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Tese de Mestrado

Mestrado em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

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Abstract

Cork oak (*Quercus suber*) is a species of high importance in the Mediterranean. Besides its ecological importance, the cork oak also has a high relevance for the Portuguese economy. This importance is due to the production and processing of cork. However, the sustainability of the cork oak is being threatened due to climate change, which will reduce the availability of water in the Mediterranean, and the occurrence of diseases that increasingly affect their normal development. The charcoal disease, caused by the fungus *Biscogniauxia mediterranea*, leads to decline, and eventually, death of the tree. *Diplodia corticola* is another pathogen of the cork oak and is involved in various diseases considered responsible for the decline of the cork oak in the Mediterranean Basin. Currently, the use of fungicides is the main technique used to combat these problems. However the environmental hazard and toxicity of this process leads to the necessity of finding a more effective method. Biological control is an appropriate strategy as an alternative to this problem. Endophytes have the potential to be used as biological agents to control diseases. In addition to being described as potentiators of plant responses to stressful situations, endophytes have the potential for use as biological control agents. To identify endophytic fungi in cork oak, four sites of continental Portugal (Bragança, Gerês, Alcobaça and Grândola), which had differences in water availability, were selected to carry out the collection of biological material. Subsequently, the community of fungal endophytes of three organs (leaves, stems, roots) was evaluated. The trees analysed were largely colonized by fungi of the phylum Ascomycota. In general, Grândola had the highest colonization frequency and diversity of endophytes, while Alcobaça had the lowest. Of all study sites, the cork oaks from Gerês showed a more distinct community, differentiating from all other locations. The roots have a more diverse fungal community than the leaves and stems. It was found that the pathogenic fungi tested (*B. mediterranea* and *D. corticola*), essentially affect the aboveground organs of the tree, however no disease symptoms were detected. Furthermore, Gerês was the only study site that did not show any signs of infection by these pathogenic fungi. *D. corticola* only infected the southern regions (Alcobaça and Grândola), where as *B. mediterranea* also infected the trees in Bragança. Taking this into account, many fungi were selected for antagonism assays of biological control. Three species (belonging to two genera) may potentially act as biocontrol agents against diseases in *Q. suber*.

Key-words: *Quercus suber*; *Biscogniauxia mediterranea*; *Diplodia corticola*; ecology; diversity; endophytic fungi; antagonism

Resumo

O sobreiro (*Quercus suber*) é uma espécie de elevada importância na região do Mediterrâneo. Para além da sua importância ecológica, o sobreiro também apresenta uma elevada relevância para a economia Portuguesa. Esta relevância provém da indústria de produção e transformação da cortiça. No entanto, a sustentabilidade do sobreiro poderá estar ameaçada devido às previstas alterações climáticas, que diminuirão a disponibilidade de água na região do Mediterrâneo, e à ocorrência de doenças que afetam cada vez mais o seu normal desenvolvimento. A doença do carvão do entrecasco, causada pelo fungo *Biscogniauxia mediterranea*, origina um acentuado declínio na árvore, podendo levar à sua morte. *Diplodia corticola* é um outro fungo patogénico do sobreiro, que está envolvido em várias doenças consideradas responsáveis pelo declínio do sobreiro na Bacia do Mediterrâneo. Atualmente, a utilização de fungicidas é a técnica mais usada para o combate destes problemas. Contudo, o risco ambiental e a toxicidade deste processo levam à necessidade da utilização de outros métodos eficazes. A luta biológica é uma estratégia adequada como alternativa a este problema. Além de se encontrarem descritos como potenciadores das respostas das plantas a situações de stresse, os organismos endófitos têm potencial para serem utilizados como agentes na luta biológica. Para identificar fungos endófitos em sobreiro, quatro locais de Portugal continental (Bragança, Gerês, Alcobaça e Grândola), que apresentavam diferenças na disponibilidade de água, foram selecionados para realizar a recolha de material biológico. A comunidade de fungos endófiticos foi avaliada em três órgãos (folhas, caules, raiz). Os sobreiros testados encontram-se maioritariamente colonizados por fungos do filo Ascomycota. Os sobreiros de Grândola apresentaram uma maior frequência de colonização e diversidade de endófitos, enquanto que os de Alcobaça apresentaram a menor. De todos os locais, os sobreiros do Gerês foram os que apresentaram uma comunidade mais distinta, diferenciando-se de todos os outros locais. As raízes apresentaram uma comunidade de fungos mais diversa do que as folhas e caules. Verificou-se ainda que os fungos patogénicos testados (*B. mediterranea* e *D. corticola*), infetaram essencialmente a parte aérea da árvore, apesar de não terem sido verificados sintomas nas mesmas. O Gerês foi o único local de estudo que não apresentou nenhum sinal de infeção por parte destes fungos. O fungo *D. corticola* infetou exclusivamente as regiões mais a sul (Alcobaça e Grândola), enquanto o fungo *B. mediterranea* foi também encontrado na região de Bragança. Tendo isto em

consideração, diversos fungos foram selecionados para estudos de antagonismo e luta biológica, tendo sido encontradas três espécies (pertencentes a dois géneros) que poderão potencialmente atuar como agentes de biocontrolo contra as doenças de *Q. suber*.

Palavras-chave: *Quercus suber*; *Biscogniauxia mediterranea*; *Diplodia corticola*; ecologia; diversidade; fungos endófitos; antagonismo

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List of Abbreviations

µL	Microliter
bp	Base pair
DNA	Deoxyribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
dNTPs	Deoxynuoside triphosphates
h	Hour
s	Seconds
min	Minute
ITS	Internal transcribed spacer
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
ºC	Degree Celsius
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
Spp.	Species (plural)
ha	Hectare
mm	Millimetre
IAA	Auxin indole-3-acetic acid
ISR	Induced systemic resistance
CF	Colonization frequency
RA	Relative abundance

1. Introduction

Cork oak (*Quercus suber* L.) is a sclerophyllous evergreen tree species from the Fagaceae family, which includes other important genera, such as *Fagus* or *Castanea*. The *Quercus* genus is one of the most important woody genera in temperate ecosystems of North America, Europe and Mediterranean Basin, where more than 300 species are currently present (Toumi and Lumaret, 2001).

The healthy development of cork oak trees requires an annual mean temperature of 13 to 18°C. Cork oak growing areas are characterized by warm to hot dry summers and cool wet winters with unpredictable amount of rainfall (Cowling *et al.*, 2005). As a result, the most extensive forest areas of *Q. suber* are found on the Atlantic coast of the Iberian Peninsula (Figure 1; Gil and Varela, 2008). The highest levels of genetic diversity have been found in Spain (Center and South), also suggesting that the Iberian Peninsula is a genetic refuge for this species (Gil and Varela, 2008). All continental Portugal has climatic conditions that allow the adaptability of the cork oak and Alentejo region has the largest sites of distribution (Natividade, 1990). The current cork oak distribution mainly results from various circumstances, like fires, excessive grazing, forestation of fast growing species, such as eucalyptus, the development of urban areas and the intensive exploration of cork (Bellarosa, 2003).

Figure 1. Global distribution of *Q. suber*. Cork oak is mainly found in the coastal regions of the western Mediterranean Basin, as well as in several islands of the Mediterranean sea and the north of Africa (Gil and Varela, 2008).

Q. suber is a strictly Mediterranean evergreen oak species with a genome comprising 24 chromosomes. The cork oak is a medium-size tree, ranging from 10 to 20 meters height (Figure 2A), able to survive to an age of 250 years (Torres, 1979). *Q. suber* has several drought avoidance adaptations, such as deep-reaching root systems (Kurz-Besson *et al*., 2006), relatively long leaf longevity (Oliveira *et al*., 1996) and a thick bark, the cork, which controls water loss from the trunk and branches (Oliveira and Costa, 2012). Cork is a highly valuable product, resulting from the activity of a secondary meristem called cork cambium (Pintus, 1996). Every seven to nine years, cork is removed from the branches and trunk throughout the tree's lifetime (Figure 2B) (Oliveira *et al*., 2002).

Cork oak has a high social and economic importance in Portugal (Costa and Oliveira, 2015). Cork products (wine bottling, fashion products, construction, etc.) represent approximately 2.3% of the total annual export of the country (Pereira *et al*., 2008). Portugal leads the global cork production sector with an average share of over 65%. In addition to being the largest producer, Portugal is also the main importer of raw cork, as well as the main country to transform cork (APCOR, 2016).

Figure 2. Adult cork oak trees (*Q. suber*). **A.** Healthy cork oak tree with an approximate height of 15 meters. **B.** Cork oak tree after the cork harvest, showing a red trunk, typical from a recently cork stripped tree.

In Portugal, cork oak trees can grow in an agroforestry system known as *montado*. This system, similar to *dehesas* in Spain, presents a low tree density (usually between 30-60 trees/ha) thus comprising open forest areas (Carreiras *et al.*, 2006; Correia *et al*., 2011). Alongside cork oak trees, plants from Fabaceae and Poaceae families are commonly observed, forming agro-silvopastoral systems (Gil and Varela, 2008). *Sobreiral* is another cork oak forestry system, characterized by a higher tree density (more than 150-200 trees/ha) and does not allow intercropping with agriculture crops (Correia and Oliveira, 1999; Carreiras *et al.*, 2006; Correia *et al*., 2011). The main cork oak forestry system found in Portugal, mainly in the southern region, is the *montado*, which is primarily used for cork production (Josep *et al.*, 2014). Currently, much of the center and southern coast of Portugal is dominated by semi-natural or discontinuous cork oak stands, cultivated in the *montado* system. Moreover, in areas where cork oaks are abundant, usually there is low anthropogenic pressure (Varela and Eriksson, 1995). Most, if not all, plants in natural ecosystems are able to form interactions with fungal endophytes and mycorrhizal fungi (Petrini, 1986). These fungi have an enormous influence on plant fitness, ecology and evolution (Rodriguez *et al.*, 2009).

1.1. Environmental relevance of endophytic microorganisms

Endophytes can be described as any microbe that can be isolated from the inside of asymptomatic plant tissue. These microorganisms, bacteria and fungi, associate with their plant host, establishing a microbial community in the plant endosphere, providing benefits like growth promotion and stress tolerance (Hardoim *et al.*, 2015). Fossilized tissue of different plant organs revealed that plant-endophyte interactions may have evolved from the period where higher plants colonized the planet (Redecker *et al.*, 2000), thus playing an important role in the evolution of life on land. The fungal group *Glomeromycota* has for a long time been the prime candidate for interaction with the first terrestrial plants, in the Ordovician era (Bidartondo *et al.*, 2011). Recently, members of the *Mucoromycotina* were also speculated to have had symbiotic interactions with the first terrestrial plants (Hardoim *et al.*, 2015). Endophytes live asymptomatically inside the plant host (Freeman *et al.*, 2001). This interaction is based on a balance between the demands of the endophyte and plant response. If the interaction is disturbed, disease symptoms could appear and the endophyte could be excluded by induced host defense reactions (Kogel *et al.*, 2006).

Recent studies have been providing techniques that significantly improved the isolation of fungal endophytes, turning their study easier (*e.g.* Greenfield *et al.*, 2015). Thanks to these advances, gaps in the knowledge of this important interaction (plant-fungal endophyte) can finally be studied.

1.2. Diversity and classification of endophytic fungi

Fungal endophytes belong to the *Glomeromycota* (40%), *Ascomycota* (31%), *Basidiomycota* (20%), *Zigomycota* (0.1%) and other unknown phyla (8%) (Hardoim *et al*., 2015). The *Glomeromycota* phylum merely comprises arbuscular mycorrhizal endophytes, 39% of which belong to the class *Glomeromycetes* (Schubler *et al.*, 2001; Hardoim *et al.*, 2015). Every member of this class forms ubiquitous endosymbioses with most land plants and have an enormous economic and ecological meaning (Jeffries *et al*., 2003).

A large number of *Ascomycota* endophytes belongs to the class *Dothideomycetes* (15%) (Hardoim *et al*., 2015). This class contains necrotrophic plant-pathogenic fungi capable of producing host-specific toxins (phytotoxic metabolites and peptides) that are required for pathogenicity and are only active against particular plant species in certain conditions (Horbach *et al.*, 2011). Secreted toxins contribute to virulence or pathogenicity by disrupting host cells, facilitating the colonization process (Stergiopoulos *et al.*, 2013). *Epicoccum* and *Alternaria* are two important genera of this class. Some *Alternaria* species can be considered pathogens, but can be detected in high abundance in healthy plants (Jumpponen and Jones, 2009). Another important class of this phylum is *Sordariomycetes* (9%), comprising the well-known *Fusarium, Verticillium, Rosellinia* and *Balansia* genera (Hardoim *et al.*, 2015). Also, in this class, the *Clavicipitaceae* includes many symbiotic species associated with grasses, sedges and rushes (Rodriguez *et al*., 2009).

Among *Basidiomycota*, the *Agaricomycetes* class comprises the largest number of endophytes, mostly mushroom-forming fungi that cause wood decay (Hardoim *et al*., 2015). Endophytes from the order *Sebacinales* (*Agaricomycetes* that usually do not produce mushrooms) tend to form mycorrhizal symbioses with a wide range of plants, such as members of *Ericaceae* and *Orchidaceae* families (Weiß *et al.*, 2011). Other *Basidiomycota* classes that contain endophytes are *Microbotryomycetes, Cystobasidiomycetes, Tremellomycetes* and *Atractiellomycetes*. The existence of several

taxa comprising strains with and without pathogenic effects indicates that the endophytic functions cannot necessarily be associated with taxonomy (Hardoim *et al*., 2015).

Besides their phylogeny, the life history traits of endophytes have been used to distinguish two major groups of endophytic fungi: the clavicipitaceous endophytes (C-endophytes) and the nonclavicipitaceous endophytes (NC-endophytes), which are subdivided into three classes. These classes have different taxonomy, evolutionary relatedness, ecological functions and plant hosts (Table 1; Rodriguez *et al*., 2009).

Table 1: Criteria used to characterize fungal endophytic classes. (Rodriguez *et al*., 2009).

	Clavicipitaceous	**Nonclavicipitaceous**		
Criteria	**Class 1**	**Class 2**	**Class 3**	**Class 4**
Host range	Narrow	Broad	Broad	Broad
Tissue(s) colonized	Shoot and rhizome	Shoot, root and rhizome	Shoot	Root
***In planta* colonization**	Extensive	Extensive	Limited	Extensive
***In planta* biodiversity**	Low	Low	High	Unknown
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Fitness benefits*	NHA	NHA and HA	NHA	NHA

*The benefits of habitat-adapted (HA) result from habitat-specific selective pressures such as salinity, temperature and pH. Nonhabitat-adapted (NHA) benefits are common among endophytes, despite their habitat of origin, and range from drought tolerance to growth enhancement.

C-endophytes (class 1 endophytes) are represented by a small percentage of phylogenetically related *Clavicipitaceae* (*Ascomycota*) species that are restricted to some cool/warm season grasses, such as sledge and rush hosts. These endophytes mainly colonize the plant shoots, but their presence in the rhizome has also been detected (Bischoff and White, 2005). Three types of class 1 endophytes have been reported. Type I comprises symptomatic and pathogenic species, whereas Type II and Type III represent asymptomatic endophytes (Clay and Schardl, 2002). These fungi are usually recognized by providing benefits to the plant host, such as increasing drought tolerance, plant biomass, and through the production of toxic metabolites able to decrease herbivory (Clay, 1988).

Ecological interactions, life history and other traits allow NC-endophytes to be subdivided into three different classes (Table 1). Despite the lack of functional studies, NC-endophytes have been shown to confer fitness benefits, such as increased growth and yields, biotic and abiotic tolerance and nutrient acquisition improvement (Rodriguez *et al.*, 2009). Differences between endophyte classes reside on their transmission patterns and colonization strategies (Rodriguez *et al.*, 2008).

1.3. Transmission and colonization strategies of endophytic fungi

Endophytes can be transmitted either vertically or horizontally. When transmitted vertically, the endophyte never leaves its host, being transferred from parent to offspring via seeds. Fungal reproduction through asexual or sexual spores leads to horizontal transmission, where endophytes may spread between plants in a population or community (Tadych *et al*., 2014). In this case, endophytes can enter the plant endosphere compartment through the caulosphere (bark), rhizosphere (root), laimosphere (belowground tissues), anthosphere (flower), spermosphere (germinating seed), phyllosphere (leaf), or carposphere (fruits) environments (Compant *et al*., 2016)**.** Many variables influence the colonization capability of endophytic fungi (Figure 3), such as plant tissue and type, biotic and abiotic environmental conditions, strain type and plant genotype (Hardoim *et al*., 2015). C-endophytes and NC-endophytes have different colonization strategies. The transmission of C-endophytes (class 1) is essentially vertical, passing on to the offspring by seed infection (Saikkonen *et al*., 2002). These endophytes colonize intercellular spaces of newly forming shoots and proliferate in the shoot meristem, systemically colonizing the plant host (Saikkonen *et al*., 2002**;** Hardoim *et al*., 2015). Despite the fact that the transmission of C-endophytes is essentially vertical, some species of *Neotyphodium* and *Epichloe* may also be transmitted horizontally via leafs falling on the soil (Tadych *et al.*, 2007; Hardoim *et al*., 2015). A study performed on *Lolium perenne* showed that *Neotyphodium* can colonize ovules and appear during infructescense development (Philipson and Christey, 1986).

Among NC-endophytes, those belonging to class 2 have a broad range of hosts and are able to colonize above and below ground plant tissues. In addition, they can be transmitted either vertically or horizontally, as those members from class 1. In contrast, class 3 endophytes are only horizontally transmitted, being restricted to above ground tissues where they form highly localized infections (Rodriguez *et al.*, 2008). This class

comprise the majority of the well-studied endophytes from several tree species, which mainly belong to the subkingdom *Dikaryomycota* (*Basidiomycota* or *Ascomycota*) (Davis and Shaw, 2008; Hardoim *et al*., 2015). Although being also transmitted horizontally, class 4 endophytes are restricted to below ground tissues, where they reside inter- and/or intracellularly in the cortical cell layers (O´Dell *et al.*, 1993; Hardoim *et al*., 2015). These fungi include dark septate endophytes, similar to mycorrhizal fungi. Both classes 2 and 4 are capable of extensive tissue colonization forming systemic infections (Rodriguez *et al*., 2009).

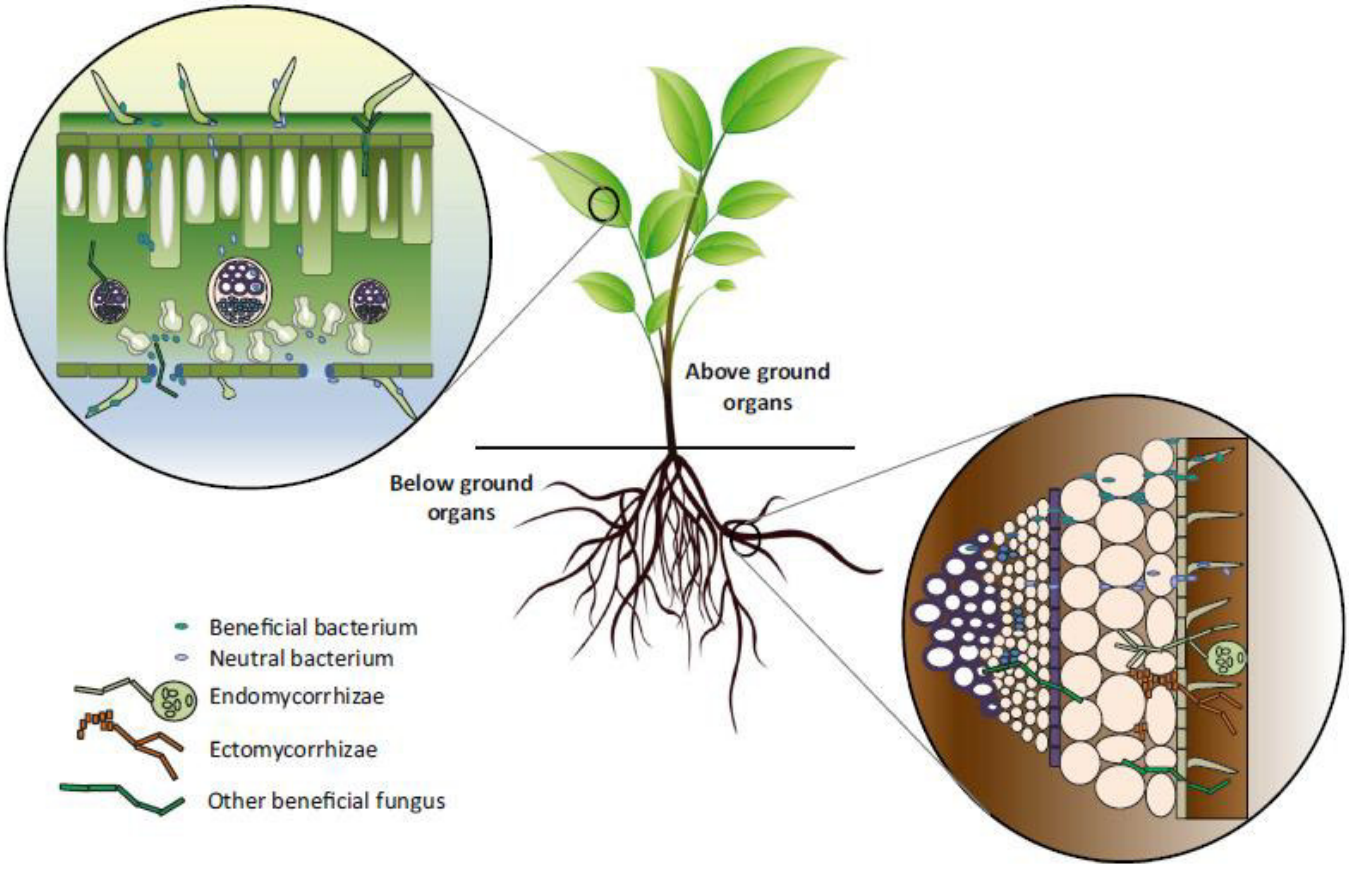

Figure 3. Niches where endophytes can reside inside plants and ways of colonization by endophytes (adapted from Compant *et al.*, 2016).

Microorganisms that need plant tissues to complete their life cycle are described as obligate. Many mycorrhizal fungi and members of the fungal genera *Epichloë, Neotyphodium* and *Balansia* (class 1 endophytes) are well-known examples of obligate microorganisms. Opportunistic endophytes could be found on the surface of plant tissues (epiphytes) and only sporadically enter the plant endosphere. Among these are fungi of the genera *Hypocrea* and *Trichoderma* (Hardoim *et al.,* 2015).

1.4. Ecologic roles of endophytic fungi

The complex interaction between endophyte and host can be described as mutualism, commensalism, saprophytism, parasitism or exploitation, and can change towards a more specialized or different interaction (Zuccaro *et al*., 2011). Commensal endophytes live on the metabolites produced by the host and have no apparent effect on plant performance. On the other hand, many endophytes can confer positive effects on their hosts performance, such as plant growth promotion, stress tolerance, secondary metabolite production and defense against pathogens and herbivores, either via antibiosis or via induced systemic resistance (ISR) (Scortichini and Loreti, 2007; Hardoim *et al*., 2015).

Endophytes are able to promote plant growth through the production of phytohormones, resulting in architectural and morphological changes in plant hosts (Khan *et al.*, 2012, 2015). Cytokinins are frequently observed in endophytes and play an important role in the promotion of plant growth. A fungal strain of the root-colonizing endophyte *Piriformospora indica*, presenting deletions in the cytokinin biosynthesis genes, prevented the plant growth promoting effect (Vadassery *et al.*, 2008). Root endophytes are also known for the production of auxins and gibberellins (Khan *et al.*, 2012). The auxin indole-3-acetic acid (IAA) is known to improve plant growth and increase colonization efficiency, interfering with the host defense system (Navarro *et al*., 2006). Besides the production of plant growth hormones, there are additional compounds produced by fungi that are able to promote plant growth. Adenine and adenine ribosides have been identified as growth promoting compounds in Scots pine endophytes (Vadassery *et al.*, 2008).

Making nutrients available to plants is another mechanism behind plant growth promotion. Some fungal endophytes produce siderophores, which are important for iron acquisition by soil microorganisms (Powell *et al*., 1980) and are also reported to play a role in the induction of ISR (van Loon *et al.*, 2008). Moreover, the interruption of the siderophore biosynthesis gene cluster in *Epichloë festucae* compromised the symbiotic association between *E. festucae* and ryegrass (*Lolium perenne)* (Johnson *et al.*, 2013).

Endophyte infected plants can also present higher plant growth due to enhanced nitrogen (N_2) fixation, which is significantly higher when endophytes are present in the plant host. For example, fungal strains of *Neotyphodium* spp. were shown to protect

plants against N_2 starvation (Ravel *et al.*, 1997). However, this process revealed to be more effective in the presence of bacterial endophytes than of fungal endophytes. Root nodules of leguminous plants are a well-known case study of this effect (Pirttilä *et al.*, 2004). Other benefits, such as protection against pests and herbivores, disease suppression (through the production of secondary metabolites) and stress tolerance, can indirectly arise as a result of plant growth promotion (Compant *et al.,* 2016).

Another well-known effect of endophytes is the plant host protection against different stresses. A recent study showed that tomato plants, colonized with systemic fungal endophytes (class 2) isolated on salinized soil, when exposed to NaCl or drought conditions, present higher root and shoot biomass, better water-use efficiency and higher photosynthetic activity than non-colonized plants (Azad and Kaminskyj, 2016). The root fungal endophyte *Piriformospora indica* was also shown to induce salt tolerance in barley (Baltruschat *et al.*, 2008) and drought tolerance in Chinese cabbage plants (Sun *et al.*, 2010). In all these cases, increases in antioxidant levels were the proposed mechanisms behind plant hosts tolerance to salt/drought stresses. In addition, endophytic association of *Paecilomyces formosus* LWL1 with *Oryza sativa* cv. *Dongjin* (Japonica rice) significantly improved plant growth attributes (such as dry and fresh plant weight or chlorophyll content) under prolonged heat stress conditions (Waqas *et al.*, 2015). Such fungal endophytes were then suggested to be helpful for sustainable crop production under high environmental temperatures. Furthermore, fungal endophytes have been shown to interfere with cold tolerance of rice plants (Redman *et al.*, 2011).

The biological control of parasites and predators of grasses, through the use of selected endophyte strains, has been considered increasingly important because chemical control of diseases and pests is becoming more restricted. Endophyte colonized plants are better protected against insects (*Arthropoda*), pathogenic fungi, bacteria and nematodes, and are less attractive to grazing animals due to the toxic compounds (secondary metabolites) released to the surrounding environment (Latch, 1993). Effective biocontrol strains of *Trichoderma virens* can induce the production of defense-related compounds against a number of different fungi on various hosts (Hanson and Howell, 2004). The endophyte, *Penicillium canescens*, was able to produce growth inhibitors against bacteria and yeasts (Peláez *et al.*, 1998). Many secondary metabolites produced by endophytes are indeed an important source of

antioxidant, immunosuppressive, antifungal, antibacterial, anti-oomycete, insecticidal, nematicidal, and antiviral agents (Hardoim *et al.*, 2015). On the other hand, the plant phytohormone ethylene is a key regulator of pant tissue colonization by some beneficial bacterial endophytes, also promoting root elongation and protection against potential pathogens (Iniguez *et al.*, 2005).

Taking into account the conditions under which the host grows, endophytes can change their effects. For example, the fungus *Fusarium verticillioides* has a dual role in maize, both as a beneficial endophyte and as a pathogen (Bacon *et al.*, 2008). Host genotype and abiotic stress factors may change the delicate balance in endophyte-host interaction, leading to disease symptoms and fungal production of mycotoxins (Bacon *et al.*, 2008; Hardoim *et al*., 2015). Host-fungus interaction studies have improved the understanding of what determines whether a fungus behaves as a parasite or as an endophyte. Colonization in different hosts can cause a fungus to switch lifestyles. For example, *Colletotrichum magna* grows asymptomatically on various species, but is a fungal pathogen in cucurbit plants, causing anthracnose (Freeman *et al.*, 2001). In addition, mutated *C. magna* strains can colonize a broader range of hosts and are able to colonize cucurbit asymptomatically (Redman *et al.*, 1999). This interaction switch and expansion of host range were thus suggested to be due to mutations in the endophyte genome. Nevertheless, loss of fungal pathogenicity does not necessarily result in asymptomatic endophyte development. Endophyte mutations could also lead to higher plant defense efficiency that would restrict fungal growth (Jansen *et al.*, 2005). There are now studies showing that most switches from mutualism to parasitism are due to mutations in a single gene. Molecular analysis of endophyte mutants have revealed a single-copy plasmid insertion into the *NoxA* gene, which encodes an NADPH oxidase. Plants inoculated with these *noxA* mutant became, severely stunted, lost their apical dominance, showed precocious senescence, and eventually died. *In planta* ROS production by *E. festucae NoxA* negatively regulates hyphal tip growth and fungal development, thereby preventing excessive colonization of the plant tissue (Tanaka *et al.*, 2006).

1.5. Studying endophytic communities in oak species

The endophytic mycota of broad-leaved trees of temperate regions has only recently started being studied (Gonthier *et al*., 2006). However, there are already relevant studies on the different relationships between endophytes and their host trees (Kwaśna *et al.*, 2016; Busby *et al*., 2015). In Northern Spain, there is an intensive monitoring of fungal populations in pine plantations, which shows that over time there are changes in the endophytic community (Martinez-Alvarez *et al*., 2012). Studies describing the endophytic mycota of several *Quercus* spp. in healthy and declining trees have also been documented. Declining trees of *Q. robur* and *Quercus cerris* showed a lower population of fungal endophytes than healthy trees (Gennaro *et al.*, 2003). In contrast, in *Quercus robur* roots subjected to flooding, the number of fungal root endophyte species was higher than standard roots (Kwaśna *et al*., 2016). In this later work, the majority of these species belong to class 2 endophytes. Due to the role that endophytes play on drought stress tolerance and their role on the biocontrol of one of the major causal agents of cork oak decline (*Phytophthora* spp.), the knowledge of endophyte communities that reside within cork oak trees could be a fundamental tool for the future of oak stands sustainability (Arnold *et al.*, 2003; Bae *et al.*, 2011; Linaldeddu *et al.*, 2013; Scanu *et al.*, 2013). Furthermore, because cork oak has an important economic relevance, more studies about its endophytic community and their relation with plant health and resistance to stress, are being described (Linaldeddu *et al.*, 2011).

Now more than ever, it is important to understand all relations between endophyte-host and endophyte-endophyte. To achieve this later goal, fungal antagonism assays are an essential tool to provide information about the biocontrol potential of certain endophytes. The endophyte antagonistic interactions can be mediated after fungal contact or at a distance (Woodward and Boddy, 2008). Several morphological, physiological, and biochemical changes happen during interactions, which are mainly influenced by the fungal species present and other conditions. These alterations could include hyphal aggregation, rapid cell division, hyphal branching, aerial growth, autolysis, pigment production, release of volatile organic compounds (which also act as info-chemicals), production of diffusible enzymes, toxins, and antifungal metabolites (Woodward and Boddy, 2008; Hiscox *et al.*, 2015).

1.6. Aims of the present work

The main goal of this study was to analyze, in quantitative and diversity terms, the fungal endophytic community of various cork oak populations with different water availability (correlation with the precipitation levels). Several cultures for the growth of endophytic fungi, from four different study sites, were establish, including one from a *montado* in Alentejo (Grândola) which comprises cork oaks with different degrees of decline. Other important step was, through the use of antagonism assays, to understand the relations between fungal endophytes and pathogens (association with decline).

2. Material and methods

2.1. Selection of sampling sites

The study was conducted between October 2015 and April 2016 in four different regions of continental Portugal, ranging from north to south of the country (Figure 4; Table 2). The selected areas include both *sobreirais* and *montados*, two types of *Q. suber* forest ecosystems. Each site had different geographical and ecological characteristics, thus allowing a better differentiation of the sampling sites.

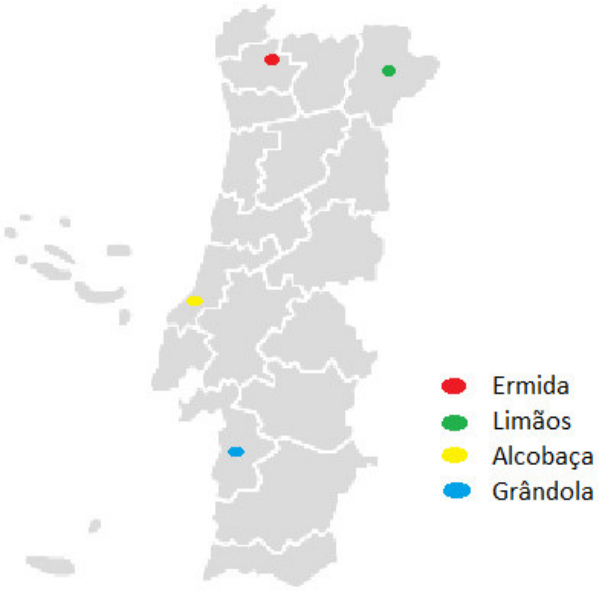

Figure 4. Geographic location of the sampling sites on continental Portugal. The exact locations and climatic conditions are listed in Table 2.

Table 2: Geographic and climatic conditions of the sampling sites.

	Bragança (Limãos)	**Gerês (Ermida)**	**Alcobaça (Casal do Rio)**	**Alentejo (Grândola)**
GPS location	41°31´51.5412”N 6°49´56.5642”W	41°42´39.7630”N 8°6´14.8649”W	39°27´41.1281”N 9°2´42.5169”W	38°11´32.3699”N 8°37´11.4058”W
Elevation (m)	600	627	78	150
Precipitation (average past 30 years) mm	64.4	120.7	54.3	61.3
Temperature (average past 30 years) ºC	15	12.7	17.0	16.6
Vegetation cover	*Cistus* sp.; *Cytisus* sp.	*Genista tridentata*; *Cistus* sp.; *Ulex* sp.	*Pistacia lenticus* ; *Ulex* sp.; *Rubus fruticosus*; *Rosa canina.*	*Cistus ladanifer*; *Cistus salvifolius.*

Bragança (Limãos) (Bra)

This *sobreiral*, located at 600 m altitude, is mainly composed by *Q. suber* in the dominant and co-dominant crown classes. Alongside the cork oak trees, plants from the genera *Cytisus* and *Cistus* are commonly observed, and nearby meadow areas are primarily used for farming and grazing (Figure 5A). This site was previously used for studying the soil microbiome and the ectomycorrhizal community, which have used the same cork oak trees (Francisca Reis, ongoing PhD project). Limãos site has a temperate climate with cold long winters and short hot summers.

Gerês (Ermida) (Ger)

The main feature about this *sobreiral* is the low anthropogenic influence, being a remote place in the Peneda-Gerês National Park (PNPG) in northwestern Portugal (Figure 5B). Cohabitating with the *Q. suber* community, there were other members of the Fagaceae family, like *Quercus robur*. Pine trees also took part in the composition of the landscape. This s*obreiral* was the highest sampled site (627 m), displaying also the highest mean annual precipitation (120.7 mm, as the average precipitation for the past 30 years) and the lowest mean annual temperature (12.7ºC, as the average temperature for the past 30 years).

Alcobaça (Casal do Rio) (Alc)

This sampled *sobreiral* (Figure 5C) is located at just 78 m, displays an average temperature for the past 30 years of 17ºC and a mean annual precipitation of 54.3 mm. Although these meteorological data pointed to the driest sampled site, the nearby Atlantic coast and the proximity of water courses turned this site one of the wettest sampling site. Accordingly, this site has the most diverse vegetation cover, causing the *Q. suber* to cohabitate with a high range of plant species, like *Pinus pinaster*.

Alentejo (Grândola) (Gra)

Grândola is the most southern sampling site, comprising a *montado* (Figure 5D). This site is situated at 150 m and displays a mean annual precipitation of 61.3 mm. Alongside the *Q. suber* trees, plants from the genus *Cistus* are frequently observed. This site comprises several cork oaks displaying decline disease symptoms.

Figure 5. Satellite images of the four sampling sites, displaying the five *Q. suber* trees sampled. Panel A: Limãos (sampled 7th October 2015). Panel B: Ermida, (sampled 20th January 2016). Panel C: Alcobaça (sampled 14th March 2016). Panel D: Grândola (sampled 5th April 2016). (A1 to A5, trees 1 to 5).

2.2. Cork oak organs collection

From each sampling site, five trees were selected separated at least 30 m away from another *Q. suber* tree or other Fagaceae or Pinaceae plants. The selected plants did not exhibited apparent disease symptoms, although in Grândola, some trees with different decline levels (tree 1: 100%; tree 2: 20%; tree3: 60%; tree 4: 10%; tree 5: 90%) were selected. From each tree, seven branches and seven root samples, without any visible lesions, were harvested. The plant material was stored in plastic bags at 4ºC and processed within two days.

2.3. Sterilization and isolation of endophytic fungi

From each branch or root, five leaves, five twigs and five root segments were randomly selected. The plant material was washed under running water (15-20 s, for leaves and stems; 30 s, for roots), and then surface-sterilised in 70% (v/v) ethanol for two minutes, followed by immersion in commercial bleach (4 min, for leaves and stems; 6 min, for roots) with agitation. Finally, the material was immersed again in 70% (v/v) ethanol for one minute. The surface sterilisation was completed by three rinsings in sterile water. This method was shown to be sensitive enough to allow the growth of endophytic fungi and to be effective in eliminating epiphytes from the plant surface.

Leaves were cut into five fragments (5 mm^2/each), distinguishing the petiole, the midrib blade, the secondary veins blade and the apex, thus allowing the study of endophytes in different leaf parts. The stems and roots were cut into five fragments and placed on the surface of potato-dextrose agar (PDA) medium. Fungal growth was promoted at room temperature in the dark. A total of 630 PDA plates [five trees x three organs x seven samples x five replicas (and one control per sample)] were prepared for each location. Fungi that outgrowth from the plant segments were recorded as endophytes and sub-cultured in fresh PDA medium for subsequent identification. Fungal colonies were grouped by their cultural characteristics, further designated as cultural morphotypes. Water from the final rinsing (150 µL) was placed in PDA medium and used as control to ascertain the efficiency of the sterilization procedure (Rubini *et al*., 2005; Banhos *et al*., 2014).

2.4. Identification of fungal isolates and DNA extraction

Fungal isolates were grouped according to their cultural feature, which were used for defining different morphotypes. In the present work, the morphotype separation was performed based on similarity in colony color (surface and underneath), margin shapes, texture, elevation, exudates and sporulating structures (if present). A total of 24 different morphotypes were identified (Table 3).

Isolates of each cultural morphotype, obtained from each sampling site, were selected for molecular identification using the sequence of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). Morphotypes with higher number of isolates had more fungi selected for identification. For this purpose, fungal DNA was extracted using the *REDExtract-N-Amp*^{TM} *Plant PCR* kit (Sigma-Aldrich, USA). About 10 mg of mycelia was placed in 25 µL of extraction solution and heated for 10 min at 95ºC. The extraction was completed by adding 50 µL of dilution solution provided by the kit. Due to the high levels of polyphenols and polysaccharides of some fungal DNA, three samples were further purified using the *ZR Fungal/Bacterial DNA MiniPrep*^{TM} kit (Zymo Research, USA).

After DNA extraction, a PCR amplification was performed using ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) as primers for the ITS regions (White et al., 1990; Gardes and Bruns, 1993) and a *Taq DNA polymerase* created in our laboratory with the PCR conditions described in Table 4. The amplification was confirmed by agarose gel (1%) electrophoresis. PCR products were sequenced using Macrogen services (Amsterdam, Netherlands). Primary sequencing analysis was performed using *Sequence Scanner* v.2.0 software. Fungal identification was performed using the BLAST algorithm in both NCBI database (http://www.ncbi.nlm.nih.gov) and UNITE database (http://unite.ut.ee). Only the identifications corresponding to blasts with the lowest E-value (nearly 0.0) and high identity score (>97%) were accepted. The identified operational taxonomic units (OTUs) were taxonomically classified according to the Index Fungorum Database (http://www.indexfungorum.org). The OTUs that showed similar results were grouped and aligned using *DNASTAR's Megalign 7.0.0.* to see if differences in same species were found between study sites.

Table 3: Description of the 24 different fungal morphotypes, distinguished by their colony features and sporulating structures (described in the text).

Fungal morphotype	Description
A	Opaque; Filamentous margin; Powdered texture; Flat elevation; Sprinkled/Hairy Surface; Whitish color.
B	Opaque; Filamentous margin and texture; Flat elevation; White surface with green splashes.
C	Opaque; Undulate margin; Filamentous texture; Flat elevation; Smooth surface; Greenish color.
D	Transparent; Filamentous margin, texture and form; Flat elevation; Bright.
E	Transparent; Undulate margin; Filamentous texture; Flat elevation; Rhizoid form; Bright.
F	Opaque; Undulate margin; Filamentous texture; Flat elevation; Rhizoid form; Hairy surface; Whitish color.
G	Opaque; Undulate margin; Consistent texture; Flat elevation; Rhizoid form; Hairy surface; Orange pink color.
X	Transparent; Undulate margin; Filamentous texture and form.
I	Opaque; Undulate margin; Filamentous texture; Flat elevation; Rhizoid form; Hairy surface; Yellow color.
J	Opaque; Creamy texture; Smooth margin; Convex elevation; Circular form; Rough surface; Whitish color.
K	Opaque; Filamentous margin and texture; Flat elevation; White surface with a brown center.
H	Opaque; Filamentous margin; Consistent texture; Flat elevation; Rhizoid form; Brown center.
O	Opaque; Filamentous margin; Hairy texture; Flat elevation; Orange tonality, with dark orange punctuation.
R	Opaque; Circular transparent margin; Hairy texture; Dark green in the center with black disperse punctuation (exudates).
Y	Opaque; Smooth margin; Hairy texture; White color, more concentrated in the center.
Z	Opaque; Bright; Creamy texture; Smooth margin; Whitish color.
T	Opaque; Bright; Creamy texture; Undulate margin; Dark red color.
U	Opaque; Smooth margin; Consistent texture; Convex elevation; Circular form; Yellow surface with white margins.
N	Opaque; Smooth margin; Consistent texture; Convex elevation; Circular form; Brown surface.
V	Transparent; Undulate margin; Filamentous texture and form; Blue green center; Signs of sporulation in the surface.
Q	Opaque; Smooth margin; Consistent texture; Convex elevation; Circular form; Dark red surface; White bottom.
Fx	Opaque; Filamentous margin; Fluffy texture; Convex elevation; White color.
L	Opaque; Filamentous margin (Ellipses) and texture; Hairy surface; Whitish color.
ÇÇ	Opaque; Creamy texture; Smooth margin; Convex elevation; Worm like form; Rough surface; Purple color.

Table 4: PCR conditions used for amplification of the ITS region.

	Temperature (ºC)	Time	Cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 s	35
Annealing	----	30 s	35
Extension	72	30 s	35
Final extension	72	10 min	1
Hold	4	Forever	----

2.5. Endophytic occurrence, diversity and statistical analysis

Fungal occurrence was measured by calculating the frequency of endophytic colonization and relative abundance found in each organ sample/tree. The endophytic colonization frequency (CF, %) was measured as the number of segments colonized by each fungal morphotype divided by the total number of plant segments examined. The relative abundance (RA, %) of each fungal morphotype was calculated by the total number of isolates of a specific morphotype divided by the total number of all isolates.

Species Diversity and Richness 4.1.2. (SDR) (Seaby and Henderson, 2007) and *EstimateS 9.1.0.* (Colwell, 2013) softwares were used to investigate species diversity and richness estimators. The number of morphotypes shared between samples was obtained by SDR software. Alpha diversity indices determine the diversity of a specific community considering both the number of species and the proportion in which each species is represented. *Simpson diversity index* (D), *Shannon-Wiener diversity index* (H'), *Fisher's alpha* as well as the species richness estimators, *Chao1* and *Jackknife 1*, were calculated.

Simpson's index (Simpson, 1949) (equation 1.) measures the probability of any two randomly chosen individuals taken from an infinitely large community belong to the same species. This index is more focused on the abundance of species in the sample than the species richness itself. *Shannon index (H')* (Shannon, 1948) (equation 2.) characterizes the species diversity in a community. This index computes the uncertainty associated with identifying species in a community. The other alpha diversity index considered was Fisher's alpha (Fisher *et al*., 1943) (equation 3.) that accepts that species abundance follows a log series distribution.

$$D = -\sum_{i=1}^{S} p_i^2 \qquad \text{Equation 1.}$$

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \qquad \text{Equation 2.}$$

where:
S, number of species
p_i, proportion of the species *i* $p_i = n_i/N$
n_i, number of individuals of specie *i*
N, total number of individuals
ln *pi*, base 2 logarithm of *pi*

$$S = \alpha \ln\left(1+\frac{N}{\alpha}\right) \qquad \text{Equation 3.}$$

where:
S, number of taxa
N, number of individuals
α – Fisher's alpha; N(1-x)/x

Regarding the richness estimators, Chao 1 (Chao, 1984) (equation 4.) uses the number of species represented by one and two individuals in the samples. Jackknife 1 (Burnham and Overton, 1978, 1979) (equation 5.) estimates the total richness using the number of species present in only one sample.

$$S_J = s + \frac{Q_J^2}{2Q_J} \qquad \text{Equation 4.}$$

$$S_J = s + Q_J\frac{n-1}{n} \qquad \text{Equation 5.}$$

where:
SJ, estimated richness
s, observed richness
QJ, number of species that occur in j samples
n, number of samples

The coefficient of similarity (*Bray-Curtis coefficient*) between soils samples was calculated using the *Community Analysis Package 4.0* software (Henderson and Seaby, 2007). The *Bray-Curtis coefficient* (Bray and Curtis, 1957) (equation 6.) is a coefficient of beta diversity that is calculated considering the differences in abundance of each species between sites. This index was used over the Jaccard and Sorensen indexes because it considers the abundance.

$$S=100\frac{\sum_{i=1}^{p} 2\min(Y_{ij},Y_{ik})}{\sum_{i=1}^{p}(Y_{ij}+Y_{ik})} \qquad \text{Equation 6.}$$

where:
Yij and Yik, measure of species i in samples j and k,
Min (Yij , Yik), minimum of Yij and Yik
p, number of species

The similarity between samples was performed by a non-metric multidimensional scaling (NMDS) performed in the *Community Analysis Package 4.0* software (Henderson and Seaby, 2007). Other graphics and ANOVA statistical analysis between diversity parameters was determined by *GraphPad Prism 5.0* software (San Diego, CA).

2.6. Antagonistic activity assays

Biscogniauxia mediterranea and *Diplodia corticola* fungi, the causal agents of two major cork oak diseases - inner bark coal disease (Vannini *et al.*, 2009) and oaks decline disease (Linaldeddu *et al*., 2014), respectively - were detected in cork oak stands, although sampled trees did not display disease symptoms. All the fungi used in antagonistic assays were isolated from apparently healthy leaves, stems and roots of cork oaks (Table 5 and Table 6). The endophytic fungal isolates obtained from Bragança were used to assess potential antagonistic activities against *Biscogniauxia mediterranea,* whereas those isolated from Alcobaça were used to assess the potential antagonistic activities against *Diplodia corticola*. These sites were chosen because they had both the pathogenic fungi and fungi with high antagonistic potential.

Table 5: Endophytic fungi used in the co-culture with *Biscogniauxia mediterranea*.

Fungi	Classe	Isolated from
Gelasinospora tetrasperma	Sordariomycetes	Leaves
Trichoderma virens	Sordariomycetes	Roots
Alternaria photistica	Dothideomycetes	Leaves
Alternaria alternata	Sordariomycetes	Leaves and Stems
Giberella pulicaris	Dothideomycetes	Roots
Leptosphaeria maculans	Sordariomycetes	Leaves
Fimetariela rabenhorstii	Sordariomycetes	Stems
Dactylonectria estremocensis	Dothideomycetes	Roots
Aspergillus niger	Eurotiomycetes	Roots
Cladosporium cladosporoides	Dothideomycetes	Leaves and Stems

Table 6: Endophytic fungi used in the co-culture with *Diplodia corticola*.

Fungi	Classe	Isolated from
Gelasinospora tetrasperma	Sordariomycetes	Leaves
Gelasinospora seminuda	Sordariomycetes	Stems
Trichoderma virens	Sordariomycetes	Roots
Neofusicoccum luteum	Dothideomycetes	Stems
Fusarium acutatum	Sordariomycetes	Roots
Saccharicola bicolor	Dothideomycetes	Roots
Rosellinia corticium	Sordariomycetes	Leaves
Diaporthe viticola	Sordariomycetes	Stems
Ilyonectria spp.	Sordariomycetes	Roots
Gnomoniopsis spp.	Sordariomycetes	Leaves

All isolates were isolated and sub-cultured onto PDA medium. Peripheral portions of each species mycelium were transferred to MMN (pH 6.6), a less nutritional medium than PDA, and incubated in the dark, at room temperature (*c.*25ºC). Small portions of peripheral mycelium of growing colonies were stamped out using an inverted sterile Pasteur pipette and were inoculated onto a fresh MMN medium (pH 6.6), at a distance of 3 cm from a *B. mediterranea* or *D. corticola* inoculum, prepared in a similar way. Antagonist assays using high-sporulating isolates were performed using spore suspensions prepared in sterile water. Spore suspensions (10 uL) were placed over

sterile filter paper discs (Ø 6 mm), introduced directly above MMN medium, at the same distances as previously referred. Co-cultures were incubated in the dark, at room temperature. For each assay, four co-culture replicas, four control *B. mediterranea* - *B. mediterranea* (or *D. corticola* – *D. corticola*) and four control replicas for the interacting fungus were established, for a total of 12 plates per assay.

The internal radius (inter-inoculants region) and external radius (diametrically opposed region) of the interacting fungal colonies were daily measured, until fungal contact occurs. Morphological changes, color of the colony on top and underside, margin, mycelium texture and halo formation were registered in both co-cultures and controls. *GraphPad Prism 5* software was used to make the graphics as well as the statics analysis with ANOVA.

3. Results and discussion

The fungal isolates were obtained from a total of 20 cork oak trees in four different sites of continental Portugal (five trees per site). These spots were chosen because they present differences in water availability, due to the climatic parameters and agroforestry conditions, and had been previously studied for the presence of mycorrhizal root tips (Francisca Reis, ongoing PhD project). Fungal endophyte isolates were obtained from three different organs (leaves, stems and roots). A similar approach has been performed for obtaining fungal endophytes from olive trees (*Olea europaeae*) in the northeastern region of Portugal (Martins *et al.*, 2016).

3.1. Structural analysis of fungal frequency and diversity

The total number of isolates obtained from all four sites was 7473 (Table 7). The isolates are mainly fast growing fungi due to the nature of the method used.

Table 7: Number of isolates of fungal endophytes by study site and by organ. Letters in lowercase represent statistic differences (at P≤0.05) between the number of isolates present in the organs. Letters in uppercase represent statistic differences (at P≤0.05) between the number of isolates present in the study sites.

	Study site	Leaves	Stems	Roots	Total per site
Number of isolates	**Bragança**	318	603	368	1289 ^{A}
	Gerês	458	609	433	1500 ^{A}
	Alcobaça	1027	814	427	2268 ^{B}
	Grândola	926	935	555	2416 ^{B}
	Total per organ	2729 ^{a}	2961 ^{a}	1783 ^{b}	7473

Alcobaça and Grândola showed significant higher infection levels than Bragança and Gerês, clearly distinguishing northern and southern sites. Indeed, all the explants removed from Alcobaça cork oaks presented fungal growth, corresponding to a fungal colonization frequency (CF) of 100%. Grândola explants also display a high frequency of colonization (91%), while Gerês and Bragança have only displayed 57% and 49% infection frequencies, respectively. Besides the geographic conditions, also the climatic conditions at sampling sites could have affected the endophyte occurrence. Both Alcobaça and Grândola were sampled in the spring (March and April, respectively) opposed to Bragança and Gerês that were sampled in autumn (October) and winter

(January), respectively. In a previous study on the influence of geographical and seasonal factors in endophyte occurrence on *Quercus ilex*, the degree of endophytic infection was also significantly higher in the spring when compared to autumn (Collado *et al.*, 1999). The authors suggested that the humidity and warm temperatures of the spring season may have contributed for a higher fungal infection, resulting in a higher number of fungal isolates (Collado *et al.*, 1999). The isolation frequency has also been suggested to be significantly dependent on season and geographical characteristics of the sampling sites when studying olive tree endophytes (Martins *et al.*, 2016). Furthermore, the colonization frequency was already reported to be higher for trees growing at sea level (as Alcobaça and Grândola sites) when compared with those growing at higher elevations (higher than 350-400 m, like Bragança or Gerês) (Ragazzi *et al.*, 2003). Moreover, trees with a higher decline degree (like those in Grândola) have presented a higher colonization frequency when compared with healthy trees (Ragazzi *et al.*, 2003). All these factors may be the cause for higher infection levels observed in Alcobaça and Grândola, when compared to Bragança and Gerês.

When comparing the cork oak organs, the roots showed significant lower degree of infection (CF of 50%) from all three organs. In contrast, the above ground organs (leaves and stems) presented higher infection levels, with the stems (84%) just above the leaves (78%). The root endophytic fungi were isolated from the fine roots, which have recently been formed and therefore would have less endophytes than the structural roots (Kwaśna *et al.*, 2016). In addition, some root endophytic fungi should be mycorrhizal and are difficult to cultivate or take months to grow (Díez *et al.*, 2000). Studies performed on several *Quercus* species have showed that stems usually have a higher colonization frequency than leaves (Kehr and Wulf, 1993; Ragazzi *et al.*, 2003).

Due to the large number of fungal isolates, the isolated endophytes were grouped together considering their morphological characteristics. The concept of morphological species (delimited based on morphological characteristics) has been used since 1996, in studies regarding fungal endophytes (Bill *et al.*, 1996). Schulthess and Faeth (1998) used the term “morphotype” to describe different morphological species of fungal endophytes found in *Festuca arizonica* leaves. A study evaluating the biodiversity and tissue occurrence of endophytic fungi in *Tripterygium wilfordii* has also separated the isolated fungi into different morphotypes (Kumar and Hyde, 2004). The major problem in sorting fungal isolates into morphotypes is that one cannot be

sure that these morphotypes truly reflect taxonomic units (Lacap *et al.*, 2003). However, this method allows an overview of the diversity and, in some cases, be sufficient to distinguish species (Mayr, 1970).

A total of 24 fungal morphotypes were distinguished in different cork oak samples from different sites (Table 8), based on their cultural features (described in detailed in Material and Methods section). The lowest number of fungal morphotypes was found in Alcobaça (15), whereas Grândola samples presented the highest number of morphotypes (20). Stems were the organs from which the lowest number of morphotypes were obtained, especially for Bragança and Gerês samples (9 in each, corresponding to 47.4% and 50% of the total morphotypes found in the respective sites). Although Alcobaça samples also revealed the lowest number of morphotypes found in stems (12), they correspond to 80% of the morphotypes found in that site. In contrast, the highest number of morphotypes was obtained from Grândola samples in stems and roots (17 each that corresponds to 85% of the morphotypes found in that site) than from leaves (10, corresponding to 50%). These results suggest a clear difference of morphotype distribution among organs and sampling sites.

Table 8: Number of fungal morphotypes found in each cork oak organ and sampling sites.

	Sampling site	Leaves	Stems	Roots	Total
Number of morphotypes	**Bragança**	12	9	11	19
	Gerês	11	9	14	18
	Alcobaça	13	12	13	15
	Grândola	10	17	17	20
Total of different morphotypes per organ		18	18	22	24

A color code chart representing the total number of isolates per morphotype in all studied sites was also created (Figure 7). From this color chart, the uniformization among replicas from each tree, and among trees from the same organ/sampling site becomes obvious. Differences between organs and sampling sites are also apparent. Such differences can be detailed when considering the colonization frequency (CF) and relative abundance (RA) of each morphotype in each organ (Table 9) and sampling site (Table 9). From all identified morphotypes, the morphotype D shows the highest colonization frequency and relative abundance (Table 9). This morphotype was present in all studied sites and organs, but its higher abundance (27.9%) in whole study is

mainly due to its high abundance in leaves (48.6%) from all sampling sites (Table 9). This morphotype was indeed the most abundant in all sampling sites, except in Alcobaça sampling site that revealed only 21.5% of morphotype D relative abundance, in contrast with morphotype F that presented 34.1% of relative abundance in this site (Table 9). The Morphotype F is also the most abundant morphotype in the stems (25.5%). In contrast, the morphotypes X and Z have the highest CF in the roots (10%), presenting very similar relative abundances (19.2% and 20.3%, respectively). In general, there is a clear dominance of morphotypes D and F in leaves and stems (that together account for more than 51.7% and 43.4% of relative abundance, respectively), whereas roots show more diversity in morphotypes. This result is corroborated by other studies that revealed that the endophytic mycota tends to be different when comparing roots with the above ground organs (Martins *et al.*, 2016).

Endophytes has been described to be organ or location specific (Gange et al., 2007; Moricca et al., 2012). Accordingly, morphotypes L, Q, U and ÇÇ revealed to be root specific, whereas morphotypes O and K were leaf specific (Figure 7). In this study, no stem specific morphotypes were found. Furthermore, some morphotypes were only found in certain sampling sites, such as morphotypes L, K and O that were specific to Bragança, morphotype Q specific to Gerês, and morphotype ÇÇ specific to Grândola. In such cases, the morphotypes that are specific to one region are also specific for the organ (Table 9).

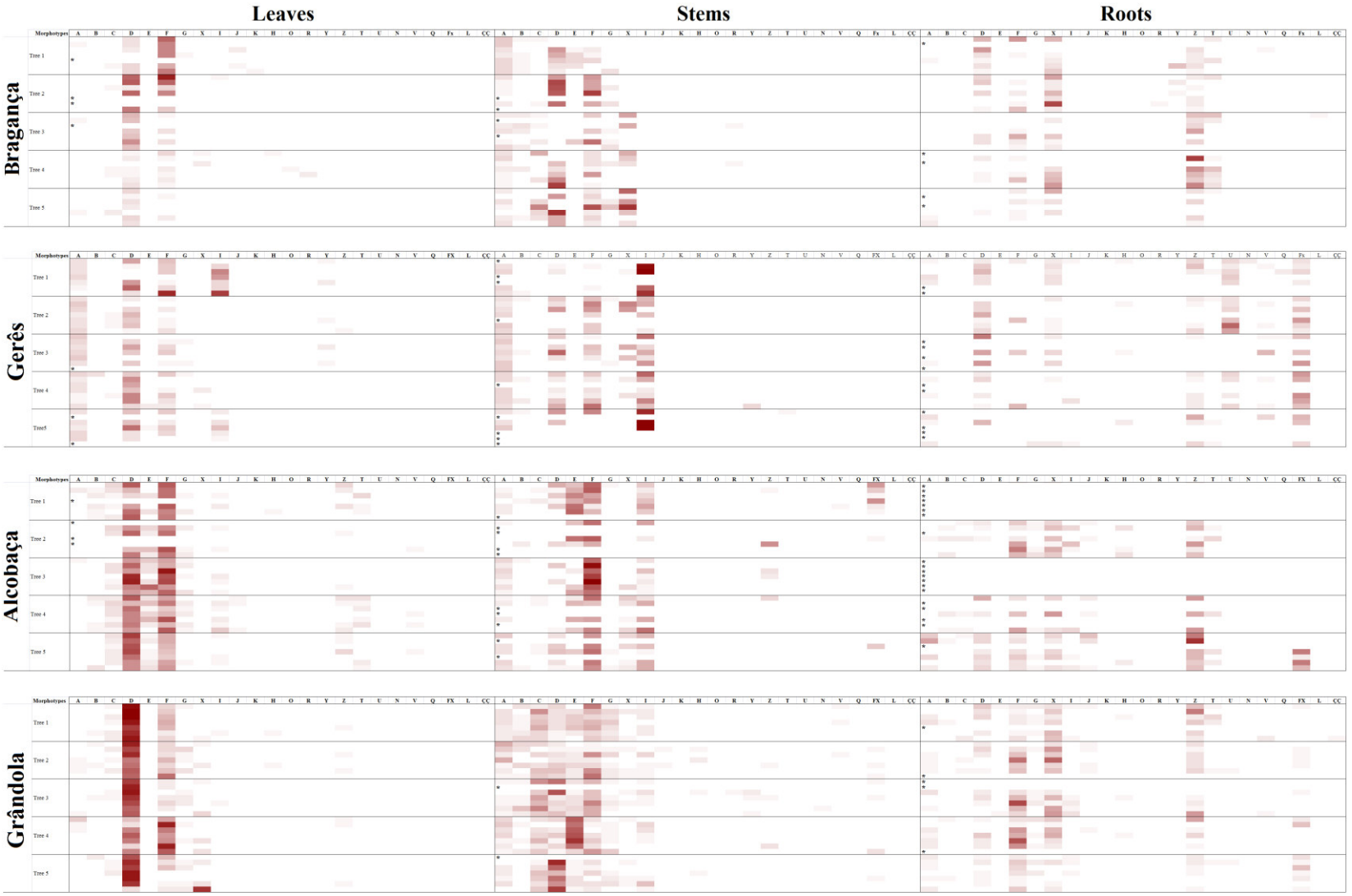

Figure 7. Color code chart of the different morphotypes (A to ÇÇ) found in all five trees of the studied sites. The color gradient indicates the number of isolates found for each morphotype (white, 0; dark red, 25 or higher). * represents samples not considered, as the negative controls presented microbial growth.

Table 9: Colonization frequency (CF) and relative abundance (Ra, %) of each fungal morphotype in relation with all organs and study sites. The value of RA is shown in brackets. Bold values represent the highest CF and RA values found in each organ or in the total.

	Bragança				**Gerês**				**Alcobaça**				**Grândola**			
	CF (RA%)				**CF (RA%)**				**CF (RA%)**				**CF (RA%)**			
	Leaves	**Stems**	**Roots**	**Total**	**Leaves**	**Stems**	**Roots**	**Total**	**Leaves**	**Stems**	**Roots**	**Total**	**Leaves**	**Stems**	**Roots**	**Total**
A	0 (0.94)	0.08 (11.97)	0 (1.09)	0.03 (6.10)	0.11 (21.18)	0.11 (16.09)	0.02 (3.46)	0.08 (14.00)	0 (0.34)	0.06 (6.02)	0.02 (4.92)	0.03 (3.22)	0.01 (1.32)	0.09 (8.99)	0.03 (5.05)	0.04 (4.76)
B	0 (0)	0.02 (2.87)	0 (0)	0.01 (1.33)	0 (0.66)	0.01 (1.15)	0 (0)	0 (0.67)	0.02 (1.72)	0.01 (0.61)	0.01 (2.11)	0.01 (1.28)	0.01 (0.72)	0.04 (4.62)	0 (0.56)	0.02 (2.00)
C	0.01 (1.88)	0.05 (6.75)	0 (0)	0.02 (3.60	0 (0.87)	0 (0)	0 (0)	0 (0.27)	0.06 (6.19)	0.01 (0.86)	0.01 (1.41)	0.03 (3.04)	0.02 (2.27)	0.15 (15.15)	0 (0.75)	0.07 (7.55)
D	0.17 (45.45)	**0.22 (32.88)**	0.06 (13.62)	**0.15 (30.49)**	**0.20 (38.65)**	0.10 (14.78)	0.10 (21.02)	**0.14 (23.87)**	**0.38 (38.03)**	0.06 (6.51)	0.04 (8.20)	0.21 (21.47)	**0.59 (61.48)**	**0.20 (20.24)**	0.05 (8.41)	**0.32 (35.06)**
E	0 (0)	0.02 (2.87)	0 (0)	0.01 (1.33)	0 (0.66)	0 (0)	0 (0)	0 (0.20)	0.09 (9.28)	0.16 (17.20)	0 (0)	0.10 (10.10)	0 (0)	0.16 (16.80)	0 (0.37)	0.06 (6.84)
F	**0.17 (47.34)**	0.16 (23.61)	0.05 (12.81)	0.13 (26.43)	0.11 (20.31)	0.12 (16.75)	0 (0)	0.07 (13.00)	0.33 (33.22)	**0.38 (40.79)**	0.09 (17.56)	**0.34 (34.13)**	0.24 (25.48)	0.18 (18.58)	**0.18 (28.97)**	0.21 (22.75)
G	0 (0.31)	0.03 (3.88)	0 (0.27)	0.01 (1.95)	0 (0.66)	0 (0.33)	0 (0.46)	0 (0.47)	0.03 (2.63)	0.01 (0.61)	0.01 (2.11)	0.02 (1.68)	0.03 (2.75)	0.06 (6.04)	0.02 (3.55)	0.04 (3.88)
X	0 (0.94)	0.10 (14.84)	**0.13 (30.25)**	0.08 (15.79)	0 (0.66)	0.05 (6.90)	0.04 (7.16)	0.03 (5.07)	0 (0)	0.04 (3.93)	0.07 (15.22)	0.04 (4.28)	0.05 (4.78)	0.01 (1.42)	0.15 (24.49)	0.07 (7.64)
I	0 (0.31)	0 (0)	0 (0)	0 (0.08)	0.08 (14.63)	**0.30 (43.19)**	0.01 (1.15)	0.13 (22.33)	0.03 (2.63)	0.15 (16.34)	0.02 (4.22)	0.08 (7.67)	0 (0.24)	0.04 (4.62)	0.01 (2.24)	0.02 (2.21)
J	0 (0.94)	0 (0)	0 (0)	0 (0.23)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.34)	0 (0)	0.02 (3.51)	0.01 (0.79)	0 (0)	0 (0.24)	0.01 (1.12)	0 (0.33)
K	0 (0.31)	0 (0)	0 (0)	0 (0.08)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
H	0 (0.31)	0 (0)	0 (0)	0 (0.08)	0 (0)	0 (0)	0.01 (1.15)	0 (0.33)	0 (0.11)	0 (0)	0.01 (2.11)	0 (0.44)	0 (0.24)	0.01 (0.59)	0 (0.56)	0 (0.42)
O	0 (0.31)	0 (0)	0 (0)	0 (0.08)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
R	0 (0.63)	0 (0.34)	0 (0.27)	0 (0.39)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.12)	0 (0)	0 (0.04)
Y	0 (0)	0 (0)	0.01 (2.45)	0 (0.70)	0.01 (1.53)	0 (0.66)	0 (0)	0 (0.73)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.47)	0 (0)	0 (0.17)
Z	0 (0)	0(0)	**0.13 (30.25)**	0.04 (8.68)	0 (0.22)	0 (0)	0.05 (10.16)	0.02 (3.00)	0.03 (2.75)	0.02 (2.58)	**0.13 (27.40)**	0.07 (7.14)	0.01 (0.72)	0.01 (0.59)	0.10 (15.89)	0.04 (4.01)
T	0 (0)	0(0)	0.04 (8.72)	0.01 (2.50)	0 (0)	0 (0.16)	0 (0.92)	0 (0.33)	0.02 (2.18)	0 (0)	0.01 (3.04)	0.01 (1.41)	0 (0)	0 (0)	0.01 (1.68)	0 (0.38)
U	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)	0.08 (16.63)	0.03 (4.80)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.37)	0 (0.08)
N	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.46)	0 (0.13)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.12)	0 (0)	0 (0.04)
V	0 (0)	0(0)	0 (0.27)	0 (0.08)	0 (0)	0 (0)	0 (0.69)	0.01 (1.13)	0 (0.46)	0 (0.12)	0 (0)	0 (0.22)	0 (0)	0 (0.36)	0 (0.56)	0 (0.25)
Q	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.69)	0 (0.20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fx	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)	**0.16 (32.79)**	0.05 (9.47)	0 (0)	0.04 (4.42)	0.04 (8.20)	0.03 (3.13)	0 (0)	0.01 (1.07)	0.03 (5.23)	0.01 (1.54)
L	0 (0.31)	0(0)	0 (0)	0 (0.08)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ÇÇ	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0.19)	0 (0.04)
Total	0.36 (100)	0.68 (100)	0.42 (100)	0.49 (100)	0.52 (100)	0.70 (100)	0.49 (100)	0.57 (100)	1 (100)	0.93 (100)	0.49 (100)	1 (100)	0.96 (100)	0.97 (100)	0.61 (100)	0.91 (100)

Besides the evaluation of fungal infection in different cork oak organs, in this work the colonization of different leaf regions was also considered. Four different leaf regions (petiole, midrib blade, secondary veins blade and apex) were analysed (Figure 8).

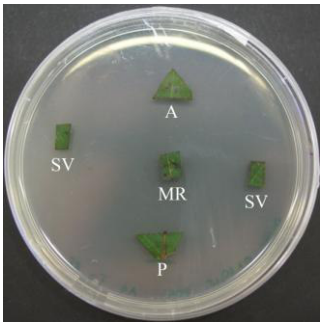

Figure 8: Petri dish with five leaf fragments representing the four different regions analysed (P-petiole; MR-mid rib blade; SV-secondary veins blade; A-apex).

The relative abundance of all isolates found in each leaf region was quite similar for all studied sites, with the secondary veins blade region presenting the highest percentage (Figure 9). Nonetheless, this percentage was only significantly different in Alcobaça and Grândola, when compared to Bragança and Gerês. A possible explanation is that secondary veins are more prone to fungal infection *via* the stomata. In contrast, a previous study performed on *Q. ilex* showed that the leaf tips were more colonized than the other leaf parts, however that difference was not significant (Fisher *et al.*, 1994).

As previously described for the whole leaf, all four leaf regions were predominately infected by morphotypes D and F (Figure 10). However, their predominance depends on the region of leaves from each sampling site. In Bragança, morphotype D is usually the most common (52-48%), except in the secondary veins, where morphotype F is dominant (60%). In Gerês and Grândola, morphotype D is also the most common in all leaf parts (41-52% in Gerês and 63-68% in Grândola), being followed by morphotype F (16-27% in Gerês and 21-16% in Grândola), while is equally abundant to morphotype F in Alcobaça (36-41% of morphotype D and 35-37% of morphotype F). In Bragança leaves, 3 to 10% of all other isolates were shared by 10 different morphotypes. In contrast, morphotype I, despite not being unique to Gerês, is the third more abundant morphotype in this site (14-27%), surpassing the relative abundance of morphotype F in the leaf apex (27% morphotype I *vs* 16% morphotype F). The same can be said for morphotype E in Alcobaça and morphotype X in Grândola,

although relative abundances were much reduced when compared to morphotypes D and F (7-11% morphotype E and 3-6% morphotype X). The only leaf region specific morphotypes were morphotypes O and K, which were specific to the midrib blade and leaf apex, respectively.

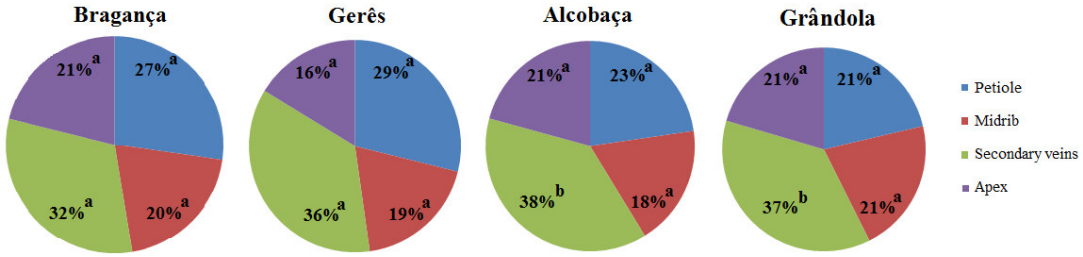

Figure 9. Percentage of isolates obtained in four different parts of the leaf (petiole, midrib blade, secondary veins blade and apex) in all four studied sites. Statistically significant (at $P \leq 0.05$) differences among leaf regions in each sampling site are denoted by different letters.

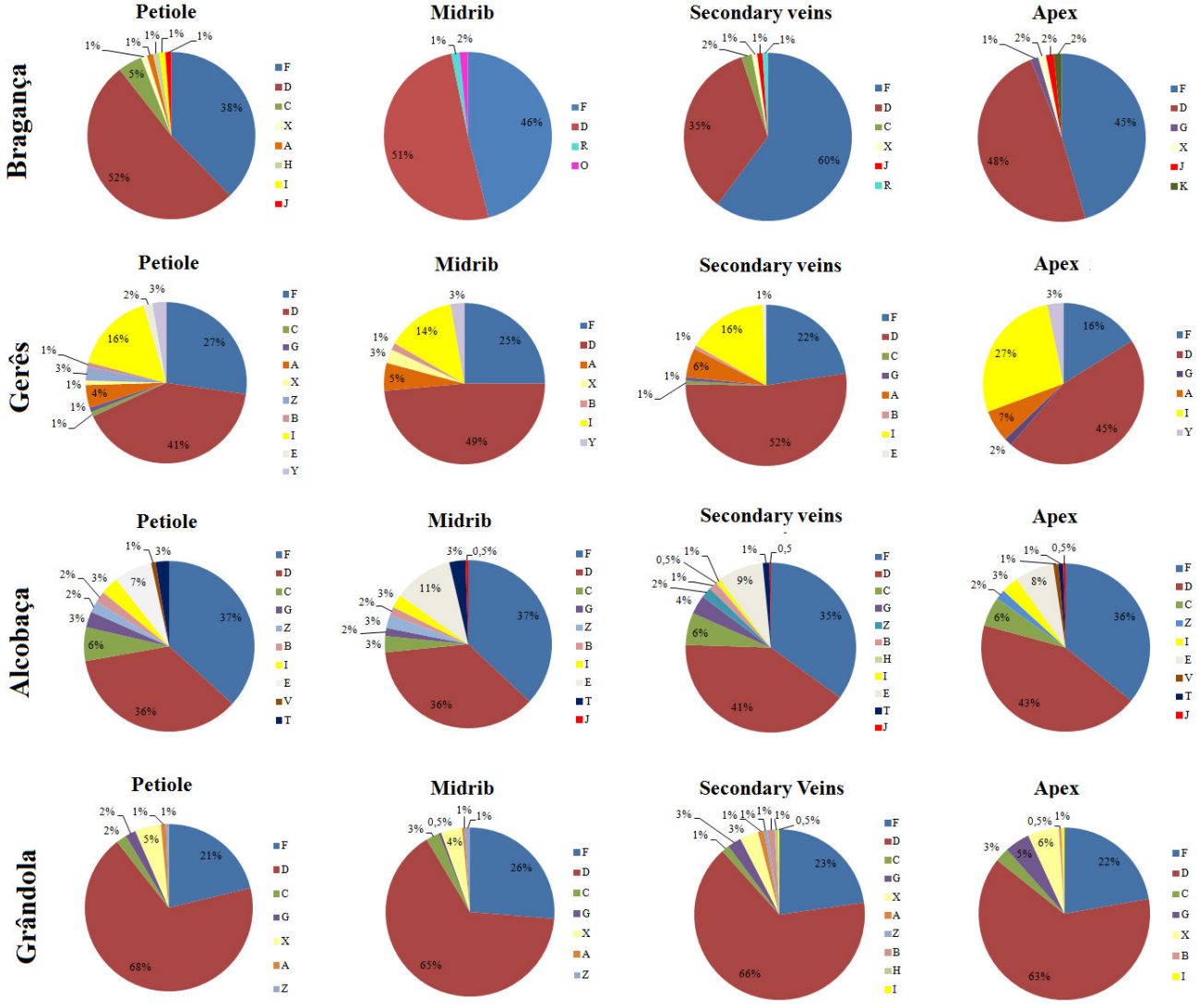

Figure 10. Relative abundance of different fungal morphotypes on the four different leaf regions (petiole; midrib; secondary veins and apex) in all four study sites.

3.2. Fungal morphotype diversity analysis

Biodiversity *is essential to the health and quality of different habitats*. For comparing the morphotype diversity in each studied organ and sampling site, rarefaction curves were determined (Figure 11 and 12). This curves revealed that the fungal community of the roots has higher diversity than stems or leaves communities (Figure 11). The rarefaction curves from all organs, except the roots reached a plateau suggesting a good representation of the fungal community. The curves of Gerês, Alcobaça and Grândola reached a plateau, suggesting that the fungal community is well represented (Figure 12). However, the curve of Bragança did not reach the plateau, suggesting that the fungal community in this study site is still not well represented. Rarefaction curves for the different study sites suggest that Grândola has the higher fungal diversity when compared with the other three regions.

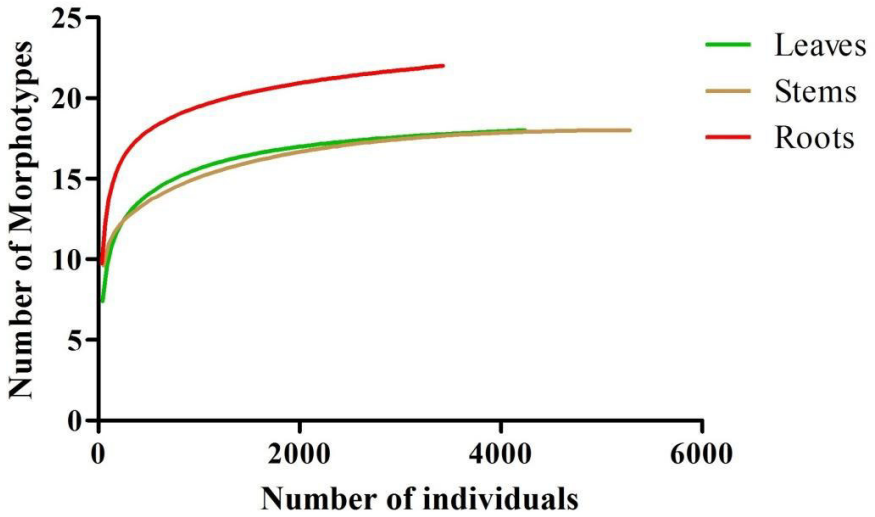

Figure 11: Rarefaction curves for the fungal community at 97% similarity. Representation of the rarefaction curves for the different organs. Rarefaction curves computed in Species Diversity and Richness 4.1.2 (Seaby and Henderson, 2006) and plotted in GraphPad 6 (GraphPad Software).

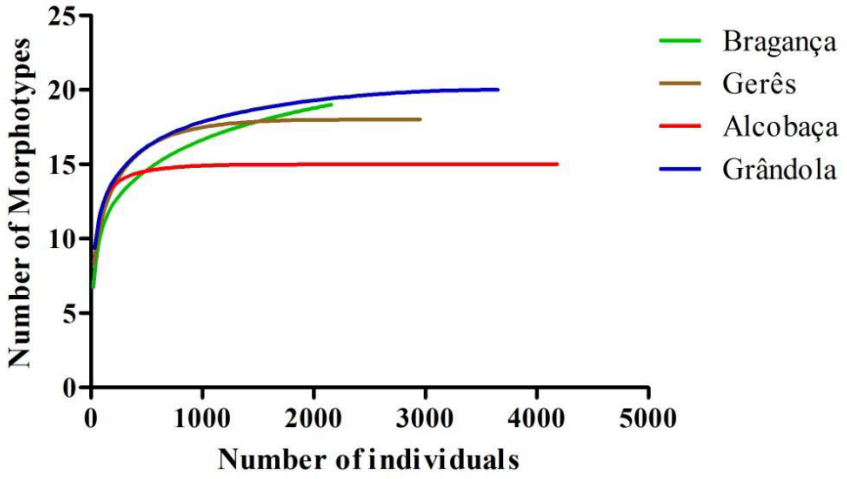

Figure 12: Rarefaction curves for the fungal community at 97% similarity. Representation of the rarefaction curves for the different study sites. Rarefaction curves computed in Species Diversity and Richness 4.1.2 (Seaby and Henderson, 2006) and plotted in GraphPad 6 (GraphPad Software).

The diversity between and among organs and sampling sites were further studied by computing several diversity indexes. Alpha diversity is used to study the species richness within a local community (Whittaker et al., 2001). In this work, several alpha diversity indexes were used, in order to study the morphological species diversity within the same specific community, namely, *Simpson diversity index* (D), *Shannon-Wiener diversity index* (H') and *Fisher's alpha* indexes. Richness estimators, such as *Chao & Lee 1* and *Jackknife 1* estimate the total number of morphological species in one specific community, based on the number of detected morphotypes in each sample.

When comparing the diversity parameters and richness estimators of fungal communities found in different organs, a pattern is distinguished between roots and stems when compared to leaves (Table 10). Between each sampling site a clear difference is detected among leaves, stems and roots. In Alcobaça and Gerês the most diverse samples were clearly the roots, presenting statistically significant differences in some diversity indexes and richness estimators, when compared to leaves or stems. Although Grândola roots were similarly diverse, the highest diversity parameters were found in stems from this sampling site (not statistically different from root indexes). Indeed, Grândola displayed the highest diversity indexes found in all stems found in

other sites (significantly different in *α Fisher* index and richness estimators). The high diversity commonly found in roots was not so clear for Bragança samples. The diversity indexes computed for roots in Bragança were always the lowest among roots from other sampling sites (statistically different in some diversity indexes and richness estimators from those found in Alcobaça and Gerês, results not shown).

The organ that displayed the less diverse fungal community varied according with the sampling site. Grândola leaves displayed a significantly reduced diversity when compared to other Grândola organs, while in Gerês the less diverse organ were the stems (although not significant). In Bragança and Alcobaça the scenario is not so clear.

Table 10: Species richness and diversity parameters for fungal communities from the four sites studied: species richness (S), Simpson's index (D) on its inverse form, Shannon index (H'), Fisher's alpha, Chao & Lee 1 and Jackknife 1 estimates. The highest values are highlighted in bold and the lowest values are underlined. Statistically significant differences (at P≤0.05) for each location's index, were denoted by different lowercase letters. Statistically significant differences (at P≤0.05) for each total were denoted by different capital letters.

		S	D	H'	α Fisher	Chao & Lee 1	Jackknife 1
Bragança	**Leaves**	**12^{a}**	2.28^{a}	1.01^{a}	**2.16^{a}**	**12.25^{a}**	**12.00^{a}**
	Stems	9^{a}	4.72^{b}	1.63^{b}	1.37^{a}	9.00^{a}	9.00^{a}
	Roots	11^{a}	**4.39^{b}**	**1.66^{b}**	1.90^{a}	11.33^{a}	11.00^{a}
	Total	19^{A}	4.88^{A}	1.88^{A}	2.87^{A}	19.03^{A}	19.00^{A}
Gerês	**Leaves**	11^{a}	3.85^{a}	1.53^{a}	1.85^{a}	11.00^{a}	11.00^{a}
	Stems	9^{a}	3.27^{a}	1.45^{a}	1.31^{a}	9.00^{a}	9.00^{a}
	Roots	**14^{b}**	**5.14^{a}**	**1.92^{a}**	**2.12^{b}**	**13.00^{b}**	**13.00^{b}**
	Total	18^{A}	6.27^{A}	2.08^{A}	2.52^{A}	18^{A}	18^{A}
Alcobaça	**Leaves**	**13^{a}**	3.42^{a}	1.56^{a}	1.93^{a}	**13.00^{a}**	**13.00^{a}**
	Stems	12^{a}	4.40^{ab}	1.80^{a}	1.76^{a}	12.00^{a}	12.00^{a}
	Roots	**13^{a}**	**5.90^{b}**	**2.09^{b}**	**2.15^{a}**	**13.00^{a}**	**13.00^{a}**
	Total	15^{B}	5.53^{A}	2.06^{A}	2.04^{B}	15^{B}	15^{B}
Grândola	**Leaves**	10^{a}	2.07^{a}	1.06^{a}	1.47^{a}	10.00^{a}	10.00^{a}
	Stems	**17^{b}**	**6.62^{b}**	**2.09^{b}**	2.72^{b}	**17.00^{b}**	**17.00^{b}**
	Roots	**17^{b}**	5.40^{b}	2.00^{b}	**2.97^{b}**	**17.00^{b}**	**17.00^{b}**
	Total	22^{C}	5.20^{A}	2.03^{A}	2.73^{A}	22.00^{C}	22.00^{C}
Total	**Leaves**	19^{a}	5.04^{a}	1.86^{a}	2.41^{a}	19.00^{a}	19.00^{a}
	Stems	18^{a}	6.46^{b}	2.08^{b}	2.42^{a}	18.00^{a}	18.00^{a}
	Roots	**22^{a}**	**7.26^{b}**	**2.24^{b}**	**3.12^{a}**	**22.00^{a}**	**22.00^{a}**

Despite not statistically different, the *Simpson* and *Shannon* indexes were higher in the roots of Alcobaça when compared with the other regions, however in this site only the roots of three trees were studied. In this case looking at the *α Fisher* index, which is independent of sample size, gives us a clear perspective on what is happening in terms of diversity in the roots. Overall, Grândola has highest root diversity. On the other hand, the roots of Bragança show the lowest values of species richness and diversity (Table 10). The diversity present in the stems is clearly higher in Grândola with statistically differences in the species richness, *α Fisher* index, *Chao & Lee 1* and *Jackknife 1* indexes. Despite having the same number of species and the same values of *Chao & Lee 1* and *Jackknife 1* than Bragança, the stems in Gerês had the lowest values of D and H'. The leaves of Grândola showed the lowest values of species richness and diversity when compared with the other regions. The only exception was the H', which was lower in Bragança, however this difference was not statistically different. On the other hand Alcobaça showed the highest values of the parameters analysed with the exception of D and *α Fisher*, which were higher in Gerês and Bragança, respectively.

For comparing the diversity between organs and sampling sites (β-diversity analysis), a similarity matrix was prepared by computing the *Bray-Curtis coefficient*, an ecological quantitative index that quantifies the dissimilarities between samples and demonstrates environmental and ecological connections. The values range from 0 to 1, where 1 indicates a maximum of dissimilarity, and 0 means that both samples share exactly the same species. The similarity of fungal morphotypes found in every organs/sampling is better perceived by a non-metric multidimensional scaling (NMDS) analysis, using the *Bray-Curtis* similarities matrix. A NMDS plot ranks fungal communities (represented by points) in ordination space in a way that the distance between two points is inversely proportional to their similarity. When using the similarity values for every organ/sampling sites, a clear distinction between organs is detected. Analysing the organs individually (Figure 13) shows dissimilarities between the roots and the aboveground organs.

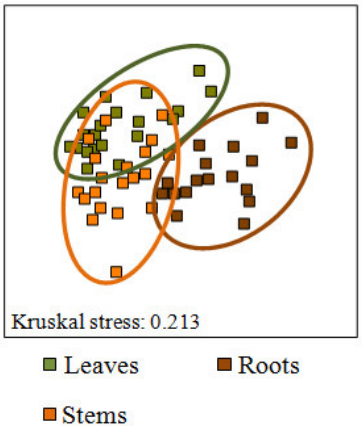

Figure 13. Non-metric multidimensional scale (NMDS) plot corresponding to the clustering analysis for the three organs (leaves, stems and roots), performed with Bray-Curtis coefficient. The different set of colors represent different trees.

That dissimilarity is more evident comparing the roots and the leaves. Despite the fact that all clusters are statistically different in dissimilarity (at P=0.001), some connection can be found between leaves and stems. Kruskal's stress values inferior to 0.2 represent good ordination plots, where as values greater that 0.3 provide a poor representation. In this case the Kruskal stress factor is above 0.2 (Figure 13), however, due to the number of samples analysed, the results can be said as significantly different.

When comparing the dissimilarities of the trees as a whole (Figure 14), Gerês clearly stands out from the other study sites, showing no similarity. The other three study sites demonstrate some similarity, however only Alcobaça and Grândola showed no statistically differences in dissimilarity (at P=0.001).

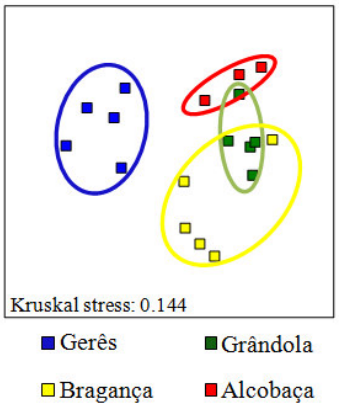

Figure 14. Non-metric multidimensional scale (NMDS) plot corresponding to the clustering analysis among samples for the all tree, performed with Bray-Curtis coefficient. The different set of colors represent different trees.

When analysing the roots, all sites seem to be clustered and significantly dissimilar (Figure 15C). However, significant statistically differences were not found between Alcobaça and Grândola (at P=0.001). All sites share some similarity except Gerês, which stands out when compared with the other study sites (Figure 15C). When analyzing the stems, no significant differences in dissimilarity (at P=0.001) can be found between Bragança and Grândola (Figure 15B). On the other hand, Gerês and Alcobaça show dissimilarity with the other study sites. The dissimilarities between Bragança and Alcobaça are mainly due to morphotypes F (26%), I (17%) and E (16%), where as between Bragança and Gerês are mainly due to morphotypes I (44%) and D (16%). The dissimilarities between Grândola and Alcobaça are mainly due to morphotypes F (23%), E (14%) and C (13%), where as between Grândola and Gerês are mainly due to morphotypes I (32%), C (14%) and E (14%). Looking at the leaves, all sites are statistically dissimilar (at P=0.001). Nevertheless, some similarity distinguishes the northern from the southern regions. When comparing all study sites, Bragança clearly shows the most dissimilarity within itself, that is mainly due to two morphotypes, D (60%) and F (35%). Furthermore, morphotypes D and F are the main cause for dissimilarity between, Bragança/Alcobaça and Bragança/Grândola with a cumulative contribution of 67% and 86%, respectively.

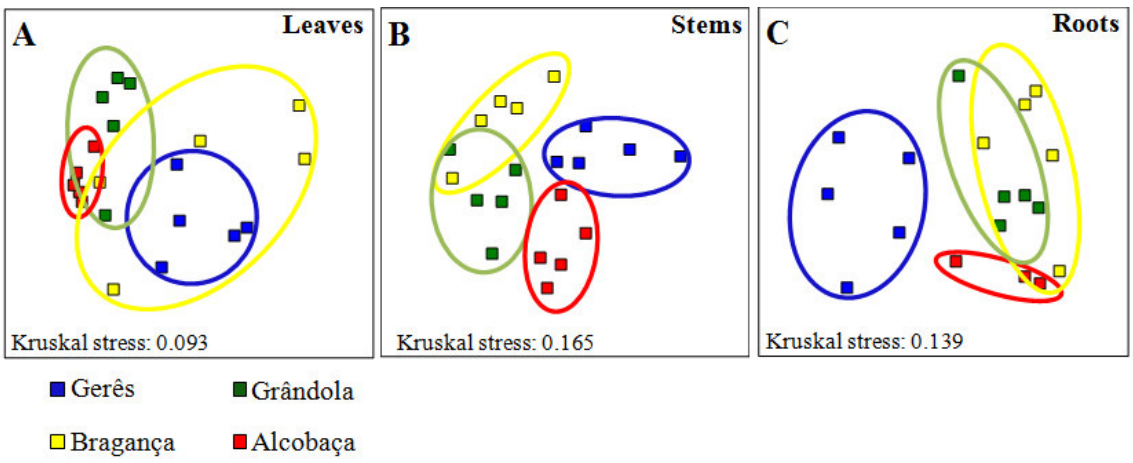

Figure 15. Non-metric multidimensional scale (NMDS) plots corresponding to the clustering analysis among samples, performed with Bray-Curtis coefficient. Panels A, B, C represent the analysis for the leaves, stems and roots, respectively. The different set of colors represent different trees.

3.3. Endophyte species identification

For determining which species could be responsible for each morphotype, different isolates were selected for molecular identification using the sequence of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). After sequencing, two databases (NCBI and UNITE) were used for the molecular identification of fungi (Table 11). From the isolates obtained from 24 morphotypes, it was not possible to sequence four morphotypes (O, Y, U, L), or the BLAST only recognized a fungal OTU not specifying further taxonomic data (J, ÇÇ). Certain fungi are clearly related with a specific morphotype, like Pezizomycetes, *Sordaria fimicola*, or *Penicillium* spp. that appear to be associated with morphotype A, B and V, respectively. *B. mediterranea* is also strongly related with morphotype D and Nectriaceae with morphotype H. However, many fungal species appear to be responsible for the same morphotype (for example, C, G, or X). On the other hand, certain species are suggested to originate different morphotypes.

The *Gnomoniopsis* genus was the most prevalent result and was mainly grouped as morphotypes E, F and I. This could suggest different species of *Gnomoniopsis*, however the alignment of all sequences showed that all *Gnomoniopsis* sequences were almost identical (Figure A 1, annex). There are three *Gnomoniopsis* with differences in one nucleotide (single nucleotide polymorphism, SNP). Those differences were detected in isolates obtained from the leaves of Bragança (Figure A 1, annex). This genus forms a well-known group of endophytes. Studies performed in *Castanea sativa* showed that they can lead to canker formation and tree rot (Rai and Dar, 2013; Dennert *et al.*, 2015).

From all the fungi identified some arouse more attention than others, mainly due to their relation with diseases in *Quercus* species. The necrotrophic activity of *B. mediterranea* and canker formation on water-stressed hosts was described in previous studies (Vannini and Valentini, 1994; Vannini *et al.*, 1996; Luchi *et al.*, 2005). This fungus appears in all studied sites except Gerês and was mainly identified as morphotype D (Table 11). However, one exception was found in Grândola, where *B. mediterranea* was identified as having morphotype I. Alignments of all sequencing results showed that several *B. mediterranea* species had SNPs between them (Figure A 2, annex). From all the studied sites, Bragança had the highest number of *B. mediterranea* isolates.

Table 11. Representation of the sequencing results obtained from the molecular identification. Fungi represents isolates that had no sequencing result.

Morphotype	Bragança	Gerês	Alcobaça	Alentejo
	Sequencing Result	Sequencing Result	Sequencing Result	Sequencing Result
A	*Pezizomycetes* *Pezizomycetes*	*Trichoderma gamsii* *Pezizomycetes* *Pezizomycetes* *Pezizomycetes* *Diaporthe passiflorae*	*Pezizomycetes* *Diplodia corticola* *Diplodia corticola* *Neofusicoccum luteum*	Fungi *Diplodia corticola* *Diplodia corticola* *Pezizomycetes* *Botryotinia* *Trichoderma virens*
B	*Sordaria fimicola* *Phomopsis columnaris*	*Gelasinospora seminuda* *Sordaria fimicola* *Sordaria fimicola*	*Diaporthales* *Diaporthales* *Gelasinospora seminuda* *Gelasinospora tetrasperma* *Diplodia corticola* *Diplodia corticola* *Sordaria fimicola* *Trichoderma virens*	*Sordaria fimicola* *Sordaria fimicola* *Sordaria fimicola* *Trichoderma atroviride* *Macrophomina phaseolina*
C	Fungi Fungi Fungi *Ascomycota* *Pleosporaceae* *Pleosporaceae* *Cladosporium cladosporioides*	Fungi *Cladosporium cladosporioides* *Botryosphaeria dothidea* *Alternaria alternata*	*Cladosporium* *Cladosporium cladosporioides*	Fungi *Penicillium brevicompactum* *Cladosporium*
D	Fungi *Umbelopsis ramanniana* *Aureobasidium pullulans* *Alternaria photistica* *Alternaria alternata* *Biscogniauxia mediterranea* *Biscogniauxia mediterranea* *Biscogniauxia mediterranea* *Biscogniauxia mediterranea* *Biscogniauxia mediterranea* *Biscogniauxia mediterranea* *Biscogniauxia mediterranea* *Daldinia* *Pyrenophora bisepiata* *Leptosphaeria maculans* *Phoma* *Chromelosporium carneum* *Neonectria*	Fungi	*Biscogniauxia mediterranea* *Biscogniauxia mediterranea*	-
E	-	-	*Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis*	*Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Mortierella* *Phomopsis velata*
F	Fungi *Umbelopsis* *Dactylonectria estremocensis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Fimetariella rabenhorstii*	Fungi *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Diaporthe passiflorae*	*Gnomoniopsis* *Gnomoniopsis* *Diaporthe viticola*	*Cryptosporiopsis* *Cryptosporiopsis* *Paecilomyces verrucosus*
G	Fungi Fungi *Gibberella pulicaris*	*Epicoccum nigrum* *Epicoccum nigrum* *Penicillium adametzii*	Fungi Fungi Fungi *Epicoccum nigrum* *Gnomoniopsis* *Fusarium acutatum*	Fungi *Fusarium oxysporum* *Fusarium acutatum* *Dactylonectria estremocensis* *Dactylonectria estremocensis* *Fusarium acutatum* *Mariannaea samuelsii* *Epicoccum nigrum* *Thelonectria veuillotiana* *Thelonectria veuillotiana* *Thelonectria veuillotiana* *Umbelopsis ramanniana* *Alternaria alternata* *Cryphonectria*
X	*Alternaria* *Phomopsis columnaris* *Phomopsis*	-	*Roselinia corticium*	Fungi Fungi *Tricholomataceae* *Cryptosporiopsis* *Mycena*
I	-	*Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis*	*Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis* *Gnomoniopsis*	*Biscogniauxia mediterranea* *Gnomoniopsis* *Gnomoniopsis*
J	-	-	-	Fungi Fungi
K	*Gelasinospora tetrasperma*	-	-	-
H	-	*Nectriaceae* *Nectriaceae* *Ascomycota*	*Ilyonectria* *Ilyonectria* *Ilyonectria* *Nectriaceae*	-
O	-	-	-	-
R	*Aspergillus niger*	-	-	-
Y	-	-	-	-
Z	Fungi Fungi *Pleosporaceae* *Pleosporaceae*	Fungi Fungi Fungi	Fungi Fungi Fungi *Gnomoniopsis*	-
T	Fungi Fungi *Alternaria*	-	-	-
U	-	-	-	-
N	-	*Umbelopsidaceae* *Umbelopsidaceae*	-	-
V	*Penicillium*	*Penicillium godlewskii*	*Penicillium canescens* *Penicillium canescens* *Saccharicola bicolor*	*Penicillium roseopurpureum* *Penicillium adametzioides*
Q	-	*Umbelopsis* *Umbelopsis* *Umbelopsis*	-	-
Fx	-	Fungi *Umbelopsis* *Umbelopsis*	*Umbelopsis versiformis* *Umbelopsis versiformis* *Umbelopsis versiformis*	*Umbelopsis versiformis* *Umbelopsis versiformis* *Umbelopsis versiformis*
L	-	-	-	-
ÇÇ	-	-	-	Fungi

Another important species for cork oak found in this study was *Diplodia corticola*, a well known agent of canker disease in several *Quercus* spp. This fungus was mainly grouped as morphotype A. In Alcobaça however two isolates of *D. corticola* were grouped in morphotype B. Alignments of all sequencing results show that all *D. corticola* show SNPs between them (Figure A 3, annex). This species was only found in the stems (Table 12).

The organs that the identified isolates infected were determined (Table 12). This revealed that only one genus (*Alternaria* spp.) is shared between the roots and the above ground organs (leaves and stems) (Table 12). A total of four species is shared between both aerial organs (highlighted in Table 12 with an asterisk). The pathogenic *B. mediterranea* isolates only appear in the above ground organs (Table 12). The *Umbelopsis* spp. is exclusive to the roots (Table 12) and the alignment of all the sequence results (Figure A 4, annex) showed that the most diversity among isolates was found in this genus.

To understand the species richness found in each study site, Krona charts comprising all well-assigned sequences of each site were created. All well-assigned sequences of Bragança were included in two phyla, five classes, 12 orders, 14 families, 22 genera and 16 species (Figure 16). The richest phyla was Ascomycota (93%) followed by Zygomycota (7%). Within Ascomycota the highest OTU number belonged to the Sordariomycetes (46%) class, followed by Dothideomycetes (35%), Eurotiomycetes (8%) and Pezizomycetes (8%), while the Zygomycota was represented by Incertae sedis (100%). Zygomycota phylum was only represented by Incertae sedis class.

All well-assigned sequences of Gerês were included in two phyla, four classes, 10 orders, 10 families, 11 genera and 10 species (Figure 17). The richest phyla was Ascomycota (92%) followed by Zygomycota (8%). Within Ascomycota the highest OTU number belonged to the Sordariomycetes (46%) class, followed by Dothideomycetes (31%), Eurotiomycetes (15%) and Pezizomycetes (8%), while Zygomycota was represented by Incertae sedis (100%). Zygomycota phylum was only represented by Incertae Sedis class.

Table 12: Species and genus identified separated by which organ they were detected in.

Organ of isolation	Isolated fungi
Leaves	*Alternaria photistica* *Botryosphaeria dothidea* *Diaporthe passiflorae* *Epicoccum nigrum* *Gelasinospora tetrasperma* *Leptosphaeria maculans* *Phomopsis velata* *Pyrenophora bisepta* *Rosellinia corticium* *Gnomoniopsis* spp. **Sordaria fimicola* **Alternaria alternata* **Biscogniauxia mediterranea* **Cladosporium cladosporioides*
Stems	*Neofusicoccum luteum* *Gelasinospora seminuda* *Fimetariella rabenhorstii* *Diaporthe viticola* *Diplodia corticola* *Chromelosporium carneum* *Aureobasidium pullulans* *Daldinia* spp. *Cryphonectria* spp. *Phoma* spp. **Sordaria fimicola* **Alternaria alternata* **Biscogniauxia mediterranea* **Cladosporium cladosporioides* **Alternaria* spp.
Roots	*Phomopsis columnaris* *Penicillium godlenskii* *Giberella pulicaris* *Fusarium acutatum* *Dactylonectria estremocensis* *Aspergillus niger* *Fusarium oxysporum* *Thelonectria veuillotiana* *Mariannaea samuelssi* *Macrophomina phaseolina* *Penicillium brevicompactum* *Penicillium roseopurpureum* *Paecilomyces verrucosus* *Saccharicola bicolor* *Trichoderma atroviride* *Trichoderma gamsii* *Trichoderma virens* *Umbelopsis ramanniana* *Umbelopsis versiformis* *Cryptosporiopsis* spp. *Umbelopsis* spp. *Mortierella* spp. *Mycena* spp. *Phomopsis* spp. *Penicillium* spp. *Ilyonectria* spp. *Botryotinia* spp. **Alternaria* spp.

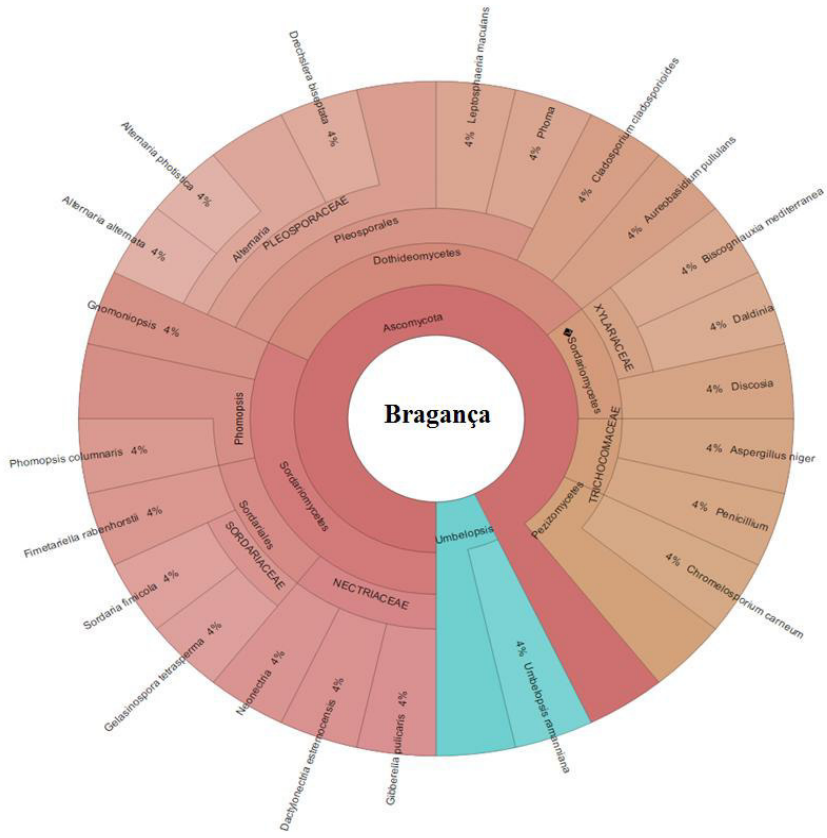

Figure 16. Representation of fungal taxonomic richness obtained from Bragança. Charts created on Krona (Ondov *et al.*, 2011).

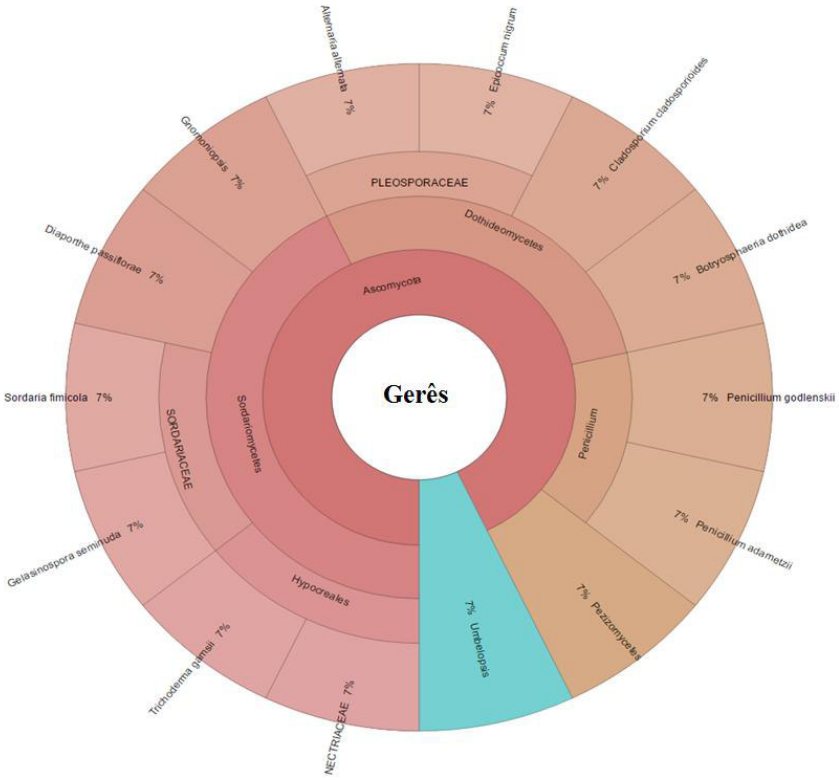

Figure 17. Representation of fungal taxonomic richness obtained from Gerês. Charts created on Krona (Ondov *et al.*, 2011).

All well-assigned sequences of Alcobaça were included in two phyla, four classes, 10 orders, 11 families, 14 genus and 13 species (Figure 18). The richest phyla was Ascomycota (94%) followed by Zygomycota (6%). Within Ascomycota the highest OTU number belonged to the Sordariomycetes (53%) class, followed by Dothideomycetes (35%), Eurotiomycetes (6%) and Pezizomycetes (6%), while Zygomycota was represented by Incertae sedis (100%). Zygomycota phylum was only represented by Incertae Sedis class.

All well-assigned sequences of Grândola were included in three phyla, seven classes, 13 orders, 17 families, 21 genus and 20 species (Figure 19). The richest phyla was Ascomycota (83%) followed by Zygomycota (10%) and Basidiomycota (7%). Within Ascomycota the highest OTU number belonged to the Sordariomycetes (50%) class, followed by Dothideomycetes (21%), Eurotiomycetes (17%) and Leotiomycetes (8%) and Pezizomycetes (4%), while Zygomycota was represented by Incertae sedis (100%) and Basidiomycota was represented by Agaricomycetes (100%). Zygomycota phylum was only represented by Incertae Sedis class, where as Basidiomycota was only represented by Agaricomycetes class.

Figure 18. Representation of fungal taxonomic richness obtained from Alcobaça. Charts created on Krona (Ondov *et al.*, 2011).

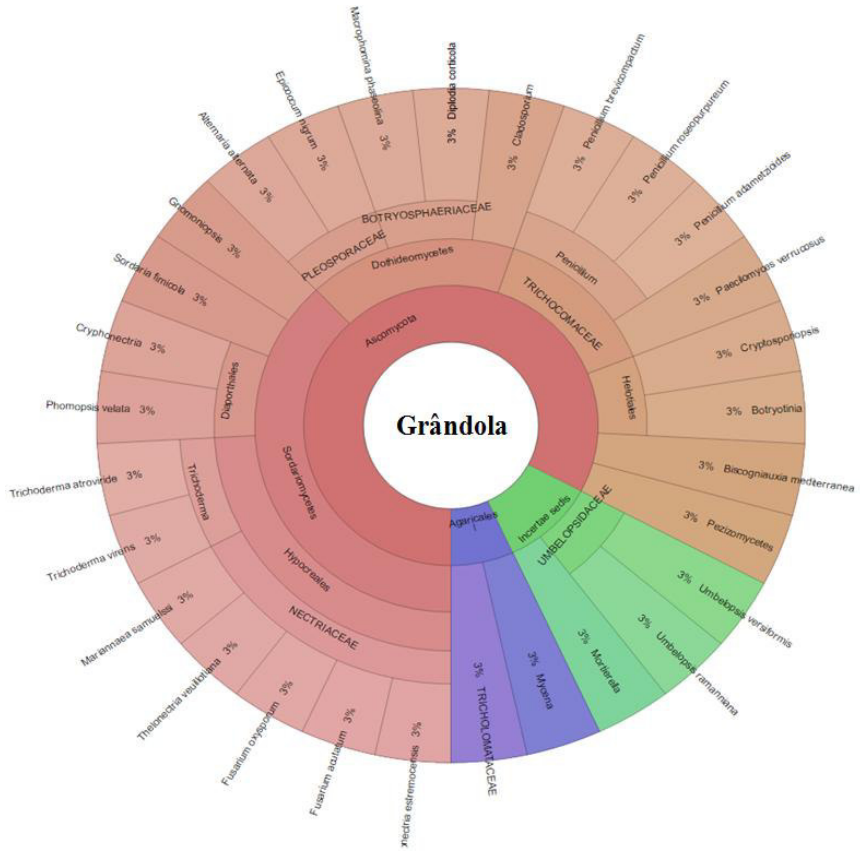

Figure 19. Representation of fungal taxonomic richness obtained from Grândola. Charts created on Krona (Ondov *et al.*, 2011).

3.4. Antagonism assays

A total of 20 fungi were selected to evaluate the potential antagonism against *Biscogniauxia mediterranea* and *Diplodia corticola*, two well-known pathogenic fungi for several *Quercus* species. In the Mediterranean Basin, *B. mediterranea* is the main agent of charcoal canker, causing cork oak decline (Vannini *et al.*, 2009). In Portugal, this disease is widespread on adult declining trees and nowadays is increasingly affecting young trees (Henriques *et al.*, 2016). On the other hand, *D. corticola* causes progressive necrosis of the bark, epicormic cambium, dieback of branches and yellowing of the leaves, ultimately leading to tree death (Dreaden *et al.*, 2016). For these reasons and to attempt to devise a potential biocontrol agent, it would be important to understand all the relations between non-harming endophytes and these disease agents. The interacting fungi to be assayed were selected based on previous described works of antagonism, endophytes or because they were not studied before (Table 13 and 14). Moreover, some of these fungi cause diseases on other plants.

Table 13: Endophytic fungi used in the co-culture with *Biscogniauxia mediterranea*, along with relevant previous studies.

Fungi	Reference
Gelasinospora tetrasperma	-
Trichoderma virens	Djonovic *et al*., 2007
Alternaria photistica	-
Alternaria alternata	Musetti *et al.*, 2006
Giberella pulicaris	-
Leptosphaeria maculans	Howlett *et al.*, 2001
Fimetariela rabenhorstii	-
Dactylonectria estremocensis	-
Aspergillus niger	Schena *et al*., 1999
Cladosporium cladosporoides	Wang *et al.*, 2013

Table 14: Endophytic fungi used in the co-culture with *Diplodia corticola*, along with relevant previous studies.

Fungi	Reference
Gelasinospora tetrasperma	-
Gelasinospora seminuda	Oki *et al*., 2016
Trichoderma virens	Djonovic *et al*., 2007
Neofusicoccum luteum	-
Fusarium acutatum	-
Saccharicola bicolor	Eriksson and Hawksworth, 2003
Rosellinia corticium	Osono *et al.*, 2012
Diaporthe viticola	-
Ilyonectria spp.	Halleen *et al*., 2004
Gnomoniopsis spp.	-

The antagonism assays were performed in MMN (pH 6.6) over a period of four days. The interactions were evaluated in regard to mycelia growth by measuring the colonies rays, more specifically the inner (inoculate inter-region) and the external (diametrically opposed region) rays.

3.4.1. Interactions with *Biscogniauxia mediterranea*

The fungi used to study the potential antagonism against *B. mediterranea* were selected from Bragança. This site showed both the pathogenic fungi and fungi with high potential for antagonism. Moreover, some fungi were selected because no information regarding their antagonistic potential is known. The progression of antagonism assays performed with *B. mediterranea* was followed by measuring the fungal growth of both interacting fungi, in inter-inocula region (internal radius, Figure 20) and in the diametrically opposed region (external radius, Figure 21).

Many interacting fungi presented higher growth rates than *B. mediterranea*, which led to a rapid occupancy of inter-inocula region after 24 hours. This aspect is visible when the mycelia growth is represented as a function of time, in which the growth of interacting fungi is several times higher than that of *B.mediterranea*. This is the case of *G. tetrasperma* (six-fold higher growth) and *Trichoderma virens* (four-fold higher growth, where no significant change in the growth of *B. mediterranea* was detected, either in the presence or absence of the interacting fungus. However, when comparing the growth of *G. tetrasperma* in the co-culture (*Gt-Bm*) and corresponding control (*Gt-Gt*), or *T. virens* in co-culture (*Tv-Bm*) and control (*Bm-Bm*), there are significant changes showing a higher growth rate of interacting fungi in both co-coltures (*Gt-Bm* and *Tv-Bm*). This suggests that the presence of *B. mediterranea* stimulates the growth of *G. tetrasperma* and *T. virens* without compromising its own growth. Four days after inoculation, when there was contact between the hyphae of interacting fungi, a hyphae barrier was formed by *B. mediterranea* in the presence of *G. tetrasperma*, while no barrier is formed when assaying *T. virens*, which simply grows over *B. mediterranea*. (Figure 22).

Alternaria photistica and *Giberella pulicaris*

The growth rate of *A. photistica* and *Giberella pulicaris* is equivalent to *B. mediterranea* growth, taking four days for the mycelia to touch. *A. photistica* is not affected nor affects the *B. mediterranea* growth. However, comparing the growth of *B. mediterranea* in co-culture (*Gp-Bm*) and in control (*Bm-Bm*), a significant increase in growth of *B. mediterranea* was detected in *Gp-Bm* internal radius. This difference was not noticeable when analysing the external radius. After mycelia contact, both interacting fungi formed a dense barrier of hyphae (Figure 23).

Leptosphaeria maculans

L. maculans does not seem to exert an inhibitory effect on the growth of *B. mediterranea*, both in the inner and external rays (Figure 20E and 21E). The growth of *B. mediterranea* in co-culture (*Lp-Bm*) was similar to the control (*Bm-Bm*). Moreover, it seems that *B. mediterranea* exerts an inhibitory effect on the growth of *L. maculans*. Four days after inoculation, when there is contact between the two fungi, no barrier is formed. *B. mediterranea* begins to grow over the mycelium of *L. maculans*, and after seven days covers the entire plate.

Fimetariela rabenhorstii

Evaluating the interaction between *F. rabenhorstii* and *B. mediterranea*, the fungus *F. rabenhorstii* does not seem to exert an inhibitory effect on both the inner and external radial growth of *B. mediterranea* (Figure 20F and 21F). Three days after inoculation, when there is contact between the two fungal hyphae, no barrier is formed and *B. mediterranea* starts to grow under the mycelium of *F. rabenhorstii* without morphological changes occurring.

Dactylonectria estremocensis

The interaction *D. estremocensis* with *B. mediterranea* does not show an inhibitory effect on the growth of *B. mediterranea* (Figure 20G and 21G), presenting a very similar behaviour with the interaction between *F. rabenhorstii* with *B. mediterranea*. After the contact of both mycelia, no barrier is formed and *B. mediterranea* starts growing over the mycelium of *D. estremocensis*, eventually covering the entire plate.

Alternaria alternata

Apparently the *A. alternata* does not exert an inhibitory effect on the growth of *B. mediterranea* as can be seen by measuring the mycelial growth (Figure 20H and 21H). Also, *A. alternata* grew similarly either in culture with *B. mediter*ranea and in control situation (*Aa-Aa*). Moreover, when there is contact between the two hyphal fungi, *B. mediterranea* does not appear to be inhibited in the contact zone, there is no change of the macroscopic hyphae nor formation of a barrier defense. *B. mediterranea* begins to grow over the mycelium of *A. alternata*, eventually covering the entire plate.

Aspergillus niger

The fungus *A. niger* apparently did not exert inhibitory effects on the growth of *B. mediterranea* (Figure 20I and 21I). There is greater growth of *B. mediterranea* in co-culture (*An-Bm*) than in the control situation (*Bm- Bm*). *A. niger* seems to be inhibited by the presence of *B. mediterranea* since its growth is significantly higher in the control situation (*An-An*) than in the co-culture (*An-Bm*). This inhibition is more evident in the internal radius growth (Figure 20I). When there is contact between the two interacting fungi, *A. niger* appears to offer some resistance in the contact region, forming a barrier separating both fungi, similar to *A. photistica*. After four days, *B. mediterranea* covers the all plate, however it does not grow over *A. niger*.

Cladosporium cladosporoides

The fungus *C. cladosporoides* does not appear to exert a inhibitory effect on *B. mediterranea* growth (Figure 20J and 21J). Moreover, the growth of *B. mediterranea* is slightly increased when in co-culture with *C. cladosporoides* (*Cc-Bm*). In this case, it seems that *B. mediterranea* exerts an inhibitory effect on the growth of *C. cladosporoides*. After four days, contact occurs between the two interacting fungi and a barrier of *C. cladosporoides* hyphae is formed, similar to *A. photistica,* preventing the progression of *B. mediterranea*, which grows around the mycelium of *C. cladosporoides*.

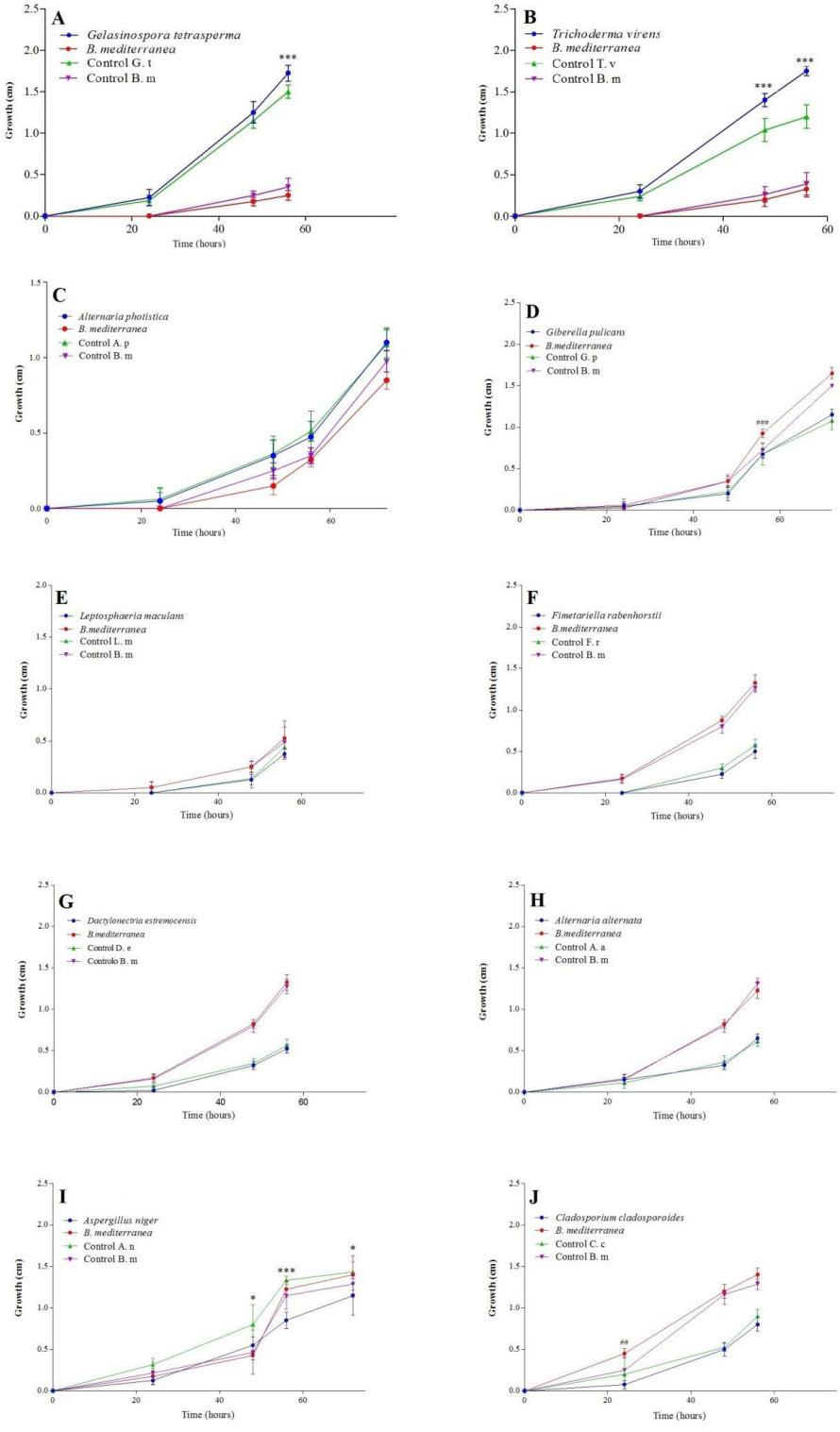

Figure 20. Evaluation of fungal interaction by measuring the internal radius (inter-inocula) during four days of co-culture with *Biscogniauxia mediterranea* and several interacting fungi: (A) *G. tetrasperma*, (B*) T. virens*, (C) *A. photistica*, (D) *G. pulicaris*, (E) *L. maculans,* (F) *F. rabenhorstii*, (G) *D. estremocensis*, (H) *A. alternata*, (I) *A. niger* and (J) *C. cladosporoides*. *,** and *** indicate that the differences between the mean values of the interacting fungus and corresponding controls are significant (P=0.05, P≤0.01 and P≤0.001, respectively). The #, ## and ### indicate the same for the mean values of *B.mediterranea* and respective controls.

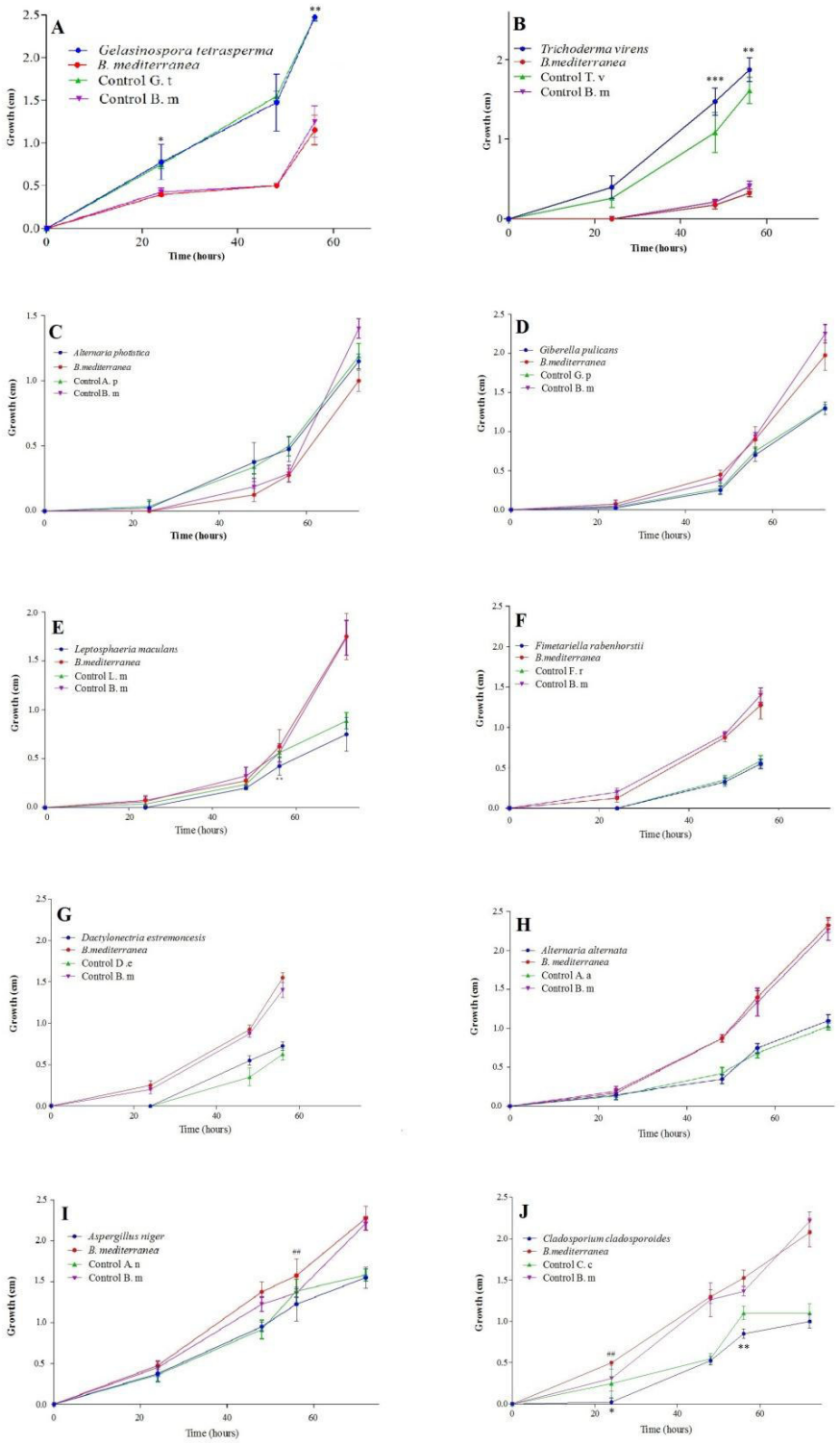

Figure 21. Evaluation of fungal interaction by measuring the external radius (diametrically opposed region) four days of co-culture with *Biscogniauxia mediterranea* and several interacting fungi: (A) *G. tetrasperma*, (B*) T. virens*, (C) *A. photistica*, (D) *G. pulicaris*, (E) *L. maculans,* (F) *F. rabenhorstii*, (G) *D. estremocensis*, (H) *A. alternata*, (I) *A. niger* and (J) *C. cladosporoides*. *,** and *** indicate that the differences between the mean values of the interacting fungus and corresponding controls are significant (P=0.05, P≤0.01 and P≤0.001, respectively). The #, ## and ### indicate the same for the mean values of *B.mediterranea* and respective controls.

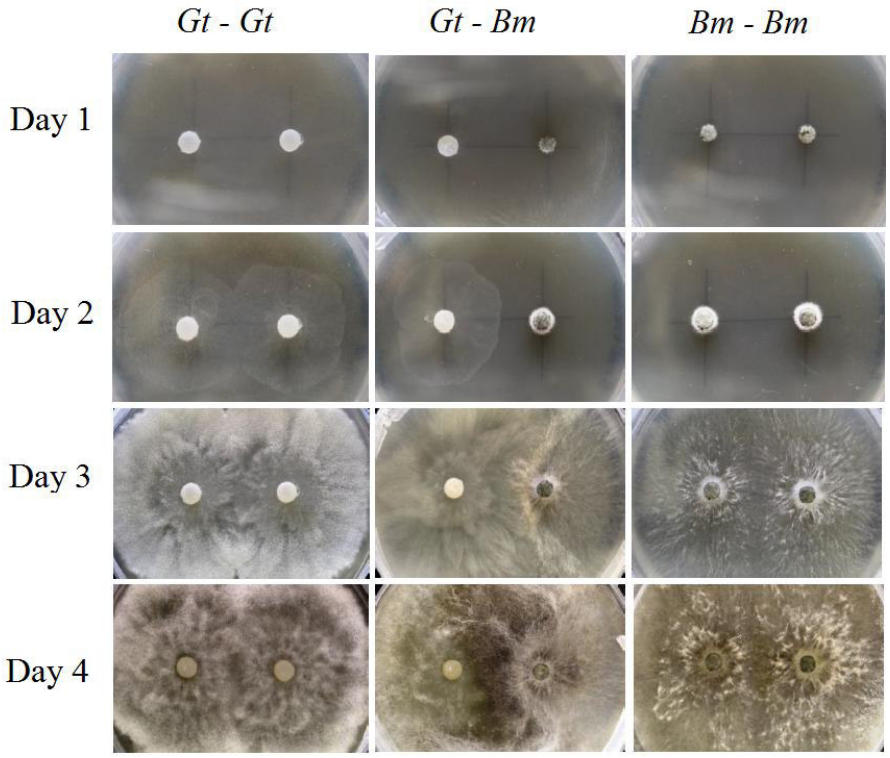

Figure 22. Morphological aspect of the co-cultures between *G. tetrasperma – G. tetrasperma* (*Gt-Gt*), *G. tetrasperma – B. mediterranea* (*Gt-Bm*) and *B. mediterranea – B. mediterranea* (*Bm-Bm*).

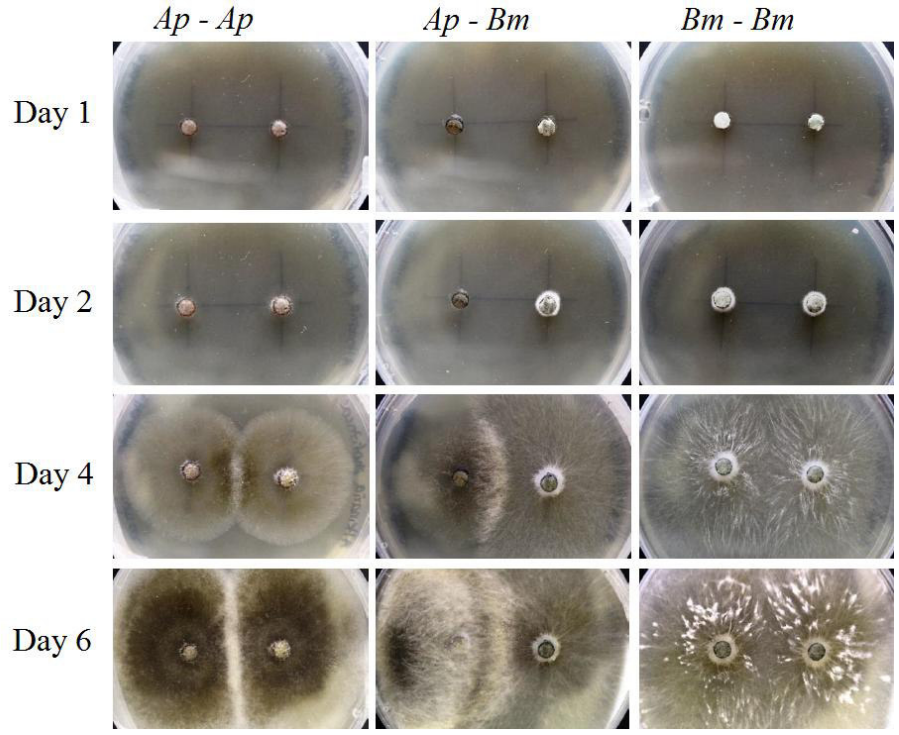

Figure 23. Morphological aspect of the co-cultures between *A. photistica – A. photistica* (*Ap-Ap*), *A. photistica – B. mediterranea* (Ap-Bm) and *B. mediterranea – B. mediterranea* (*Bm-Bm*).

Table 15 summarizes the main features observed during the interactions with *B. mediterranea.* Some tests showed that the growth of *B. mediterranea* was higher in the presence of the interacting fungi. However, only two interacting fungi showed growth stimulation when in presence of *B. mediterranea* (*G. tetrasperma* and *T. virens*). In many cases several fungi were able to react by forming a hyphal barrier. The format of this barrier may indicate which of the interacting fungi is reacting to the presence of the other. Many fungi form a barrier when in presence of *B. mediterranea* (*A. photistica*, *G. Pulicaris*, *D. estremocensis*, *A. niger* and *C. cladosporoides*), only *G. tetrasperma* is able to promote a similar reaction in *B. mediterranea*.

Table 15: Summary of antagonism assays against *B. mediterranea*.

Interacting fungi	*B. m* inhibition	Interacting fungi inhibition	Hyphal barrier formation (formed by)	Type of antagonism
Gelasinospora tetrasperma	-	- (stimulates)	+ (*B. mediterranea*)	Mycelial contact and distance antagonism
Trichoderma virens	-	- (stimulates)	-	-
Alternaria photistica	-	-	+ (*A. photistica*)	Mycelial contact
Giberella pulicans	- (stimulates)	-	+ (G. pulicans)	Mycelial contact and distance antagonism
Leptosphaeria maculans	-	-	-	-
Fimetariela rabenhorstii	-	-	-	-
Dactylonectria estremocensis	-	-	-	Mycelial contact
Alternaria alternata	-	-	-	-
Aspergillus niger	- (stimulates)	+	+ (*A. niger*)	Mycelial contact and distance antagonism
Cladosporium cladosporoides	- (stimulates)	+	+ (*C. cladosporoides*)	Mycelial contact and distance antagonism

3.4.2. Interaction between *Diplodia corticola*

The fungi used to study the potential antagonism against *D. corticola* were selected from Alcobaça. This site showed both the pathogenic fungi and fungi with high potential for antagonism. Moreover, some fungi were selected because no information regarding their antagonistic potential is known. The progression of antagonism assays performed with *D. corticola* was followed by measuring the fungal growth of both interacting fungi, in inter-inocula region (internal radius, Figure 24) and in the diametrically opposed region (external radius, Figure 25).

Trichoderma virens

When analysing the internal radius of the interaction *T. virens* with *D. corticola* a clear inhibition on the growth of *D. corticola* is showed (Figure 24A). Furthermore, a significant growth in *T. virens* growth is noticed. However, that difference in growth is not noticeable when looking at the external radius (Figure 25A). Also, the growth of the external radius of *D.corticola*, despite not being very significant, is slightly higher than the control culture (*Dc-Dc*). In this interaction there is no noticeable formation of hyphal barriers.

Gelasinospora tetrasperma

The interaction *D. corticola* with G. tetrasperma is very similar to the interaction *D. corticola* with *T. virens* (*Tv-Dc*). The internal radius of *D. corticola* is significantly affected when in co-culture with *G. tetrasperma* (Figure 24B). Moreover, the growth of the internal radius of *G. tetrasperma* is significantly increased when in culture with *G. tetrasperma* (*Gt-Dc*). However, when analysing the external radius (Figure 25B), that difference is not noticeable and *D. corticola* has a slightly higher growth in co-culture compared with the control. After four days of interaction *D. corticola* forms a barrier protecting itself against *G. tetrasperma* (Figure 26).

Gelasinospora seminuda

G. seminuda significantly decreases the growth of *D.corticola* either in the internal and the external rays (Figure 24C and 25C). A significant increase in *G. seminuda* growth, when compared with control (*Gs-Gs*), can be seen when measuring the internal radius. On the other hand, that difference is not noticeable when analysing the external radius.

However, the external radius of *D. corticola* is, like the internal radius, significantly decreased (Figure 25C). In this interaction no visible barrier is formed with *G. seminuda* growing around *D. corticola*.

Neofusicoccum luteum

N. luteum does not appear to negatively affect the growth of D. corticola when in co-culture (Figure 24D and 25D). On the contrary, in co-culture with *N. luteum*, *D. corticola* demonstrated increased growth in both internal and external rays. This suggests that the presence of this interacting fungus promotes the growth of *D. corticola* not affecting its own growth. In this interaction *N. luteum* forms a barrier of hyphae, blocking the progress of *D corticola* (Figure 27).

Fusarium acutatum

The fungus *F. acutatum* shows decreased growth in the internal radius, compared with control (*Fa-Fa*), when in co-culture (*Fa-Dc*), while *D. corticola* has a significant increase in growth (Figure 24E). However, when analysing the external radius, a different result can be observed. F. acutatum has slightly higher growth than the control, almost like it is growing away from *D. corticola* (Figure 25E). On the other hand, *D. corticola* still grows more compared with the control (*Dc-Dc*). After the mycelial contact of both fungi, *D. corticola* forms a dark barrier between *F. acutatum* and itself.

Saccharicola bicolor

The interaction *S. Bicolor* with *D. corticola* is similar to the interaction *F. acutatum* with *D.corticola*. Again, the growth of *D. corticola* internal radius is significantly increased when in co-culture with *S. bicolor*, where as the growth of S. bicolor internal radius is significantly decreased (Figure 24F). Although statistically significant, the growth of the external radius of S. bicolor is not very noticeable (Figure 25F). Almost no changes can be noticed when comparing the external radius of *D. corticola* both in co-culture (*Sb-Dc*) and in control (*Dc-Dc*). After four days, *D.corticola* surround *S. bicolor* leaving a small space between the two and forms a barrier of hyphae. This characteristic also appears when *D. corticola* is in co-culture with *Ilyonectria* spp.

Rosellinia corticium

When analysing the interaction *R. corticium* with *D. corticola* (*Rc-Dc*) after two days, a clear increase in growth can be seen in the internal radius of *D.corticola* as well as a decrease in the internal radius of *R. corticium* (Figure 24G). However, almost no significant differences can be observed when comparing the external radius (Figure 25G). Only after four days a significant growth increase in *R. corticium* external radius can be observed. In this interaction there is no noticeable formation of a barrier by any of the fungi. After four days *D. corticola* surrounds *R. corticium*, eventually covering the all plate.

Diaporthe viticola

Evaluating the interaction *D. viticola* with *D. corticola*, the fungus *D. corticola* shows a significant increase in internal radius growth when compared with the control (*Dc-Dc*) (Figure 24H). On the other hand, the internal radius of *D. viticola* is significantly decreased when in co-culture (*Dv-Dc*). The growth of the external radius of *D.viticola* does not appear to be affected by the co-culture with *D. corticola* (Figure 25H). However, a slight increase in the growth of the external radius of *D. corticola* can be observed almost after two days. Just like *S. bicolor* and *Ilyonectria* spp., in this interaction *D. corticola* surrounds *D. viticola* forming a barrier that separates both fungi, leaving a small space between the two.

Ilyonectria spp.

Ilyonectria spp. shows decreased growth in the internal radius when in co-culture with *D. corticola*. On the other hand, the internal radius of *D. corticola* significantly increases, especially after the first 24 hours (Figure 24I). When analysing the external radius (Figure 25I), no significant differences in growth were observer in for *D. corticola*. The same happens when observing the external radius of *Ilyonectria* spp., except for the last measurement, where the growth in co-culture (*I spp.-Dc*) is decreased. After four days *D. corticola* stars to surround *Ilyonectria* spp. leaving a small space between the two (Figure 28). By the sixth day of observation, *D. corticola* has almost totally surrounded *Ilyonectria* spp., never touching each it.

Gnomoniopsis spp.

The interaction *Gnomoniopsis* spp. with *D. corticola* shows a significant increase in the internal radius of *D. corticola*, as well as a significant decrease of the internal radius of *Gnomoniopsis* spp. (Figure 24J). Analysing the growth of the external radius, no significant differences in the growth of *D. corticola* can be found, however when looking at the growth of *Gnomoniopsis* spp., when in co-culture, the growth rate after two days seems to be higher than the control (Figure 25J). As time passes, *D. corticola* starts to surround *Gnomoniopsis* spp. eventually covering it and the entire plate.

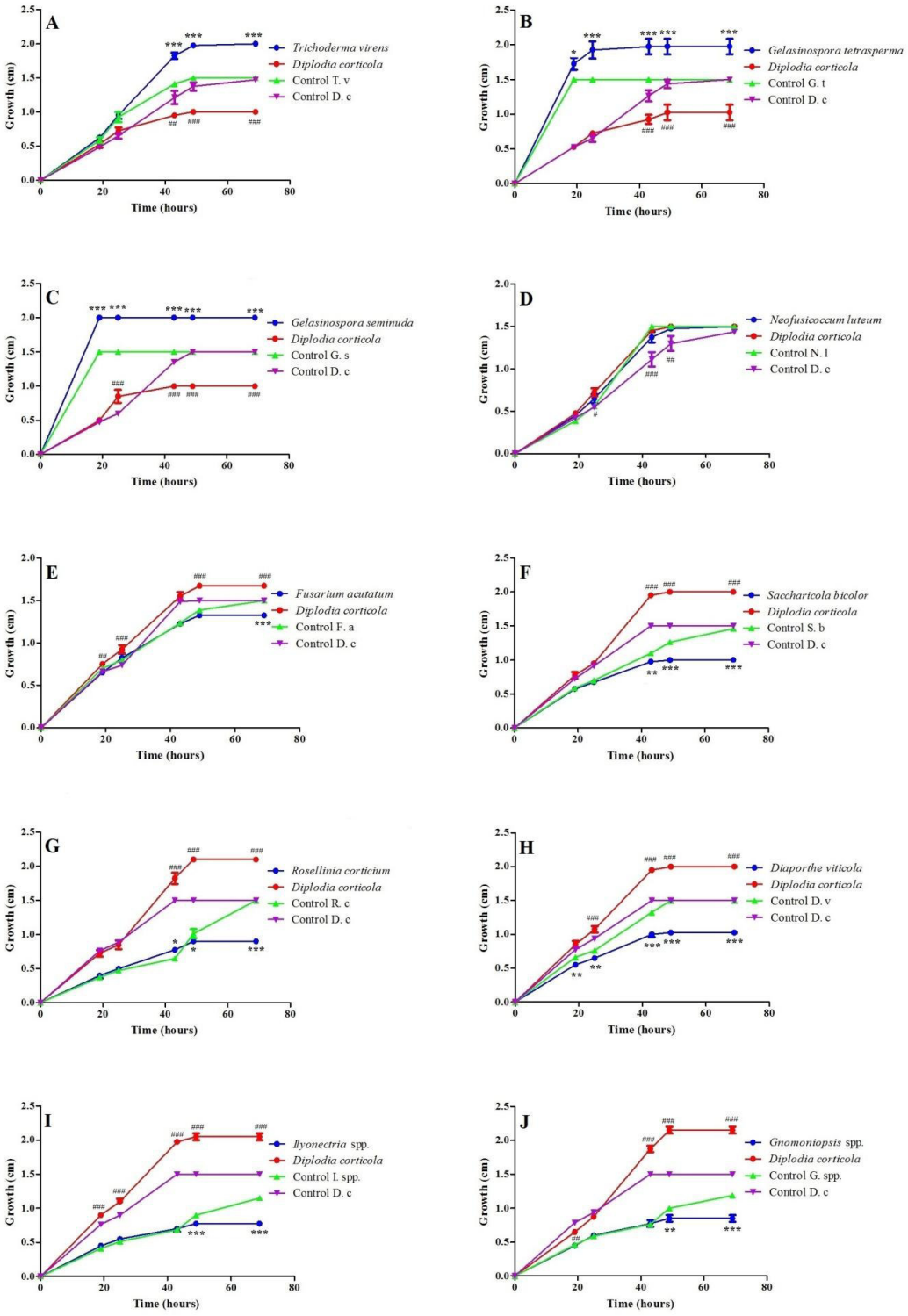

Figure 24. Evaluation of fungal interaction by measuring the internal radius (inter-inocula) during four days of co-culture with *Diplodia corticola* and several interacting fungi: (A) *T. virens*, (B) *G. tetrasperma*, (C) *G. seminuda*, (D) *N. luteum*, (E) F. acutatum, (F) *S. bicolor*, (G) *R. corticium*, (H) *D. viticola*, (I) *Ilyonectria spp*. and (J) *Gnomoniopsis* spp. *,** and *** indicate that the differences between the mean values of the interacting fungus and corresponding controls are significant (P=0.05, P≤0.01 and P≤0.001, respectively). The #, ## and ### indicate the same for the mean values of *B.mediterranea* and respective controls.

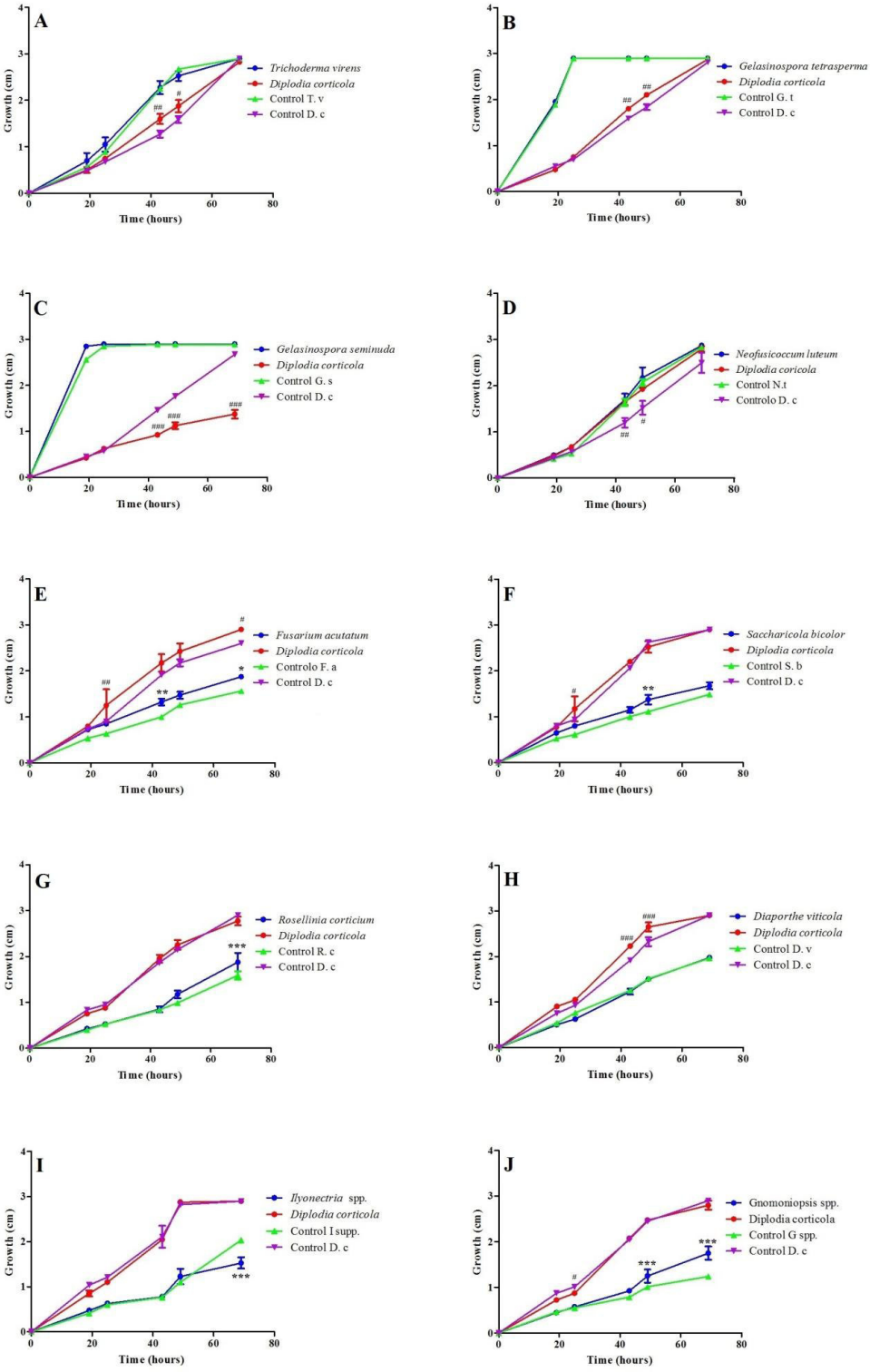

Figure 25. Evaluation of fungal interaction by measuring the external radius (diametrically opposed region) during four days of co-culture with *Diplodia corticola* and several interacting fungi: (A) *T. virens*, (B) *G. tetrasperma*, (C) *G. seminuda*, (D) *N. luteum*, (E) F. acutatum, (F) *S. bicolor*, (G) *R. corticium*, (H) *D. viticola*, (I) *Ilyonectria spp*. and (J) *Gnomoniopsis* spp. *,** and *** indicate that the differences between the mean values of the interacting fungus and corresponding controls are significant (P=0.05, P≤0.01 and P≤0.001, respectively). The #, ## and ### indicate the same for the mean values of *B.mediterranea* and respective controls.

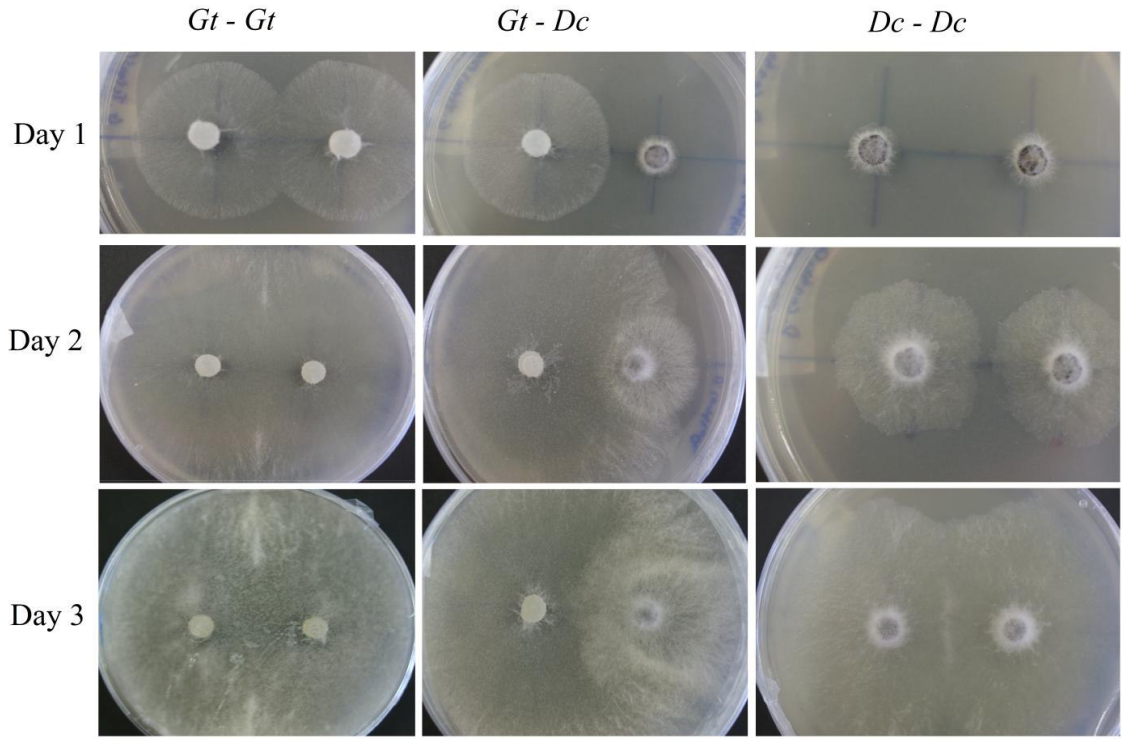

Figure 26. Morphological appearance of the co-cultures between *G. tetrasperma – G. tetrasperma* (*Gt-Gt*), *G. tetrasperma – D. corticola* (*Gt-Dc*) and *D. corticola – D. corticola* (*Dc-Dc*).

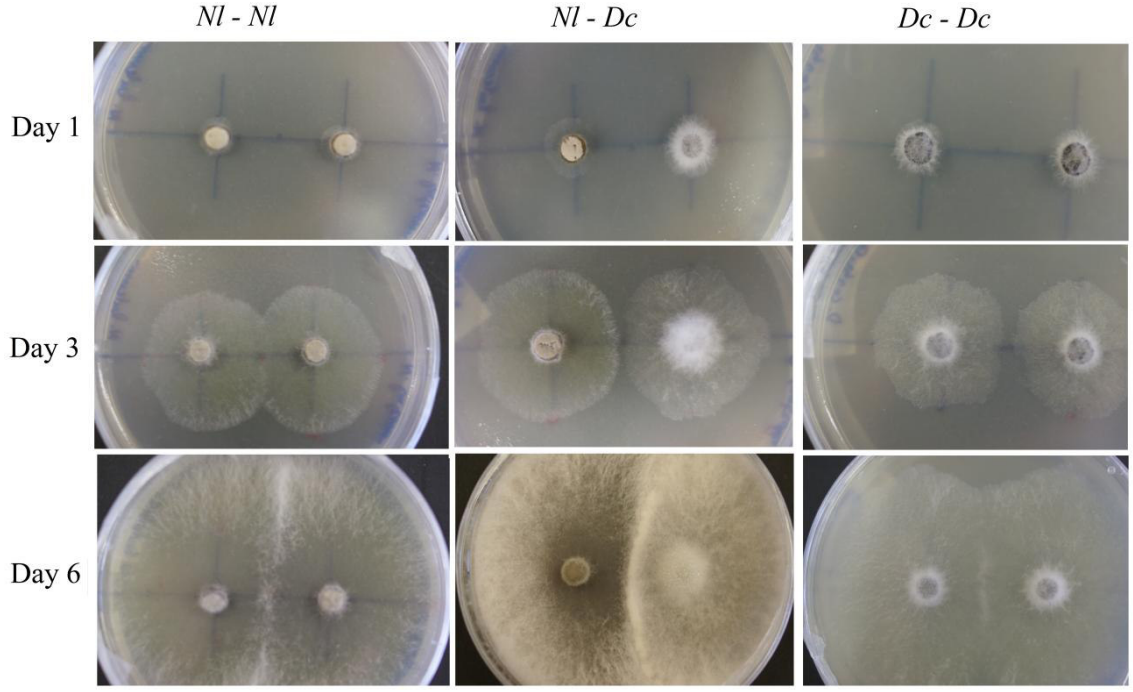

Figure 27. Morphological appearance of the co-cultures between *N. luteum – N. luteum* (*Nl-Nl*), *N. luteum – D. corticola* (*Nl-Dc*) and *D. corticola – D. corticola* (*Dc-Dc*).

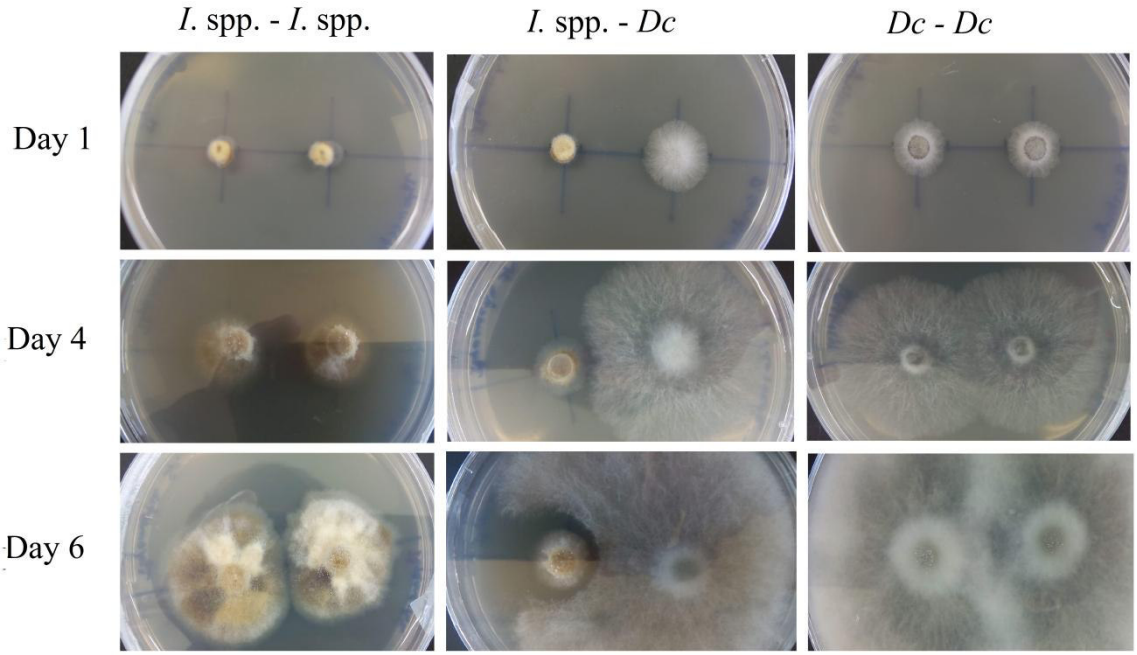

Figure 28. Morphological appearance of the co-cultures between *Ilyonectria* spp. - *Ilyonectria* spp. (*I.* spp.-*I.* spp.), *Ilyonectria* spp. – *D. corticola* (*I.* spp.-*Dc*) and *D. corticola* – *D. corticola* (*Dc*-*Dc*).

Table 16 summarizes the main features observed during the interactions with *D. corticola*. Most interactions stimulate the growth of *D. corticola* inhibiting the development of the interacting fungi. Three fungi (*T. virens*, *G. tetrasperma* and *G. seminuda*) showed significant inhibition of *D. corticola*. However, only in the interaction with *G. tetrasperma* a defensive barrier was created by *D. corticola*. In the interactions *D. corticola* with *S. bicolor, D. viticola* and *Ilyonectria* spp. the two interacting fungi never touch each other. Despite forming a barrier, *D. corticola* always distances itself at least 2mm from the interacting fungi. Despite having the growth of the internal radius inhibited, *F. acutatum*, *Gnomoniopsi*s spp., *R. corticium* and *S. bicolor* show a significant increase in the external radius growth, compared with the controls. This suggests that this interacting fungi are growing away from *D. corticola*.

Table 16: Summary of the results obtained in the antagonism assays against *D. corticola.*

Interacting fungi	*D. c* inhibition	Interacting fungi inhibition	Hyphal barrier formation (formed by)	Type of antagonism
Trichoderma virens	+	- (stimulates)	-	-
Gelasinospora tetrasperma	+	- (stimulates)	+ (*D. cortiola*)	Mycelial contact
Gelasinospora seminuda	+	- (stimulates)	-	-
Neofusicoccum luteum	- (stimulates)	+	+ (*N. luteum*)	Mycelial contact
Fusarium acutatum	- (stimulates	+	+ (*D. corticola*)	Mycelial contact and distance antagonism
Saccharicola bicolor	- (stimulates)	+	+ (*D. corticola*)	Distance antagonism
Rosellinia corticium	- (stimulates)	+	-	-
Diaporthe viticola	- (stimulates)	+	+ (*D. corticola*)	Distance antagonism
***Ilyonectria* spp.**	- (stimulates)	+	+ (*D. corticola*)	Distance antagonism
***Gnomoniopsis* spp.**	- (stimulates)	+	-	-

4. Conclusions

The study of endophytes in *Quercus* spp. has been growing over the last years (Ragazzi *et al.,* 2003; Gennaro *et al.*, 2003; Kwaśna *et al.*, 2016), however few studies focusing on the endophytic community in *Quercus suber* have been published. Recently, the variation of endophytic cork oak-associated fungal communities in relation to plant heath and water stress was evaluated (Linaldeddu *et al.*, 2011). The study concluded that water stress reduces the endophytic mycobiota diversity and promotes the proliferation of some potentially pathogenic endophytes. However, this study has only evaluated one study site and artificially subjected the trees to water stress. Taking this into account, the present study characterizes the fungal endophytic community from three different organs of *Q. suber* (leaves, stems and roots) in four different sites (Bragança, Gerês, Alcobaça and Grândola) of continental Portugal, displaying different edaphoclimatic conditions, including different precipitation levels and plant water relations.

In general, Grândola showed to be the richest and more diverse community, when compared with the other three sites. The fungal community in each tree was found to be distinct from the other sites, suggesting that the reduced water availability and geographical conditions could have an impact on this community. In general, roots and stems were the organs with highest richness and diversity, followed by the leaves. These results are in agreement with other studies, performed in other plant species, that reported a higher number of endophytes in roots when compared with aboveground organs (Zheng *et al.*, 2016; Martins *et al.*, 2016).

Taking into account that *Biscogniauxia mediterranea* appears in all study sites, except in Gerês, and that *Diplodia corticola* appears in the southern regions (Alcobaça and Grândola), the most promising fungal endophytes for biological control were selected from Bragança and Alcobaça respectively. Antagonist assays revealed that most tested fungi did not exert any antagonistic effect against the pathogenic agents. Some of them even promoted the growth of both fungal agents. However, *Trichoderma virens* and *Gelasinospora tetrasperma* showed to antagonize both *B. mediterranea* and *D. corticola*. On the other hand, *Gelasinospora seminuda* has also antagonized *D. cortiola*.

This work contributed for the better understanding of fungal communities in *Q. suber*. Potential biocontrol agents were found to antagonize two causal agents of disease, *B. mediterranea* and *D. corticola*, namely *Trichoderma virens* and *Gelasinospora* spp. (*tetrasperma* and *seminuda*). *T. virens* has already been described as an effective biocontrol agent (Hanson, 2000; Hanson and Howell, 2004). On the other hand, the antagonistic activity of *Gelasinospora* spp. has not been described. Further studies are still required for testing these fungi to act as biological control against those diseases.

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Annex

Table A 1: GPS coordinates of the 5 *Q. Suber* (Trees) in all four sampling sites.

	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5
Limãos	41º 31' 51.3058''N 6º 49' 57.0932''W	41º 31' 53.4504''N 6º 49' 56.3121''W	41º 31' 53.2750''N 6º 49' 58.5627''W	41º 31' 55.3767''N 6º 49' 59.2197''W	41º 31' 56.4899''N 6º 50' 3.5295''W
Ermida	41º 42' 25.361''N 8º 6' 33.330''W	41º 42' 31.419''N 8º 6' 53.662''W	41º 42' 31.872''N 8º 6' 55.277''W	41º 42' 27.087''N 8º 6' 41.541''W	41º 42' 25.495''N 8º 6' 23.577''W
Alcobaça	39º27´41.1281''N 9º2'42.5169''W	39º27'42.7501''N 9º2'42.4900''W	39º27'43.4101''N 9º2'42.1402''W	39º27'44.0423''N 9º2'42.0612''W	39º27'44.9154''N 9º2'41.6802''W
Grândola	38º11'32.1699''N 8º37'11.9900''W	38º11'32.9172''N 8º37'11.5940''W	38º11'33.1898''N 8º37'9.9461W	38º11'30.2649''N 8º37'8.3232''W	38º11'29.2945''N 8º37'7.8274''W

Table A 2: Species richness and diversity parameters for fungal communities from the roots of the four sites studied: species richness (S), Simpson's index (D) on its inverse form, Shannon index (H'), Fisher's alpha, Chao & Lee 1 and Jackknife 1 estimates. Statistically significant differences (at P≤0.05) for each location's index, were denoted by different letters.

Roots

Study sites	Tree	S	D	H'	α Fisher	Chao & Lee 1	Jackknife 1
Bragança	1	8	5	1,75	1,89	8	8
	2	7	3,01	1,4	1,64	7	7
	3	6	3,98	1,51	1,45	6	6
	4	5	2,97	1,28	0,92	5	5
	5	7	3,86	1,51	1,88	7	7
	Total	11^{a}	4,39^{a}	1,66^{a}	1,9^{a}	11,33^{a}	11^{a}
Gerês	1	12	6,61	2,03	2,89	12	12
	2	8	3,86	1,51	1,77	8,99	8
	3	8	3,41	1,42	1,55	8	8
	4	8	2,77	1,39	1,72	8	8
	5	10	5,52	1,93	2,25	10	10
	Total	14^{b}	5,14^{a}	1,915^{ab}	2,12^{abc}	13^{b}	13^{b}
Alcobaça	2	12	4,87	1,88	2,54	12	12
	4	10	5,19	1,87	2	10	10
	5	13	6,16	2,05	2,71	13	13
	Total	13^{b}	5,9^{a}	2,093^{b}	2,15^{bc}	13^{b}	13^{b}
Grândola	1	16	6,04	2,11	4,18	16	16
	2	11	4,32	1,74	2,5	11	11
	3	11	4,34	1,75	2,46	11	11
	4	10	4,34	1,75	2,2	10	10
	5	11	6,25	2	3,14	13,97	11
	Total	17^{b}	5,4^{a}	2,003^{b}	2,97^{c}	17^{b}	17^{b}

Table A 3: Species richness and diversity parameters for fungal communities from the stems of the four sites studied: species richness (S), Simpson's index (D) on its inverse form, Shannon index (H'), Fisher's alpha, Chao & Lee 1 and Jackknife 1 estimates. Statistically significant differences (at P≤0.05) for each location's index, were denoted by different letters.

Stems							
Study sites	**Tree**	**S**	**D**	**H'**	**α Fisher**	**Chao & Lee 1**	**Jackknife 1**
Bragança	1	7	3,99	1,54	1,63	7	7
	2	6	2,4	1,04	1,07	6	6
	3	8	5,27	1,8	1,79	8	8
	4	7	4,06	1,59	1,5	7	7
	5	7	4,55	1,63	1,33	7	7
	Total	9[a]	4,72[a]	1,63[a]	1,37[ab]	9[a]	9[a]
Gerês	1	6	1,54	0,73	1,09	6	6
	2	7	4,8	1,64	1,38	7	7
	3	7	4,53	1,58	1,46	7,99	7
	4	7	4,41	1,62	1,37	7	7
	5	6	2,25	1,11	1,02	6	6
	Total	9[a]	3,27[a]	1,45[a]	1,31[a]	9[a]	9[a]
Alcobaça	1	11	4,9	1,77	2,14	11	11
	2	8	3,73	1,54	1,51	8	8
	3	8	2,39	1,28	1,52	8	8
	4	8	4,35	1,65	1,47	8	8
	5	10	4,47	1,78	1,95	10	10
	Total	12[b]	4.4[a]	1,8[a]	1,76[b]	12[b]	12[b]
Grândola	1	10	6,52	2,02	2,05	10	10
	2	14	7,08	2,16	3,23	15	14
	3	15	5,55	1,98	3,24	15	15
	4	11	3,83	1,75	2,23	11	11
	5	10	3,03	1,56	2,05	10	10
	Total	17[c]	6,62[a]	2,09[a]	2,72[c]	17[c]	17[c]

Table A 4: Species richness and diversity parameters for fungal communities from the leaves of the four sites studied: species richness (S), Simpson's index (D) on its inverse form, Shannon index (H'), Fisher's alpha, Chao & Lee 1 and Jackknife 1 estimates. Statistically significant differences (at P≤0.05) for each location's index, were denoted by different letters.

Leaves

Study sites	Tree	S	D	H'	α Fisher	Chao & Lee 1	Jackknife 1
Bragança	1	7	1,7	0,84	1,5	7	7
	2	4	2,08	0,78	0,68	4	4
	3	3	1,42	0,53	0,61	3	3
	4	7	3,86	1,51	2,4	7,98	7
	5	4	1,74	0,81	1,21	4	4
	Total	12^{ac}	$2,28^{ac}$	$1,01^{a}$	$2,16^{a}$	$12,25^{a}$	12^{ac}
Gerês	1	5	3,71	1,38	0,91	5	5
	2	6	2,99	1,24	1,46	6,99	6
	3	5	3,25	1,29	1,1	5	5
	4	8	2,56	1,26	1,83	8,99	8
	5	5	3,66	1,38	0,98	5	5
	Total	11^{a}	$3,85^{ab}$	$1,53^{b}$	$1,85^{a}$	11^{a}	11^{a}
Alcobaça	1	11	3,34	1,56	2,19	11	11
	2	8	3,23	1,41	1,48	8	8
	3	8	3,11	1,32	1,44	9	8
	4	11	4,36	1,79	2,16	11	11
	5	10	2,73	1,34	2,03	11	10
	Total	13^{b}	$3,42^{b}$	$1,56^{b}$	$1,93^{a}$	13^{a}	13^{b}
Grândola	1	9	1,62	0,75	1,75	23,95	9
	2	9	2,15	1,11	1,8	9	9
	3	8	1,41	0,69	1,62	10,99	8
	4	6	2,32	1,03	1,09	6	6
	5	7	1,99	1,04	1,32	7	7
	Total	10^{bc}	$2,07^{c}$	$1,06^{a}$	$1,47^{a}$	10^{a}	10^{bc}

Table A 5: Species richness and diversity parameters for fungal communities from the organs of Bragança: species richness (S), Simpson's index (D) on its inverse form, Shannon index (H'), Fisher's alpha, Chao & Lee 1 and Jackknife 1 estimates. Statistically significant differences (at P≤0.05) for each location's index, were denoted by different letters.

Bragança							
Organ	**Tree**	**S**	**D**	**H'**	**α Fisher**	**Chao & Lee 1**	**Jackknife 1**
Roots	1	8	5	1,75	1,89	8	8
	2	7	3,01	1,4	1,64	7	7
	3	6	3,98	1,51	1,45	6	6
	4	5	2,97	1,28	0,92	5	5
	5	7	3,86	1,51	1,88	7	7
	Total	11[a]	4,39[a]	1,66[a]	1,9[a]	11,33[a]	11[a]
Stems	1	7	3,99	1,54	1,63	7	7
	2	6	2,4	1,04	1,07	6	6
	3	8	5,27	1,8	1,79	8	8
	4	7	4,06	1,59	1,5	7	7
	5	7	4,55	1,63	1,33	7	7
	Total	9[a]	4,72[a]	1,63[a]	1,37[a]	9[a]	9[a]
Leaves	1	7	1,7	0,84	1,5	7	7
	2	4	2,08	0,78	0,68	4	4
	3	3	1,42	0,53	0,61	3	3
	4	7	3,86	1,51	2,4	7,98	7
	5	4	1,74	0,81	1,21	4	4
	Total	12[a]	2,28[b]	1,01[b]	2,16[a]	12,25[a]	12[a]

Table A 6: Species richness and diversity parameters for fungal communities from the organs of Gerês: species richness (S), Simpson's index (D) on its inverse form, Shannon index (H'), Fisher's alpha, Chao & Lee 1 and Jackknife 1 estimates. Statistically significant differences (at $P \leq 0.05$) for each location's index, were denoted by different letters.

Gerês							
Organ	**Tree**	**S**	**D**	**H'**	**α Fisher**	**Chao & Lee 1**	**Jackknife 1**
Roots	1	12	6,61	2,03	2,89	12	12
	2	8	3,86	1,51	1,77	8,99	8
	3	8	3,41	1,42	1,55	8	8
	4	8	2,77	1,39	1,72	8	8
	5	10	5,52	1,93	2,25	10	10
	Total	13[a]	5,14[a]	1,915[a]	2,12[a]	13[a]	13[a]
Stems	1	6	1,54	0,73	1,09	6	6
	2	7	4,8	1,64	1,38	7	7
	3	7	4,53	1,58	1,46	7,99	7
	4	7	4,41	1,62	1,37	7	7
	5	6	2,25	1,11	1,02	6	6
	Total	9[b]	3,27[a]	1,45[a]	1,31[b]	9[b]	9[b]
Leaves	1	5	3,71	1,38	0,91	5	5
	2	6	2,99	1,24	1,46	6,99	6
	3	5	3,25	1,29	1,1	5	5
	4	8	2,56	1,26	1,83	8,99	8
	5	5	3,66	1,38	0,98	5	5
	Total	11[b]	3,85[a]	1,53[a]	1,85[b]	11[b]	11[b]

Table A 7: Species richness and diversity parameters for fungal communities from the organs of Alcobaça: species richness (S), Simpson's index (D) on its inverse form, Shannon index (H'), Fisher's alpha, Chao & Lee 1 and Jackknife 1 estimates. Statistically significant differences (at P≤0.05) for each location's index, were denoted by different letters.

Alcobaça							
Organ	**Tree**	**S**	**D**	**H'**	**α Fisher**	**Chao & Lee 1**	**Jackknife 1**
Roots	2	12	4,87	1,88	2,54	12	12
	4	10	5,19	1,87	2	10	10
	5	13	6,16	2,05	2,71	13	13
	Total	13[a]	5,9[a]	2,093[a]	2,15[a]	13[a]	13[a]
Stems	1	11	4,9	1,77	2,14	11	11
	2	8	3,73	1,54	1,51	8	8
	3	8	2,39	1,28	1,52	8	8
	4	8	4,35	1,65	1,47	8	8
	5	10	4,47	1,78	1,95	10	10
	Total	12[a]	4.4[ab]	1,8[b]	1,76[a]	12[a]	12[a]
Leaves	1	11	3,34	1,56	2,19	11	11
	2	8	3,23	1,41	1,48	8	8
	3	8	3,11	1,32	1,44	9	8
	4	11	4,36	1,79	2,16	11	11
	5	10	2,73	1,34	2,03	11	10
	Total	13[a]	3,42[b]	1,56[b]	1,93[a]	13[a]	13[a]

Table A 8: Species richness and diversity parameters for fungal communities from the organs of Grândola: species richness (S), Simpson's index (D) on its inverse form, Shannon index (H'), Fisher's alpha, Chao & Lee 1 and Jackknife 1 estimates. Statistically significant differences (at P≤0.05) for each location's index, were denoted by different letters.

Grândola							
Organ	**Tree**	**S**	**D**	**H'**	**α Fisher**	**Chao & Lee 1**	**Jackknife 1**
Roots	1	16	6,04	2,11	4,18	16	16
	2	11	4,32	1,74	2,5	11	11
	3	11	4,34	1,75	2,46	11	11
	4	10	4,34	1,75	2,2	10	10
	5	11	6,25	2	3,14	13,97	11
	Total	17[a]	5,4[a]	2,003[a]	2,97[a]	17[a]	17[a]
Stems	1	10	6,52	2,02	2,05	10	10
	2	14	7,08	2,16	3,23	15	14
	3	15	5,55	1,98	3,24	15	15
	4	11	3,83	1,75	2,23	11	11
	5	10	3,03	1,56	2,05	10	10
	Total	17[a]	6,62[a]	2,09[a]	2,72[a]	17[a]	17[a]
Leaves	1	9	1,62	0,75	1,75	23,95	9
	2	9	2,15	1,11	1,8	9	9
	3	8	1,41	0,69	1,62	10,99	8
	4	6	2,32	1,03	1,09	6	6
	5	7	1,99	1,04	1,32	7	7
	Total	10[b]	2,07[b]	1,06[b]	1,47[b]	10[b]	10[b]

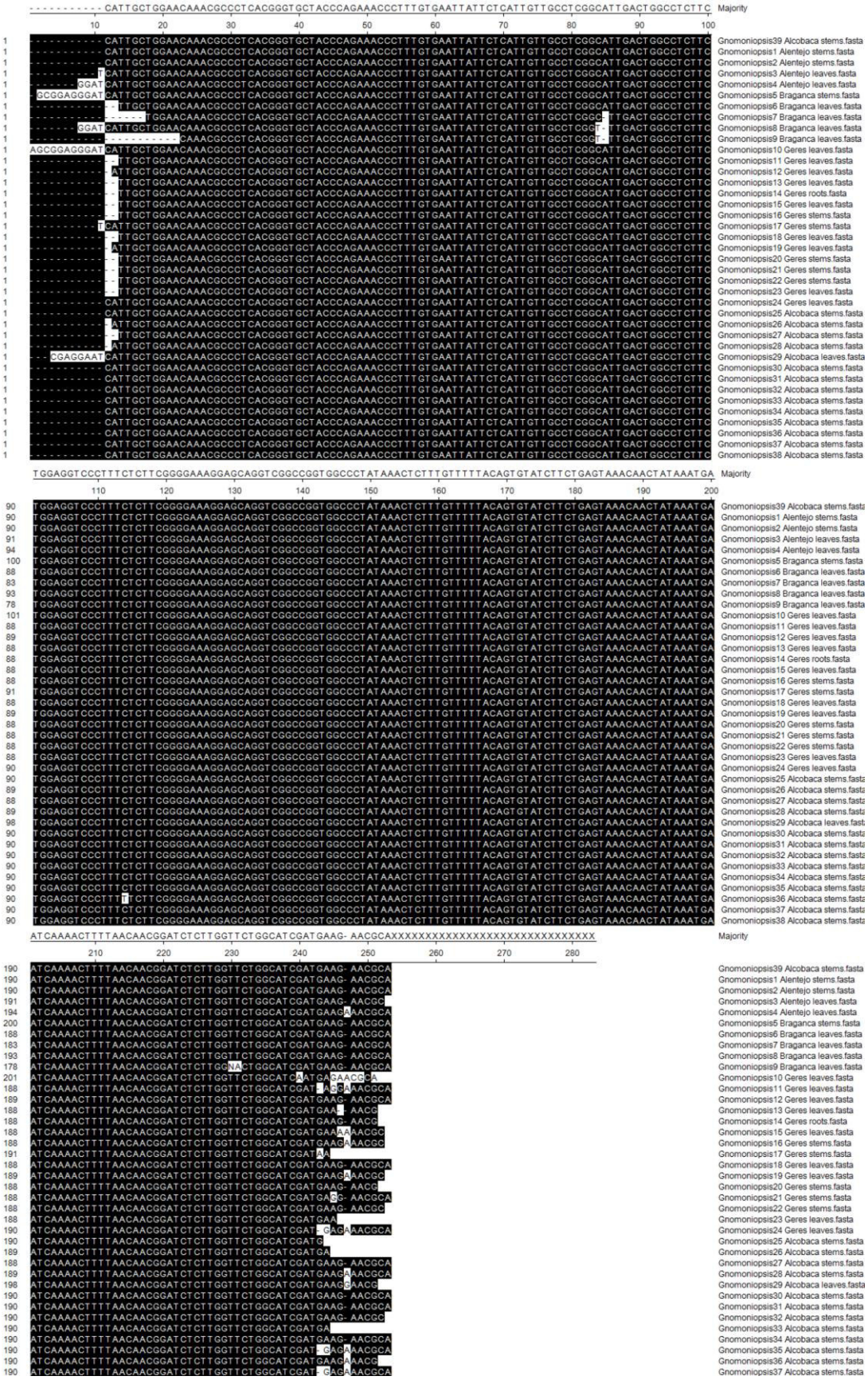

Figure A 1: Alignments of the several *Gnomoniopsis* spp. identified in the molecular identification.

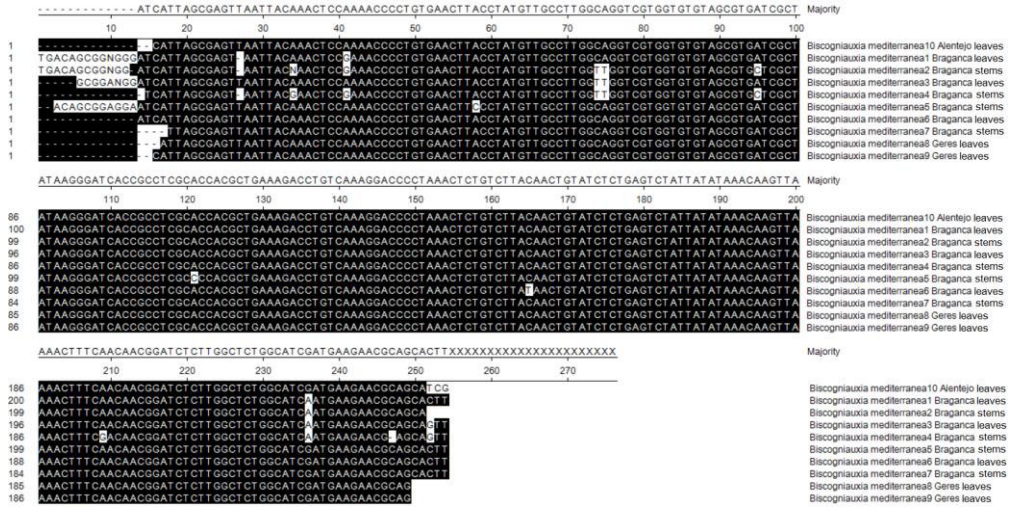

Figure A 2: Alignments of the several *Biscogniauxia mediterranea* identified in the molecular identification.

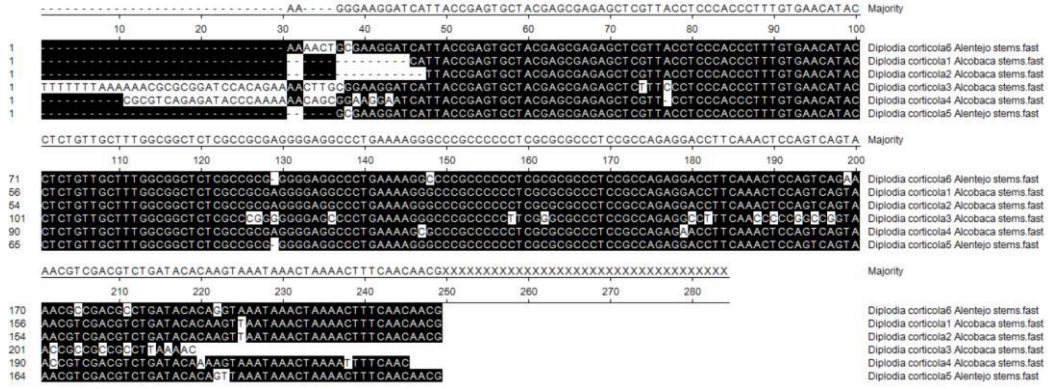

Figure A 3: Alignments of the several *Diplodia corticola* identified in the molecular identification.

Figure A 4: Alignments of the several *Umbelopsis* spp. identified in the molecular identification.