampling effort in spring than in autumn. For both fungal communities (especially for endophytes), overall species accumulation curves obtained in autumn appeared to be more close to an asymptote than the curves obtained in spring (Fig. 2.3). For epiphytic community, there was a 2.9-fold significant increase (P<0.001) on fungal abundance (n° of isolates) from autumn to spring (Fig. 2.6B).

NMDS plots, based on Jaccard's and Bray-Curtis indexes, and ANOSIM analysis showed that whole fungal communities composition differ significantly between seasons (R=0.674, P =0.001). This dissimilarity was greater for epiphytes (R=0.940; P =0.001) than for endophytes (R=0.540; P =0.001). In fact, only 44 species out of the 290 recovered in this study were isolated in both seasons; 164 species were isolated only in spring and 82 were recovered only in autumn (Fig. S2.1B). There was also some variation in the most abundant families found in both seasons, which accounted for differences in the composition of fungal communities in spring and autumn (Fig. 2.4). For example, endophytic OTUs belonging to families Leptosphaeriaceae and Trichocomaceae were preferentially isolated in autumn, while endophytes from Pyronemataceae, Pleosporaceae and Pezizaceae families were more frequent and/or were exclusively isolated (*e.g.* Pezizaceae) in spring. Epiphytes belonging to Psathyrellaceae were more frequently isolated in autumn, whereas in spring were those from Davidiellaceae family.

2.4.3 Leaves and twigs have similar microbiota, but diversity of fungal endophyte communities is different

The total number of epiphytic OTUs found in twigs was higher than that in leaves (185 vs. 163, Table S2.1), but there were no significant differences in fungal abundance (n° isolates), richness and diversity (1/D) in both the organs (Fig. 2.6B). This pattern was observed in either autumn or spring (Table S2.1). In contrast, within endophytic communities, twigs exhibited significantly (P <0.001) higher diversity (up to 1.1-fold), richness (up to 1.4-fold) and fungal abundance (up to 3.1-fold) as compared to leaves (Fig. 2.6B; Table S2.1). This pattern was particularly observed in autumn season, where an increase on diversity (up to 2.7 and 1.1-fold for H' and 1/D, respectively), richness (up to 2.8-fold) and endophyte abundance (up to 4.4-fold) was

significantly greater (P< 0.001) in twigs than in leaves. In spring, only endophytic richness of twigs was 1.4-fold significantly higher (P<0.001) than that of leaves.

According to NMDS plots (Fig. 2.5B) and ANOSIM analysis, fungal communities composition of twigs was very similar to leaves either for endophytes (R=0.090, P =0.049) or epiphytes (R=0.133, P =0.057). Out of total fungal taxa, around 43% was shared between both organs of olive trees (Fig. S2.1C).

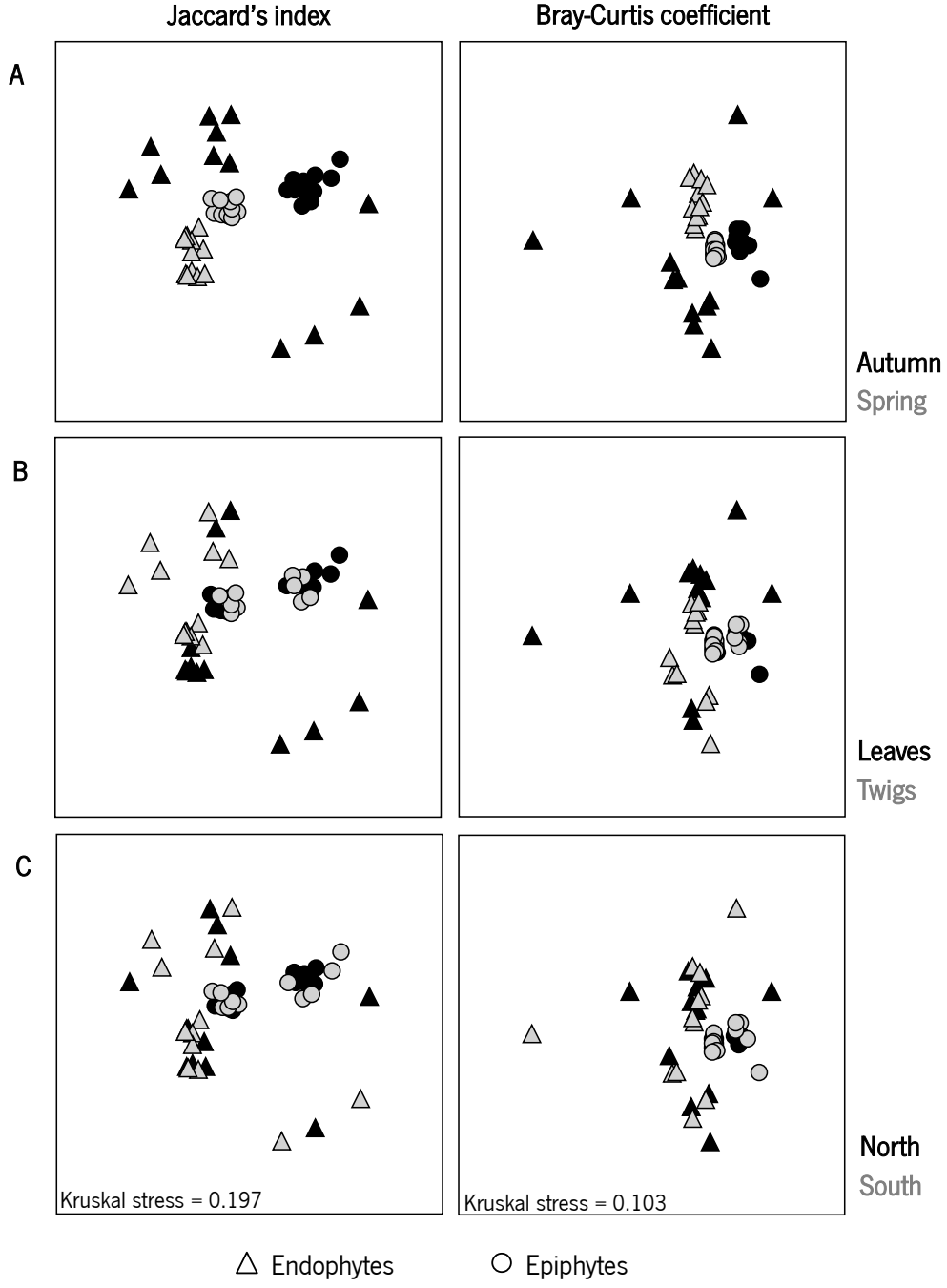

Fig. 2.5 Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of endophytic (**Δ**) and epiphytic (**O**) communities grouped by (**A**) season (autumn and spring), (**B**) olive tree organ (leaves and

twigs) and (**C**) cardinal direction (north and south). Cluster analysis was performed with two different community similarity measures, namely, Jaccard's index (binary data) and Bray-Curtis coefficient (raw abundance data). Kruskal's stress values less than 0.2 represent good ordination plots.

2.4.4 Phyllosphere fungal communities from north and south organs did not differ in diversity and composition, but depends on season

When organ tissues were taken from both cardinal directions (north and south), both endophytic and epiphytic communities showed no significant differences with respect to fungal abundance, diversity and richness (Fig. 2.6). Differences became evident when considering samples collected in different seasons (Table S2.2). In spring, the abundance of fungal endophytes was higher in north than in south direction (up to 1.2-fold; P <0.05). In autumn, diversity (H'), richness and abundance of fungal epiphytes was higher in north than in south direction (up to 1.8-fold; P < 0.01) (Table S2.2).

According to NMDS plots (Fig. 2.5C) and ANOSIM analysis, a high degree of overlap of fungal taxa was observed for epiphytes (R = 0.132, P = 0.053) and especially for endophytes (R = 0.042, P = 0.882) in north and south directions. Almost 49% of the total fungal taxa found were shared between north and south (Fig. S2.1D). Therefore, taxonomic composition of fungal communities inhabiting plant tissues located in north and south directions of the olive trees is not show.

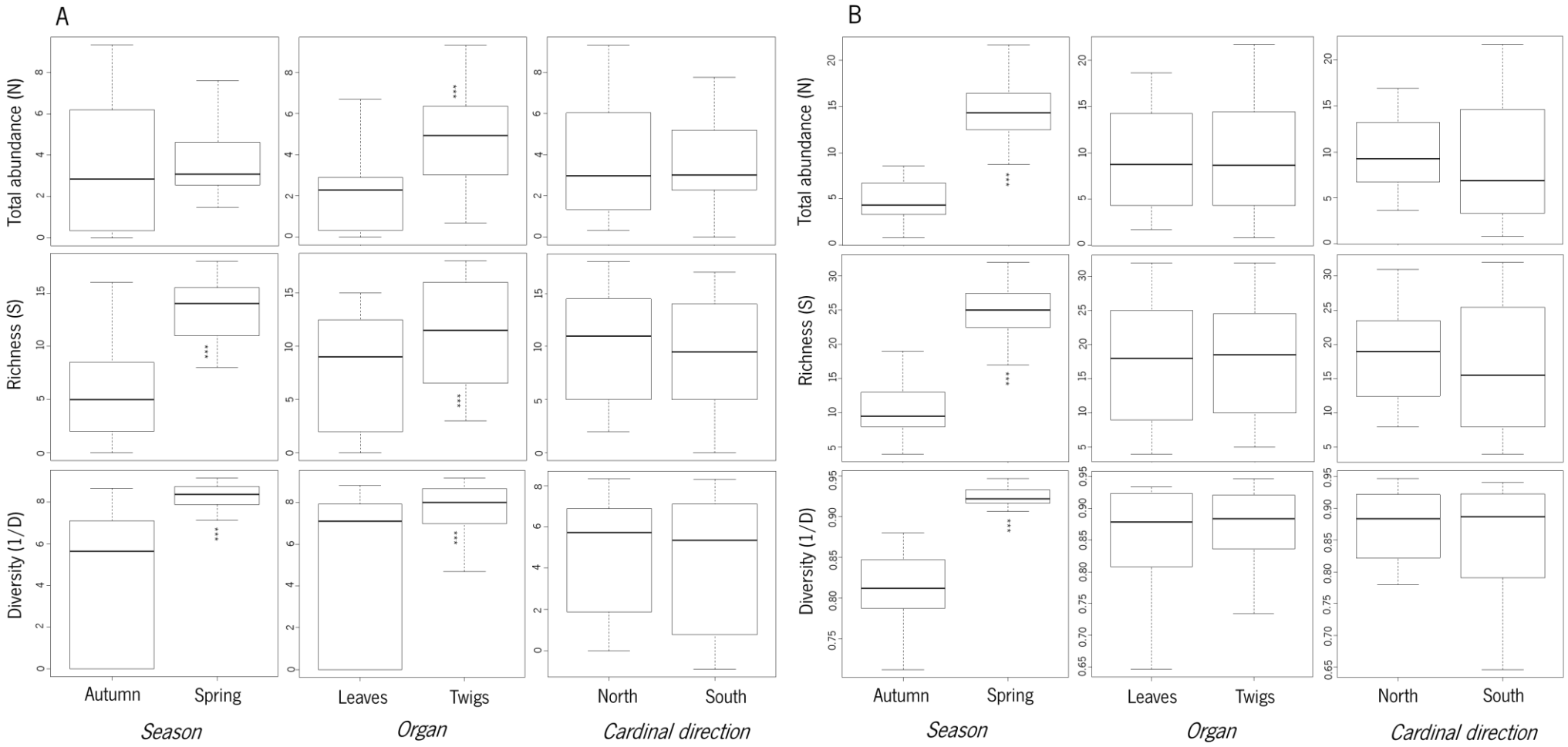

Fig. 2.6 Multiple linear regression regarding the total abundance (N), richness (S) and diversity (1/D), within endophytic (**A**) and epiphytic (**B**) fungal communities in order to evaluate their significance with season (autumn *vs.* spring), plant organs (leaves *vs.* twigs), and cardinal directions (north *vs.* south).

2.4.5 Season is the most important parameter for shaping the fungal microbiota

Co-inertia analysis was performed to determine global similarity of fungal community structures observed in each season, plant organ and cardinal direction (Fig. 2.7). This analysis was also used to compute the contribution of each of these parameters to fungal structure and to identify fungal genera that contributed most to the total co-variance of samples. The structure of either endophytic (F = 8.47, P = 0.001) or epiphytic (F = 51.15, P = 0.001) communities were significantly affected by seasons (spring and autumn). In contrast, plant organ only had a significant effect on fungal endophytic composition (F = 5.04, P = 0.002), but not on epiphytic community (F = 1.74, P = 0.122). Cardinal direction had no effect on both fungal community structures (F = 0.37, P = 0.860, for endophytes; F = 2.27; P = 0.072 for epiphytes). In fact, the majority of endophytic and epiphytic communities structure variations could be explained by season (12% and 49%, respectively), and in a lesser extent by plant organs (5% and 0.7%, respectively) and cardinal directions (0% and 1.2%, respectively). By performing co-inertia analysis, seasons were confirmed to strongly affect the structure of both fungal communities (Fig. 2.7). Indeed, fungal community composition observed in spring was distinctly different from that observed in autumn, mainly due to the presence/abundance of certain genera. The endophytes *Chromelosporium*, *Tricharina*, *Fusarium*, *Neofabraea* and *Pyronema*, and epiphytes *Fusarium*, *Hyalodendriella*, *Phoma*, *Biscogniauxia* and *Coniozyma* were positively correlated with spring. The endophytes *Penicillium*, *Lewia*, *Nemania* and *Phaeosphaeria*, and epiphytes *Coprinopsis*, *Coprinellus* and *Ulocladium* were positively correlated with autumn. Although not so evident as for the season, both plant organs (twigs and leaves) also slightly influenced the fungal endophytic composition. *Pyronema*, *Biscogniauxia* and *Penicillium* endophytic genera were positively correlated with twigs, while *Pseudocercospora* endophytic genus was positively correlated with leaves.

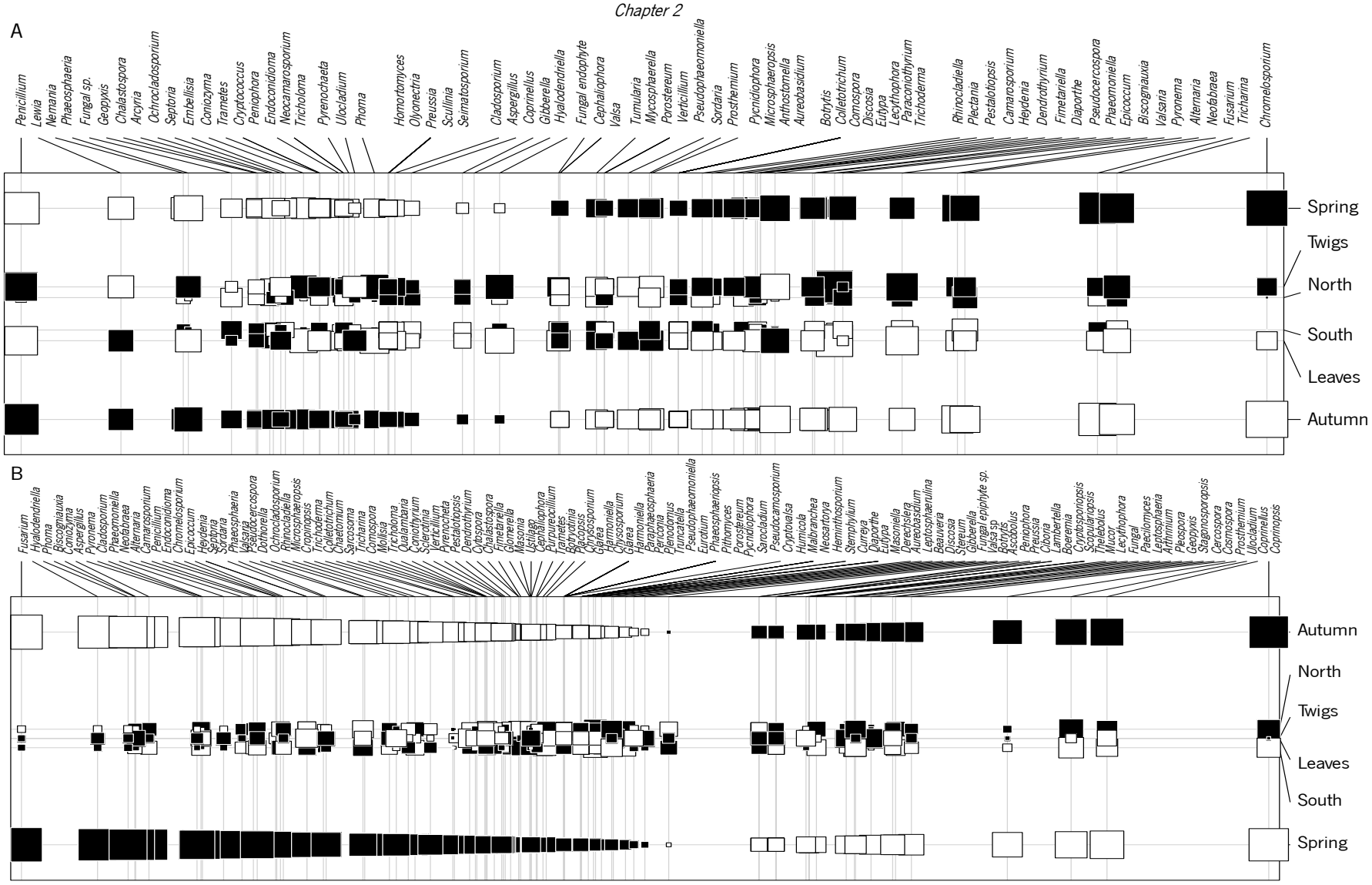

Fig. 2.7 Co-inertia factorial map showing positive (■) and negative (□) relationships between endophytic (**A**) or epiphytic (**B**) fungal communities and season (spring *vs.* autumn), olive tree organ (twigs *vs.* leaves) and cardinal direction (north *vs.* south).

2.4.6 Microclimate conditions affect fungal community structures, mainly the epiphytic

Our results indicate that the composition of phyllosphere fungi was greatly affected by seasons. This suggests that these microbial communities could be affected by climatic conditions. Using distance-based linear models (DistLM), the effect of several microclimate variables (mean temperature, maximum and minimum relative humidity, cumulative rainfall, and mean wind speed) on fungal assemblage structure was assess. To perform this analysis, weather data was collected 3-, 5-, 10- and 20-days before the sampling date. The results of marginal tests indicate that all climatic variables individually had a significant effect on endophytic and particularly on epiphytic community structures (Table 2.1). Sequential tests revealed that wind speed (9.3%) caused the greatest amount of variation within epiphytic community, especially when occurring 5 to 20 days before sampling date (8.9%, Tables 2.1 and S2.3). Variation was also caused by mean temperature (4.8%), mainly when considering 20 days before sampling date (0.6%), followed by cumulative rainfall (4.2%), which occurred 3, 10 and 20 days before sampling date.

Variation of endophytic community structure was caused by rainfall (6.3%), especially occurring from 5 to 20 days before sampling date (6.1%, Tables 2.1 and S2.3). Other factors that caused variation were mean temperature (3.4%, mainly when occurring 10 days before sampling date), wind speed (1.4%, mainly when occurring 5 to 10 days before sampling date), and maximum relative humidity occurring 5 days before sampling (0.9%).

Table 2.1 - Results of distance based linear model (DistLM) using fungal endophytic/epiphytic data and (**A**) each climatic variable (ignoring other variables) or (**B**) stepwise selection of variables, where the amount of variation explained by each variable added to the model is conditional on variables already considered.

A) Marginal tests	**Climatic variable**	**F**	**P**	**Prop. (%)**
Epiphytic fungi	Mean temperature (°C)	9.92	0.001	7.37
	Max. relative humidity (%)	10.01	0.001	7.42
	Min. relative humidity (%)	10.94	0.001	8.06
	Cumulative rainfall (mm)	8.17	0.001	6.15
	Mean wind speed (m/s)	12.79	0.001	9.30
Endophytic fungi	Mean temperature (°C)	7.69	0.001	5.81
	Max. relative humidity (%)	3.20	0.001	2.50
	Min. relative humidity (%)	6.54	0.001	4.98
	Cumulative rainfall (mm)	8.41	0.001	6.31
	Mean wind speed (m/s)	3.51	0.001	2.74
B) Sequential tests	**Climatic variable**	**F**	**P**	**Prop. (%)**
Epiphytic fungi	Mean temperature (°C)	6.94	0.001	4.82
	Mean wind speed (m/s)	12.80	0.001	9.30
Endophytic fungi	Mean temperature (°C)	4.60	0.001	3.36
	Cumulative rainfall (mm)	8.41	0.001	6.31

F = statistic; P = probability; Prop. = proportion of total variation explained.

2.5 Discussion

This study provided significantly new insights of endophytic and epiphytic fungal communities associated with olive tree leaves and twigs, and the way they could be modulated by different biotic and abiotic factors. The fungal community of olive tree phyllosphere was found to be highly diverse (290 OTUs), supporting numerous genera (149) and families (68). Using a metabarcoding approach, Abdelfattah et al. (2015), have previously revealed a rich fungal community (195 OTUs) associated with leaves, flowers and fruits of olive trees growing in a Mediterranean ecosystem (Italy). Using an approach similar to our study, 13 endophytic fungal taxa were found in the leaves and twigs of olive trees growing in another Mediterranean ecosystem (Portugal) (Martins et al., 2016). However, epiphytic and endophytic fungal communities were not compared in these studies. Epiphytes inhabiting phyllosphere are under a set of selective pressures, which are distinct from those that endophytes are facing (Rastogi et al., 2013). However, the implications of such differences have not yet been properly explored.

In our study, epiphytic community was found to be significantly richer and more abundant than endophytic community, as described for the phyllosphere of other woody plant systems (Santamaria and Bayman, 2005; Osono, 2008; Zambell and White, 2014). On the surface of olive tree leaves and twigs, the composition of fungal communities was completely different from that in internal olive tissues. In previous studies, the same pattern was observed in leaves (Osono, 2007; Osono, 2008) and stems (Zambell and White, 2014) of other plant species. Epiphytic community was dominated by fungal species with melanized hyphae/spores belonging to the families Davidiellaceae (mostly *Cladosporium* spp.), Pleosporaceae (mostly *Alternaria* spp. and *Ulocladium* spp.) and Psathyrellaceae (mostly *Coprinellus* spp. syn. *Coprinus* spp.). In endophytic community, these species were found to be less abundant than non-pigmented fungal groups. Melanin is considered to confer tolerance to environmental stresses, such as UV radiation and desiccation (Gessler et al., 2014). Melanin accumulation seems to be advantageous in areas with high levels of radiation and extreme temperatures, such as Mediterranean regions (Kembel and Mueller, 2014). Thus, the enrichment of melanized fungal groups on leaves and twigs surface of olive tree is likely driven by environmental climatic conditions.

The difference between surface and inner tissues is evident by the small fraction (32%) of identified OTUs shared between both fungal communities. A similar conclusion was reached by Kharwar et al. (2010) in *Eucalyptus* leaves, and by Kembel and Mueller (2014) in greenbrier (*Smilax rotundifolia*) stems. As both endophytic and epiphytic communities had been

simultaneously evaluated, our results suggest that endophytes outcompeted other fungi for colonization of leaves/twigs or that host plant selects for fungal species or both. We hypothesize that phyllosphere fungal endophytes could have been epiphytes that penetrated into plant tissues (Porras-Alfaro and Bayman, 2011; Kharwar et al., 2010). This hypothesis is also reinforced by the low similarity found between endophytic and epiphytic communities composition on twigs when compared to leaves. Compared to twigs, leaves are more prone to be infected by epiphytes due to their extensive hairy surface, tenderness and presence of natural opening points (*e.g.* stomata). On olive tree phyllosphere, 38% of fungi were confined only to internal tissues of host plant. This suggests that they probably have originated from the soil and migrated through roots to aerial parts. Indeed, soil has been previously proposed as a potential endophytic inoculum for aboveground organs of grapevine (Zarraonaindia et al., 2015).

Few studies have determined the influence of environmental and biological factors on the fungal composition of phyllosphere in Mediterranean crops, particularly when all these factors are simultaneously considered. In our study, season (spring *vs.* autumn) was the main factor shaping fungal community composition of olive tree phyllosphere. Likewise, seasonal variations were found to affect fungal endophytic communities in phyllosphere of other plant species growing in tropical (such as *Tectona grandis*; 38), temperate (such as *Pinus* spp.; 39), and in Mediterranean (such as *Quercus ilex*; Collado et al., 1999; Peñuelas et al., 2012) areas. A similar effect was reported within epiphytic fungal community inhabiting the leaf surface of *Camellia japonica* (Osono, 2015) or Q. *ilex* (Peñuelas et al., 2012), growing in temperate and Mediterranean areas, respectively. As expected, seasonality had greater influence on epiphytic than on endophytic fungal community structure. Furthermore, seasonal shifts were found to be partly related to climatic factors, as previously observed in fungal phyllosphere of Q. *ilex* in a mixed Mediterranean forest (Peñuelas et al., 2012). Unlike epiphytes, endophytes are confined within host plant tissues; therefore, they are likely to be less exposed to rapid changes in external environment (Santamaria and Bayman, 2005). In our study, wind speed (for epiphytic) and rainfall (for endophytic) occurring 5 to 20 days before sampling date were the climatic variables that better explain total variation of community structures. Thus, wind might be important for dispersal of epiphytic propagules (as reviewed by Vacher et al., 2016) and could be responsible for increasing diversity of epiphytic community. On the other hand, as rainfall mainly contributed for shaping endophytic community, rainfall might be important for fungal spore's dispersion and colonization of endophytes (as reviewed by Vacher et al., 2016). In fact, rainfall has been previously identified as a key factor for the colonization pattern

of endophytes (Martinez-Alvarez et al., 2012). Fungal community structure was also driven by mean temperatures at 10- (for endophytes) or 20- (for epiphytes) days before sampling date. At moderate temperatures (average temperature of 14.4 °C in spring and 8.1 °C in autumn), fungal propagules would have higher viability, thus increasing successful colonization of plant tissues (Collado et al., 1999). Therefore, the highest abundance and richness of fungi observed during spring could be due to the warmer temperatures and higher wind speed (for epiphytes) or rainfall (for endophytes) observed during this season in comparison with autumn. Similarly, other studies have reported that richness and abundance of fungal endophytes (Collado et al., 1999) and epiphytes (Osono, 2008) was higher in spring than in autumn. All these findings suggest that seasonal shifts among phyllospheric fungi may be due to climatic conditions that favor propagule production or dispersal and/or to different abilities exhibited among fungal species. For example, spring preference of sporulation and differential capacity of fungi to adapt to climate have been described by Jumpponen and Jones (2010) or Martinez-Alvarez et al. (2012). Indeed, a great number of endophytic and epiphytic fungal genera were found to be positively associated with either spring or autumn.

Olive plant organ was found to influence the endophytic fungal composition, but not the epiphytic. To the best of our knowledge, no previous studies have investigated the specificity of epiphytic fungi towards different host organs. In this study, colonization and composition of fungal community on the surface of twigs and leaves was not greatly different, suggesting no organ-specificity among epiphytes. This is not surprising because the canopy of woody evergreen plants, such as olive trees, is exposed to the same aerial inoculum. Both plant organs are reasonably close to each other, so they may even be in direct contact with each other, leading to a high number of shared OTUs. In what concerns endophytes, our study demonstrates that twigs harbor more species and strains than leaves, as described for other plant systems growing in Mediterranean climate, such as *Quercus* spp. and *Pinus* spp. (Collado et al., 1999; Martinez-Alvarez et al., 2012; Moricca et al., 2012). On the other hand, in tropical trees the endophytic fungal richness in leaves was reported to be higher (Singh et al., 2017) or lower (Verma et al., 2014) when compared to stems. Although endophytic composition was similar in leaves and twigs, several endophytic fungal genera were found to be more correlated either with leaves or twigs, suggesting signs of organ specificity by certain genera. This hypothesis is also supported by the fact that a higher number of endophytes is observed in twigs than in leaves. As leaves are soft tissues, they are more exposed to external environment than woody twigs. Therefore, climatic factors influence more the endophytic community on leaves than twigs, resulting in the lower endophyte richness found in

leaves. This result is in agreement with previous studies indicating the possible organ-specificity of fungal endophytes *(e.g.* Collado et al., 1999; Moricca et al., 2012; Sun et al., 2012).

Exposure to ultraviolet radiation depends on organ orientation (north or south) within canopies, and is expected to influence the colonization of epiphytic and endophytic phyllosphere fungi (Laforest-Lapointe et al., 2016). However, our results revealed no significant differences in the diversity and composition of fungal communities inhabiting leaves or twigs at different canopy locations (north and south). Few studies have compared phyllospheric fungi inhabiting plant organs oriented in north and south directions. In the phyllosphere microbiota of beech (*Fagus crenata*), Osono and Mori (2003) reported that the composition and richness of species inhabiting shade leaves differed from that of sun leaves. They detected that endophytes and epiphytes showed higher richness in sun and shade leaves, respectively. In contrast, Laforest-Lapointe et al. (2016) found that canopy location did not have a significant influence on the epiphytic bacterial community structure in leaves of several temperate forest tree species, including angiosperms and gymnosperms. Although phyllosphere fungi did not differ significantly with respect to the location of plant organs that they inhabit, we found that both canopy location and season jointly affected the diversity and abundance of fungal phyllosphere community. In spring, fungal endophytes were more affected by canopy location than epiphytes. An opposite pattern was observed in autumn, being epiphytes more susceptible to the effect of canopy location. Therefore, although canopy orientation does not have a great influence on fungal assemblage, it does have a more pronounced effect on fungal epiphytic and endophytic communities during autumn and spring, respectively.

In conclusion, endophytic and epiphytic fungal communities of olive tree phyllosphere are distinct and are not driven by the same factors. While epiphytic fungal assemblage appeared to be largely influenced by environmental factors (mostly season, but also wind speed and mean temperature), endophytic fungal assemblage is driven by both environmental factors (mostly season and slightly by rainfall and mean temperature) and plant organs. The potential or ecological significance of both phyllosphere fungal communities is unknown, and further investigations are still required to identify the functional role of these microorganisms.

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2.7 Supporting Information

The following Supporting Information is available for this chapter:

Table S2.1 Abundance and diversity of fungal endophytes and epiphytes detected on each olive tree organ, during autumn and spring surveys. Species diversity indices (1/D, H') are presented as mean values ± SE (n=63) and as total number (in brackets). Different superscript lowercase letters in each row denote a statistically significant differences (P <0.05). Differences between endophytic and epiphytic communities are denoted by asteristics (*P<0.05; ** P<0.01; ***P<0.001).

	Parameters	Autumn			Spring			Total		
		Leaf	Twigs	Total	Leaf	Twigs	Total	Leaf	Twigs	Total
Endophytes	Average n° of isolates/tree	1.6±1.5^{c}	7.1±3.2^{a}	4.4±2.7^{b}	3.2±1.1^{b}	5.3±1.8^{a,b}	4.2±1.5^{b}	2.0±1.4^{c}	6.1±2.7^{a}	4.3±2.2^{b}
	Total number of OTUs	22	50	58	58	72	90	71	102	125
	Average n° of OTUs/tree	1.2±0.8^{c}	3.4±1.1^{a}	2.5±1.0^{b}	2.7±2.2^{b}	3.7±2.5^{a}	3.2±2.4^{a}	2.0±0.8^{b,c}	3.7±1.2^{a}	2.9±1.7^{b}
	Shannon-Wiener index (H')	0.3±0.2 (2.0)^{c}	0.8±0.5 (2.2)^{a}	0.6±0.5 (2.3)^{b}	0.8±0.5 (2.2)^{a}	0.9±0.5 (2.3)^{a}	0.9±0.5 (2.3)^{a}	0.5±0.3 (2.4)^{b}	0.9±0.5 (2.5)^{a}	0.7±0.5 (2.6)^{a,b}
	Simpson's index (1/D)	3.0±1.3 (3.6)^{b}	3.3±1.0 (3.6)^{a}	3.2±1.1 (3.6)^{a,b}	3.6±1.3 (3.7)^{a}	3.4±1.0 (3.7)^{a}	3.5±1.1 (3.8)^{a}	3.4±1.3 (3.8)^{a}	3.3±0.9 (3.7)^{a}	3.5±1.0 (3.8)^{a}
Epiphytes	Average n° of isolates/tree Log_{10} (CFU/cm^{2})	5.2±1.8^{c}	5.1±1.7^{c}	5.2±1.7^{c}	15.2±2.8^{a}	15.2±3.7^{a}	15.2±3.2^{a}	10.2±2.8^{b}	10.1±3.3^{b}	10.2±2.4^{b}
	Total number of OTUs	56	66	87	127	144	185	163	185	242
	Average n° of OTUs/tree	3.4±3.0^{c}	3.5±3.1^{c}	3.4±3.1^{c}	7.4±3.5^{a}	7.1±4.4^{a}	7.3±4.0^{a}	5.4±3.9^{b}	5.3±4.1^{b}	5.4±4.0^{b}
	Shannon-Wiener index (H')	0.8±0.6 (2.1)^{b}	0.9±0.6 (2.4)^{b}	0.9±0.6 (2.3)^{b}	1.4±0.5 (2.7)^{a}	1.4±0.5 (2.7)^{a}	1.4±0.5 (2.7)^{a}	1.1±0.6 (2.7)^{a,b}	1.1±0.7 (2.8)^{a,b}	1.1±0.7 (2.8)^{a,b}
	Simpson's index (1/D)	3.5±0.6 (3.7)^{a,b}	3.7±0.9 (3.8)^{a}	3.7±0.8 (3.7)^{a}	3.7±0.2 (3.9)^{a}	3.7±0.3 (3.9)^{a}	3.7±0.3 (3.9)^{a}	3.5±0.5 (3.9)^{a,b}	3.7±0.7 (3.9)^{a}	3.6±0.6 (3.9)^{a}

Table S2.2 Abundance and diversity of fungal endophytes and epiphytes isolated from northern or southern olive tree tissues, in the autumn and spring. The results of species diversity indices (1/D, H') are present as the mean value ± SE (n=63) and as total number (in brackets). Different superscript lowercase letters in each row denote a statistically significant differences (P<0.05). Differences between endophytic and epiphytic communities are denoted by asteristics (* P<0.05; ** P<0.01; *** P<0.001)

	Parameters	Autumn			Spring			Total		
		North	South	Total	North	South	Total	North	South	Total
Endophytes	Average n° of isolates/tree	4.5±2.9^{a}	4.3±2.5^{a}	4.4±2.7^{a}	4.5±1.7^{a}	3.9±1.2^{b}	4.2±1.5^{a}	4.5±2.3^{a}	4.1±1.9^{a}	4.3±2.2^{a,}
	Total number of OTUs	50	41	58	82	62	90	110	90	125
	Average n° of OTUs/tree	2.5±1.1^{b}	2.4±1.0^{b}	2.5±1.0^{b}	3.3±2.4^{a}	3.1±2.3^{a}	3.2±2.4^{a}	2.9±1.8^{b}	2.8±1.7^{b}	2.9±1.7^{b}
	Shannon-Wiener index (H')	0.6±0.5(2.3)^{c}	0.6±0.5 (2.2)^{c}	0.6±0.5(2.3)^{c}	0.9± (2.5)^{a}	0.8±0.6 (2.3)^{a}	0.9±0.5(2.3)^{a}	0.7±0.1 (2.6)^{b}	0.7±0.1 (2.5)^{b}	0.7±0.5 (2.6)^{b}
	Simpson's index (1/D)	3.1±1.1(3.6)^{b}	3.2±1.1(3.6)^{a,b}	3.2±1.1 (3.6)^{a,b}	3.5±1.2(4.0)^{a}	3.5±1.0(3.8)^{a}	3.5±1.1 (3.8)^{a}	3.3±1.2 (4.0)^{a,b}	3.4±1.1 (3.8)^{a}	3.5±1.1 (3.8)^{a}
Epiphytes	Average n° of isolates/tree Log_{10} (CFU/cm^2)	6.9±5.4^{c}	3.4±1.4^{d}	5.2±1.7^{c,d}	14.6±2.9^{a}	15.7±3.6^{a}	15.2±3.2^{a}	10.7±4.1^{b}	9.6±2.5^{b}	10.2±2.4^{b}
	Total number of OTUs	74	49	87	131	141	185	177	173	242
	Average n° of OTUs/tree	4.4±3.1^{b}	2.5±0.9^{c}	3.4±3.1^{b,c}	7.1±3.7^{a}	7.4±4.2^{a}	7.3±4.0^{a}	5.8±3.4^{b}	5.0±2.6^{b}	5.4±4.0^{b}
	Shannon-Wiener index (H')	1.1±0.6(2.2)^{b}	0.6±0.5 (2.2)^{c}	0.9±0.6(2.3)^{b}	1.4±0.5(2.6)^{a}	1.4±0.5 (2.7)^{a}	1.4±0.5(2.7)^{a}	1.0±0.6 (2.7)^{b}	1.0±0.5 (2.8)^{b}	1.1±0.7 (2.8)^{b}
	Simpson's index (1/D)	3.6±0.8 (3.7)^{a}	3.7±0.7 (3.7)^{a}	3.7±0.8 (3.7)^{a}	3.7±0.2 (3.9)^{a}	3.7±0.5 (3.9)^{a}	3.7±0.3 (3.9)^{a}	3.6±0.6 (3.9)^{a}	3.7±0.6 (3.9)^{a}	3.6±0.6 (3.9)^{a}

Table S2.3- Sequential test results for the distance based linear model (DistLM) explaining the contribution of climatic variables to the total endophytic and epiphytic fungal variation. This analysis was performed using the weather data collected 3-, 5-, 10- and 20-days before sampling. The displayed climatic variables were selected by DistLM as part of the best model; '–' indicate those climatic variables that were not selected as part of the best model.

Climatic variables		Epiphytic fungi			Endophytic fungi		
		F	P	Prop. (%)	F	P	Prop. (%)
Mean temperature (°C)	3 days	-	-	-	-	-	-
	5 days	-	-	-	-	-	-
	10 days	-	-	-	5.83	0.001	1.09
	20 days	3.59	0.001	0.63	-	-	-
Max. relative humidity (%)	3 days	-	-	-	-	-	-
	5 days	-	-	-	4.80	0.001	0.88
	10 days	-	-	-	-	-	-
	20 days	-	-	-	-	-	-
Min. relative humidity (%)	3 days	-	-	-	-	-	-
	5 days	-	-	-	-	-	-
	10 days	-	-	-	-	-	-
	20 days	-	-	-	-	-	-
Cumulative rainfall (mm)	3 days	8.48	0.001	1.47	-	-	-
	5 days	-	-	-	7.99	0.001	1.50
	10 days	9.79	0.001	1.74	10.70	0.001	2.04
	20 days	5.48	0.001	0.99	13.08	0.001	2.54
Mean wind speed (m/s)	3 days	-	-	-	-	-	-
	5 days	9.35	0.001	1.70	5.37	0.001	0.99
	10 days	7.22	0.001	1.34	2.21	0.019	0.40
	20 days	31.47	0.001	5.90	-	-	-

F = statistic; P = probability; Prop. = proportion of total variation explained.

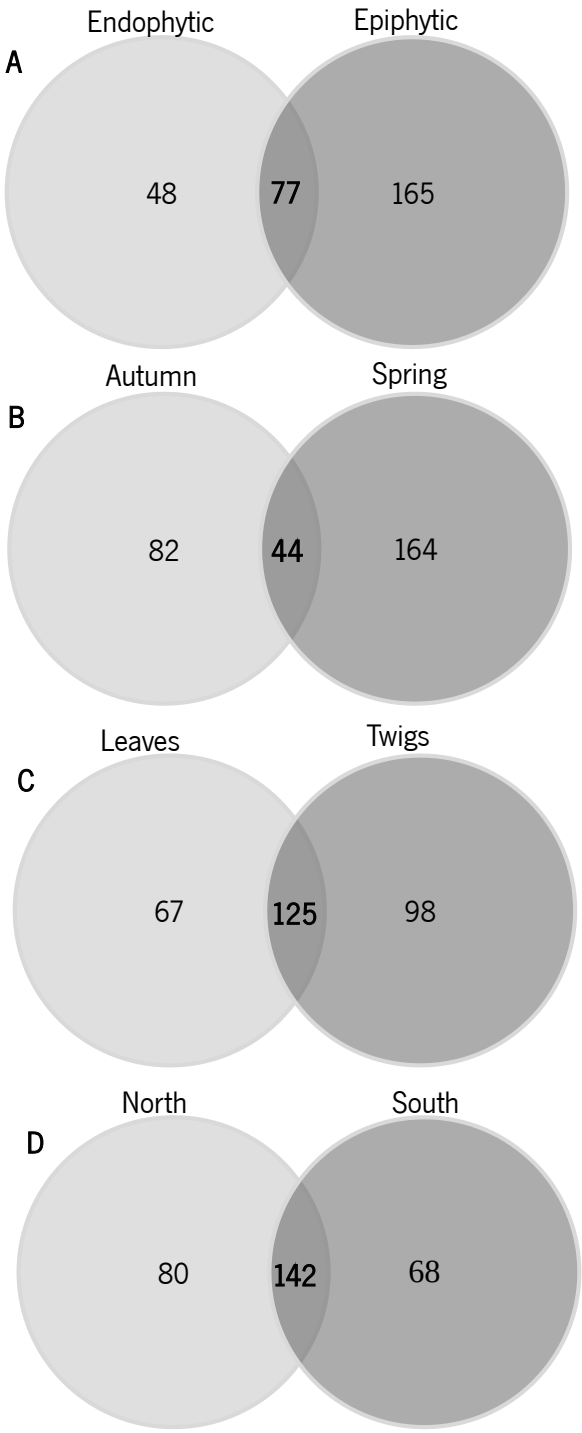

Fig. S2.1 - Venn diagrams representing the total number of fungal OTUs shared between (**A**) epiphytic and endophytic communities, (**B**) season (autumn and spring), (**C**) olive tree organ (leaves and twigs), and (**D**) plant tissue direction (north and south).

Chapter 3

Does plant disease alter host microbiota?

Does plant disease alter host microbiota?

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3.1 Abstract

Microbial interactions taking place in phyllosphere are known to fulfil important functions for plant health. Yet, little is known about the influence of pathogen infections on plant microbiota and if the outcome of microbial interactions is dependent on host plant genotypes. In this work, the endophytic and epiphytic fungal communities of leaves displaying or not disease symptoms were compared. Fungal assemblages differed in size and composition between olive leaf spot (OLS) asymptomatic and symptomatic olive tree leaves, especially within the epiphytic community. While disease symptoms seem to play an important role in the epiphytic community structure, endophytic community assemblage is likely to be more influenced by additional factors. The comparison of leaves taken from three olive cultivars with different susceptibilities to OLS revealed the structuring effect of host genotype on the composition of fungal communities in asymptomatic leaves, but not in leaves displaying symptoms. Taken together, the results suggest that the combined effect of microbe-microbe and microbe-host interactions for fungal assembly in diseased leaves. Some fungal taxa were found to be specific to either asymptomatic or symptomatic leaves, suggesting their competitive or cooperative activity with pathogen, within and outside leaf tissues. This issue should be investigated in the future.

3.2 Introduction

In nature, the above-ground parts of plants (the phyllosphere) harbor a diverse and complex microbial community, living either on the surface (epiphytes) or in the inner (endophytes) plant tissues (Kumar et al., 2017). These microorganisms establish complex interactions with each other and with plants, and could frequently lead to plant infection by pathogens (Rastogi et al., 2013). Although such pathogenic interactions are ubiquitous in nature, prior research mainly used reductionist approaches by focusing on either the plant-pathogen (Gupta et al., 2015) or the pathogen-microbe (Heydari and Pessarakli, 2010) interactions. Moreover, phyllosphere microbial interactions were viewed from the perspective of how specific beneficial microorganisms inhibit the growth or activity of pathogenic microorganisms (Stromberg et al., 2000; Saraiva et al., 2014; Jiang et al., 2015), ignoring the vast majority of plant microbiota (Finkel et al., 2017). The possible relationship and interspecies interaction between the incoming pathogens and the resident microbiota of phyllosphere also remains to be elucidated (Venturi and Passos da Silva, 2012). Can incoming pathogens change the composition of resident microorganisms? Are there any

interactions (cooperative or competitive) between resident microorganisms and incoming pathogens? Are the outcomes of these interactions dependent on the plant genotype?

Changes in microbial composition upon pathogen infection (Zhang et al., 2011; Trivedi et al., 2012; Chapelle et al., 2016) and their relation to competitive and cooperative microbial interactions (Galiana et al., 2011) have already been reported in the rhizosphere. We hypothesized that such interactions may also exist in the phyllosphere. To test this hypothesis, we will use the olive tree and one of the most important foliar diseases affecting it, the olive leaf spot (OLS), as a model system.

Among cultivated plants, olive (*Olea europaea* L.) is the sixth most important oil crop in the world, being Mediterranean countries the greatest olive oil producers (Kostelanos and Kiritsakis, 2017). OLS is the most common and damaging leaf disease of this crop (Belfiore et al., 2014), resulting in high yield losses estimated up to 20% (cited by Rongai et al., 2012). OLS is caused by a subcuticular growing fungus, *Venturia oleaginea* (=*Spilocaea oleaginea*), which commonly affects mostly leaves being a non-systemic disease (Gonzales-Lamonthe et al., 2002). Symptoms usually appear on the upper surface of leaves, as circular lesions (Gonzales-Lamonthe et al., 2002). As most infected leaves fall prematurely, olive trees become weakened and flower formation is inhibited (Belfiore et al., 2014).

Previous research revealed that the naturally occurring saprophytic fungi on olive leaves affect the conidial production by V. *oleaginea* (cited by Viruega et al., 2013), suggesting that the resident microflora might interact with pathogen biology. Thus, OLS-symptomatic leaves provide a special niche for studying the relationships between the pathogen and other resident fungi, including both epiphytes and endophytes. An added benefit of this system is cultivars with different susceptibility levels to OLS (Mahfoud et al., 2018) that host both OLS-symptomatic and asymptomatic leaves within the same olive tree.

In light of the above, this study aims to answer the following questions: i) Are fungal endophyte and epiphyte communities similar? ii) What is the effect of disease on leaf fungal communities? iii) Does host genotype (at cultivar level) and disease susceptibility influence fungal endophyte and epiphyte communities? iv) Can we determine indicator communities associated with disease and/or other factors (*e.g.* host genotype/susceptibility)? Taking these questions into account, we used a culture dependent PCR-based identification approach to characterize the endophytic and epiphytic fungal communities associated with OLS-symptomatic and asymptomatic

leaves of three distinct olive cultivars. This methodology was chosen to test the fungal cultures isolated and identified as potential biocontrol agents against V. *oleaginea*.

3.3 Material and methods

3.3.1 Study site and leaves collection

We sampled leaves during the spring 2014 (from March to May) in three olive orchards located in Mirandela (Northeast of Portugal), at coordinates N 41° 32.593'; W 07° 07.445'(orchard 1), N 41° 32.756'; W 07° 07.590'(orchard 2) and N 41° 29.490'; W 07° 15.413' (orchard 3). Management of selected orchards followed integrated production guidelines (Malavolta and Perdikis, 2012). In each orchard trees were planted at 7 × 7 m spacing, and included three Portuguese olive cultivars with different susceptibilities to OLS (cvs. *Verdeal Transmontana* and *Madural* are susceptible, while cv. *Cobrançosa* is less susceptible) (Malheiro et al., 2015). In each orchard, seven olive trees of each cultivar were randomly selected. In each tree, asymptomatic and OLS-symptomatic leaves were collected from two cardinal orientations (north and south) at the heights of 1.5–2 m above ground, and used to isolate fungal epiphytes and endophytes. Incidence of OLS was assessed in the same olive trees, simultaneously to sample collection, by determining the percentage of infected leaves (Teviotdale and Sibbett 1995). According to their susceptibility, the levels of disease incidence (%) varied between cultivars, being lower in cv. *Cobrançosa* (1.1%±0.8) compared to cvs. *Verdeal Transmontana* (7.3%±1.5) and *Madural* (8.2%±1.7).

3.3.2 Isolation of fungal epiphytes and endophytes

From each tree, five asymptomatic and five OLS-symptomatic leaves were selected and used to isolate epiphytes and endophytes. All symptomatic leaves had a similar level of infection, showing lesions covering 20-50% of leaf area. Fungal epiphytes were isolated by the plate dilution method, onto Potato Dextrose Agar (PDA, Difco) and Plate Count Agar (PCA, Himedia) media supplemented with 0.01% (w/v) chloramphenicol (Oxoid), following the procedure described by Gomes et al. (2018). Results of epiphytes were expressed as log CFU/cm^2, *i.e.* the number of individual colonies of fungi adhered to leaf surface. We approximated leaf surface area using an ellipse equation ($A=\pi ab^*2$), in which A is the area, and *a* and *b* are the longitudinal and the transverse axes of the leaf, respectively. The average leaf area of the cvs. *Cobrançosa*, *Verdeal Transmontana* and *Madural* was 12.7±0.6, 12.6±0.5 and 13.9±0.4 cm^2, respectively.

Endophytic fungi were isolated from the same leaf fragments used to isolated epiphytes. After surface disinfection (Martins et al., 2016), each leaf was cut in segments (*ca.* 5 x 5 mm), which were transferred to the same culture media used to isolate epiphytes. Validation of surface sterilization procedure was done by imprinting the surface of sterilized plant tissues onto PDA and PCA media. A total of 3,720 plant tissue segments were surveyed for each symptomatic and asymptomatic leaf. Fungal colonies were subcultured on fresh medium until pure epi/endophytic cultures were obtained.

3.3.3 Identification of fungal epiphytes and endophytes

Pure fungal cultures were sorted into groups according to morphological characteristics (both colony and microscopic morphology) and then three representative isolates of each morphotype were identified by sequencing the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). Total genomic DNA was extracted from harvested mycelium or spores using the REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK). The ITS region (ITS1, 5.8S, ITS2) was amplified using ITS1/ITS4 or ITS5/ITS4 primers sets (White et al., 1990) in a PCR protocol described previously by Oliveira et al. (2012). The amplified products (≈650 bp) were purified and sequenced using Macrogen Inc. (Seoul, South Korea) services. The obtained DNA sequences were analyzed with DNASTAR v.2.58 software, and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST algorithm (Gomes et al., 2018). The obtained sequences are available at GenBank with the following accession numbers: KU324941-KU325040; KU325041-KU325240; KU325241-KU325457. Each operational taxonomic unit (OTU) was taxonomically classified according to the Index Fungorum Database (www.indexfungorum.org). Pure cultures of each identified isolate were preserved and deposited in the culture collection of the Polytechnic Institute of Bragança (School of Agriculture).

3.3.4 Effect of OLS disease and host genotype on fungal diversity

The effect of OLS disease or host genotype in leaf fungal diversity was evaluated at both community and functional group levels. At *community* level, the fungal diversity was determined by evaluating the abundance (number of isolates), richness (number of different OTUs) and by computing Shannon–Wiener (H') index in *Species Diversity and Richness* v. 4.0 (Seaby and Henderson, 2006). Results are presented as the mean of replicates (= 21, for each cultivar). At *functional group* level, identified fungi were firstly classified into functional based categories through

the knowledge of their ecology. Considered functional groups were previously described for fungal endophytes by Hardoim et al. (2015), and included commensal, beneficial and pathogen roles. The commensal group comprised fungi that do not have an apparent effect on host plants. The fungi conferring beneficial effects to host plants, such as protection against pathogens and pests and plant growth promotion, were classified as beneficial microbes. The third group, pathogens, included latent plant pathogens. Fungal OTUs belonging to other groups or not able to be identified into a functional group were categorized as "other" and "unknown fungi", respectively. After grouping, the proportional abundance and richness of each functional group across asymptomatic and OLS-symptomatic leaves was determined. To determine differences on fungal diversity/functional group among leaves, a one-way analysis of variance (ANOVA) with *SPSS* v.20 was performed, with multiple comparisons according to Tukey ($P<0.05$).

3.3.5 Data analysis

Multivariate statistical analysis was performed to describe differences in fungal community composition between cultivars or asymptomatic/symptomatic leaves, and to identify potential fungal core OTUs associated to each of them. The pathogen V. *oleaginea* was excluded from all of these analyses. All statistical analyses were performed in *R* (R Core Team, 2014).

Effect of OLS disease and host genotype on fungal community structure. A Canonical Correlation Analysis (CCA) was used to explore the correlation of cultivar or presence/absence of OLS-leaf symptoms with the composition of endophytic and epiphytic fungal communities. All data were $\log_2(x+1)$ transformed for standardization. CCA was performed using the *CCorA* function in the *vegan* package (Oksanen et al., 2017). To test for significant differences between fungal community groupings obtained in the CCA, an ANOVA was conducted with the *anova* function. Variation partitioning was then performed with the *vegan* package using *varpart* function, to estimate the relative contribution of cultivar and presence/absence of OLS-leaf symptoms in shaping either the endophytic or epiphytic fungal community composition. Additionally, an analysis of similarity (ANOSIM) was performed to further assess the differences in the fungal composition among OTU groups previously identified by the CCA approach. This analysis was performed using the *anosim* function with *vegan* package and the Bray–Curtis dissimilarity index (Oksanen et al., 2017).

Identification of core fungi associated to OLS disease and/or host genotype. Indicator species analysis was performed to identify fungal OTUs associated with (or indicative of) particular

olive cultivars and asymptomatic/symptomatic leaves. This analysis was conducted with presence/absence data using the *multipatt* function from *indicspecies* package (Cáceres et al., 2012). The use of presence/absence data implies that the obtained indicator species are specific and frequent, but not dominant, and are diagnostic on their own "not only when exceeding a certain cover value" (Willner et al., 2009). This analysis generates an Indicator Value (*IndVal*) index and a P-value for each fungal species. The *IndVal* expresses the species importance in the group and is defined as the product of two components: "A" and "B"; where "A" is the *specificity* (i.e. uniqueness) and "B" is the *sensitivity* (i.e. frequency) of the species to a particular habitat (Cáceres et al., 2012). The *IndVal* ranges from 0 (no indication) to 1 (perfect indication), and significant (P <0.05) values greater than 0.5 indicate species that are deemed characteristic of a group (Cáceres et al., 2012).

To examine the interrelationships between fungal endophytes or epiphytes recovered from a particular cultivar and the presence/absence of OLS symptoms, we performed a Principal Component Analysis (PCA) by using the *psych* package (Revelle, 2017). For this analysis only the endophytic and epiphytic fungal species with the greatest power to separate both types of leaves (asymptomatic and symptomatic) were used, as preselected from the Random Forests analysis using R *RandomForests* package. Random Forests is a statistical classifier analysis with high accuracy, allowing the determination of variable importance of a data set through artificial intelligence algorithms (Cutler et al., 2007). For each tree grown on a bootstrap sample, the error rate for observations left out of the bootstrap sample is monitored. This is called the "out-of-bag" (OOB) error rate, indicating the analysis accuracy (Breiman, 2011). The mean decrease in *Gini coefficient* indicates the variable contribution for the presence/absence of OLS-disease. This measure allows us to identify the ranking of fungal species with higher relevance, explaining the non-occurrence/occurrence of OLS-symptoms, as well as, variable selection (Breiman, 2011; Cutler et al., 2007). Error rate (OOB) estimate was 31.87% and 53.29% within endo- and epiphytic communities, respectively.

3.4 Results

3.4.1. The effect of disease on fungal diversity

Analysis of fungal communities revealed a total of 183 OTUs, belonging to 111 genera and 47 families, all from the Ascomycota (166 OTUs, 96.6%) and Basidiomycota (17 OTUs) phyla (Fig.

S3.1 and S3.2). The most representative genera were *Cladosporium* (14.9% of the total isolates), followed by *Alternaria* (10.5%) and *Fusarium* (9.7%) (Fig. S3.1).

The diversity and abundance of fungal epiphytes was significantly ($P<0.001$) higher when compared to endophytes (Table S3.1). Both fungal communities were different between asymptomatic and OLS-symptomatic leaves. Fungal richness and abundance in asymptomatic leaves relative to OLS-symptomatic leaves was especially greater within the epiphytic community (Fig. 3.1A). In fact, epiphytes were 2.1-fold significantly more abundant and 1.9-fold more rich and diverse in asymptomatic than in OLS-symptomatic leaves. In particular, there was a reduction in isolates from asymptomatic to symptomatic leaves belonging to Davidiellaceae, Pleosporaceae, Nectriaceae, Phaeomoniellaceae, Xylariaceae and Trichocomaceae, and eighteen families of fungi disappeared (Fig. S3.2). In contrast, within endophytic communities no significant differences in fungal richness, abundance or diversity were detected between leaf statuses (Fig. 3.1A). Despite this, some endophytes decreased (Pezizaceae and Pyronemataceae) their abundance in symptomatic leaves, while others (Dermateaceae) increased (Fig. S3.2). Additionally, endophytic fungi of nine families, which appeared in asymptomatic leaves, disappeared in symptomatic leaves, contrasting with specific families only found in symptomatic leaves (Psathyrellaceae and Stereaceae).

Endophytic and epiphytic fungal communities also displayed a different proportion of each functional group between asymptomatic and symptomatic leaves (Fig. 3.1B). The effect of OLS disease on functional fungal assemblages was more intense for the epiphytic community, where a significant decrease in fungal abundance and richness was observed in most functional groups in symptomatic leaves. A similar, but somewhat weaker, reduction was observed for endophytic fungal abundance. However, a significant richness increase in pathogenic, beneficial and "other" endophytic fungi was detected in asymptomatic leaves, while commensal fungi remained unchanged. Among the identified pathogens, only two [*Neofabraea alba* (=*Neofabraea vagabunda*) and *Pseudocercospora cladosporioides*] are well known olive fungal pathogens, while the others are pathogens of other plant species. *Neofabraea alba* (more common as an epiphyte) and P. *cladosporioides* (more common as an endophyte) were isolated from both asymptomatic (2.4% and 2.1%, respectively) and symptomatic leaves (2.4% and 3.2%, respectively) (data not shown).

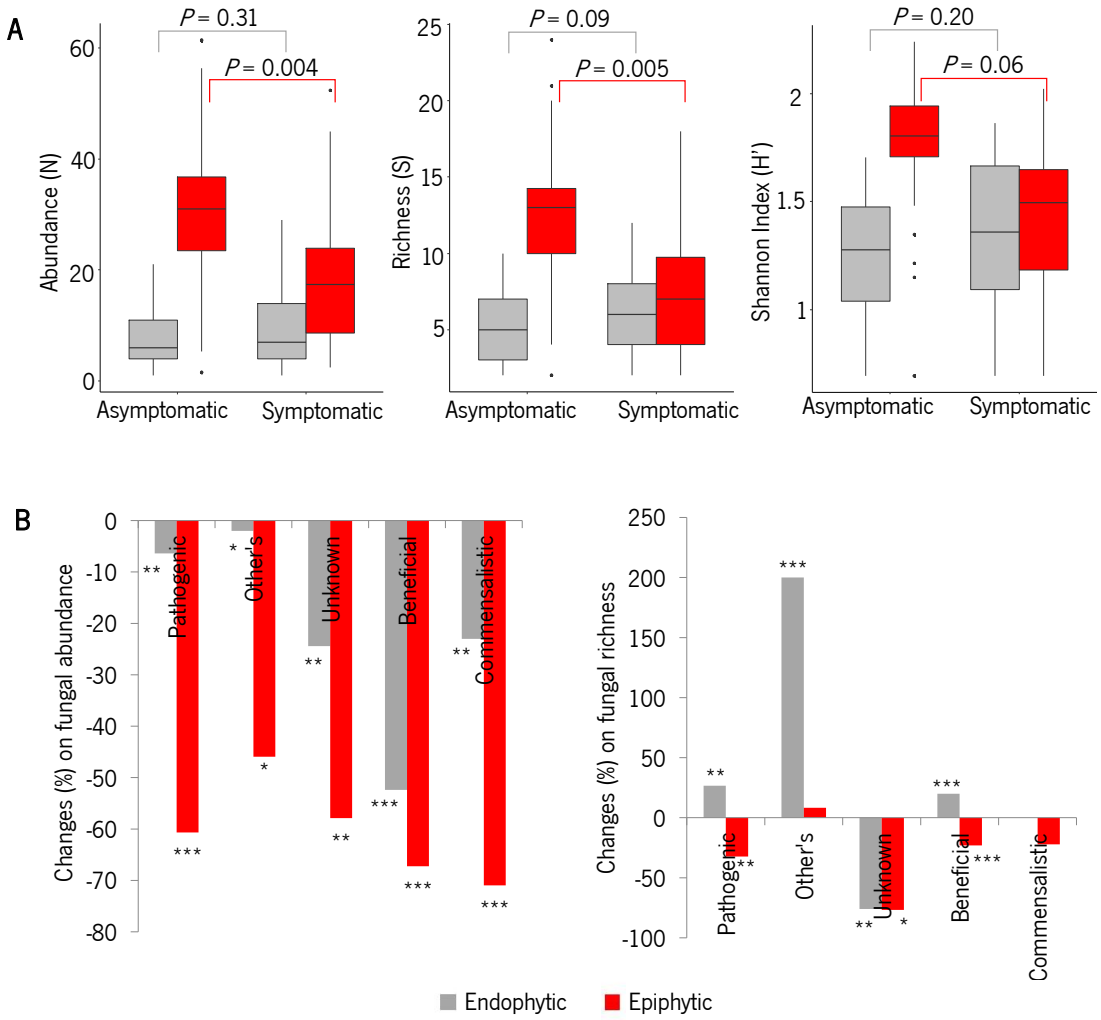

Fig. 3.1 – Comparison of fungal diversity between asymptomatic and OLS-symptomatic leaves, either within endophytic or epiphytic communities. (**A**) Diversity at community level by using abundance, richness and Shannon–Wiener index. Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (black dots). Statistically differences between pairs of values are showed over horizontal lines. (**B**) Changes on fungal abundance and richness detected in asymptomatic/OLS-symptomatic leaves for each functional group. Asterisks indicate statistically significant differences showed in asymptomatic/OLS-symptomatic values (n = 21, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

3.4.2 The effect of host genotype on fungal diversity

OLS disease caused a greater loss of both fungal abundance and richness in cv. *Cobrançosa* (up to 1.9-fold) than in cvs. *Madural* or *Verdeal Transmontana* (both up to 1.1-fold) (Fig. 3.2A). Most of the isolates lost with disease symptoms in all three cultivars belong to Davidiellaceae, followed by Pleosporaceae (for cv. *Cobrançosa*) or by Nectriaceae, Phaeomoniellaceae and Pyronemataceae (for cv. *Madural*) families (Fig. S3.2Fig,). Despite this,

the Shannon's diversity index did not significantly differ between asymptomatic and symptomatic leaves (Fig. 3.2A). When OTUs were divided into functional categories, differences were also detected between host genotypes (Fig. 3.2B). For all the three cultivars, a significant decline in fungal abundance and richness was observed in symptomatic leaves for most functional groups. However, these changes were generally greater in cv. *Cobrançosa*, with exception of pathogenic richness.

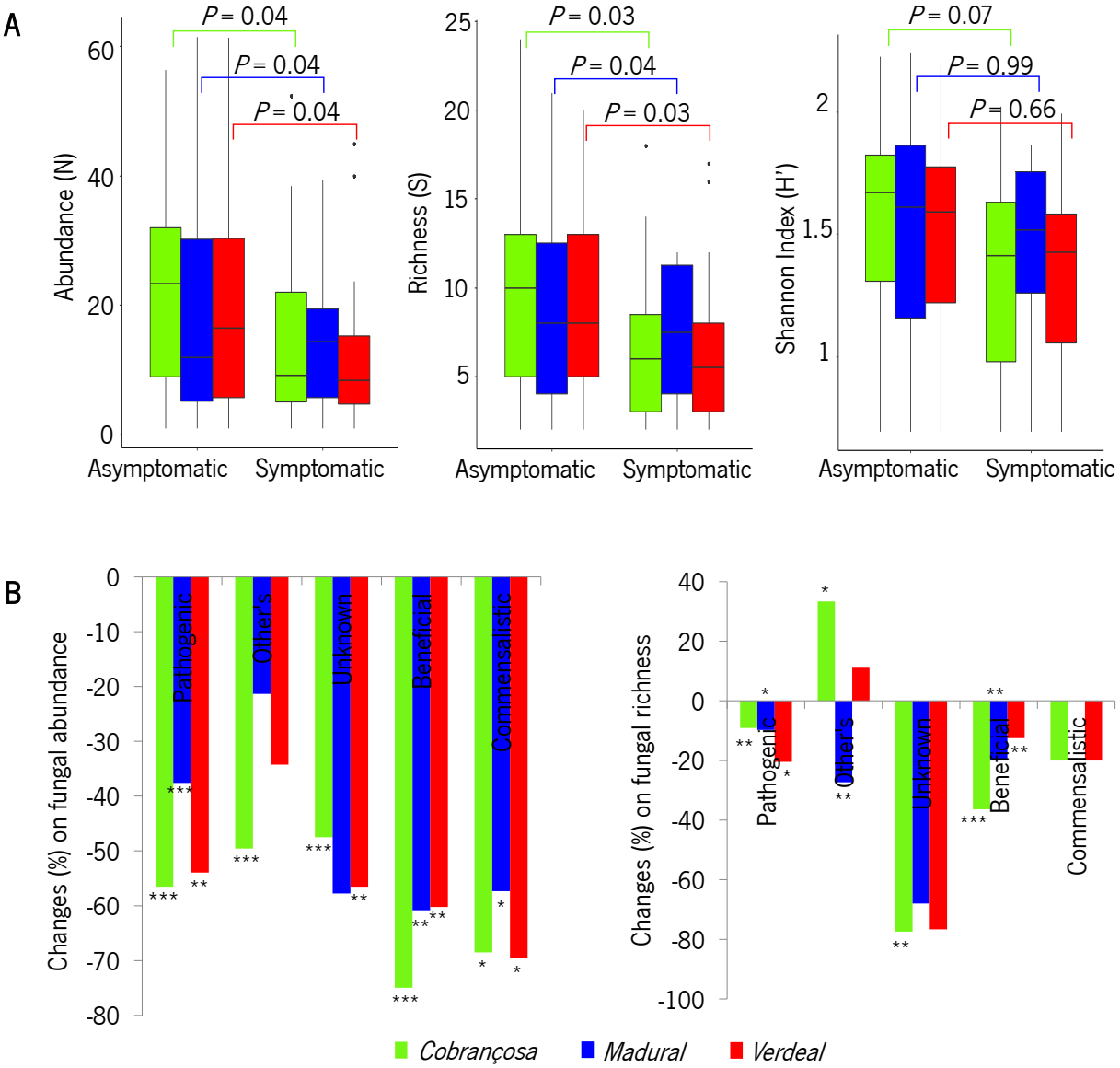

Fig. 3.2 - Comparison of fungal diversity between asymptomatic and OLS-symptomatic leaves within each olive tree cultivar (*Cobrançosa*, *Madural* and *Verdeal Transmontana*). (**A**) Diversity at community level by using abundance, richness and Shannon–Wiener index. Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (black dots). Statistically differences between pairs of values are showed over horizontal lines. (**B**) Changes on fungal abundance and

richness detected in asymptomatic/OLS-symptomatic leaves for each functional group. Asterisks indicate statistically significant differences between pairs of values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.4.3 The effect of disease and host genotype on fungal communities' composition

The effect of disease and host genotype on the composition of endophytic and epiphytic fungal communities was assessed using a CCA biplot analysis. While a clear separation of fungal communities was detected based on the presence/absence of OLS-leaf symptoms in sampled leaves, olive cultivar only had a notable effect on fungal communities from asymptomatic leaves (Fig. 3.3). These observations were supported by ANOVA (Table S3.2) and ANOSIM (Table S3.3) analyses. In fact, significant differences were found on the composition of total ($P < 0.005$), endophytic ($P < 0.005$) and epiphytic ($P < 0.005$) fungal communities inhabiting asymptomatic and OLS-symptomatic leaves (Table S3.2). However, differences between leaves were greater within epiphytes (R = 0.367, $P = 0.001$) than within endophytes (R = 0.151; $P = 0.020$), and greater in cv. Cobrançosa (R = 0.312, $P = 0.001$) than in cvs. Madural (R = 0.149, $P = 0.020$) or Verdeal Transmontana (R = 0.236, $P = 0.005$) (Table S3.3). Despite the significant effect of OLS disease on fungal community composition, the variation partitioning revealed that this factor only explained 2.1%, 4.2% and 4.4% of total, endophytic and epiphytic communities variance, respectively (Table S3.2). The same analyses revealed that host genotype significantly affected the composition of total ($P < 0.003$), endophytic ($P < 0.005$) or epiphytic ($P < 0.001$) fungal communities of asymptomatic leaves; but failed to explain the significant variation in symptomatic leaves ($P = 0.280$, $P = 0.264$ and $P = 0.453$, respectively for total, endophytic and epiphytic communities). The host genotype explained 13% of total fungal species composition variation in asymptomatic leaves (Table S3.2).

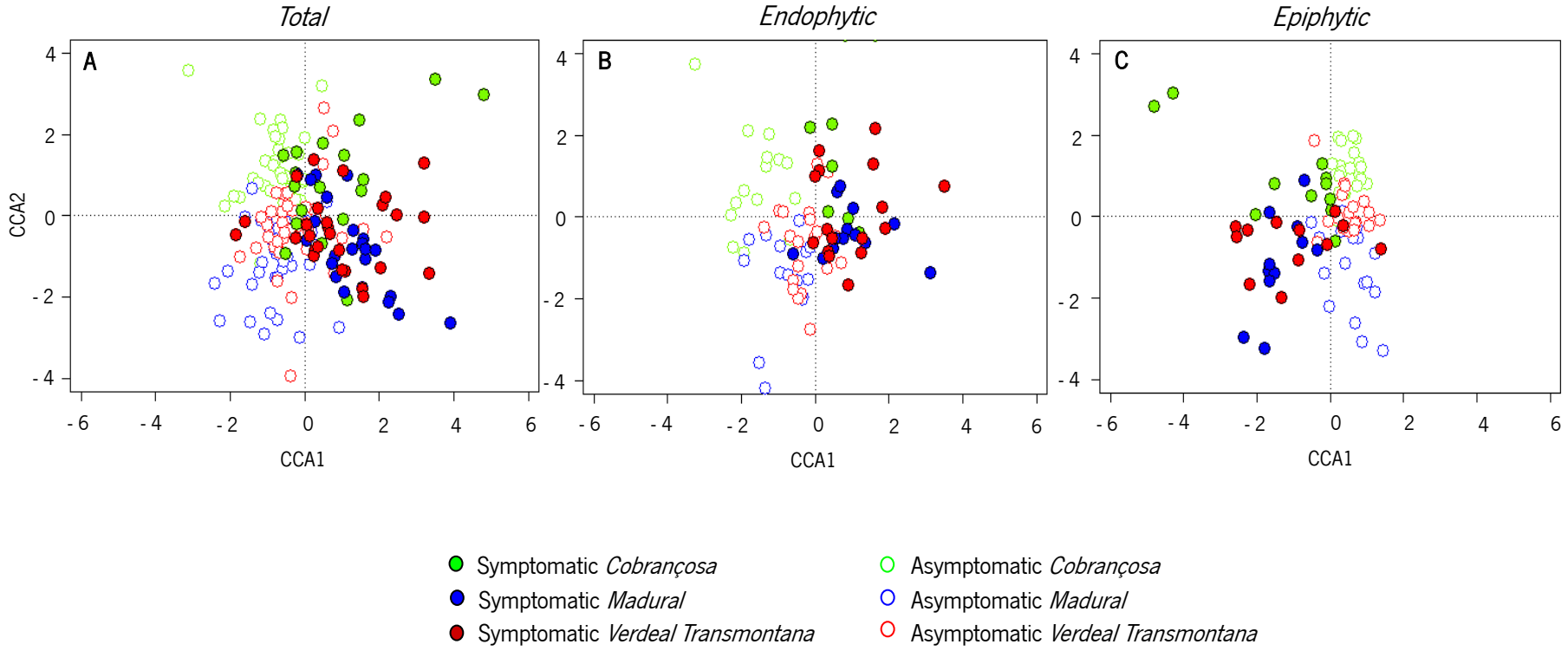

Fig. 3.3 – Canonical correspondence analysis (CCA) ordination showing the dispersion of individual OTUs within total (**A**), endophytic (**B**) and epiphytic (**C**) fungal communities relative to the presence/absence of OLS-leaf symptoms and olive tree cultivar (*Cobrançosa*, *Madural* and *Verdeal Transmontana*).

3.4.4 Foliar fungal consortia associated to host genotypes and disease symptoms

In order to determine indicator communities associated with tested factors, such as disease symptom occurrence or host genotype/susceptibility, a core endophytic and epiphytic fungal communities was first defined as the 10 most abundant OTUs present on each cultivar and asymptomatic/symptomatic leaves. This analysis resulted in 36 different fungal OTUs (Fig. 3.4). The global fungal community inhabiting asymptomatic leaves of cv. *Cobrançosa* was mainly represented by the endophytes *Pseudocercospora cladosporioides*, *Chromelosporium carneum*, and the epiphyte *Cladosporium cladosporioides* (accounting together for 12.1% of the total isolates), while in symptomatic leaves, the endophyte P. *cladosporioides* dominated (6.9%). In cvs. *Madural* and *Verdeal Transmontana*, *Alternaria* sp. was the most abundant either in asymptomatic (common as endophyte and epiphyte) or symptomatic (as endophyte) leaves, accounting for 6.7% and 8.5% of total isolates, respectively for each cultivar.

Species indicator analyses were further used on the whole community to discover which fungal OTUs better characterize the cultivar/leaf symptoms. Results revealed five indicator OTUs of asymptomatic leaves (one endophyte and four epiphytes), and five indicators of OLS-symptomatic leaves (two endophytes and three epiphytes) (*IndVal* > 0.4*, P* < 0.05; Table3.1). From these, the best indicator fungal OTUs that characterized asymptomatic leaves were epiphytes associated to cvs. *Cobrançosa* (*Alternaria* sp., *IndVal* = 0.70) and *Verdeal Transmontana* (*Biscogniauxia mediterranea*, *IndVal* = 0.73). In symptomatic leaves, the best species indicators were endophytes associated with cvs. *Cobrançosa* (B. *mediterranea*, *IndVal* = 0.68) and *Madural* (*Phaeosphaeria avenaria*, *IndVal* = 0.63) (Table 3.1).

Despite the species indicator analysis allow the identification of the fungal OTUs that characterize cultivar/leaf statuses, it was important to understand which specific fungal OTUs were the higher contributors to distinguish a set of leaves (asymptomatic or symptomatic) and cultivar. A PCA analysis was performed to address these questions (Fig. 3.5). This analysis was done by using only the preselected fungal OTUs by the random forest analysis (Fig. S3.3). Results revealed that specific fungal OTUs were indeed associated with a particular set of leaves (asymptomatic or symptomatic) and cultivar. This was particularly noticed within endophytes (Fig. 3.5A). Indicator endophytes colonizing asymptomatic and symptomatic leaves were completely distinct, according to PC2 (which represents around 29% of total endophytic variation). Similarly, indicator endophytes inhabiting leaf samples of cvs. *Madural* and *Cobrançosa* are different from those of cv. *Verdeal Transmontana*, according to PC1 that accounted for 40% of total endophytic variation. Within

epiphytic community, no clear clustering was observed according to the presence/absence of leaf symptoms, as only few epiphytic OTUs were highly associated to either asymptomatic or symptomatic leaves (Fig. 3.5B). Leaves of all three cultivars revealed clear differences on epiphytic fungal community composition. Altogether, the results indicate the existence of a complex fungal consortia associated with either asymptomatic leaves (being the most important the endophytes *Tricharina striispora*, C. *carneum*, *Heydenia alpina*, and the epiphytes C*. carneum*, *Penicillium brevicompactum*, *Epicoccum nigrum*, *Fusarium solani* and *Alternaria alternata*), or symptomatic leaves (being the most important the endophytes *Cladosporium herbarum*, *Phaeosphaeria* sp. and *Neofabraea alba*, and the epiphytes *Discosia fraxinea* and *Trichoderma* sp.), the composition of which changes according to the olive cultivar (Fig. 3.5).

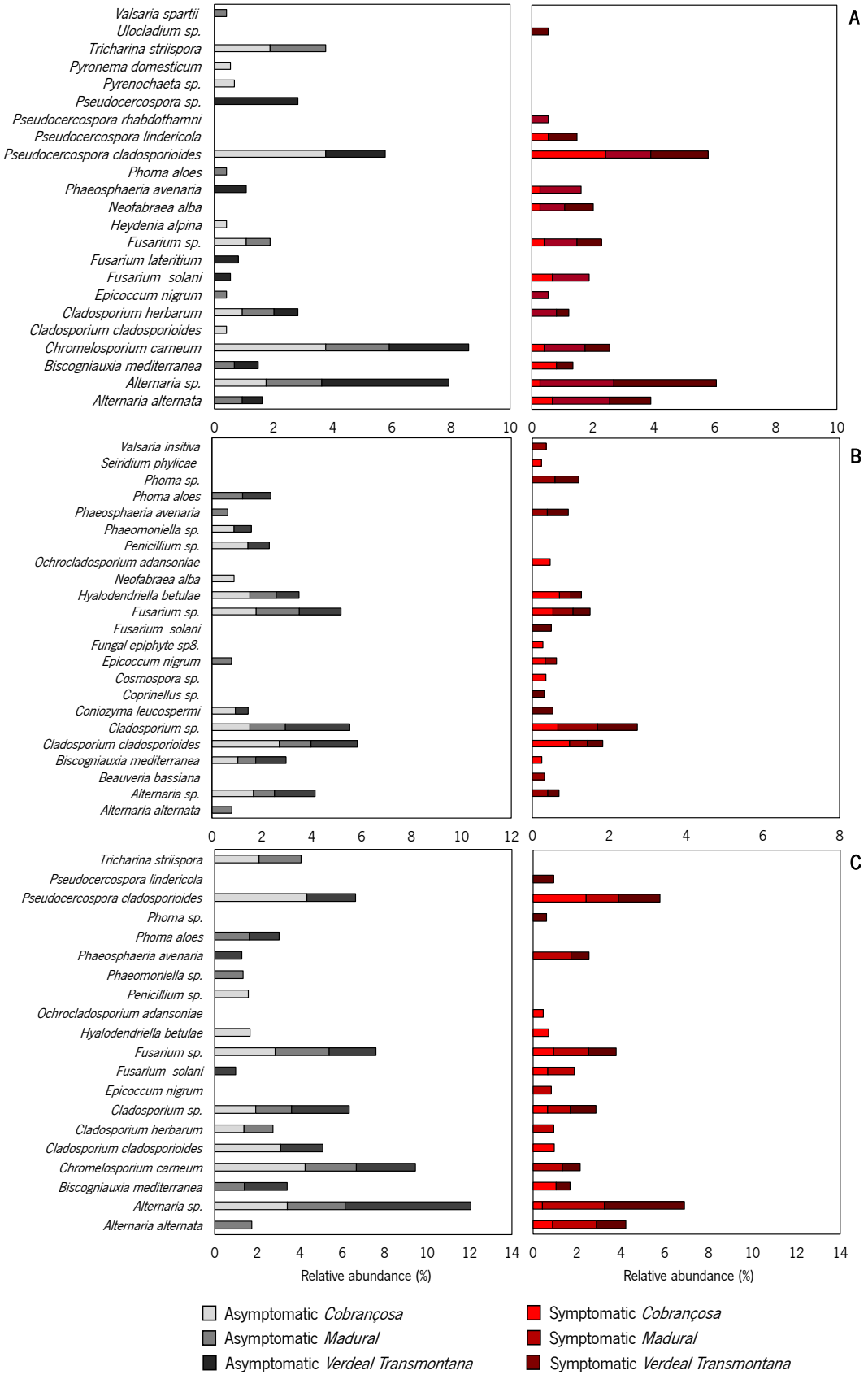

Fig. 3.4 – Relative abundance of the most abundant endophytic (**A**), epiphytic (**B**) and total (**C**) fungal OTUs in asymptomatic (left panels) and OLS-symptomatic (right panels) leaves of different olive cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*).

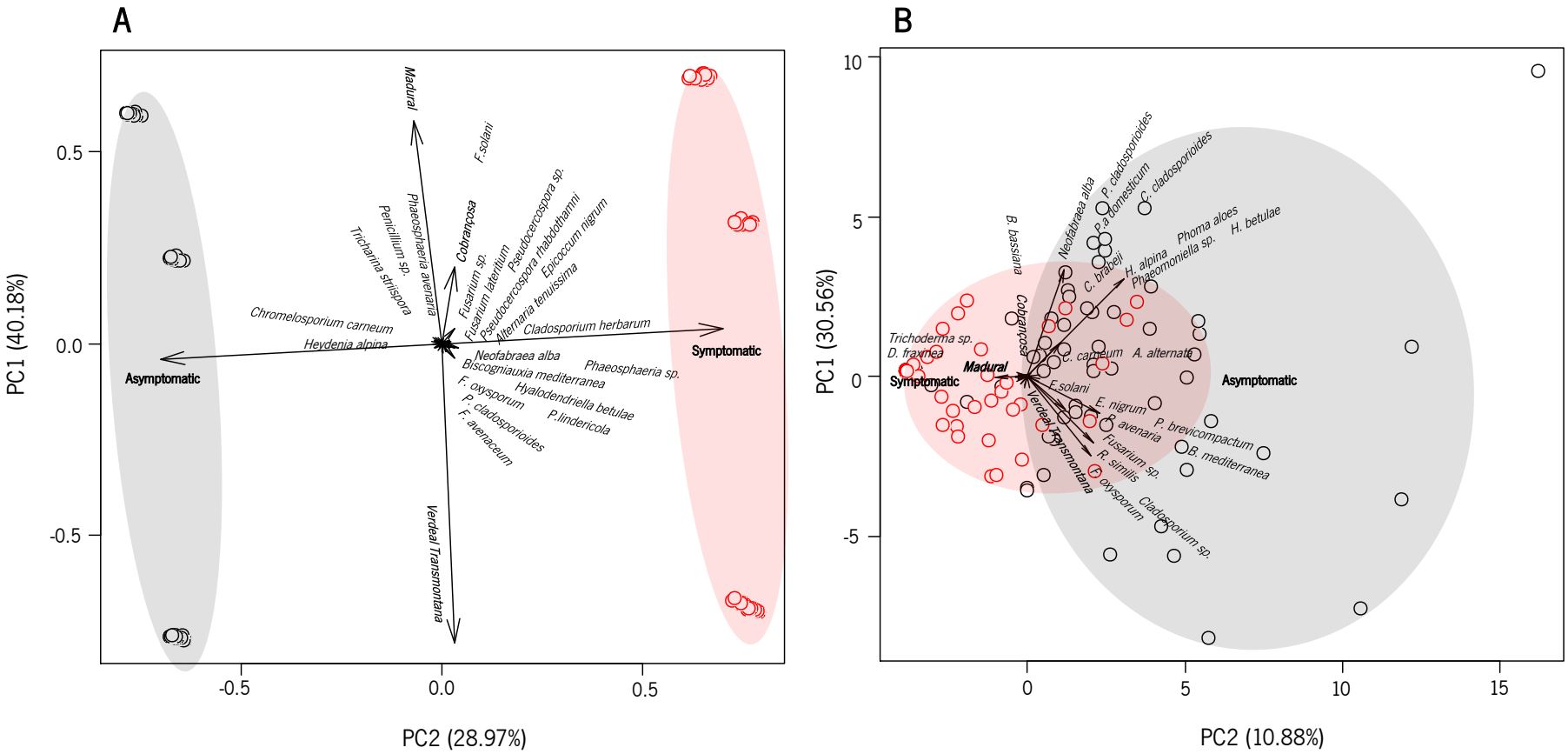

Fig. 3.5 – Principal component analysis (PCA) of endophytic (**A**) and epiphytic (**B**) fungal communities, inhabiting asymptomatic and OLS-symptomatic leaves from different olive cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*). This analysis was performed with preselected fungal OTUs by the random forest analysis.

Table 3.1 – Endophytic and epiphytic fungal indicator OTUs in asymptomatic and OLS-symptomatic leaves from olive tree cultivars *Cobrançosa*, *Madural* and *Verdeal Transmontana*. A - *Specificity* (*i.e.* uniqueness to a particular habitat), B - *Sensitivity* (*i.e.* frequency within that particular habitat).

	Cultivar	Leaf status	Indicator fungal OTUs	A	B	Frequency of occurrence (%)	IndVal	*P*-value
Endophytic	*Cobrançosa*	Asymptomatic	-			-	-	-
		Symptomatic	*Biscogniauxia mediterranea*	0.93	0.50	0.81%	0.68	0.0182
	Madural	Asymptomatic	*Tricharina striispora*	1.00	0.31	1.88%	0.56	0.0474
		Symptomatic	*Phaeosphaeria avenaria*	0.86	0.46	1.34%	0.63	0.0364
	Verdeal	Asymptomatic	-			-	-	-
		Symptomatic	-			-	-	-
Epiphytic	*Cobrançosa*	Asymptomatic	*Alternaria* sp.	0.84	0.58	1.66%	0.70	0.0084
			Chromelosporium carneum	0.86	0.39	0.48%	0.58	0.0180
		Symptomatic	*Ochrocladosporium adansoniae*	1.00	0.16	0.46%	0.40	0.0306
	Madural	Asymptomatic	*Phoma aloes*	1.00	0.47	1.22%	0.69	0.0132
		Symptomatic	*Beauveria bassiana*	0.91	0.36	0.32%	0.58	0.0374
			Phoma sp.	0.86	0.36	0.59%	0.56	0.0428
	Verdeal	Asymptomatic	*Biscogniauxia mediterranea*	0.89	0.60	1.21%	0.73	0.0056
			Alternaria sp.	0.80	0.60	1.62%	0.69	0.0328
		Symptomatic	*Phaeosphaeria avenaria*	0.85	0.43	0.54%	0.54	0.0498
			Phoma sp.	0.92	0.33	0.61%	0.55	0.0340

3.5 Discussion

3.5.1 Does disease affect fungal endophyte and epiphyte communities in a similar way?

Our findings suggest that epiphytic communities are more sensitive to disease, specifically diseases with surface symptoms like OLS-disease, than endophytic communities. This study shows that endophytic and epiphytic fungal communities are differentially impacted by OLS disease in olive tree leaves. Symptomatic leaves exhibited a reduction in richness and abundance of epiphytes, as well as, a shift in community composition, while endophyte communities were relatively unchanged. The decline in leaf surface fungal community might be related to the competitive exclusion of microorganisms promoted by the pathogen V. *oleaginea* on symptomatic leaves (Meyer and Leveau, 2012). In inner leaf tissues, the interactions established between endophytes may be more complex, since living host tissues are also involved (reviewed by Hardoim et al., 2015). The composition of fungal endophytes is likely to be determined by a combination of factors or host plant attributes, rather than plant disease alone, as reported in other studies (Pan et al., 2008). Indeed, the fungal species that compose this community should have the ability to overcome or circumvent host plant defenses and to interact with established microorganisms (reviewed by Braga et al., 2016). Previous studies support our findings by showing a decrease in the diversity and richness of epiphytic bacterial (Manching et al., 2014) and fungal (Zhang et al., 2017) assemblages, respectively in maize and pumpkin leaves, showing symptoms of high levels of pathogen infection, as well as reduced variations in endophytic bacterial diversity in wood tissues of esca-foliar symptomatic and asymptomatic grapevines (Bruez et al., 2015).

Changes in functional groups of phyllosphere fungi have been rarely documented. All functional groups revealed a substantial change on both fungal abundance and richness between asymptomatic and OLS-symptomatic leaves. Such compositional shifts are particularly evident for the epiphytic communities, confirming their higher vulnerability to OLS disease, when compared to endophytic communities. Significant reductions (on average up to 67%) in the abundance of pathogenic, beneficial and commensal epiphytes were observed in symptomatic leaves, indicating that all these functional groups are similarly affected by OLS disease. Functional groups within endophyte communities were comparatively less affected by disease, although certain groups, such as beneficial fungi, were more susceptible to disease. Indeed, a strong decline in the abundance (up to 52%) and an increase (up to 20%) in the richness of beneficial endophytes was noticed in symptomatic leaves. Some of the identified beneficial fungi were members of *Epicoccum*, *Trichoderma* and *Penicillium* genera that have been reported to inhibit the growth of several plant

pathogens (Carrero-Carrón et al., 2016; Musetti et al., 2011; Javadi et al., 2012). Their defensive role on olive tree against OLS disease remains a topic for further research.

3.5.2 Does host genotype and disease susceptibility influence fungal communities?

Fungal composition of asymptomatic leaves from different olive cultivars was significantly different. The observed patterns may be explained by the ability of each cultivar to selectively recruit foliar fungal strains (especially endophytes) from the environment (Bálint et al., 2013). In addition, the leaves of surveyed cultivars showed differences in chemical and physical features (Malheiro et al., 2015), which are known to influence fungal community assembly (reviewed by Hardoim et al., 2015). In contrast with asymptomatic leaves, host cultivar had no effect on both epiphytic and endophytic communities in symptomatic leaves, which also displayed a similar fungal composition among the three cultivars, irrespective to their susceptibility to OLS. Although this variation between asymptomatic and symptomatic leaves provides no information regarding the pathogen cause and effect, the role of V. *oleaginea* as a "niche constructor" in symptomatic leaves seems to be reasonable. This pathogen could shape and change the leaf environment either to the benefit of certain microorganisms or to the detriment of others, leading to alterations on leaf-fungal composition, as previously suggested by McNally and Brown (2015). Since no distinct fungal communities were identified among cultivars after occurrence of disease symptoms, we hypothesized that this new fungal community exhibits some degree of specificity to the environment created by the pathogen. Indeed, the presence of a single pathogen has been already reported to modify the plant host environment to the advantage/disadvantage of other pathogens, affecting their abundance within microbial community (Perefarres et al., 2014). Similarly, the presence of specific microbes, often defined as "keystone" species, was recently suggested to be highly connected to the presence of other microbes within networks (Hassani et al., 2018). Such microbes are likely to exert a high influence on the structure of microbial communities. However, the role played by host-fungal interactions can not be completely neglected, since in symptomatic leaves some specific fungi were still highly associated to a particular cultivar. Besides microbe-microbe interactions, host-fungal interactions may also play an important role in the establishment of leaf-associated fungi (Hassani et al., 2018).

The effect of OLS infection on fungal communities depended on olive tree cultivar. A greater variation in richness, abundance and composition of fungal communities in leaves with and without OLS-symptoms was detected in the tolerant cv. *Cobrançosa*, when compared to both susceptible

cvs. *Madural* and *Verdeal Transmontana*. Specifically, a highly significant decrease on beneficial fungal abundance and richness was observed in symptomatic leaves of tolerant cultivar, suggesting that this fungal group may play a role in conferring host tolerance to OLS disease. Thus, the intrinsic characteristics of native communities that inhabit leaf tissues and their ability to shape the proper environment for pathogen colonization might be a determinant for host susceptibility. The pathogen establishment may then create niche opportunities for other microbes and disturb previously established microbe networks, resulting in a more similar fungal community among cultivars. This assumption still needs to be confirmed with further work.

3.5.3 Is there an indicator fungal community associated with disease and/or host susceptibility?

Based on indicator value analysis, a set of fungal OTUs that best characterized asymptomatic and symptomatic leaves of each cultivar was identified. The indicator OTUs of asymptomatic leaves included several plant pathogens from other crops (B. *mediterranea*, P. *avenaria*, *Alternaria* sp., *Phoma* sp.), and two fungi with unknown function (T. *striispora* and C. *carneum*). Potential pathogenic microorganisms are frequently detected in high abundance on symptomless plants (*e.g.* Pan et al., 2008; Abdelfattah et al., 2015; Zhao et al. 2017). For example, *Cladosporium*, *Alternaria*, *Biscogniauxia* or *Fusarium* have been isolated as endophytes from many host plant species (Raja et al., 2015; Paul et al., 2006, Silva-Hughes et al., 2015). Although some identified indicator OTUs are considered generalist pathogens, such as *Alternaria* spp., many of these ame fungi are also known to produce antimicrobial compounds active against bacteria and yeasts (Gu, 2009; Yagi et al., 1993; Vaz et al., 2009). These microorganisms may then be potential biocontrol resources for preventing OLS disease, but their impact on olive trees, a non-host plant, still remains a topic for further study. Similarly, the indicator OTUs that best characterize symptomatic leaves comprised mostly pathogens from other crops, but also include the fungus *Beauveria bassiana*, which has a recognized role in protecting plants against pests and diseases (Vega et al., 2009). The role of these fungi as "pathogen antagonist" or "pathogen facilitator" should be studied in future work.

The clustering of some fungal OTUs together with asymptomatic or symptomatic leaves of each cultivar suggests their high affinity for specific tissues. These results indicate that some fungal OTUs have only colonized a precise niche, determined by the presence/absence of OLS symptoms and also by cultivar specificity. Thus, our data suggests fungal niche differentiation and adaptation

of fungal communities to asymptomatic/symptomatic leaves of each cultivar, that seem to serve as environmental filters. Whether these patterns reflect facilitation/antagonism effects of foliar fungi on OLS disease is not known yet.

In summary, this study demonstrates that fungal communities could play a role in plant disease susceptibility. When infected by V. *oleaginea,* leaves of olive tree change their associated fungal assemblages, particularly in OLS-tolerant cultivars. Due to the reduction in the importance of host genotype in fungal community assembly in symptomatic leaves, we hypothesize that these changes may be facilitated by V. *oleaginea* itself. We also identified cultivable fungal OTUs specific to either asymptomatic or symptomatic leaves, suggesting their role as "pathogen antagonists" or "pathogen facilitators", respectively. Future research should be carried out in order to identify if these fungal OTUs can be utilised in olive tree defense against OLS disease.

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3.7 Supporting Information

The following Supporting Information is available for this chapter:

Table S3.1 - Occurrence and diversity of fungal endophytes and epiphytes from asymptomatic and OLS-symptomatic leaves. For each parameter, different letters mean significant differences ($P < 0.05$).

	Parameters	Asymptomatic leaves				Symptomatic leaves			
		Cobrançosa	*Madural*	*Verdeal Transmontana*	Total	*Cobrançosa*	*Madural*	*Verdeal Transmontana*	Total
Endophytic	Average no. of isolates/tree	7.0±5.8^{a}	5.1±4.7^{b}	7.1±6.2^{a}	6.9±6.5^{a}	2.9±2.3^{c}	7.2±6.1^{a}	6.0±5.2^{a,b}	6.2±3.4^{a,b}
	Total no. of OTUs	37	33	30	61	20	41	39	60
	Average no. of OTUs/tree	4.1±2.8^{a,b}	4.0±3.1^{a}	4.8±2.7^{a}	4.6±2.9^{a}	1.9±1.7^{c}	4.9±4.8^{a}	4.3±3.3^{a,b}	3.7±2.3^{a,b}
	Freq. of colonization (%)	11.8%	8.6%	11.9%	21.9%	4.8%	12.0%	10.0%	17.6%
Epiphytic	Average no. of isolates/tree Log10 (CFU/cm^{2})	34.4±9.9^{a}	25.8±16^{b}	28.9±13.5^{a,b}	29.8±13.2^{a,b}	10.7±9.2^{c}	9.2±8.1^{c}	9.9±8.3^{c}	9.6±8.4^{c}
	Total no. of OTUs	74	72	75	127	45	36	37	66
	Average no. of OTUs/tree	13.8±3.2^{a}	10.2±6.6^{b}	11.5±4.6^{a,b}	11.8±4.8^{a,b}	4.0±3.9^{c}	3.7±3.5^{c}	4.1±3.8^{c}	7.3±4.3^{b,c}
	Freq. of occurrence (%)	17.7%	13.4%	24.9%	18.9%	16.8%	14.7%	15.2%	15.1%

Occurrence of fungal endophytes and epiphytes was measured by determining the frequency of colonization (FC, %) and relative abundance (RA, %), respectively. The frequency of colonization was calculated as the total number of leaf tissue segments colonized by each endophyte divided by the total number of leaf segments surveyed. The relative abundance was determined as the total number of isolates of an OTU/genus divided by the total number of isolates.

Table S3.2 - ANOVA analysis to test significant differences (*P*-value) between fungal community groups obtained in the Canonical Correlation Analysis (CCA). Variation partitioning (*Varpart*) was calculated to achieve the total variance explained by each factor. *P*-values in bold are significant.

Factors			*P*-value	Varpart (%)
Total	Cultivar		0.110	0.6%
	Leaf status		**0.005**	2.1%
	Fungal community		**0.005**	8.1%
Endophytic	Cultivar		0.180	2.0%
	Leaf status		**0.005**	4.2%
Epiphytic	Cultivar		0.065	2.1%
	Leaf status		**0.005**	4.4%
Total	Cultivar	Asymptomatic	**0.003**	13.0%
		Symptomatic	0.280	2.0%
Endophytic	Cultivar	Asymptomatic	**0.005**	-
		Symptomatic	0.264	-
Epiphytic	Cultivar	Asymptomatic	**0.001**	-
		Symptomatic	0.453	-

Table S3.3 - Analysis of similarity (ANOSIM), based on Bray-Curtis distance, between the fungal communities inhabiting asymptomatic and OLS-symptomatic leaves. Included are the variables, and corresponding R-statistics (R) and *P*values.

Variables		ANOSIM	
		R	*P*-value
All cultivars	*Cobrançosa*	0.312	**0.001**
	Madural	0.149	**0.020**
	Verdeal Transmontana	0.236	**0.005**
Endophytic	*Cobrançosa*	0.257	**0.003**
	Madural	0.058	0.094
	Verdeal Transmontana	0.135	**0.020**
	All cultivars	0.151	**0.020**
Epiphytic	*Cobrançosa*	0.367	**0.001**
	Madural	0.239	**0.005**
	Verdeal Transmontana	0.336	**0.001**
	All cultivars	0.367	**0.001**

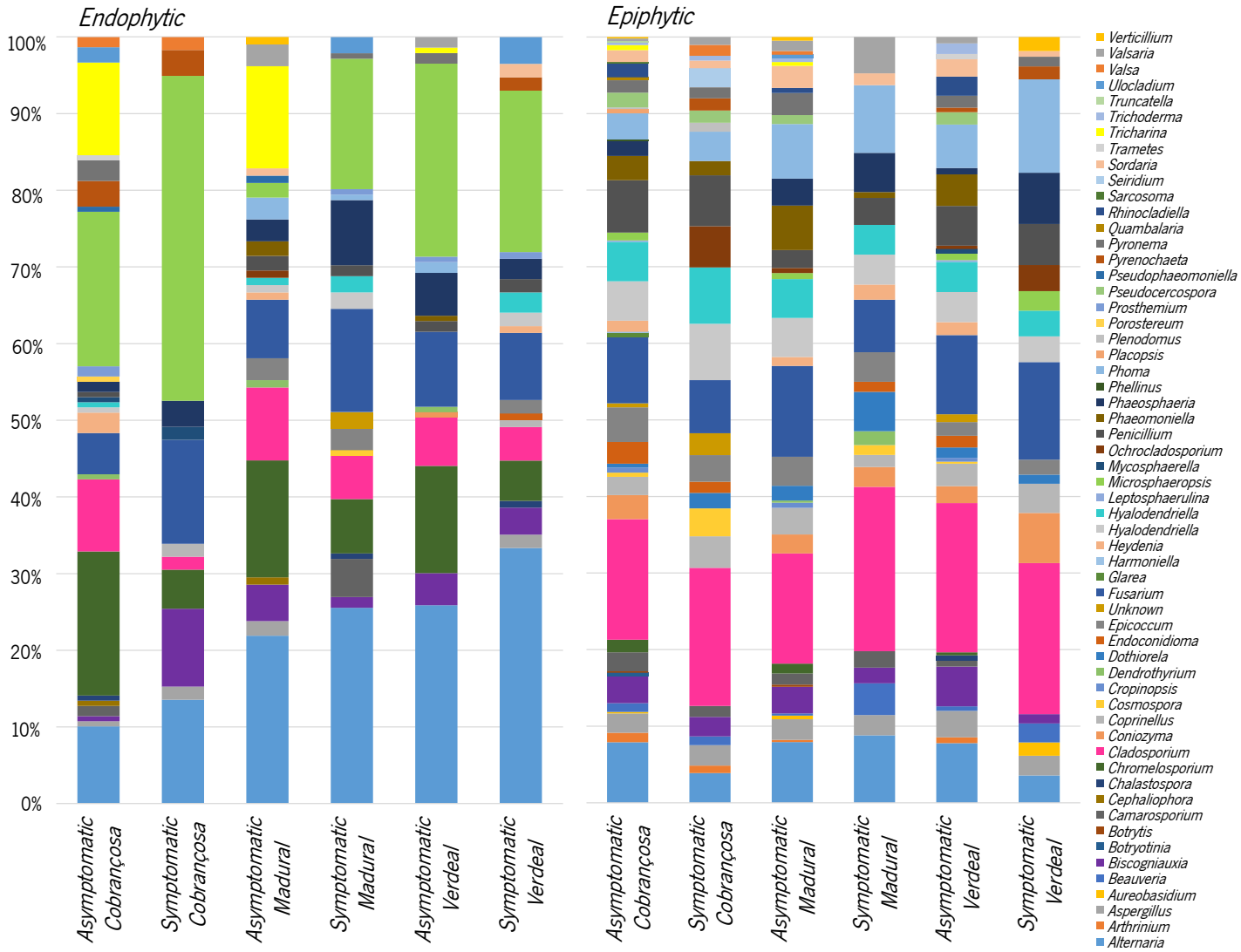

Fig. S3.1 - Frequency (%) of fungal endophytic and epiphytic genera present in asymptomatic and OLS-symptomatic leaves from olive cultivars *Cobrançosa, Madural* and *Verdeal Transmontana*.

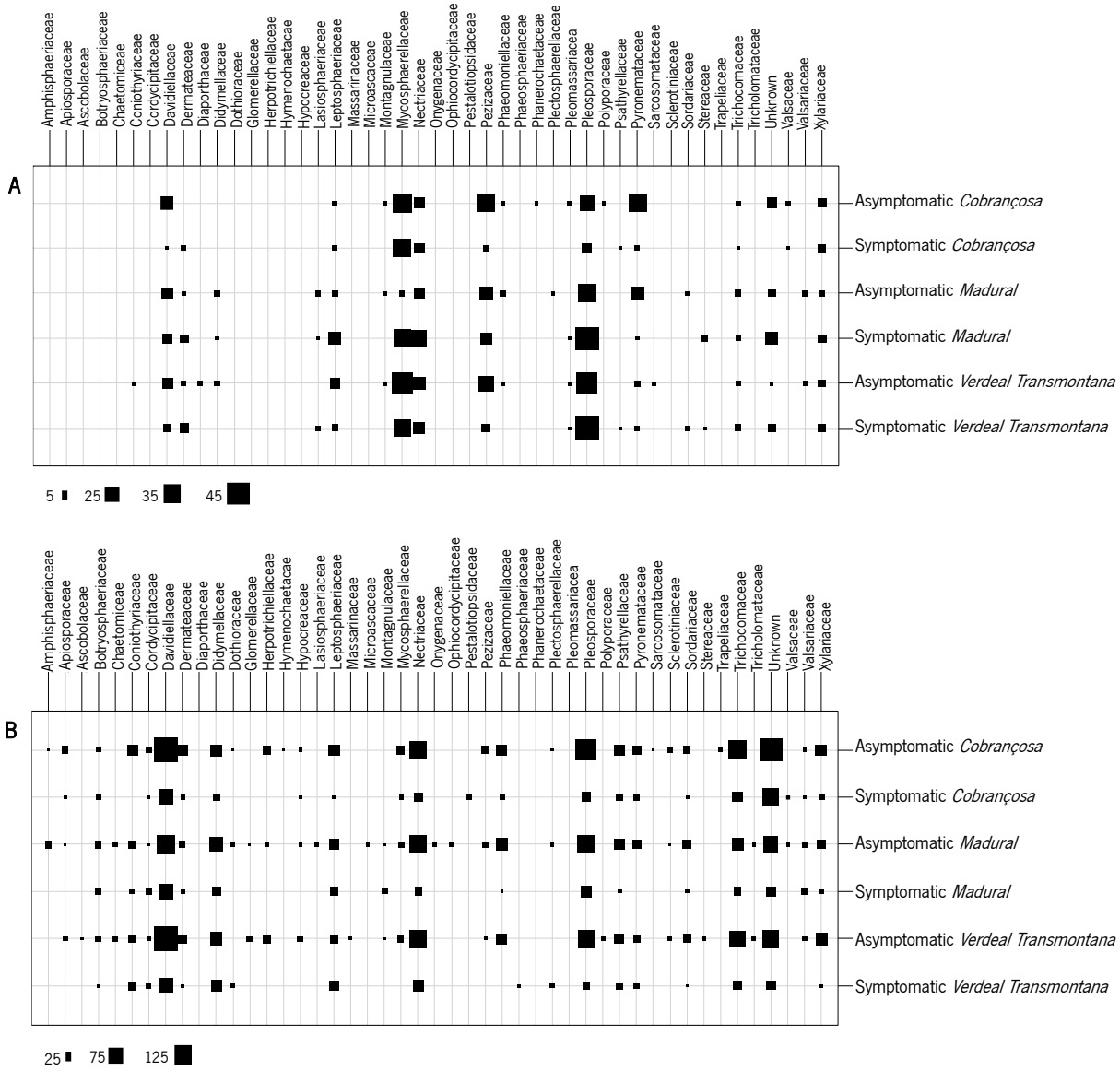

Fig. S3.2 - Distribution of families in endophytic (**A**) and epiphytic (**B**) fungal communities present in asymptomatic and OLS-symptomatic leaves of olive cultivars *Cobrançosa*, *Madural* and *Verdeal Transmontana*. Square sizes represent the number of fungal isolates (abundance) present in each leaf sample.

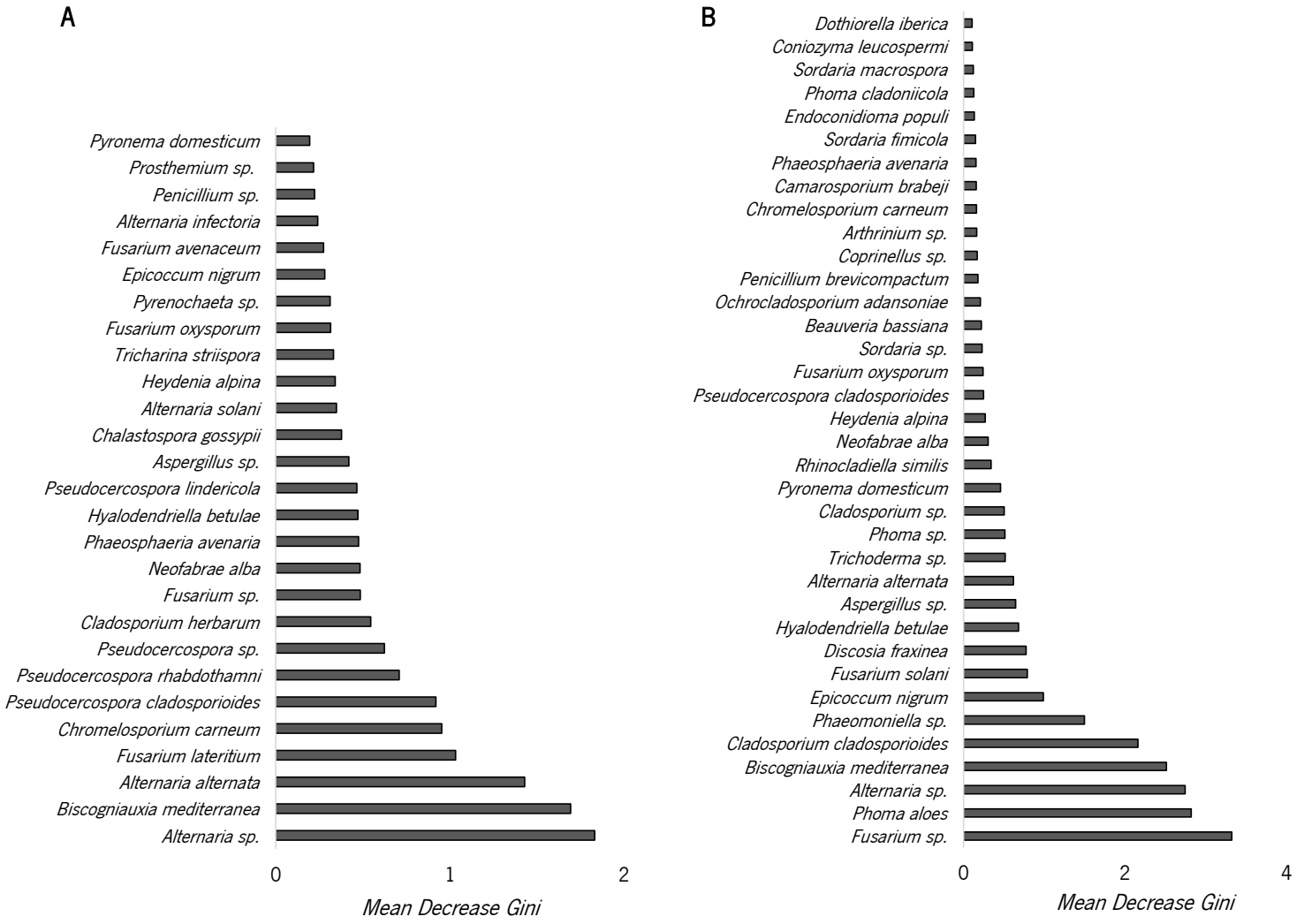

Fig. S3.3 – Ranking of fungal OTUs importance according to the presence/absence of OLS-symptoms within endophytic (**A**) and epiphytic (**B**) communities. *Mean Decrease Gini* value is a measure of fungal OTU importance for the presence/absence of leaf symptoms. The highest values represent the best predictors.

Chapter 4

Does bacterial disease shape local fungal communities in twigs?

Does bacterial disease shape local fungal communities in twigs?

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4.1 Abstract

In nature, pathogens live and interact with other microorganisms on plant tissues. Yet, the research area exploring interactions between bacteria-fungi and microbiota-plants, within the context of pathobiome, is still scarce. In this study, the impact of olive knot (OK) disease caused by the bacterial pathogen *Pseudomonas savastanoi* pv. *savastanoi* (Psv) on the epiphytic and endophytic fungal communities of olive tree twigs from three different cultivars, was investigated in field conditions. The ITS-DNA sequencing of cultivable fungi, showed that OK disease perturbs the resident fungal communities, which may reflect changes made by the Psv to the habitat. In particular, a reduction on epiphytic fungal abundance and diversity, as well as changes on epiphytes composition, were observed. Compared to epiphytes, endophytes were less sensitive to OK disease, but their abundance was increased by disease, in particular of potential pathogens. Host genotype, at cultivar level, contributed to plant fungal assembly particularly upon OK disease establishment. Therefore, besides fungi - Psv interactions, the combination of cultivar - Psv also appeared to be critical for the composition of fungal communities in olive knots. Specific fungal OTUs were associated to the presence and absence of OK disease, and their role in the promotion or suppression of this disease should be studied in the future. Our results can improve the knowledge of the complex interactions between host-fungal-Psv bacteria interactions, which could be used for controlling OK disease.

4.2 Introduction

The above-ground parts of plants (phyllosphere) are naturally inhabited by a great diversity of microbes (Vorholt, 2012). Plant health is strongly dependent on the presence of plant pathogens in such microbial community, but some microbes could also provide direct or indirect pathogen protection (reviewed by Hassani et al., 2018). Indeed, many plant pathogens do not act alone (Rovenich et al., 2014). Their disease potential is mediated by different pathogen interactions occurring within the pathobiome, including microbial cooperation or antagonism relations (Kemen, 2014; Vayssier-Taussat et al., 2014). The importance of phyllosphere microbiota have been mostly recognized from the interaction studies involving microorganisms from the same kingdom, either bacteria or fungi (reviewed by Hassani et al., 2018). The importance of bacterial-fungal interactions for host health has been widely demonstrated for humans (Morales and Hogan, 2010; Arvanitis and Mylonakis, 2015), but their relevance for plant health is still scarce. Few examples indicate that bacterial-fungal interactions can result in enhanced or decreased pathogenicity of one partner

towards their host (reviewed by Frey-Klett et al., 2011). Thus, both microbial kingdoms are expected to work in association, through competition or cooperation, for a more effective interaction with plant host (Hacquard, 2017). Previous studies have only considered a single plant genotype (reviewed by Frey-Klett et al., 2011), even though plant genotype has been increasingly recognized to be a key determinant of phyllosphere microbiota composition (Bodenhausen et al., 2014). This raises the question whether host plants can affect the outcome of microbial interactions occurring between the incoming pathogen and the resident microbiota. Also, previous studies were mainly performed using artificial and simplified bioassays, which only partially reflect the complex phyllosphere conditions, and were exclusively focused on interactions among endophytic or epiphytic communities (reviewed by Frey-Klett et al., 2011).

In the present study, we used the olive knot disease caused by the bacterial pathogen *Pseudomonas savastanoi* pv. *savastanoi* (Psv) as a model for decipher interactions between pathogenic bacteria and epi- or endophytic fungal communities, taking place in field host plants (olive trees). Olive knot (OK) is one of the most important worldwide olive tree (*Olea europaea* L.) diseases, causing serious losses in terms of production and olive oil quality (Quesada et al., 2010; Ramos et al., 2012). This disease is characterized by the formation of overgrowth tissues (knots) in the aerial part of olive trees, mainly on twigs and branches (Ramos et al., 2012). These knots caused by Psv were chosen as a model for studying bacterial-fungal interactions because they have already showed to provide an especial niche to study microbial multispecies interactions (Buonaurio et al., 2015). Indeed, recent studies indicated that some non-pathogenic bacteria, namely *Erwinia toletana*, *Pantoea agglomerans* and *Erwinia oleae*, are frequently associated with Psv in olive knots and effectively cooperate with the pathogen for increasing disease severity (Hosni et al., 2011; Passos da Silva et al., 2014). Compared with bacterial communities, the fungal community composition in olive knots remains unknown, as well as the way the bacterial pathogen interacts and impacts this fungal community. Using this model system, the simultaneous study of interactions occurring within members of epiphytic or endophytic microbial communities would be possible, due to the recognized ability of Psv to live as an epiphyte or endophyte on olive phyllosphere (Quesada et al., 2010). The availability of olive cultivars with different susceptibility levels to olive knot (Godena et al., 2009), which could simultaneously present asymptomatic twigs and knots in the same olive tree, also makes this pathosystem a good model for studying microbial interactions.

With this work, we specifically want to answer the following questions: i) What is the effect of olive knot disease, tree genotype (at cultivar level), and their interaction on fungal communities of twigs? ii) Are these effects identical on epi- and endophytic fungal communities? iii) Is there any fungal consortia associated with olive knot disease and/or host susceptibility? To accomplish this, the composition of both epiphytic and endophytic fungal communities, associated to symptomless twigs and knots from three distinct olive cultivars, were investigated under field conditions. Fungal communities were assessed by DNA sequence analysis of isolates. This work is the first step for ascertaining the role of such fungi on OK disease establishment/development in olive tree.

4.3 Material and methods

4.3.1 Plant sampling

Plant collection was performed during spring 2014, in three olive orchards located in Mirandela, Northeast of Portugal, at coordinates N 41° 32.593'; W 07° 07.445' (orchard 1), N 41° 32.756'; W 07° 07.590' (orchard 2) and N 41° 29.490'; W 07° 15.413' (orchard 3). Each orchard comprised olive trees from three cultivars, planted at 7 × 7 m spacing, with different levels of susceptibility to olive knot disease: cv. *Cobrançosa* is moderately tolerant, cv. *Madural* is moderately susceptible and cv. *Verdeal Transmontana* is the most susceptible. Their susceptibility was confirmed by estimating OK disease incidence simultaneously to sample collection. The levels of disease incidence (%), determined by the percentage of infected twigs, were indeed lower in cv. *Cobrançosa* (7.3±3.2) when compared to cvs. *Madural* (13.4±6.7) and *Verdeal Transmontana* (17.2±9.8). Twigs were sampled from randomly selected 7 olive trees of each cultivar, from which asymptomatic and OK-symptomatic (with knots) twigs were collected from two cardinal orientations (north and south), at 1.5–2 m above ground height. The collected symptomatic twigs presented a similar level of severity, by showing 3-4 knots.

4.3.2 Fungal isolation

From each tree, stem and knots segments (N=5 each, with around 1 gram of weight/each) were randomly selected from asymptomatic and symptomatic twigs, respectively. These plant tissues were used for isolation of fungal epiphytes and endophytes. Fungal epiphytes were isolated by the plate dilution method, onto Potato Dextrose Agar (PDA, Difco) and Plate Count Agar (PCA, Himedia) media, supplemented with 0.01% (w/v) chloramphenicol (Oxoid), following the procedure described by Gomes et al. (2018). The number of epiphytes were expressed as log CFU/cm^2, *i.e.*

the number of individual colonies of fungi adhered to stem/knot surface. To estimate plant tissues surface, a cylinder equation [$A=2\pi r^2 + h\ (2\pi r)$] was used, in which A is the area, *r* and *h* are the radius and height of the stem/knot segments, respectively. The average stem/knot segments area of cvs. *Cobrançosa*, *Madural* and *Verdeal Transmontana* was 11.2±0.9, 10.8±1.7 and 11.9±1.8 cm^2, respectively.

Endophytic fungi were isolated from the same stem/knot segments used to isolated epiphytes. After surface disinfection (Martins et al., 2016), each stem/knot was cut in segments (*ca.* 4-5 mm), which were transferred to the same culture media used for epiphytes isolation. Validation of surface sterilization procedure was done by imprinting the surface of sterilized plant tissues onto PDA and PCA media. Endophytic fungal community was isolated from a total of 7,440 plant tissue segments. Fungal colonies were subcultured on fresh medium until pure epi/endophytic cultures were obtained.

4.3.3 Fungal identification

Firstly, groups of fungal isolates were formed according to their morphological features (both colony and microscopic morphology). Then, three representative isolates of each morphotype were selected for molecular identification, by sequencing the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA). Total genomic DNA was extracted from harvested mycelium or spores using the *REDExtract-N-Amp™ Plant PCR kit* (Sigma, Poole, UK). The ITS region (ITS1, 5.8S, ITS2) was amplified using ITS1/ITS4 or ITS5/ITS4 primers sets (White et al., 1990) in a PCR protocol previously described by Oliveira et al. (2012). The amplified products (≈650 bp) were purified and sequenced using Macrogen Inc. (Seoul, South Korea) services. The obtained DNA sequences were analyzed with *DNASTAR v.2.58* software, and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST algorithm (Gomes et al., 2018). The obtained sequences are available at GenBank with the following accession numbers: KU324941-KU325040; KU325041-KU325240; KU325241-KU325457. Each operational taxonomic unit (OTU) was taxonomically classified according to the *Index Fungorum Database* (www.indexfungorum.org). Pure cultures of each identified isolate were preserved and deposited in the culture collection of the Polytechnic Institute of Bragança (School of Agriculture).

4.3.4 Effect of OLS disease and host genotype on fungal diversity

The effect of OK disease or host genotype in twig fungal diversity was achieved at both community and functional group levels. The *community level* was evaluated through the fungal diversity evaluation, namely by determination of abundance (number of isolates) and richness (number of different OTUs), and by computing Shannon–Wiener (H') index in *Species Diversity and Richness* v. 4.0 (Seaby and Henderson, 2006). Results are presented as the mean of replicates (N=21, for each cultivar). The *functional group* level was evaluated after previous classification of identified fungi into functional categories (commensal, beneficial and pathogenic), according to the previous description of fungal endophytes by Hardoim et al. (2015). The commensal group is comprised by fungi that do not have an apparent effect on host plants. The beneficial fungi can protect host plants against pathogens and pests, as well as presenting a plant growth promotion effect. The pathogens group includes latent plant pathogens. Fungal OTUs belonging to *uncertain sedis* or other functional groups, were categorized as "unknown fungi" or "other", respectively. After grouping, the relative abundance and richness of each functional group across asymptomatic and OK-symptomatic twigs was determined. To determine differences on fungal diversity/functional group among twigs, a one-way analysis of variance (ANOVA) with *SPSS* v.20 was performed, with multiple comparisons according to Tukey (P<0.05).

4.3.5 Data analysis

A combination of univariate and multivariate methods were used to identify potential fungal OTUs that can differentiate olive tree cultivars (cvs. *Cobrançosa*, *Madural* and *Verdeal-Transmontana*) and twig status (asymptomatic and symptomatic). All statistical analyses were performed in *R* (R Core Team, 2014).

Effect of OK disease and host genotype on fungal community structure. A canonical correlation analysis (CCA) was used to find possible correlations among surveyed cultivars or twig status with the identified fungal communities (endophytes, epiphytes and total). All data were $\log_2$ (x+1) transformed for standardization. The CCA was performed using "*CCorA*" function in the *vegan* package (Oksanen et al., 2017). One-way analysis of variance (ANOVA) was performed with "*anova*" function to determine statistical significant differences among cultivars and twig status, on endophytic, epiphytic and total fungal communities. Variation partitioning was used to calculate communities dissimilarity (%), according to different explanatory variables (olive cultivar and presence/absence of OK-symptoms). This analysis was also performed with the *vegan* package

using "*varpart*" function. A one-way analysis of similarity (ANOSIM) was used to test statistic differences between fungal groups separated by the CCA, using the Bray–Curtis distance matrices. This analysis was performed using the "anosim" function in the *vegan* package (Oksanen et al., 2017).

Identification of fungal OTUs associated to OK disease and/or host genotype. Random forests analysis was performed to identify the ranking importance of fungal OTUs for distinguishing asymptomatic from OK-symptomatic twigs (Breiman, 2011; Cutler et al., 2007). This analysis was set through artificial intelligence algorithms (Cutler et al., 2007). For each tree grown on a bootstrap sample, the error rate for observations left out of the bootstrap sample was monitored. The *Gini coefficient* indicates the variable contribution for distinguishing between asymptomatic and OK-symptomatic twigs. The ranking species importance was explained by 81.4% and 84.2% for endophytes and epiphytes, respectively. Then, a principal component analysis (PCA) and indicator fungal species analysis were performed using the pre-selected species. Both analyses were used to explore the potential associations between fungal OTUs and cultivar/twig status. The PCA was performed by using the *psych* package (Revelle, 2017). The indicator fungal species analysis was conducted using the function "*multipatt*" from *indicspecies* package (Cáceres, 2013). The Indicator Value Index (*IndVal*) was used as the statistical index (*IndVal* > 0.5 represents the most constant and specific species) and is defined as the product of two components: "A", that is the *specificity* of the species as indicator of the site group and "B"; that is the *sensitivity* of the species as indicator of the site group (Cáceres, 2013).

4.4 Results

In this study, 179 fungal OTUs belonging to two phyla, 47 families, and 89 genera were identified as inhabitants of olive tree twigs. Ascomycota was the most abundant phylum, accounting for 97.2% of the isolates (Fig. S4.1). The remaining isolates belonged to Basidiomycota. *Fusarium* (Nectriaceae), *Alternaria* (Pleosporaceae), and *Cladosporium* (Cladosporiaceae) were the most abundant genera, accounting together with 23% of total isolates (Fig. S4.2). Distinct species were found as the most abundant in both endo- and epiphytic communities. Indeed, *Alternaria* was the most frequently isolated in the endophytic community, whereas *Cladosporium* was dominant in the epiphytic community (Fig. S4.2). Epiphytes were also found in significant (P <0.002) greater abundance and diversity than endophytes (data not shown).

4.4.1 Effect of OK disease on fungal diversity

The diversity of epiphytic and endophytic fungal communities varied significantly between asymptomatic (stems) and OK-symptomatic (knots) twigs, but differences were greater for epiphytes (Fig. 4.1A). Epiphytic fungi showed a greater decline in abundance (up to 1.7-fold), richness (up to 1.9-fold) and diversity (up to 1.3-fold) in symptomatic twigs (in relation to asymptomatic twigs) than endophytic fungi. In particular, a reduction in abundance of epiphytes belonging to Pleosporaceae, Chaetomiaceae, and Aspergillaceae was evident in symptomatic twigs, and in a lesser extent to Pyronemataceae, Phaeomoniellaceae, Hypocreaceae and Valsariaceae families (Fig. S4.3). In addition, thirteen epiphytic families disappeared in symptomatic twigs and no exclusive families were found in knots. Endophytes revealed a greater fungal abundance (up to 1.2-fold) in asymptomatic than OK-symptomatic twigs, but showed a smaller reduction in richness and diversity (up to 1.1-fold) (Fig. 4.1A). An increase on abundance was mainly observed for the Nectriaceae family (Fig. S4.3), in particular for the *Fusarium* genus (Fig. S4.2). Additionally, seven endophytic families, which appeared in asymptomatic twigs, disappeared in symptomatic twigs, contrasting with the five exclusive families from symptomatic twigs (Dothideaceae, Herpotrichiellaceae, Leptosphaeriaceae, Pleomassariaceae and Stereaceae).

Fungal community responses to OK disease were also assessed in terms of fungal guilds. The abundance of most epiphytic trophic groups decreased significantly on symptomatic twigs, while the endophytic ones increased (Fig. 4.1B). Changes in the richness of fungal trophic groups promoted by OK disease were greater for the epiphytic community, where a significant decrease in the pathogenic group was observed. Within endophytes, a significant decrease in the richness of beneficial fungi was observed in symptomatic twigs.

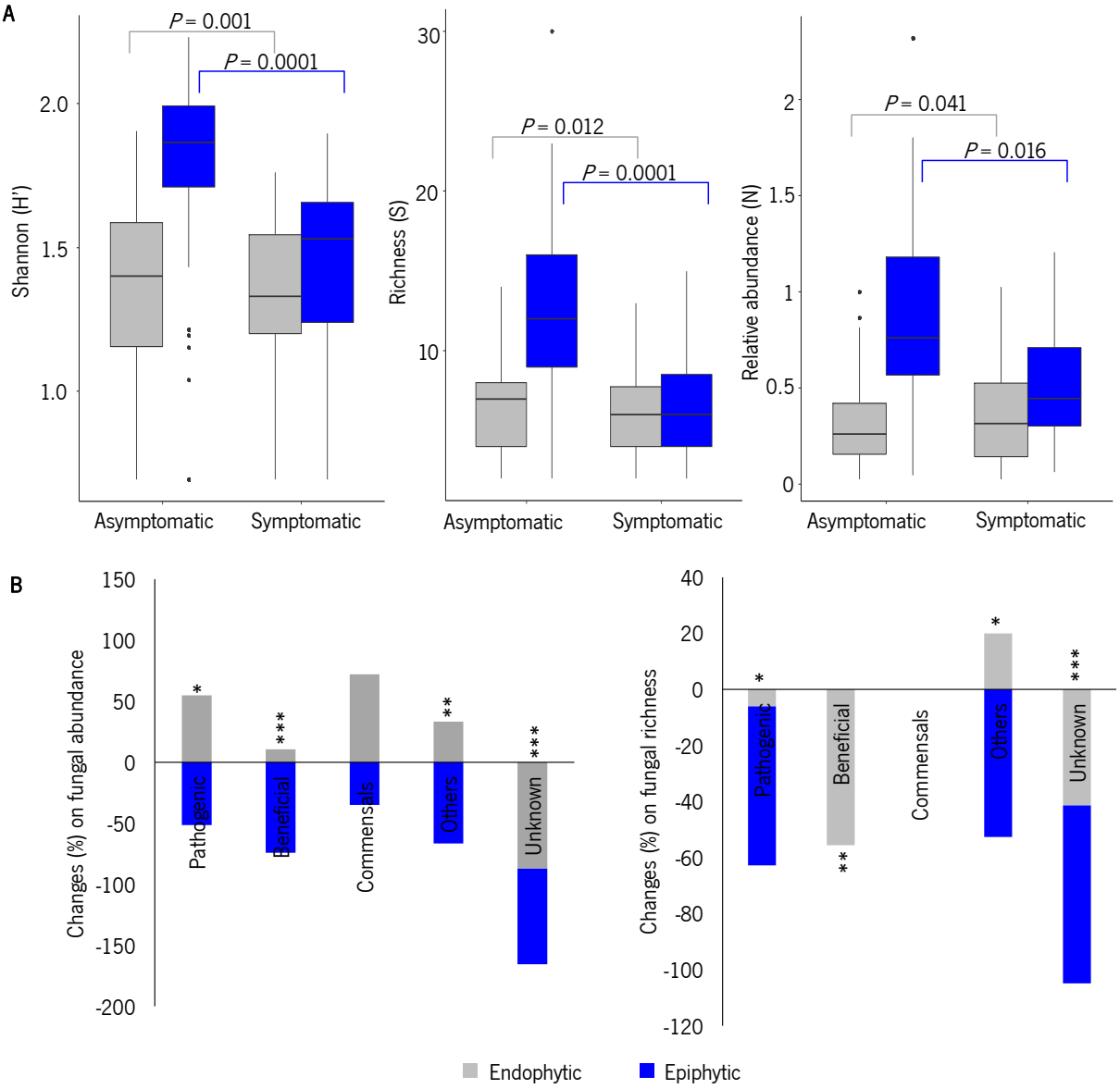

Fig. 4.1 – Comparison of fungal diversity between asymptomatic and OK-symptomatic twigs, either within endophytic or epiphytic communities. (**A**) Diversity at community level evaluated by determining abundance, richness and by using Shannon–Wiener index. Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (black dots). Significant differences between pairs of values are represented over horizontal lines. (**B**) Changes (%) on fungal abundance and richness for each functional group, occurring on OK-symptomatic twigs in relation to asymptomatic twigs. Asterisks indicate significant differences between pairs of values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

4.4.2 Effect of host genotype on fungal diversity

Fungal communities inhabiting asymptomatic twigs presented higher abundance and fungal diversity ($P < 0.05$) in the most OK tolerant cultivar (cv. *Cobrançosa*) than cvs. *Madural* and *Verdeal*

Transmontana (Fig. 4.2A). Fungal communities of each cultivar were differentially affected by OK disease. Knots of cv. *Cobrançosa* presented a significantly greater loss of both fungal abundance and richness (up to 1.6-fold, in relation to asymptomatic twigs) than cvs. *Madural* (up to 1.4-fold) or *Verdeal Transmontana* (up to 1.1-fold). Most of the lost isolates in knots belong to Pezizaceae (for cv. *Cobrançosa*), Chaetomiaceae (for cv. *Madural*) and Gnomoniaceae (for cv. *Verdeal Transmontana*) families (Fig. S4.3). An increase in abundance of Nectriaceae and Pestalotiopsidaceae families was observed in the symptomatic samples of all cultivars, as well as an increase of Mycosphaerellaceae family for the most OK-susceptible cultivars (cv. *Verdeal Transmontana*).

When fungal OTUs were divided into functional categories, host genotype differences were also detected (Fig. 4.2B). For all cultivars, a significant decline on abundance and richness of all functional groups was observed in symptomatic twigs in relation to asymptomatic ones, with exception of commensals. Abundance of beneficial fungi and richness of pathogens underwent greater declines on cv. *Cobrançosa*, while pathogenic and commensal fungal abundance, as well as richness of beneficial fungi, were greatly reduced in cv. *Madural*.

4.4.3 Effect of disease, host genotype and their interaction on the composition of fungal communities

As revealed by the fungal clustering in CCA analysis, the fungal composition in twigs appeared to be primarily drive by OK disease and, to a lesser extent, by host genotype (Fig. 4.3). Results from ANOVA (Table S4.1) and ANOSIM (Table S4.2) confirm that the presence/absence of OK symptoms had the greatest influence on fungal species composition (P =0.005). Disease accounts for 3.9%, 5.6% and 5.9% of total fungal species, endophytic and epiphytic variation, respectively; whereas host genotype only explained 0.5%, 1.1% and 1.3% of fungal communities variance, respectively (Table S4.1). The effect of host genotype on species composition was greater in symptomatic (P=0.005) than in asymptomatic (P =0.049) twigs, explaining 2.8% and 1.5% of species composition variance, respectively (Table S4.1). Differences on fungal species composition between asymptomatic and symptomatic twigs (Table S4.2) were especially noticed in fungal communities of cv. *Cobrançosa*, in particular for epiphytic community (R = 0.498, P = 0.001). There was also a significant relation between OK disease and host genotype for the overall composition of the fungal community (P =0.010) or for the composition of endophytic fungal

community (P =0.005). In contrast, epiphytes composition was not impacted by the interaction between OK disease and host genotype (data not shown).

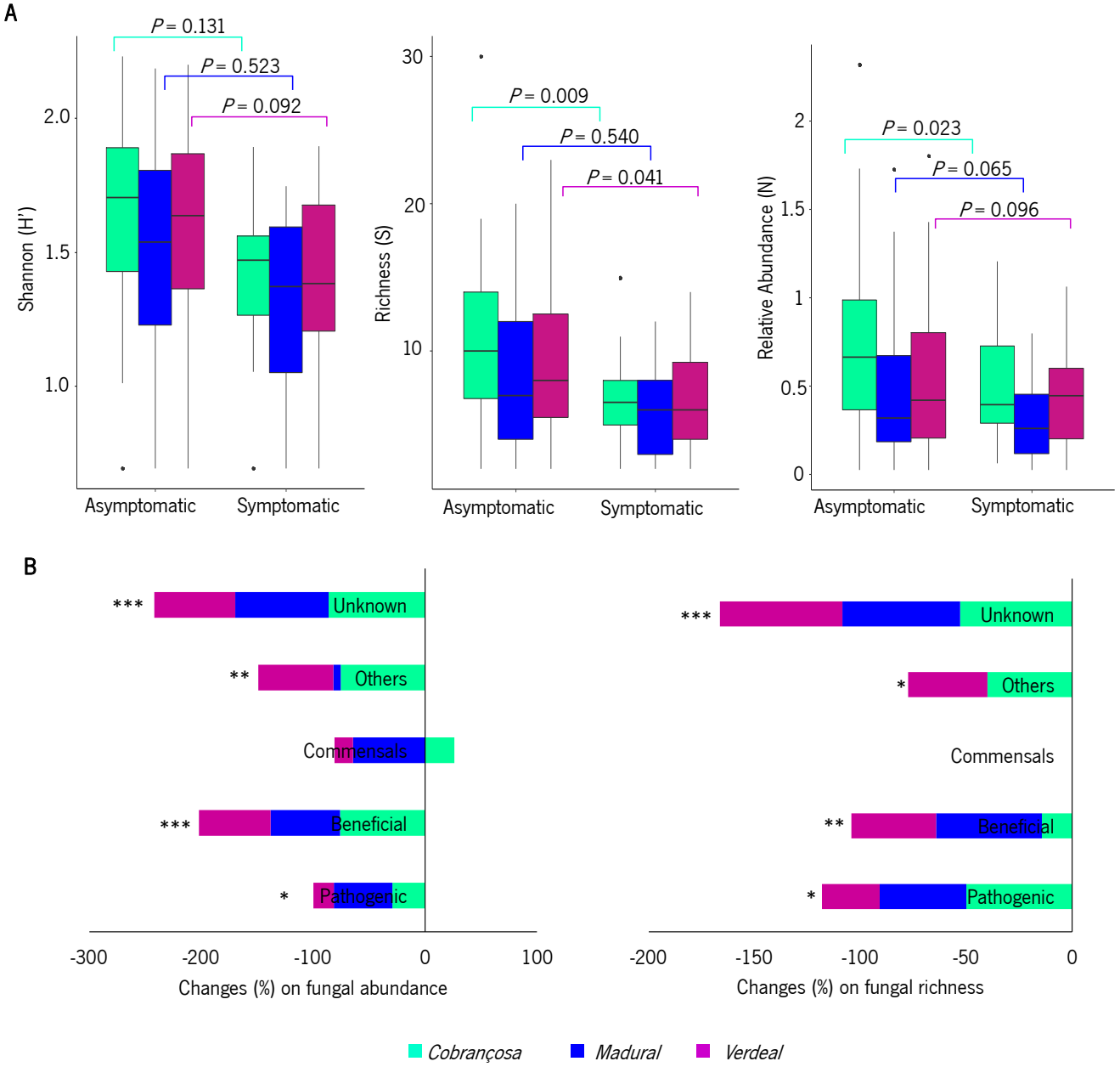

Fig. 4.2 - Comparison of fungal diversity between asymptomatic and OK-symptomatic twigs within each olive tree cultivar (*Cobrançosa*, *Madural* and *Verdeal Transmontana*). (**A**) Diversity at community level by determining abundance, richness and by using Shannon–Wiener index. Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (black dots). Statistically differences between pairs of values are showed over horizontal lines. (**B**) Changes (%) on fungal abundance and richness between asymptomatic and OK-symptomatic twigs for each functional group. Asterisks indicate statistically significant differences between pairs of values (* P< 0.05, ** P< 0.01, *** P< 0.001).

4.4.4 Associations between fungal OTUs and disease symptoms or host genotype

Previous analyses indicate that OK disease plays an important role on fungal community assemblage, suggesting the existence of a fungal consortium associated to either asymptomatic or OK-symptomatic twigs. To test this hypothesis, a random forest analysis was performed to provide a ranking of the relative importance of each endophyte and epiphyte OTU for distinguishing asymptomatic from OK-symptomatic twigs (Fig. S4.4). The most distinguishing fungal OTUs were selected and then used to perform a PCA, in order to identify which could be potentially related to OK disease and cultivar (Fig. 4.4). Among the selected OTUs, some were specifically associated with a particular set of twigs, either asymptomatic or symptomatic. *Heydenia* sp., *Phaeomoniella* sp., *Plectania rhytidia*, *Pyronema domesticum*, and *Chromelosporium carneum*, in the endophytic community, as well as *Alternaria alternata*, *Pyronema domesticum*, *Hyalodendriella betulae*, *Epicoccum nigrum*, *Cladosporium cladosporioides*, *Coprinellus radians*, *Neofabraea alba*, *Trichoderma* sp. in the epiphytic community, often occur together and are highly associated with asymptomatic twigs. On the other hand, *Fusarium* sp., *Fusarium lateritium*, *Biscogniauxia mediterranea*, *Fusarium oxysporum*, *Neofabraea alba*, *Alternaria* sp., and *Alternaria tenuissima*, in the endophytic community, as well as *Penicillium spinolosum*, *Penicillium canescens* and *Comospora* sp., in the epiphytic community, were simultaneously found on symptomatic twigs (knots). However, a clear association of these fungal OTUs and olive cultivars was not found. In order to discover significant associations between fungal OTUs of asymptomatic/symptomatic twigs and olive cultivars, a species indicator analysis was carried out using preselected OTUs by the random forest analysis. The best indicator OTUs (*IndVal* > 0.70) of asymptomatic and symptomatic twigs were found in cv. *Cobrançosa* (Table 4.1). Curiously, H. *betulae* was identified as an epiphyte indicator of asymptomatic twigs for the three cultivars. The endophyte *Fusarium* was found to be a good indicator of symptomatic twigs for the three cultivars.

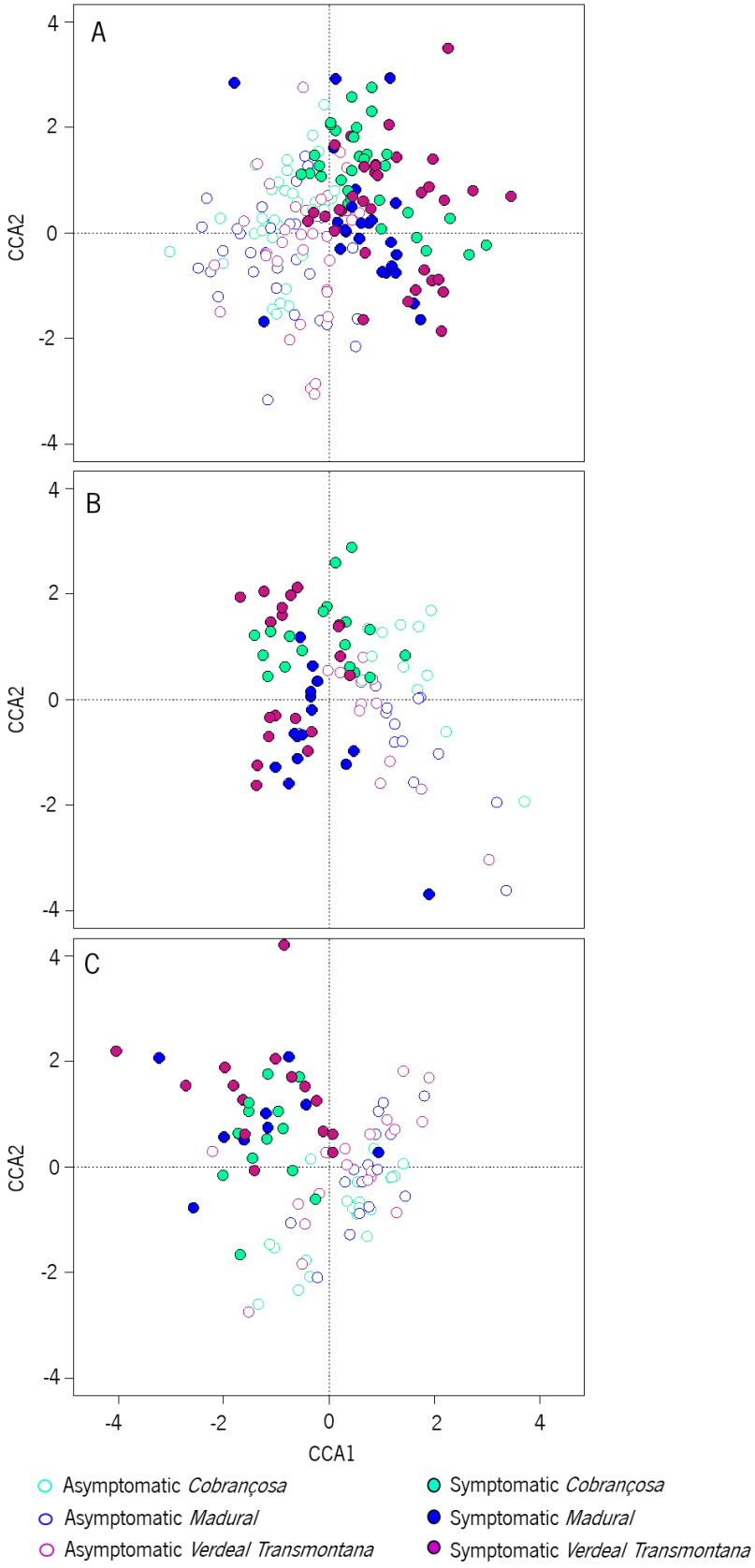

Fig. 4.3 – Canonical correspondence analysis (CCA) ordination showing the dispersion of individual OTUs within total (**A**), endophytic (**B**) and epiphytic (**C**) fungal communities relative to the presence/absence of OK-twig symptoms and olive tree cultivar (*Cobrançosa*, *Madural* and *Verdeal Transmontana*).

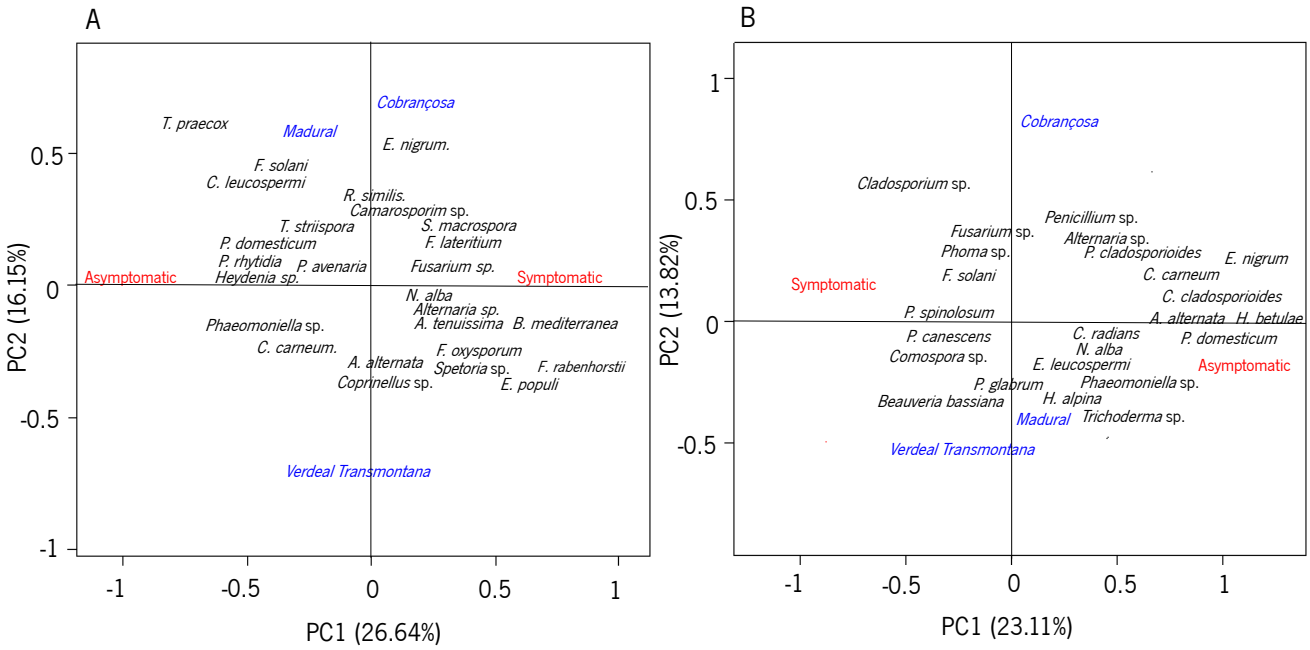

Fig. 4.4 – Principal component analysis (PCA) of endophytic (**A**) and epiphytic (**B**) fungal communities, inhabiting asymptomatic and OK-symptomatic twigs from different olive cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*). This analysis was performed with preselected fungal OTUs by the random forest analysis.

Table 4.1 - Endophytic and epiphytic fungal indicator OTUs in asymptomatic and OK-symptomatic twigs from olive tree cultivars *Cobrançosa*, *Madural* and *Verdeal Transmontana*. A - Specificity (*i.e.* uniqueness to a particular habitat), B – Sensitivity (*i.e.* frequency within that particular habitat).

			Indicator species	A	B	*IndVal*
Endophytic	*Cobrançosa*	Asymptomatic	*Chromelosporium carneum*	0.97	0.72	0.84
		Symptomatic	*Fusarium lateritium*	0.98	0.91	0.95
			Fusarium sp.	0.81	0.66	0.73
	Madural	Aymptomatic	*Pyronema dosmesticum*	1.00	0.52	0.72
		Symptomatic	*Alternaria alternata*	0.90	0.60	0.74
			Fusarium lateritium	1.00	0.30	0.55
	Verdeal	Asymptomatic	*Chromelosporium carneum*	1.00	0.55	0.74
			Pyronema dosmesticum	1.00	0.27	0.52
		Symptomatic	*Fusarium oxysporum*	1.00	0.44	0.66
Epiphytic	*Cobrançosa*	Asymptomatic	*Cladosporium cladosporioides*	0.87	0.85	0.87
			Alternaria sp.	0.72	0.81	0.77
			Hyalodendriella betulae	0.88	0.57	0.71
			Biscogniauxia.mediterranea	0.81	0.52	0.65
			Alternaria alternata	1.00	0.38	0.61
			Pyronema dosmesticum	1.00	0.38	0.61
		Symptomatic	*Cladosporium* sp.	0.76	0.78	0.77
	Madural	Asymptomatic	*Coniozyma leucospermi*	1.00	0.43	0.66
			Hyalodendriella betulae	1.00	0.43	0.66
			Phoma sp.	0.91	0.33	0.55
	Verdeal	Asymptomatic	*Penicillium* sp.	0.95	0.50	0.69
			Hyalodendriella betulae	0.86	0.55	0.69
			Phoma aloes	1.00	0.35	0.59
			Heydenia alpina	1.00	0.30	0.54
			Phaeomoniella sp.	1.00	0.30	0.54
		Symptomatic	*Beauveria bassiana*	0.83	0.37	0.56
			Comospora sp.	1.00	0.33	0.56

4.5 Discussion

4.5.1 Olive knot disease and host genotype affect mostly the epiphytic fungal diversity

Comparative community analysis of fungi between asymptomatic and OK-symptomatic twigs showed that OK disease, caused by the bacterium Psv, affects mostly epiphytic fungal community, by decreasing their abundance, richness, and diversity. This result suggested that Psv may prevent fungal colonization and proliferation on the knots surface. Antifungal activity displayed by *Pseudomonas* has been already reported, mostly in the context of biocontrol of fungal phytopathogens (Maheshwari, 2013). Mechanisms involved in such antifungal activity included the production of antibiotics and fungal cell wall degrading enzymes, as well as the competition for space and nutrients sources, among others (Maheshwari, 2013). Compared to biocontrol studies using *Pseudomonas* spp., very few studies have explicitly examined the antifungal activity promoted by the phytopathogenic *Pseudomonas* (Frey-Klett et al., 2011). Despite this, there are some evidences suggesting their ability to inhibit and extract nutrients from filamentous fungi, in environments where few nutrients occur, such as the phyllosphere (Wichmann et al., 2008). Thus, it is likely that Psv would also trigger similar mechanisms to suppress fungal colonization/proliferation on olive knots surface. Compared to epiphytes, endophytic fungal diversity and richness in knots decreased less, suggesting their less sensitivity to OK disease. Probably, other biotic factors (*e.g.* host plant), rather than the single factor of OLS disease, may affect the assembly of endophytic fungi in knots. Indeed, numerous studies showed that colonization of plant tissues by fungal endophytes result from a complex interplay between the fungi, the plant and its indigenous microbiota (Compant et al., 2016). In contrast to epiphytes, the abundance of fungal endophytes increased in olive knots, suggesting an unexpected stimulatory effect of Psv on the number of fungal isolates. In particular, there was an enrichment in olive knots of *Pseudocercospora* spp. (Mycosphaerellaceae family), which are well recognized plant pathogens on a wide range of plant hosts, including olive tree (Crous et al., 2013). This increasing abundance in the presence of Psv bacteria could result from the production of specific compounds by the pathogenic bacteria that could benefit endophytic fungi (Frey-Klett et al., 2011). Effects of *Pseudomonas* on fungal growth and density stimulation have been already demonstrated in a number of settings, including mushroom formation (Rainey et al, 1990; Cho et al., 2003) and human infections (Briard et al., 2016).

The composition of fungal community of olive twigs was primarily impacted by OK disease. McNally and Brown (2015) reported that this could be partly due to the Psv ability to shape and

change the shared environment, either to the benefit or detriment of certain fungal species, leading to alterations on fungal composition. There are also some evidences suggesting that OK disease is a result of a Psv biofilm formation (Rodríguez-Moreno et al., 2009). In many cases, such microbial biofilms, containing both fungi and bacteria, represent a hotspot for microbial interactions that locally shape microbial assemblages (Frey-Klett et al., 2011; Hassani et al., 2018). Bacterial-Psv interactions have already been reported to occur in olive knots, where the presence of Psv is suggested to be essential for the creation and maintenance of a core group of bacterial genera observed in olive knots (Passos da Silva et al., 2014). These multispecies interactions among bacteria are also likely to occur within Psv bacteria and fungi.

4.5.2 Host genotype effect is more pronounced in symptomatic twigs

Cultivar had minor effects on the fungal community composition in the total dataset. By splitting the data into asymptomatic/symptomatic twigs, we observed a greater effect of the cultivar on fungal community composition, being this effect more pronounced in symptomatic twigs (*i.e.* knots). The effect of OK disease on fungal richness and abundance was greater in the OK-tolerant cv. *Cobrançosa*, when compared to both OK-susceptible cultivars. Probably, after being able to form knots, the antifungal activity displayed by Psv is more effective in cv. *Cobrançosa*, either due to the cultivar chemical composition or simply because this cultivar comprises more-sensitive species or display a different community context. A strong decrease on *Chromelosporium carneum* (Pezizaceae family) abundance was observed, suggesting that this species is highly sensitive to OK disease. Future work is needed to validate *C. carneum* inhibition by Psv and to elucidate the importance of *C. carneum* abundance in knot environment. This indicates that, upon OK disease establishment, the cultivar contribution in fungal assemblage was greater. Therefore, besides fungi - Psv interactions, also the combination of cultivar - Psv appeared to be critical for the establishment of fungal communities in olive knots. Probably, the host plant could play a significant role in recruiting fungi upon OK disease establishment, as previously showed in the *Arabidopsis thaliana* rhizosphere upon foliar pathogen attack (Berendsen et al., 2018). The composition of endophytic fungal community in olive twigs was affected by the interaction between OK disease and cultivar but the epiphytic fungal community was not, which reinforced our hypothesis.

4.5.3 Fungal community composition is primarily affect by OK disease

The decline on both fungal richness and abundance between asymptomatic and symptomatic twigs occurred mostly within fungal OTUs that have not been studied or have been poorly studied, and there is little or no information regarding their biological role. The genera *Heydenia, Hyalodendriella*, *Masonia*, *Ochrocladosporium* or *Prosthemium* are some examples of such fungi (data not shown). These results open the field for exploring an untapped diversity with potential to benefit olive tree health.

Beneficial and pathogenic fungi were also affected by OK disease, but in different ways depending on the type of fungal community (endophytic or epiphytic). In knots surface, pathogenic fungi decreased in abundance in almost the same proportion as the beneficial fungi, indicating that OK disease restrict the growth of both functional groups in the same degree. In contrast, in the interior of knots, the increase on pathogenic fungal abundance was accompanied by a decrease in beneficial fungal richness. Some of the lost beneficial fungi were members of *Epicoccum*, *Cladosporium*, and *Penicillium* genera that included antagonists and disease-protective fungi (Dzoyem et al., 2017; Khan et al., 2016; Jouda et al., 2016). The decline of these fungal genera in olive knots suggested that they can potentially limit or prevent OK disease. Pathogens with increased abundance included mostly non-pathogens of olive tree. The potentially role of these beneficial and pathogenic groups in the development of OK disease must be studied in future works.

Our data revealed that a consortium of fungal OTUs is associated with asymptomatic or symptomatic twigs of each cultivar. The most parsimonious assumption is that these consortia probably have relevance to olive tree health. Otherwise, they would not be maintained highly associated to these different plant samples. The best indicator OTUs of asymptomatic twigs are antagonists toward plant pathogens (*C. cladosporioides* in cv. *Cobrançosa*; Köhl et al., 2015), and fungi with unknown biological function (*e.g. P. domesticum* in cv. *Madural*, *C. carneum* in cv. *Verdeal Transmontana*, and *H. betulae* in the three cultivars). *Alternaria* sp. was also found to be indicative of asymptomatic twigs in cv. *Cobrançosa*. The genus *Alternaria* includes both plant-pathogenic and saprophytic species, and is one of the most well-known fungal genera producers of diverse secondary metabolites, including toxins (Dang et al., 2015) and antimicrobial compounds (Vaz et al., 2009).

The best indicator OTUs for olive knots comprise members of *Fusarium*, a genus including common plant pathogens (Santori et al., 2010; Duan et al., 2016). This taxonomic group has been

described as "pathogen facilitator" by helping pathogens to successfully infect the plant host or increase disease severity (Rodriguez-Estrada et al. 2012). A strong association was also detected between the well-known phytopathogen *A. alternata* (Ning et al., 2016) and knots of cv. *Madural*. *Fusarium* and *Alternaria* genera include fungal species that have been recently reported to cause olive tree diseases with minor importance (Trabelsi et al., 2017; Basim et al., 2017). The role of these fungal taxa associated to either asymptomatic or symptomatic twigs, on olive tree's defense against OK disease remains a topic for further study.

The obtained data indicated that OK disease caused by the bacterium Psv alters the resident fungal community of olive twigs in terms of species composition (abundance and richness). This effect was most notorious within epiphytes than within endophytes. In the interior of olive knots, Psv seems to shape fungal assemblages, mainly by favoring the development of pathogens. Host plant is also likely to structure olive knot-associated fungal communities. Specific fungal signatures were detected for each asymptomatic/symptomatic twigs, suggesting an important role of fungal community in OK disease establishment/development. The results represent an important step forward in understanding the complexity of interactions between bacteria-fungi, and host-microbe interactions, which is needed for predicting and manipulating OK disease suppression.

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4.7 Supporting Information

The following Supporting Information is available for this chapter:

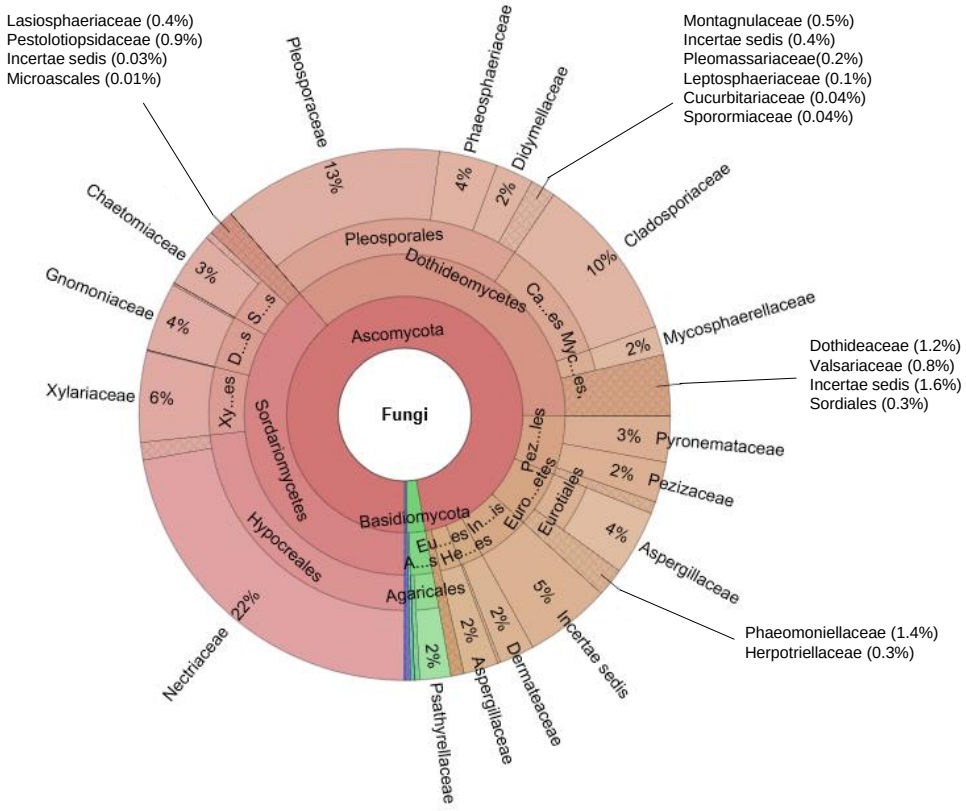

Fig. S4.1 - Krona chart of the taxonomic affiliation down to family level of the global fungal community found in olive twigs.

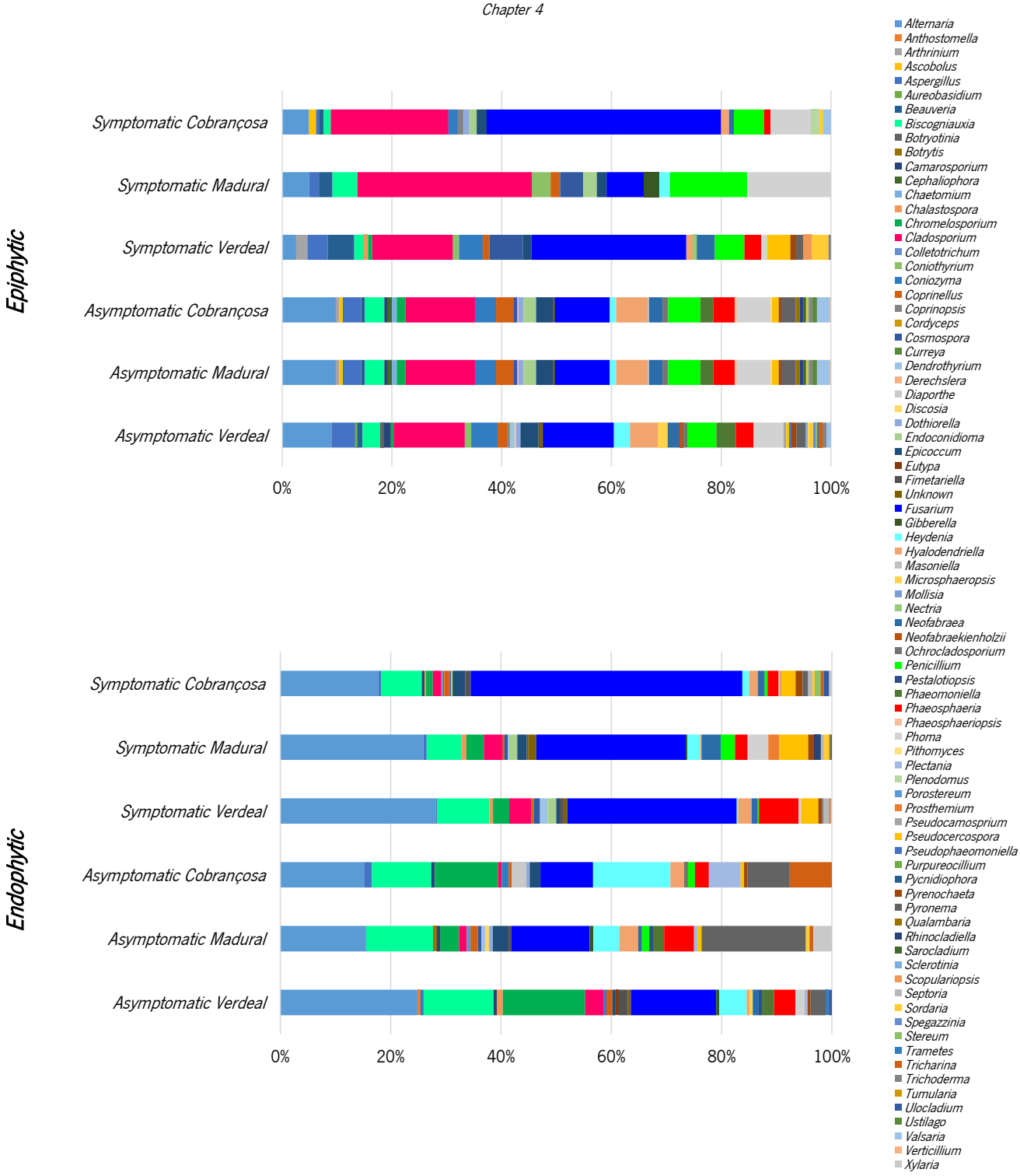

Fig. S4.2 - Frequency (%) of fungal epiphytic and endophytic genera present in asymptomatic and OK-symptomatic twigs from *Cobrançosa*, *Madural* and *Verdeal Transmontana* olive cultivars.

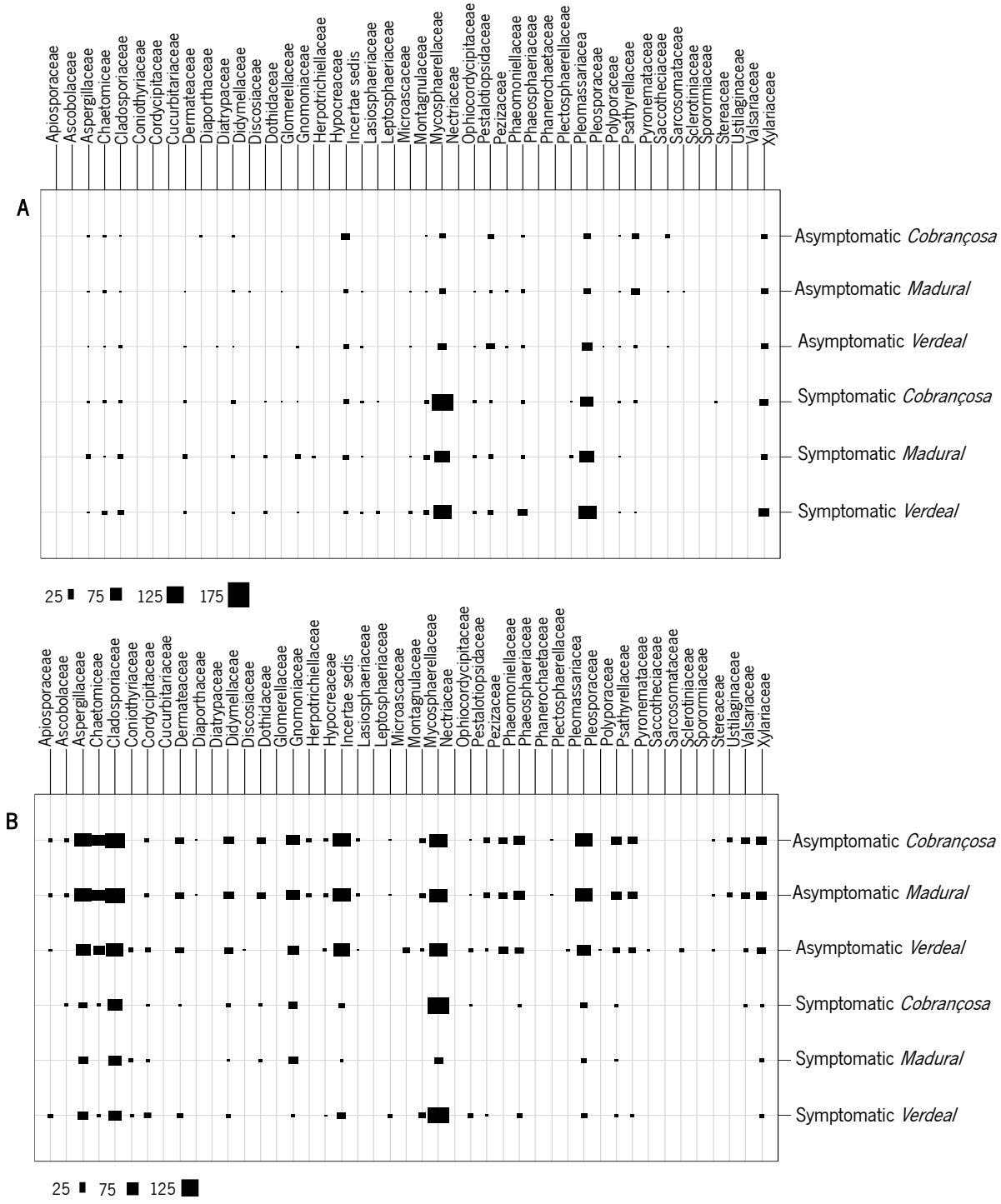

Fig. S4.3 - Distribution of families in epiphytic (**A**) and endophytic (**B**) fungal communities present in asymptomatic and OK-symptomatic twigs of *Cobrançosa*, *Madural* and *Verdeal Transmontana* olive cultivars. Square sizes represent the number of fungal isolates (abundance) present in each plant tissue sample.

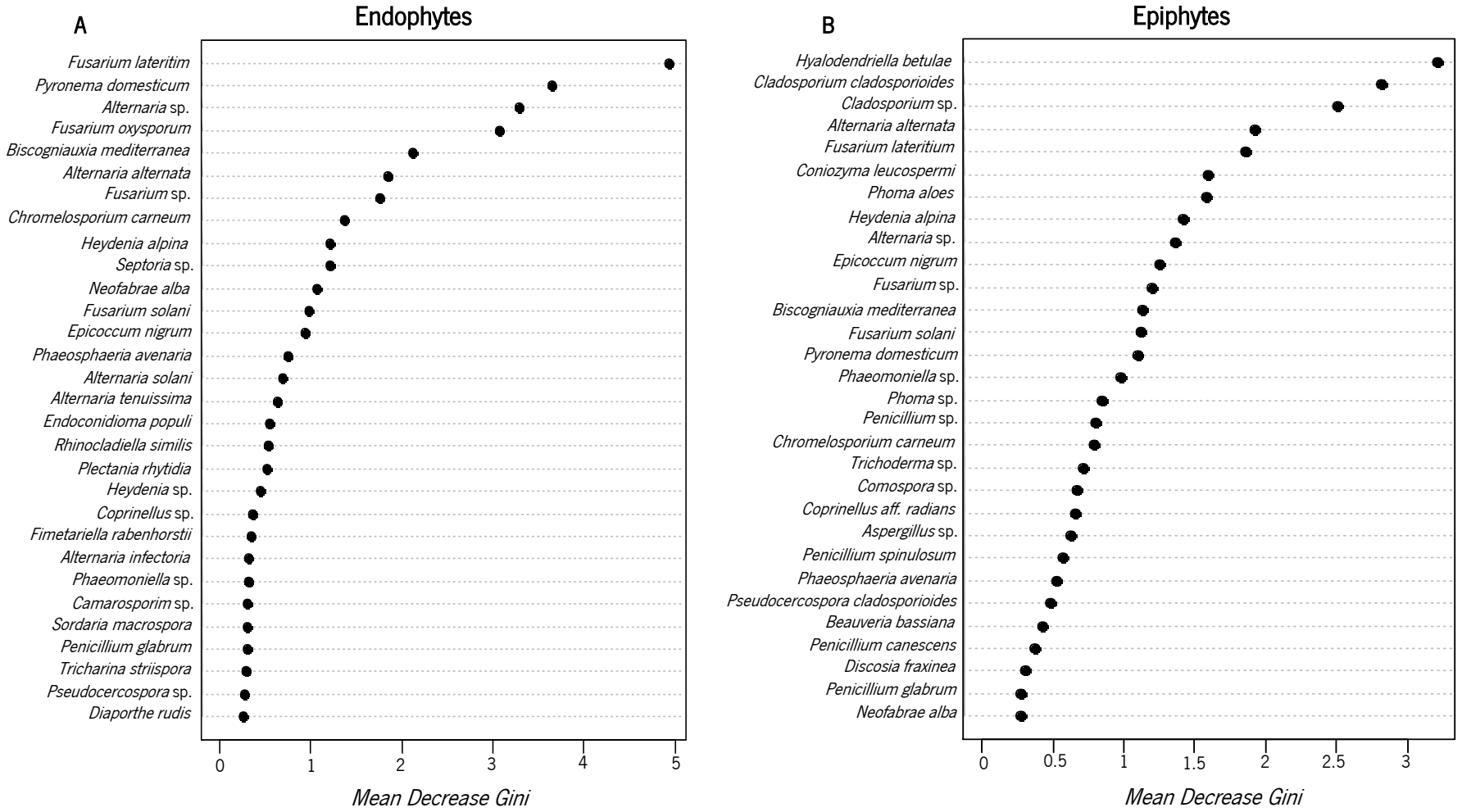

Fig. S4.4 – Ranking of relative importance of each fungal OTUs in distinguishing among asymptomatic and OK-symptomatic twigs within endophytic (**A**) and epiphytic (**B**) communities. *Mean Decrease Gini* value predicts fungal OTUs that distinguish asymptomatic from OK-symptomatic twigs. The highest values represent the best predictors.

Table S4.1 - ANOVA analysis to test significant differences (*P*-value) between fungal community groups obtained in the Canonical Correlation Analysis (CCA). Variation partitioning (*Varpart*) was calculated to achieve the total variance explained by each factor. *P*-values in bold are significant.

Factors			*P*-value	*Varpart* (%)
	Cultivar		**0.005**	**0.5%**
Total	Twig statuses		**0.005**	**3.9%**
	Fungal community		**0.005**	**5.2%**
Endophytic	Cultivar		**0.005**	**1.1%**
	Twig statuses		**0.005**	**5.6%**
Epiphytic	Cultivar		**0.025**	**1.3%**
	Twig statuses		**0.005**	**5.9%**
Asymptomatic	All Cultivars		**0.049**	**1.5%**
	Endophytic	All cultivars	**0.010**	-
	Epiphytic	All cultivars	0.050	-
Symptomatic	All Cultivars		**0.005**	**2.8%**
	Endophytic	All cultivars	**0.005**	-
	Epiphytic	All cultivars	**0.005**	-

Table S4.2 - Analysis of similarity (ANOSIM), based on Bray-Curtis distance, between fungal communities inhabiting asymptomatic and OK-symptomatic twigs. R-statistics (R) and *P* -values of each variable are included.

Variables		ANOSIM	
		R	*P*-value
Endophytic	All cultivars	0.221	0.001
	Cobrançosa	0.246	0.001
	Madural	0.232	0.006
	Verdeal Transmontana	0.237	0.001
Epiphytic	All Cultivars	0.239	0.000
	Cobrançosa	0.498	0.001
	Madural	0.211	0.020
	Verdeal Transmontana	0.136	0.005
Total	*Cobrançosa*	0.222	0.001
	Madural	0.160	0.002
	Verdeal Transmontana	0.009	0.001

Chapter 5

Disease incidence is related to fungal community and secondary metabolites composition of host plant

Disease incidence is related to fungal community and secondary metabolites composition of host plant

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5.1 Abstract

Plants comprise a complex niche for microorganisms with beneficial or harmful effects. The mechanisms behind the dynamics of microbial communities is still poorly understood, but plant secondary compounds (*e.g.* volatiles and phenolics) have been recognized as key driving factors in shaping microbial communities. Using olive trees under field conditions, displaying different olive leaf spot (OLS) incidences, the relationship between fungal communities and metabolic profiles was determined in different cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*). The main goal was to determine whether OLS disease incidence was related to differences in leaf fungal communities and/or secondary metabolites, associated with distinct olive cultivars. Variations on fungal communities and metabolite composition were detected in leaves from trees of different OLS disease incidences, but were dependent on host genotype. Changes were particularly noticed for the OLS-tolerant cv. *Cobrançosa*, in contrast to OLS-susceptible cv. *Verdeal Transmontana*, suggesting that disease development is dependent on fungal microbiota present on leaves and produced secondary metabolites. Furthermore, the OLS-tolerant cultivar was linked to a lower OLS incidence, whereas OLS-susceptible cultivar was more associated to higher disease incidence levels. A set of fungi/metabolites was also identified that could explain cultivar resistance/susceptibility to OLS disease. Some metabolites (mainly α-farnesene, *p*-cymene) and fungal OTUs (*Fusarium* sp. and *Alternaria* sp.) were related with disease incidence, while *Pyronema domesticum* was suggested to suppress OLS disease. Differences in cultivar response to OLS disease are then suggested to be driven by fungal community composition on leaves, foliar volatile and phenolic properties of host leaves, and interactions of both. A deeper understanding of these complex interactions within each host genotype may unravel plant defensive responses.

5.2 Introduction

In natural ecosystems, the aboveground parts of plants come across with a myriad of fungal species that can colonize the surface (epiphytes) or internal (endophytes) plant tissues, with beneficial or detrimental outcomes (Zeilinger et al., 2016). Despite the ecological and agricultural importance of these plant-fungal associations, the way plants control fungal populations on their aerial tissues still remains to be elucidated (Vorholt, 2012). This knowledge will be useful for managing foliar fungal communities, which will be important, for example, in terms of plant health.

Plant genotype has been recognized as an important factor for determining the structure of fungal communities in the leaves (*e.g.* Bálint et al., 2013). Although the mechanisms for these genotypic effects are still unknown, plant secondary metabolites could be responsible for the variation on composition of foliar fungal communities (Zambell and White, 2017). This suggestion is supported by studies showing *in vitro* antifungal effects of some plant secondary metabolites (El-Khateeb et al., 2013). Several other studies also revealed the influence of specific secondary compounds on fungi from leaves (Sanchez-Azofeifa et al., 2012) and twigs (Bailey et al., 2005). From the wide range of plant secondary metabolites, both phenolic and volatile organic compounds (VOCs) seems to be particularly important, due to their recognized antimicrobial activity and defense role against pathogens in plants (Lattanzio et al., 2006; Ansari et al., 2013; Farré-Armengol et al., 2016). However, identifying the implicated host chemical factors when considering fungal assemblages in field experiments is challenging. In fact, plant-infecting microorganisms are ubiquitous in nature (Partida-Martínez and Heil, 2011) and these microorganisms can contribute to metabolite production in different ways: by producing their own secondary metabolites which will be mixed with the ones produced by the plant; by altering the biosynthesis of plant metabolites (Etalo et al., 2017; Chagas et al., 2018); and by metabolizing plant secondary metabolites (Farré-Armengol et al., 2016). Probably due to these complex interactions, few studies have focused on the selective pressures exerted by plant secondary compounds on fungal foliar communities (Zambell and White, 2017). Studies have been performed under *in vitro* conditions (Saunders and Kohn, 2008), and those performed under field conditions have only focused on endophytic fungal communities (Bailey et al., 2005; Sanchez-Azofeifa et al., 2012).

In this work, the relationship between plant secondary metabolites and composition of epi- and endophytic fungal communities on leaves was investigated under field conditions. With the aim to exploit this knowledge for improving plant health, a particular attention was given to the presence of pathogenic fungi. Olive leaf spot (OLS) is a disease caused by *Venturia oleaginea* (syn. *Fusicladium oleagineum*) that is specific of olive (*Olea europaea* L.) (González-Domínguez et al., 2017). Development of this fungus is mostly restricted to olive leaf tissues, including leaf surface and subcuticular areas, causing scab lesions and leaf-drop symptoms and occasionally the tree death (Viruega et al., 2013). Under the same agro-climatic conditions, the olive leaf spot disease is more severe in some cultivars (*e.g.* *Madural* and *Verdeal Transmontana*) than in others (*e.g.* *Cobrançosa*) (Gomes T., Per. Com.), suggesting a contribution of plant genotype to disease incidence. The contribution of plant secondary metabolites to prevent this pathogen *in planta* is not

known. Here, we tested whether OLS disease incidence was related to differences in leaf secondary metabolites (at cultivar level) and determine the contribution of epi- and endophytic fungal communities to such differences. Indeed, the presence of fungi may alter the chemical proprieties of olive tree leaves, as previously reported for other plant species (Farré-Armengol et al., 2016). We hypothesized that plant interactions with fungi could modify plant secondary metabolites composition and, consequently, affect the incidence of plant disease OLS. Specifically we addressed the following questions: (1) Is OLS incidence related to host associated epi- and endophytic fungal communities composition in leaves? (2) Is OLS incidence related to host plant composition on phenolic and volatile compounds? (3) Is there any relationship between secondary metabolites composition and fungal consortia associated to different incidence levels of OLS disease? As far as we known, no previous investigation have addressed the study of fungal communities and chemical composition of olive tree leaves as a whole. The understanding of these complex associations (*i.e.* host plant, phytochemistry, fungal community and disease incidence) might improve our knowledge on the role of different fungal taxa and metabolites in OLS disease incidence. Our ultimate goal is being able to determine specific interventions that improve the plant health.

5.3 Material and methods

5.3.1 Study site and olive leaves collection

The study was conducted in two olive orchards at Mirandela region (Northeast of Portugal), at coordinates N 41° 32.593' W 07° 07.445' (orchard 1) and N 41° 29.490' W 07° 15.413' (orchard 2). Each orchard comprises olive trees from three olive cultivars, *i.e. Cobrançosa*, *Madural* and *Verdeal Transmontana*, at spacing of 7 x 7m, and is managed through integrated production guidelines (Malavolta and Perdikis, 2012). In each orchard, five trees per cultivar were selected in close proximity to each other, with each plant representing an experimental unit. Leaves were randomly collected on four orientations of tree canopy, at 1.5 meter above the ground, from March to May 2015. The collected leaves were used to assess OLS disease incidence (% infected leaves), to determine their epi- and endophyte fungal communities, as well as their chemical constituents (*i.e.* phenolic and volatile compounds). For fungal and chemical evaluations, and to mimic natural conditions within tree canopy, a mixture of ten randomly selected leaves per tree were used, comprising five leaves with visible spots (OLS-symptomatic leaf) and five leaves without visible spots (asymptomatic leaf). All evaluations were performed using fresh leaves (immediately upon their

collection), with exception of phenolic compounds that used lyophilized leaves. For this, leaves were stored in a deep freezer at -20°C, lyophilized, ground to a fine powder using an analytical mill, and stored in a dark room until phenolic analyzes.

5.3.2 Disease incidence assessment

The levels of disease incidence (%) were determined by determining the percentage of infected leaves simultaneously to sample collection. The number of leaves with visible spots was recorded and used to determine the percentage of infected leaves per tree.

5.3.3 Foliar fungal communities assessment

Fungal isolation. Both epiphytic and endophytic fungal communities in olive tree leaves were evaluated based on culture-dependent methods. The isolation of fungal epiphytes was performed by the dilution plate method, by using around 1-gram weight of leaf samples in 9 mL of sterile potassium phosphate buffer pH 7.0, according to the procedure described by Gomes et al. (2018). Aliquots (1 ml) of the resulting microbial suspension were separately plated in triplicate onto Potato Dextrose Agar (PDA, Difco) and Plate Count Agar (PCA, Himedia) media, supplemented with 0.01% (w/v) chloramphenicol (Oxoid, Basingstoke, Hampshire, UK). Plates were incubated at 25 ± 2°C in the dark for fungal growth and colonies counting. The number of epiphytes, *i.e.* the number of individual colonies of fungi adhered to leaf surface, was expressed as log CFU/cm^2. For estimating the leaf surface an ellipse equation ($A=\pi ab^*2$) was used, being A the area, whereas *a* and *b* were the leaf longitudinal and transverse axes, respectively. The average leaf area of cvs. *Cobrançosa*, *Madural* and *Verdeal Transmontana* was 10.4±1.6, 14.1±2.3 and 11.4±3.5 cm^2, respectively.

Endophytic fungi were isolated from the same leaf segments used to isolate epiphytes. After removing epiphytes by surface disinfection using the procedure described by Martins et al. (2016), each leaf was cut in fragments (*ca*. 5 x 5 mm), which were transferred to the same culture media used to isolate epiphytes. Endophytic fungi were isolated from a total of 1,800 leaf tissue segments. Validation of surface sterilization procedure was done by imprinting the surface of sterilized leaf tissues onto PDA and PCA media. Emerging fungal colonies were subcultured on fresh medium until pure epi/endophytic cultures were obtained.

Fungal identification. Each fungal colony was identified by using morphological and molecular approaches according to Gomes et al. (2018). Briefly, fungal colonies were grouped

according to their morphological similarity and three representative isolates of each morphotype were further selected for molecular identification, using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA). Total genomic DNA was extracted from harvested mycelial/spores using the *REDExtract-N-Amp™ Plant PCR* kit (Sigma, Poole, UK). The ITS region (ITS1, 5.8S, ITS2) was amplified using ITS1/ITS4 or ITS5/ITS4 primers sets (White et al., 1990), using a PCR protocol previously described by Oliveira et al. (2012). The amplified products (~ 650 bp) were purified and sequenced using Macrogen Inc. (Seoul, South Korea) services. The obtained DNA sequences were analysed with *DNASTAR v.2.58* software and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST algorithm, according the procedure described by Gomes et al. (2018). The obtained sequences are available at GenBank with the following accession numbers: KU324941-KU325040; KU325041-KU325240; KU325241-KU325457. Each operational taxonomic unit (OTU) was taxonomically classified according to the *Index Fungorum Database* (www.indexfungorum.org). Pure cultures of each identified isolate were preserved and deposited in the culture collection of the Polytechnic Institute of Bragança (School of Agriculture).

5.3.4 Phenolic compounds identification and quantification

Standards and reagents. Used standards were from Sigma (St. Louis, MO, USA) or Extrasynthèse (Genay, France). Methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) before use.

Extraction of phenolic compounds. Each lyophilized leaf samplewas powered to pass a 900 µm sieve, before the extraction of phenolic compounds. The extraction was performed as previously described by Vinha et al. (2002). Briefly, about 1.5 g of the powdered leaf samples were weighed in quadruplicates, which were separately mixed with 50 mL of methanol (99.96%, Aldrich) at 150 rpm during 1 h (room temperature), and filtered through a Whatman No.4 paper. Methanolic extracts were evaporated (Stuart RE3000, UK) to dryness under reduced pressure (35°C) and redissolved in 2 ml methanol (99.96%, Aldrich). After filtration (Whatman No. 2), an aliquot of 20 µl of the obtained extracts was analysed by HPLC.

Analysis of phenolic compounds. Chromatographic separation was performed as previously reported by Vinha et al. (2002), with an analytical HPLC unit (Knauer Smartline), equipped with a Knauer Smartline autosampler 3800, and a Knauer Diode Array Detector (DAD).

A reversed-phase Spherisorb ODS2 column was used during analysis (250 x 4.6 mm, 5 µm particle size, Merck, Darmstadt, Germany). The used solvent system was a gradient of A) water–formic acid (19:1) and B) methanol, applied at a flow rate of 0.9 mL min−1 , as follows: 5% B at 0 min, 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 40% B at 39 min, 45% B at 42 min, 45% B at 45 min, 47% B at 50 min, 48% B at 60 min, 50% B at 64 min and 100% B at 66 min. Spectral data from all peaks were accumulated within the 200–400 nm range. Chromatograms were recorded at 280, 320 and 350 nm, and data were managed on *ClarityChrom*® software (Knauer, Berlin, Germany). Phenolic compounds were quantified through the absorbance recorded in the chromatograms relative to external standards: hydroxytyrosol, oleuropein, chlorogenic acid and rutin were quantified at 280 nm, caffeic acid at 320 nm, and verbascoside, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside and luteolin at 350 nm. HPLC analyses were performed using two technical replicates for each extract and thereafter the means of the four replicates for each leaf sample were calculated. Phenolic compounds were expressed as the amount of phenolics per dry weight (DW) of leaf extract (mg/g of DW).

5.3.5 Volatile identification and quantification

The extraction and analysis of volatile compounds from fresh leaves were performed according to the methodology described by Malheiro et al. (2015), with some modifications.

Extraction of volatile compounds. The extraction of leaf volatiles was performed by headspace solid-phase microextraction (HS-SPME). Around 1-gram weight of fresh leaves was placed in 50 ml vials, containing 10 µl of 4-methyl 2-pentanol (10.65 ppm dissolved in methanol), which was used as an internal standard. The vials were then sealed with a polypropylene cap with silicon septum, and were placed in an ultrasonic bath at 40°C for 10 min. After that, the divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; 50/30 µm) fiber was inserted into the vial headspace for more 30 minutes, at 40°C, for volatiles adsorption. The volatiles were desorbed by placing the fiber into the gas chromatographic (GC) injection port for 10 min, at 280°C. The HS-SPME analyses were performed using five technical replicates for each sample.

Gas chromatography-mass spectrometry (GC-MS) conditions. Chromatographic analysis was performed on an *Agilent 6890 series* GC (Agilent, Avondale, PA, USA), with splitless injection, coupled to a MS detector (Agilent 5973), according to the conditions described by Malheiro et al. (2015). Volatile compounds were identified by comparing the experimental spectra with spectra from NIST data bank (NIST/EPA/NISH Mass Spectral Library, version 1.6, U.S.A.) and also by

comparison of their GC retention index. Retention indices were determined as reported by Malheiro et al. (2015). Concentration of identified compounds were calculated by the ratio of each individual base ion peak area to the area of the internal standard and converted to mass equivalents on the basis on the internal mass added. Volatiles were represented as the amount of volatile compound per fresh weight (FW) of leaf tissue (mg/kg of FW).

5.3.6 Data analysis

Based on OLS disease assessment results, three ranges of disease incidence were defined: 0-5%, 5-10%, 10-15%. Data was analysed for each group of disease incidence and also for each cultivar.

Differences on fungal communities and metabolite profiles among OLS incidence ranges and cultivars. The total number and abundance of fungal OTUs and metabolites (phenolic and volatiles compounds) for each olive tree were first determined in *Species Diversity and Richness* v. 4.0 (Seaby and Henderson, 2006). Results are presented as the mean of trees for each OLS disease incidence range (0-5%, 5-10%, 10-15%) and cultivar (*Cobrançosa*, *Madural, Verdeal Transmontana*). Differences between means were evaluated by one-way analysis of variance (One-way ANOVA) with *SPSS v.20*, followed by Tukey's post hoc test (P <0.05). Non-metric Multidimensional Scaling (NMDS) plots, based on Bray–Curtis distance, were performed to assess the variation in the composition of foliar fungal communities and metabolite profiles, among different ranges of OLS disease incidence (0-5%, 5-10%, 10-15%). The Bray-Curtis similarity index compares the presence or absence of fungal OTUs/metabolite, as well as their abundance among samples (Bray and Curtis, 1957). Kruskal's stress was used to estimate goodness of fit (commonly acceptable when <0.2). A one-way analysis of similarity (ANOSIM) was used to determine significant differences in fungal (or metabolite) compositions among OLS incidence groupings, using Bray–Curtis distance matrices. ANOSIM generates a P-value (significant level below to 0.05) and Rvalue (gives the degree of discrimination between groups), which ranges from 0 (indistinguishable) to 1 (completely dissimilar) (Clarke and Gorley, 2015). NMDS plots and ANOSIM analyses were performed using *Community Analysis Package* v. 4.0 (Henderson and Seaby, 2007). Subsequent analyses were performed in R (R Core Team, 2014). Using the '*heatmap 2'* function of *gplots* package, with the Euclidean distance (Gentleman et al., 2004), heatmaps with hierarchical clustering were constructed in order to group host cultivars and OLS incidence ranges, according to the most abundant fungal OTUs (abundance >10) and metabolites (abundance >12).

Each sample was transformed into a row Z-score and high relative values were colored differently from those with low relative values.

Relationship between OLS disease incidence, host cultivar, fungal OTUs and metabolites. Random forests analysis was firstly performed to identify the ranking importance of variables (fungal OTUs and metabolites) for predicting OLS incidence (Breiman, 2011; Cutler et al., 2007). This analysis was set through artificial intelligence algorithms, using the R *RandomForest* package (Cutler et al., 2007). For each tree grown on a bootstrap sample, the error rate for observations left out of the bootstrap sample was monitored. The ranking variable importance was explained by 74.1% and 85.2% for fungal OTUs and metabolites, respectively. The *Gini coefficient* indicates the variable contribution for OLS disease incidence. *Spearman* correlations and redundancy analyses (RDA) were then performed using the most important fungal OTUs and metabolites, which have been pre-selected by the random forest analysis (*Gini index* >100). The *Spearman* correlations were computed using the R *corrplot* package (Wei, 2017), in order to check the correlation between fungal OTUs, metabolites and OLS incidence. RDA was performed using R *vegan* package (Oksanen et al., 2017), in order to find relationships among cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*), OLS disease incidence ranges (0-5%, 5-10%, 10-15%), fungal OTUs and secondary metabolites. One-way analysis of variance (ANOVA) was carried out with '*anova*' function to test significant differences between cultivars or OLS incidence groupings, previously obtained by RDA ordination based on fungal OTUs and metabolites.

5.4 Results

5.4.1 Differences in diversity and abundance of fungal community and metabolite profiles

In this study, a total of 154 fungal OTUs, 18 phenolic and 73 volatile compounds were identified from all analyzed olive leaves (Figs. S5.1-S5.3). Among the identified fungal genera, *Cladosporium*, *Alternaria* and *Fusarium* were the most frequently isolated, representing 35% of the total isolates. In what concerns metabolites, the phenolics compounds oleuropein, apigenin-7-*O*-glucoside, rutin and verbascoside, as well as the volatiles Z3-hexen-1-ol-acetate and Z3-hexen-1-ol, were the most abundant, accounting together for 78% and 82% of the total phenolic and volatile fraction, respectively. In general, the number of both fungal OTUs and detected metabolites did not change significantly across the three levels of OLS disease incidence (Fig. S5.4). Only the abundance of fungal isolates retrieved from the most OLS-susceptible cultivar (*Verdeal*

Transmontana) showed a 2-fold significant increase (P<0.05) in trees with the highest OLS disease incidence. Differences were also detected among cultivars for the volatiles content. With an increase of OLS disease incidence, the levels of volatiles decreased significantly (P<0.05) in leaves of cv. *Cobrançosa*, while in the cv. *Madural* they increased significantly (P<0.05). The endophytic and epiphytic variations were different among olive trees displaying distinct OLS incidence (Fig. S5.5). Endophytic fungi displayed a greater increase in abundance (up to 1.4-fold, P<0.05) and richness (up to 1.1-fold, P<0.05) in trees with the highest OLS disease incidence than epiphytic fungi. Only a significant increase in epiphytes abundance was detected in trees with highest OLS incidence (up to 1.3-fold, P<0.05).

The composition of foliar fungal communities and metabolite profiles differs significantly among trees with distinct disease incidence levels, being the highest differences observed between trees with the lowest and the highest OLS incidence (Fig. 5.1, Table S5.1). Hierarchical cluster analysis based on the most abundant fungal OTUs also separated fungal communities into two main groups, corresponding to the communities found in trees with low OLS incidence and communities with higher incidence levels (Fig. 5.2A). However, this separation was dependent upon the olive cultivar, as *Verdeal Transmontana* fungal communities clustered together regardless of tree disease incidence. Accordingly, the fungal composition of cvs. *Cobrançosa* and *Madural* is more distinct between trees exhibiting high and low OLS incidence levels (ANOSIM, R=0.40, P=0.001) than cv. *Verdeal Transmontana* trees (ANOSIM, R=0.33, P=0.002). Nevertheless, the ANOSIM analysis could still distinguish the three incidence ranges for this cultivar (R=0.31, P=0.016). Differences on fungal community composition between cultivars also revealed to be lower when OLS disease incidence increased, which was particularly observed for cvs. *Cobrançosa* and *Madural*. Indeed, the highest differences on fungal composition of trees from the three cultivars were observed in those trees with the lowest disease incidence level (ANOSIM, R=0.72, P=0.001).

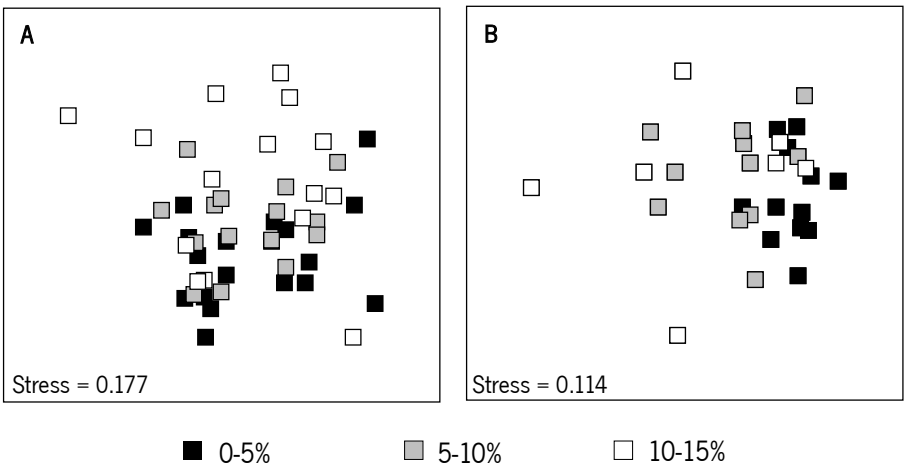

Fig. 5.1 - Non-metric multidimensional scaling (NMDS) plots of foliar fungal communities (**A**) and metabolite profiles (**B**) detected on olive trees displaying different levels of OLS disease incidence (0-5%, 5-10%, 10-15%). Clustering analysis was performed with Bray-Curtis distance. Kruskal's stress values are displayed.

The metabolite profiles of trees from the three cultivars are distinct (ANOSIM, R=0.32, *P*=0.001), being these differences greater between cvs. *Madural* and *Verdeal Transmontana* (ANOSIM, R=0.35, *P*=0.001). Although less relevant, leaf metabolite composition within each cultivar also varied with OLS disease incidence, being the greatest differences observed between trees with the lowest and highest disease incidence levels. This result was particularly observed for trees from cvs. *Madural* (ANOSIM, R=0.970, *P*=0.002) and *Cobrançosa* (ANOSIM, R=0.88, *P*=0.002), while cv. *Verdeal Transmontana* exhibited a similar metabolite composition among all trees (ANOSIM, R=0.125, *P*=0.079).

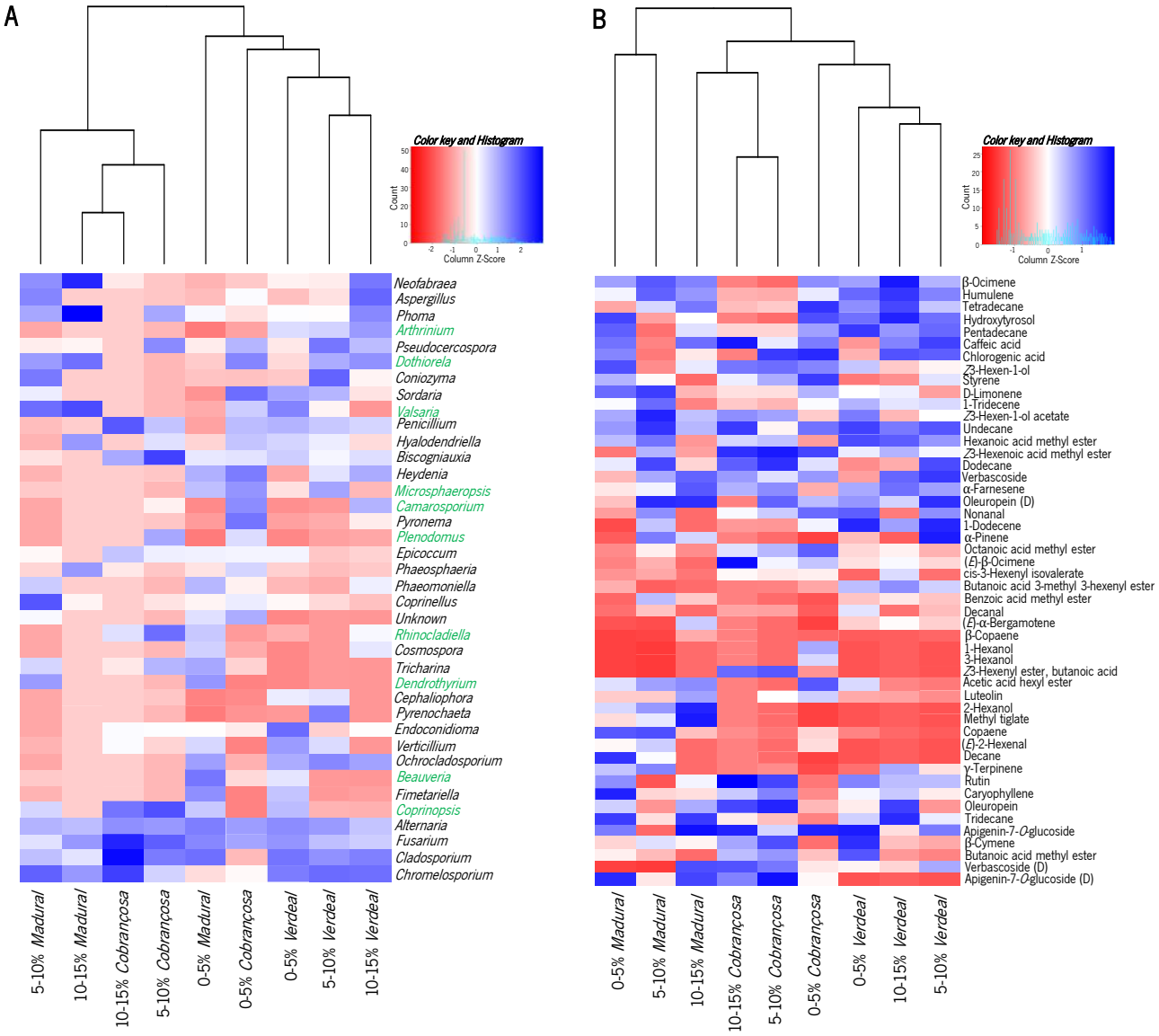

Fig. 5.2 - Variation of fungal communities (**A**) and metabolites profiles (**B**) in leaves of olive trees from distinct cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*), displaying different levels of OLS disease incidence (0-5%, 5-10% and 10-15%). Heat maps indicate differences in the relative abundances of the most abundant fungal OTUs and metabolites. The color-scale ranges from red z < -3 to blue z >3, indicating the abundance of fungal OTUs and metabolites. Fungal isolates exclusively found on the episphere (leaf surface) and endosphere (leaf interior) are shown in green and purple color, respectively.

5.4.2 Relationship between host cultivar, foliar fungal community, metabolite profile and disease incidence

One of the goals of this study was the identification of a set of fungal OTUs and metabolites that could explain differences in susceptibility of different olive tree cultivars to OLS disease. The complexity of this biological system, in which correlation and interaction effects between host plant,

fungi, and metabolites should be considered, as well the large amount of microbial/metabolite data, increases the difficulty of this task. Thus, to more accurately predict such relationships a random forest analysis was employed to select the variables (*i.e.* fungi/metabolite) that were most relevant for the prediction of OLS incidence. This is an important tool for reducing the number of variables for further data analysis. The random forest ranks the importance of input variables measured by a *Gini coefficient* value. A higher *Gini coefficient* value represents a greater variable importance (Cutler et al., 2007; Breiman, 2011). Eight fungal OTUs and ten metabolites (four phenols and six volatiles) were identified to be the most important variables for determining OLS disease incidence (*Gini coefficient* > 100; Fig. S5.6). These variables were then used to perform *Spearman* correlations, to test the association between fungi, metabolite and OLS disease incidence (Fig. 5.3). We found that the volatiles (*E*)-α-bergamotene, α-farnesene and *p*-cymene, the phenolic luteolin, and the fungal OTUs *Alternaria* sp., exhibited significant positive correlations with disease incidence. In contrast, *Cladosporium cladosporioides* and *Pyronema domesticum* were negatively correlated with OLS disease incidence. Some fungal OTUs were also found to be either negatively or positively correlated with some metabolites, as well as with other fungal OTUs. Specific significant inter- and intra-group metabolites correlations also existed, being particularly observed a strong positive correlation between the volatiles α-farnesene and *p*-cymene, and between these two compounds and 1-octanol.

Since *Spearman* correlations only test correlation between two variables, we have additionally performed a redundancy analysis (RDA) that can be applied to a set of variables. This analysis expresses how much of the variance in the set of response variables (fungal OTUs and metabolites) is explained by the set of explanatory variables (host cultivar and disease incidence level). For RDA, only the most important variables preselected by the random forest analysis were used. RDA clearly separated the three OLS disease incidence ranges (P <0.01) and olive cultivars (P <0.01) based on foliar fungal community and metabolite profiles (Fig. 5.4). Several correlations were also detected between metabolites, fungal OTUs, host cultivars and OLS disease incidence. The strength of such correlations were assessed by the arrow length and angle between arrows and axes. The lowest OLS disease incidence range is more closely associated to cv. *Cobrançosa*, and to the presence of *P. domesticum* on leaves. In contrast, higher OLS disease incidence is more associated to cv. *Verdeal Transmontana*. The segregation of trees with higher OLS disease incidence is based on metabolite and fungal profiles. Disease incidence of 5-10% was mostly related with the metabolites α-farnesene, *p*-cymene, apigenin-7-*O*-glucoside and to the presence of

Alternaria sp.; while the highest disease incidence (10-15%) was more closely associated to verbascoside, *Z*3-hexenoic acid, methyl ester, butanoic acid, methyl ester, and to the presence of *Fusarium* sp..

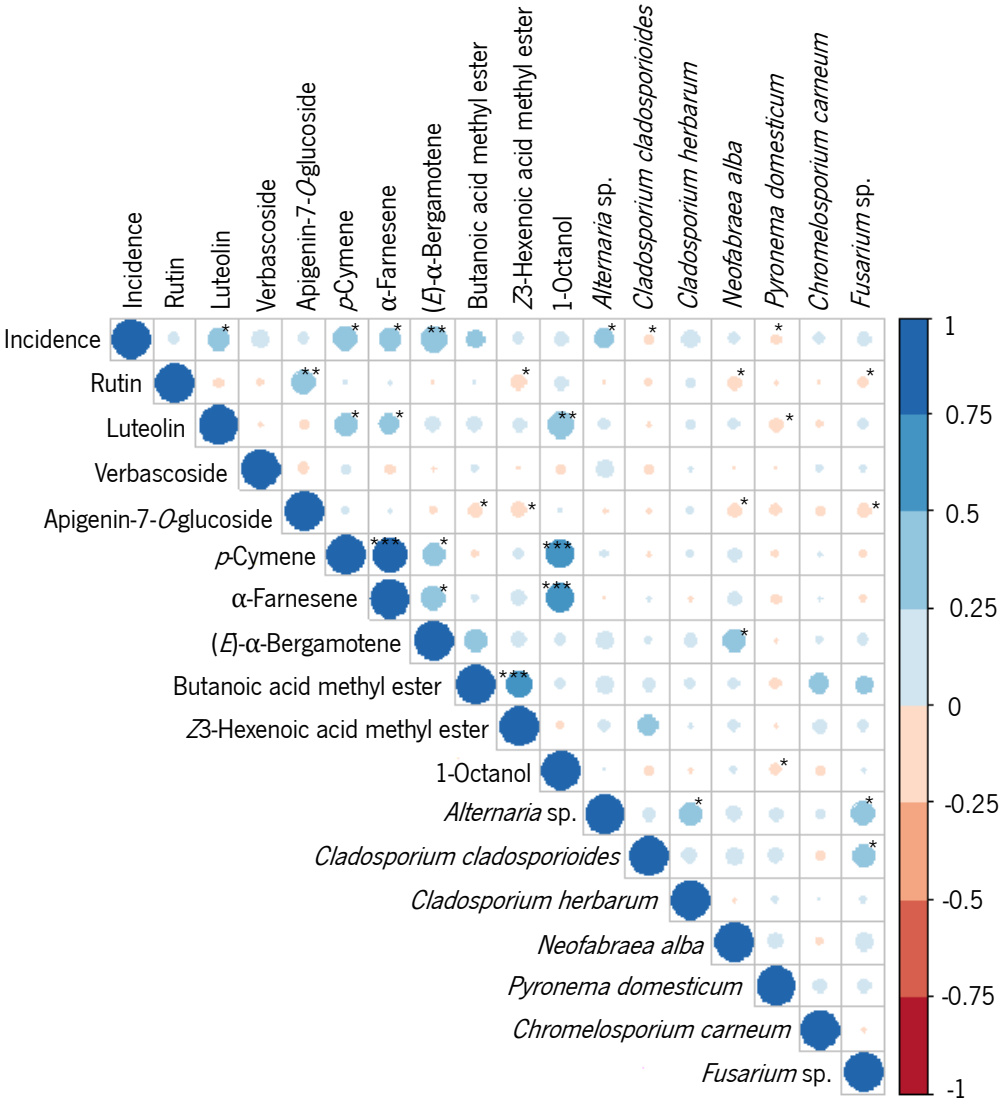

Fig. 5.3 - *Spearman* correlations between fungal OTUs abundance, metabolites concentration and OLS disease incidence. These correlations were only performed with variables (fungal OTUs and metabolites) preselected by the random forest analysis. Blue color represents positive correlations, while red color represents negative correlations. Circle size and color shading indicate correlation coefficient values. High coefficient values (maximum = 1) are represented by larger and darker circles, while smaller and lighter circles represent lower coefficient values (minimum = 0). Asterisks indicate statistically significant correlations at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

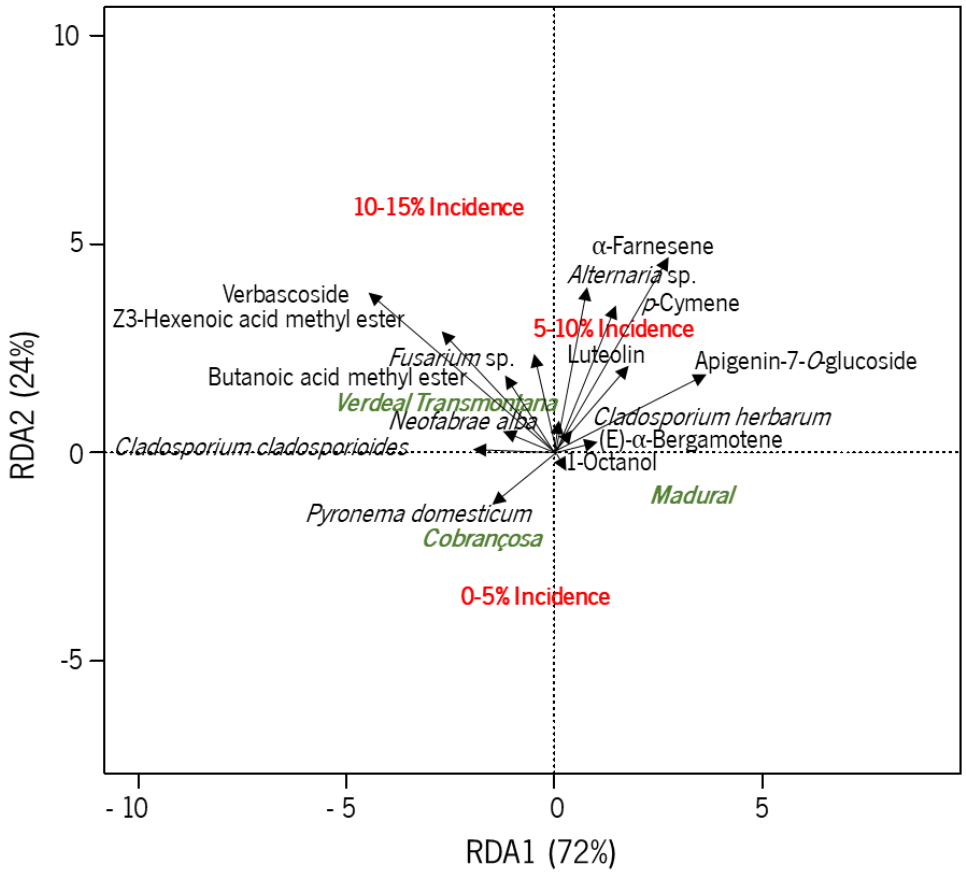

Fig. 5.4 - Redundancy analysis (RDA) ordination plot showing the association between the OLS disease incidence levels (0-5%, 5-10% and 10-15%), olive tree cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*), leaf fungal community and metabolites composition. This analysis was only performed with variables (fungal OTUs and metabolites) preselected by the random forest analysis. In RDA analysis, a positive correlation between two variables is expressed by relatively long vectors pointing approximately in the same direction, whereas a negative correlation is indicated by arrows pointing in opposite directions. The longer the variable decline, the stronger the relationship of that parameter with the olive cultivar/disease incidence. The percentage of variation is explained by each axis.

5.5 Discussion

To the best of our knowledge, the present study is the first work to address the relationship between host plant, foliar fungal communities, metabolic profiles and plant disease incidence under field conditions. We attempted to determine whether differences in susceptibility of different olive tree cultivars to OLS disease is linked to fungal communities and/or metabolite composition of host plant leaves.

5.5.1 Is OLS incidence related to host associated fungal communities composition in leaves?

In general, our results underline the importance of fungal communities inhabiting the leaves of each olive tree as an important determinant of disease incidence. Several significant correlations between specific fungi (*Alternaria* sp., *C. cladosporioides* and *P. domesticum*) and incidence of OLS disease were observed. Moreover, the relation between disease incidence and fungal composition was found to be dependent on host cultivar (and thereby on genotype susceptibility). A greater variation on foliar fungal composition occurred in the OLS-tolerant cv. *Cobrançosa* when analyzing the three levels of OLS disease incidence, in comparison with the susceptible cv. *Verdeal Transmontana*. Therefore, we hypothesize that fungal community changes could have important consequences for OLS disease incidence. In the most tolerant cultivar (cv. *Cobrançosa*), a reduction on the abundance of fungi able to provide host plant protection against OLS disease could determine higher disease incidence. In accordance, the presence of OLS symptoms in leaves of cv. *Cobrançosa* caused a significant shift on fungal community from healthy leaves, including a reduction on the abundance of beneficial fungi (Gomes et al., *submitted*). Therefore, the importance of fungal communities (in particular beneficial fungi) for OLS disease suppression should not be neglected, but this assumption still needs to be confirmed with further work. In the most OLS-susceptible cultivar (cv. *Verdeal Transmontana*), the pathogen is probably more adapted to the leaf fungal community, and less modifications on the fungal community composition of the sharing niche are needed for disease development. Accordingly, few changes were observed in fungal composition of leaves of cv. *Verdeal Transmontana* when analyzing leaves from trees with different levels of OLS incidence. We also found that in trees with the highest OLS disease incidence, the foliar fungal community composition mas more similar among the three cultivars, thereby providing evidences that the pathogen *V. oleaginea* itself alter fungal community of leaves during disease development. A similar pattern was previously observed by our team in the same pathosystem when studying fungal communities on asymptomatic and symptomatic leaves (Gomes et al., *submitted*). The results from the present study are in line with the now accepted idea that disease development is dependent on the adaptation of pathogen to the new environment, as well as on the interactions outcomes established with other microorganisms in the shared niche (McNally and Brown, 2015; Gomes et al., *submitted*). However, further work is still required to confirm this.

5.5.2 Is OLS incidence related to host plant composition on phenolic and volatile compounds?

Phenolic and volatile compounds are two relevant groups of secondary metabolites involved in plant resistance against pathogens (Quintana-Rodriguez et al., 2015; Lattanzio et al., 2006). These compounds may perform direct defensive functions by inhibiting the development of pathogens, or indirectly by signaling the activation of plant defense responses (Quintana-Rodriguez et al., 2015; Lattanzio et al., 2006). Despite their recognized importance in plant protection against pathogens, the impact of plant secondary metabolites production on disease incidence is less known (Niinemets et al., 2013).

In the present study, we demonstrated that leaf volatile emissions changed both quantitatively and qualitatively in leaves from trees exhibiting different incidences of OLS disease. Detected variations were different according to the host cultivar: the most OLS-tolerant cultivar (cv. *Cobrançosa*) displayed greater changes with OLS incidence than the most OLS-susceptible (cv. *Verdeal Transmontana*). This variation in cultivar response to OLS disease incidence suggests that volatile compounds can probably contribute to plant OLS-resistance/tolerance. As far as we know, this is the first time that such differences on cultivar response have been reported, leaving us to speculate on the underlying mechanism. Cultivars differences can be caused by the activation of different plant defense pathways. Indeed, in a recent meta-analysis on induced plant volatiles, the diverse effects of pathogenic fungi were attributed to differences in induced defense pathways (Ameye et al., 2017). Curiously, the observed response of the most OLS-tolerant cultivar to the increase of disease incidence was associated with a suppression rather than an induction of volatile emissions. Similarly, a pathogen attack has been shown to reduce volatile emissions in maize (Seidl-Adams et al., 2015). Reduction on volatile emissions has sometimes been associated with enhanced defense responses, suggesting that volatiles may also act as disease suppressors (Erb, 2018). However, little is known about such volatile capacity and mechanisms involved in the process (Erb, 2018). In the present work, some volatile compounds (*i.e.* α-farnesene, *p*-cymene and 1-octanol) were also found to be positively correlated with each other and with OLS incidence, suggesting that they may be integrated in a specific pathway to yield a higher OLS incidence. Given the capacity of volatiles to regulate different signaling cascades involved in plant defense, the integration of these volatile compounds through a signaling crosstalk is likely (Erb, 2018).

The phenolic composition of olive tree leaves also changed with OLS disease incidence levels, displaying a variable pattern depending on the cultivar. As for volatile compounds, the observed differences on phenolics might reflect the variation of olive tree cultivars, with consequences on their tolerance/susceptibility to disease. A relation between phenolic composition and susceptibility to infection was also found in Norway spruce, when attacked by the needle bladder rust (Ganthaler et al., 2017). The possible contribution of phenolic compounds to the OLS-resistance/tolerance of the host cultivar was further reinforced by the positive correlation found between some phenolic compounds (*i.e.* luteolin, rutin, verbascoside and apigenin-7-*O*-glucoside) and OLS disease incidence. However, further studies are still needed to confirm the role of these phenolics in the susceptibility of olive tree cultivars to OLS disease.

5.5.3 Is there any combination between secondary metabolites and fungal consortia associated to different OLS disease incidences?

Previous works on plant defense responses to pathogen attacks mainly used reductionist approaches, by focusing on host plant protection conferred by either fungal endophytes (*e.g.* Lugtenberg et al., 2016) or plant secondary metabolites (*e.g.* Du Fall and Solomon, 2011). In the present study, disease incidence was interlinked for the first time to host cultivar, to fungal communities inhabiting leaves and to leaf metabolite composition. Different olive tree cultivars, grown in the same field, exhibited distinct fungal communities on their leaves and displayed diverse leaf metabolite compositions. Thus, host cultivar appears to affect not only leaf fungal composition, but also metabolite profiles. Similarly, previous studies reported the contribution of host genotype to the microbial assemblage and composition of secondary metabolites of their leaves in natural environments (Wagner et al. 2016; Misran et al., 2018). Beyond host cultivar, the interaction effects between fungi and metabolite compounds could also play an important role on the composition of each other. Accordingly, changes on fungal and metabolite composition in leaf samples from trees with different incidence levels of OLS disease showed a similar trend, suggesting a possible link between fungi and metabolites. This relationship is further reinforced by the significant correlations found between some fungal OTUs and various metabolites. Despite further analysis is required, we hypothesized that fungal communities residing in olive leaf could influence the metabolites of host plant, as suggested by Farré-Armengol et al. (2016) in *Brassica nigra.* Reciprocally, leaf metabolites could also affect fungal communities on olive leaves (Zambell and White, 2017).

A strength of our work is the identification of fungal OTUs and secondary metabolites strongly associated with OLS disease incidence. The lowest level of OLS incidence, which was found to be associated to the most OLS-tolerant cultivar (cv. *Cobrançosa*), was linked to the presence of *P. domesticum* that appears to suppress OLS disease. Indeed, a significant negative correlation was found between this fungus and OLS disease incidence. This possibility is an interesting finding that is worth investigating. Although *P. domesticum* has been already described to colonize the inner tissues of other plant species (Hammami et al., 2016), information about their role in conferring host plant protection against biotic stress is completely absent. The highest level of OLS incidence, which was associated to the most OLS-susceptible cultivar (cv. *Verdeal Transmontana*), was found to be positively correlated with various fungal OTUs and metabolites. Among the fungal taxa positively correlated with OLS incidence, *Alternaria* sp. and *Fusarium* sp., are already known to be important plant pathogens causing various diseases in several plant species (Wei et al., 2018; Hernandez-Escribano et al., 2018). In what concerns olive tree, only few reports described their capacity to infect olive fruits, causing fruit-rot (Trapero-Casas et al., 2009; Moral et al., 2008). However, both genera have been described as part of synergistic pathogen-pathogen interactions that often lead to increased disease severity (Lamichhane and Venturi, 2015). Both fungi are likely to play a similar role in our pathosystem. The positive correlation of specific secondary metabolites with OLS incidence could be due to their role on OLS disease development or as part of plant defense responses. Metabolites could be produced by pathogen for disease progression or improving the growth of other fungi to enhance disease incidence, but can also be produced by plants as a defense mechanism. Among the positively correlated metabolites, both α-farnesene and *p*-cymene seem to be the most important volatiles produced in leaves from trees with higher OLS disease incidence. These two sesquiterpenes have been described as important players on plant defenses against pathogen attacks (Holopainen and Gershenzon, 2010; Kessler et al., 2006). Other phenolic compounds, apigenin-7-*O*-glucoside and verbascoside, could also play a role on OLS plant responses, since their levels were previously described to increase after bacterial infection of olive tree (Schmidt et al., 2014; Markakis et al., 2010). In addition, other phenolics (*e.g.* flavonoids and cinnamic acid derivatives) and volatile (e*.g.* ester) compounds were also positively correlated with OLS disease incidence, although without significance. Therefore, the role of those positively correlated metabolites with OLS disease incidence is more likely to be part of plant defense responses to pathogen attack.

From this study we conclude that fungal communities and metabolite compositions can govern disease incidence, in association with plant genotype. The OLS-tolerant cv. *Cobrançosa* displayed greater variation in fungal and metabolite assemblages among trees with different OLS incidence, when compared to OLS-susceptible cv. *Verdeal Transmontana*. Thus, it is likely that OLS-susceptibility differences of cultivars could be in part related with their composition on fungi and metabolites (both phenolic and volatile compounds). These differences in host cultivar proprieties seem to result from complex interactions between host plant, fungi and metabolite composition, which together influence OLS disease incidence. Our work also identified key fungi and metabolites that could play an important role in the susceptibility/tolerance of cultivars to OLS disease. Their role on OLS disease development should be considered in future research works.

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5.7 Supporting Information

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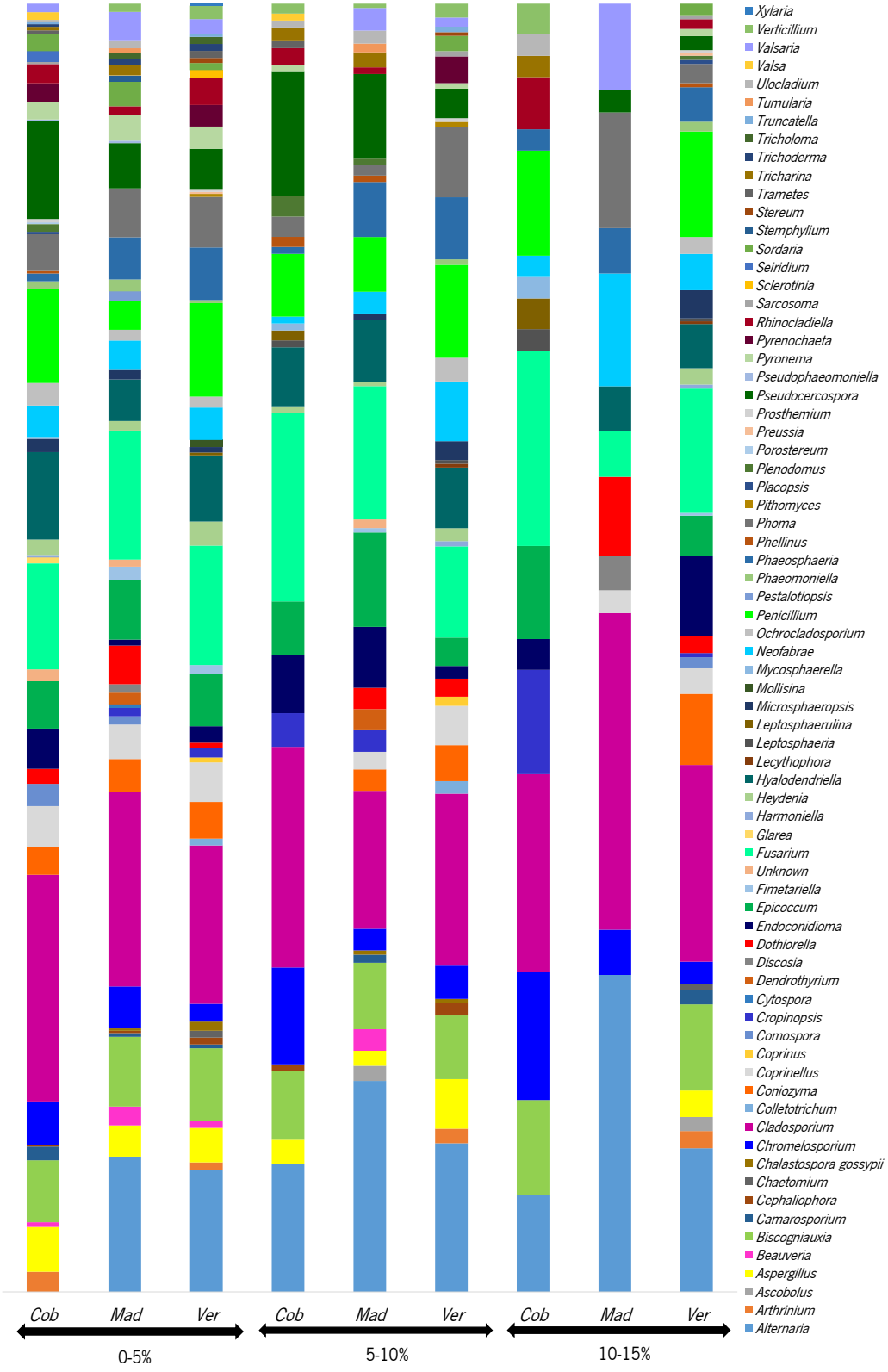

Fig. S5.1 – Relative abundance (%) of fungal genera isolated from leaves of olive trees from distinct cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*), displaying different levels of OLS disease incidence (0-

5%, 5-10% and 10-15%). Fungal isolates exclusively found on the episphere (leaf surface) and endosphere (leaf interior) are shown in green and purple color, respectively.

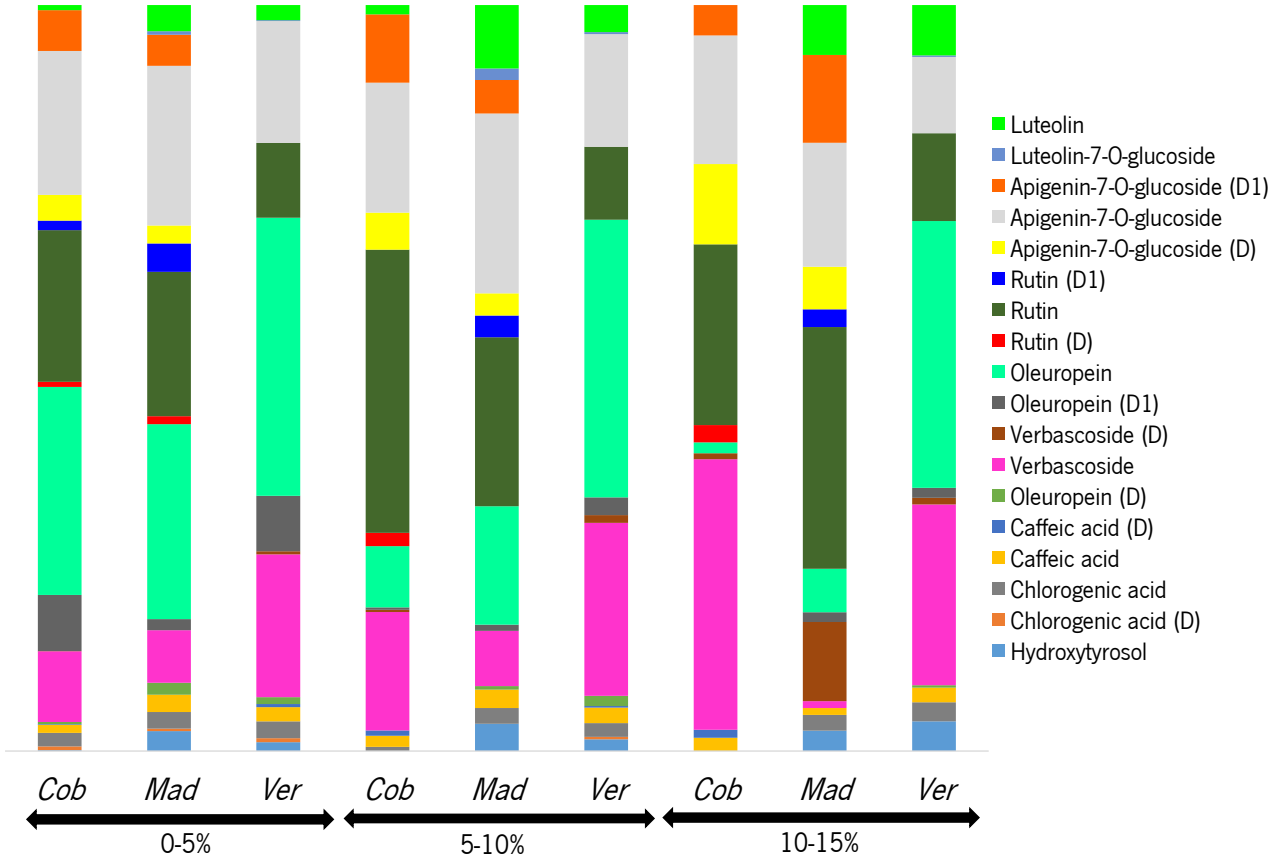

Fig. S5.2 – Relative contents of phenolic compounds (%) detected in leaves of olive trees from distinct cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*), displaying different levels of OLS disease incidence (0-5%, 5-10% and 10-15%).

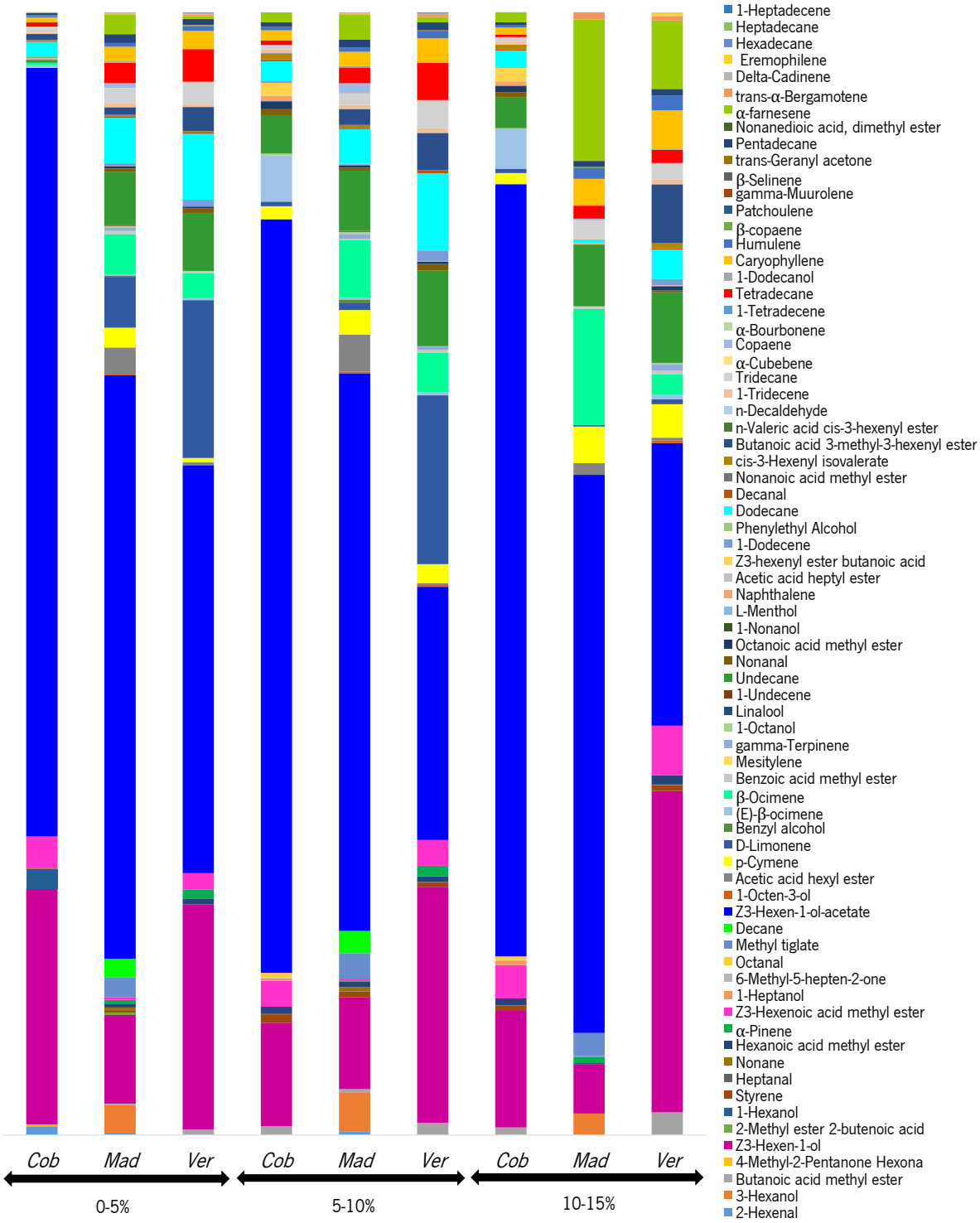

Fig. S5.3 – Relative contents of volatile compounds (%) detected in leaves of olive trees from distinct cultivars (*Cobrançosa*, *Madural* and *Verdeal Transmontana*), displaying different levels of OLS disease incidence (0-5%, 5-10% and 10-15%).

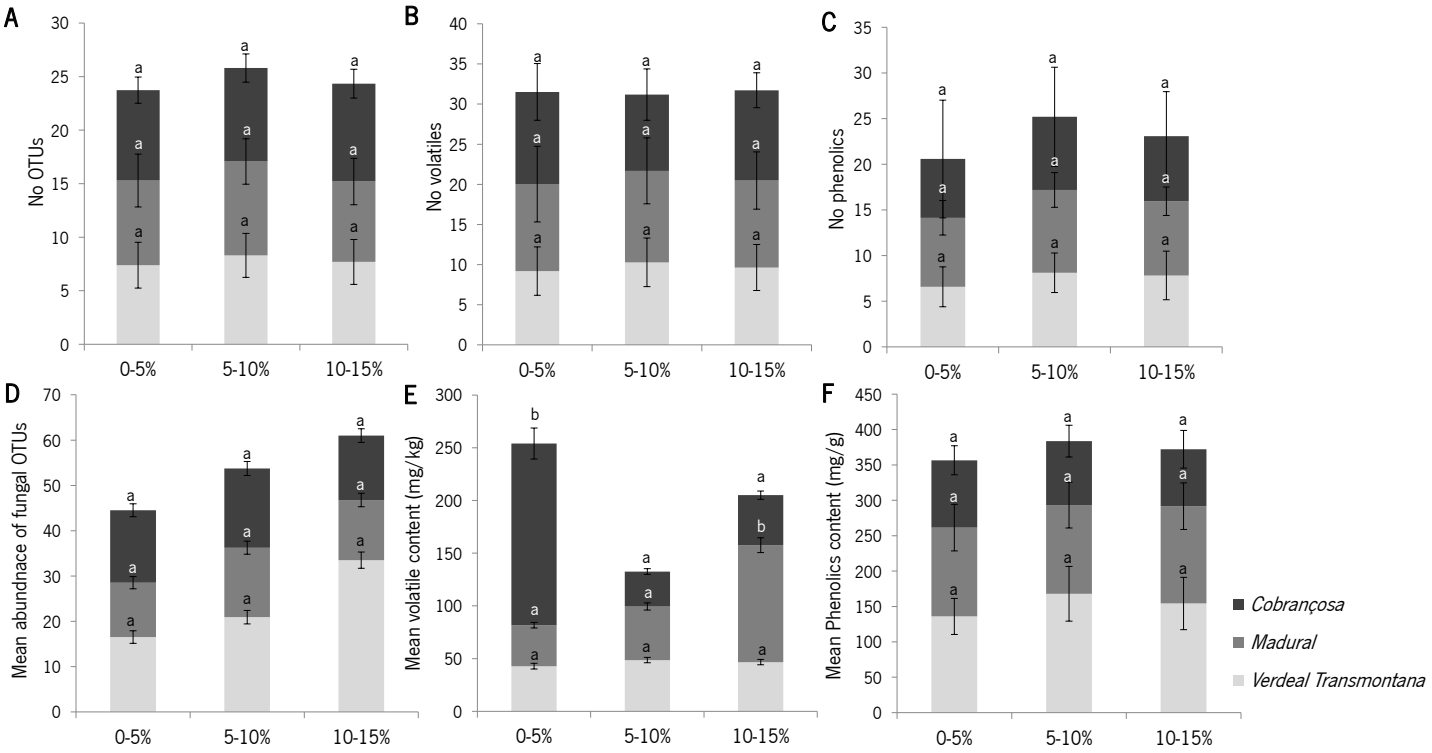

Fig. S5.4 – Number and abundance of foliar fungal OTUs and metabolites, among different OLS disease incidence levels (0-5%, 5-10%, 10-15%) within each olive tree cultivar (*Cobrançosa*, *Madural* and *Verdeal Transmontana*). (**A**) Mean number of fungal OTUs; (**B**) Mean number of volatiles; (**C**) Mean number of phenols; (**D**) Mean abundance of fungal OTUs; (**E**) Mean volatile contents; (**F**) Mean phenol contents. For each parameter, different letters mean significant differences ($P < 0.05$) within each olive tree cultivar.

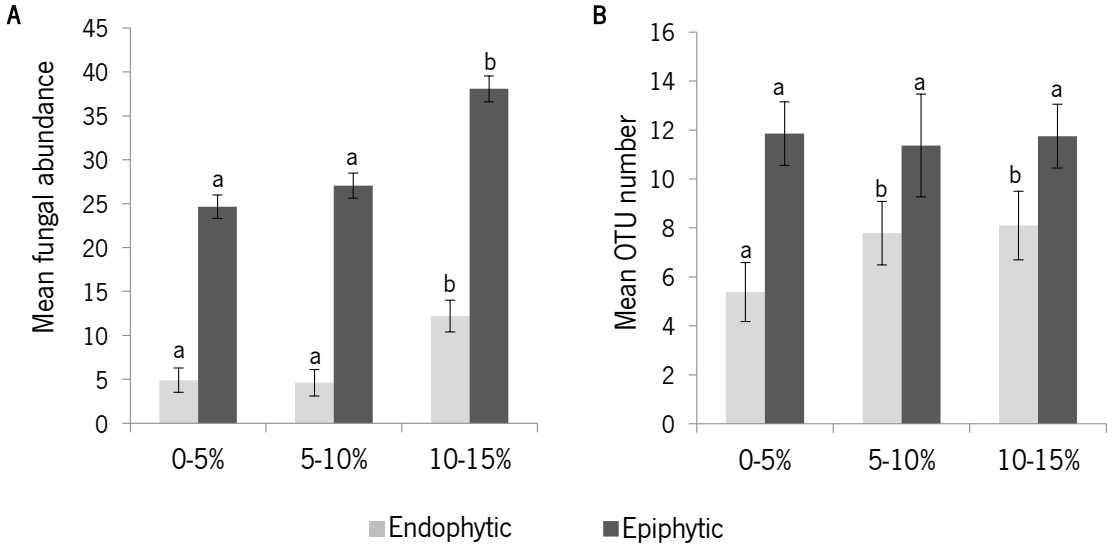

Fig. S5.5 – Number (**A**) and abundance (**B**) of foliar epiphytic and endophytic fungal OTUs in trees displaying different levels of OLS disease incidence (0-5%, 5-10%, 10-15%). Different letters mean significant differences ($P < 0.05$) within each fungal community.

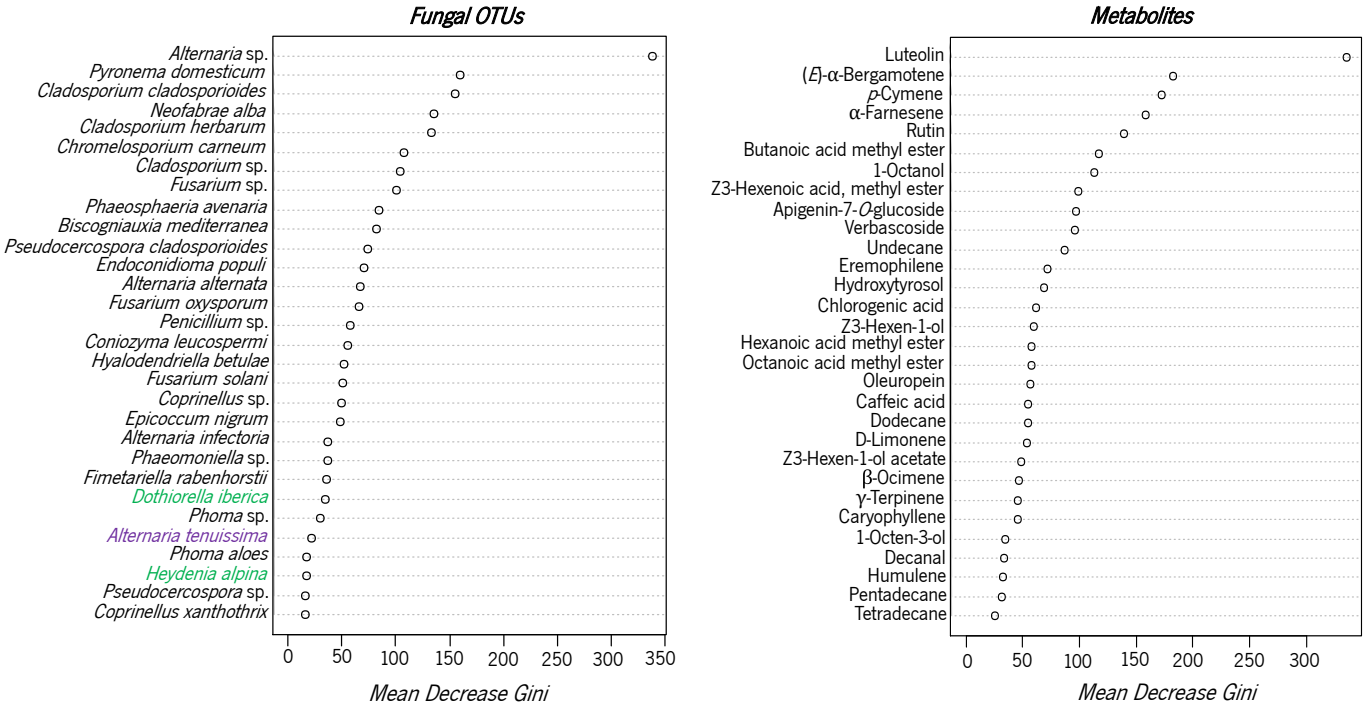

Fig. S5.6 - Ranking of fungal OTUs and metabolite compounds (phenols and volatiles) that displayed the most important impact for explaining OLS-disease incidence on olive trees. *Gini coefficient* value is a measure for variables importance on OLS-disease. The highest values represent the best predictors. Fungal isolates exclusively found on the episphere (leaf surface) and endosphere (leaf interior) are shown in green and purple color, respectively.

Table S5.1 - Analysis of similarity (ANOSIM) of foliar fungal communities (total, endophytic and epiphytic) and metabolites profiles present in olive trees displaying different levels of OLS incidence (0-5%, 5-10%, 10-15%). This analysis was performed with Bray-Curtis distance and considering trees from all cultivars. Numbers refers to R-values, being *P*-values represented in brackets.

	OLS disease incidence ranges comparison		
	0-5% *vs.* 5-10%	5-10% *vs.* 10-15%	0-5% *vs.* 10-15%
Fungi (total)	0.35 (0.001)	0.39 (0.001)	0.41 (0.001)
Endophytes	0.39 (0.002)	0.16 (0.018)	0.44 (0.001)
Epiphytes	0.33 (0.006)	0.35 (0.001)	0.37 (0.017)
Metabolites	0.33 (0.001)	0.16 (0.021)	0.55 (0.001)

Chapter 6

Concluding remarks and future perperctives

6.1. Concluding remarks and future perspectives

The olive leaf spot (OLS) and olive knot (OK) are, among the diseases affecting olive trees, the most serious constrains to worldwide olive crop production (Obanor et al., 2008; Quesada et al., 2010; Salman et al., 2011; Marchi et al., 2005). Crop losses caused by OLS mainly result from defoliation of infected leaves, while OK produces tumorous galls or knots, mostly on the stems and branches of infected trees. The main aim of this work was to elucidate the role of fungal communities, present in leaves and twigs of olive trees, in conferring host protection to both diseases. Specifically, studies were performed to know how fungal microorganisms residing in (endophytes) and on (epiphytes) olive tree phyllosphere interact among themselves and with their plant hosts, and how these interactions influence the host plant protection to both diseases. Thus, this work was initiated by a characterization of fungal communities of olive tree phyllosphere ("Who is there?") and assessing the factors that are responsible for microbial composition ("Which factors contribute to their shaping?"). Finally, fungal communities were related with plant susceptibility to both diseases ("What can they do?").

6.2 Who is there?

The fungal community associated with leaves and twigs of olive tree was rich and abundant, comprising species belonging mainly to Ascomycota phyla, which were included in 63 families, and 127 genera. A greater diversity and abundance of fungal epiphytes was found when compared to endophytes. Both fungal communities also differed in terms of species composition. Davidiellaceae, Psathyrellaceae, Pleosporaceae, Nectriaceae and Trichocomaceae were the most dominant fungal groups within epiphytic communities, whereas Leptosphaeriaceae, Pleosporaceae, Pyronemataceae, Trichocomaceae, and Pezizaceae were the most abundant within endophytic fungal communities. The level of dissimilarity on fungal composition between epiphytic and endophytic communities also differed according to the season and olive tree organ.

6.3 Which factors contribute to their shaping?

The significance of different abiotic (climate-related) and biotic (plant- and microbe-related) factors on phyllosphere fungal assemblages was investigated. Results from **chapter 2** revealed that season was a key factor structuring olive phyllosphere fungal communities, especially epiphytic community. Moreover, endophytic and epiphytic communities were found to be driven by different factors. Wind speed and temperature were important in shaping epiphytes, whereas plant organ,

rainfall and temperature were the major drivers of endophytic community. Results from **chapters 3 to 5** also indicated that plant genotype (at cultivar level) and phenotype (related with volatile and phenolic composition), microbial interactions occurring in plant, as well as disease presence, contribute to the shaping of fungal communities in olive tree phyllosphere. These factors were also found to distinctly affect endophytic and epiphytic communities.

6.4 What can they do?

To better understand plant-microbe and microbe-microbe interactions with respect to their impact on plant health, fungal communities were compared between asymptomatic and symptomatic organs (leaves or twigs) of olive cultivars displaying different susceptibilities to OLS and OK diseases (**chapters 3 and 4**, respectively). Results indicate that OLS and OK diseases disturb the resident fungal communities of leaves and twigs, respectively. This may reflect changes made by the corresponding pathogens to the habitat, probably as a result of their interaction with the resident fungi (pathogen-fungal interaction). This effect was most notorious within epiphytes than within endophytes. In particular, a reduction on epiphytic fungal abundance and diversity, as well as changes on epiphytes composition, was observed in symptomatic leaves/twigs. Host genotype (at cultivar level) was also found to structure phyllosphere fungal community, but with distinct effects according to the pathosystem. Indeed, in the OK pathosystem a more pronounced effect of the cultivar was detected in symptomatic (*i.e.* knots) than in asymptomatic twigs, whereas in OLS pathosystem, the host genotype was an important driver of fungal communities of asymptomatic but not in OLS-symptomatic leaves. Moreover, when became infected by pathogen, asymptomatic leaves/twigs drastically change their associated fungal assemblages, particularly in tolerant when compared to susceptible cultivars to both diseases. Therefore, the complex interplay between pathogen, host plant and its indigenous microbiota, seems to be critical for the establishment of fungal communities (and the underlying pathogen) in olive phyllosphere. But, how does phyllosphere fungal communities influence olive tree tolerance to diseases? **In chapter 5**, this question was addressed by examining the relationship between host cultivar, foliar fungal community, foliar metabolite (volatile and phenolic) profile, and OLS disease incidence. The results indicated that cultivar susceptibility to OLS might be in part related with the composition on fungi and metabolites of their leaves. Indeed, only taking into account fungal and metabolites composition of leaves, the most tolerant cultivar was found to be associated to the lowest OLS-disease incidence level, whereas the most susceptible cultivar was more correlated with the highest

OLS-disease incidence levelCultivars differences on fungal and metabolite composition seem to be determined by plant itself and also by plant interactions with their associated fungi, along with interactions occurring between fungi-metabolite, fungi-fungi and metabolite-metabolite. Our work also identified key fungi and metabolites (volatile and phenolic compounds) that could play an important role for the susceptibility/tolerance of cultivars to OLS disease.

Despite the contribution of these results to decipher previously undescribed microbe-olive tree and microbe-microbe relationships, the full understanding of the diverse interactions that can take place in the olive phyllosphere is still remote. Future research needs to decipher these complex and dynamic microbial interaction networks and assess their role in plant health. In particular, the exact function of identified key fungi in olive tree health should be studied and carefully examined by using metatranscriptome sequencing, metaproteome and metabolome analysis. These results would be of much relevance for plant health and for designing new strategies for the biological control of OK and OLS diseases.

6.5 References

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“Life doesn’t require that we be the best, only that we try our best.”

H. Jackson Brown Jr.