UNIVERSIDADE TÉCNICA DE LISBOA

INSTITUTO SUPERIOR DE AGRONOMIA

FUNGI ASSOCIATED WITH *PLATYPUS CYLINDRUS* (COLEOPTERA: PLATYPODIDAE) AND THEIR RELATION TO CORK OAK DECLINE

TESE APRESENTADA PARA OBTENÇÃO DO GRAU DE DOUTOR
EM ENGENHARIA FLORESTAL E DOS RECURSOS NATURAIS

Maria de Lurdes Nunes Silva Inácio

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TABLE OF CONTENTS

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ABSTRACT

The ambrosia beetle *Platypus cylindrus* establishes symbiotic relationships with fungi. *P. cylindrus* may act as a vector for the dispersal of phytopathogenic fungi in the Mediterranean cork oak stands. The aim of this study was to identify the fungi associated with *P. cylindrus* in Portugal and to understand the role and potential impacts on cork oak stands of both insect and fungi. A complex of fungi was found in association with the insect, namely species of cosmopolitan genera whose exact role in the interaction still remains under discussion. *Biscogniauxia mediterranea*, the causal agent of charcoal canker disease was also found associated with *P. cylindrus* which contributes to disease spread in cork oak stands. A total of fourteen representative isolates belonging to Ophiostomatales where selected. Fungal strains were morphologically and molecularly characterized. Three rDNA contiguous regions SSU, ITS and LSU, were profiled for each strain and contrasted with pre-existent Ophiostomatales species worldwide. In addition, pathogenicity tests were conducted. A new *Ophiostoma* species, with *Hyalorhinocladiella* anamorph, and a *Raffaelea* species closely related to *R. canadensis* were noticed for the first time and in association with the ambrosia beetle. Molecular evidence suggests that a different phylogenetic lineage of Ophiostomatales exists in Portugal. *R. montetyi* was found to be the primary ambrosia fungus of *P. cylindrus*. The pathogenicity of *R. montetyi* towards cork oak seedlings was confirmed and its role as a cork oak declining agent determined.

Key words: cork oak pinhole borer, ambrosia beetle, ambrosia fungi, *Quercus suber*, *Raffaelea* spp., Ophiostomatales, *Hyalorhinocladiella*, mycangia, interaction, symbiosis

FUNGOS ASSOCIADOS A *PLATYPUS CYLINDRUS* (COLEOPTERA: PLATYPODIDAE) E SUA RELAÇÃO COM O DECLÍNIO DO MONTADO

RESUMO

Platypus cylindrus é um insecto ambrósia que estabelece relações simbióticas com fungos, podendo actuar como vector na dispersão de fungos fitopatogénicos nos povoamentos de sobreiro no Mediterrâneo. O objectivo deste estudo foi identificar os fungos associados a *P. cylindrus* em Portugal e compreender o potencial impacto nos montados, tanto dos ataques do insecto como dos fungos. Foi identificado um complexo de fungos associado ao insecto, nomeadamente espécies de géneros cosmopolitas cujo papel na interacção permanece ainda em discussão. O agente causal do carvão do entrecasco, *Biscogniauxia mediterranea*, foi também associado a *P. cylindrus* que contribui para a dispersão da doença em montados de sobro. Catorze isolados de fungos Ophiostomatales foram seleccionados, tendo sido caracterizados morfologicamente e por técnicas de biologia molecular. Para cada isolado, analisaram-se as três regiões contíguas do rDNA, SSU-ITS-LSU, efectuando a comparação com Ophiostomatales de outros países. Uma nova espécie de *Ophiostoma*, com *Hyalorhinocladiella* como anamorfo, e uma espécie de *Raffaelea* muito próxima de *R. canadensis*, foram detectadas pela primeira vez nesta interacção. Evidências moleculares apontam para a existência em Portugal de uma linhagem filogenética diferente de Ophiostomatales. A espécie *R. montetyi* foi identificada como o fungo ambrósia principal de *P. cylindrus*. Foi avaliada a patogenicidade de *R. montetyi* em plântulas de sobreiro e determinado o seu papel como agente de declínio do montado.

Palavras-chave: plátipo, insecto ambrósia, fungo ambrósia, *Quercus suber*, *Raffaelea* spp., Ophiostomatales, *Hyalorhinocladiella*, micângio, interacção, simbiose

RESUMO ALARGADO

Uma das características mais notáveis da floresta portuguesa reside no facto de incluir a maior área de montado do mundo. Os povoamentos de sobreiro (*Quercus suber* L.) em território nacional perfazem cerca de um terço da área mundial ocupada por esta espécie, sendo responsáveis por mais de metade da produção de cortiça do planeta.

Os montados e sobreirais da bacia mediterrânica têm vindo a sofrer do declínio generalizado dos carvalhos observado na Europa, na América e também no continente Asiático. Em Portugal, o fenómeno começou a manifestar-se no início do século XX e representa actualmente um sério problema que ameaça a sobrevivência deste importante recurso florestal.

Nas últimas décadas, têm sido amplamente debatidas as causas da perda de vigor dos montados nacionais. As características do solo e a escassa disponibilidade hídrica em grande parte do ciclo vegetativo são apontados como factores desencadeantes do processo. Para além disso, pragas e doenças, actuando em sobreiros já debilitados, constituem factores de aceleração que culminam, com frequência, na morte das árvores.

O insecto *Platypus cylindrus* (plátipo) foi desde cedo associado ao declínio do sobreiro mas inicialmente como uma praga secundária. Atacando árvores de todas as idades, manifesta preferência por aquelas com maiores diâmetros, sobretudo se recém-descortiçadas. Nas últimas décadas, tem-se assistido ao aumento dos ataques deste insecto em sobreiros aparentemente sãos que podem sucumbir ao fim de poucos meses. Os ataques são massivos e, a par de uma intensa actividade de escavação de galerias no tronco, os insectos inoculam fungos que revestem as galerias aonde a descendência se desenvolve. *P. cylindrus* é designado um insecto ambrósia ou xilomicetógafo e os fungos que transporta e de que depende para sobreviver denominam-se fungos ambrósia.

Os estudos realizados para caracterização da micoflora associada a *P. cylindrus* e determinação do seu papel no declínio do montado permitiram confirmar os resultados de trabalhos anteriores. O insecto transporta um cortejo de fungos que foi

possível isolar do exosqueleto, do conteúdo intestinal e dos micângios, órgãos especializados para o transporte e acondicionamento dos fungos ambrósia, assim como das galerias do insecto. Isolaram-se fungos dos géneros *Acremonium*, *Aspergillus*, *Beauveria*, *Biscogniauxia*, *Botryosphaeria*, *Geotrichum*, *Gliocladium*, *Fusarium*, *Paecylomyces* e *Penicillium*, *Scytalidium* e *Trichoderma*. Muitos destes fungos também se obtiveram nos isolamentos efectuados a partir da madeira sã de árvores não afectadas, comprovando a sua ubiquidade no material biológico em estudo. Para além destes, e com recurso a meios selectivos, foi possível isolar com grande frequência fungos Ophiostomatales, nomeadamente fungos do género *Raffaelea*, que inclui os fungos ambrósia actualmente conhecidos, num total de 24 espécies.

Considerando a frequência de isolamento e a informação dos trabalhos da especialidade publicados nos últimos anos, foi seleccionado um conjunto de isolados para aprofundamento dos estudos, designadamente os fungos dos géneros *Acremonium*, *Biscogniauxia* e *Botryosphaeria*, assim como dos Ophiostomatales obtidos. Foi efectuada a caracterização morfologia e cultural de representantes de cada espécie provável e, quando considerado importante, o estudo molecular de um subconjunto de isolados. Com o objectivo de averiguar o papel dos diferentes fungos no sucesso de colonização do hospedeiro pelo insecto, foram efectuados testes de patogenicidade em plântulas de sobreiro. Os isolados do género *Botryosphaeria* nunca produziram esporos em meio de cultura e a sua identificação específica está ainda em curso, atendendo ao facto de que podem incluir espécies patogénicas para o sobreiro. Do mesmo modo, os fungos do género *Acremonium*, género considerado como endófito de sobreiro, serão alvo de estudos posteriores.

Os isolados do género *Biscogniauxia* foram identificados como pertencendo à espécie *B. mediterranea*, causadora do carvão do entrecasco. Esta espécie revelou-se patogénica em plântulas de *Q. suber* e com acção fitotóxica em plantas indicadoras de tabaco. A confirmação da sua identidade foi efectuada com recurso à amplificação por PCR da região ITS do DNA ribossomal (rDNA).

Os fungos Ophiostomatales obtidos manifestaram grande tolerância a doses crescentes do antibiótico cycloheximide, característica que confirma o seu enquadramento como formas anamórficas de *Ophiostoma* sp.. Estes fungos têm caracteres morfológicos pouco distintivos e que se sobrepõem muito entre espécies diferentes. Por isso, actualmente, a sua identificação baseia-se sobretudo na sequenciação de regiões do rDNA. Ainda que desejável, a análise multigenética é de difícil execução neste grupo de fungos dado que a região dos ITS é rica em homopolímeros, impossibilitando a sua extracção. Apesar destas dificuldades, foram efectuadas tentativas de sequenciação das regiões SSU, LSU e ITS do rDNA.

A utilização conjugada dos parâmetros morfológicos e culturais, de técnicas moleculares e de estudos de patogenicidade permitiu concluir que o principal fungo ambrósia transportado por *P. cylindrus* é *Raffaelea montetyi*, muito semelhante a *R. quercivora*, quer morfológica quer geneticamente, causadora de extensa mortalidade em espécies de *Quercus* no Japão. *R. montetyi* manifestou-se patogénica em plântulas de sobreiro.

Foi também encontrada uma espécie de *Raffaelea* muito próxima de *R. canadensis*. Ainda que não causando a morte das plântulas inoculadas induziu, no entanto, amarelecimento e queda das folhas e lesões no caule.

Para além destas duas espécies de fungos ambrósia do género *Raffaelea*, foi possível isolar com grande frequência a partir do insecto, sobretudo dos micângios, e das galerias, uma nova espécie de *Ophiostoma*, geneticamente diferente de outros *Ophiostoma* reportados até hoje, com *Hyalorhinocladiella* como anamorfo. Este fungo foi igualmente isolado de árvores com sinais de ataques abortados de *P. cylindrus*. A ocorrência simultânea do fungo nos micângios e no conteúdo intestinal do insecto leva a que esta associação simbiótica estável seja uma hipótese muito plausível. Este facto põe em causa a definição de fungo ambrósia actualmente aceite em que todos os simbiontes são agrupados no género mitospórico *Raffaelea*.

Em síntese, com a realização deste trabalho contribuímos para o aumento do conhecimento acerca da micoflora associada ao insecto *P. cylindrus* e da sua participação no declínio do montado. Do conjunto de isolados obtidos, pela sua frequência e atendendo ao actual conhecimento de fungos ambrósia patogénicos para as árvores que os albergam, seleccionámos os fungos Ophiostomatales para melhor caracterização e estudo do seu potencial como agentes de declínio. Foram encontradas três espécies de fungos Ophiostomatales, duas das quais possivelmente novas para a ciência, uma espécie do género *Raffaelea* muito próxima de *R. canadensis* e *Ophiostoma* sp., e *R. montetyi*. A espécie *R. montetyi*, revelando-se como patogénica em plântulas de sobreiro, foi associada pela primeira vez ao declínio do montado.

Para além destes fungos Ophiostomatales, a espécie *B. mediterranea* também é transportada e inoculada por *P. cylindrus.* Se tivermos em conta que se trata de um dos mais importantes patogénios dos nossos montados, o plátipo ao inoculá-lo directamente no hospedeiro pode assumir um papel relevante na sua dispersão.

Assim, estes resultados poderão ser utilizados para melhorar a gestão dos povoamentos de sobreiro, nomeadamente através do controlo das populações de *P. cylindrus*, uma vez que uma possível redução dos efectivos da praga contribui para, em simultâneo, limitar a disseminação dos patogénios que transporta.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. The importance of cork oak

Cork oak (*Quercus suber* L.) is an evergreen oak of the family *Fagaceae,* described by Linneo in 1753. The species can be distinguished from other oak species by the presence of a conspicuous thick and furrowed bark with a continuous layer of cork in its outer part. The remarkable and profuse production of cork has given notoriety and economic importance to cork oak (Natividade 1950, Pereira 2007). The unique properties of cork have been known since antiquity. However, it was the high density of the wood from cork oak which first triggered the interest on this species that travelled around the world as bows and keels in the hulls of Portuguese caravels. The cork layers could be manually stripped off from the stem, without endangering the tree vitality, and a new cork layer subsequently regrows. This process constitutes the basis for the sustainable production of cork during the cork oak's long life, which still persists at present time. Currently cork is a trademark of excellence for the wine bottle stopper industry, recognized worldwide (Pereira 2007).

Cork oak has a very restricted distribution in the world, spreading across the western Mediterranean basin and the adjoining Atlantic coasts, within a total area of *ca.* 2 million hectares. The largest continuous area of cork oak stands is located in the southwest of the Iberian Peninsula (Portugal and Spain). It is also widely present in the northern African countries of Morocco, Algeria and Tunisia (Capelo and Catry 2007). In addition, cork oak is also found in the Landes region of France. The easternmost distribution of the species reaches Sardinia, Sicily and the Calabria region in Italy (Silva and Catry 2006). Occasionally it vegetates in the eastern Mediterranean (the former Yugoslavia, Albania and Greece).

In Portugal, cork oak is mostly concentrated south of Tagus river, in the provinces of Alentejo and Ribatejo, at Tagus valley, forming mono-specific stands. It is also present in the rest of the country, often mixed with other species. Several attempts were made to establish the species in diverse countries, ranging from the United States, South America, Bulgaria and Australia. However, the difficult seed conservation and field establishment of the seedlings, coupled with the species slow growth, did not facilitate more than a few scattered plots, mainly ornamental (Pereira 2007).

Cork oak has a relevant contribution to the economy, and ecological balance, of the areas where it is present. Portugal is the main producer, with 736 700 ha, representing about 33% of the total area of cork oak forests, producing 190 000 ton of cork per year, which account for more than 50 % of the world cork production (Pausas *et al.* 2009, AFN 2010). The remaining production is divided between Spain, with *ca.* 30% of the total production, Italy, Algeria, Morocco, Tunisia and France (Costa and Pereira 2007).

The value generated by Portuguese cork exports is considerable, standing at approximately 2.3% of the Portuguese exports' total value and about 30% of the Portuguese forestry exports. The importance of the cork sector is even more relevant at the regional level, supporting a socio-economic chain in regions where other economic activities and sources of income are scarce. This is the case in the Alentejo region, where cork oak sector represents 72% of the whole gross added value of cork national production (Evangelista 2010).

Cork oak stands, unlike the case of timber-production species, are present in a wide range of structures and densities. The main reason for this variety relies on the existence of different types of land use associated with cork oak (Silva and Catry 2006). Natividade (1950) distinguished typical forest stands, i.e. denser stands which are mainly used for the production of cork (*sobreiral*), from those which are part of an agro-forestry system, named *montado* in Portugal and *dehesa* in Spain.

This system comprises scattered *Q. suber/Q. rotundifolia* Lam. (evergreen oak) trees sharing the same space with agriculture or grazing land. This combination offers economical and ecological advantages, where trees are a source of additional income to the landowner (cork, firewood and fodder provided by foliage and acorns) and a guarantee of soil protection. The most associated crops are wheat, and other winter cereals and the most common livestock are cattle and sheep. Other relevant land uses, which have been gaining importance in the *montado* areas in the last few years, are hunting and the breeding of the renowned Black Iberian Pig (*porco preto*). This latter activity was the main factor linked with the expansion of the *montado* system, mainly the evergreen oak stands, starting in the 17th century (Coelho 1996, 2007). Another important cause for the conservation of this agro-forestry system in vast areas of Portugal is the existence of an ancient legislation (13th century) protecting cork oak stands (Mendes 2002). This fact has avoided the extensive depletion of stands by farmers, forcing them to keep the trees when reconverting areas to agriculture (Silva and Catry 2006).

Overall, the *montado* systems are ancient agro-ecosystems recognized by a remarkable and unique biodiversity and ecological value (Pereira and Fonseca 2003, Onofre 2007). Cork oak landscapes exhibit a variety of aromatic and medicinal plants (Costa and Pereira 2007) and wild mushrooms (Barrico *et al.* 2010). The recognition of the ecological importance of cork oak in the regions of southern Europe and northern Africa, as a buffer to soil erosion and desertification, with a remarkable resistance to fire (Varela 2004, Silva and Catry 2006) and a undeniable action in the compensation of CO_2 emissions and water resources (Pereira 2007), is determinant for its conservation and for the inclusion of cork oak stands, as protected habitats, in the framework of the Natura 2000 Network, established by the European Union, since 1993 (Directive no. 92/43/CEE).

1.2. The decline of cork oak

The progressive decay of cork oak forests and woodlands follows the general oak decline that has been noticed for several oak species around the globe, namely in the United States (Oak *et al.* 1996), Europe (Brasier *et al.* 1992, Degreef 1992, Osazko 2000, Barros *et al.* 2002), North Africa (Bakry and Abourouh 1996) and Japan (Kubono and Ito 2002, Matsuda *et al.* 2010), both in extent and severity of the process. Since the last decades, the main causes of oak decline have been studied and discussed, with particular emphasis in the detection and characterization of populations of harmful agents, aiming to find relationships between the incidence of these agents and the onset of symptoms of decline. However, there are still many knowledge gaps in areas as relevant as the interactions in the system insects-fungi-host, among others (Sousa *et al.* 2007).

The loss of vigour of Portuguese cork oak stands has been a long lasting cause of ecological and economical concern (Pimentel 1953, Cabral *et al.* 1992, David *et al.*

1992, Cadima *et al.* 1995, Santos 1995). Unusual mortality of cork oak was first recorded in the late 19th century and first decades of the 20th century (Câmara-Pestana 1898, Baeta-Neves 1944). In the 1940's severe attacks of different pests caused heavy economic losses whose control only came effective in the 60's, after successive campaigns with DDT (Silva 2002).

Adverse climatic factors, mainly long periods of drought were pointed out as responsible for large scale mortality of cork oak trees (Kurz-Besson *et al.* 2006), being considered as trigger factors for the attack of pests and diseases (Brasier 1996, Costa *et al.* 2002). Moreover, for Natividade (1950) the inappropriate management practices, with deep plowing, understorey removal and exhausting crop production, pruning and overexploitation of cork, constituted the main causes for the decline of the *montado* system. In the last decade, tree mortality increased. In the year 2000 the highest cork oak mortality was recorded (Barros *et al.* 2002), raising a growing concern amongst producers, industries and research institutions, which actively pursue to endeavour of determining the causes, consequences and solutions for the decay of cork oak (Sousa *et al.* 2007, Costa *et al.* 2009, Costa *et al.* 2010).

The main symptoms of cork oak decline can be summarized as follows: (i) rarefaction of the crown, which becomes "transparent", bearing only one-year old leaves; (ii) premature desiccation of leaves, remaining attached to the tree even after this dies, for a period usually superior to one year; leave discoloration; (iii) conspicuous bunches of leaves in the middle of the branches; (iv) drying of the tips of the branches; (v) appearance of dark spots later becoming whitish outside the cork, sign of the presence of fungi and insects on the trunk and branches (Cabral and Sardinha 1992).

However, two kinds of symptoms could be noticed, i.e. (i) *sudden decline* in trees of all ages and sizes, which might cause tree death one week since the initial yellowing of leaves; this event is identifiable between September and December; (ii) *progressive decline* is observed in older and larger trees, being characterized by partial defoliation and branch death, lack of leaf canopy renewal and crown transparency. Both manifestations are accompanied by secondary attacks of pests and diseases (Pereira *et al.* 1999). Similar symptoms have been referred in other countries and are generally included in the widespread process of oak decline (Bakry and Abourouh 1996, Oak *et al.* 1996, Luque *et al.* 2000, Riziero *et al.* 2002).

The decay of cork oak stands is a complex phenomenon due to the interaction of multiple biotic and abiotic factors, which individually have not been determined to be primary causes of decline (Cabral *et al.* 1993).

Cabral and Sardinha (1992) and Sousa (1995) gathered several factors influencing cork oak decline into three groups. Table 1 summarizes them according to (i) *predisposing factors*, i.e., if they act during the tree lifetime; (ii) *inducing factors*, i.e., if they act independently of the vigour of the tree, but with more serious impact on weaker trees; (iii) *accelerating factors*, i.e., if they only act on previously weakened trees.

Table 1. Factors influencing the decline of cork oak forests and woodlands

Predisposing factors	**Inducing factors**	**Accelerating factors**
• overexploitation of resources • inappropriate management • intensive agricultural and livestock occupation • disturbance of soil structure and fertility	• climatic variations (drought) • excessive harvesting and pruning • interventions in the understorey and lack of protection	• insects • fungi • fire

(Adapted from Cabral & Sardinha, 1992; Sousa, 1995)

Pests and diseases involved in cork oak decline

The attack of insects in cork oak stands can be related to ecological or vegetative changes in these ecosystems associated with the symptoms of decline mentioned herein. According to Ferreira and Ferreira (1986), 92 species of insects are known in Portugal with potential to cause damages on cork oak. However, only some are considered capable of causing economic damages. It is generally assumed that the defoliators such as the lepidopteran *Lymantria dis*par L. (oak caterpillar), *Euproctis chrysorrhoea* L. and *Tortrix viridana* L., and the hymenopteran *Periclista* spp. act as primary factors, while the xylophagous are considered secondary agents (Sousa 1995).

The first references of attacks of defoliators on Portuguese cork oak stands date from the late 19th century due to the action of *L. dispar.* In 1945-48 their population levels raised above the economic threshold, causing severe damage, especially south of Tagus river. The causes of this outbreak were unclear (Nogueira 1967). The intensification of agricultural development program focusing on the wheat crop in the *montado* system, with deep plowing and excessive fertilization may have contributed to the ecological unbalance of the ecosystem, hence, opening the path for the outbreak of pests and diseases (Silva 1944). Following these populations outbreaks chemical control was widely used to control the oak caterpillar, which instead led to the increase of other defoliators (Baeta-Neves 1944).

Since the 1980's, coinciding with the gradual decline of cork oak stands, increasing attacks of the coleopteran *Coroebus florentinus* (Herbst.), *C. undatus* (Fab.) and the wood borer *Platypus cylindrus* Fab. were reported (Ferreira and Ferreira 1986, Sousa 1995, Cabral & Ferreira 1999). The most important pests of cork oak in Portugal, the main symptoms and signs displayed, and the principal factors which favour them, are summarized in Table 2.

Table 2. The most important pests of cork oak in Portugal

Scientific name	Order / Family	Symptoms/ Signs	Favourable factors
Defoliators			
Euproctis chrysorrhoea	Lepidoptera / Lymantriidae	nests of leaves and silk; egg laying on leaf underside, defoliation	poor soils dry and very hot summer
Lymantria dispar	Lepidoptera / Lymantriidae	egg laying cream-colored in the trunk, crown defoliation	weakened trees with cracks in the bark
Tortrix viridana	Lepidoptera / Tortricidae	rolled leaves with silk webs, buds destruction, defoliation	weakened trees (poor soils and drought) and isolation
Pericista spp.	Hymenoptera / Tenthredinidae	chewed leaves defoliation	unfavourable climate and soil conditions
Bark beetles/ Xylofagous			
Coroebus florentinus	Coleoptera / Buprestidae	reddish leaves and dry branches, bark blistering	isolated trees
C. undatus	Coleoptera / Buprestidae	larval galleries in the inner bark, yellowish exudates in the cork	trees with thin bark, sandy soils, poor and acid, drought, excessive pruning and debarking
Platypus cylindrus	Coleoptera / Platypodidae	brown leaves which then fall, galleries in the wood, holes and orange sawdust on the trunk	Larger trees, Trees recently debarked, stressed trees

Several fungi have been associated with cork oak forests in recent decades (Sousa *et al.* 1997, Santos *et al.* 1999, Luque *et al.* 2000, Moreira 2002, Franceschini *et* *al.* 2005, Luque *et al.* 2008, Linaldeddu *et al.* 2009, Maddau *et al.* 2009). The main pathogenic agents considered relevant in the process of Portuguese cork oak decline are species of *Bothryosphaeria* (Fr.) Mont, acting at the trunk and crown levels (Alves *et al.* 2004, Linaldeddu *et al.* 2007, Luque *et al.* 2008), *Biscogniauxia mediterranea* (de Not.) Kuntze at the trunk (Santos and Martins 1992, Collado *et al.* 2001, Mazzaglia *et al.* 2001), and *Armillaria mellea* (Fr.) Kummer, Santos *et al.* 1999, Bragança *et al.* 2004) and *Phytophthora cinnamomi* Rands. at the roots (Brasier *et al.* 1992, Moreira 2002, Tuset *et al.* 2002, Moreira *et al.* 2006). *P. cinnamomi* is pointed out as a determinant key factor of the "sudden death" of oak trees (Cobos *et al.* 1992, Moreira *et al.* 1993, Tuset *et al.* 1996, Sanchez *et al.* 2002) (Table 3).

Table 3. The most important diseases of the cork oak in Portugal

Scientific name	Phylum / Order	Symptoms/ Signs	Favourable factors
Armillaria mellea	Basidiomycota / Agaricales	progressive "dieback" of the crown, presence of mycelium, carpophores, rhizomorphs	weakened trees
Biscogniauxia mediterranea	Ascomycota / Xylariales	transparency of the crown, cracks and carbonaceous stroma, powdery brown mass of spores	periods of drought, excessive debarking and pruning
Botryosphaeria spp.	Ascomycota/ Botryosphaeriales	partial yellowing of the crown canker, necrosis, pycnidia	weakened trees
Phytophthora cinnamomi	Oomycota (Chromista Kingdom) Peronosporales	tree decline, crown transparency, black exudations, stem rot	Mild winters, flooded soil

1.3. *Platypus cylindrus* as agent of cork oak decline

P. cylindrus was initially considered a secondary pest in Portuguese *montados* since its attacks were generally limited to dead or weakened trees (Seabra 1939, Baeta-Neves 1950, Español 1964). Elsewhere, sporadic attacks were also described in apparently healthy trees (Balachowsky 1949). Conversely, in Morocco this beetle is considered an important pest of *Q. suber* (Villemant & Fraval 1993, Sousa *et al.* 2005).

In Portugal, since the 1980's, severe infestations were observed in apparently healthy cork oaks (Ferreira and Ferreira 1989, Sousa 1996; Sousa & Débouzie 2002) causing widespread tree death within three months to one year and a half after the attack, depending on the host vigour and resistance.

According to Sousa *et al.* (1995), *P. cylindrus* could be considered as part of a succession of biotic agents involved in the process of cork oak decline in Portugal. *P. cylindrus* population outbreaks would directly result from a large number of weakened trees. Disturbances in the soil (acidification, reduced levels of calcium and potassium and high concentrations of aluminium and zinc) are favourable for the decay of the *montado*, creating good conditions for the settlement of the species. *P. cylindrus* seems to mainly attack trees with low foliar concentrations of calcium, iron and aluminium, vegetating in soils exhibiting reduced levels of organic matter and total carbon. Soils with low concentrations of calcium and high levels of sodium and zinc also seem to favour the selection of hosts by *P. cylindrus* (Sousa and Débouzie 2002).

P. cylindrus attacks trees of all ages, especially those recently decorked or weakened, but maintaining a certain degree of wood humidity. Mortality occurs in isolated trees or in small groups scattered throughout the cork oak stand (Ferreira and Ferreira 1991, Sousa and Débouzie 1999).

The attacks of *P. cylindrus* are located in the trunk and branches of larger diameter. The main signs of the attack are the presence of small entrance circular holes (about 2 mm) produced by the insects on the trunk, and orange sawdust coming out of these holes in spring, summer and autumn. If the attack is intense, the soil near the tree is also covered with this sawdust. Unlike the fibrous bore frass produced by the adult beetles, the larval stages produce powdery sawdust which is lighter in early stages of the attack (Sousa 1996).

The crown of trees exhibits green leaves and leaves with pale, reddish-brown colour and finally the entire crown turns reddish-brown (Fig. 1). The leaves gradually fall, but the majority remains in the tree for an extended period (Ferreira and Ferreira 1991, Sousa and Débouzie 1993, Cabral and Ferreira 1999). Into the heartwood, an extensive systems of galleries at different plans, with surrounding wood staining brown, can be noticed when symptomatic trees are cut down (Fig. 2a-b).

In early infestations, the tunnels run across the grain but later, branches run in any direction (Sousa and Débouzie 1999) which suggests that female are looking for the most suitable conditions of wood humidity and sapwood concentration (Sousa 1996).

Fig. 1 - Symptoms on *Quercus suber* after the attack of *Platypus cylindrus*; arrowed, sign of the presence of *Platypus cylindrus* on the trunk: orange sawdust coming out of the entry holes.

In cross section numerous holes constitute very conspicuous signs of *P. cylindrus* attack. Pinholes caused by *P. cylindrus* do not significantly affect the properties of the wood, but the cork is devalued because it loses weight and quality (Sousa and Inacio 2005).

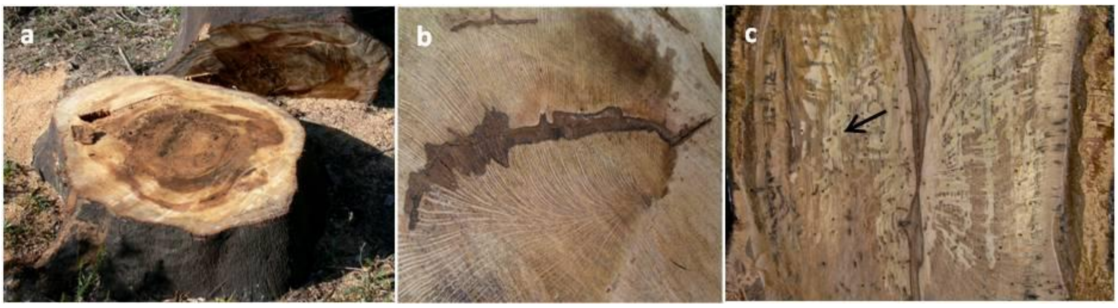

Fig.2 - Internal lesions caused by *Platypus cylindrus* in the trunk of *Quercus suber* trees: a) wood staining accompanying the galleries system b) detail of a gallery section with surrounding wood staining brown c) bore holes in cork oak heartwood in cross-section (arrow).

Climatic factors, including successive years of drought and heat, as excess of water, causing suffocation of the roots, are factors favourable to the development of *P. cylindrus.* Insect attacks are also triggered by inappropriate management practices such as severe pruning and debarking and injuries in the tree. Furthermore, the permanence in the stand of infested or diseased trees, and wood derived from pruning, provides new foci for insect dispersal (Sousa and Inacio 2005).

P. cylindrus is a wood borer insect of the order Coleoptera, family Platypodidae, described by Fabricius in 1801. *P. cylindriformis* Reitter and *P. bimaculatus* Duft could be retained as its synonyms (Cabral and Ferreira, 1999). The common name of *P. cylindrus* is the oak pinhole borer (Hickin 1963) and in Portugal is known as the "plátipo" (Sousa 1996, Ferreira and Ferreira 1991).

The genus *Platypus* comprises about 1000 species, from which only *P. cylindrus* and *P. oxyurus* Duf. are in the Palaearctic region (Espagnol 1964, Ferreira and Ferreira 1989). The majority of species within this genus is tropical (Dajoz 1980, Barbosa and Wagner 1988, Farrel *et al.* 2001). The geographic distribution of *P. cylindrus* includes Southern Europe (Portugal, Spain, France and Italy), West Germany, Morocco, Algeria, Tunisia, Japan, the Caucasus, Armenia and Iraq. Besides cork oak, *P. cylindrus* is hosted by other oaks *(Q. rotundifolia* Lam, *Q. robur* L.), chestnut *(Castanea sativa* Miller), eucalyptus *(Eucalyptus globulus* Labill.) poplar *(Populus* spp.), ash *(Fraxinus spp.),* plane (*Platanus* spp.) elm *(Ulmus* spp.), beech *(Fagus sylvatica* L.) and cherry *(Prunus avium* L.) (Balackowsky *et al.* 1963, Espagnol 1964, Graham 1967, Ferreira and Ferreira 1991, Villemant and Fraval 1993, Cabral and Ferreira 1999, Sousa and Débouzie 2002, Ocasio-Morales 2007).

The Platypodidae and Scolytidae (bark beetles), two families within the weevils (Curculionoidea) (Wood 1982, Marvaldi *et al.* 2002) are closely related, but can be distinguished by the elongate body form, short abdomen (shorter than metathorax in lateral view), and elongate first tarsal segment longer than the remaining segments combined. Males of all *Platypus* species have more developed armature (spineous or chitinous processes) of the elytral declivity (sloping area) than females (Atkinson 2004).

The adults of *P. cylindrus* reach 5 to 7 mm length and 1.5 mm wide, with an elongate body, pitchy-brown to almost dark-brown in colour and golden yellow pubescence. The pronotum is bright with very fine points on the disk and coarser on the side and laterally. Elytra sub-parallel with stria and interstria punctuated, carinated, with denticles on the upper slope. The males have the third interstria tuberculated and the eighth completed with terminal spines; apical border of elytra slope and very hairy. The females have elytra unarmed, very hairy at the apex (Fig. 3).

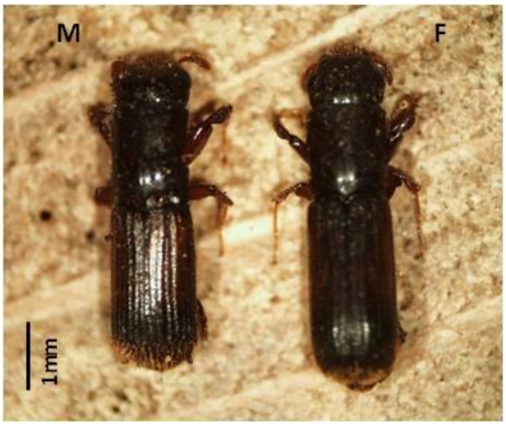

Fig. 3 - *Platypus cylindrus* male (M) and female (F).

The eggs are white, oval and translucent, and are deposited in groups of 3-4 at the end of the galleries (Fig. 4a).

The apodan larvae are white and blind. In the first instars, they have a flat body with pleural tubercles equipped with stout setae as false legs to allow them to move along the tunnel wall (Fig. 4b, c). Independently of the time after the last molting, they have poorly sclerotized mandibles, adapted to feed upon fungi (Korolyov 1989). The fourth instar larva has a body as thick as the tunnel diameter and is nearly cylindrical and hypognata (Fig. 4d).

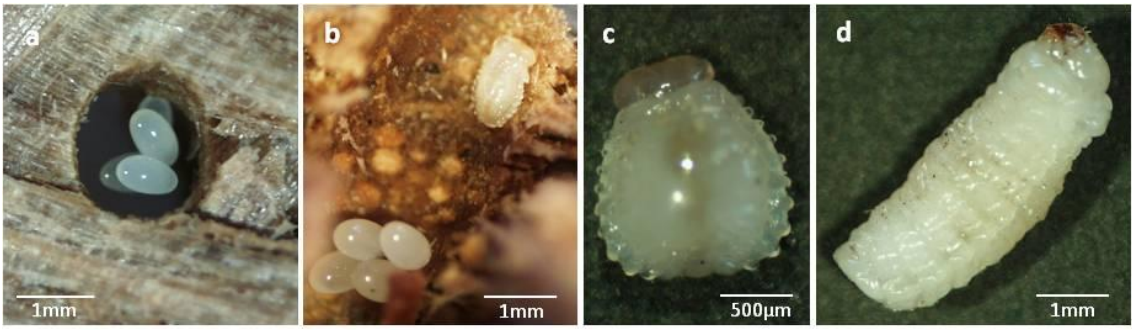

Fig. 4 - *Platypus cylindrus* eggs and larvae: a) eggs in the gallery, b) newly hatched larva and egg laying, c) first instar flattened larva with pleural tubercles, d) fourth instar cylindrical larva.

Mandibles of the fifth instar larvae have a chisel-like cutting edge, probably an adaptation for cutting through hard wood. Baker (1963) suggests that wood particles (wood floor) in tunnel walls lining with mycelium undergo constant cutting by larvae, serving as additional substrate for fungal growth. As a result, most of the wood released during tunnel excavation by adults and larvae is utilized. The rest is flushed out of the tunnel by the wood boring larva using a flattened tergite of the segment in the anal part of the abdomen (Koryolov 1989, Tío *et al.* 1993). The pupae are white, with the appendages of the imago (Ferreira & Ferreira, 1991, Sousa and Débouzie 2002).

Adult flight and foraging period of *P. cylindrus* occurs during the warm seasons. Emergence takes place approximately from May to October in Portugal (Sousa 1996). Nonetheless, sporadic emergence of mature beetles has been reported to occur throughout the year, but it is thought that probably only the beetles emerging from late spring until early autumn are able to survive and breed successfully. The dispersal and lifespan of adult insects are directly influenced by a combination of environmental and populational factors (Sousa and Inacio 2005).

The mechanism for tree selection by the insect is not fully understood and probably obeys to complex stimuli, particularly host volatiles like ethanol and terpenes (Shore and McLean 1983), and the silhouette of the trees (Chararas 1979, Byers *et al.* 1985). The insect preference for a host most likely takes into consideration wood moisture, osmotic pressure, sap flow and tree leaf composition, among others (Sousa *et al.* 1995; Yamasaki & Futai 2008). Additionally, a kairomone mediating this primary attraction to *P. cylindrus* has been described (Algarvio *et al.* 2002, Teixeira *et al.* 2003). Analysis of the evolution of *P. cylindrus* attacks reveals a preference for the largest hosts (height and perimeter), mainly for those recently decorked (Sousa and Débouzie 1999).

The males begin the colonization of trees by selecting the most suitable host, based in the parameters listed herein, and initiate gallery excavation (1-2 cm). The male will not accept a female until the tunnel is long enough to accommodate both beetles (Baker 1963). The secondary attraction begins by the aggregation of insects of the same sex followed by the attraction of the other sex (Ytsma 1986, Atkinson 2004). A high density of *P. cylindrus* attacks in the same tree confirms the existence of this secondary attraction mechanism initiated by the appeal of the other males through an aggregation pheromone (Algarvio *et al.* 2002), similarly to other Platypodidae (Renwick *et al.* 1977, Milligan *et al.* 1988, Tokoro *et al.* 2007, Kim *et al.* 2009). Each male is joined by a single female whose attraction is possibly mediated by a sexual pheromone (Allegro and Della Beffa 2001) and the couple initiates the single act of mating throughout his life cycle. This species reproduces during two consecutive years, producing 150 to 200 eggs per female (Sousa and Débouzie 2002). The female then continues to excavate the galleries from the entrance hall, inoculating them with ambrosia fungi, while the male removes sawdust to the outside. The gallery system can become quite complex due to profuse branching, in different plans, from the initial one (Sousa and Débouzie 1999). Egg laying occurs from autumn until the following spring. Eggs, larvae, pupae and adults could be simultaneously found in the galleries. The behaviour and fecundity of females depends on various factors such as availability of space and food in the gallery and the number of developing larvae (Sousa 2006). Egg hatching takes place two to six weeks after laying, being followed by five to six larval stages and whose exact number is not clear. During larval development, males remain in the top surface of the gallery, protecting it and preventing outflow of larvae. The nymphosis starts after the insect closes the gallery with sawdust and excrements. The development of larvae and pupae is slow, taking about five months from egg to adult. The emergence period in the first year of the insect life cycle is wider than the second since in the second year, as the galleries are already built, the laying period is less prolonged (Baker 1963, Sousa 1996, Sousa and Inacio 2005). Adults emerge through the original entry hole (Atkinson 2004).

1.3.1. The symbioses of *Platypus cylindrus* with fungi

P. cylindrus is an ambrosia beetle, or xylomycetophagous beetle, since its larvae and adults feed mainly upon fungi that cover the walls of their galleries in the host, the ambrosia fungi. The designation ambrosia derivates from the Greek mythology, meaning "food of the gods" (Baker 1963, Batra 1963, Beaver 1989).

Ambrosia beetles (almost all members of Platypodidae and some genera of the Scolytidae, *i.e. Corthylus*, *Gnathotricus*, *Tripodendron*, *Xyleborus* and *Xylosandrus*) are a polyphyletic, ecologically defined group of about 3.400 species derived from bark beetles (Farrell *et al.* 2001, Marvaldi *et al.* 2002, Kim *et al.* 2011).

The term ambrosia was first used in 1836 by Schmidberger who found a viscous material covering the galleries of *Xyleborus dispar* (Fab.) Hartig (1844) recognized the substance as a fungus but kept the idea of spontaneous generation, assuming that these fungi derived from the sap along with insect excretions. The work of Hubbard (1897) on ambrosia beetles of the United States boosted further investigation on this issue. However, only in 1960-80's more literature was published on the subject (Batra, 1963, 1966, 1967, Franck-Grosmann 1967, Norris & Baker, 1968; Subramanian, 1983; Batra, 1985). Nowadays, the interactions between ambrosia fungi and forestry pests are of major concern (Gebhardt *et al.* 2004, Inacio *et al.* 2005, Henriques *et al.* 2006, Fraedrich *et al.* 2008, Massoumi Alamouti 2009, Matsuda *et al.* 2010, Harrington *et al.* 2011).

According to Francke-Grosmann (1967), the interactions ambrosia beetle-ambrosia fungi are considered ectosymbioses: fungi can live in special organs in the insect or live outside its body, stored in special organs of ectodermal origin called mycangia. The purpose of such specialized structures is the storage, cultivation and transportation of fungi.

Some mycangia are complex and include secretory cells while others are less-well developed as simple pits in the exoskeleton of the head, pronotum or elytra (Harrington 1993). These special spore-carrying sacs can be found in one or both adult sexes. Fungal symbionts are transported from one tree to the next in these sacs (Batra 1963, Francke-Grosmann 1967, Beaver 1989). Glandular secretions into the mycangium may facilitate growth of specific fungi (Norris 1976).

Being an ambrosia beetle, *P. cylindrus* ensures the transportation and storage of fungal propagules in their mycangia (Baker 1963). Mycangia are more numerous in the female (330±104) than in male (5-25) (Fig. 5a-d). Mycangia are ovoid structures, located at the top of the prothorax and consisting of semi-spherical cavities for fungal transportation, linked with several glandular cells (Cassier *et al.* 1996). Their ultrastrutural study revealed the presence of a large bundle of interlaced microtubular structures across the cuticle from the cavity of the glandular cell to the spheroidal cavity containing the spores. The secreting glands might play an important role controlling the growth of undesirable fungi, as well as the maintenance of favourable conditions for the fungi during the flight and dispersion of insects (Cassier *et al.* 1996).

In dying insects, the fungal development is no longer controlled and the mycelium spreads over the insect body (Fig. 5e).

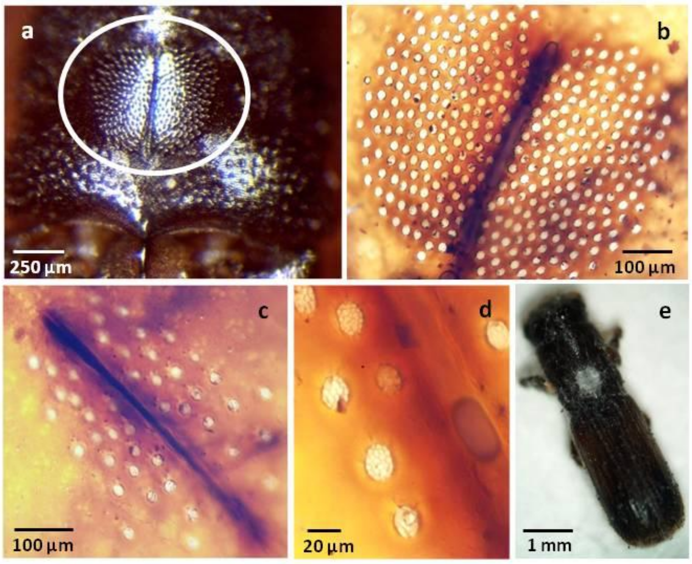

Fig. 5 - Mycangia of *Platypus cylindrus*: a) female mycangia, b) micrograph of female mycangia, c-d) micrographs of male mycangia, d) detail of integumentary pits filled with tightly packed spores of ambrosia fungi, e) dying insect covered by the ambrosial mycelium coming out of the mycangia.

Advantages of the symbioses

The symbiosis with fungi offers several advantages for the insects: (i) fungi are a food source for ambrosia beetles which do not feed exclusively on wood. Wood is a poor substrate for nutrition of insects, since they are not able to digest lignin, cellulose and hemicelluloses, the main constituents of the xylem, and it is deficient in essential B vitamins which the insect cannot synthesize for itself (Baker 1963, Graham 1967, Beaver 1989). Ambrosia beetles have overcome this problem through the ectosymbiosis with fungi (Francke-Grosmann 1963, Graham 1968) as the hyphae are a richer source in protein than wood, being able to concentrate nitrogen from the substrate on which it occurs at very low concentrations (Martin 1979, Swift and Boddy 1984). Besides nitrogen compounds, fungi produce sterols, essential for the development of several phases of the insect life cycle (Norris 1976). According to Kok (1979) ergosterol is the most essential and critical nutrient constituting the chemical basis of insect-fungus symbiosis; (ii) fungal symbionts participate in the decomposition of wood tissue thus facilitating the construction of galleries and creating favourable conditions for offspring development (Francke-Grosmann 1967, Berryman 1982, Six 2003); (iii) fungi might contribute to trees decline and debilitating host defences hence facilitating insects establishment; (iv) fungi can induce the production of pheromones (Christansen and Hornvedt 1983).

Fungi also benefit from the interaction with ambrosia beetles: (i) the insect vector provides the means for the transportation and dissemination of fungi, overcoming the spatial discontinuities between plant hosts (Malloch and Blackwell 1994, Harrington 2005); (ii) the insect ensures direct fungal inoculation into susceptible host; (iii) inside the galleries, fungi find favourable conditions for the development and growth within the host tree (Subramanian 1983, Beaver 1989). This association is rather obligate since the fungi are highly adapted to dissemination by insects and apparently wholly dependent on the beetle for dispersal (Blackwell and Jones 1997).

Apart from the advantages that both parts benefit from the mutual relation, there are also some costs associated with the loss of independence of each organism: ambrosia spores are not exposed in fruiting bodies on the surface of the wood and these fungi must entirely rely on the new generation of insects for their survival, dispersal and inoculation. Overall, ambrosia fungi fail to control their own vegetative growth and usually forego sexual reproduction. During co-evolutionary processes the sexual stage might have been lost (Gebhardt and Oberwinkler 2005). The offspring of ambrosia beetles depend totally on the growth of fungi in the galleries as they are exclusively mycetofagous. In the same way, after pupation, teneral adults feed for a time on the ambrosia fungi and then cease feeding. After the arrival at the new host, they do not eat until ambrosia fungi have established themselves. As soon as the fungi are established in the galleries, the beetles resume feeding (Baker 1963). There is also an additional energy cost for the adults in the production and maintenance of specialized mycangia (Beaver 1989).

The insect-fungus association should not be considered independently of the host where they both live. For most ambrosia beetles, the host tree is essentially a passive host since it is already declining at the time of attack. However, when the tree is vigorous, it has an active defense against attacks by insects and fungi (Sousa 1996). Many insects ambrosia are thus classified as secondary pests, attacking dead or decaying hosts that are already weakened (Harrington 2005). However, there are several examples of species that attack healthy trees exhibiting an active defense against the attack of insects and fungi (Külnholz *et al.,* 2003, Kubono and Ito 2002, Harrington *et al.* 2010). The relationship between insect and fungi and host tree can be considered antagonistic (Beaver 1989).

Ecology of ambrosia fungi

Typically, ambrosia fungi are dimorphic, i.e., they grow in the ambrosia form (yeast form) or as mycelium. The mycelium ramifies in the xylem and phloem but usually sporulates only in the beetle galleries. The secretions of the larvae and adults appear to be responsible for the induction of the ambrosia form that develops after physical contact with the insect. Under appropriate conditions, ambrosial cells can produce vegetative mycelium and vice versa. This phenomenon is known as pleomorphism (Batra 1967, 1985). The ambrosial form that could originate within the host galleries is one of the diagnostic features of these fungi. Colour can be variable and usually consists of chains of cells that form a compact palisade covering the main tunnel and chambers (Batra 1963). The observation of gallery walls confirms the existence of a very thin and light-coloured coat, consisting of mycelium and ambrosial cells of the fungal symbiont. This ambrosial mat lining the galleries becomes darker as the galleries get older (Inacio *et al.* 2005, Sousa *et al*. 2005) (Fig. 6).

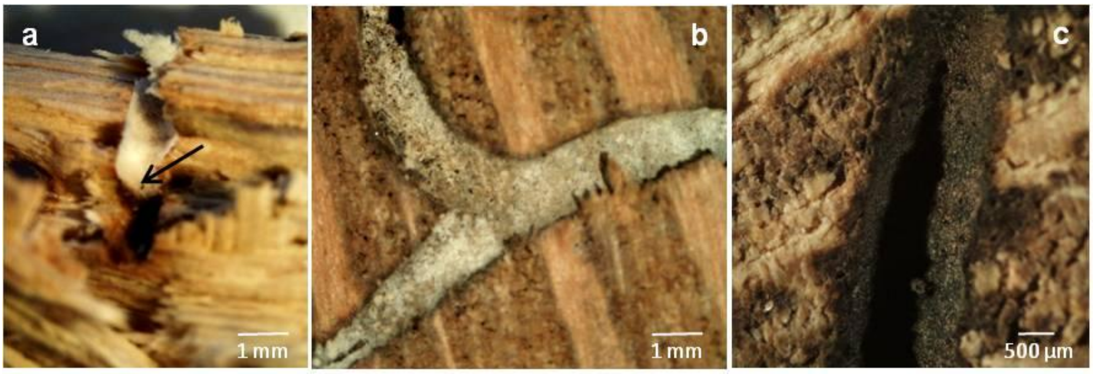

Fig. 6 – Galleries of *Platypus cylindrus* in cork oak: a) cross section of a pupal chamber lined by a silky white mycelium (arrow), b-c) transversal cut showing ambrosial mat, c) older gallery.

The ambrosia fungi were classified by Batra (1985) as primary and auxiliary. The primary ambrosia fungi are highly specific, so its distribution corresponds to that of their insect symbionts. They are present and dominant in the galleries where they are consumed and can often be isolated from beetle's mycangia or during the opening of the galleries. The primary ambrosia fungi are obligatory mutualistic and extremely susceptible to dryness, hardly found outside the mycangia or insect galleries (Batra, 1966). The auxiliary ambrosia fungi are transient and relatively non-specific to the insect symbiont. They may appear after the complete development of insects or optionally in the larval chambers or adult insects (Batra 1985). Many of these opportunistic fungi produce mucilaginous spores which allow them to be externally transported by insects, such as fungi of the genera *Gliocladium* and *Acremonium*. Many auxiliary fungi are easily isolated and cultured, being erroneously considered primary ambrosia fungi (Lévieux and Cassier 1994). More recent studies proved that the ambrosia fungi are not species-specific to the beetles and the natural food of the adults and their brood is probably the whole mutualistic microbial complex (Beaver 1977, Cassier *et al.* 1996, Gebhardt and Oberwinkler 2005). Most ambrosia beetles are polyphagous and breed in a wide range of hosts because the fungi on with they feed upon are polyphagous. So far as is known, the beetle feeds on the same fungi whatever host it is attacking (Beaver 1989, Harrington *et al.* 2011)

The inoculation of fungi occurs passively during the excavation of galleries: the excavation process of females causes an increase of glandular secretions from the mycangia, stimulating spores to bud and to ooze out of the integumentary pits (Francke-Grossmann 1967, Farris and Funk 1965). Females also prepare the galleries, with a mixture of sawdust and exudates, for the inoculation and development of fungi. Favourable conditions of temperature and humidity are maintained inside the galleries (Baker 1963).

Typically, ambrosia fungi are asexual and form wet droplets of conidia in small conidiophores projected into the insect passageways that effectively force the insects into contact with the spore masses as they pass through the restricted spaces. This process is known as the ‘paint brush’ method employed by slimy-spored fungi (Beaver 1989). However, the insects could also be dusted with spores in some dry-spored fungi. In both ways, spore dispersal is achieved through adherence to insects moving throughout the fungus colonized substratum (Harrington 2005).

After completion of the life cycle inside the tree, while awaiting the right conditions to start the search for new hosts, imagos move along the galleries, contributing to the random dispersion of non-specific fungi inhabiting these galleries. The newly hatched adults also feed on the fungi and emerge without further boring (Inacio *et al.* 2005).

Taxonomy of ambrosia fungi

The taxonomy of ambrosia fungi was unclear and unstable as their reduced morphological features led to ambiguous classification systems, until the common application of DNA sequence analyses (Cassar and Blackwell 1996, Jones and Blackwell 1998, Rollins *et al.* 2001, Massoumi Alamouti 2009, Harrington *et al.* 2010).

A comprehensive taxonomic revision of fungi associated with ambrosia beetle has not been conducted since Batra (1967), who placed most of the species in the mitosporic genera *Ambrosiella* Brader ex Arx & Hennebert emend. T.C. Harr. and *Raffaelea* Arx & Hennebert. Phylogenetic analyses currently places the type species of these genera within the ascomycete genera *Ceratocystis* Ellis & Halst. and *Ophiostoma* Syd. & P. Syd., respectively (Cassar and Blackwell 1996, Jones and Blackwell 1998). *Ambrosiella* and *Raffaelea* were originally distinguished based on annellidic vs. sympodial proliferation of the conidiogenous cells, respectively (Batra 1967). However, many *Raffaelea* species have a combination of sympodial and percurrent (annellidic) proliferation of conidiogenous cells (Gebhardt and Oberwinkler 2005) and Batra's distinction lost its taxonomic value in the last few years and is not tenable (Massoumi Alamouti 2009, Harrington *et al.* 2010, 2011).

The asexual symbionts of ambrosia beetles occur in a monophyletic clade within the genus *Ophiostoma*, and *Raffaelea* is proposed as the proper mitosporic genus for members of this group (Harrington *et al.* 2010). Massoumi Alamouti *et al.* (2009) found two subclades within *Raffaelea* to have bootstrap support and suggested to separate them into two subclades. However, no phenotypic character distinguishes these two subclades.

Conidiophores and conidia of *Raffaelea* species could fit the concept of *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr., a common anamorph of *Ophiostoma* species (Gebhardt & Oberwinkler 2005, Zipfel *et al.* 2006, Massoumi Alamouti *et al.* 2009). The presence of sporodochia was proposed to distinguish *Raffaelea* from *Hyalorhinocladiella* but Harrington *et al.* (2008) proposed that *Raffaelea* species are better distinguished by symbiotic relationship with ambrosia beetle (Gebhardt and Oberwinkler 2005, Harrington *et al.* 2010) which is as ecological character rather than a taxonomical placement, thus needing further clarifications.

Together with other ascomycetes, *Ophiostoma*, *Ceratocystis, Ceratocystiopsis* as well as related asexual fungi in the genera *Leptographium*, *Pesotum*, *Hyalorhinocladiella, Sporothrix* and *Raffaelea* are known as "ophiostomatoid fungi" (Kirisits 2007). Ophiostomatoid fungi associated with bark beetles are also commonly called "blue-stain fungi", referring to the damage these fungi cause, namely blue or even black discoloration of the sapwood of trees, mostly on conifers (Kim *et al.* 2003, Zhou *et al.* 2006). However, hardwoods are also affected, exhibiting vascular wilt and vascular stain diseases (Delatour *et al.* 1992, Nkuekam *et al.* 2011).

Ceratocystis, Ceratocystiopsis and *Ophiostoma* and their related anamorphs could be distinguished based on the chemical composition of their cell walls. Besides chitin, cell walls of *Ophiostoma* also contain cellulose and rhamnose which is quite unusual for ascomycete fungi. In contrast, the cell walls of *Ceratocystis* and *Ceratocystiopsis* fungi consist mainly of chitin. The cell walls of *Ophiostoma* fungi make them highly tolerant to cycloheximide that inhibits the protein synthesis of most eukaryotic organisms (Harrington 1981) while *Ceratocystis* and *Ceratocystiopsis* are very sensitive to even low concentrations of this antibiotic (Kirisits *et al.* 2002, Plattner *et al.* 2009, Reid *et al.* 2010). *Raffaelea* sp., as a mitosporic *Ophiostoma,* is tolerant to high concentrations of cycloheximide (Harrington and Fraedrich 2010). Conversely, species of *Raffaelea* are very difficult to distinguish since they do not exhibit distinctive morphological characteristics. The conidiophores lack pigmentation, are mostly simple, and the mode of conidiogenesis is barely visible with light microscopy. Conidia also lack pigmentation, separation and other special features (Gebhardt and Oberwinkler 2005). Thus, identification is primarily based on nuclear rDNA sequences (Jones and Blackwell 1998, Massoumi Alamouti 2009). Thus far, the only complete and sufficiently variable dataset of DNA sequences for identification of *Raffaelea* species is the large subunit (LSU, 26S) rDNA dataset. The small subunit (SSU, 18S) sequences do not show sufficient variation to discriminate all of the known species of *Raffaelea* and *Ophiostoma* (De Beer *et al.* 2003, Harrington *et al.* 2010, 2011). Unfortunately, the more variable internal transcribed spacer (ITS) regions of rDNA are difficult to amplify in most of the *Raffaelea* species (Fraedrich *et al.* 2008), and in several *Ophiostoma* species, due to their high CG nucleotide content (Mullineux and Hausner 2009, Tsui *et al.* 2010, Nkuekam *et al.* 2011).

Main ambrosia fungi associated with Platypus cylindrus

P. cylindrus is associated with several fungal species whose exact role has yet to be fully understood. The primary ambrosia fungi isolated from *P. cylindrus* belong to the genus *Raffaelea,* namely *R. ambrosiae* Arx & Hennebert (Arx and Hennebert 1965, Sousa *et al.* 1997) and *R. montetyi* Morelet (Cassier *et al.* 1996, Morelet 1998). The former has been considered the primary specific ambrosia fungus of *P. cylindrus* while *R. montetyi* was also identified as the primary ambrosia fungus of *Xyleborus monographus* (Fabr.) and *X. dryographus* (Ratzb.) (Gebhardt *et al.* 2004).

Apart from these primary ambrosia fungi, others have been isolated both from the galleries of *P. cylindrus* in *Q. suber*, and directly from the adults mycangia, exoskeleton and gut content (Cassier *et al.* 1996, Sousa 1996, Sousa *et al.* 1997). The isolates mainly comprised species of *Acremonium*, *Aspergillus*, *Fusarium*, *Gliocladium*, *Nodulisporium*, *Paecilomyces*, *Penicillium*, *Scytalidium*, *Trichoderma* and *Trichothecium*. The specific role of these secondary fungi associated with *P. cylindrus* was even less clear. Species identification and ecological role, in the interaction with the oak pinhole borer, suffered from a lack of investigation prior to the earlier works of Sousa (1996) and Sousa *et al.* (1997).

Since *P. cylindrus* carries many fungal species besides the referred cosmopolitan species, it is difficult at first to assess which fungi could be phytopathogenic and thus responsible for participating in cork oak decline. However, *Raffaelea* species, as a mitosporic *Ophiostoma* should be taking into consideration, especially because several *Raffaelea* species were noticed provoking tree mortality (Kubono and Ito 2002, Fraedrich *et al.* 2008, Eskalen and McDonald 2011). The interaction between *R. quercivora* Kubono & Shin and *Platypus quercivorus* (Murayama) in *Quercus* spp. is of particular interest given the similarity with the Portuguese case. *R. quercivora* has been responsible for mass mortality of fagaceous trees in Japan since the 1980s and is now widespread (Murata *et al.* 2005, 2007, 2009, Matsuda *et al.* 2010). In the southeastern USA, *R. lauricola* T.C.Harr., Aghayeva & Fraedrich, transported by the exotic redbay ambrosia beetle, *Xyleborus glabratus* Eichh. is currently causing lethal vascular wilt disease on Lauraceae members (Fraedrich *et al.* 2008, Harrington *et al.* 2010, Harrington and Fraedrich 2011).

Evolutionary aspects of the symbioses Platypus cylindrus-ambrosia fungi

Batra (1966) suggested that the association between beetles and fungi was initially fortuitous. Fungi growing below the bark ramified into the frass-filed larval galleries of bark beetles, and were occasionally eaten along with the phloem. Phloeophagy is certainly the primitive feeding habit in Scolytidae and Platypodidae (Wood 1982, Kirkendall *et al.* 1997). Once beetles began to depend on the fungi for food, they were able to colonize the less nutritious but less well defended xylem. The exploitation of a new ecological niche led in turn to extensive species radiations in some groups, particularly the Xyleborini, Corthylini and Platypodidae (Atkinson 2004).

Ambrosia beetles evolved from bark beetles in at least seven separate events, each origin following a shift to angiosperms (Farrell *et al.* 2001). So it is not surprising that most of the symbionts of ambrosia beetles are found within the large genus *Ophiostoma*, which hosts the majority of bark beetles associated fungi and may be more than 85 million years old (Cassar and Blackwell 1996, Jones and Blackwell 1998, Farrell *et al.* 2001). It is surprising to find, however, that all the asexual symbionts in the *Ophiostoma* clade that are associated with ambrosia beetles may have evolved from a single ancestor. The ancestor of *Raffaelea* may have uniquely been successful in both serving as food for ambrosia beetles and reproducing in the mycangia of the insects. It would have had an ophiostomatoid sexual state and anamorph with annellidic conidiogenesis similar to extant species of *Hyalorhinocladiella* or *Pesotum*. Studies from Gebhardt *et al.* (2004) placed *Raffaelea* species in a clade together with some *Ophiostoma* species, with anamorphs assignable to *Pesotum* and *Leptographium.* The congruence between the DNA-based phylogeny and morphological characters of *Raffaelea* species is additionally supported by knowledge of the vector relationship within the genus *Ophiostoma*. Even these fungi depend on dispersal by arboreous insects.

During co-evolutionary processes the sexual state might have been lost in *Raffaelea* species, since the conidia could be preserved and transported within mycangia of the symbiotic beetles, although there are some examples of bark beetles mycangial fungi that retain teleomorphic states (Gebhardt and Oberwinkler 2005). In addition, species of the relatively young tribe Xyleborini have ambrosia fungi both from the *Ophiostoma* and *Ceratocystis* groups. It appears that various species of ambrosia beetles have independently acquired their symbionts from several species in these relatively old insect-associated genera (Harrington 2005). A gradation in the tightness of the association, from species transported by a wide range of different insects to those that consistently interact with one or a few species, is evident (Francke-Grosmann 1967, Gebhardt and Oberwinkler 2005). In addition, some species of bark beetles have obtained mycangia through evolutionary processes, hence allowing the fungi to serve as a source of nutrition. Like the evolution of mycophagy within the ambrosia beetles, a number of independent evolutionary events were required to explain the diversity of fungi that are adapted to being fed upon and transported by mycophagous bark beetles.

It is noteworthy that several beetle species can inhabit the same host tree, allowing exchange of associated symbiotic fungi between the galleries of different beetle species or even between bark and ambrosia beetles (Gebhardt *et al.* 2004). The regularity with which blue-stain fungi have been found in ambrosia beetles tunnels clearly denotes that they have a close association with the ambrosia beetles, even though the evidence is against their being primary ambrosia fungi (Baker 1963, Harrington *et al.* 2011). Thus, host switching of ambrosia fungi may account for points of incongruence between the beetles and fungal phylogenies.

It seems probable that evolutionary process of mycophagy occurred in tropical rain forest, in which the high temperatures and humidity are particularly favourable to fungal growth, and where associations between beetles and fungi would frequently have occurred. Those contacts between symbionts may have arisen from competition between beetles and fungi for the same substrate (Beaver 1989).

If the ambrosia-feeding habit is basically a response to high humidity and temperatures, it might be expected that it would occur more frequently in regions with these climatic conditions. A general correlation with latitude was shown by Beaver (1989). Tropical regions have a higher percentage of xylomycetophagous than temperate areas. However, in a scenario of climatic changes coupled with the intercontinental movement of infected material and insects associated with tree pathogens, the Mediterranean climate would offer propitious conditions for such evolutionary events (Desprez-Loustau *et al.* 2006, Henriques *et al.* 2011, Nkuekam *et al.* 2011).

1.3.2. Control of *Platypus cylindrus* attacks in cork oak stands

The control of *P. cylindrus* in Portuguese cork oak stands is based on the biology of this insect. Usually, it involves the use of one of the following control measures: cultural, chemical and biotechnical control.

Cultural control

General sanitary practices have a place in integrated pest control, e.g., removal and/or burning of obvious inoculum sources such as infested logs, old branchwood or firewood, or even severely infested trees, before the emergence of adult insects. Logs and trees can be attacked within days from cutting. Once the beetle has established in the timber, early conversion to accelerate the drying process, perhaps coupled with kiln drying, is the only way to prevent further activity, if the use of wood is desired. Once the moisture content falls below 30-40%, beetle activity is minimal but it will not cease completely until the moisture content reaches 25% or less. At this point the ambrosia fungi can no longer survive in the galleries and *P. cylindrus* perishes (Tilbury 2009).

In stands with *P. cylindrus* attacks, decorking should be delayed for two years. Even outside of the functional beetle flight period, it is advisable to carry out regular inspections for signs of attack since warmer winter and spring temperatures might increase beetles survival and successful breeding (Sousa and Inacio 2005).

Appropriate production techniques that create healthier trees and prompt removal of declining trees from cork oak stands contribute to a decrease in *P. cylindrus* populations. In this context, the improvement of soil composition and fertility may also contribute to an increase in trees health condition, and consequently to the control of new infestations. In the same way, the establishment of new stands should make use of mycorrhized seedlings.

Chemical control

Once beetles have tunnelled into trunks or logs they cannot be successfully controlled with insecticide. Chemical treatment is, therefore, only of value to kill adult *P. cylindrus* as they attempt to bore out of infested logs left in the stand or in yards. In Portugal, spraying logs at the felling site is not allowed, but in some countries such as the United Kingdom, this practice is widely used to treat logs of oak trees (Tilbury 2009). However, more desirable and environmentally friendly means of protection must be undertaken, namely covering or burying logs and stumps.

The control of the ambrosia fungi inside the host tree is not easily feasible due to the high restrictions for the use of pesticides in cork oak stands, mainly in certified FSC (Forest Stewardship Council) forests (FSC 2005). Overall, the application of fungicides on the trunks of adult trees capable of moving systemically and being able to control fungal development inside galleries has not been fully studied (Pires 2007).

Biotechnical control

The attractiveness of fresh cut logs has been used to attract adults during its flight period (Ferreira and Ferreira, 1991). However, working with *P. quercivorus,* Ueda and Kobayashi (2004) noticed that it was the presence of males in the entrance holes rather than the odour of the logs themselves that was responsible for the attraction.

The development of traps baited with attractive semiochemicals in the control of *P. cylindrus* shows great potential as it emerges as a possible alternative to the use of pesticides and possesses a high degree of specificity (Romeiras 1995). The primary attraction involving both sexes of *P. cylindrus* is mediated by kairomones from cork oak such as α -thujene, β-pinene and camphene (Algarvio 2000, Casas-Novas 2001). These compounds should be part of a broader fragrant blend that may be related to the physiological weakness of the host tree (Algarvio 2000). The mass attraction of conspecific insects for the same host allows them to overcome tree defences. The aggregation pheromone of *P. cylindrus* consists of a mixture of three compounds: hexanol, sulcatol and sulcatone (Algarvio 2000, Correia 2003, Teixeira *et al.* 2003), being able to attract simultaneously males and females, allowing the direct control of the xylomycetophagous (Algarvio *et al.* 2002, Barata *et al.* 2002, Teixeira *et al.* 2003). The components proportions and concentrations in the final mixture are still under study for both attractives (Henriques *et al.* 2010).

The effect of predation for control of *P. cylindrus* is very limited in Portuguese cork oak stands being only known an insect predator *Colydium elongatum* F., while in Morocco are known *Corticeus pini* Panz. and *Platysoma oblongum* F. , both existing in Portugal but associated with other host trees (Sousa *et al.* 2005).

1.4. Aims of the thesis

Since a few decades ago, *P. cylindrus* has been associated with cork oak decline (Ferreira and Ferreira 1989, Sousa 1995, Sousa and Débouzie 1999). The combined action of extensive tunneling into the heartwood and the inoculation of ambrosia fungi, on which beetles and their offspring depend upon for host colonization and survival, leads to an increase in tree mortality over the past few years (Sousa and Inacio 2005, Sousa *et al.* 2005). The lifecycle of the oak pinhole borer was thoroughly studied in countries of the Mediterranean basin in which it occurs (Villemant and Fraval 1991, 1993, Sousa *et al.* 2005), namely in Portuguese oak stands (Sousa 1996, Sousa and Débouzie 2002). Despite the importance of *P. cylindrus* as a vector of fungi that beetles inoculate and spread in cork oak stands, the whole symbiont complex had not been studied since the early studies of Sousa (1996). Overall, the potential of the primary ambrosia symbionts as pathogens of *Q. suber* was never assessed both for Portugal and the rest of the *P. cylindrus* distribution range. Specific roles for non-pathogenic fungi associated with this species were even less well understood.

Thus, the goals of this research project were i) to precise which fungi are consistently carried by *P. cylindrus* in Portuguese cork oak stands, as well as their frequency and location in the insect's body, in order to understand the role of the main vectored fungi in the success of tree host colonization; ii) to identify the primary ambrosia fungi associated with *P. cylindrus* taking into consideration the more recent studies on ambrosia fungi worldwide (Gebhardt and Oberwinkler 2005, Massoumi Alamouti 2009, Matsuda *et al.* 2010, Harrington *et al.* 2010, 2011). Identifications were strengthened with a phylogenetic and morphological characterization of these primary symbionts; iii) to ascertain the pathogenic role of the main ambrosia fungi and to discuss their role in cork oak decline. Questions like the doubtless presence of *P. cylindrus*'symbionts in dying cork oak trees without visible beetles attack, aborted attacks and disease spreading, as well as, new and diverse fungal associations were studied for the first time in Portugal and in the entire cork oak production area.

The main premise of the research was to improve current scientific knowledge in the causes of cork oak decay aiming to understand the contribution of *P. cylindrus*' attacks in the decline of the unique Portuguese cork oak forests and woodlands.

1.5. Thesis structure

The current research comprises six chapters, with the results presented in the form of six scientific papers, either already published (two), in press (three) or submitted for publication (one) to peer-reviewed journals. Each paper follows the journal's specific guidelines consisting of an introduction, material and methods, results, discussion of results and literature cited.

Chapter 1 reviews the current knowledge in cork oak decline in Portugal, the role of *P. cylindrus* in the phenomenon and the multiple aspects of the symbiosis between the beetle and the ambrosia fungi, along with the presentation of the main objectives of this thesis.

Chapter 2 presents studies on the mycobiota vectored by *P. cylindrus* and is divided into three sub-chapters:

- Sub-chapter 2.1 studies the whole complex of fungi transported by *P. cylindrus*, found on the insect's body and within its galleries in cork oak.

- Sub-chapter 2.2 focuses on the *Raffaelea* fungi associated with the oak pinhole borer discussing their role as a nourishment source and the possibility of being pathogenic agents.

- Sub-chapter 2.3 investigates the contribution of the main fungi associated with *P. cylindrus* in beetles' host tree colonization, as part of a broader strategy of colonization through the production of pheromones and massive attacks.

Chapter 3 presents the studies on the symbiotic fungi playing a role in the process of loss of vigor of Portuguese cork oak stands and is divided in three sub-chapters:

- Sub-chapter 3.1 presents evidences for the transportation and inoculation by *P. cylindrus* of *Biscogniauxia mediterranea,* the causal agent of cork oak charcoal canker, thus proving its role of dispersal disease agent on cork oak stands.

- Sub-chapter 3.2 addresses the pathogenic role to cork oak seedlings of two *Raffaelea* species associated with *P. cylindrus*

- Sub-chapter 3.3 studies the Ophiostomatales associated with *P. cylindrus* pointing out their systematic position within known ambrosia fungi commonly found associated with ambrosia beetles and that are pathogenic to host trees.

Finally, in chapters 4 and 5, the results of the six publications/ manuscripts are summarized and reviewed in a discussion presenting the most important conclusions, and suggesting future lines of research. Chapter 6 groups all literature cited, excluding the references of the papers which are integrated in each publication.

CHAPTER 2

FUNGI TRANSPORTED BY *PLATYPUS CYLINDRUS*

SUB-CHAPTER 2.1

MYCOBIOTA ASSOCIATED WITH *PLATYPUS CYLINDRUS* ON CORK OAK IN PORTUGAL

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Mycobiota associated with *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) on cork oak in Portugal

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Abstract: *Platypus cylindrus* populations outbreaks observed in the last decades are related to cork oak decline in Portugal, as in other Mediterranean countries. Being an ambrosia beetle, it feeds on fungi that carries and inoculates in galleries excavated in host trees. To study this mycobiota, fungi were isolated from the beetles (mycangia, intestine and exoskeleton) and cork oak galleries. Several fungi were identified, namely *Raffaelea* (Ascomycota, Ophiostomatales) and *Nodulisporium* (asexual stage of *Biscogniauxia mediterranea*), possibly implicated in host weakness. The other isolated fungi, particularly *Gliocladium*, *Scytalidium* and *Trichoderma* genera, might be involved in processes such as insect feeding, wood degradation and fungal antagonism in the galleries.

Key words: *Quercus suber*, decline, ambrosia fungi, ambrosia insect

Mycobiote associé à *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) dans les subéraies du Portugal

Résumé: L'explosion des populations de *Platypus cylindrus* observée au cours des dernières décennies est liée au processus de dépérissement des peuplements de chêne-liège, au Portugal comme dans d'autres pays méditerranéens. Étant un coléoptère ambrosia, le platype se nourrit des champignons qu'il transporte et inocule dans les galeries qu'il creuse dans les arbres. Pour étudier ce mycobiote, on a isolé des champignons à partir des insectes (mycangia, contenu intestinal et exosquelette) et des galeries de chêne-liège. Plusieurs champignons ont été isolés, notamment *Raffaelea* (*Ascomycota*, *Ophiostomatales*) et *Nodulisporium* (forme asexuée de *Biscogniauxia mediterranea*), qui sont probablement impliqués dans l'affaiblissement de l'hôte. Les autres champignons isolés, surtout ceux appartenant aux genres *Gliocladium*, *Scytalidium* et *Trichoderma*, pourraient être impliqués dans des processus tels que l'alimentation de l'insecte, la dégradation du bois et l'antagonisme entre champignons à l'intérieur des galeries.

Mots-clé: *Quercus suber*, dépérissement, champignons ambrosia, insectes ambrosia

Introduction

Platypus cylindrus Fab. (Coleoptera: Platypodidae) is known to attack mainly dead or weakened trees. However, since the 1980's, its population outbreaks have been related to cork oak decline in Portugal and other Mediterranean countries (Ferreira & Ferreira 1989; Chakali et al. 2002; Tiberi et al. 2002; Sousa et al 2005). This insect establishes symbioses with fungi that he carries in specialized pouches – mycangia – as well as in the intestine and on the body surface (Sousa et al. 1995; Henriques et al. 2006). Such fungi are so called *ambrosia* as they act as a food source for the beetle descendants after being inoculated and cultivated in the galleries. The observation of those galleries confirms the existence of a light-coloured, thin wall cover, constituted by mycelium of the symbiotic fungi (Sousa & Inácio 2005).

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Ambrosia fungi definition includes a set of concepts whose interception allows the classification of several fungi as ambrosia: i) direct participation in insect nourishment; ii) presence inside insect tunnels into the host; iii) dimorphism, that is, the ability to grow either as hyphae forming an extended mycelium or in yeast-like form; iv) probable specificity in insect-fungi-host (Beaver 1989). Batra (1985) grouped ambrosia fungi as primary and secondary or auxiliary. Primary ambrosia fungi are strictly entomochoric and highly insect species specific. They are usually dominant in the galleries and frequently isolated from beetle's mycangia. The auxiliary ambrosia fungi are transitory and non-specific with respect to symbiotic insect. More often, they are not present in larval cradles or teneral adults and their distribution range is unrestricted and unrelated to that of the ambrosia beetles. Together they form a symbiotic microbial complex (Lévieux & Cassier 1994).

In addition to fungi directly related to insect nourishment, others could be found associated with insects, such as pathogenic fungi that may play an essential role in insect selection and tree colonization. Those fungi could play both roles, thus contributing to the establishment of insect populations since they help to reduce the host defence mechanisms in the vicinity of the insect attack. Among those are *Ceratocystis*, *Graphium*, *Leptographium* and *Ophiostoma.* Studies of oak decline in Europe showed that the complex *Ophiostoma/ Ceratocystis* is pathogenic to *Quercus* trees (Badler 1992; Degreef 1992; Delatour et al. 1992).

Several fungi have already been isolated from *P. cylindrus* and its galleries in *Quercus* spp. (Baker 1963; Cassier et al. 1996; Sousa et al. 1997; Morelet 1998; Henriques et al. 2006).

The aim of the present study was to characterise the mycobiota associated with *P. cylindrus* in weakened cork oak and to discuss its contribution for insects establishment and host decline.

Material and methods

Twelve infested logs by *P. cylindrus* of cork oak that exhibit decline symptoms were collected from main productive Portuguese regions. The logs were settled in the laboratory and the associated insects captured in fabric traps, attached to the log with a silicone joint. Those samplings were repeated during 2005, 2006 and 2007.

A total of 100 insects per location and per year were aseptically dissected to obtain their mycangia, intestine and parts of the exoskeleton (elytra). The logs were cut in order to identify the different gallery sections: cork, inner-bark, pre-parental, larval and gallery end. One complete gallery was observed from each log and six samples (fragments of $1cm^2$) of each section were collected. At the same time, isolations from the cork, inner-bark and wood of symptomless hosts were also performed in order to identify the natural fungal community of these tissues.

All the pieces, both from insects and wood, were surface sterilised with a sodium hypochlorite solution (1%) for 1 min and rinsed with sterilised distilled water. They were plated into 9cm diameter Petri dishes with malt extract agar (MEA, Difco, USA) added with 500mg/l of streptomycin (Sigma-Aldrich, USA), a large spectrum antibiotic, and MEA added with 500mg/l of cycloheximide (Sigma-Aldrich, USA), an antibiotic useful to distinguish fungi of the *Ophiostoma* genus (Harrington 1981; Hawksworth et al. 1981). Cultures were incubated at 25±1ºC in darkness.

Pure cultures of each fungus were obtained and grouped according to their macroscopic characteristics. Fungal identification at the genus level was based on cultural and morphological features according to Ellis (1971, 1976), Kiffer & Morelet (1997) and Barnett

& Hunter (1998). Macroscopic characters of colonies were described after 21 days of growth on potato dextrose agar (PDA, Difco, USA); colour names are from Saccardo (1891).

Results

Several fungi (belonging to *Zygomycota* and *Ascomycota*) and a *Streptomycetales* were obtained from the insect and cork oak tissues. Most of the isolated fungi, except those belonging to *Mucorales* order, were classified in different orders included in the *Ascomycota*: *Eurotiales, Heloliates, Hypocreales, Ophiostomatales, Pleosporales, Saccharomycetales* and *Xylariales*.

Table 1. Cultural characteristics of the *Ascomycota* isolates, after 21 days of growth on PDA

Isolates	Upper surface				Lower surface	Observations
	Cultural aspect	Density	Color	Zonation		
Acremonium	effuse, felted	high	white	absent	idem upper face	
Aspergillus	effuse, powdery	high	white, yellow, several green tonalities or black	weak or absent	idem to upper face except the color (yellowish)	
Beauveria	effuse, cotton-like to powdery	high	white	absent	idem upper face	
Botrytis	effuse, felted	light	grey	absent	idem upper face	
Chaetomium	effuse, felted	high	initially white turning grey to olive and reddish with maturation	absent	idem upper face except the color (dark brown)	
Fusarium	effuse, cotton-like	high	white	absent	idem to upper face except the color (carmine to yellow)	media's carmine pigmentation
Geotrichum	effuse, powdery	light	white	absent	idem upper face	
Gliocladium	felted	high	sulfur yellow to straw yellow	light concentric	idem to upper face except the color (citric yellow)	
Nodulisporium	effuse, cotton-like	high	white to grey with green emergences in the colony center	absent	idem upper face except the color (fuliginous)	
Paecilomyces	effuse, powdery	high	green-brownish	absent	idem upper face	
Penicillium	effuse, powdery	high	green to brown	radial	idem upper face except the color (yellowish)	produces coloured oozing
Raffaelea	effuse, yeast-like, some with a felted mycelium in the colony center	light to media	cream-colored, light olive-green or fuliginous	light-concentric or absent	idem upper face	
Scytalidium	effuse, felted to farinaceous in the colony edge	light	hazelly	radial	idem upper face	
Trichoderma	effuse, floccose	media	green-yellowish with white to grey flakes	media concentric	idem upper face	media's green-yellowish pigmentation

In some fungi, it was not possible to observe structures that allowed their identification; they were thus reported as unidentified group. The numerous fungi included in this group presented very distinct morphological features, and could thus probably be categorized in different taxa. All the obtained genera but *Chaetomium* were isolated in the mitosporic state. Two isolate sets were identified as *mycelia sterilia*. In table 1 are described the main cultural features of the *Ascomycota* isolates.

The localization of the isolates in *P. cylindrus'* different organs could be seen in Figure 1. The genera obtained in host's healthy tissues and along the insect galleries on cork oak are presented in Figures 2 and 3.

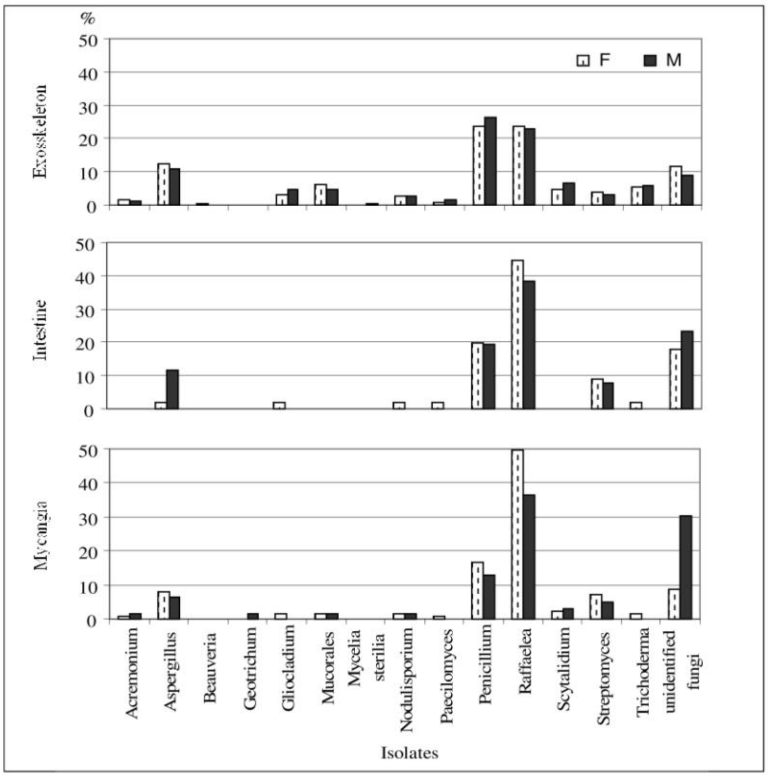

Figure 1. Isolates from the different insect parts (exoskeleton, intestine and mycangia) of *Platypus cylindrus* females (F) and males (M).

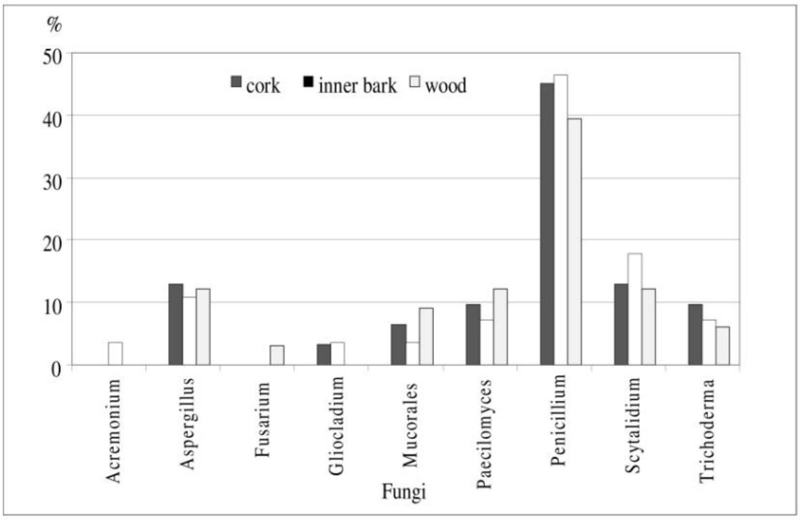

Figure 2. Fungi isolated from healthy cork oak tissues (cork, inner bark and wood).

In respect to fungi obtained in insects' isolements, the exoskeleton exhibits a major diversity of genera, namely cosmopolitan fungi such as *Penicillium*, *Aspergillus*, *Trichoderma* and *Gliocladium*. Several *Mucorales* isolates were also retrieved. In female's mycangia and intestine, almost half of the isolated fungi were *Raffaelea* species (49.6% and 44.6%, respectively). In males, *Raffaelea* are also the main isolated genus both on intestine (38.5%) and mycangia (36.5%). *Nodulisporium* sp., asexual stage of *Biscogniauxia mediterranea* (De Not.) Kuntze, causal agent of cork oak charcoal disease (Collado et al. 2001), was isolated of all parts, although in reduced number.

Along *P. cylindrus* galleries as in healthy cork oak tissues, the main isolated genus was *Penicillium,* highlighting his cosmopolite and saprophytic behavior. In the same way, *Trichoderma*, *Gliocladium* and *Scytalidium* were found in all gallery sections.

Raffaelea was isolated in a considerable percentage in all sections, particularly in the pre-parental section (16%) being absent in wood from trees not attacked by *P. cylindrus*.

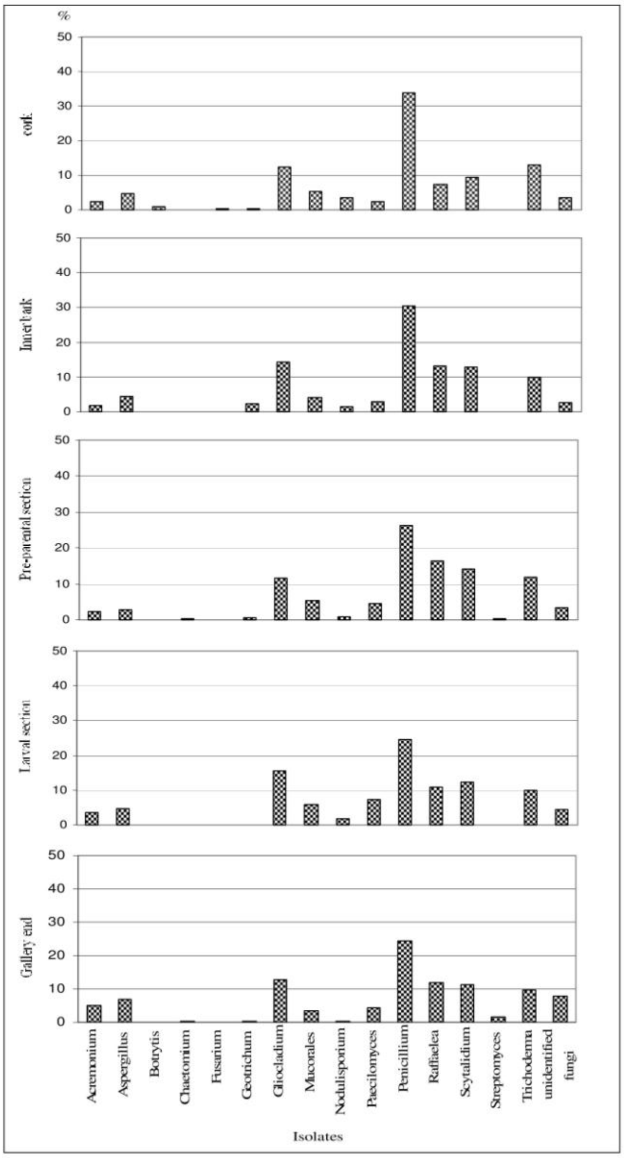

Figure 3. Isolates from the different parts of *Platypus cylindrus'* galleries on cork oak (cork, inner bark, pre-parental section, larval section and gallery end).

Discussion

The fact that in the present study *Raffaelea* isolates were found closely associated to *P. cylindrus* enhances their known importance as primary ambrosia fungi. Given that several isolates of *Raffaelea* were obtained both from *P. cylindrus*' intestine and mycangia, it is doubtless their direct participation in insect nourishment and their entomochoric nature. At the same time, they were also isolated from insect galleries, thus fulfilling the set of concepts that allows to classify them as ambrosia fungi. This genus includes twelve species, the majority associated with ambrosia beetles (Jones & Blackwell 1998; Kubono & Ito 2002). Two species (*R. ambrosiae* and *R. montetyi*) are identified as *P. cylindrus* primary ambrosia fungi (Arx & Hennebert 1965; Sousa et al. 1995; Morelet 1998) and have been isolated both from the insect and its galleries in cork oak (Sousa et al. 1997; Morelet 1998). Although *Raffaelea* sexual state is lacking, observations of conidial development support its placement within the *Ophiostomatales* (Gebhardt & Oberwinkler 2005) and 18S ribosomal DNA sequences analysis resolves *Raffaelea* as a monophyletic lineage which forms a sister group to species of the genus *Ophiostoma* (Jones & Blackwell 1998). The *Ophiostomatales* are economically important sapstaining fungi occurring worldwide on hardwoods. In fact, *R. quercivora* Kubono & Ito, involved in an interaction with *Platypus quercivorus,* was proven to be pathogenic to fagaceous trees in Japan, being associated with mass mortality of adult trees, particularly *Quercus serrata* and *Q. mongolica* (Kubono & Ito 2002; Kinnura & Kobayashi 2006). These results raise interesting questions that must be answered, namely the correct phylogenetic relationship between the several species within this genus and the *Ophiostomatales* and its pathogenicity towards cork oak trees. The pathogenicity of *Nodulisporium* isolates must be ascertained too.

Although the unidentified group presented a high frequency in the insect, the individual proportion of the numerous isolated fungi is very low as they belong to different taxa. Nevertheless, being carried out in mycangia, they might be important and therefore remain under study. Concerning the other genera associated with *P. cylindrus*, some could act as secondary ambrosia agents as they are present in the mycangia and intestine, namely *Penicillium* and *Streptomyces*. Fungi such as *Gliocladium* and *Trichoderma*, known for their antagonistic activity, were isolated from the insect tunnels leading us to suppose that they might contribute to control fungi growth inside the galleries (Henriques 2007). Simultaneously, these genera and *Scytalidium*, also found along the galleries, have a proven degradative wood action (Kiffer & Morelet 1997; Szakacs & Tengerdy 1997), being possibly able to play a pioneering role in host colonization and insect establishment.

References

Arx, J. A. von & Hennebert, G. L. 1965: Deux champignons ambrosia. Mycopathol. Mycologia Applic. 25: 309-315.

Badler, H. 1992: Pathogenecity of *Ceratocystis* spp. in oaks under strees. Proceedings of an International Congress "Recent Advances in Studies on oak decline". Selva di Fasano (Brindisi), Italy: 31-37.

Baker, J. M. 1963: Ambrosia beetle and their fungi, with particular reference to *Platypus cylindrus* Fab. Symposia Soc. Gen. Microbiol. 13: 323-354.

Barnett, H. L. & Hunter, B. B. 1998: Illustrated Genera of Imperfect Fungi. APS Press, Minnesota, USA, 218 pp.

Batra, L. R. 1985: Ambrosia beetle and their associated fungi: Research trends and techniques. Proc. Indian Acad. Sci. 49: 137-148.

Beaver, R. A. 1989: Insect-fungus relationships in the bark and ambrosia beetles. In: Wilding, Collins, Hammond and Webber (eds.): Insect-Fungus Interactions. Academic Press, London: 121-143.

Cassier, P., Lévieux, J., Morelet, M. & Rougon, D. 1996: The mycangia of *Platypus cylindrus* Fab. and *P. oxyurus* Dufour (Coleoptera: Platypodidae). Structure and associated fungi. J. Insect Physiol; 42: 171-179.

Chakali, G., Attal-Bedreddine, A. & Ouzani, H. 2002: Insect pests of the oaks *Quercus suber* and *Q. ilex* in Algeria. IOBC/WPRS Bull. 25(5): 93-100.

Collado J., Platas, G. & Peláez, F. 2001: Identification of an endophytic *Nodulisporium* sp. from *Quercus ilex* in central Spain as the anamorph of *Biscogniauxia mediterranea* by rDNA sequence analysis and effect of different ecological factors on distribution of the fungus. Mycologia 93: 875-886.

Degreef, J. 1992: Isolation of *Ophiostoma querci* (Georgev.) from declining oaks: selection techniques and pathogenicity test. Proc. Internatl. Congr. "Recent Advances in Studies on oak decline". Selva di Fasano (Brindisi), Italy: 471-473.

Delatour, C., Menard, A., Vautrot, A. & Simonin, G. 1992: Pathogenicity assessment of Ophiostomatales: *Ophiostoma querci* on oak compared to *O. novo-ulmi* on elm. Proc. Internatl. Congr. "Recent Advances in Studies on oak decline". Selva di Fasano (Brindisi), Italy: 59-65.

Ellis, M. B. 1971: Dematiaceous Hyphomycetes. CAB, England, 608 pp.

Ellis, M. B. 1976: More Dematiaceous Hyphomycetes. CAB, England, 507 pp.

Ferreira, M. C. & Ferreira, G. W. S. 1989: *Platypus cylindrus* F. (Coleoptera: Platipodidae) Plaga de *Quercus suber*. Bol. Sanid. Veg. Plag. 4: 301-305.

Gebhardt, H. & Oberwinkler, F. 2005: Conidial development in selected ambrosial species of the genus *Raffaelea*. Antonie van Leeuwenhoek 88: 61-66.

Henriques, J. 2007: Fungos associados a *Platypus cylindrus* Fab. e sua relação com o declínio do sobreiro em Portugal. Tese Mestrado, Universidade Évora, 118 p.

Henriques, J.; Inácio, M.L. & Sousa, E. 2006: Ambrosia fungi in the insect-fungi symbiosis in relation to cork oak decline. Revista Iberoam. Micol. 23: 185-188.

Jones, K. G. & Blackwell, M. 1998: Phylogenetic analysis of ambrosial species in the genus *Raffaelea* based on 18S rDNA sequences. Mycol. Res. 102: 661-665.

Kiffer, E. & Morelet, M. 1997: Les Deutéromycètes – classification et clés d´identification générique. INRA Editions, Paris, 306 pp.

Kinuura, H. & Kobayashi, M. 2006: Death of *Quercus crispula* by inoculation with adult *Platipus quercivorus* (Coleoptera: Platypodidae). Appl. Entomol. Zool. 41(1): 123-128.

Kubono, T. & Ito, S. 2002: *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). Mycosci. 43: 255-260.

Lévieux, J. & Cassier, P. 1994: Vection de champignons pathogènes des résineux par les xylophages forestiers. Ann. Biol. 33: 19-37.

Morelet, M. 1998: Une espèce nouvelle de *Raffaelea*, isolée de *Platypus cylindrus*, coléoptère xylomycétophage des chênes. Ann. Soc. Sci. Nat. Archéol. Toulon Var 50: 185-193.

Saccardo, P. A. 1891: *Chromotaxia seu nomenclator colorum polyglottus adclitis speciminibus coloratis ad botanicorum et zoologorum*. Patavii, 22 pp.

Sousa, E.; Debouzie, D. & Pereira, H. 1995: Le rôle de l'insecte *Platypus cylindrus* F. (Coleoptera, Platypodidae) dans le processus de dépérissement des peuplements de chêne-liège au Portugal. IOBC/WPRS Bull. 18(6): 24-37.

Sousa, E. & Inácio, M. L. 2005: New Aspects of *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) Life history on cork oak stands in Portugal. In: Entomological Research in Mediterranean Forest Ecosystems, Lieutier and Ghaioule (eds.). INRA Editions, Paris, 280 pp.

Sousa, E., Inácio, M. L., El. Antry, S., Bakry, M. & Kadiri, Z. A. 2005: Comparaison de la bio-écologie et comportement de l'insecte *Platypus cylindrus* Fab (Coléoptère, Platypodidae) dans les subéraies portugaises et marocaines. IOBC/WPRS Bull. 28(8): 137-144.

Sousa, E., Tomaz, I. L., Moniz, F. A. & Basto, S. 1997: La répartition spatiale des champignons associés à *Platypus cylindrus* Fab. (Coleoptera: Platypodidae). Phytopathol. Medit. 36: 145:153.

Szakacs, G. & Tengerdy, R. P. 1997: Lignocellulolytic enzyme production on pretreated poplar wood by filamentous fungi. W. J. Microbiol. Biotechnol. 13: 487-490.

Tiberi, R., Ragazzi A., Marianelli, L.; Sabbatini, P. & Roversi, P. F. 2002: Insects and fungi involved in oak decline in Italy. IOBC/WPRS Bull. 25(5): 67-74.

SUB-CHAPTER 2.2

FUNGI OF RAFFAELEA GENUS (ASCOMYCOTA: OPHIOSTOMATALES) ASSOCIATED WITH PLATYPUS CYLINDRUS (COLEOPTERA: PLATYPODIDAE) IN PORTUGAL

INACIO ML, HENRIQUES J, LIMA A & SOUSA E (2008)

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FUNGI OF *RAFFAELEA* GENUS (ASCOMYCOTA: OPHIOSTOMATALES) ASSOCIATED TO *PLATYPUS CYLINDRUS* (COLEOPTERA: PLATYPODIDAE) IN PORTUGAL

FUNGOS DO GÉNERO *RAFFAELEA* (ASCOMYCOTA: OPHIOSTOMATALES) ASSOCIADOS A *PLATYPUS CYLINDRUS* (COLEOPTERA: PLATYPODIDAE) EM PORTUGAL

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ABSTRACT

In the study of the fungi associated to *Platypus cylindrus,* several fungi were isolated from the insect and its galleries in cork oak, among which three species of *Raffaelea*. Morphological and cultural characteristics, sensitivity to cycloheximide and genetic variability had been evaluated in a set of isolates of this genus. On this basis *R. ambrosiae* and R. *montetyi* were identified and a third taxon segregated witch differs in morphological and molecular characteristics from the previous ones. In this work we present and discuss the parameters that allow the identification of specimens of the three *taxa*. The role that those ambrosia fungi can have in the cork oak decline is also discussed taking into account that *Ophiostomatales* fungi are pathogens of great importance in trees, namely in species of the genus *Quercus*.

Key-words: Ambrosia beetle, ambrosia fungi, cork oak, decline.

RESUMO

No estudo dos fungos associados ao insecto xilomicetófago *Platypus cylindrus* foram isolados, a partir do insecto e das suas galerias no sobreiro, diversos fungos, entre os quais três espécies de *Raffaelea*. Avaliaram-se características morfológicas e culturais, sensibilidade à ciclohexamida e variabilidade genética num conjunto de isolados do género. Foram identificados *R. ambrosiae* e *R. montetyi* e segregou-se um terceiro táxone que difere em características morfológicas e moleculares dos dois anteriores. No presente trabalho são apresentados e discutidos os parâmetros que permitem identificar espécimes dos três táxones. É ainda discutido o papel que estes fungos ambrósia podem ter no declínio do sobreiro, sabido que fungos Ophiostomatales são patogénios de grande importância em plantas lenhosas, nomeadamente em espécies do género *Quercus*.

Palavras-chave: Declínio, fungo ambrósia, insecto ambrósia, sobreiro.

INTRODUCTION

Many insects use vegetal resources, from herbaceous plants to frondose trees. Some constitute primary pests for their hosts, attacking vigorous plants and over-

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coming its defences, while others do not have such ability, colonizing only weakened plants and carrying allies that break these barriers. Fungi, viruses and nematodes are frequently involved with insects in those relations, weakening the hosts and thus leaving them accessible to the insects. The microorganisms, in turn, find a way to overcome distances between the hosts (Tainter & Baker, 1996).

In the forest, there are several examples of insects that establish symbioses with other organisms causing severe damages in the attacked trees, namely the Dutch elm disease caused by *Ophiostoma ulmi* and *O. novo-ulmi* (Buisman) Nannf, vectored by *Scolytus* spp. bark beetles (Jacobi *et al.*, 2007; Six *et al.*, 2005) or *Ophiostoma* spp. of maritime pine, carried by *Ips sexdentatus* (Lieutier & Levieux, 1985; Levieux *et al.*, 1989).

The insect *Platypus cylindrus* Fab. is known to attack mainly dead or weakened trees. However, since the 1980's, its population outbreaks seemed to be related to the cork oak decline in Portugal and other Mediterranean countries. This beetle establishes symbioses with fungi that are carried in specialized organs – mycangia – as well in the intestine and on the body surface (Sousa *et al.*, 1995; Henriques *et al.,* 2006). Such fungi are so called ambrosia as they act as a nourish source for the insect descendants after being inoculated and cultivated in the galleries. The observation of those galleries confirms the existence of a light-coloured, thin wall cover, constituted by mycelium of the symbiotic fungi (Inácio *et al*., 2005; Sousa & Inácio, 2005).

The taxonomy of ambrosia fungi is somewhat confused and the general papers on this issue were published a long time ago. Those works placed ambrosia fungi within four mitosporic genera, *Ambrosiella, Monacrosporium, Phialosphoropsis* and *Raffaelea* but is clear that many more genera are involved including *Acremonium*, *Candida*, *Fusarium* and *Graphium* (Batra, 1963; Baker 1963).

In addition to fungi directly related to insect nourishment, others have been found, such as pathogenic fungi that may play an essential role in insect selection and tree colonization. Those fungi could play both roles, thus contributing to the establishment of insect populations. Among those are *Botryodiplodia*, *Ceratocystis*, *Graphium*, *Leptographium* and *Ophiostoma* (Badler, 1992). Cladistic studies have shown that ambrosia fungi such as species of *Ambrosiella* are closely related to Ascomycetes species of either *Ophiostoma* or *Ceratocystis* (Cassar & Blackwell, 1996) and species of *Raffaelea* are related to *Ophiostoma* genus (Henriques, 2007), based on rDNA sequences and confirmed by patterns of cycloheximide sensitivity. According to Harrington *et al.* (2008), *Raffaelea* fungi do not form a sexual state, and thus the rules of nomenclature do not allow describing them as species of *Ophiostoma*. Nevertheless species of *Raffaelea* could be described as a genus of ambrosia beetle symbionts within the genus *Ophiostoma*. Also, the results of the sequence analysis of 18S-rDNA, if *R. hennebertii* Scott & du Toit is excluded, revealed that *Raffaelea* resolves a monophyletic lineage which forms a group very close to species of *Ophiostoma* (Jones & Blackwell, 1998).

Studies of oak decline in Europe showed that the complex *Ophiostoma/ Ceratocystis* is pathogenic to *Quercus* trees (Badler, 1992; Degreef, 1992; Delatour *et al.*, 1992). In addition, *R. quercivora* Kubono & Ito was proven to be pathogenic to fagaceous trees in Japan, being associated with mass mortality of adult trees, particularly *Q. serrata* and *Q. mongolica* (Kubono & Ito, 2002).

The aim of the present study was to determine the correct identity of *Raffaelea*-like isolates occurring in association with *P. cylindrus* on cork oak and to discuss its pathogenicity on host trees. To accomplish this goal, fungi isolated both from insects and their galleries were morphologically characterised and subjected to DNA analyses of their small subunit region of rDNA (SSU-rDNA). An additional test of cycloheximide sensitivity was also performed.

MATERIAL AND METHODS

Twelve infested logs of cork oak trees that exhibit decline symptoms from the regions Chamusca (Ribatejo province), Montemor and Grândola (Alentejo province) were collected and the associated insects captured in fabric traps, attached to the log with a silicone joint. Those samplings were repeated during 2005, 2006 and 2007.

A total of 100 insects per location were aseptically dissected to obtain their mycangia, intestine and parts of the exoskeleton (elytra). The logs were cut in order to identify the different gallery sections: cork, inner-bark, pre-parental section, larval section and gallery end. One complete gallery was observed from each log (fragments of wood with 1 cm^2) and six samples (fragments of wood with 1 cm^2) of each section were collected. All the pieces were surface sterilised with a sodium hypochlorite solution (1%) for 1 min and rinsed with sterilized distilled water. They were plated into 9 cm diameter Petri dishes with malt extract agar (Difco MEA, USA) added with streptomycin (Sigma-Aldrich, USA) (500 mg/l) and MEA added with cycloheximide (Sigma-Aldrich, USA) (500 mg/l). The former is a large spectrum antibiotic and the latter has both antibacterial and antifungal action and could be used to distinguish fungi of the *Ophiostoma* genus (Harrington, 1981; Hawksworth *et al*., 1981). Cultures were incubated at 25±1ºC in darkness. Pure cultures of each fungus were obtained and the isolates were grouped according to their macroscopic characteristics. In the present work only representative isolates of *Raffaelea*-like cultures were chosen to continue the studies.

Morphological characterisation

Fungal identification was based on cultural and morphological features according to Ellis (1971, 1976), Lanier *et al.* (1978), Kiffer & Morelet (1997) and Barnett & Hunter (1998). Conidia biometry of the different isolates was assessed on cultures grown on potato-dextrose agar (Difco PDA, USA) after five to ten days, in the darkness at 25±1 ºC. Structures were mounted in sterilized distilled water, and 40 measurements at x600 magnification were made for each isolate. The 95% confidence levels were calculated and the extremes of spore measurements were given. Images were taken from slides mounted in sterilized distilled water. Macroscopic characters of colonies were described after 21 days of growth; colour names are from Saccardo (1891).

Cycloheximide sensitivity

The effect of different concentrations of cycloheximide (0, 5, 10, 100, 500 and 1000 ppm) was tested on isolates of each *Raffaelea* group. The appropriate amount of cycloheximide was added to autoclavated MEA. Media were dispensed into 9 cm diam Petri dishes (20 ml/plate). The center of each plate was inoculated with a 5 mm diam mycelial plug from the advancing margin of a MEA-grown culture and incubated at 27,5±1ºC in darkness for five days (Harrington, 1981; M. Wingfield, pers. com). One isolate of *Ophiostoma ulmi* (GU81158) from the UIPP Forestry Fungi Collection was used at the same time as a positive control.

Molecular analysis

The molecular analysis of the isolates was based on the amplification and sequencing of the 18S rDNA region, according to Cassar & Blackwell (1996), Jones & Blackwell (1998) and Rollins *et al.* (2001). DNA was extracted using the PuregeneDNA® kit. PCR amplification and sequencing of the SSU-rDNA was performed as described by Henriques (2007). Homologous sequences were obtained from GenBank (NCBI) using Basic Local Alignment Search Tool (BLAST). Sequences were aligned using BioEdit v. 7.0.5.3. The phylogenetic analysis was performed with MEGA v.4.0 (Tamura *et al.*, 2007). In the phylogenetic tree, downloaded sequences are indicated by their GenBank accession numbers. A member of Ophiostomatales was used as an outgroup (*Sporothrix schenckii* Hekt. & Perkins).

RESULTS AND DISCUSSION

One of the most frequent fungi isolated from *P. cylindrus* and its galleries belong to *Raffaelea* genus, obtained in particular from the intestinal content and from the mycangia (approximately 40% and 30% of all the isolated fungi, in both organs, in females and males, respectively) (Henriques, 2007). Four apparently different groups were obtained according to their macroscopic characteristics and representative isolates from each group were chosen to pursue the studies: PC05.005, PC05.006, PC05.007and PC06.001, integrated into the Forestry Fungi Collection of the Instituto Nacional de Recursos Biológicos (INRB). Other isolates from the same work collection were also used in molecular assays.

Morphological characterisation

The cultural characterisation of the colony's surface and reverse is shown in Table 1 and their observed aspects are in Figure 1.

Table 1 – Macroscopic description of the Raffaelea cultures on PDA

Raffaelea Isolate	Upper surface				Colony reverse	Observations
	Cultural aspect	Density	Colour	Zonation		
PC05.005	effuse, yeast-like, some with aerial floccose mycelium in the colony center	media	cream-colored, few with a light olive-green central zone	light-concentric	idem surface	
PC05.006	effuse, yeast-like; some isolates with aerial mycelium in the colony center	light to media	fuliginous or light olive-green	light-concentric or absent	idem surface	growth variability
PC05.007	effuse, yeast-like, some isolates with aerial mycelium in the colony center	media	dark olive-green to black with a white central zone	absent	idem surface	
PC06.001	effuse, yeast-like to farinaceous with long, sparse, vigorous aerial mycelium	light	pale brown	absent	idem surface	spreading rapidly

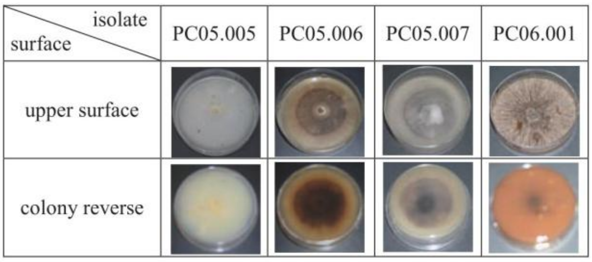


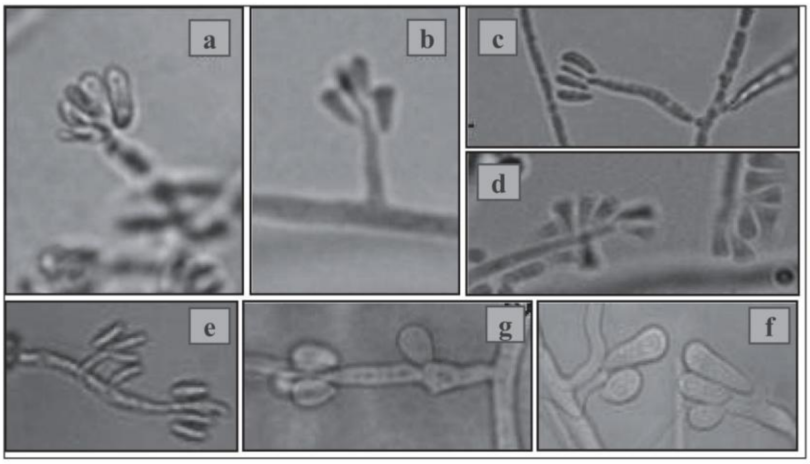
Figure 1 – Typical cultural aspect (surface, reverse) of *Raffaelea* isolates.

Isolate PC05.005. Hyphae hyaline and septate that bound together forming compact hyphae ropes. Conidiophores macronematous and mononematous, erect, septate, slender with a tapered apex, producing simpodulosporic conidiae that leave cicatricial scars in the conidiogenous cells. Conidiae unicellular, hyaline, with variable forms (triangular, oval and allantoid) and measuring 5,0-(5,8)-8,4 x 1,7-(2,1)-3,3 µm.

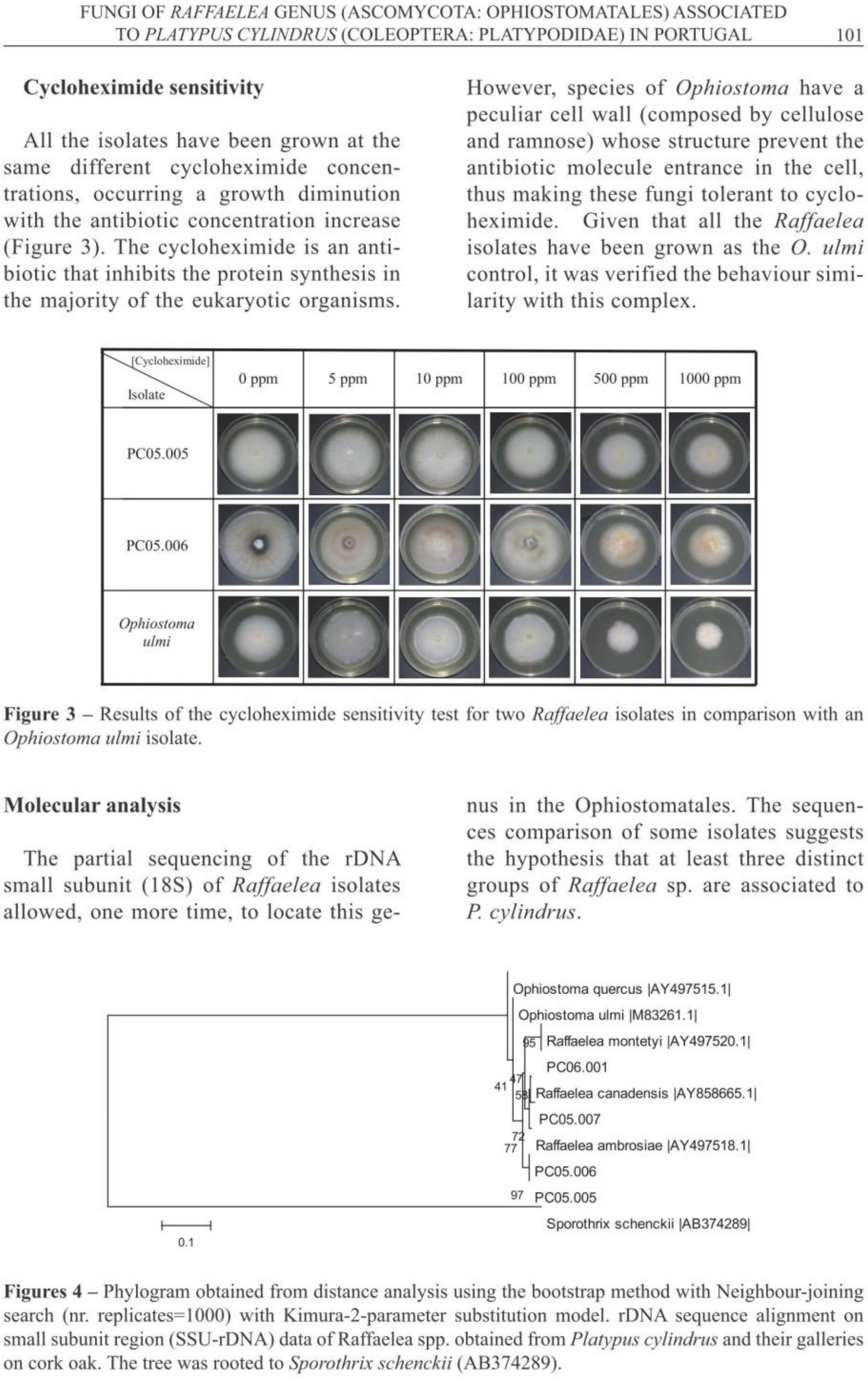
Isolate PC05.006. Hyphae hyaline and septate that bound together forming compact hyphae ropes. Conidiophores macronematous and mononematous, erect, septate, slender with a tapered apex, producing simpodulosporic conidiae that leave cicatricial scars in the conidiogenous cells but not so pronounced as in isolate I5.05. Conidiae unicellular and hyaline, with variable forms (triangular, oval and fusiform) and measuring 3,3-(3,7)-5,0 x 1,7-(1,8)-3,3 µm.

Isolate PC05.007. Hyphae hyaline and septate repeatedly branched and interlocked, hyphal ends sometimes developing into torulose swellings. Conidiophores unbranched, distinct from hyphae bearing them, hyaline, solitary or clustered together to form sporodochia. Conidiae blastosporic, unicellular and hyaline, usually solitary but sometimes upon germination *in situ* appear to be in monilioid chains, smooth-walled with variable forms (triangular, oval or fusiform), measuring 5-(9)-20x2,5-(3,7)-7,5 µm.

Isolate PC06.001. Hyphae hyaline and septate, long, ascendant, erect and vigorous, simple or feebly ramified. Conidiophores macronematous and mononematous, erect, septate, slender with a tapered apex, producing simpodulosporic. Conidiae adherent in a mucilaginous droplet, leaving a discreet cicatricial scars in the conidiogenous cells. Conidiae unicellular and hyaline, smooth-walled, rounded apex and truncated base, with variable forms (pyriform, claviform and cuneiform) and measuring 5,0-(7,8)-10,0 x 2,5-(3,7)-5,8 µm.



Figures 2 – Conidiophores and conidia of fungi of *Raffaelea* genus, a-b) isolate PC05.005: a) conidia allantoid (x600), b) conidia triangular (x600); c-e) isolate PC05.006: c) conidia fusiform to allantoid (x600), d) conidia triangular (x600); e) conidia fusiform (x600); f) isolate PC05.007: conidia pyriforme to globose (x1000); i) isolate PC06.001: conidia pyriform truncated (x1000).



Cycloheximide sensitivity

All the isolates have been grown at the same different cycloheximide concentrations, occurring a growth diminution with the antibiotic concentration increase (Figure 3). The cycloheximide is an antibiotic that inhibits the protein synthesis in the majority of the eukaryotic organisms. However, species of *Ophiostoma* have a peculiar cell wall (composed by cellulose and ramnose) whose structure prevent the antibiotic molecule entrance in the cell, thus making these fungi tolerant to cycloheximide. Given that all the *Raffaelea* isolates have been grown as the *O. ulmi* control, it was verified the behaviour similarity with this complex.

[Cycloheximide] Isolate	0 ppm	5 ppm	10 ppm	100 ppm	500 ppm	1000 ppm
PC05.005						
PC05.006						
Ophiostoma ulmi						

Figure 3 – Results of the cycloheximide sensitivity test for two *Raffaelea* isolates in comparison with an *Ophiostoma ulmi* isolate.

Molecular analysis

The partial sequencing of the rDNA small subunit (18S) of *Raffaelea* isolates allowed, one more time, to locate this genus in the Ophiostomatales. The sequences comparison of some isolates suggests the hypothesis that at least three distinct groups of *Raffaelea* sp. are associated to *P. cylindrus*.

Figures 4 – Phylogram obtained from distance analysis using the bootstrap method with Neighbour-joining search (nr. replicates=1000) with Kimura-2-parameter substitution model. rDNA sequence alignment on small subunit region (SSU-rDNA) data of Raffaelea spp. obtained from *Platypus cylindrus* and their galleries on cork oak. The tree was rooted to *Sporothrix schenckii* (AB374289).

The phylogram analysis also suggests that isolate PC06.001 is close to *R. montetyi* and PC05.007 is related to *R. canadensis*. Isolates PC05.005 and PC05.006 appear to be very similar originating a separate group also close to *Ophiostoma* spp.. Nevertheless, conjugating molecular and morphological analysis is possible to suggest that PC05.005 is next to *R. ambrosiae*.

Twelve species were described within *Raffaelea*, the majority being associated with ambrosia insects (Kubono & Ito, 2002; Bisby *et al*., 2006). Concerning *P. cylindrus, R. ambrosia* and *R. monteyi* had been already identified as the main ambrosia fungi (Arx & Hennebert, 1965; Morelet, 1998). In bibliographical terms it is also necessary to consider *Sporothrix* sp. described for Baker (1963) and later classified as *R. ambrosiae* by Arx & Hennebert (1965). In the same way, isolates of *Cephalosporium* sp. obtained by Baker (1963) and one brownish fungus not identified by Cassier *et al*. (1996) but later classified as *R. montetyi* by Morelet (1998) must be considered.

Raffaelea is a mitosporic genus poorly studied perhaps for its cryptic nature: although cosmopolite (Kiffer & Morelet, 1998), living in symbiosis with insects they are not commonly observed. Even if their sexual phase is still unknown, observations of the conidial development of *Raffaelea* spp. are concordant with the position of this genus within the Ophiostomatales group (Gebhardt & Oberwinkler, 2005).

Studies on oak decline in Europe show that fungi of the complex *Ophiostoma/ Ceratocystis* are frequent pathogens of species of *Quercus* (Badler, 1992; Degreef, 1992; Delatour et al., 1992). Santos *et al.* (1999) registered the occurrence of *Ophiostoma* sp. in *Q. suber* in Portugal. The effect of *R.* ambrosiae and *R. montetyi* in *Q. suber* is still unknown; however, in Japan the pathogenicity of *R. quercivora* was proven (Kubono & Ito). This primary ambrosia fungus of *P. quercivorus* Murayama was associated with a mass mortality of fagaceous, especially *Q. serrata* Thunb., *Q. mongolica* Fich. and *Q. crispula* Blume (Kubono & Ito, 2002; Kinuura & Kobayashi, 2006). A recently identified *Raffaelea* species associated with the ambrosia beetle *Xyleborus glabratus* Eichhoff was related with a new devastating disease of *Lauraceae* plants in South Carolina (USA) (Fraedrich *et al.*, 2007).

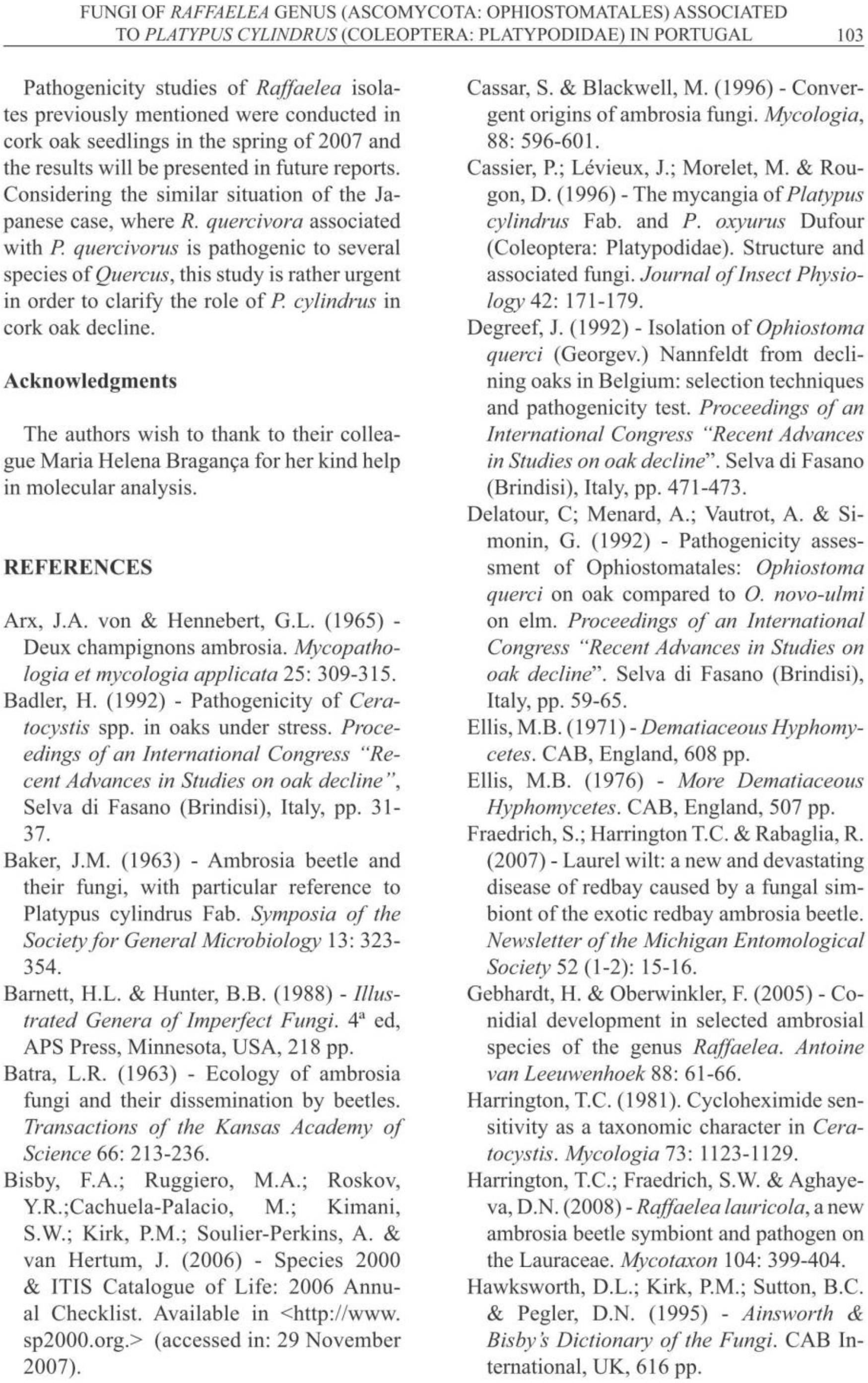
CONCLUSION

In the last decade, the insect *P. cylindrus* has been considered one of the most important biotic agents directly involved in cork oak decline. Being an ambrosia beetle, it establishes symbioses with fungi that it carries and inoculates in the host tree to favour its settlement.

The relative importance of the isolated fungi is quite variable: besides those involved in insect nourishment, some could be potentially pathogenic to cork oak, while others could have an antagonistic action or be simply saprobes that are involved in a commensalist relation with the host tree.

In this work, *Raffaelea* spp. were the fungi most frequently isolated, especially from the mycangia and the intestinal content both of female and male insects, thus leading to the conclusion that this genus includes the primary ambrosia fungi associated with *P. cylindrus*. All the conducted essays pointed out that *Raffaelea* spp. are closely related to Ophiostomatales. Although the relations inside each group are not satisfactorily clear their morphological characterisation and rDNA sequence comparison corroborate the hypothesis that at least three distinct species of *Raffaelea* sp. are associated with *P. cylindrus*: *R. ambrosiae*, *R. montetyi* and probably *R. canadensis*. This last one was never clearly associated to that interaction.

To fully clarify the taxonomic status of *Raffaelea* species associated with *P. cylindrus*, either in Portugal or in the other Mediterranean countries, additional sequence data need to be generated. Also, many more isolates must be brought into this study.



Pathogenicity studies of *Raffaelea* isolates previously mentioned were conducted in cork oak seedlings in the spring of 2007 and the results will be presented in future reports. Considering the similar situation of the Japanese case, where *R. quercivora* associated with *P. quercivorus* is pathogenic to several species of *Quercus*, this study is rather urgent in order to clarify the role of *P. cylindrus* in cork oak decline.

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REFERENCES

Arx, J.A. von & Hennebert, G.L. (1965) - Deux champignons ambrosia. *Mycopathologia et mycologia applicata* 25: 309-315.

Badler, H. (1992) - Pathogenicity of *Ceratocystis* spp. in oaks under stress. *Proceedings of an International Congress "Recent Advances in Studies on oak decline"*, Selva di Fasano (Brindisi), Italy, pp. 31-37.

Baker, J.M. (1963) - Ambrosia beetle and their fungi, with particular reference to Platypus cylindrus Fab. *Symposia of the Society for General Microbiology* 13: 323-354.

Barnett, H.L. & Hunter, B.B. (1988) - *Illustrated Genera of Imperfect Fungi*. 4ª ed, APS Press, Minnesota, USA, 218 pp.

Batra, L.R. (1963) - Ecology of ambrosia fungi and their dissemination by beetles. *Transactions of the Kansas Academy of Science* 66: 213-236.

Bisby, F.A.; Ruggiero, M.A.; Roskov, Y.R.;Cachuela-Palacio, M.; Kimani, S.W.; Kirk, P.M.; Soulier-Perkins, A. & van Hertum, J. (2006) - Species 2000 & ITIS Catalogue of Life: 2006 Annual Checklist. Available in <http://www.sp2000.org.> (accessed in: 29 November 2007).

Cassar, S. & Blackwell, M. (1996) - Convergent origins of ambrosia fungi. *Mycologia*, 88: 596-601.

Cassier, P.; Lévieux, J.; Morelet, M. & Rougon, D. (1996) - The mycangia of *Platypus cylindrus* Fab. and *P. oxyurus* Dufour (Coleoptera: Platypodidae). Structure and associated fungi. *Journal of Insect Physiology* 42: 171-179.

Degreef, J. (1992) - Isolation of *Ophiostoma querci* (Georgev.) Nannfeldt from declining oaks in Belgium: selection techniques and pathogenicity test. *Proceedings of an International Congress "Recent Advances in Studies on oak decline"*. Selva di Fasano (Brindisi), Italy, pp. 471-473.

Delatour, C; Menard, A.; Vautrot, A. & Simonin, G. (1992) - Pathogenicity assessment of Ophiostomatales: *Ophiostoma querci* on oak compared to *O. novo-ulmi* on elm. *Proceedings of an International Congress "Recent Advances in Studies on oak decline"*. Selva di Fasano (Brindisi), Italy, pp. 59-65.

Ellis, M.B. (1971) - *Dematiaceous Hyphomycetes*. CAB, England, 608 pp.

Ellis, M.B. (1976) - *More Dematiaceous Hyphomycetes*. CAB, England, 507 pp.

Fraedrich, S.; Harrington T.C. & Rabaglia, R. (2007) - Laurel wilt: a new and devastating disease of redbay caused by a fungal simbiont of the exotic redbay ambrosia beetle. *Newsletter of the Michigan Entomological Society* 52 (1-2): 15-16.

Gebhardt, H. & Oberwinkler, F. (2005) - Conidial development in selected ambrosial species of the genus *Raffaelea*. *Antoine van Leeuwenhoek* 88: 61-66.

Harrington, T.C. (1981). Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* 73: 1123-1129.

Harrington, T.C.; Fraedrich, S.W. & Aghayeva, D.N. (2008) - *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the Lauraceae. *Mycotaxon* 104: 399-404.

Hawksworth, D.L.; Kirk, P.M.; Sutton, B.C. & Pegler, D.N. (1995) - *Ainsworth & Bisby's Dictionary of the Fungi*. CAB International, UK, 616 pp.

Henriques, J.; Inácio, M.L. & Sousa, E. (2006) - Ambrosia fungi in the insect-fungi symbiosis in relation to cork oak decline. *Revista Iberoamericana Micologia* 23: 185-188.

Henriques, J. (2007). *Fungos associados a Platypus cylindrus Fab. (Coleoptera: Platypodidae e sua relação com o declínio do sobreiro em Portugal*. Dissertação de mestrado, Universidade de Évora, Évora, 118 pp..

Inácio, M.L.; Henriques, L. & Sousa, E. (2005) - As relações mutualistas entre fungos e insectos: sua influência no estado sanitário da floresta em Portugal. *Actas das comunicações do 5º Congresso Florestal Nacional*, Instituto Politécnico de Viseu, Viseu. (digital format)

Jacobi, W.R.; Koshi, R.D.; Harrington, T.C. & Witcosky, J. (2007) - Association of *Ophiostoma novo-ulmi* with *Scolytus shevyrewi* (Scolytidae) in Colorado. *Plant Disease* 91: 245-247.

Jones, K.G. & Blackwell, M. (1998) - Phylogenetic analysis of ambrosial species in the genus *Raffaelea* based on 18S rDNA sequences. *Mycologycal Research* 102: 661-665.

Kiffer, E. & Morelet, M. (1997) - *Les Deutèromycetes – classification et clés d´identification générique.* INRA Editions, Paris, 306 pp.

Kinuura, H. & Kobayashi, M. (2006) - Death of *Quercus crispula* by inoculation with adult *Platypus quercivorus* (Coleoptera: Platypodidae). *Applied Entomology and Zoology* 41, 1: 123-128.

Kubono, T. & Ito, S. (2002) - *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* 43: 255-260.

Lanier, L.; Joly, P.; Bondoux, P. & Bellemère; A. (1978) - *Mycologie et pathologie forestières. Tome I - Mycologie forestière.* Masson, Paris. 487 pp.

Lieutier, F. & Levieux, J. (1985) - Les relations conifères-scolytides: Importance et perspectives de recherches. *Annales des sciences forestières* 42: 359-370.

Levieux, J. ; Lieutier, F. ; Moser, J.C. & Perry, T.J. (1989) - Transportation of phytopathogenic fungi by the bark beetle *Ips sexdentatus* Boerner and associated mites. *Journal of applied Entomology* 108: 1-11.

Morelet, M. (1998) - Une espèce nouvelle de *Raffaelea*, isolée de *Platypus cylindrus*, coléoptère xylomycétophage des chênes. *Extrait des Annales de la Société des Sciences Naturelles et d'Archeologie de Toulon et du Var* 50: 185-193.

Rollins, F.; Jones, K.; Krokene, P.; Solheim, H. & Blackwell, M. (2001) - Phylogeny of asexual fungi associated with bark and ambrosia beetles. *Mycologia* 93: 992-996.

Saccardo, P.A. (1891) - *Chromotaxia seu nomenclator colorum polyglottus adclitis speciminibus coloratis ad botanicorum et zoologorum*. Patavii, 22 pp.

Santos, M.N.; Machado, M.H.; Bragança, M.H.; Ramos, H.; Sousa E. & Tomaz, I. (1999) - Mycoflora associated with cork oak (*Quercus suber* L.) in Portugal. IOBC/*wprs Bulletin* 22 (3): 25-28.

Six, D.L; Beer, Z.W.; Beaver, R.A., Visser, L. & Wingfield, M. (2005) - Exotic invasive elm bark beetle, *Scolytus kirschii*, detected in South Africa. *South African Journal of Science* 101: 229-232.

Sousa , E.; Debouzie, D. & Pereira, H. (1995) - Le rôle de l'insecte *Platypus cylindrus* F. (Coleoptera, Platypodidae) dans le processus de dépérissement des peuplements de chêne-liège au Portugal. *IOBC/ wprs Bulletin* 18: 24-37.

Sousa, E. & Inácio, M.L. (2005) - *New Aspects of Platypus cylindrus Fab. (Coleoptera: Platypodidae) Life History on Cork Oak Stands in Portugal.* In: F. Lieutier & D. Ghaioule (Eds.) Entomological Research in Mediterranean Forest Ecosystems. INRA Editions, Paris, 280 pp.

Tainter, F.H. & Baker, F.A. (1996) - *Principles of Forest Pathology*. John Wiley & Sons, Inc., New York, 805 pp.

Tamura K.; Dudley J.; Nei M. & Kumar S. (2007) - MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24:1596-1599.

SUB-CHAPTER 2.3

CONTRIBUTION OF SYMBIOTIC FUNGI TO CORK OAK COLONIZATION BY *PLATYPUS CYLINDRUS* (COLEOPTERA: PLATYPODIDAE)

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Contribution of symbiotic fungi to cork oak colonization by *Platypus cylindrus* (Coleoptera: Platypodidae)

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Platypus cylindrus Fab. (Coleoptera: Platypodidae) has changed its status from uncommon to pest contributing to cork oak decline. Besides its massive attacks, *P. cylindrus* is associated with fungi on which it depends for survival and host colonization. Isolations from beetles yielded seven genera with a potential role on insects' establishment: *Acremonium, Biscogniauxia, Botryosphaeria, Gliocladium, Raffaelea, Scytalidium* and *Trichoderma. Raffaelea* spp. were the most frequent fungi mainly in insect's mycangia and gut confirming their role as primary symbionts and possibly capable of weaken the host. Similarly *Biscogniauxia* and *Botryosphaeria* genera may act to overwhelm tree defenses. The genera *Scytalidium, Gliocladium* and *Trichoderma* are known to have a degradative wood ability and play a pioneering role in host colonization. These results demonstrate the close association between *P. cylindrus* and its ambrosia fungi. These are mainly from the *Raffaelea* genus and also the auxiliary ambrosia fungi, whose presence is part of the insect's strategy for host colonization.

Key words: ambrosia beetle, ambrosia fungi, *Quercus suber*, *Raffaelea* spp., Ophiostomatales.

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Introduction

Among the most successful wood-inhabiting insects are the Scolytidae and Platypodidae which cause damage of economic significance to timber and trees (Cassier *et al.* 1996). *Platypus cylindrus* Fab., the oak pinhole borer, is the most common Platypodid beetle in southern Europe. It mainly attacks oaks (Balackowsky *et al.*, 1963) but it is also described on chestnut, beech, ash, elm and wild cherry trees (Espagnol, 1964; Graham, 1967).

P. cylindrus attack is usually limited to dead or weakened trees (Seabra 1939; Baeta-Neves 1950; Español 1964). Sporadic attacks were also described on apparently healthy trees (Balachowsky 1949) and in Morocco this beetle is considered an important pest of cork oak (*Quercus suber* L.) (Villement & Fraval 1993; Sousa *et al.* 2005). In Portugal, since the 1980's, severe infestations were observed in apparently healthy cork oaks (Sousa 1992; Sousa & Debouzie 2002) causing widespread tree death within three months to one year and a half after the attack, depending on the host vigour and resistance (Sousa & Inacio 2005).

In the host colonization process, primary attraction of the Platypodidae was associated with certain tree volatiles like ethanol and terpenes (Shore & McLean 1983). Analysis of the temporal evolution of *P. cylindrus* attacks reveals a preference for the biggest hosts (height and perimeter) mostly for those recently decorked (Sousa & Débouzie 1999). However, the insect preference for a host probably results from a combination of stimuli such as wood moisture, osmotic pressure, sap flow and tree leaf composition, among others (Chararas 1979; Sousa *et al.* 1995; Yamasaki & Futai 2008). In addition, a kairomone to *P. cylindrus* has been described (Algarvio *et al.* 2002; Teixeira *et al.* 2003).

The establishment of insects on a host is the last step of the attack process. The secondary attraction begins by the appeal of insects of the same sex followed by the attraction of the other sex insects (Ytsma 1986; Atkinson 2004). The high density of *P. cylindrus* attacks on the same tree confirms the existence of these secondary attraction mechanisms initiated by the appeal of the other males (aggregation pheromone) (Algarvio *et al.* 2002), similarly to other Platypodidae (Renwik *et al.* 1977; Milligan *et al.* 1988; Tokoro *et al.* 2007; Kim *et al.* 2009). Each male is joined to a single female whose attraction is probably mediated by sexual pheromones (Allegro & Della Beffa 2001). Mated couples tunnel into the heartwood and introduce ectosymbiotic fungi into their galleries on which they and their offspring feed. These insects are so called ambrosia beetles since the larvae and adults feed mainly on the fungal mycelium lining the sinuous tunnels (Batra, 1963; Beaver, 1989). For the transport and maintenance of fungal inoculum, ambrosia beetles developed specialized organs – mycangia - which provide suitable conditions for fungi storage during flight and spreading of the insects (Francke-Grossman 1963; Cassier *et al.* 1996). The mycobiota associated with the insects allows them to be nutritionally independent of the host (Külnholz *et al.* 2003). Early studies carried out on symbiosis with *P. cylindrus* described several fungi and yeasts that are important in insect nourishment. The main symbiotic fungus is the mitosporic Ophiostomataceae *Raffaelea ambrosiae* v. Arx & Hennerbert, (Baker 1963; Uchastnova 1985; Sousa *et al.* 1995). Other fungi were also identified in this association but their exact roles have yet to be fully clarified (Sousa & Inacio 2005; Henriques *et al.* 2006). Thus, the aim of this study is to determine what fungi are carried consistently by *P. cylindrus* in Portuguese cork oak stands. Furthermore, their frequency and location in the insect's body was determined in order to understand the role of the main vectored fungi on the success of tree host colonization.

Material and Methods

Collection

Twelve logs from cork oak severely infested by *P. cylindrus* and exhibiting decline symptoms were collected from Alentejo and Ribatejo province, two main cork producing regions of Portugal. The logs were settled in the INRB, I.P. laboratories at Oeiras and the emerged adults captured in fine mesh nets, attached to the log with a silicone joint. The samplings were repeated during 2005, 2006 and 2007.

Beetles were observed under a binocular microscope to confirm their identity. Excised mycangia from 200 insects, half males and females were mounted on microscope slides in clear lactophenol. Preparations were observed under a Olympus BX41TF microscope and the mycangial pits were counted. For scanning electron microscopy, 10 specimens of *P. cylindrus* (5 males, 5 females) previously ultrasound cleaned were sputter coated with gold palladium (98:2) (Henriques 2007) and examined using a JOEL 35 scanning electron microscope.

Fungal isolation and identification

A total of 100 insects per year were aseptically dissected with iris scissors to obtain their mycangia, intestine and parts of the exoskeleton (elytra). All the pieces were surface sterilized with a sodium hypochlorite solution (1%) for 1 min and rinsed with sterilized distilled water. They were plated into 9 cm diameter Petri dishes with malt extract agar (MEA, Difco, USA) added with 500 mg/l of streptomycin (Sigma-Aldrich, USA), a large spectrum antibiotic, and MEA added with 500 mg/l of cycloheximide (Sigma-Aldrich, USA), inhibitory to most fungi except those belonging to the genus *Ophiostoma* (Harrington 1981; Hawksworth *et al.* 1995). Some yeasts, however, and species of filamentous fungi, including *Penicillium*, also may grow on these media (Harrington 1992). Cultures were incubated at 25±1ºC in darkness. Pure cultures of each fungus were obtained and grouped according to their macroscopic characteristics. Fungal identification at the genus level was based on cultural and morphological features in accordance to Batra (1967), Ellis (1971, 1976), Kiffer & Morelet (1997) and Barnett & Hunter (1998). Fungi were scored as either present or absent on a Petri dish, regardless on the number of colonies of each fungi on the plate.

Statistical analysis

Results were analyzed through analysis of variance (ANOVA) after the angular transformation in to arcsin√x of the fungi frequencies expressed in percentage. Significant means were compared through a LSD test. In all cases $p < 0.001$. The analyses were made using the software Statistica 6.0 (Statsoft).

Results

Specialized organs for transporting fungi

Observations of adults confirmed the presence of mycangia, ovoid in shape, located in both sexes on the flat middle upper part of the prothorax. This cuticular plate was perforated by numerous pits and the male has a less developed mycangium with 15±11 integumentary pits (min = 0; max = 53) separated by the straight cuticular line. In *P. cylindrus* females, 370±26 cavities were observed (min = 326; max = 406) (Figure 1A-D).

In both sexes, perforations were apparently filled with the same type of fungal structures. On a specimen, a growing mycelium expanding on the cuticular surface and protruding from the perforations was observed (Figure 1E).

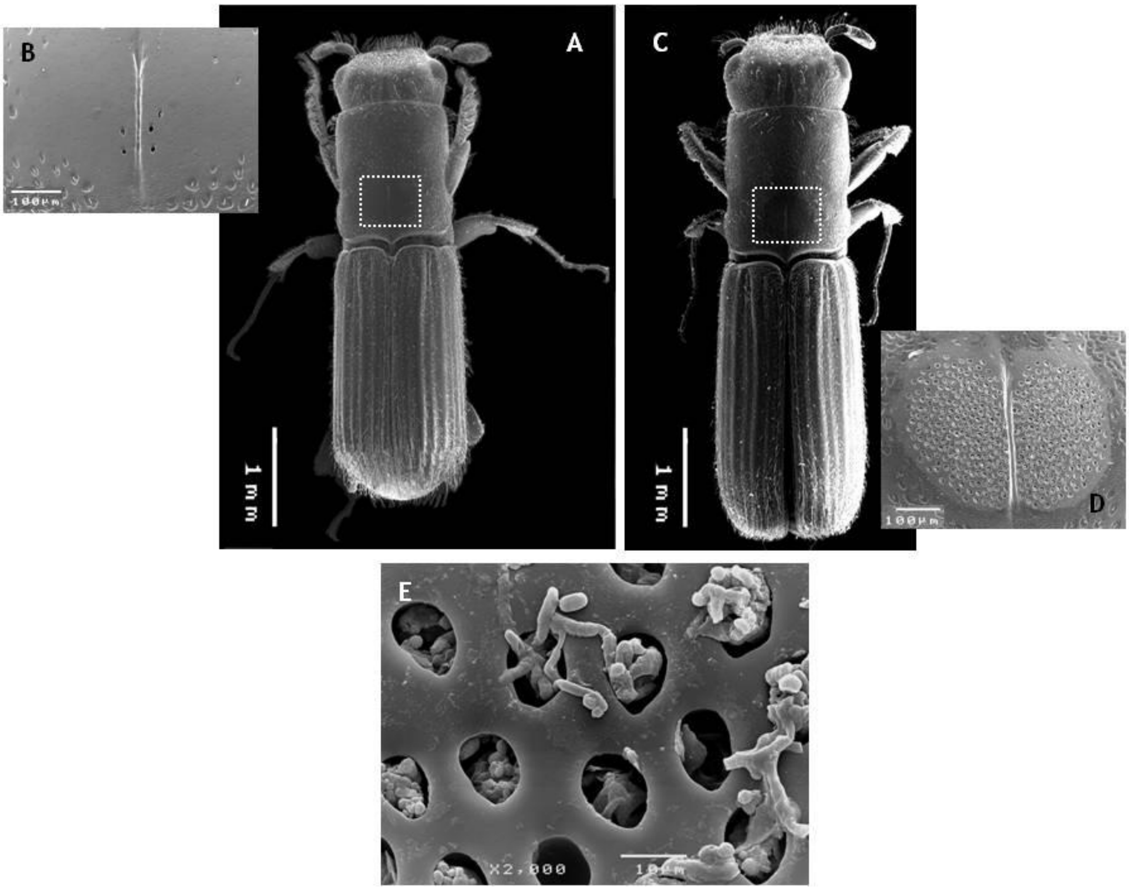


Figure 1. Scanning electron micrographs of *Platypus cylindrus* adults. A. Male. B. Male mycangia. C. Female. D. Female mycangia. E. Growing mycelium and spores on female mycangia cavities.

Fungal isolation and identification

Out of the 300 insects observed (142 males and 158 females), 258 yielded at least one fungal isolate in any insect's body location. From this 86% that contained fungi, 116 were male and 142 were female. Fungi belonging to seven genera were obtained: *Acremonium, Biscogniauxia, Botryosphaeria, Gliocladium, Raffaelea, Scytalidium and Trichoderma.* More than one species was isolated from the genera *Gliocladium, Raffaelea* and *Trichoderma.* Phoretic, intestinal and mycangial fungi obtained from individual *P. cylindrus* are summarized in Table 1.

Table 1. Fungal isolates from the intestinal content (Ic), mycangia (My) and exoskeleton (Ex) of *Platypus cylindrus* males (M) and females (F)

Year	sex	N	Part	Isolate						
				Acrem	*Biscogn*	*Botryos*	*Gliocl*	*Rafael*	*Scytal*	*Trichod*
2005	M	62	Ic	0	0	0	0	2	0	0
			My	1	0	0	0	3	1	0
			Ex	2	8	12	13	53	17	16
	F	38	Ic	0	1	0	1	4	0	0
			My	0	0	0	1	6	0	0
			Ex	4	1	8	4	27	8	10
2006	M	40	Ic	0	0	1	0	2	0	0
			My	0	1	11	0	3	0	0
			Ex	2	1	12	3	13	6	3
	F	60	Ic	0	0	3	0	7	0	1
			My	0	2	6	0	25	2	2
			Ex	0	4	13	4	17	4	3
2007	M	40	Ic	0	0	5	0	6	0	0
			My	0	0	8	0	17	1	0
			Ex	0	1	10	0	15	0	2
	F	60	Ic	0	0	7	0	14	0	0
			My	1	0	6	1	37	1	0
			Ex	0	2	10	0	19	0	1
Total		300		10	21	112	27	270	40	38

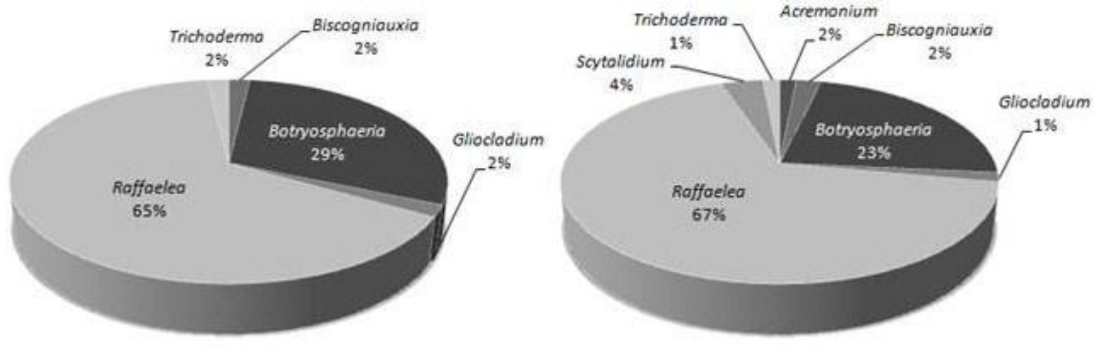
Note: *Acrem, Acremonium* sp.; *Biscogn, Biscogniauxia* sp.; *Botryos – Botryosphaeria* sp.; *Gliocl, Gliocladium* spp.; *Rafael., Raffaelea* spp.; *Scytal, Scytalidium* sp.; *Trichod, Trichoderma* spp..

The mycobiota obtained from individual *P. cylindrus* did not significantly differ in the three years of fungal isolation ($F_{2,123} = 0.2255$; $p = 0.7985$).

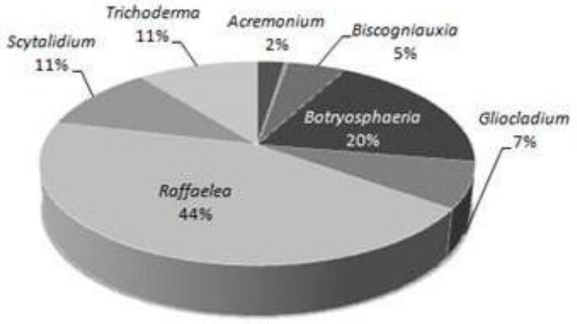
Although females may transport large amounts of fungal propagules, in terms of frequency of vectored fungi there were no statistically significant differences between males and females ($F_{1,124} = 0.0708$; $p = 0.7906$). Therefore, results for both sexes were pooled.

The ophiostomatoid *Raffaelea* genus was the most frequently isolated, in particular from the mycangia and the intestinal content. Different putative species of *Raffaelea* were obtained but their identification requires a multigene phylogeny and will be the subject of a future work.

The second most frequent genera was *Botryosphaeria* which together with *Raffaelea* species scored more than 90% of presence in the gut and in mycangia of the insects (Fig. 2).



Intestinal content **Mycangia**



Exoskeleton

Figure 2. Genera of fungi (%) isolated from the intestine, mycangia and exoskeleton of *Platypus cylindrus.*

The proportions of fungi found in mycangia and in the intestine versus phoretic on the exoskeleton were significantly different ($F_{2,123} = 16.5784$; $p < 0.001$). Besides *Botryosphaeria* sp. several non-ophiostomatoid fungi were isolated mainly from exoskeleton surfaces. In the mycangia these fungi were the rarest and the insects' gut showed lower diversity of fungi. Species of *Biscogniauxia*, *Gliocladium* and *Trichoderma* genera were found in all the insect organs. *Scytalidium* sp. and *Acremonium* sp. were not found in the intestinal content.

Several saprobes fungi of the genera *Alternaria*, *Aspergillus*, *Geotrichum*, *Paecylomyces* and *Penicillium* were frequently isolated as well as species of *Streptomyces* and Mucorales but they fall outside the scope of this paper.

Discussion

In this study *Platypus cylindrus* was found to transport several fungi, out of which the most frequent was the ophiostomatoid species of *Raffaelea*. *Raffaelea ambrosiae* was considered the main ambrosia fungi and several authors reported this species as the principal symbiont of the oak pinhole borer (Baker 1963; Arx & Hennerbert 1965; Sousa *et al.* 1995). However, and according to more recent work, *P. cylindrus* is associated with other *Raffaelea* species namely *R. montetyi,* an ambrosia fungus that decays wood (Morelet 1998, Inacio *et al.* 2008), and *R. canadensis* (Inacio *et al.* 2008). The Ophiostomatales are economically important sapstaining fungi that occur worldwide on hardwoods. Moreover, some species of *Raffaelea* that are closely associated with ambrosia insects cause serious outbreaks in healthy trees (Kubono & Ito, 2002; Murata *et al.* 2005; Fraedrich *et al.* 2008; Kim *et al.* 2009; Harrington *et al.* 2010).

P. cylindrus, although a wood borer, is not a wood feeder. Our results clearly show that adults feed on fungi, mainly on *Raffaelea* species thus confirming them as the primary ambrosia fungi. *Botryosphaeria* sp. is also very frequent in all the isolations, even from the intestine. This genus comprises the widespread and virulent species, *B. corticola* (Alves *et al.* 2004; Luque *et al.* 2008; Linaldeddu *et al.* 2009). The ingested thick-walled spores may pass through the gut unchanged and germinate on the walls of the galleries, but the hyphae are digested by the beetles and their larvae, hence providing a richer source of protein than wood (Beaver 1989).

Aside from these two most frequent fungi, others were isolated either from the exoskeleton or from the mycangia. Although these fungi may be significant components of the insect fungal flora, they were usually considered to be weed fungi with no more than a commensal relationship with the insects (Harrington 2005). Nevertheless, *Biscogniauxia* sp. and specifically *B. mediterranea* (Henriques 2007), the causal agent of cork oak charcoal canker (Collado *et al.* 2001; Linaldeddu *et al.* 2010), was consistently present in all the insect organs, even if in small fractions. Given both its epizoic and endozoic dispersal by the insect, it could be hypothesized that *P. cylindrus* contributes to the spreading of its spores in cork oak stands. Likewise, *Acremonium* sp. and in particular *A. crotocinigenum* has shown to be pathogenic towards cork oak seedlings (Inacio *et al.* 2010a).

The association of *P. cylindrus* with cosmopolitan fungi is well documented by others (Baker 1963; Cassier *et al.* 1996; Sousa *et al.* 1997). In the present study, they were consistently isolated from the exoskeleton and mycangia, even after an accurate and thorough disinfection by fractional sterilization (Francke-Grosmann 1956) to avoid saprobes growth (data not shown). It is possible that they might play a role in the insect-fungi interaction and thus in the establishment of the insect. It has been emphasized that these secondary symbionts may act as wood degrading agents to facilitate galleries excavation. *Gliocladium* sp., *Trichoderma* sp. and *Scytalidium* sp., as producers of lignocellulolytic enzymes might have this pioneer role (Kiffer & Morelet 1997; Szakacs & Tengerdy 1997; Maddau *et al.* 2009; Inacio *et al.* 2010b). Moreover, species of *Trichoderma* and *Gliocladium* are known for their antagonistic activity and may possibly control fungal growth inside the galleries (Henriques 2007).

The increase of *P. cylindrus* attacks in Portuguese cork oak stands suggests that behaviour changes may have happened and new strategies of host colonization may have arisen. The identification of chemical attractants such as kairomones and aggregation pheromones and possibly sexual pheromones mediating *P. cylindrus* attraction to host explains the massive attacks of the insect (Algarvio *et al.* 2002; Teixeira *et al.* 2003; Henriques *et al.* 2010). Our studies confirmed the phoretic transportation of fungi on the exoskeleton surfaces and the intimate association with fungi housed in the insect's mycangia. The final role of the ambrosia fungi, which is the base of this insect-fungi interaction, is the nourishment of the larvae and adults. In fact, a more restrict range of fungi was found associated with *P. cylindrus* feeding habits. Future work will be carried out in order to identify the several fungal species isolated within each genus.

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References

Algarvio R, Teixeira C, Barata E, Pickett J, Casas Novas P, Figueiredo D, 2002. Identification of a putative aggregation pheromone from males *Platypus cylindrus* (Coleoptera: Platypodidae). *In Proceedings of the 19th Annual Meeting International Society of Chemical Ecology*, Univ. Hamburg, Germany: 151.

Allegro G, Della Beffa G, 2001. Un nuovo problema entomologico per la pioppicoltura italiana: *Platypus mutatus* (Chapuis) (Coleoptera, Platypodiade). Sherwood – *Foresti ed Alberi Oggi* **66**: 31-34.

Alves A, Luque J, Phillips A, 2004. *Botryosphaeria corticola* sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph, *Diplodia mutila*. *Mycologia* **96**: 598-613.

Arx JA von H, 1965. Deux champignons ambrosia. *Mycopathol. Mycol. Appl.* **25**: 309-315.

Atkinson TH, 2004. Ambrosia Beetles. *Platypus* spp. (Insecta: Coleoptera: Platypodidae) Univ. Florida, Gainsville, FL. Available on line at http://edis.ifas.ufl.edu/IN331 accessed 22.10.2010.

Baeta-Neves C, 1950. *Introdução à Entomologia Florestal Portuguesa. A Serra e o Homem*. Lisboa.

Baker JM, 1963. Ambrosia beetle and their fungi, with particular reference to *Platypus cylindrus* Fab. *Symp. Soc. General Microbiol*. **13**: 323-354.

Balachowsky AS, 1949. *Faune de France : coléoptères scolytides*. Lechevalier, Paris.

Balachowsky AS, Chevalier M, Cuillé J, Grison P, Hoffmann A, Jourdheuil P, Labeyrie V, Remaudière G, Steffan JR, Touzeau J, Vilardebo A, 1963. Famille des Platypodidae. *In* Balachowsky AS (Ed.), *Entomologie appliquée à l'agriculture". Tome II. Coleoptères.* Paris, pp. 1289-1291.

Barnett HL, Hunter BB, 1998. *Illustrated Genera of Imperfect Fungi*. APS Press, Minnesota, USA.

Batra LR, 1963. Ecology of ambrosia fungi and their dissemination by beetles. *Trans. Kansas Acad. Sci*. **66**: 213–236.

Batra LR, 1967. Ambrosia fungi: A taxonomic revision and nutritional studies of some species. *Mycologia* **59**: 976–1017.

Beaver RA, 1989. Insect-fungus relationships in the bark and ambrosia beetles. *In* Wilding N, Collins, NM, Hammond, PM, Webber, JF (Eds.), *Insect-Fungus Interactions*. Academic Press, London, pp. 121-143.

Cassier P, Lévieux J, Morelet M, Rougon D, 1996. The Mycangia of *Platypus cylindrus* Fab. and *P. oxyurus* Dufour (Coleoptera: Platypodidae). Structure and associated fungi. *J. Insect Physiol.* **42**: 171-179.

Chararas C, 1979. *Écophysiologie des insectes parasites des forêts.* Chararas C. (Ed.), Paris.

Collado J, Platas G, Peláez F, 2001. Identification of an endophytic *Nodulisporium* sp. from *Quercus ilex* in central Spain as the anamorph of *Biscogniauxia mediterranea* by rDNA sequence analysis and effect of different ecological factors on distribution of the fungus. *Mycologia* **93**: 875-886.

Ellis MB, 1971. Dematiaceous *Hyphomycetes*. CAB, England.

Ellis MB, 1976. *More Dematiaceous Hyphomycetes*. CAB, England.

Español F, 1964. Los Platipodidos de Cataluña (Col. Phytophagoidea). *Bol. Ser. Plagas For.* **7**: 115-117.

Fraedrich SW, Harrington TC, Rabaglia RJ, Ulyshen MD, Mayfield AE, Hanula JL, Eickwort JM, Miller DR, 2008. A fungal symbiont of the redbay ambrosia beetle causes a lethal wilt in redbay and other *Lauraceae* in the southeastern United States. *Plant Dis.* **92**: 215–224.

Francke-Grosmann H, 1956. Hautdrüsen als Träger der Pilzsymbiose bei Ambrosiakäfern. *Z. Morphol. Öekol. Tiere* **45**: 275-308.

Francke-Grosmann H, 1963. Some New Aspects in Forest Entomology. *Ann. Rev. Entomol.* **8**: 415-438.

Graham K, 1967. Fungal-insect mutualism in trees and timber. *Ann. Rev. Entomol.* **12**: 105-126.

Harrington TC, 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* **73**: 1123-1129.

Harrington TC, 1992. *Leptographium. In* Singleton LL, Mihail, JD, Rush, CM (Eds.), *Methods for Research on Soilborne Phytopathogenic Fungi*. APS Press, St. Paul, Minnesota, pp. 129–133.

Harrington TC, 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. *In* Vega FE, Blackwell, M. (Eds.), *Insect-Fungal Associations: Ecology and Evolution*. Oxford University Press, Inc. New York, pp. 257–292.

Harrington TC, Aghayeva DA, Fraedrich SW, 2010. New combinations in *Raffaelea, Ambrosiella* and *Hyalorhinocladiella*, and four new species from the red-bay ambrosia beetle, *Xyleborus glabratus*. *Mycotaxon* **111**: 337–361.

Hawksworth DL, Kirk PM, Sutton BC, Pegler DN, 1995. *Ainsworth & Bisby's Dictionary of the Fungi*. CAB International, UK.

Henriques J, Inacio ML, Sousa E, 2006. Ambrosia fungi in the insect-fungi symbiosis in relation to cork oak decline. *Rev. Iberoam. Micol*. **23**:185-188.

Henriques J, Inacio ML, Pires S, Sousa E, 2010. *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) control strategies. *IOBC/wprs Bulletin* **57**: 103-106.

Henriques J, 2007. *Fungos associados a Platypus cylindrus Fab. (Coleoptera: Platypodidae e sua relação com o declínio do sobreiro em Portugal*. Dissertação de mestrado, Universidade de Évora, Évora, Portugal.

Inacio ML, Henriques J, Lima A, Sousa E, 2008. Fungi of *Raffaelea* genus (Ascomycota: Ophiostomatales) associated to *Platypus cylindrus* (Coleoptera: Platypodidae) in Portugal. *Rev. de Ciências Agrárias* **31**: 96-104.

Inacio ML, Henriques J, Guimarães L, Azinheira H, Lima A, Sousa E, 2010a. The ambrosia fungus *Acremonium crotocinigenum* causes canker on cork oak. I*n Actas do VI Congresso da Sociedade Portuguesa de Fitopatologia.* Univ. Évora, Évora, Portugal: 191.

Inacio ML, Henriques J, Sousa E, 2010b. Mycobiota associated with *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) on cork oak in Portugal. *IOBC/wprs Bulletin* **57**: 87-95.

Kiffer E, Morelet M, 1997. *Les Deutèromycetes – classification et clés d´identification génériques.* INRA Editions, Paris.

Kim KH, Choi YJ, Seo ST, Shin HD, 2009. *Raffaelea quercus-mongolicae* sp. nov. associated with *Platypus koryoensis* on oak in Korea. *Mycotaxon* **110**: 189–197.

Kubono T, Ito S, 2002. *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* **43**: 255-260.

Külnholz S, Borden JH, Uzonovic A, 2003. Secondary ambrosia beetles in apparently healthy trees: Adaptations, potential causes and suggested research. *Integrated Pest Management Reviews* **6**: 209-219.

Linaldeddu BT, Sirca C, Spano D, Franceschini A, 2009. Physiological responses of cork oak and holm oak to infection by fungal pathogens involved in oak decline. *Forest Pathology* **39**: 232–238.

Linaldeddu BT, Sirca C, Spano D, Franceschini A, 2010. Variation of endophytic cork oak-associated fungal communities in relation to plant health and water stress. *Forest Pathology,* 2010, *on line early,* doi: 10.1111/j.1439-0329.2010.00652.x.

Luque J, Pera J, Parladé J, 2008. Evaluation of fungicides for the control of *Botryosphaeria corticola* on cork oak in Catalania (NE Spain). Forest *Pathology*, **38**: 147-155.

Maddau L, Cabras A, Franceschini A, Linaldeddu BT, Crobu S, Roggio T, Pagnozzi D, 2009. Occurrence and characterization of peptaibols from *Trichoderma citrinoviride*, an endophytic fungus of cork oak, using electrospray ionization quadrupole time-of-flight mass spectrometry. *Microbiology* **155**: 3371–3381.

Milligan RH, Osborne GO, Ytsma G, 1988. Evidence for an aggregation pheromone in *Platypus gracilis* (Coleoptera: Platypodidae). *J. Appl. Entomol.* **106**: 20-24.

Morelet M, 1998. Une espèce nouvelle de *Raffaelea*, isolée de *Platypus cylindrus*, coléoptère xylomycétophage des chênes. *Extrait des Annales de la Société des Sciences Naturelles et d'Archeologie de Toulon et du Var* **50**: 185-193.

Murata M, Yamada T, Ito S, 2005. Changes in water status in seedlings of six species in the Fagaceae after inoculation with *Raffaelea quercivora* Kubono et Shin-Ito. *J. For. Res.* **10**: 251–255.

Renwick JA, Vité JP, Billings RF, 1977. Aggregation pheromones in the ambrosia beetle *Platypus flavicornis*. *Naturwissenschaften* **64**: 226.

Seabra AF, 1939. Contribuição para a história de Entomologia em Portugal. *Publicações da D.G.S.F.A.* **6**: 1-20.

Shore TL, Mclean JA, 1983. Attraction of *Platypus wilsoni* Swaine (Coleoptera: Platypodidae) to traps baited with sulcatol, ethanol and a-pinene. *Can. For. Serv. Bi-Mon. res. Notes* **3**: 24-25.

Sousa E, 1992. Alguns dos factores responsáveis pelo declínio do montado de sobro na Herdade da Chaminé. *In Actas do 2º Encontro sobre os Montados de Sobro e Azinho*, Évora: 324-335.

Sousa E, Debouzie D, 1999. Spatio-temporal distribution *of Platypus cylindrus* F. attacks in cork oak stands in Portugal. *IOBC/ wprs Bull.* **22**: 47-58.

Sousa E, Debouzie D, 2002. Contribution à la bioecologie *de Platypus cylindrus* F. au Portugal. *IOBC/ wprs Bull.* **25** : 75-83.

Sousa E, Debouzie D, Pereira H, 1995. Le rôle de l'insecte *Platypus cylindrus* F. (Coleoptera, Platypodidae) dans le processus de dépérissement des peuplements de chêne-liège au Portugal. *IOBC/ wprs Bull*. **18**: 24-37.

Sousa E, Inacio ML, 2005. New Aspects of *Platypus cylindrus* Fab. (Coleoptera: Platypodidae): Life History on Cork Oak Stands in Portugal. *In* Lieutier F, Ghaioule, D. (Eds.), *Entomological Research in Mediterranean Forest Ecosystems,* INRA Editions, pp. 147-168.

Sousa E, Inacio ML, El Antry S, Bakry M, Kadiri ZA, 2005. Comparaison de la bio-écologie de l'insecte *Platypus cylindrus* Fab. (Col., Platypodidae) dans les subéraies portugaises et marocaines. *IOBC/ wprs Bull* **28**: 137-144.

Sousa E, Tomaz IL, Moniz FA, Basto S, 1997. La répartition spatiale des champignons associés à *Platypus cylindrus* Fab. (Coleoptera: Platypodidae*). Phytopath. Medit.* **36**:145-153.

Szakacs G, Tengerdy RP, 1997. Lignocellulotytic enzyme production on pretreated poplar wood by filamentous fungi. *World Journal of Microbiology & Biotechnology* **13**: 487-490.

Teixeira C, Algarvio RM, Casas-Novas P, Barata EN, 2003. Actividade biológica de análogos de três componentes da putativa feromona de agregação de *Platypus cylindrus* (Coleoptera: Platypodidae). *In Actas V Congresso Nacional de Etologia*. Universidade do Algarve, Faro: 29.

Tokoro M, Kobayashi M, Saito S, Kinuura H, Nakashima T, Shoda-Kagaya E, Kashiwagi T, Tebayashi S, Kim C.S, Mori K, 2007. Novel aggregation pheromone, (1*S*,4*R*)-*p*-menth-2-en-1-ol, of the ambrosia beetle, *Platypus quercivorus* (Coleoptera: Platypodidae). *Bull. For. For. Prod. Res. Inst.* **6**: 49–57.

Uchastnova LN, 1985. The Complex of fungi associated with the galleries of *Platypus cylindriformis* (Coleoptera, Platypodidae) and *Xyleborus monographus* (Coleoptera, Scolytidae). *Biol. Nauki.* **2**: 47-50.

Villemant C, Fraval A, 1993. La faune entomologique du chêne-liège en forêt de la Mamora (Maroc). *Ecol. Mediterr*. **19**: 89-98.

Yamasaki M, Futai K, 2008. Host selection by *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae) before and after flying to trees. *Appl. Entomol. Zool*. **43**, 249–257.

Ytsma G, 1986. Inducing attack by male *Platypus* (Col.: Platypodidae) on wood billets in the laboratory. *J. Appl. Entomol.* **102**: 210-212

CHAPTER 3

FUNGI ASSOCIATED WITH *PLATYPUS CYLINDRUS* RELATED TO CORK OAK DECLINE

SUB-CHAPTER 3.1

PLATYPUS CYLINDRUS TRANSPORTS *BISCOGNIAUXIA MEDITERRANEA*, CAUSAL AGENT OF CORK OAK CHARCOAL DISEASE

INACIO ML, HENRIQUES J , GUIMARÃES L, AZINHEIRA H., LIMA A & SOUSA E (2011)

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Platypus cylindrus Fab. (Coleoptera: Platypodidae) transports *Biscogniauxia mediterranea*, agent of cork oak charcoal canker

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Abstract

Severe outbreaks of the ambrosia beetle *Platypus cylindrus* have been reported in Portuguese cork oaks stands for nearly three decades, mainly in trees recently decorked. Simultaneously, the incidence of charcoal canker caused by *Biscogniauxia mediterranea* became alarming. The great abundance of inoculum on colonized parts of host trees and the likelihood that spores might be vectored and inoculated by insects constitutes the basis of the hypothesized association between the oak pinhole borer and *B. mediterranea*. This assay undoubtedly proves the specific transport of the fungal propagules by *P. cylindrus*. *B. mediterranea* has a proven pathogenicity against *Quercus suber* seedlings and produces phytotoxic compounds causing necroses on indicator plants.

Key words: ambrosia beetle, charcoal disease, *Quercus suber*, mycangia, symbiosis.

INTRODUCTION

Platypus cylindrus Fab., the oak pinhole borer, used to be regarded as a secondary pest of cork oak stands in Portugal and Mediterranean basin countries (SEABRA, 1939; BAETA-NEVES, 1950). However, since the 1980s its numbers increased and attacks have been noticed in apparently healthy trees, especially in those recently decorked (SOUSA 1992; SOUSA & DÉBOUZIE, 2002). The time between onset of the first symptoms to death can be as short as 3 months to one year and a half, depending on the host vigour and resistance (SOUSA & INÁCIO, 2005).

P. cylindrus bore galleries and lay eggs in the nutrient-poor sapwood of selected hosts and introduce into their tunnels ambrosia fungi which grow on the tunnel walls and serve as the main source of food for the adults and their offspring. The principal ambrosia fungi are *Raffaelea* spp. and the inoculation of some *Raffaelea* species caused wilting and/or dead of cork oak seedlings and thus would be pathogenic fungi (INÁCIO *et al.*, 2008; INÁCIO *et al.,* 2011). Nevertheless, the recent modification of insects' behavior leads us to suppose that new associations may have arisen. Taking into consideration that charcoal canker caused by *Biscogniauxia mediterranea* (de Not.) Kuntze is increasing in Portuguese cork oak stands, namely in young trees which exhibit a sudden decline process (SOUSA *et al.,* 2007), *P. cylindrus* could be hypothesized as a disease vector spreading the inoculum in oak stands. Thus, the goal of this work was to ascertain the transportation of *B. mediterranea* by *P. cylindrus* and to verify its pathogenicity towards cork oak and the effect of phytotoxic compounds on disease development.

MATERIAL AND METHODS

Twelve logs of cork oak severely infested by *P. cylindrus* and exhibiting decline symptoms were collected in Alentejo and Ribatejo province, two main cork producing regions of Portugal. The logs were settled in the INRB, I.P. laboratories at Oeiras and the emerged adults were captured in fine mesh nets, attached to the log with a silicone joint. Each log was cut to follow one complete gallery. The samplings were repeated during 2005, 2006 and 2007. One hundred insects per year were aseptically dissected with iris scissors under a stereo binocular microscope to obtain their mycangia and parts of the exoskeleton (elytra). For isolating the fungi from tunnel walls, sections of the gallery system where the larvae graze on the fungus were taken. All the pieces were surface sterilized and plated in malt extract agar (MEA, Difco, USA) with 500mg/l of streptomycin sulfate (Sigma-Aldrich, USA), a large spectrum antibiotic. Cultures were incubated at 25±1ºC in darkness.

Fungal identification was based on cultural and morphological features. Genomic DNA was extracted from pure cultures and the ITS rDNA region was amplified according to the protocol of COLLADO *et al.* (2001) using the primer pairs ITS4/ITS5. The sequences of both strands were determinate using the PCR-primers. For a preliminary taxonomical placement, each sequence was submitted to BLAST against the NCBI nucleotide databases.

Inoculation experiments were conducted in May 2008 on 1 year-old seedlings and repeated in October 2008. 16 seedlings were wound inoculated with a mycelial plug and the same number for the wound control plants, in both seasons. Symptoms (wilting or death) were assessed daily for 4 months after inoculation. The length of stem lesions was measured after removal of the outer bark. Fungal isolations of the discolored sapwood of each newly dead or wilted seedling were carried out.

Toxicity of fungi filtrate solutions was evaluated by a modification of the Klement's injection infiltration method (KIRÁLY *et al.,* 1970), on tobacco plants (*Nicotina tabacum* var. Samsun) and consisted of injecting 100µl of fungi culture filtrates into the intercellular space of leaves with a syringe. The control leaves were infiltrated with water and with culture medium (MEA) and kept at 60% RH. The tests were repeated twice with two replicates each. Symptoms were assessed on tobacco leaves two weeks after infiltration using a scale based on the presence of lesions from chlorotic to necrotic (0- symptomless; 1- chlorotic spots; 2- almost necrotic spots; 3- necrotic spots). Results were analyzed through the non-parametric Kruskal-Wallis test or analysis of variance (ANOVA), with values presented as means ±SD. Statistical analyses were carried out using the software Statistica 6 (StatSoft, Inc. 2003).

RESULTS

The characteristics of the fungi obtained from the mycangia and the exoskeleton of individual *P. cylindrus* (Fig.1) and its galleries place them near *Nodulisporium*-like genus (Fig. 2). *Nodulisporium* sp. is referred to include the anamorphs of *Biscogniauxia* sp. (HSIEH & ROGERS, 2005). COLLADO *et al.* (2001) and GIAMBRA *et al.* (2009) identified *Nodulisporium* sp. as the asexual form of *B. mediterranea.*

The comparisons with the published sequences of the GenBank nucleotide sequences databases confirmed the relation of the isolates under study with *B. mediterranea*: the highest similarity (maximum identity – 100%) was found with *B. mediterranea* isolated from *Quercus* *ilex* in Spain (AF280625.1 and AF280624.1) and *Q. cerris*, in Italy (AJ246222.1). No significant length variation was observed in ITS amplicons, whose average length was 560 nucleotides. The sequence data that were successfully sequenced were submitted to the GenBank nucleotide sequences databases as accessions FR734186 and FR734187.

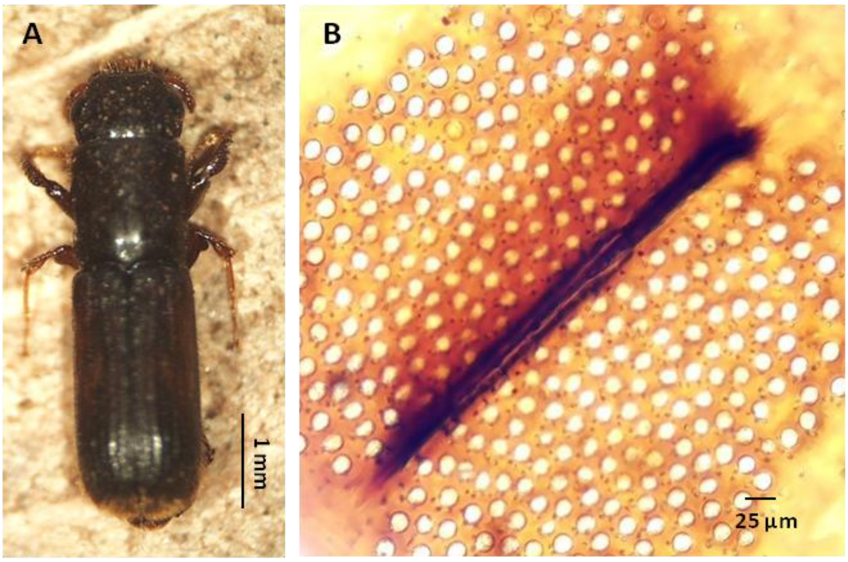

Fig. 1 - *Platypus cylindrus*: A. adult female, B. female mycangia with pits filled with fungal spores.

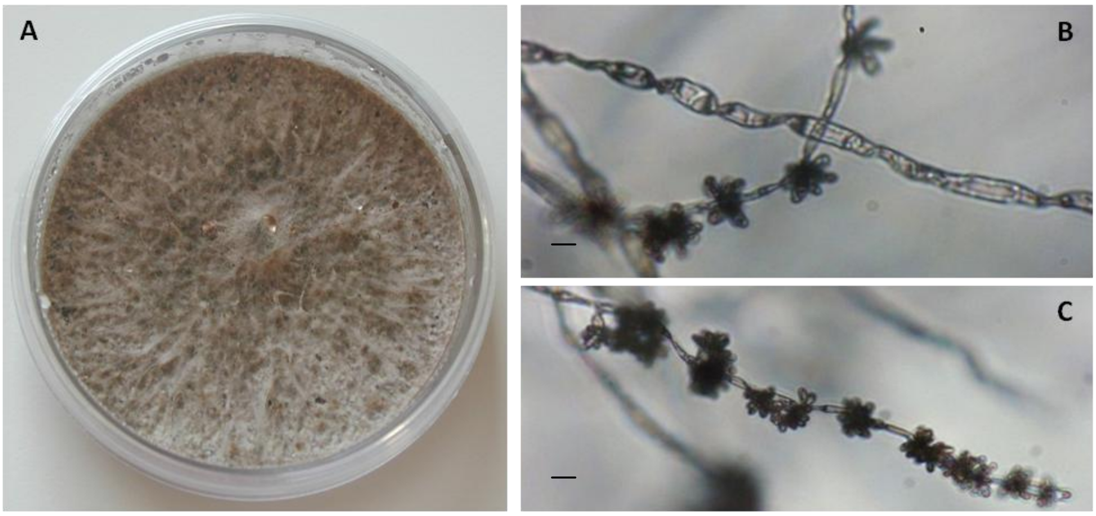

Fig. 2 - *Biscogniauxia mediterranea* anamorph: A. colony morphology after 10 days on malt extract agar in a 90 mm diameter plate, B-C. light micrographs; B. verticillate conidiogenous cells, C. conidia and conidiophore (bar = 10µm).

Control seedlings remained asymptomatic during the trial. No statistically significant differences between season inoculation and symptoms appearance were found ($F_{1,28}$=0,0039; p=0,9506), and therefore the results for both seasons were pooled. All but two seedlings inoculated with the pathogen showed bark and xylem lesion at the end of the experiment (average length lesion=1,8±0,3 cm) (Fig. 3). These results agree with the assays of LINALDEDDU *et al.* (2009). The fungus was re-isolated from the stems of all symptomatic plants.

In the tobacco leaf puncture assay, the compounds obtained from *B. mediterranea* proved to have phytotoxic activity causing symptoms on the injected leaves and no lesions were seen both with water and media (Kruskal Wallis test: H=33,4328, d.f=2, p<0,001). One half of the plants presented "chlorotic" to "almost necrotic" lesions and the other half exhibited "necrotic" lesions in all the punctures.

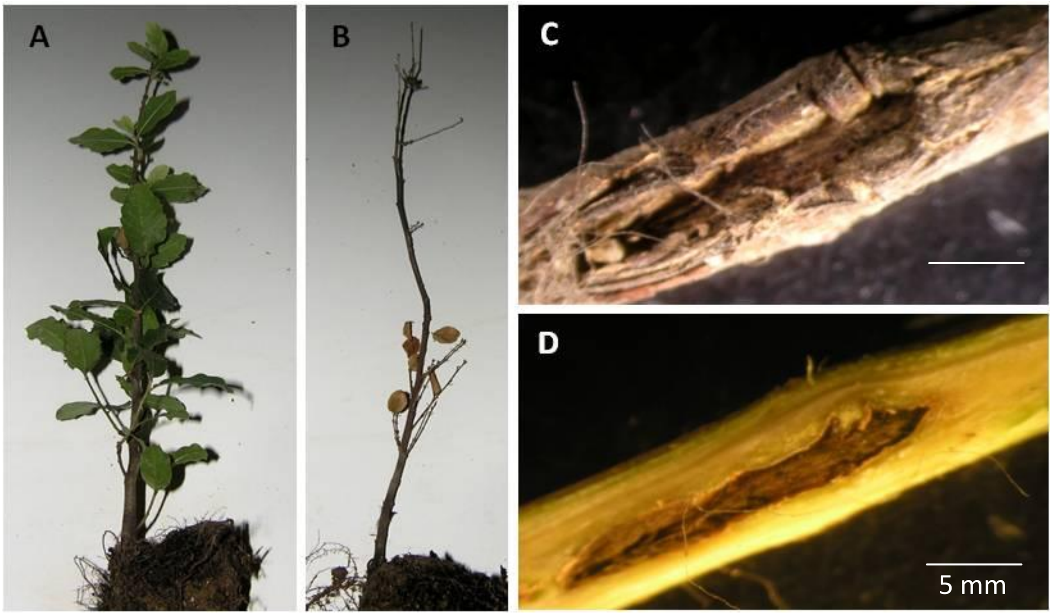

Fig. 3 - Pathogenicity tests on 1-year-old cork oak seedlings: A. control seedling, B-D. symptoms four months after inoculation with *Biscogniauxia mediterranea;* B. inoculated seedling, C. canker in the stem, D. bark and xylem necrosis.

DISCUSSION

Results obtained in the present study confirm the specific transport of *B. mediterranea* by the ambrosia beetle *P. cylindrus* as besides its phoretic association the fungus was found closely associated with insect's mycangia. Furthermore, it was possible to isolate the symbiotic fungus from the beetle galleries. Morphological and molecular features refer the obtained *Nodulisporium*-like isolates to *B. mediterranea* which proved to be pathogenic towards cork oak seedlings. Fungal extracts exhibited phytotoxic activity in tobacco indicator plants as obtained by EVIDENTE *et al.* (2005) in *Q. suber* cuttings.

Charcoal canker is one of the most frequent disorders of Portuguese cork oak stands, *B. mediterranea* being considered an endophyte in oak tissues (SANTOS, 2003). However, its presence has increased over the last years especially in young cork oak trees without other symptoms of decline. The entomochoric nature of its spores and the close association with *P. cylindrus* lead us to conclude that the insect outbreaks might contribute to disease spread, as stated by others (VANNINI & VALENTINI 1994). Being already inside the host, the fungus can rapidly spread from several infection points (MAZZAGLIA *et al.* 2001). So, the direct inoculation of the pathogen into new hosts may increase disease incidence in cork oak stands, even when the attacks of the beetles yielding the fungus are aborted.

Platypus cylindrus **Fab. (Coleoptera: Platypodidae) transporta** ***Biscogniauxia mediterranea*****, el agente del chancro carbonoso del alcornoque**

Resumen

Se han reportado en Portugal ataques severos del insecto ambrosia *Platypus cylindrus* desde hace casi tres décadas, principalmente en los árboles recién descortezados. Al mismo tiempo, la incidencia de la enfermedad causada por *Biscogniauxia mediterranea* se ha tornado alarmante. La abundancia del inoculo en las partes colonizadas de las plantas huéspedes y la posibilidad de que las esporas puedan ser vectorizadas e inoculadas por insectos son la base de la hipótesis de asociación entre el insecto y *B. mediterranea*. Este estudio demuestra sin duda el transporte específico de propagules del hongo por *P. cylindrus*. *B. mediterranea* ha demostrado su patogenicidad en plántulas de *Quercus suber* y produce compuestos fitotóxicos que causan necrosis en plantas indicadoras.

Palabras clave: insecto ambrosia, chancro carbonoso, *Quercus suber*, micangios, simbiosis.

References

BAETA-NEVES, C. 1950. Introdução à Entomologia Florestal Portuguesa. *A Serra e o Homem*. Lisboa, 120 pp.

COLLADO J., PLATAS, G., PELÁEZ, F. 2001. Identification of an endophytic *Nodulisporium* sp. from *Quercus ilex* in central Spain as the anamorph of *Biscogniauxia mediterranea* by rDNA sequence analysis and effect of different ecological factors on distribution of the fungus. *Mycologia*, **93**: 875-886.

EVIDENTE, A., ANDOLFI, A., MADDAU, L., FRANCESCHINI, A., FRANCESCO, M. 2005. Biscopyran, a Phytotoxic Hexasubstituted Pyranopyran Produced by *Biscogniauxia mediterranea,* a Fungus Pathogen of Cork Oak. *J. Nat. Prod*, **68**: 568-571.

GIAMBRA, S., TORTA, L., SCOPEL, C., CAUSIN, R., BURRUANO, S. 2009. Primi studi su *Biscogniauxia mediterranea* in Sicilia Occidentale. In *Atti del Terzo Congresso Nazionale di Selvicoltura*. Taormina (ME), Italy, 1394-1396.

HSIEH, H., JU, Y., ROGERS, J.D. 2005. Molecular phylogeny of *Hypoxylon* and closely related genera. *Mycologia*, **97**(4): 844-865.

INÁCIO, M.L, HENRIQUES, J., LIMA, A., SOUSA, E. 2008. Fungi of *Raffaelea* genus (Ascomycota: Ophiostomatales) associated with *Platypus cylindrus* (Coleoptera: Platypodidae) in Portugal. *Revista Ciências Agrárias*, **31**: 96-104.

INÁCIO, M.L, HENRIQUES, J., LIMA, A., SOUSA, E. 2011. Ophiostomatoid fungi associated with cork oak mortality in Portugal**.** IOBC / wprs Bull. *(in press)*.

KIRÁLI, Z., KLEMENT, Z., VOROS J. 1970. *Methods in Plant Pathology*. Akadémiai Kiadó, Budapest, 509 pp.

LINALDEDDU, B.T., SIRCA, C., SPANO, D., FRANCESCHINI, A. 2009. Physiological responses of cork oak and holm oak to infection by fungal pathogens involved in oak decline. *For. Path*. **39**: 232-238.

MAZZAGLIA, A.; ANSELMI, N.; GASBARRI, A., VANNINI, A. 2001. Development of Polymerase Chain Reaction (PCR) assay for the specific detection of *Biscogniauxia mediterranea* living as an endophyte in oak tissues. *Mycol. Res*. **105**: 952-956.

SANTOS, M.N. 2003. Contribuição para o conhecimento das relações *Quercus suber - Biscogniauxia mediterranea*. *Silva Lusitana*, **11**: 21-29.

SEABRA, A. F.1939. Contribuição para a história da Entomologia em Portugal. *Publicações D.G.S.F.A.*, **6**: 1-20.

SOUSA, E. 1992. Alguns factores responsáveis pelo declínio do montado de sobro na Herdade da Chaminé. *In Actas do 2º Encontro sobre os Montados de Sobro e de Azinho*, Évora: **324**-335.

Sousa, E., Debouzie, D. 2002. Contribution à la bioecologie de *Platypus cylindrus* Fab. au Portugal. *IOBC / wprs Bull*., 25: 75-83.

SOUSA, E., INÁCIO, M. L. 2005. New Aspects of *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) Life History on Cork Oak Stands in Portugal. *In* Lieutier, F., Ghaioule, D., eds., *Entomological Research in Mediterranean Forest Ecosystems*. INRA Editions, France: 147-168.

SOUSA, E., SANTOS, N., VARELA, C., HENRIQUES, J. 2007. *Perda de vigor dos montados sobro e azinho: análise da situação e perspectivas*, DGF, Lisboa, 80 pp.

VANNINI, A. & VALENTINI, R. 1994. Influence of water relations on *Quercus cerris - Hypoxylon mediterraneum* interactions: a model of drought-induced susceptibility to a weakness parasite. *Tree Physiology*, **14**: 129-139.

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SUB-CHAPTER 3.2

OPHIOSTOMATOID FUNGI ASSOCIATED WITH CORK OAK MORTALITY IN PORTUGAL

INACIO ML, HENRIQUES J , LIMA A & SOUSA E (2011)

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Ophiostomatoid fungi associated with cork oak mortality in Portugal

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Abstract: Two Ophiostomatoid fungi consistently isolated from declining cork oaks attacked by the ambrosia beetle *Platypus cylindrus* in Portugal are described as *Raffaelea canadensis* and *R. montetyi*. A molecular analysis based on DNA sequences classified these species within the Ophiostomatales. The investigation of conidiogenesis of *R. canadensis* and *R. montetyi* by SEM supported this taxonomic placement and showed the formation of conidia by sympodial and annellidic percurrent proliferation, respectively. Also, their pathogenicity was proven through inoculations experiments. The fungi have been isolated both from wood and the body surface and mycangia of the beetle. It is certain that the pathogens were transferred to cork oak by *P. cylindrus*, consequently, the previous hypothesis of a species-specific association of a single ambrosia fungus with a single beetle species is questioned.

Key word: *Quercus suber; Platypus cylindrus; ambrosia fungi; Raffaelea*

Introduction

Decline of cork oak (*Quercus suber* L.) has increased in Portugal since the 1980's. Symptomatic or dead trees are frequently found to be infested by the ambrosia beetle *Platypus cylindrus* Fab. (Sousa et al. 1995; Sousa & Inacio 2005). Ambrosia beetles and larvae feed on symbiotic fungi that grow in the otherwise nutrient-poor sapwood (Batra 1963). The fungal symbionts produce small conidiophores in tight cluster (sporodochia) which are suitable for grazing by ambrosia beetle larvae and adults (Harrington et al. 2008). Propagules of the fungi are carried in one or both sexes of adult insects in specialized organs called mycangia (Batra 1963; Beaver et al. 1989).

The fungal symbionts of the ambrosia beetles are asexual and their pleomorphic nature in culture has led to ambiguous identifications until the application of DNA sequence analysis (Cassar & Blackwell 1996; Massoumi Alamouti 2009; Matsuda & Ito 2010).

It was generally believed that only one or a few fungal symbionts are closely associated with a particular ambrosia beetle species (Batra 1963). However, our isolations resulted in more than one species of *Raffaelea* (Sousa et al. 1995; Inacio et al. 2008; 2010), being in accordance with others (Harrington et al. 2010).

Here we describe two species of *Raffaelea* isolated from the galleries in the trunk and from *P. cylindrus* recovered from cork oak. Analyses of rDNA sequences infer that these two species are members of a monophyletic group of ambrosia beetle symbionts that are asexual species of *Ophiostoma* (Gebhardt et al 2004; Harrington et al. 2008).

Species of *Raffaelea* have been reported to cause a vascular wilting in trees of the *Lauraceae* (Harrington et al. 2008) and in oak trees (Kubono & Ito 2002). In this study, we investigate the effect of inoculation with *Raffaelea* species on cork oak seedlings.

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Material and Methods

Material was collected from Alentejo and Ribatejo province, two main cork producing regions of Portugal. Logs of cork oak trees (25-30cm in diameter) infested by *P. cylindrus* were cut. Cross-sections were sawn and the tunnel systems of the beetles in the sapwood were immediately open.

Wood chips containing beetle tunnel sections were plated into malt extract agar (Difco MEA, USA) amended with cycloheximide (Sigma-Aldrich, USA) (500mg/l) a semi-selective medium for the *Ophiostoma* genus (Harrington 1981). Adult females and males collected from the galleries were also collected for fungal isolation. Pure cultures of each fungus were obtained. For comparison, a culture of *Raffaelea montetyi* Morelet from CBS (strain CBS 451.94) was used. Samples were mounted on slides and observed under a light microscope. For scanning electron microscopy (SEM), small wood blocks bearing fungal structures were fixed, critical point dried, coated with gold palladium (Lee et al. 2003; Massoumi Alamouti 2009) and examined using a JOEL 35 scanning electron microscope.

The molecular analysis of the representative isolates was based on the amplification and sequencing of the 18S rDNA region using the primer pairs NS1/NS4, according to Gebhardt et al. (2004) and Massoumi Alamouti et al. (2009). The six sequences obtained were submitted to BLAST against the NCBI nucleotide databases to confirm the identifications.

Inoculation experiments were conducted in May 2008 on 1 year-old seedlings. Inoculations were repeated in October 2008. For each fungal species, 16 seedlings were wound inoculated with a mycelial plug and the same number for the wound control plants, in both seasons. Symptoms (wilting or death) were assessed daily for 4 months after inoculation. Fungal isolations of the discolored sapwood of each newly dead or wilted seedling were carried out as well for the surviving plants inoculated with both *Raffaelea* species. Statistical analyses (ANOVA) were carried out using the software Statistica 6 (StatSoft, Inc. 2003).

Results and discussion

Raffaelea montetyi Morelet and *Raffaelea canadensis* Batra were isolated both from the galleries and the body of insects. While the former species is a well known *Platypus* and *Xyleborus* symbiont (Gebhardt et al. 2004; Morelet 1998, Inacio et al. 2008), *R. canadensis* was never associated to *P. cylindrus*.

Observations by light microscopy of these species do not allow clearly distinguishing conidial proliferation, a usual distinctive character for *Raffaelea* species. In contrast, SEM observations revealed annellidic and sympodial conidiogenesis in *R. montetyi* and *R. canadensis*, respectively. These findings are in accordance with others (Gebhardt et al. 2004; Massoumi Alamouti et al. 2009). Table 1 summarized morphological features of both *Raffaelea* species.

The comparison of the sequences obtained with the *Raffaelea*'s published sequences from the GenBank nucleotide sequences databases confirmed the species identity of the two *Raffaelea* fungi with a high level of sequence identity to *R. canadensis* (99%; E value 0) and to *R. montetyi* (99%; E value 0), though the latter also have a very close relationship with *R. quercivora*. The position of the two *Raffaelea* species was consistent since they were placed in the Ophiostomatales clade, agreeing with Massoumi Alamouti et al. (2009), Harrington et al. (2010) and Matsuda et al.

(2010). Detailed results of the phylogenetic analyses using parsimony analyses and bootstrapping will be published elsewhere.

Table 1. Comparison of morphological characters of *Raffaelea montetyi* and *R. canadensis* isolated from *Platypus cylindrus* on cork oak

Species	Morphology and frequency of sporodochia	Conidia (µm)	Conidia shape	Conidiogenesis
Raffaelea montetyi	Rare; when present, not a typical sporodochia (fascicles)	5.01-10.20x2.51-5.85	Obovoide to pyriform truncated	Annellidic percurrent proliferation
Raffaelea canadensis	Frequently absent	6. 68-10.20x2.51-5.01	Globose to pyriform	Sympodial with flat lateral scars in the conidiogenous cells

There was a clear and significant difference between isolates in the number of days necessary to wilting symptoms onset both in spring ($F_{1,17}$=167.60, p<0.0001) and in autumn ($F_{1,11}$=127.22, p<0.0001) with spring symptoms appearing soon (Table 2). Death of symptomatic seedlings infected by *R. montetyi* occurred in approximately 2 months, and such a pattern did not depend on the season of the inoculation ($F_{1,20}$=1.35, p=0.2587). No mortality was observed in *R. canadensis* tests until the end of the experiment. Both isolates were recovered from all the symptomatic or dead plants but none was reisolated neither from asymptomatic seedlings nor from controls.

Table 2. Number of days before wilting or mortality was observed after cork oak seedlings inoculation with *Raffaelea montetyi* and *R. canadensis* (mean±SD); number of wilted or dead plants (N) of the total of inoculated seedlings (n), per treatment

Isolate	Inoculation season		Wilting		Death	
		n	N	Days	N	Days
Raffaelea montetyi	Spring	16	14	37,0±2,5	14	58,0±2,2
	Autumn	16	10	43,3±2,4	8	56,1±5,4
Raffaelea canadensis	Spring	16	5	54,0±2,5	0	-
	Autumn	16	3	63,7±4,0	0	-

The gallery system of declining cork oaks attacked by *P. cylindrus* showed a discoloration of the tunnel walls. Different fungi, particularly ophiostomatoid fungi were invariably present. In this study, cultures of the ambrosia fungi were identified as *R. montetyi* and *R. canadensis*. The occurrence of these two species in association with this ambrosia beetle challenges the hypothesis of a species-specific association of a single ambrosia fungus with a single beetle species being in accordance with recent works (Massoumi Alamouti 2009; Harrington et al. 2010).

Taking into consideration that *R. montetyi* and *R. canadensis* were isolated from declining and dead cork oaks and from the bodies and mycangia of *P. cylindrus*, it is likely that these fungi were transferred to oak trees by this insect. Phylogenetic data placed these isolates in the *Ophiostoma* clade, an important group of forestry pathogens. Until now only *R. lauricola* and *R. quercivora* were known to kill woody plants. In the present study, we confirmed the pathogenicity of *R. montetyi* and possibly of *R. canadensis* towards cork oak. To gain further insight into a relationship between phylogenetic and pathological traits of these fungal species, additional sequences and other members of the genus *Raffaelea* must be brought into a multigene phylogenetic analysis. The β-tubulin gene would be a suitable candidate for next sequencing to achieve such discrimination (Matsuda et al. 2010).

References

Batra, L.R. 1963: Ecology of ambrosia fungi and their dissemination by beetles. Transactions of the Kansas Academy of Science 66: 213-236.

Beaver, R.A. 1989: Insect-fungus relationships in the bark and ambrosia beetles. In : Wilding, N.; Collins, N.M.; Hammond, P.M. & Webber, J.F. (Eds.) Insect-Fungus Interactions. Academic Press, London: 121-143.

Cassar, S. & Blackwell, M. 1996: Convergent origins of ambrosia fungi. Mycologia 88: 596–601;

Gebhardt, H., Begerow D. & Oberwinkler F. 2004: Identification of the ambrosia fungus of *Xyleborus monographus* and *X. dryographus* (Coleoptera: Curculionidae, Scolytinae). Mycol prog 3: 95–102;

Harrington, T.C. 1981: Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. Mycologia, 73: 1123-1129.

Harrington, T.C., Aghayeva, D.N. & Fraedrich, S.W. 2010: New combinations in *Raffaelea*, *Ambrosiella*, and *Hyalorhinocladiella*, and four new species from the readbay ambrosia beetle, *Xyleborus glabratus*. Mycotaxon 111: 337– 361.

Harrington, T.C., Fraedrich, S.W. & Aghayeva, D.N. 2008: *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. Mycotaxon 104: 399– 404.

Inácio, M.L., Henriques, J., Lima, A. & Sousa, E. 2008: Fungi of *Raffaelea* genus (Ascomycota: Ophiostomatales) associated with *Platypus cylindrus* (Coleoptera: Platypodidae) in Portugal. Revista de Ciências Agrárias, 31(2): 96-104.

Inácio, M.L., Henriques, J. & Sousa, E. 2010: Mycobiota associated with *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) on cork oak in Portugal. IOBC/ wprs Bulletin, 57: 87-95.

Kubono, T. & Ito, S. 2002: *Raffaelea quercivora sp. nov*. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). Mycoscience 43: 255-260.

Lee, S., Kim, J.J., Fung, S. & Breuil, C. 2003: A PCR-RFLP marker distinguishing *Ophiostoma clavigerum* from morphologically similar *Leptographium* species associated with bark beetles. Can J Bot 81: 1104–1112.

Massoumi Alamouti, S., Tsui, C.M. & Breuil, C. 2009: Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. Mycol Res 113:822–835.

Matsuda, Y., Kimura, K. & Ito, S. 2010: Genetic characterization of *Raffaelea quercivora* isolates collected from areas of oak wilt in Japan. Mycoscience 51: 310–316.

Morelet, M. 1998 : Une espèce nouvelle de *Raffaelea*, isolée de *Platypus cylindrus*, coléoptère xylomycetétophage des chênes. Annal. Soc. Sci. Nat. Arch. de Toulon et Var. 50: 185–193.

Sousa, E., Debouzie, D. & Pereira, H. 1995: Le rôle de l'insecte *Platypus cylindrus* F. (Coleoptera, Platypodidae) dans le processus de dépérissement des peuplements de chêne-liège au Portugal. IOBC/ wprs Bulletin, 18: 24-37.

Sousa, E. & Inácio, M.L. 2005: New Aspects of *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) Life History on Cork Oak Stands in Portugal. In: F. Lieutier & D. Ghaioule (Eds.) Entomological Research in Mediterranean Forest Ecosystems. INRA Editions, Paris, 280 pp.

SUB-CHAPTER 3.3

NEW ASSOCIATIONS OF *RAFFAELEA* AND *OPHIOSTOMA* SPECIES WITH *PLATYPUS CYLINDRUS* IN PORTUGAL INFERRED FROM MOLECULAR AND MORPHOLOGICAL EVIDENCE

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Mycologia (submitted)

New associations of *Raffaelea* and *Ophiostoma* species with *Platypus cylindrus* in Portugal inferred from molecular and morphological evidence

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Abstract

Severe *Quercus suber* decline prevails in the unique Portuguese cork oak stands. Attacks of the oak pinhole borer *Platypus cylindrus* follow the spread of oak loss of vigor. We contribute to clarify the etiology of cork oak decline in Portugal describing the complex of fungi associated with the ambrosia beetle. Adults of *P. cylindrus* were screened for the presence of ambrosia fungi in their mycangia and gut. Isolations were also made from the exoskeleton. Two *Raffaelea* species were recovered: *R. montetyi* and a *Raffaelea* species closely related to *R. canadensis*. *R. montetyi* was the dominant ambrosia fungus associated with *P. cylindrus.* In addition, a new *Ophiostoma* species with a *Hyalorhinocladiella* anamorph was consistently found in the mycangia. This species was also isolated from the insect gallery system and from declining cork oak trees. Although morphologically similar, phylogenetic analyses of SSU and LSU rDNA regions indicate that the new *Ophiostoma* species is genetically distinct from previously reported *Ophiostoma* spp.. Moreover, constant and identical length ITS1 regions in all Portuguese isolates under study, set apart from pre-existing Ophiostomatales sequenced for this region, together with the phylogenetic relationships found for the 5.8S-ITS2 region corroborate the existence of a distinct lineage of Ophiostomatales in Portugal. The ophiostomatoid fungi reported herein are observed for the first time in association with *P. cylindrus* in Portuguese cork oak stands.

Key words: *Raffaelea montetyi*, *Raffaelea* sp., *Ophiostoma* sp., *Hyalorhinocladiella* sp., cork oak pinhole borer, cork oak decline.

INTRODUCTION

In Portugal, severe cork oak decline has become evident since the 1980s and is now widespread in the main cork-producing regions (Sousa 1995, Sousa and Inacio 2005). Cork oak has a restricted worldwide distribution and possesses a relevant socio-cultural and economic relevance in the Mediterranean basin (Silva and Catry 2006, Pereira 2007). Following the spread of the cork oak decline, attacks of the ambrosia beetle *Platypus cylindrus* Fab (Coleoptera: Platypodidae) increased (Sousa et al. 1995, Sousa and Débouzie 2002). Scolytid and platypodid ambrosia beetles are a polyphyletic, ecologically defined group of about 3400 species derived from bark beetles in at least seven evolutionary events (Farrel et al. 2001). Ecologically, ambrosia beetles are distinguished from bark beetles by laying eggs in the nutrient-poor sapwood of declining trees, while bark beetles lay their eggs along galleries in the nutrient-rich inner bark (phloem) of host trees (Six 2003, Harrington 2005). The beetles cannot use wood as a primary nutritional substrate. Hence adults and larvae feed upon symbiotic fungi carried in one or both sexes of adults in specialized organs called mycangia (Batra 1963, Beaver 1989, Francke-Grosmann 1967, Fraedrich et al. 2008). Budding spores of the symbiont ooze out of the mycangium as the adult female constructs their tunnels and lay eggs (Harrington and Fraedrich 2010). The symbionts are mitosporic fungi that typically produce small conidiophores in tight clusters (sporodochia) in beetle galleries which are suitable for grazing by larvae and teneral adults (Batra 1967).

Many of the associated symbionts have been described in the asexual genera *Raffaelea* Arx and Hennerbert emend Batra and *Ambrosiella* Brader emend. L.R. Batra, which are phylogenetically placed within the sexual genera *Ophiostoma* and *Ceratocystis*, respectively (Harrington and Fraedrich 2010). *Ambrosiella* and *Raffaelea* were originally distinguished based on annellidic *vs.* sympodial proliferation of the conidiogenous cells, respectively (Arx and Hennebert 1965, Batra 1967). However, this early important taxonomic character has been challenged because conidial fungi often develop more than one pattern of conidiogenesis and can be assigned to different anamorphic genera (Gebhardt and Oberwinkler 2005, Tsuneda and Currah 2006).

Most of the ambrosia beetle symbionts related to *Ophiostoma* species have been described as species of *Raffaelea* (Kubono and Ito 2002). Zipfel et al. (2006) redefined *Ophiostoma* and distinguished *Ophiostoma* species (with *Pesotum*, *Hyalorhinocladiella* and *Sporothrix* anamorphs) from *Ceratocystiopsis* (with *Hyalorhinocladiella* anamorphs) and *Grosmannia* (with *Leptographium* anamorphs). Later, Harrington et al. (2008) emended *Raffaelea* to include all ambrosia beetle symbionts related to *Ophiostoma*. In addition, *Ambrosiella* species associated with bark beetles were transferred to *Hyalorhinocladiella* (Harrington et al. 2010).

Species of *Raffaelea* are difficult to distinguish as they lack clearly defining morphological features, so identification is primarily based on rDNA sequences (Gebhardt et al. 2004, Matsuda et al. 2010). Currently, the only complete and variable dataset of DNA sequences for identification of *Raffaelea* species is the large subunit (LSU, 26S) rDNA dataset (Harrington et al. 2010). The small subunit (SSU, 18S) rDNA sequences do not show sufficient variation to distinguish all of the 24 known species of *Raffaelea*, although should be considered in a multigene phylogeny of filamentous ambrosia fungi (Massoumi Alamouti et al. 2007). Attempts to infer phylogenetic relationships in Ophiostomatales using the hypervariable internal transcribed spacer region (ITS-rDNA) have been recently reported (Wingfield et al. 1999, Gorton et al. 2004, Roets et al. 2008, Zipfel et al. 2006, Massoumi Alamouti et al. 2009, Zanzot et al. 2010).

The aim of this study is to analyse and discern the relationship between *P. cylindrus* and Ophiostomatales in Portugal in an attempt to contribute to clarify the etiology of cork oak decline in Portuguese cork oak stands. We analyze the phylogenetic relationships of the three main rDNA regions commonly used in molecular analyses of Ophiostomatales fungi, mentioned herein, and comprehensively described the morphology of fungal species obtained in this study. Comparisons with representative pre-existent Ophiostomatales worldwide were made.

MATERIAL AND METHODS

Beetle collection and fungal isolation – In order to obtain a comprehensive representation of the ambrosia pinhole cork oak borer populations in Portuguese cork oak stands (*Quercus suber* L.) three hundred adult freshly emerged *P. cylindrus*, 158 females and 142 males, were collected live in fine mesh nets attached to a silicone joint on logs of cork oak obtained from two main cork producing regions of Portugal, Ribatejo and Alentejo provinces. A total of twelve logs with ca. 30 cm diameter and 0.5 m length were used in the sampling. Collections were assembled during the month of May from 2005 to 2007. Each beetle was dissected aseptically with iris scissors in order to obtain their mycangia, intestine and exoskeleton parts (i.e., elytra) aiming to determine fungal presence within these morphological structures. All biological material was surface sterilized for 1 min in 1% sodium hypochlorite and then rinsed in sterile dH2O and plated in malt extract agar (10 g Difco MEA, 15 g agar) amended with 500 mg/L cycloheximide and 500 mg/L streptomycin. Cycloheximide media are semi-selective for *Ophiostoma* species and related anamorphs such as *Raffaelea* (Harrington 1981, 1992, Harrington and Fraedrich 2010). Plates were incubated at room temperature in the dark for 1 to 2 weeks depending on fungal growth. Number of isolates was determined afterwards. Colonies of different size, color and mycelial pattern (concentric rings, yeasty growth, hyaline margins, etc.) were considered putative species. Axenic cultures of each fungal strain were obtained and voucher specimens of each mycelial phenotype were deposited in the LISFA fungal collection from the National Forestry Station of INRB, (primary collection; PC acronyms TABLE I). In addition, mycelial plugs of at least three isolates of each putative fungal species were placed in the voucher specimen collection at Iowa State University (secondary collection; C acronyms TABLE I) for storage and subsequent DNA extraction and sequencing.

DNA extraction and sequencing – Fourteen axenic fungal strains representing all the putative fungi species retrieved from *P. cylindrus* using the methodologies mentioned herein, were processed for genomic DNA extraction with Puregene® DNA Purification Kit (Gentra Systems Inc., Minneapolis, USA) following the manufacturer's protocol. Mycelium plugs scraped with a sterile scalpel from the surface of individual cultures were used as biological sample material. For comparison purposes, cultures of *Raffaelea montetyi* Morelet from Centraalbureau voor Schimmelcultures (strain CBS 451.94) and *R. quercivora* Kubono & Shin (isolates MAFF919 and MAFF921) from Mie University, Dep. Forest Pathology and Mycology, Japan, were also used for DNA extraction and sequencing.

Table I. Fungal species included in this stud

Species	Source[a]	Location	Associated insect	GenBank accession nrs.[b]			
				nSSU rDNA	nLSU rDNA	ITS1-5.8S-ITS2	5.8S-ITS2
Raffaelea sp.	PC05.007, C2512	Portugal	*Platypus cylindrus*	**JF909509**	**JF909537**	**JF946762**	**JF909525**
	PC05.041, C2516	Portugal	*P. cylindrus*	**JF909510**	**JF909538**	**-**	**-**
	PC05.042, C2513	Portugal	*P. cylindrus*	**JF909511**	**JF909539**	**-**	**-**
R. albimanens		South Africa	*P. externedentatus*	EU170269	EU984296		
R. ambrosiae		UK	*P. cylindrus*	EU170278	EU984297		
R. amasae		Taiwan	*Amasa concitatus*	AY858661			
R. arxii		South Africa	*Xyleborus torquatus*	EU170279	EU984298		
R. brunnea		USA	*Monarthrum* sp.	EU170280	EU177457		
R. canadensis		Canada	*P. wilsonni*	EU170270	EU177458		
		Canada	*Gnathotrichus sulcatus*	EU170281			
R. ellipticospora		USA	*X. glabratus*		EU177444		
R. fusca		USA	*X. glabratus*		EU177447		
R. gnathotrichi		USA	*G. retusus*	EU170282	EU177460		
R. lauricola		USA	*X. glabratus*	EU123076	EU123077		
R. montetyi	PC06.001, C2506	Portugal	*P. cylindrus*	**JF909512**	**JF909540**	**JF946763**	**JF909526**
	PC07.003, C2505	Portugal	*P. cylindrus*	**JF909513**	**JF909541**	**JF946764**	**JF909527**
	PC06.038, C2504	Portugal	*P. cylindrus*	**JF909514**	**JF909542**	**-**	**-**
	PC06.039, C2514	Portugal	*P. cylindrus*	**JF909515**	**JF909543**	**-**	**JF909529**
	CBS451.94, C2220	Portugal	*P. cylindrus*	**-**	EU177461		
		Portugal	*P. cylindrus*		EU984301		
		France	*P. cylindrus*				AB496432
		Germany	*X. dryographus*	AY497522			
R. quercivora	PC10.919, MAFF919	Japão	*P. quercivorus*	**JF909517**	**JF909547**	**JF960136**	**JF909531**
	PC10.921, MAFF921	Japão	*P. quercivorus*	**JF909516**	**JF909546**	**-**	**JF909530**
		Japão	*P. quercivorus*		AB552937		AB496452
		Japão	*P. quercivorus*	AB496428	AB496454		AB496433
R. santoroi		Argentina	*Platypus* sp.	EU984261	EU984302		
R. subalba		USA	*X. glabratus*		EU177441		
R. subfusca		USA	*X. glabratus*	EU170268			
R. sulcati		Canada	*G. sulcatus*	EU170271	EU177462		
R. sulphurea		USA	*X. saxeseni*	EU170272			
R. tritirachium		USA	*M. mali*	EU170273	EU984303		

Table I. (continued)

Species	Source[a]	Location	Associated insect	GenBank accession no.[b]			
				nSSU rDNA	nLSU rDNA	ITS1-5.8S-ITS2	5.8S-ITS2
Ophiostoma sp.	PC05.005, C2510	Portugal	*P. cylindrus*	**JF909502**	**JF909532**	**JF946755**	**JF909518**
Ophiostoma sp.	PC05.006, C2511	Portugal	*P. cylindrus*	**JF909503**	**JF909533**	**JF946756**	**JF909519**
Ophiostoma sp.	PC06.022, C2519	Portugal	*P. cylindrus*	**JF909504**	**JF909534**	**JF946757**	**JF909520**
Ophiostoma sp.	PC06.032, C2508	Portugal	*P. cylindrus*	**JF909505**	**JF909535**	**JF946758**	**JF909521**
Ophiostoma sp.	PC06.034, C2507	Portugal	*P. cylindrus*	**JF909506**	**JF909544**	**JF946759**	**JF909522**
Ophiostoma sp.	PC07.004, C2517	Portugal	*P. cylindrus*	**JF909507**	**JF909545**	**JF946760**	**JF909523**
Ophiostoma sp.	PC07.007, C2509	Portugal	*P. cylindrus*	**JF909508**	**JF909536**	**JF946761**	**JF909524**
Ophiostoma sp.	PC96.021, C2515	Portugal	*P. cylindrus*	-	**JF946765**	-	-
O. aurorae		South Africa	*Hylurgus angustatus*			DQ396798	DQ396798
O. abieticola		Japan	*Ips subelongatus*				GU134156
O. abietinum		Canada	*Dendroctonus ponderosae*	EU984276		AY924382	AY924382
O. bicolor		Germany	*Ips typographus*	AY497512			
O. canum		Canada	-	EU984277	AJ538342		
O. cucullatum		Germany	*Dryocoetes autographus*	AY497513			
O. fusiforme		Austria	-			AY280497	AY280497
O. floccosum		Canada	-	AF139810	AJ538343		
O. gossypinum var. robustum		Spain	-			AY924388	AY924388
O. ips		Canada	*Ips* sp.	AY172021	AY172022		
O. lanatum		Austria	-			AY280486	AY280486
O. montium		Canada	*Ddr. ponderosae*	EU984278	AY194947		
O. nigrocarpum		Japan	*Ddr. ponderosae*		EF506941	AF484457	AF484457
O. piceaperdum		Germany	*Cryptugus cinereus*	AY497514			
O. piceae		-	-	AB007663	AJ538341		
O. quercus		-	-	AF234835			
O. stenoceras		South Africa	-	M85054	DQ836904	AF484460	AF484460
		South Africa	-			AF484459	AF484459
		South Africa	-				AF484464
O. triangulosporum		Spain	-				AY934525
O. ulmi		USA	-	M83261			

Table I. (continued)

Species	Source[a]	Location	Associated insect	GenBank accession no.[b]			
				nSSU rDNA	nLSU rDNA	ITS1-5.8S-ITS2	5.8S-ITS2
Sporothrix sp.		México	*Ips calligraphus*			AY546722	AY546722
		México	*I. calligraphus*			AY546719	AY546719
		USA	NA				HQ630984
S. shenckii		Japan	NA		AB363791		AF484471
S. variecibatus		South Africa	-			DQ821568	DQ821568
Leptographium abietinum		unknown	*Ddr. pseudotsugae*		DQ097852		
L. frutticetum		USA	*I. perturbatus*		DQ097848		
Hyalorhinocladiella ips		USA	*Ips* sp.	EU170276	EU984289		
H. macrospora		Sweden	*I. acuminatus*	EU170284	EU984290		
H. tingens		Sweden	*I. sexdentatus*		EU984293		
Grossmania davidsonii		-	-				EU879134
Ceratocystis adiposa		-	-		EU984304		
C. coerulescens		-	-		AY214000		
C. monilliformis		-	-		EU984305		
Ceratocystiopsis sp.		-	*I. perturbatus*				EU913717
Cop. manitobensis		USA	*I. perturbatus*	EU984266	DQ268609		DQ268610
Cop. minima		Canada	-		EU913661		
Cop. ranunculosa		USA	*Ddr. frontalis*		DQ 268617		
Ambrosiella ferruginea		-	*Trypodendron* spp.		EU984285		
A. hartigii		-	*X. dispar*		EU984288		
A. xylebori		-	*Xylosandrus compactus*		EU984294		
Aspergillus fumigatus		-	NA		AFU2846		
Penicillium expansum		-	NA	DQ126698			
Taphrina pruni		-	NA			AB505449	AB505449
T. wiesneri		-	NA	AY548293	AF492075		

a PC, LISFA fungal collection of the National Forestry Station/ INRB,I.P, Portugal; C, Iowa State University, Dept. of Plant Pathology, USA; CBS, Centraalbureau voor Schimmelcultures, Uthecht, the Netherlands; MAFF, Mie University, Dep. Forest Pathology and Mycology, Japan

b Accession numbers of sequences newly produced (bold) or downloaded from GenBank

NA non applicable

The LSU rDNA region was amplified with primers NL1 and NL4 (Matsuda et al. 2010) and LROR and LR5 being the PCR products sequenced with primers LROR and LR3 (Harrington et al. 2010). Primers for amplification and sequencing of the SSU rDNA included NS1, NS3, NS4 and NS6 (White et al. 1990). The ITS region was amplified using nested PCR. The first PCR was conducted with primers ITS5/ NL4 (O'Donnell 1993), the second PCR using ITS1F (Gardes and Bruns 1993) and ITSp3 (Kusaba and Tsuge 1995). Template DNA was amplified in a 50 µl single reaction volume, containing 0.2 units of Taq Dream DNA polymerase (MBI Fermentas, Lithuania), 1xPCR reaction buffer, 20 µl dNTPs, 4% (V/V) DMSO and 2 µl of each primer. Cycling conditions were initial denaturation at 95 C for 5 min, 35 cycles of annealing at 50 C for SSU and 40 cycles at 55 C for the LSU and ITS regions, and primer elongation at 72 C for 1.5 min. When single DNA bands, with the target bp length, were present in gel electrophoresis, PCR products were purified with QIAquick PCR Purification kit (Quiagen Inc., Valencia, California, USA) following the manufacturer's instructions and sequenced at STABVIDA facilities, Caparica (Portugal) using the DNA analyzer ABI PRISM 3730xl (Applied Biosystems) and at the DNA Synthesis and Sequencing Facility at Iowa State University.

Chromatograms were edited and consensus sequences were generated with Sequencher^{TM} (Gene Codes Corp., Ann Arbor, Michigan). Sequences were analyzed with all closely related sequences obtained from GenBank using BLAST (i.e., Maximum Identity of database segments against the subject sequence above 97%) and previously reported sequences (Gebhardt et al. 2004, Massoumi-Alamouti et al. 2009, Harrington et al. 2010, Matsuda et al. 2010). Sequences were aligned individually with the aid of Clustal W (Chenna et al. 2003) and retrieved with Jalview software (Clamp et al. 2004). When necessary, subsequent manual adjustments were made. Sequences generated in this study were deposited in GenBank (accession numbers are included in TABLE I). Primers were excluded from published sequences and sequence alignments.

Phylogenetic analysis - Phylogenetic analysis was conducted separately for the three rDNA regions (nSSU, nLSU and 5.8S-ITS2) with sequences listed in TABLE I and all related sequences in GenBank. A concatenated dataset analysis of the three adjacent regions was not done since the high degree of variance, and length, of the ITS1 region with sequences of Ophiostomatales available in GenBank, led us to opt for a discrete phylogenetic analysis of the 5.8S-ITS2 regions, and partial nucleotide sequences from the large and small subunit rDNA, LSU and SSU, respectively. The complete ITS region obtained in this study for Ophiostomatales species was also made available in GenBank for informative purposes (TABLE I). For all the genes listed herein only sequence fragments that could be aligned with certainty were used to generate alignments.

Outgroup sequences were selected based on their genetic distance to Ophiostomatales used in the phylogenetic analyses (Gebhardt et al. 2005; Luo et al. 2010, Matsuda et al. 2010). Phylogenetic trees were estimated under maximum parsimony (MP) using PAUP 4.0b10 (Swofford 2003). For parsimony analyses, all characters were equally weighted and gaps treated as missing data (Swofford 2003). Bootstrap analyses were performed with 1000 bootstrap replications with 30 random stepwise additions of taxa and tree-bisection-reconnection branch-swapping algorithms. Multiple equally parsimonious trees were combined into a single strict-consensus tree. Only bootstrap values above 70 were considered as well supported in the final consensus tree.

Fungal morphology – Cultures were grown on 1.5% MEA medium at 25 C in the dark. Colonized agar plugs (5 mm diam) were excised from actively growing 1 wk old cultures of three different isolates of each putative species. These discs were transferred to the centers of fresh dishes containing 20 mL 1.5% MEA. Growth rates were determined at temperatures ranging from 5-35 C, at five-degree intervals, three and ten days after inoculation, in the dark. Colony diameter of six replicates cultures were calculated by averaging the twelve measurements. Mycelial colors are described using the terminology from Saccardo (1891). Tolerance to cycloheximide was assessed by measuring fungal growth on MEA amended with 100, 500 and 1000 ppm cycloheximide after autoclaving. For fungal morphological characterization 3 to 5-day-old slide cultures (Riddel 1950) mounted in lactophenol were examined with light microscopy with differential interference contrast microscopy (Olympus BX-41 with Olympus DP11). Fifty measurements were obtained for each taxonomically informative structure. For scanning electron microscopy (SEM), small wood blocks (5x2x5mm) bearing fungal structures were fixed according to Lee et al. (2003) and Massoumi-Alamouti et al. (2009). After fixation, samples were critical point dried, sputter coated twice with gold palladium (98:2) and examined using a JOEL 35 scanning electron microscope.

RESULTS

Isolation of fungi from Platypus cylindrus – A total of 300 adults were sampled for the presence of Ophiostomatales and 270 individual Ophiostomatales isolates were obtained on the cycloheximide media. Of the 300 beetles, 249 (83%) yielded a least one species of Ophiostomatales and only 4% yielded more than one species.

The isolated Ophiostomatales were grouped into three putative morphological species based on cultural features, microscopic characteristics and growth rate. Each of the three fungal species tolerated high concentrations of cycloheximide and two of them had SSU and LSU sequences similar to those of described species of *Raffaelea* (ie, Raffaelea sp. Y and *R. montetyi*).

The remaining species had sequences similar to other members of the genus *Ophiostoma* but not to *Raffaelea*, being described here as new (*Ophiostoma* sp. X). The species isolated were distinguished from each other by analysis of LSU and SSU sequences (FIG. 1-4) and by mycelial and conidial morphology (FIG. 5-7).

Phylogenetic analyses - A total of 2010 base pairs were sequenced in the genetic analyses of this study. Parsimony informative characters for the rDNA regions used in the analysis were: LSU (40%), ITS (18.4%) and SSU (15%). Conserved nuclear rDNA regions (i.e., SSU and LSU, FIG. 1 and 2 respectively) showed high resolution for the topology of deeper nodes and terminal branches but weaker at the base nodes.

Both regions clearly differentiate monophyletic groups of *Raffaelea* species, namely, *R. quercivora* and *R. montetyi*.

A strongly supported (97% BS) monophyletic clade of mostly *R. montetyi* species was also found at the terminal node of the SSU region (FIG. 1). The *R. montetyi* isolated from *P. cylindrus* in Portugal (JF909512-JF909515) are part of the latter monophyletic group. The same taxa for the LSU region (JF909540-JF909543, FIG. 2) are nested with the holotype *R. montetyi* and are sibling to the *R. quercivora* group. A strong support (100% BS) at the deeper node and weak (56% BS) at the base node, observed for the LSU phylogeny, discriminates *R. montetyi*, *R. quercivora*, and all other *Raffaelea* species.

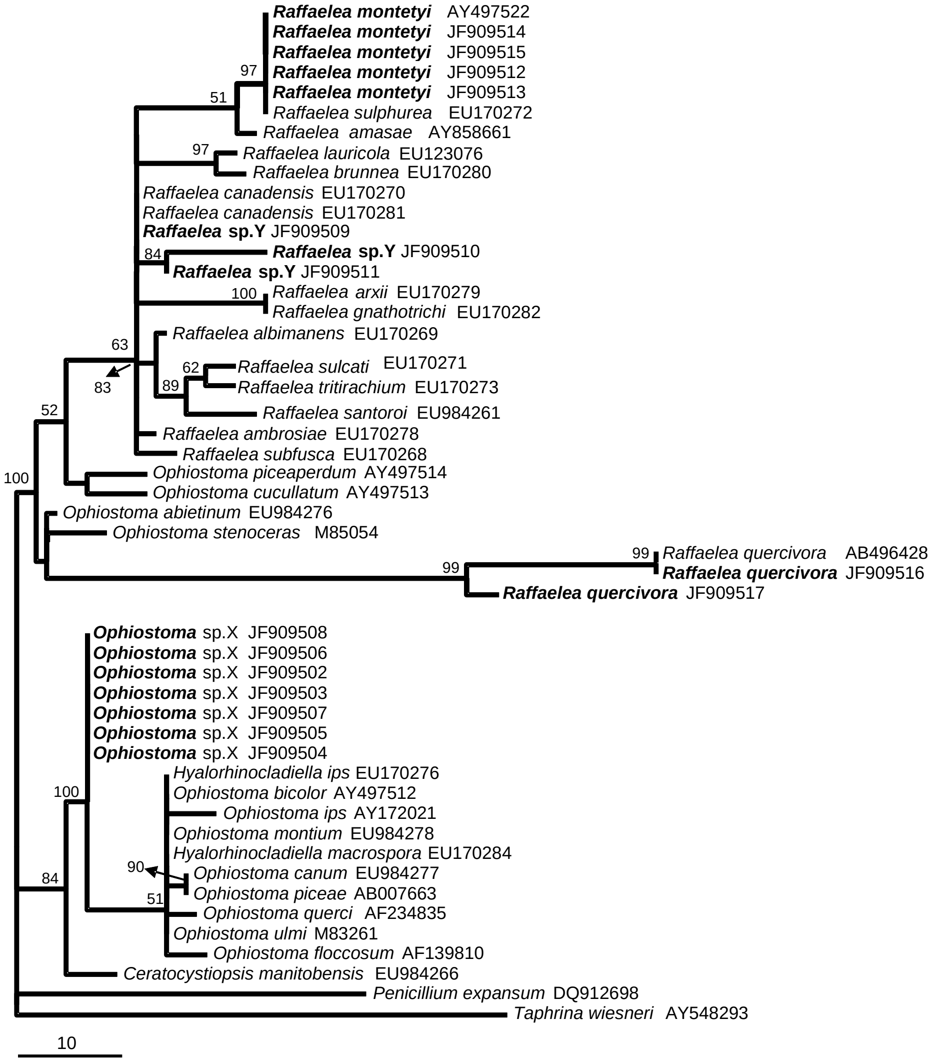

Fig. 1 - Phylogenetic relationships (Inferred MP) of Ophiostomatales species retrieved from *Platypus cylindrus* in Portugal with previously reported Ophiostomatales genera and species retrieved from GenBank. Consensus of 217 parsimonious trees inferred from the heuristic analysis of the partial sequence (1028 bp) of the small subunit (SSU) ribosomal DNA region. Fungal strains obtained in this study are shown in bold. Numbers above nodes are bootstrap values based on 1000 iterations with 30 random additions.

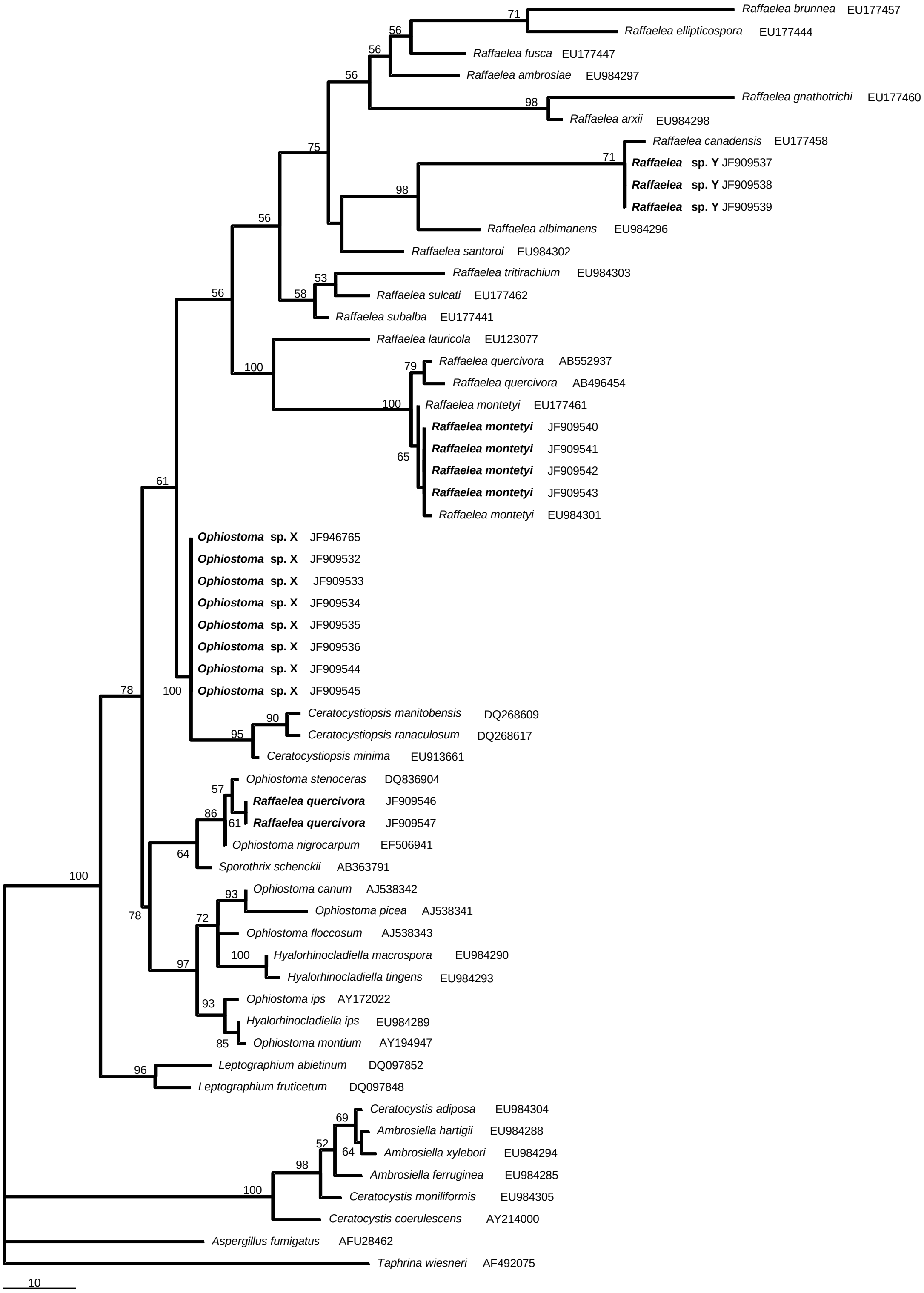

FIG. 2 - Phylogenetic relationships (Inferred MP) of Ophiostomatales species retrieved from *P. cylindrus* in Portugal with previously reported Ophiostomatales genera and species retrieved from GenBank. Consensus of 100 parsimonious trees inferred from the heuristic analysis of the partial sequence (560 bp) of the large subunit (LSU) ribosomal DNA region. Fungal strains obtained in this study are shown in bold. Numbers above nodes are bootstrap values based on 1000 iterations with 30 random additions.

For the SSU region an evident and strongly supported (99% BS) monophyletic group of *R. quercivora* from Asia corroborated the findings of Matsuda et al (2010), suggesting that *R. quercivora* may be phylogenetically distinct from previously known *Raffaelea* species. Although this monophyletic clade is well resolved in the SSU phylogenetic tree, the relationship is not so clearly inferred for the LSU phylogeny. The position of *R. quercivora* isolates originated in Asia for this latter region (JF909546 and JF909547) seems ambiguous as they cluster in a subclade of an external *Sporothrix* clade and a basal *Ophiostoma* major clade. In addition, they are the only *Raffaelea* taxa to be outside of the complex of *Raffaelea* in the major clade, including other *R. quercivora*. This pattern also occurs in the 5.8S-ITS2 phylogeny (FIG. 3) for one of the isolates (*R. quercivora* JF909531), whereas another isolate had no support in the phylogeny (JF909530). Again, these isolates are set apart from other holotype *R. quercivora* in the analysis.

The position of putative *R. canadensis* species found in Portugal (JF909537-JF909539, for LSU) is monophyletic with the holotype (EU177458, 543 of 554 bp matching), however they appear genetically distinct, as evidenced in the LSU clade. The SSU region (JF909509-JF909511) is more ambiguous for this comparison, although a similar pattern with the LSU region is clear (EU170270, *c.a.* 1% to 2% bp divergence with holotype *R. canadensis* sequence).

A major monophyletic clade of symbiotic ambrosia fungi (i.e., *Raffaelea* spp.) consistently grouped species in the genus in well supported internal subclades. This clade is separated from another major clade of non ambrosial symbiont species, in both SSU and LSU regions. The support for the two major clades was 100% BS and 61% BS for the SSU region and the LSU region, respectively.

Portuguese *Ophiostoma* sp. for both SSU region (JF909502-JF909508) and LSU region (JF909532-JF909536, JF909544 and JF909545) grouped separately (100% BS for both rRNA regions). The closest match for the SSU sequence was *H. ips* (EU170276) and *O. ulmi* (M83261), both with 3.2% bp divergence. Their LSU sequences most closely match *Ceratocystiopsis minima* (EU913661, 517 of 547 bp matching) and *C. manitobense* (DQ268609, 513 of 547 bp matching) and *C. ranaculosa* (DQ268617, 512 of 547 bp matching). Since our *Ophiostoma* sp. isolates were highly tolerant to cycloheximide they could not be assigned to *Ceratocystiopsis* sp. whose members are sensitive to this antibiotic (Plattner et al. 2009).

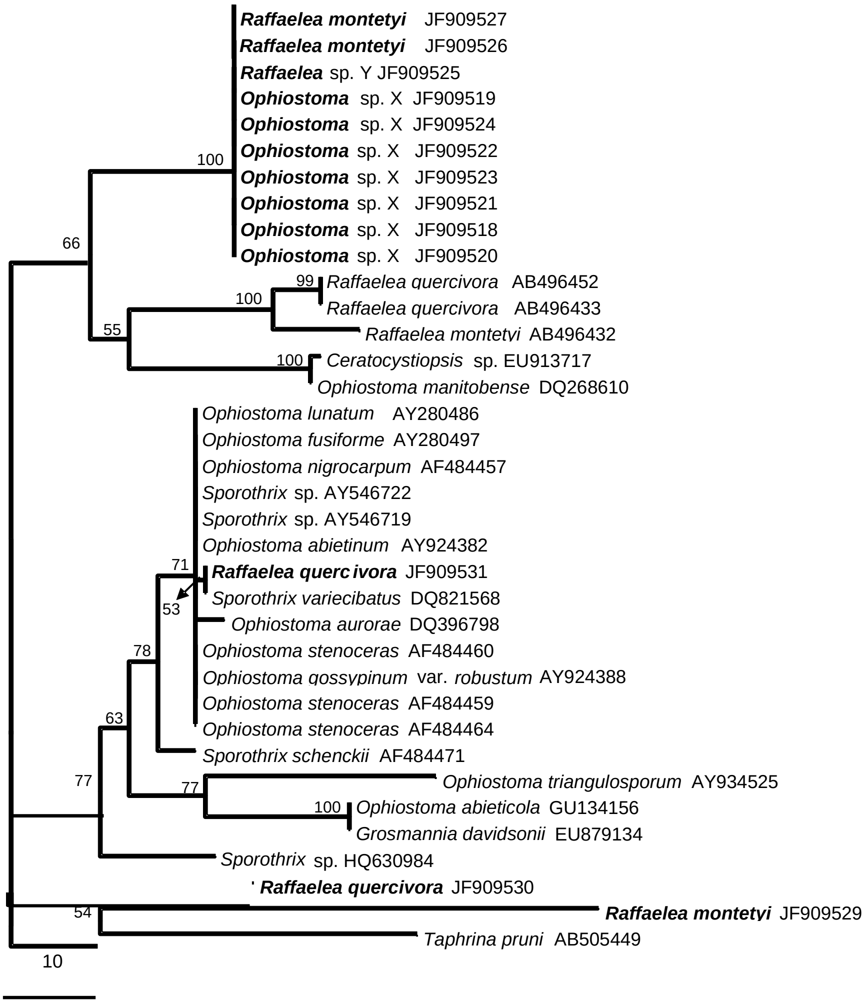

FIG. 3 - Phylogenetic relationships (Inferred MP) of Ophiostomatales species retrieved from *Platypus cylindrus* in Portugal with previously reported Ophiostomatales genera and species retrieved from GenBank. Consensus of 10 parsimonious trees inferred from the heuristic analysis of the partial sequence (422 bp) of the 5.8S-ITS2 ribosomal DNA region. Fungal strains obtained in this study are shown in bold. Numbers above nodes are bootstrap values based on 1000 iterations with 30 random additions.

The new Portuguese *Ophiostoma* species presents an unstable position in the inferred phylogenetic relationships since, depending on the region profiled, it is a basal sister group to the large monophyletic *Raffaelea* clade (in the LSU region) or sibling to the *Ophiostoma* species major clade (in the SSU region). The sibling relationship with *Raffaelea* is also observed for the 5.8S-ITS2 region sequenced (66% BS). This region did not substantially discriminated relationships within Portuguese isolated species but rather a divergence between these and other genera, and species previously reported.

There was a high resolution for the topology of the deeper node grouping all Portuguese isolates found in this study (BS 100%). This might indicate that a different monophyletic lineage of ambrosia fungi associated with *P. cylindrus* independently evolved in Portuguese cork oak stands. Different lineages of Ophiostomatales have been previously reported elsewhere (Wingfield et al. 1999). We observed that the ITS1 region for all genetic sequences derived from Portugal had consistent nucleotide lengths and sequence, although set apart from all outputs in GenBank BLAST (Table I).

A MP analysis of the ITS1-5.8S-ITS2 region evidences a clear phylogenetic divergence between Portuguese isolates and Ophiostomatales sequences retrieved from GenBank (Fig. 4).

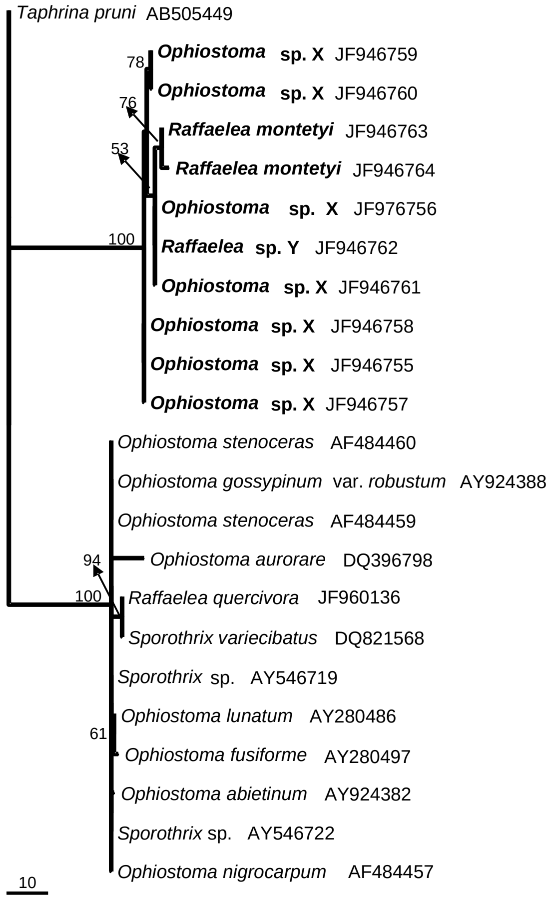

Fig. 4 - Phylogenetic relationships (Inferred MP) of Ophiostomatales species retrieved from *Platypus cylindrus* in Portugal with previously reported Ophiostomatales genera and species retrieved from GenBank. Consensus of 15 parsimonious trees inferred from the heuristic analysis of the partial sequence (681 bp) of the complete ITS ribosomal DNA region. Fungal strains obtained in this study are shown in bold. Numbers above nodes are bootstrap values based on 1000 iterations with 30 random additions. Informative characters 134.

Morphological investigation - *Raffaelea montetyi* Morelet was the most frequently recovered species from *P. cylindrus* (122 individual isolates corresponding to 45% of the isolates), mostly from the exoskeleton and mycangia (65.6% and 27, 9%, respectively).

R. montetyi was easily morphologically distinguishable from other fungi due to the presence of brown mycelium, with long, sparse and vigorous aerial hyphae and fast growth rate, reaching 70 mm diameter on MEA after 3 d at 25 C. The isolates showed a range of colony color from tawny (32) to yellowish-white (28) (FIG. 5A). The conidiophores are hyaline and mononematous or macronematous, erect, septate, slender with a tapered apex. Conidia adherent in a mucilaginous droplet, leaving a discreet cicatricial scar in the conidiogenous cells. Scanning electron micrographs showed tightly packed annellations at the apices of the conidiogenous cells (FIG. 5B-E). Conidia unicellular and hyaline, smooth-walled, (5.0-)7.4-8.1(-10) x (2.5-)3.5-3.8(-5.8) µm, rounded apex and truncated base, with variable forms (pyriform, claviform and cuneiform).

Raffaelea sp. Y was morphologically closely related to *R. canadensis* L.R. Batra and the less frequent (33 individual isolates corresponding to 12.2% of the isolates) species found, being mainly present in the exoskeleton and mycangia (57.6% and 27, 3%, respectively). *Raffaelea* sp. Y presented colonies effuse, yeast-like, with aerial brownish green floccose mycelium in the center, attaining a diam of 45-46 mm after 10 days on MEA, at 25 C, similar to those described for *R. canadensis* (Batra 1967, Funk 1970). Colony color from cream (27) to fuliginous (11) (FIG. 6A). Hyphae hyaline and septate repeatedly branched and interlocked, hyphal ends sometimes developing into torulose swellings. Conidiophores unbranched, hyaline, solitary or clustered together to form hyaline sporodoquia. Conidiogenous cells without conspicuous scars from conidial dehiscence. Conidia blastosporic (sprout cells), unicellular and hyaline, usually solitary but sometimes in monilioid chains after germination *in situ*, smooth-walled, triangular to oval or fusiform, (6.7-)7.7-8.4(-10.9) x (2.5-)3.2-3.6(-5.0) µm (FIG. 6B-D).

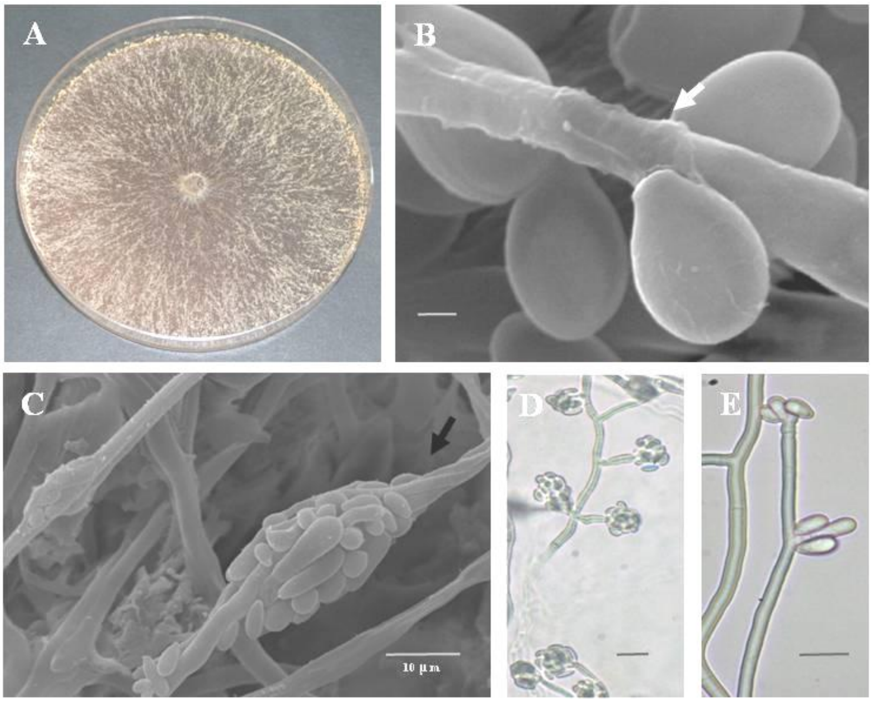

FIG. 5. *Raffaelea montetyi* culture, conidiophores and conidia. (isolate PC06.001=C2506) (A) Colony morphology after 5 days on malt extract agar in a 90 mm diameter plate. (B-C) scanning electron micrographs of conidiogenous cells with tightly packed annellations (white arrow) and conidia clusters (black arrow) (B bar = 1µm). (D-E) Light micrographs of the conidia clustering at the tip of conidiophores and conidiogenous cell (top in E) with annellations (bar=10 µm).

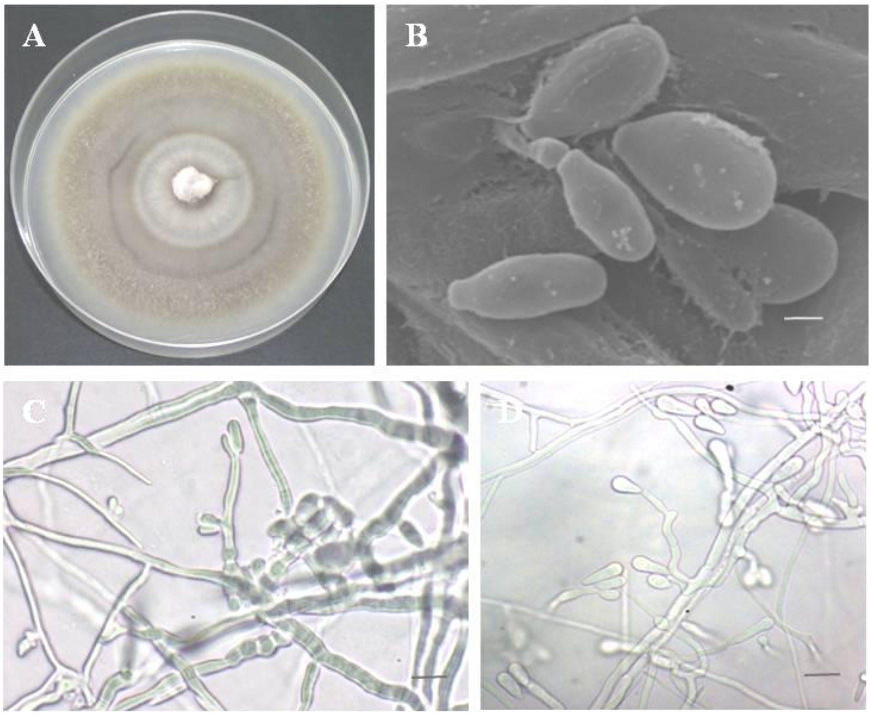

FIG. 6. Culture, conidiophores and conidia of *Raffaelea* sp. Y. (isolate PC05.041=C2516) (A) Colony morphology after 2 weeks on malt extract agar in a 90 mm diameter plate. (B) Scanning electron micrograph showing ovoid conidia (bar =1µm) (C-D). Light micrographs of conidia and conidiogenous cells without conspicuous scars from conidial dehiscence (bar=10µm).

Ophiostoma sp. X was the second most frequent species (115 individual isolates corresponding to 43% of the isolates, from which 113 were found alone), being mainly present in the mycangia and exoskeleton (41.7% and 39.1%, respectively).

Colonies were effuse, yeast-like, ivory-white (1) to cream-colored (27), smooth, later mucilaginous, with light concentric zonation, few with a light olive-green mottling appearing in the center or a sporodochium-like both in culture and in wood (FIG. 7A-D), corresponding to a *Hyalorhinocladiella* anamorph which formed protoperithecia in culture that did not developed necks, even when isolates were paired (Fig. 7E-F). Colonies grow slowly on MEA, 37-38 mm after 10 d at 25 C. Hyphae hyaline and septate that bound together forming compact hyphal ropes with cluster of conidia. Conidiophores micronematous and mononematous or synnematous, erect, septate, slender with a tapered apex. Conidial development occurring through both annellidic percurrent or sympodial proliferation but not leaving conspicuous scars (FIG. 7G-K). Conidia with various shape being the triangular the most prevalent, with (4.2-)5.2-5.8(-8.4) x (1.7-)1.8-2.2(-3.3)µm (FIG 7L-M). Our SEM micrographs do not help in the clarification on the mode of conidial development since we found hyaline conidiophores and primary annellidic conidiogenous cells as well as proliferation sympodial. These findings agree with Gebhardt and Oberwinkler (2005) and Massoumi Alamouti et al (2009).

Morphological comparisons and growth data indicate that this *Ophiostoma* species, with *Hyalorhinocladiella* anamorph, retrieved from *P. cylindrus* in *Q. suber* is different from any *Ophiostoma* previously described. This fungus could also be distinguished from previously described *Ophiostoma* and *Raffaelea* species based on DNA comparisons.

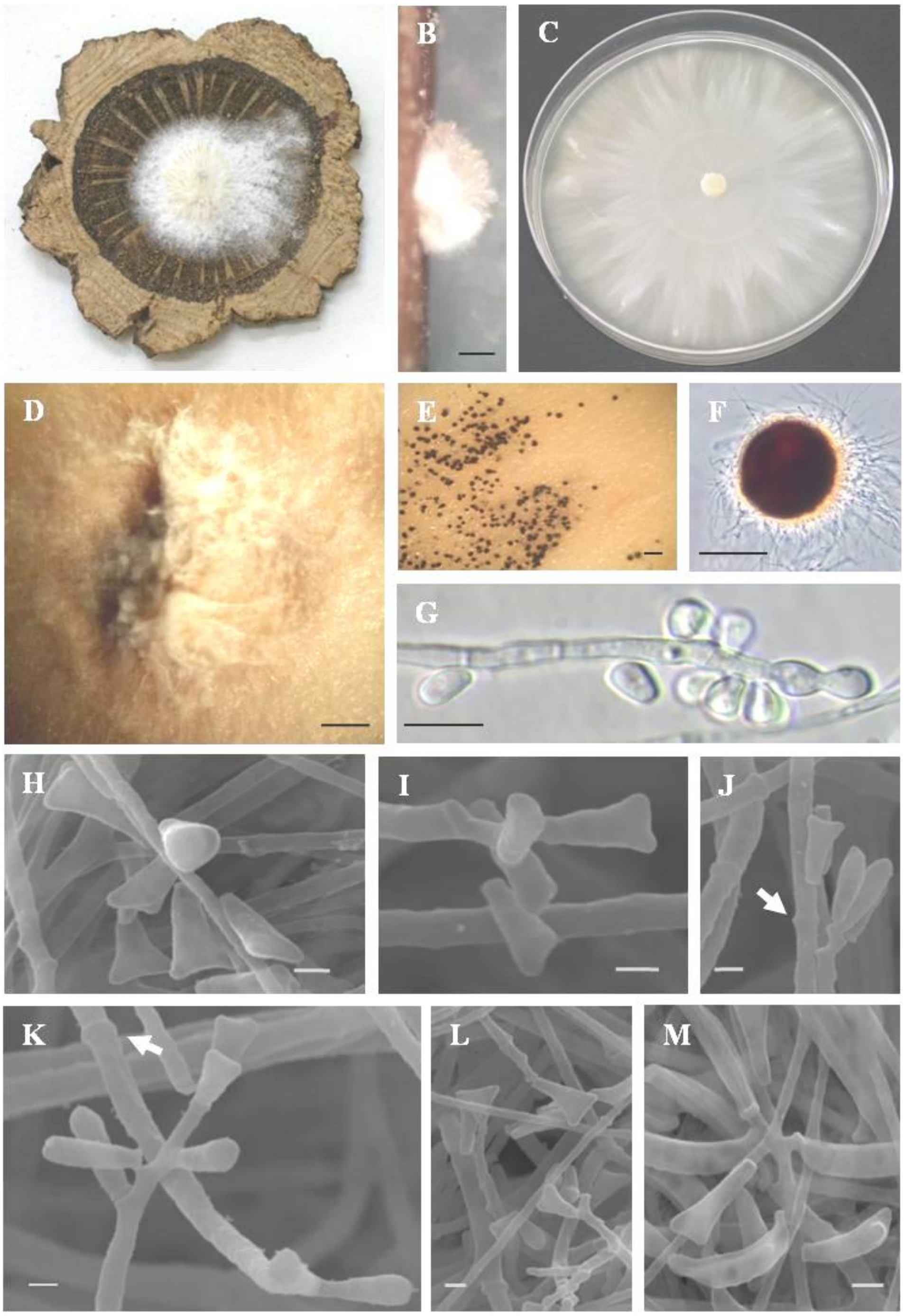

FIG. 5. Culture, conidiophores and conidia of *Ophiostoma* sp. X. (isolate PC06.032=C2508). (A) Culture growing on a wood disc of *Quercus suber*. (B) Sporodochia-like of conidiophores on wood (bar=250µm). (C) Colony morphology after 2 weeks on malt extract agar in 90 mm diameter plate. (D) Mass of conidiophores, conidia and yeast-like growth on malt extract agar (D-E bar=250µm). (E-F) Protoperithecia formed on malt extract agar. (F) Differential interference contrast (bar=50µm). (G) Newly formed conidia through percurrent proliferation without conspicuous scars (annellations) at the point of conidial dehiscence (bar=10 µm). (H-M) Scanning electron micrographs of conidia of various shape and conidiogenous with percurrent and sympodial proliferation and some conidiogenous cells showing annellations (arrows in J and K) (bar = 1µm).

DISCUSSION

Raffaelea montetyi was the predominant *Raffaelea* species isolated from *Platypus cylindrus* in Portugal, consistent with the results of Morelet (1998) and Gebhardt et al. (2004). Isolations from adults yielded *R. montetyi* in about 45% of the asexual cycloheximide-tolerant symbionts retrieved in this study. In contrast to the slow growth of most of ambrosia fungi, *R. montetyi* grows rapidly being able to colonize the galleries system much faster than the other symbionts. Taking into consideration previous studies on *P. cylindrus* mycoflora (Sousa 1996, Sousa et al. 1997) where *R. montetyi* was not identified, its presence on the symbiosis appears to constitute an ecological adaptation aiming faster and efficient host colonization.

Besides its association with *P. cylindrus*, *R. montetyi* was described as the main ambrosia fungi associated with *Xyleborus monographus* (Fabr.) and *X. dryographus* (Ratzeb.) in *Quercus robur* L. (Gebhardt et al. 2004). The former belongs to the natural entomofauna of Portuguese cork oak stands (Baeta Neves 1964, Lombardero 1996). As cross contamination of fungal symbionts may occur when more than one beetle species inhabits an individual tree (Gebhardt et al. 2004, Kim et al. 2011), it could be hypothesized that exchange of symbiotic fungi might have occurred between the galleries of *P. cylindrus* and *X. monographus*, where the insect tunnels cross to one another. Hence, this event prevents specificity of the fungi with their symbiotic beetle.

In Great Britain *P. cylindrus* is associated with *R. ambrosiae* (Baker 1963, Arx and Hennebert 1965), while in France and in Portugal it transports *R. montetyi* (Morelet 1998). However, isolates from Sousa et al. (1997) also contained *R. ambrosiae*. In the present study, the latter species was not found either associated with the insect or in the galleries. Therefore, several ambrosia fungi could be found associated with a particular ambrosia beetle in different geographical areas and the associations seem to evolve over time.

Putative Portuguese *R. canadensis* isolates retrieved from *P. cylindrus* in cork oak were identical to the holotype *R. canadensis*, both morphologically and genetically. Pairwise *p* distances between Portuguese and holotype strains available in GenBank, showed nucleotide differences of *ca.* 2% for the more discriminate nuclear rDNA LSU regions. This far, *R. canadensis* had been solely identified in association with *Platypus wilsonii* Swaine and *Gnathotrichus sulcatus* Lec. (as *A. sulcati*) in *Psedotsuga menziesii* (Douglas fir) (Funk 1970). However, a recent association of an unidentified *Raffaelea* species similar to *R. canadensis* (1.9% bp divergence for the LSU region) was also reported from another ambrosia beetle, *Xyleborus glabratus* Eichh. in *Persea borbonia* (redbay) hosts (Harrington et al. 2011). Based on the comprehensive molecular and morphological data from our study we believe the new associations recently found with the ambrosia beetles *P. cylindrus* and *X. glabratus* may consist of unreported *Raffaelea* closely related to *R. canadensis* with a selective preference for associations with a specific insect species. This new *Raffaelea* species is therefore newly described both in association with *P. cylindrus* and with cork oak declining trees.

It has generally been accepted that one or few fungal species are associated with a particular ambrosia beetle species (Batra 1963, Funk 1970) but recent studies pointed out that the symbionts of ambrosia beetles are more diverse, more promiscuous and more competitive than previously assumed and *Raffaelea* species may compete among each other for entrance to and growth within the mycangium (Harrington et al. 2010, 2011).

Ophiostoma sp. X was found closely associated with the mycangia of *P. cylindrus* being the second most frequent isolated species. The SSU and LSU sequences of all the cycloheximide-tolerant *Hyalorhinocladiella* isolates did not closely match available sequences. The closest match for the SSU sequence was *H. ips* and *O. ulmi*, (3.2% bp divergence) and for the LSU region, *Ceratocystiopsis minima, C. manitobense* and *C. ranaculosa* (up to 6.3% bp divergence). These divergences coupled with the differences found in the morphology with the absence of sprout cells or sporodochia typical of *Raffaelea* species. Based on the data herein we believe this species is likely to be new being thus the first *Ophiostoma* associated with the ambrosia beetle *P. cylindrus*. To date, only *Raffaelea* spp. and *Ambrosiella* spp. are clearly important food mycangial symbionts and a sexual state has not been found for any ambrosia beetle symbiont since it would not likely be an advantage for dispersal by an ambrosia beetle with mycangia (Harrington 2005, Harrington et al. 2010). It is probable that species of *Raffaelea* have derived from an ancestor with an ophiostomatoid sexual state and conidiogenesis similar to extant species of *Hyalorhinocladiella* or *Pesotum* (Gebhardt and Oberwinkler 2004).

The accurate isolation technique from the mycangia and intestinal content of the beetles employed (Henriques et al. 2006, Inacio et al. 2008) and the use of cycloheximide in the isolation medium allows better recovery of the asexual *Ophiostoma* symbionts. The Ophiostomatales found in the interaction with *P. cylindrus* were also isolated from their galleries in host tree (Inacio et al. 2010). Moreover, *Ophiostoma* isolates besides being retrieved from the insect tunnels were obtained from declining oaks, with no visible attack of insects. However, such infections apparently could be aided by the frequent aborted attacks of *P. cylindrus* in the trunk of newly debarked trees. Thus, all the complexities of such interaction rather need an exhaustive investigation and much work is needed in studying genetic variation and species limits of fungal symbionts of ambrosia beetles.

Ophiostoma is a known variable genus including some monophyletic lineages of species that share similar morphological characters (Zipfel et al. 2006, Zanzot el at. 2010). The use of the ITS regions to discern species in closely related fungi, namely Ophiostomatales is recurrent (Kim et al. 1999, De Beer et al. 2003, Zhou et al. 2006). Unfortunately, the sequencing of the ITS region in ophiostomatoid fungi presented a technical challenge due to high GC content (Mullineux and Hausner 2009). Spliceosomal introns of recent origin have been reported to be widespread in Ascomycetes and could be related to the variability found in ITS1 (Bhattacharya et al. 2000). In our case we found a constant, similar ITS1 region for all Portuguese isolates. This pattern was also present for the remaining regions. No match where found in GenBank for the ITS1 region, but rather the remaining 5.8S-ITS2 regions. In order not to enforce alignment with the large ITS1 region, we opted to only use the region that could be accurately compared with Ophiostomatales representatives in GenBank for our analyses. Alignments of large and unique single taxon nucleotide length, or intron sequences, could bias the phylogenetic results and hence not accurately reflect the taxonomical relationships in analysis. Similar approaches have been used for the ITS regions, adjacent SSU and LSU gene regions and β-tubulin in Ophiostomatales (Wingfield et al. 1999, Gorton et al. 2004, Zipfel et al. 2006, Massoumi Alamouti et al. 2009, Zanzot et al. 2010). Nonetheless the constant ITS1 region in all Portuguese isolates together with the phylogenetic inferences from the 5.8S-ITS2 region in this study, further substantiates the presence of a distinct lineage of Ophiostomatales in Portugal.

Species of *Raffaelea* have been recently related with mass mortality of oak trees in Japan (Kubono and Ito 2002, Matsuda et al. 2010) and laurel wilt of redbay (Fraedrich et al. 2008) as well as avocado in the United States (*Persea americana*) (Eskalen & McDonald 2011). Likewise, Portuguese *Raffaelea* isolates have a proven pathogenicity against cork oak seedlings (Inacio et al. 2011) and therefore could have a significant role in cork oak decline. A comprehensive morphological and phytopathological assessment, coupled with a robust phylogenetic analysis, of the complex of fungi associated with *P. cylindrus* populations across a trans-European and north African wide-range number of cork oak stands could further clarify the relationships of ambrosia beetles and their associated fungi with cork oak decay and contribute to halt the spread of the decline in the unique cork oak landscapes enclosed in the Mediterranean basin.

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LITERATURE CITED

Arx JA von, Hennebert GL. 1965. Deux champignons ambrosia. Mycopathol et Mycologia Applic 25:309–315

Baeta Neves CM. 1964. Sobre a representação da família Scolytidae (Coleoptera) na entomofauna florestal indígena. Agros XXVII:190-196.

Baker JM. 1963. Ambrosia beetle and their fungi, with particular reference to *Platypus cylindrus* Fab. Symposia Soc. Gen. Microbiol. 13:323-354.

Batra LR. 1963. Ecology of ambrosia fungi and their dissemination by beetles. Trans Kansas Acad Sci 66:213–236.

—. 1967. Ambrosia fungi: A taxonomic revision and nutritional studies of some species. Mycologia 59:976-1017.

Beaver RA. 1989. Insect-fungus relationships in the bark and ambrosia beetles. In: Wilding N, Collins NM, Hammond PM, Webber JF, eds. Insect-fungus interactions. London: Academic Press. p 121–143.

Bhattacharya D, Lutzoni F, Reeb V, Simon D, Nason J, Fernandez F. 2000. Widespread occurrence of spliceosomal introns in the rDNA genes of ascomycetes. Mol Biol Evol 17:1971–1984.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acid Res 31:3497–500.

Clamp M, Cuff J, Searle SM, Barton GJ. 2004. The Jalview java alignment editor. Bioinformatics 20:426–427.

De Beer W, Wingfield BD, Wingfield M. 2003. The *Ophiostoma* piceae complex in the Southern Hemisphere: a phylogenetic study. Mycol Res 107:469–476.

Eskalen A, McDonald V. 2011. First Report of *Raffaelea canadensis* Causing Laurel Wilt Disease Symptoms on Avocado in California. Plant Dis doi: 10.1094/PDIS-03-11-0203.

Farrell BD, Sequeira AS, O'Meara BC, Normark BB, Chung JH, Jordal BH. 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). Evolution 55:2011–2027.

Fraedrich SW, Harrington TC, Rabaglia RJ, Ulyshen MD, Mayfield AE, Hanula JL, Eickwort JM, Miller DR. 2008. A fungal symbiont of the redbay ambrosia beetle causes a lethal wilt in redbay and other Lauraceae in the southeastern United States. Plant Dis 92:215–224.

Francke-Grosmann H. 1967. Ectosymbiosis in wood-inhabiting insects. In: Henry SM, ed. Symbiosis vol. 11. New York: Academic Press. p 142–206.

Funk A. 1970. Fungal symbionts of the ambrosia beetle *Gnathotrichus sulcatus*. Can J Bot 48:1445–1448.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for Basidiomycetes - application to the identification of mycorrhiza and rusts. Molecular Ecology 2:113–118.

Gebhardt H, Begerow D, Oberwinkler F. 2004. Identification of the ambrosia fungus of *Xyleborus monographus* and *X. dryographus* (Coleoptera: Curculionidae, Scolytinae). Mycol Progress 3:95–102.

—, Oberwinkler F. 2005. Conidial development in selected ambrosial species of the genus *Raffaelea*. Antonie van Leeuwenhoek 88:61–66.

—, Weiss M, Oberwinkler F. 2005. *Dryadomyces amasae*: a nutritional fungus associated with ambrosia beetles of the genus *Amasa* (Coleoptera: Curculionidae, Scolytinae). Mycol Res 109:687–696.

Gorton C, Kim SH, Henricot B, Webber J, Breuil C. 2004. Phylogenetic analysis of the bluestain fungus *Ophiostoma minus* based on partial ITS rDNA and β-tubulin gene sequences. Mycol Res 108:759–765.

Harrington TC. 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. Mycologia 73:1123–1129.

—. 1992. *Leptographium*. In: Singleton LL, Mihail JD, Rush CM, eds. Methods for research on soilborne phytopathogenic fungi. St. Paul, Minnesota: APS Press. p 129–133.

—. 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. In: Vega FE, Blackwell M., eds. Insect-fungal associations: ecology and evolution. New York: Oxford University Press, Inc. p 257–292.

—, Aghayeva DN, Fraedrich SW. 2010. New combinations in *Raffaelea*, *Ambrosiella*, and *Hyalorhinocladiella*, and four new species from the redbay ambrosia beetle, *Xyleborus glabratus*. Mycotaxon 111:337–361.

—, Fraedrich SW. 2010. Quantification of propagules of the laurel wilt fungus and other mycangial fungi from the redbay ambrosia beetle, *Xyleborus glabratus*. Phytopathology 100:1118–1123.

—, —, Aghayeva DN. 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the Lauraceae. Mycotaxon 104:399–404.

—,Yun H.Y, Lu S., Goto H.; Aghayeva D., Fraedrich S. 2011. Isolations from the redbay ambrosia beetle, *Xyleborus glabratus*, confirm that the laurel wilt pathogen, *Raffaelea lauricola*, originated in Asia. Mycologia doi:10.3852/10-417.

Henriques J, Inacio ML, Sousa E. 2006. Ambrosia fungi in the insect-fungi symbiosis in relation to cork oak decline. Rev Iberoam Micol 23:185-188.

Inacio ML, Henriques J, Lima A, Sousa EM 2008. Fungi of *Raffaelea* genus (Ascomycota: Ophiostomatales) associated to *Platypus cylindrus* (Coleoptera: Platypodidae) in Portugal. *Rev. Ciências Agrárias* **31**: 96-104.

—, Henriques J, Sousa E. 2010. Mycobiota associated with *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) on cork oak in Portugal. IOBC/wprs Bull 57:87-95.

—, Henriques J, Lima A, Sousa E. 2011. Ophiostomatoid fungi associated with cork oak mortality in Portugal. IOBC/wprs Bull 58 (*in press).*

Kim KH, Choi YJ, Seo ST, Shin HD. 2009. *Raffaelea quercus-mongolicae* sp. nov. associated with *Platypus koryoensis* on oak in Korea. Mycotaxon 110: 189–197.

Kim S, Harrington TC, Lee JC, Seybold SJ. 2011. *Leptographium tereforme* sp. nov. and other Ophiostomatales isolated from the root-feeding bark beetle *Hylurgus ligniperda* in California. Mycologia 103:152–163.

Kubono T, Ito S. 2002. *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). Mycoscience 43:255-260.

Kusaba M, Tsuge T. 1995. Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal transcribed spacer of ribosomal DNA. Curr Genet 28:491–498.

Lee S, Kim J-J, Fung S, Breuil C. 2003. A PCR-RFLP marker distinguishing *Ophiostoma clavigerum* from morphologically similar *Leptographium* species associated with bark beetles. Can J Bot 81:1104–1112.

Lombardero MJ. 1996. The species of Xyleborini (Coleoptera: Scolytidae) in the Iberian Peninsula. Boln Asoc esp Ent 20:173-191.

Luo A-R, Zhang Y-Z Qiao H-J, Shi, W-F, Murphy RW, Zhu C-D. 2010. Outgroup selection in tree reconstruction: a case of the family Halictidae (Hymenoptera: Apoidea). Acta Entomol Sinica 53:192-201.

Massoumi Alamouti S, Kim J-J, Humble L, Uzunovic A, Breuil C. 2007. Ophiostomatoid fungi associated with the northern spruce engraver, *Ips perturbatus*, in western Canada. Antonie van Leeuwenhoek 91:19–34.

Massoumi Alamouti S, Tsui CM, Breuil C. 2009. Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. Mycol Res 113:822–835.

Matsuda Y, Kimura K, Ito S. 2010. Genetic characterization of *Raffaelea quercivora* isolates collected from areas of oak wilt in Japan. Mycoscience 51:310–316

Morelet M. 1998. Une espèce nouvelle de *Raffaelea*, isolée de *Platypus cylindrus*, coléoptère xylomycetophage des chênes. Ann Soc Sci Nat Archeol Toulon et Var 50:185–193.

Mullineux T, Hausner G. 2009. Evolution of rDNA ITS1 and ITS2 sequences and RNA secondary structures within members of the fungal genera *Grosmannia* and *Leptographium*. Fungal Genetics and Biology 46:855–867.

O'Donnell K. 1993. *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The Fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematic. CAB International, Wallingford, p 225–233.

Pereira H. 2007. Cork, biology, production and uses. 1 Ed. Amsterdam: Elsevier Publications. 336 p.

Riddell RW. 1950. Permanent stained mycological preparations obtained by slide culture. Mycologia 42:265–270.

Plattner A, Kim J-J, Reid J, Hausner G, Lim YW, Yamaoka Y, Breuil C. 2009. Resolving taxonomic and phylogenetic incongruence within species *Ceratocystiopsis minuta*. Mycologia 101:878-887.

Roets F, De Beer ZW, Crous PW, Dreyer LL, Wingfield MJ. 2008. *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea infructescences* in South Africa. Mycologia 100:496-510.

Saccardo PA. 1891. *Chromotaxia seu nomenclator colorum polyglottus adclitis speciminibus coloratis ad botanicorum et zoologorum*. Patavii, 22 p.

Silva JS, Catry F. 2006 Forest fires in cork oak (*Quercus suber* L.) stands in Portugal. International Journal of Environmental Studies, 63:235–257.

Six DL. 2003. Bark beetle–fungus symbioses. In: Bourtzis K, MillerTA (eds), Insect Symbioses. CRC Press, Boca Raton, FL, p 97–114.

Sousa EM. 1995. Les principaux ravageurs du chêne liege au Portugal. Leurs relations avec le déclin dês peuplements. IOBC/ wprs Bull 6:18-22.

Sousa EM. 1996. Sousa, E.M. (1996). Contribution à l'étude de la biologie de populations de *Platypus cylindrus* (Coleóptera: Platypodidae) dans des peuplements de chênes liège au Portugal. Thèse de Doctorat, Lyon, France, 153 p.

Sousa EM, Débouzie DD. 2002. Biological characteristics of *Platypus cylindrus* F. in Portugal. IOBC/ wprs Bull. 25:75-83.

Sousa EM, Débouzie DD, Pereira H. 1995. The role of the insect *Platypus cylindrus* F. (Coleoptera, Platypodidae) in the decline of cork oak stands in Portugal. IOBC/ wprs Bull. 18:24-37.

Sousa EM, Inacio ML. 2005. New Aspects of *Platypus cylindrus* Fab. (Coleoptera: Platypodidae): Life History on Cork Oak Stands in Portugal. In*:* Lieutier F, Ghaioule, D. (Eds.), Entomological Research in Mediterranean Forest Ecosystems, INRA Editions, p 147-168.

Sousa EM, Tomaz IL, Moniz FA, Basto S. 1997. Distribution of fungi associated to *Platypus cylindrus* Fab. (Coleoptera: Platypodidae*)*. Phytopath Medit 36:145-153.

Swofford DL. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods), Version 4.0b 10. Sinauer Associates, Sunderland, MA.

Tsuneda A, Currah R. 2006. Toward a deeper understanding of the nature of pleomorphism in conidial fungi. Reports of the Tottori Mycological Institute 44:1-52.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), PCR Protocols: genes for phylogenetics. Academic Press, San Diego, p 315–322.

Wingfield BD, Viljoen CD, Wingfield MJ. 1999. Phylogenetic relationships of ophiostomatoid fungi associated with *Protea infructescences* in South Africa. Mycol Res 103:1616-1620.

Zanzot JW, de Beer ZW, Eckhardt LG, Wingfield MJ. 2010. A new *Ophiostoma* species from loblolly pine roots in the southeastern United States. Mycol Progress 9: 447-457.

Zhou X, de Beer ZW, Wingfield MJ. 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. Stud Mycol 55:269–277.

Zipfel RD, de Beer ZW, Jacobs K, Wingfield BD, Wingfield MJ. 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Stud Mycol 55:75–97.

FOOTNOTES

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CHAPTER 4

DISCUSSION

4. DISCUSSION

Cork oak forests are a very specific, delicately-balanced ecosystem which only persists in the Mediterranean basin. It is therefore of major concern that over the last three decades an alarming decline of trees has increased across its distribution area, namely in the representative Portuguese cork oak stands. Being cork oak decline a multifactorial process, several causes have been pointed out as contributors to tree mortality and loss of vigour, namely biotic factors (Cabral and Sardinha 1992). *Platypus cylindrus* emerged as a determinant factor in the decline of stands and its population outbreaks in the last decades have caused heavy economical damages since cork loses its quality and ultimately trees death overcomes (Sousa, 1995, Sousa and Débouzie 2002).

The symptoms and signs exhibited by cork oaks attacked by *P. cylindrus,* including the presence of numerous entry holes and profuse sawdust emerging from these holes, do not reveal the real dimension of the attack intensity within the trunk. Only cutting the attacked tree and following the insect tunnels, is possible to ascertain the extent of the galleries into the sapwood. According to Sousa and Débouzie (1999) a variable number of branches, above and below the initial entry hole, is excavated. The galleries system often becomes quite complex. Coupled with this extensive boring activity, the inoculation of ambrosia fungi is part of the insect strategy to establish its offspring in the host trees (Sousa *et al.* 1995, Sousa and Inacio 2005). *P. cylindrus* has been associated with a diverse number of fungal species (Baker 1989, Cassier *et al.* 1996, Sousa *et al.* 1997). The exact role of most of these fungi is either unknown or not fully understood. The research work presented by this thesis improves the knowledge on the mycobiota associated with the oak pinhole borer in Portuguese cork oak stands and opens new perspectives to understand the decline of *montado.*

P. cylindrus is closely associated with several fungal species, the most abundant and important of which belong to the Ophiostomatales group of ascomycetous fungi. Ophiostomatoid fungi have been isolated from *P. cylindrus*' mycangia, gut, body surfaces and adult and juveniles galleries. The fungal species diversity was similar among sites and did not vary in the three years of sampling.

Concerning the whole assemblage of fungi vectored by *P. cylindrus* in Portuguese cork oak stands, the early works of Sousa (1996) and Sousa *et al.* (1997) pointed out the presence of *Raffaelea ambrosiae* as the single primary ambrosia fungus, and several secondary associated species. Taking into consideration the recent insect outbreaks, the hypothesis of more effective fungal associations was established (Sousa *et al.* 1997). In this context, our initial works aimed to ascertain the identity of the complex of fungi vectored by *P. cylindrus.* As previously reported, several cosmopolitan fungi were retrieved from the insect besides the recurrent Ophiostomatales isolates (Henriques *et al.* 2006, Inacio *et al.* 2008, 2010a, b). The specific role of fungi from the genera *Gliocladium*, *Trichoderma* and *Scytalidium* remains unclear and their wood degrading capacity still remains under discussion. The former fungi are still regarded as antagonistic fungi (Henriques 2007). However, considering more recent results (Plattner 2008, Harrington *et al.* 2010), these fungi together with some *Acremonium*, *Aspergillus*, *Beauveria*, *Geotrichum*, *Fusarium*, *Paecylomyces*, *Penicillium* species, among others, are considered ubiquitous fungi with no more than a commensal relationship with the insects. Although these fungi may not constitute a significant component of the beetle mycoflora, most of them present a profuse growth pattern which overwhelms other fungi such the Ophiostomatales (Harrington 2005).

Some isolates that did not produce spores were later identified, with the support of A. Franceschini and B. Linaldeddu from the Stazione Sperimentale del Sughero (Sardinia, Italy), as belonging to *Botryosphaeria* genus. This genus comprises species potentially pathogenic to *Q. suber* (Alves *et al.* 2004, Linaldeddu *et al.* 2007, Luque *et al.* 2008). The specific identification of these isolates is still under study to ascertain the possibility of *P. cylindrus* being a vector of cork oak canker.

Biscogniauxia mediterranea, the causal agent of cork oak charcoal canker was consistently present in all the insect organs and galleries. It was also shown that *Nodulisporium* sp. associated with *P. cylindrus* (Sousa *et al.* 1997) corresponds to the *B. mediterranea* anamorph, based on molecular evidence from the ITS region (Inacio *et al.* 2011a). These isolates have proven pathogenicity towards cork oak seedlings and produce phytotoxic compounds causing necroses in tobacco indicator plants. These findings are in accordance with the results of Evidente *et al.* (2005). The great abundance of *B. mediterranea* inoculum on colonized organs of host trees (Collado *et al.* 2001, Jiménez *et al.* 2005, Henriques *et al.* 2011), and the association to *P. cylindrus,* undoubtedly contributes to spread the disease in Portuguese oak stands, namely in young trees which have been increasingly affected by this pathogen (Henriques 2007, Sousa *et al.* 2007).

Although more clearly defined than a decade ago, the taxonomy of fungi in the Ophiostomatales remains in a state of change. As these fungi lack clear morphological defining features, their identification mainly relies on genetic characterization (Massoumi Alamouti 2009, Harrington *et al.* 2010). The conidial development previously assumed as an important characteristic in defining species within Ophiostomatales lost their value since in some cases, the anamorphic state showed a continuum of conidial formation from sympodial to annellidic with intermediate forms (Matsuda *et al.* 2010). There is emerging evidence that the genus *Ophiostoma*, which comprise the largest number of species within Ophiostomatales, is a generic aggregate including groups of species that represent distinct monophyletic lineages (DeBeer and Wingfield 2006, Nkuekam *et al.* 2011). In our initial works, and taking into consideration the available literature (Cassar and Blackwell 1996, Jones and Blackwell 1998, Rollins *et al.* 2001), it was sequenced the SSU rDNA of the Ophiostomatalean fungi retrieved from *P. cylindrus*. The species were then identified as *R. ambrosiae*, *R. canadensis* and *R. montetyi* (Inacio *et al.* 2008). Since early studies on ambrosia fungi, the first species was considered as the primary *P. cylindrus* symbiont (Francke-Grosmann 1967, Beaver 1989, Sousa 1996). *R. canadensis* was now identified for the first time in association with *P. cylindrus* in cork oak. *R. montetyi* had already been associated with the oak pinhole borer (Morelet 1998).

R. montetyi and *R. canadensis* showed pathogenicity towards cork oak seedlings, although the latter did not cause plant mortality (Inacio *et al.* 2011b). Due to heavy restrictions and the law protection given to *Q. suber* (DL nº 11/97, 14 January) it will not be possible to do pathogenic test in adult trees, hence the use of inoculation of seedlings.

Finally, considering the more recent studies on Ophiostomatales ambrosial fungi (Massoumi Alamouti *et al.* 2009, Harrington *et al.* 2010, Kim *et al.* 2011), we performed a multigene analysis with fourteen representative morphotypes, followed by a morphological characterization. Thus, rather than the SSU rDNA sequencing, attempts to obtain the sequences of rDNA LSU and ITS, and the β-tubulin were made. While routine sequencing of amplicons from the ITS region, from the LSU and SSU regions of rDNA as well as homology comparison with sequence databases facilitate rapid species identification for most fungi (Nilsson *et al.* 2008), the sequencing of ITS region in the Ophiostomatoid fungi usually presents a technical problem due to the presence of several homopolymers (Mullineux and Hausner 2009, Tsui *et al.* 2010, Nkuekam *et al.* 2011), *Raffaelea* genus suffering particularly with this phenomenon (Fraedrich *et al.* 2008, Harrington *et al.* 2011). Despite these difficulties, we succeed to sequence the ITS1 and the 5.8-ITS2 regions of almost all isolates under study. Β-tubulin sequences were impossible to be obtained. Prior to our study, the only complete and variable dataset of DNA sequences for identification of *Raffaelea* species was the LSU rDNA dataset (Harrington *et al.* 2010).

As the sequenced SSU region does not show sufficient variation to discriminate among species of Ophiostomatoid (DeBeer *et al.* 2003, Plattner *et al.* 2009, Harrington *et al.* 2010), the multigene analysis comprising the LSU and ITS sequences, allowed us to refine the previous results. The primary ambrosia fungus of *P. cylindrus* species was confirmed to be *R. montetyi*. This species, unlike most *Raffaelea* spp., grows very quickly hence establishing itself rapidly in the host and facilitating colonization by insect vectors. *R. montetyi* had previously been isolated from *P. cylindrus* (Morelet 1998) but confirmation of its implication with declining oaks was not assessed until now. In our study, it was possible to isolate *R. montetyi* from *P. cylindrus'* galleries exhibiting discoloration. Since Koch's postulates were confirmed (Inacio 2011b) the role of *R. montetyi* on host colonization and weakening was clearly demonstrated. Cultural, morphological and genetic analyses reveled that our isolates are very similar to *R. quercivora* and the complete characterization and discrimination of these two species needs to be further substantiated (Matsuda *et al.* 2010). *R. montetyi* has also been previously found to be the single primary ambrosia of *Xyleborus monographus* and *X. dryographus* in south-west Germany attacking *Quercus robur* (Gebhardt *et al.*

2004). The occurrence of cross contamination between the galleries of *P. cylindrus* and *X. monographus* inhabiting the same tree emerges as a possibility for the new association of fungi with *P. cylindrus*. Host switching of ambrosia symbionts may contribute to points of incongruence between the beetle and fungal phylogenies. Overall, the intercontinental movement of these fungi, most likely through infected plant material and insects often associated with them, is also a hypothesis to be considered (Nkuekam *et al.* 2011).

Taking into account the phylogenetic results others than the SSU analysis, *P. cylindrus* also establishes new symbioses with a species of *Raffaelea* closely related to *R. canadensis,* differing only in 11/554 bp in the LSU sequences. In addition, LSU and ITS phylogenetic analysis, coupled with morphological results, allow concluding that a new *Ophiostoma* species with a *Hyalorhinocladiella*-anamorph is present in Portugal, in association with *P. cylindrus*. In order to achieve the description of this new species, we have to evolve the protoperithecia in the culture media and obtain the mature perithecia. Numerous isolates from this species are being paired in MEA 1% with an oak twig in the midst (Harrington, pers. com.) in cross fertilization tests. Pathogenicity tests were also conducted during this research. The new *Ophiostoma* species caused extensive seedling mortality (data unpublished). We observed that *Ophiostoma* isolates obtained from the insect and its galleries were also isolated from declining cork oaks with visible aborted attacks of *P. cylindrus.* Thus, even if insects did not succeed to colonize the tree they are able to inoculate pathogens into a susceptible host. Without *P. cylindrus* to serve as a vector, it would be virtually impossible for these fungal species to reach new hosts. In addition, fungi in the Ophiostomatales require pre-existing wounds in order to infect their hosts, hence the beetles carrying them either visit wounds on trees or produce these wounds themselves (Nkuekam *et al.* 2011). As such, without *P. cylindrus*, it would not be possible for the fungal species to continue their life cycle, just as without the fungi, the oak pinhole borer would have an extremely difficult time colonizing new trees. It is, indeed, an obligatory symbiosis. Inside the host, the fungus can rapidly spread from several points.

In the wood of their host trees, ambrosia fungi usually penetrate only a few mm into the xylem and their growth is usually restricted to areas surrounding the galleries (Kirisits 2007). However, this new *Ophiostoma* species penetrates several cm into the sapwood of its cork oak hosts and causes a brown discoloration in the xylem. It is likely that as for the case of *R. lauricola*, in aborted attacks in the sapwood of healthy trees, thousands of spores of the fungus may ooze from the mycangia and infect the severed vessels, resulting in systemic colonization of the host (Fraedrich *et al.* 2008, Kim *et al.* 2011). Solheim and Krokene (1998) noted that one beetle gallery was probably a more severe challenge to the tree's defenses compared to one cork borer inoculation. This new strategy allows the insect to spread the wilt disease in cork oak stands facilitating beetles establishment. In this way, they do not need to wait another decorking cycle to establish new populations, and trees could be potentially colonized from the decorking until they rebuilt a thick cork layer. Besides that, the beetle's recent success probably also relied on climate changes, where continuously mild winters caused less offspring mortality and where summer droughts stressed trees, thus making host trees more susceptible to attack (Desprez-Loustau *et al.* 2006).

Ambrosia beetles have generally been thought to be tightly associated with one or only a few symbiotic fungi (Batra 1967, Funk 1970, Gebhardt *et al.* 2004). Contrary to these early ideas, the ambrosia fungi are not species-specific to the beetles. Our new *P. cylindrus*' associations challenge the theory of a species-specific symbiont but are in accordance with more recent studies. In some cases, up to fifteen Ophiostomatales were isolated from one single *Dendroctonus ponderosae* Hopkins (Plattner 2008). Others obtained more than six species belonging to *Raffaelea* and *Ophiostoma* genera from the ambrosia beetle *Xyleborus glabratus* (Harrington and Fraedrich 2008, Harrington *et al.* 2011). It will be thus expected that more species could be isolated from *P. cylindrus*, especially if collected in other parts of its distribution range. If we employ a combination of isolation techniques, we will facilitate better recovery of Ophiostomatoid fungi than other isolation techniques used in the past. This is the case of the thorough isolations of the mycangia that we used and the serial dilution plating technique (Harrington *et al.* 2010). As better isolation methods and DNA sequencing are applied, it is likely to be found that many ambrosia symbionts are vectored by a single ambrosia beetle (Plattner 2008, Plattner *et al.* 2009, Harrington *et al.* 2010). Many studies on the mycobiota of European and North American bark beetles have shown that the species spectrum and frequency of fungal associates varies depending on the sources and the methods of isolation. Thus, the application of several isolation methods and the examination of various niches increase the change to discover the complete assemblage of fungi associated with a specific ambrosia beetle species.

Overall, we believe the concept of ambrosia fungi of Harrington *et al.* (2008), which emended *Raffaelea* to include all the ambrosia fungi, must be re-evaluated. The new *Ophiostoma* species identified in this study clearly falls out the *Raffaelea* clade. However, it is an ambrosia fungus as it provides a supplementary source of food for *P. cylindrus*' larvae and teneral adults, since it was isolated from mycangia and intestinal content both from adults and larvae, thus, fulfilling the set of concepts that allows classifying it as ambrosia fungus. Given that Harrington's definition has an ecological value rather than taxonomical, and considering that several authors have isolated blue-stain fungi from ambrosia beetles (Beaver 1989, Plattner 2008, Kirisits *et al.* 2002, Harrington *et al.* 2011, Kim *et al.* 2011), we believe that staining fungi closely associated with ambrosia insects can constitute the ambrosial palisades and we propose the definition of ambrosia fungi as in its original form, i.e., "*pleomorphic fungi serving as adults and larvae nourishment and transported in specialized mycangia*", (Batra 1967, Francke-Grosmann 1967) with an additional remark "*and belonging to the Ophiostomatales*".

A common trait to all Ophiostomatales species reported in this work is that they have a constant, similar ITS1 region. This fact coupled with the phylogenetic inferences from the 5.8S-ITS2 region in this study, substantiates the presence of a distinct lineage of Ophiostomatales in Portuguese cork oak stands.

While these associations benefit both the beetle and its associated fungi, trees are negatively impacted by the presence of the beetle and the fungi. The pathogenic effects of the beetle-fungi complex result in a decrease in tree health, typically resulting in tree death, helping the beetle in successfully colonizing and reproducing in host trees (Six and Paine 1998). By helping to exhaust the trees' defenses, by blocking water flow and by consuming carbon reserves stored in parenchyma rays, fungi reduce the host's defense capabilities against beetle invasion (Paine 1984, Paine *et al.* 1997).

In terms of role in oak decline, the combined action of *P. cylindrus* massive attacks and extensive boring with the inoculation of ambrosia fungi, leads to an increase of tree mortality enhanced in these past two decades through these new associations with more aggressive and wilt causing fungi. The ambrosia beetles are considered to be much more efficient transmitting the vascular wilt pathogens to susceptible trees than bark beetles, such as *Ophiostoma novo-ulmi* vectored by Scolytidae (Harrington and Fraedrich 2010).

Understanding the ecology and population dynamics of *P. cylindrus*-associated fungi is important for the surveillance and management of the beetle-fungal complex, and could improve prediction and modeling. Biological control of the pathogens may prove possible through manipulation of the mycangial mycoflora. However, with such consistently isolations of Ophiostomatales from each beetle, incidence of cork oak wilt would appear to be limited only by the population level of *P. cylindrus*, on which disease management should focus.

The ambrosia beetles and their associated fungi constitute a small part of a much larger food web, the complexities of which we have barely start to understand. There are indeed many unanswered questions about the extraordinary complexity between these wood-inhabiting beetles, the assembly of fungi which they transmit, and the tree which supports the whole community. We believe the research described herein contributed to clarify focal aspects of the pathology of cork oak decline, which could help to avoid costly mistakes in the future and preserve the economic and cultural heritage of the unique cork oak stands and landscapes present in the Mediterranean.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The main objectives of the present research work were the identification of the complex of fungi transported by *Platypus cylindrus* in Portugal and to ascertain the pathogenic role of the main ambrosia fungi and their role in cork oak decline. Since the presence of *P. cylindrus* in Portuguese cork oak stands has drastically increase in the past few decades, the initial hypothesis was that *P. cylindrus* would have established fungal associations which would enable it to better colonize host trees by compromising its defenses. The different studies conducted herein had the endeavour of better understanding the complex cork oak decline phenomenon in Portugal by increasing current scientific knowledge on key aspects as the role of *P. cylindrus* on the decline of Portuguese cork oak forests and woodlands, as well as the nature of the pathogens it could vector.

Fungi were isolated directly from adult beetles and their galleries in cork oak and a tentative identification of isolates based on the morphology was made. Several fungi were obtained, namely the cosmopolitan *Acremonium*, *Aspergillus*, *Beauveria*, *Geotrichum*, *Gliocladium*, *Fusarium*, *Paecylomyces*, *Penicillium, Trichoderma* and *Scytalidium* species, among others. Some of them might act as secondary ambrosia fungi participating in wood degradation, hence facilitating beetles establishment in host trees. In the same manner, some species might have an antagonistic action preventing fungi overgrowth inside the tunnels. These putative roles still remain under discussion.

More detailed studies are needed to final identification of isolates of *Botryosphaeria* genus obtained in the present study since this genus comprises species potentially pathogenic to cork oak. *Biscogniauxia mediterranea*, a pathogen widely present in Portuguese cork oak stands and woodlands, was consistently identified in association with *P. cylindrus*. The fungus pathogenicity towards cork oak seedlings was proven confirming *B. mediterranea* as a *P. cylindrus*' symbiont possibly involved in cork oak decline.

A holistic approach to the study of the Ophiostomatales obtained, combining the application of cultural, morphological and molecular techniques, allowed the identification of the primary ambrosia fungi associated with the oak pinhole borer. *Raffaelea montetyi* is the most important or among the most important fungal associates of *P. cylindrus*. Besides *R. montetyi*, a new species of *Raffaelea* closely related to *R. canadensis,* and a new *Ophiostoma* species were consistently retrieved from *P. cylindrus* mycangia and insect galleries in *Q. suber*. To our best knowledge, these ophiostomatoid fungi were identified for the first time, both in association with *P. cylindrus* and in cork oak stands.

The mutual network of beetle galleries and ambrosia fungi disrupts the movement of water within the tree and rapidly kills it. Since the isolated fungi were pathogenic to cork oak seedlings and are associated with wood discoloration and wilt symptoms, they were considered as declining agents, being transported and vectored by *P. cylindrus* during adult flight and directly inoculated in the host tree while foraging.

We determined that the new *Ophiostoma* species is genetically different from previously reported species in the literature and is an undescribed species of the genus *Ophiostoma,* anamorph *Hyalorhinocladiella*. The complete characterization of this species is underway requiring additional molecular data and mating studies.

The concept of ambrosia fungi may require a re-evaluation. Current findings, corroborated by previous research in other areas of the globe, do not fit the current definition grouping all ambrosia symbionts related to *Ophiostoma* species in the *Raffaelea* major clade. Moreover, the phylogenetic relationship found in our multigene analyses corroborates the existence of a distinct lineage of Ophiostomatales in Portugal.

Considering the outcomes of the current research work, we believe the mutualistic complex of *P. cylindrus* could be further clarified in the entire ecological zone where cork oak is established through the extrapolation of the holistic approach develop in this work, combining isolation techniques with genetic characterization of the Ophiostomatales retrieved from the ambrosia beetle. In addition, data robustness could be further substantiated by, in addition, molecularly characterizing β-tubulin genes. The expression, or involvement, of pathogenic genes in the infection and colonization process of the fungal agent in the host could be discerned. In addition, a multigene analysis comprising all the genes listed herein will be the best approach to identify and define the relationships between ophiostomatoid symbionts and *P. cylindrus* in the future.

The identification and molecular characterization of Ophiostomatoid fungi in Portuguese cork oak stands is established as an active research line at the Forestry Protection and Genetic departments of the INRB, I.P. (Instituto Nacional dos Recursos Biológicos, I.P.). Axenic isolates are routinely contrasted with ambrosia wilting fungi worldwide, namely Japanese and North American strains. Restriction fragment length polymorphism (RFLP) are used to initially genetically profile homologous fungal strains from different localities and detect homologies or discrepancies in the isolates found. Isolates from other countries such as Algeria and Tunisia are currently being profiled. Isolates from other countries will be incorporated into the ongoing research line.

Since the role of ophiostomatoid fungi in cork oak decline appears to be relevant and they are intimately associated with *P. cylindrus,* the discrimination of these fungi with expedient oligonucleotide probes (padlock probe) capable of detecting sets of genes with nucleotide differences, and the use of hyperbranched rolling circle amplification (HRCA) for sensitive species identification could facilitate the identification process when profuse number of fungi are found. The detection of fungi could be done directly from beetle DNA with a rapid, sensitive and reproducible methodology. These methodologies are currently under analysis. Ultimately, this methodology could be applied to a wide range of beetle pests for fungal detection without the need for fungal isolation and cultivation and represents a promising tool to better understand pathogen population dynamics in the insect-fungi interaction.

CHAPTER 6

REFERENCES

6. REFERENCES

AFN 2010. *Inventário Florestal Nacional Portugal Continental (IFN5, 2005-2006).* Autoridade Florestal Nacional. Lisboa, Portugal, 209 pp.

Allegro G, Della Beffa G 2001. Un nuovo problema entomologico per la pioppicoltura italiana: *Platypus mutatus* (Chapuis) (Coleoptera, Platypodiade). *Foresti ed Alberi Oggi* 66, 31-34.

Algarvio R 2000. *Feromonas de agregação em Platypus cylindrus Fab. (Coleoptera: Platypodidae): Evidência Electrofisiológica*. Trabalho de Fim de Curso da Licenciatura em Biologia. Universidade de Évora, Évora, 87 pp.

Algarvio R, Teixeira C, Barata E, Pickett J, Casas-Novas P, Figueiredo D 2002. Identification of a putative aggregation pheromone from males *Platypus cylindrus* (Coleoptera: Platypodidae). *In Proceedings of the 19th Annual Meeting International Society of Chemical Ecology*. University of Hamburg, Germany, 151.

Alves A, Luque J, Phillips A 2004. *Botryosphaeria corticola* sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph, *Diplodia mutila*. *Mycologia* 96, 598-613.

Arx JA, Hennebert GL 1965. Deux champignons ambrosia. *Mycol. Mycopath. Appl.* 25, 309-315.

Atkinson TH 2004. *Ambrosia Beetles*. *Platypus* spp. (Insecta: Coleoptera: Platypodidae) Univ. Florida, Gainsville, FL. (available on line at http://edis.ifas.ufl.edu/IN331, accessed 22.10.2010).

Baeta-Neves CM 1944. Problemas suberícolas na zona pliocénica ao sul do Tejo. *Bol. da Junta Nacional da Cortiça **65***, 193-197.

Baeta-Neves CM 1950. Lista dos insectos da biocenose do sobreiro (*Quercus suber* L.). *Bol. Soc. Port. Ciên. Nat.* 111, 33-46.

Baker JM 1963. Ambrosia beetle and their fungi, with particular reference to *Platypus cylindrus* Fab. *Symp. Soc. General Microbiol.* 13, 323-354.

Bakry M, Abourouh M 1996. Nouvelles données sur le dépérissement du chêne-liège (*Quercus suber* L.) au Maroc. *Ann. Rech. Maroc* 29, 24-39.

Balachowsky AS 1949. *Faune de France, Coléoptères Scolytides*. Lechevalier Ed. , Paris, 320 pp.

Balachowsky AS, Chevalier M, Cuillé J, Grison P, Hoffmann A, Jourdheuil P, Labeyrie V, Remaudière G, Steffan JR, Touzeau J, Vilardebo A 1963. Famille des Platypodidae. *In* Balachowsky AS, ed., *Entomologie appliquée à l'agriculture*. *Coleoptères.* Ed. Masson et Cie, Paris, 1289-1291.

Barata E, Casas-Novas P, Correia S, Algarvio R, Baltazar N, Figueiredo D 2002 Cork oak male beetles (*Platypus cylindrus*) release a putative aggregation pheromone that attracts both sexes. *In Proceedings of the 19th Annual Meeting, International Society of Chemical Ecology*. University of Hamburg, Germany, 152.

Barbosa P, Wagner MR 1988 *Introduction to forest and shade tree insects*. Academic Press, London, 104 pp.

Barrico L, Rodríguez-Echeverría S, Freitas H 2010. Diversity of soil basidiomycete communities associated with *Quercus suber* L. in Portuguese *montados. Eur. J. Soil Biol., 2010, on line early, doi: 10.1016/j.ejsobi.2010.05.001.*

Barros, MC, Mateus F, Rodrigues JM 2002. The main regions of cork oak decline in Portugal. *IOBC/wprs Bull*. 25, 1-4.

Batra LR 1963. Ecology of ambrosia fungi and their dissemination by beetles. *Trans. Kansas Acad. Sci.* 66, 213-236.

Batra LR 1966. Ambrosia fungi: extent of specificity to ambrosia beetles. *Science* 153, 193-195.

Batra LR 1967. Ambrosia fungi: a taxonomic revision, and nutritional studies of some species. *Mycologia* 59, 976-1017.

Batra LR 1985. Ambrosia beetle and their associated fungi: Research trends and techniques. *Proc. Indian. Acad. Sci. Plant. Sci.* 49, 137-148.

Beaver RA 1977. Bark and ambrosia beetles in tropical forests. *Biotrop. Spec. Publ.* 2, 133-147.

Beaver RA 1989. Insect-fungus relationships in the bark and ambrosia beetles. *In* Wilding N, Collins NM, Hammond PM, Webber JF, eds., *Insect-Fungus Interactions*. Academic Press, London, 121-143.

Berryman AA 1982. Population dynamics of bark beetles. *In* Mitton JB, Sturgeon KB, eds., *Bark Beetles in North American Conifers*. University of Texas Press, Austin, USA, 264-314.

Blackwell M, Jones K 1997. Taxonomic diversity and interactions of insect-associated ascomycetes. *Biodiversity and Conservation* 6, 689-699.

Bragança H, Tenreiro R, Santos N 2004. Identification of Portuguese *Armillaria* isolates by classic mating-tests and RFLP-PCR analysis of the ITS1 region of ribosomal DNA. *Silva Lusitana* 12, 67–75.

Brasier CM 1996. *Phytophthora cinnamomi* and oak decline in southern Europe. Environmental constraints including climate change. *Ann. For. Sc.* 53, 347-358.

Brasier CM, Moreira AC, Ferraz JF, Kirk S 1992. High mortality of cork oak in Portugal associated with *Phytophthora cinnamomi*. *In Proceedings of the International Congress Recent Advances in Studies on Oak Decline.* Selva di Fasano, Italy, 461-462.

Byers JA, Lanne BS, Löfqvist J, Schlyter F, Bergström G 1985. Olfactory recognition of host-tree susceptibility by pine shoot beetles. *Naturwissenschaften* 72, 324-326.

Cabral, MT, Ferreira MC 1999. *Pragas dos Montados*. Lisboa, Estação Florestal Nacional, 94pp.

Cabral, MT, Ferreira MC, Moreira T, Carvalho EC, Diniz AC 1992. Diagnóstico das causas da anormal mortalidade dos sobreiros a Sul do Tejo. *Scientia gerundensis* 18, 205-214.

Cabral MT, Lopes F, Sardinha RM 1993. Determinação das causas da morte do sobreiro nos concelhos de Santiago do Cacém, Grândola e Sines. *Silva Lusitana* 1, 7-24.

Cabral MT, Sardinha RM 1992. Perspectiva integrada do declínio dos montados de sobro alentejanos. *In Actas do II Encontro sobre montados de sobro e azinho.* Évora, Portugal, 217-231.

Cadima IS, Capelo J, Gomes AA 1995. Relação entre as variáveis ambientais, tipo de condução dos povoamentos e a mortalidade do sobreiro nos concelhos de Santiago do Cacém, Grândola e Sines. *Silva Lusitana* 1, 7-24.

Câmara-Pestana C 1898. Doenças do sobreiro. *Arquivo rural* 36, 297-298.

Capelo JC, Catry F 2007. A distribuição do Sobreiro em Portugal. *In Árvores e Florestas de Portugal - Os Montados, muito para além das árvores.* Ed. LPN, Lisboa, Portugal, 107-113.

Casas-Novas PM 2001. *Feromona de agregação em Platypus cylindrus Fab. (Coleoptera: Platypodidae): Evidência Comportamental em Túnel de Vento.* Trabalho de Fim de Curso da Licenciatura em Biologia. Universidade de Évora, Évora, Portugal, 63 pp.

Cassar S, Blackwell M 1996. Convergent origins of ambrosia fungi. *Mycologia* 88, 596-601.

Cassier P, Lévieux J, Morelet M, Rougon D 1996. The Mycangia of *Platypus cylindrus* Fab. and *P. oxyurus* Dufour (Coleoptera: Platypodidae). Structure and associated fungi. *J. Insect Physiol.* 42, 171-179.

Chararas C 1979. *Écophysiologie des insectes parasites des forêts*. Chararas Ed. , Paris, 297 pp.

Christiansen E, Horntvedt R 1983. Combined *Ips/ Ceratocystis* attack on Norway spruce, and defensive mechanisms of the trees. *Z. ang. Entomol.* 96, 110-118.

Cobos JM, Montoya R, Tuset JJ 1992. New damages to the *Quercus* woodlands in Spain. Preliminary evaluation of the possible implication of *Phytophthora cinnamomi*. *In Proceedings of the International Congress Recent Advances in Studies on Oak Decline.* Selva di Fasano, Italy, 163-169.

Coelho IS 1996. O Montado, a economia e o desenvolvimento do Alentejo. *Silva Lusitana* 4, 39–48.

Coelho IS 2007. A silvopastorícia, uma perspectiva histórica. *In Árvores e Florestas de Portugal-Os Montados, muito para além das árvores*. Ed. LPN, Lisboa, Portugal, 177-210.

Collado J, Platas G, Peláez F 2001. Identification of an endophytic *Nodulisporium* sp. from *Quercus ilex* in central Spain as the anamorph of *Biscogniauxia mediterranea* by rDNA sequence analysis and effect of different ecological factors on distribution of the fungus. *Mycologia* 93, 875-886.

Correia S 2003. *Feromona de agregação em Platypus cylindrus Fab. (Coleoptera: Platypodidae): Teste de atracção de conspecíficos para fonte de odor de machos em túnel de vento.* Trabalho de Fim de Curso na Licenciatura em Biologia. Universidade de Évora, Évora, Portugal, 79 pp.

Costa A, Pereira H 2007. Montados e sobreirais: uma espécie, duas perspectivas. *In Árvores e Florestas de Portugal - Os Montados, muito para além das árvores.* Ed. LPN, Lisboa, Portugal, 17-37.

Costa A, Pereira H, Madeira M 2009. Landscape dynamics in endangered cork oak woodlands in Southwestern Portugal (1958-2005). *Agroforest Syst* 77, 83-96.

Costa A, Pereira H, Madeira M 2010. Analysis of special patterns of oak decline in cork oak woodlands in Mediterranean conditions. *Ann. For. Sci., 2010, on line early, doi:* 10.1007/s10457-009-9212-3.

Costa A, Pereira H, Oliveira AC 2002. Influence of climate on the seasonality of radial growth of cork oak during a cork production cycle. *Ann. For. Sc.* 59, 429 – 437.

Dajoz R 1980. *Écologie des Insectes Forestiers*. Coll. Écologie Fondamentale et Appliquée. Gauthiers-Villars, Paris, 489 pp

David TS, Cabral MT, Sardinha RM 1992. A mortalidade dos sobreiros e a seca. *Finisterra* XXVII, 17-24.

David TS, Henriques MO, Kurz-Besson C, Nunes J, Valente F, Vaz M, Pereira JS, Siegwolf R, Chaves M, Gazarini LC, David JS 2007. Water-use strategies in two co-occurring Mediterranean evergreen oaks: surviving the summer drought. *Tree Physiology* 27, 793-803.

De Beer ZW, Wingfield MJ 2006. Emerging lineages, genera and ecological patterns in the Ophiostomatales. *In Proceedings of the International Symposium: "Ceratocystis and Ophiostoma: Expanding Frontiers".* Stradbroke Island, Australia, 17.

De Beer ZW, Wingfield BD, Wingfield M 2003. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycol Res* 107, 469–476.

Degreef J 1992. Isolation of *Ophiostoma querci* (Georgev.) Nannfeldt from declining oaks in Belgium: selection techniques and pathogenicity test. *In Proceedings of an International Congress Recent Advances in Studies on oak decline*. Selva di Fasano, Italy, 471-473.

Delatour C, Menard A, Vautrot A, Simonin G 1992. Pathogenicity assessment of Ophiostomatales: *Ophiostoma querci* on oak compared to *O. novo-ulmi* on elm. *In Proceedings of an International Congress Recent Advances in Studies on oak decline.* Selva di Fasano, Italy, 59-65.

Desprez-Loustau M, Marçais B, Nageleisen L-M, Piou D, Vannini A 2006 Interactive effects of drought and pathogens in forest trees. *Ann. For. Sci*. 63, 597-612.

Eskalen A, McDonald V 2011. First Report of *Raffaelea canadensis* causing laurel wilt disease symptoms on avocado in California. *Plant Dis., 2011, on line early, doi:* 10.1094/PDIS-03-11-0203

Español F 1964. Los Platipodidos de Cataluña (Col. Phytophagoidea). *Bol. Ser. Plagas For.* 7, 115-117.

Evangelista M 2010. *Relatório de Caracterização da Fileira Florestal 2010*. AIFF, S. Mª Lamas, Portugal, 80 pp.

Evidente A, Andolfi A, Maddau L, Franceschini A, Marras F 2005. Biscopyran, a phytotoxic hexasubstituted pyranopyran produced by *Biscogniauxia mediterranea*, a fungus pathogen of cork oak*. J. Nat. Prod*. 68, 568–571.

Farrell BD, Sequeira AS, O'Meara BC, Normark BB, Chung JH, Jordal BH 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). *Evolution* 55, 2011-2027.

Ferreira MC, Ferreira GW 1986. Notas sobre os insectos nocivos à *Quercus suber* L. em Portugal. *In Actas do 1º Encontro sobre os Montados de Sobro e Azinho*. Évora, Portugal, 405-422.

Ferreira MC, Ferreira GW 1989. *Platypus cylindrus* F. (Coleóptera: Platipodidae) Plaga de *Quercus suber*. *Bol. San. Veg. Plagas* 4, 301-305.

Ferreira MC, Ferreira GW 1991. *Pragas das folhosas.* série Divulgação 5, MAPA, Lisboa, 191 pp.

Fraedrich SW, Harrington TC, Rabaglia RJ, Ulyshen MD, Mayfield AE, Hanula JL, Eickwort JM, Miller DR 2008. A fungal symbiont of the redbay ambrosia beetle causes a lethal wilt in redbay and other Lauraceae in the southeastern United States. *Plant Dis* 92, 215–224.

Franceschini A, Linaldeddu BT, Marras F 2005. Occurrence and distribution of fungal endophytes in declining cork oak forests in Sardinia (Italy). *IOBC⁄wprs Bull.* 28, 67–74.

Francke-Grosmann H 1963. Some New Aspects in Forest Entomology. *Ann. Rev. Entomol.* 8, 415-438.

Francke-Grosmann H 1967. Ectosymbiosis in wood-inhabiting insects. *In* Henry SM, ed., *Symbiosis, its physical and biochemical significance.* Academic Press, New York, USA, 141-203.

Farris S H, Funk A 1965. Repositories of symbiotic fungus in the ambrosia beetle *Platypus wilsoni*. *Can. Entomol.* 97, 527-532.

FSC 2005. *Forest Stewardship Council, pesticides policy: proposed revisions*. FSC, Germany, 29 pp.

Funk A 1970. Fungal symbionts of the ambrosia beetle *Gnathotrichus sulcatus*. *Can J Bot* 48, 1445–1448.

Gebhardt H, Begerow D, Oberwinkler F 2004. Identification of the ambrosia fungus of *Xyleborus monographus* and *X. dryographus* (Coleoptera: Curculionidae, Scolytinae). *Mycol Progress* 3, 95–102.

Gebhardt H, Oberwinkler F 2005. Conidial development in selected ambrosial species of the genus *Raffaelea*. *Antoine van Leeuwenhoek*, 88: 61-66.

Graham K 1967. Fungal-insect mutualism in trees and timber. *Ann. Rev. Entomol.* 12, 105-126.

Graham K 1968. Anaerobic induction of primary chemical attractancy for ambrosia beetles. *Can. J. Zool.* 46, 905-908.

Harrington TC 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia 73*, 1123-1129.

Harrington TC 1993. Biology and taxonomy of fungi associated with bark beetles. *In* Schowalter RD, Filip GM, eds., *Beetle-pathogen interactions in conifer forests*. Academic Press, New York, USA, 37-58.

Harrington TC 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. *In* Vega FE, Blackwell M, eds., *Insect-fungal associations: ecology and evolution*. Oxford University Press, New York, USA, 257–292.

Harrington TC, Aghayeva DN, Fraedrich SW 2010. New combinations in *Raffaelea, Ambrosiella*, and *Hyalorhinocladiella*, and four new species from the redbay ambrosia beetle, *Xyleborus glabratus*. *Mycotaxon* 111, 337–361.

Harrington TC, Fraedrich SW 2010. Quantification of propagules of the laurel wilt fungus and other mycangial fungi from the redbay ambrosia beetle, *Xyleborus glabratus*. *Phytopathology* 100, 1118–1123.

Harrington TC, Fraedrich SW, Aghayeva DN 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the Lauraceae. *Mycotaxon* 104, 399–404.

Harrington, TC, Yun HY, Lu S, Goto H, Aghayeva D, Fraedrich S 2011. Isolations from the redbay ambrosia beetle, *Xyleborus glabratus*, confirm that the laurel wilt pathogen, *Raffaelea lauricola*, originated in Asia*. Mycologia, 2011, on line early, doi:* 10.3852/10-417.

Hartig T 1844. Ambrosia des *Bostrichus dispar*. *Allgem. Forst – Jagdztg.* 13, 73. (cit. Francke-Grosmann 1967).

Henriques J 2007. *Fungos associados a Platypus cylindrus Fab. (Coleoptera: Platypodidae e sua relação com o declínio do sobreiro em Portugal*. Tese de Mestrado em Protecção das Plantas. Universidade de Évora, Évora, Portugal, 118 pp.

Henriques J, Inacio ML, Sousa E 2006. Ambrosia fungi in the insect-fungi symbiosis in relation to cork oak decline. *Rev. Iberoam. Micol*. 23, 185-188.

Henriques J, Inácio ML, Lima A Sousa E 2011. New outbreaks of charcoal disease on young cork oaks in Portugal. *IOBC Bulletin (in press).*

Henriques J., Inacio ML, Pires S, Sousa E 2010. *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) control strategies. *IOBC/wprs Bulletin* 57: 103-106.

Hickin NE 1963. *The insect factor in wood decay.* Rentokil Lybrary. Hutchinson, London, 344 pp.

Hubbard HC 1897. The ambrosia beetles of the United States. *Div. Entomol. Bull.*, 7, 9-35 (cit. Francke-Grosmann 1967).

Inacio ML, Henriques J, Lima A, Sousa E 2008. Fungi of *Raffaelea* genus (Ascomycota: Ophiostomatales) associated with *Platypus cylindrus* (Coleoptera: Platypodidae) in Portugal. *Revista de Ciências Agrárias* 31, 96-104.

Inacio ML, Henriques J, Sousa E 2005. As relações mutualistas entre fungos e insectos: sua influência no estado sanitário da floresta em Portugal. *In Actas das comunicações do 5º Congresso Florestal Nacional*. Instituto Politécnico Viseu, Viseu, Portugal. (*digital format*).

Inacio ML, Henriques J, Guimarães L, Azinheira H, Lima A, Sousa E 2010a. The symbiotic fungus *Acremonium crotocinigenum* causes canker on cork oak. *In Proceedings of the 9th*

Conference of the European Foundation for Plant Pathology and VI Congresso da Sociedade Portuguesa de Fitopatologia. Évora, Portugal, 191.

Inacio ML, Henriques J, Guimarães L, Azinheira H, Lima A, Sousa E 2011a. *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) transports *Biscogniauxia mediterranea*, agent of cork oak charcoal canker. *Bol. San. Veg. Plagas* 37: 181-186 *(in press).*

Inacio ML, Henriques J, Lima A, Sousa E 2011b. Ophiostomatoid fungi associated with cork oak mortality in Portugal. *IOBC/wprs Bull* 58 (*in press).*

Inacio ML, Henriques J, Sousa E 2010b. Mycobiota associated with *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) on cork oak in Portugal. *IOBC/wprs Bull* 57:87-95.

Jiménez JJ, Sánchez ME, Trapero A 2005. El chancro carbonoso de *Quercus* I: Distribuición y caracterización del agente casual. *Bol. San. Veg. Plagas* 31, 549-562.

Jones KG, Blackwell M 1998. Phylogenetic analysis of ambrosial species in the genus *Raffaelea* based on 18S rDNA sequences. *Mycol. Res.* 102, 661-665.

Kim KH, Choi YJ, Seo ST, Shin HD 2009. *Raffaelea quercus-mongolicae* sp. nov. associated with *Platypus koryoensis* on oak in Korea. *Mycotaxon* 110, 189–197.

Kim JJ, Kim SH, Lee S, and Breuil C 2003. Distinguishing *Ophiostoma ips* and *Ophiostoma montium*, two bark beetle-associated sapstain fungi. *FEMS Microbiology Letters* 222, 187-192.

Kim S, Harrington TC, Lee JC, Seybold SJ 2011*. Leptographium tereforme* sp. nov. and other Ophiostomatales isolated from the root-feeding bark beetle *Hylurgus ligniperda* in California. *Mycologia* 103, 152–163.

Kirisits T 2007. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. *In* Lieutier F, Day KR, Battisti A, Grégoire J-C, Evans HF, eds., *Bark and Wood Boring insects in living trees in Europe, a synthesis*. Springer, Netherlands, 181-235.

Kirisits T, Wingfield MJ, Chhetri DB 2002. *Studies on the association of blue-stain fungi with the Eastern Hymalayan spruce bark beetle (Ips schmutzenhoferi) and with other bark betles in Bhutan.* Yusipang Report. Vienna, Austria, 55 pp.

Kirkendall LR, Kent DS, Raffa KF 1997. Interactions among males, females, offspring in bark and ambrosia beetles: The significance of living in tunnels for the evolution of social behaviour. *In* Choe JC, Crespi BJ, ed., *The evolution of Social Behaviour in Insects and Arachnids*. Cambridge University Press, Cambridge, UK, 181-215.

Kok LT 1979. Lipids of ambrosia fungi in the life of the mutualistic beetles. *In* Batra LR, ed., *Insect-Fungus symbioses.* Halsted Press, Sussex, UK, 33-52.

Korolyov SG 1989. The morphology of the larvae of *Platypus* beetles Herbst (Coleoptera, Platypodidae) in connection with the characteristics of their ecology. *Entomol. Obozr*. 68, 353-360.

Kubono T, Ito S 2002. *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* 43, 255-260.

Külnholz S, Borden JH, Uzunovic F 2003. Secondary ambrosia beetles in apparently healthy trees: Adaptations, potential causes and suggested research. *Integrated Pest Management Reviews* 6, 209-219.

Kurz-Besson C, Otieno D, Vale RL, Siegwolf R, Schmidt M, Herd A, Nogueira C, David TS, Tenhunen J, Pereira JS, Chaves M 2006. Hydraulic lift in cork oak trees in a savannah-type Mediterranean ecosystem and its contribution to the local water balance. *Plant & Soil* 282, 361-378.

Lévieux J, Cassier P 1994. Vection de champignons pathogènes des résineux par les xylophages forestiers. *Ann. Biol.* 33, 19-37.

Linaldeddu BT, Franceschini A, Luque J, Phillips AJ 2007. First report of canker disease caused by *Botryosphaeria parva* on cork oak trees in Italy. *Plant Dis* 91, 324.

Linaldeddu BT, Sirca C, Spano D, Franceschini A 2009. Physiological responses of cork oak and holm oak to infection by fungal pathogens involved in oak decline. *For. Path*. 39, 232–238.

Luque J, Parladé J, Pera J 2000. Pathogenicity of fungi isolated from *Quercus suber* in Catalonia NE Spain). *For. Path.* 30, 247-263.

Luque J, Pera J, Parladé J 2008. Evaluation of fungicides for the control of *Botryosphaeria corticola* on cork oak in Catalania (NE Spain). *For. Path* 38, 147-155.

Maddau L, Cabras A, Franceschini A, Linaldeddu BT, Crobu S, Roggio T, Pagnozzi D 2009. Occurrence and characterization of peptaibols from *Trichoderma citrinoviride*, an endophytic fungus of cork oak, using electrospray ionization quadrupole time-of-flight mass spectrometry. *Microbiology* 155, 3371–3381.

Malloch D, Blackwell M 1994. Dispersal biology of the ophiostomatoid fungi. *In* Wingfield MJ, Seifert KA, Webber JF, eds., *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity*. APS Press, Minnesota, USA, 195-206.

Martin, MM 1979 Biochemical implications of insect mycophagy. *Biol. Rev. Cambridge Philos. Soc*. 54, 1-21.

Marvaldi AE, Sequeira AS, O'Brien CW, Farrell BD 2002. Molecular and morphological phylogenetics of weevils (Coleoptera: Curculionoidea): do niche shifts accompany diversification? *Systematic Biology* 51, 761-785.

Massoumi Alamouti S, Tsui CM, Breuil C 2009. Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. *Mycol. Res.* 113, 822–835.

Matsuda Y, Kimura K, Ito S 2010. Genetic characterization of *Raffaelea quercivora* isolates collected from areas of oak wilt in Japan. *Mycoscience* 51, 310–316.

Mazzaglia A, Anselmi N , Gasbarri A, Vannini A 2001. Development of Polymerase Chain Reaction (PCR) assay for the specific detection of *Biscogniauxia mediterranea* living as an endophyte in oak tissues. *Mycol. Res*. 105, 952-956.

Mendes A 2002. A economia do sector da cortiça em Portugal. Evolução das actividades de produção e transformação ao longo dos séculos XIX e XX. *In Actas do XXII Encontro da Associação Portuguesa de História Económica e Social.* Universidade de Aveiro, Aveiro, Portugal, 268-273.

Milligan RH, Osborne GO, Ytsma G 1988. Evidence for an aggregation pheromone in *Platypus gracilis* (Coleoptera: Platypodidae). *J. Appl. Entomol*. 106, 20-24.

Moreira C 2002. Distribution of *Phytophthora cinnamomi* in cork oak stands in Portugal. *IOBC/wprs Bull*. 25, 41-48.

Moreira AC, Caetano P, Correia S, Brasier CM, Ferraz JF 1993. *Phytophthora cinnamomi* associated with cork oak decline in southern Portugal. *In Proceedings of the 6th International Congress of Plant Pathology*. Montreal, Canada, 154.

Moreira A, Madeira C, Maia I, Quartin V, Matos MC, Cravador A 2006. Studies on the association of the Quercus suber decline disease with *Phytophthora cinnamomi* in Portugal. *Bol. Inf. CIDEU* 1, 31-38.

Morelet M 1998. Une espèce nouvelle de *Raffaelea*, isolée de *Platypus cylindrus*, coléoptère xylomycétophage des chênes. *Annales de la S.S.N.A.T.V.* 50, 185-193.

Mullineux T, Hausner G 2009. Evolution of rDNA ITS1 and ITS2 sequences and RNA secondary structures within members of the fungal genera *Grosmannia* and *Leptographium*. *Fungal Genetics and Biology* 46, 855–867.

Murata M, Yamada T, Ito S 2005. Changes in water status in seedlings of six species in the Fagaceae after inoculation with *Raffaelea quercivora* Kubono et Shin-Ito. *J. For. Res*. 10, 251–255.

Murata M, Matsuda Y, Yamada T, Ito S 2007. Discolored and non-conductive sapwood among six Fagaceae species inoculated with *Raffaelea quercivora*. *For. Path*. 37, 73–79.

Murata M, Matsuda Y, Yamada T, Ito S 2009. Differential spread of discoloured and non-conductive sapwood among four Fagaceae species inoculated with *Raffaelea quercivora*. *For. Path*. 39, 192–199.

Natividade JV 1950. *Subericultura*. 2ª ed. Ministério da Agricultura, Pescas e Alimentação. Direcção-Geral das Florestas. Lisboa, Portugal, 387 pp.

Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson KH 2008. Intraspecific ITS variability in the kingdom fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evol. Bioinform*. 4, 193–201.

Nkuekam GK, DeBeer WZ, Wingfield MJ, Roux J 2011. A diverse assemblage of *Ophiostoma* species, including two new taxa on eucalypt trees in South Africa. *Mycol Progress* (*in press*).

Nogueira CS 1967. *Panorama sanitário dos maciços florestais a sul do Tejo*. Folhetos de divulgação. Direcção Geral dos Serviços Florestais e Aquícolas, Lisboa, Portugal, 12 pp.

Norris DM, Baker JM 1968. A complex of fungi mutualistically involved in the nutrition of the ambrosia beetle *Xyleborus ferrugineus*. *Journal of Invertebrate Pathology* 11, 246-250.

Norris DM 1976. Chemical interdependencies among *Xyleborus* spp. ambrosia beetles and their symbiotic microbes. *Beith. Mater. Org*. 3, 479-488.

Oak S, Tainter F, Williams J, Starkey D 1996. Oak decline risk rating for the southeastern United States, Ann. Sci. For. 53, 721-730.

Ocasio-Morales RG, Panaghiotis T, Harrington TC 2007. Origin of *Ceratocystis platani* on Native *Platanus orientalis* in Greece and Its Impact on Natural Forests. *Plant Dis.* 91, 901-904.

Onofre N 2007. A fauna dos montados de azinho. *In Árvores e Florestas de Portugal- Os Montados, muito para além das árvores.* Ed. LPN, Lisboa, Portugal, 131-159.

Oszako T 2000. Oak declines in Europe's forest – history, causes and hypothesis. *In* Osazko T, Delatour C, eds., *Recent Advances on oak health in Europe*. Warsaw, Poland, 199-208.

Paine TD 1984. Influence of the mycangial fungi of the western pine beetle on water conduction through ponderosa pine seedlings. *Can. J. Bot.* 62, 556–58.

Paine TD, Raffa KF, Harrington TC 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annu. Rev. Entomol*. 42, 179-206.

Pausas JG, Pereira JS, Aronson J 2009. Cork oak trees and Woodlands – The tree. *In* Aronson J, Pereira JS, Pausas JG , eds., *Cork oak woodlands on the egde: ecology adaptive management and restoration.* Island Press, Washington, USA, 11-20.

Pereira H 2007. *Cork, biology, production and uses*. 1 Ed. Elsevier Publications. Amsterdam, Netherland, 336 pp.

Pereira PM, Fonseca MP 2003. Nature vs. narture: the making of the montado ecosystem. *Conserv Ecol* 7, 7-37.

Pereira JS, Conceição M, Rodrigues JM 1999. As causas da mortalidade do sobreiro revisitadas. *Revista Florestal*. XII, 20-23.

Pimentel L 1953. *Uma ameaça para os montados nacionais, Estudos e Informação*. Direcção Geral dos Serviços Florestais e Aquícolas, Ministério da Economia, Lisboa, Portugal, 1-9.

Pires S 2007. *Avaliação da eficácia de medidas de controlo das populações do insecto Platypus cylindrus Fab. em sobreiro*. Trabalho de Fim de Curso da Licenciatura em Biotecnologia. Instituto Piaget, Almada, Portugal, 71 pp.

Plattner A 2008. *Pathogenicity and taxonomy of fungi associated with the mountain pine beetle in British Columbia.* Msc. Dissertation submitted at the University of British Columbia, Toronto, 116 pp.

Plattner A, Kim J-J, Reid J, Hausner G, Lim YW, Yamaoka Y, Breuil C 2009. Resolving taxonomic and phylogenetic incongruence within species *Ceratocystiopsis minuta*. *Mycologia* 101, 878-887.

Reid J, Iranpour M, Rudski SM, Loewen PC, Hausner G 2010. A new conifer-inhabiting species of *Ceratocystis* from Norway. *Botany* 88, 971–983.

Renwick JA, Vité JP, Billings RF 1977. Aggregation pheromones in the ambrosia beetle *Platypus flavicornis*. *Naturwissenschaften* 64, 226.

Riziero T, Ragazzi A, Marianelli L, Sabbatini P, Roversi PF 2002. Insects and fungi involved in oak decline in Italy. *IOBC/wprs Bull.* 25, 67-74.

Rollins F, Jones K, Krokene P, Solheim H, Blackwell M 2001. Phylogeny of asexual fungi associated with bark and ambrosia beetles. *Mycologia* 93, 992-996.

Romeiras M 1995. *Protecção do montado de sobro: Estudo de alguns aspectos da bioecologia de Platypus cylindrus F. (Coleoptera, Platypodidae)*. Tese de Mestrado em Engenharia Agronómica. Instituto Superior de Agronomia, Lisboa, Portugal, 116 pp.

Sánchez ME, Caetano P, Ferraz J, Trapero A 2002. *Phytophthora* disease of *Quercus ilex* in south-western Spain. *For. Path*. 32, 5-18.

Santos MN 1995. Phytopathological situation of cork oak (*Quercus suber* L.) in Portugal. *IOBC/wprs Bull.* 18, 38-42.

Santos MN, Machado MH, Bragança MH, Ramos H, Sousa E, Tomaz I 1999. Mycoflora associated with cork oak (*Quercus suber* L.) in Portugal. *IOBC/wprs Bull.* 22, 25-28.

Santos MN, Martins AM 1992. Cork oak decline in Portugal. Notes regarding damages observed and incidence of *Hypoxylon mediterraneum*. *In Proceedings of the International Congress Recent Advances in Studies on Oak Decline*. Selva di Fasano, Italie, 115-121.

Seabra AF 1939. Contribuição para a história da Entomologia em Portugal. *Publicações da D.G.S.F.A.* 6, 1-20.

Shore TL, Mclean JA 1983. Attraction of *Platypus wilsoni* Swaine (Coleoptera: Platypodidae) to traps baited with sulcatol, ethanol and a-pinene. *Can. For. Serv. Bi-Mon. res. Notes* 3, 24-25.

Silva FA 1944. Ensaio de delimitação da área atacada por *Lymantria díspar* L. em Portugal. Trabalho de Fim de Curso da Licenciatura em Silvicultura. Instituto Superior de Agronomia, Lisboa, Portugal, 63 pp.

Silva RO 2002. O montado de sobro em Portugal. Que futuro? *INIA* 4, 16-17.

Silva JS, Catry F 2006. Forest fires in cork oak (*Quercus suber* L.) stands in Portugal. *International Journal of Environmental Studies* 63, 235–257.

Six DL 2003. Bark beetle–fungus symbioses. *In* Bourtzis K, Miller TA, eds., *Insect Symbioses*. CRC Press, Florida, USA, 97–114.

Six DL, Paine TD 1998. Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Environ Entomol* 27, 1393–1401.

Solheim H, Krokene P 1998 Growth and virulence of mountain pine beetle associated blue-stain fungi, *Ophiostoma clavigerum* and *Ophiostoma montium*. *Can J Bot* 76, 561-566.

Sousa EM 1995. Les principaux ravageurs du chêne-liège au Portugal. Leurs relations avec le déclin des peuplements. *IOBC/ wprs Bull.* 18, 18-22.

Sousa EM 1996. *Contribuition à l'étude de la biologie de populations de Platypus cylindrus (Coleoptera: Platypodidae) dans des peuplements de chênes liège au Portugal*. Thèse pour l'obtention du diplôme de Doctorat. Lyon, France, 153 pp.

Sousa EM, Débouzie D 1993. Contribution à la connaissance de quelques variables sylvicoles es écologiques au coléoptère *Platypus cylindrus* F., ravageur du chêne liège au Portugal. *Silva Lusitana* 1, 183-197.

Sousa EM, Débouzie D 1999. Distribution spatio-temporelle des attaques de *Platypus cylindrus* F. (Coleoptera: Platypodidae) dans des peuplements de chêne-liège au Portugal. *IOBC/wprs Bull.* 22, 47-58.

Sousa EM, Débouzie D 2002. Caractéristiques bioécologiques de *Platypus cylindrus* au Portugal. *IOBC/wprs Bull.* 25, 75-83.

Sousa EM, Débouzie D, Pereira H 1995. Le rôle de l'insecte *Platypus cylindrus* F. (Coleoptera, Platypodidae) dans le processus de dépérissement des peuplements de chêne-liège au Portugal. *IOBC/wprs Bull.* 18, 24-37.

Sousa EM, Inacio ML 2005. New Aspects of *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) Life History on Cork Oak Stands in Portugal. *In* Lieutier F, Ghaioule D, eds., *Entomological Research in Mediterranean Forest Ecosystems*. INRA Editions, Paris, France, 280 pp.

Sousa EM, Inacio ML, El-Antry S, Bakry M, Kadiri ZA 2005. Comparaison de la bio-écologie et du comportement de l'insecte *Platypus cylindrus* Faber. (Coléoptère, Platypodidae) dans les subéraies Portugaises et Marocaines. *IOBC/wprs Bulletin* 28, 137-144.

Sousa EM, Santos MN, Varela MC, Henriques J 2007. *Perda de vigor dos montados de sobro e azinho: análise da situação e perspectivas, Documento síntese*. INRB/DGRF, Lisboa, Portugal, 91 pp**.**

Sousa EM, Tomaz IL, Moniz FA, Basto S 1997. La répartition spatiale des champignons associés à *Platypus cylindrus* Fab. (Coleoptera: Platypodidae). *Phytopath. Medit*. 36, 145: 153.

Subramanian CV 1983. *Hyphomycetes. Taxonomy and biology*. Academic Press, London, UK, 502 pp.

Swift MJ, Boddy L 1984. Animal-microbial interactions in wood decomposition. *In* Anderson JM, Rayner AD, Walton DW, eds., *Invertebrate-Microbial Interactions*. Cambridge Univ. Press, Cambridge, USA, 89-131.

Teixeira C, Algarvio RM, Casas-Novas P, Barata EN 2003. Actividade biológica de análogos de três componentes da putativa feromona de agregação de *Platypus cylindrus* (Coleoptera: Platypodidae). *In Actas do V Congresso Nacional de Etiologia*. Universidade do Algarve, Faro, Portugal, 29.

Tilburi C 2009. *Oak pinhole borer, Platypus cylindrus (Coleoptera: Curculionidae). Tree pest, Advisory note*. Available on line at http://www.forestresearch.gov.uk/pdf, accessed 11.08.2009.

Tío R, Soria FJ, Ocete ME 1993. Etudio morfológico de la larva de *Platypus cylindrus* Fabricius (Coleoptera: Platypodidae). *Elytron* VII, 37-47.

Tokoro M, Kobayashi M, Saito S, Kinuura H, Nakashima T, Shoda-Kagaya E, Kashiwagi T, Tebayashi S, Kim CS, Mori K 2007. Novel aggregation pheromone (1*S*,4*R*)-*p*-menth-2-en-1-ol, of the ambrosia beetle, *Platypus quercivorus* (Coleoptera: Platypodidae). *Bull. For. Prod. Res. Inst.* 6, 49–57.

Tsui CK, Wang B, Khadenpour L, Massoumi Alamouti S, Bohlmann J, Murray BW, Hamelin R 2010. Rapid identification and detection of pine pathogenic fungi associated with mountain pine beetles by padlock probes. *J. Microbiol. Methods., 2010, on line early, doi:* 10.1016/j.mimet.2010.07.016.

Tuset JJ, Hinarejos C, Mira JL, Cobos JM 1996. Implicación de *Phytophthora cinnamomi* Rands en la enfermedad de la "seca" de encinas y alcornoques. *Bol. San. Veg. Plagas* 22, 491-499.

Tuset JJ, Hinarejos C, Mira JJ, Cobos JM 2002. Distribution of the isolations of *Phytophthora cinnamomi* in the Spanish *Quercus* areas with oak decline disease. *IOBC/wprs Bull*. 25, 49-52.

Ueda A, Kobayashi M 2004. Long-term attractiveness of autoclaved oak logs bored by male *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae) to male and female beetles. *Bulletin of FFPRI* 391, 99-107.

Varela MC 2004 Le chêne-liège et les incendies de forêts : le cas portugais. *In Actes colloque international Vivexpo - Le chêne-liège face au feu.* Vivès, France, 1-9.

Villemant C, Fraval A 1991. *La faune do Chêne liège*. Actes Edissions. Institute Agronomique et Vétérinaire Hassan II, Rabat, Morocco, 360 pp.

Villemant C, Fraval A 1993. La faune entomologique du chêne-liège en forêt de la Mamora (Maroc). *Ecologia Mediterranea* 19, 89-98.

Wood SL 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *Great Basin Naturalist Memoirs* 6, 42-50.

Yamasaki M, Futai K 2008. Host selection by *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae) before and after flying to trees. *Appl. Entomol. Zool*. 43, 249–257.

Ytsma G 1986. Inducing attack by male *Platypus* (Col.: Platypodidae) on wood billets in the laboratory. *J. Appl. Entomol.* 102, 210-212.

Zipfel RD, de Beer ZW, Jacobs K, Wingfield BD, Wingfield MJ 2006. Multigene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Stud Mycol.* 55, 75–97.

Zhou X, de Beer ZW, Wingfield MJ 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Stud Mycol.* 55, 269-277.