INSTITUTO SUPERIOR DE AGRONOMIA
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CHARCOAL CANKER (*BISCOGNIAUXIA MEDITERRANEA*) IN CORK OAK DECLINE IN PORTUGAL

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

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ABBREVIATIONS LIST

ANOVA - Analysis Of Variance
AT - Asymptomatic Tree
BIC - Bayesian Information Criterion
BLAST - Basic Local Alignment Search Tool
CBS - Centraalbureau voor Schimmelcultures
dH_2O - Distilled water
DNA - Deoxyribonucleic Acid
dNTPs - Deoxynucleotides
DT - Declined Tree
EMB - Embryo
END - Endosperm
INIAV – Instituto Nacional de Investigação Agrária e Pescas
IPCC - Intergovernmental Panel on Climate Change
IS - Inner Seed
ISTA - International Seed Testing Association
ITS-rDNA - Internal Transcribed Spacers of rDNA
LSD - Least Significant Difference
MCL - Maximum Composite Likelihood
MEAN - Micoteca da Estação Agronómica Nacional
MEGA- Molecular Evolutionary Genetic Analysis
ML - Maximum Likelihood
MSP-PCR - Microsatellite-Primed PCR
NCBI - National Center for Biotechnology Information
NTSYSpc - Numerical Taxonomy and Multivariate Analysis System - Version 2.02h
NUV -near-Ultra Violet light
PCR - Polymerase Chain Reaction
PDA - Potato Dextrose Agar
PDAA - PDA acidified with lactic acid
PDYA - PDA supplemented with yeast extract
PT - Protection Tissue
PWP - predawn leaf water potential
RAPD -Random Amplified Polymorphic DNA
rDNA - ribosomal DNA
RNA - Ribonucleic Acid
RT-PCR – Real Time PCR
SM - Simple Matching coefficient
TBE - Tris-Borate-EDTA (Ethylenediamine tetraacetic acid)
TEF- Translation Elongation Factor 1-α
TUB1 - β-Tubulin 1
TUB2 - β-Tubulin 2
UPGMA -Unweighted Pair Group Method with Arithmetic Mean
USA – United States of America

ABSTRACT

Charcoal canker is a disease caused by *Biscogniauxia mediterranea* that affects *Quercus suber* and other hardwood species. The fungus develops endophytically in the hosts becoming pathogenic under stress conditions. However, it has become more frequent, particularly in young trees expressing atypical symptoms. This work intends to clarify the agent involved in these disease outbreaks and its variability in different situations, as well as dispersion in natural conditions. The fungal anamorph was identified as *Nodulisporium* sp. in young hosts. In the stands, ascospores airborne dispersal is predominant, occurring after precipitation periods, and vertical contamination of plants doesn't happen. A collection of isolates obtained from cork oak in Portugal and other Mediterranean countries, from other hosts and from trees with different ages and disease expression were analyzed by microsatellite-primed PCR, multigene sequencing, morpho-cultural characteristics and growth rates at different temperatures. All the approaches revealed high level of intraspecific polymorphism among isolates, not allowing relating the disease development with any considered feature. The results highlighted the variability and adaptation ability of this fungus in special in the Mediterranea region, in the present worrying scenario of climatic change. All the conditions are gathered to favor the aggravation of the disease in cork oak stands.

Key-Words: dispersion, intraspecific variability, Mediterranean ecosystem, *Nodulisporium*, *Quercus suber*

O carvão do entrecasco (*Biscogniauxia mediterranea*) no processo de perda de vigor do montado de sobro em Portugal

RESUMO

O carvão do entrecasco é uma doença causada por *Biscogniauxia mediterranea* que afeta *Quercus suber* e outras espécies lenhosas. O fungo desenvolve-se endofiticamente nos hospedeiros tornando-se patogénico sob condições de stresse. Tem-se tornado mais frequente, particularmente em árvores jovens que expressam sintomas atípicos. Este trabalho pretende clarificar o agente envolvido nestes surtos da doença e a sua variabilidade em diferentes situações, assim como a sua dispersão em condições naturais. Identificou-se o anamorfo *Nodulisporium* sp. em hospedeiros jovens. No campo, a dispersão aérea dos ascósporos é predominante, ocorrendo após períodos de precipitação, e não existe contaminação vertical de plantas. Uma coleção de isolados obtidos a partir de sobreiro em Portugal e outros países mediterrânicos, de outros hospedeiros, árvores com diferentes idades e expressão da doença foram analisados por *microsatellite-primed PCR*, sequenciação multigénica, características morfo-culturais e crescimentos a diferentes temperaturas. Todas as abordagens revelaram um elevado nível de polimorfismo intraespecífico entre os isolados, não permitindo relacionar o desenvolvimento da doença com nenhum dos fatores considerados. Estes resultados enfatizam a variabilidade e capacidade de adaptação deste fungo em especial na região mediterrânica, na atual conjuntura de alterações climáticas. Encontram-se reunidas as condições para favorecer o agravamento da doença no montado de sobro.

Palavras-chave: dispersão, ecossistema mediterrânico, *Nodulisporium*, *Quercus suber*, variabilidade intraespecífica

RESUMO ALARGADO

O carvão do entrecasco é uma doença provocada pelo fungo *Biscogniauxia mediterranea* frequente em *Quercus suber* e outras espécies lenhosas. Esta doença está diagnosticada em Portugal desde 1930, sendo considerada um agente patogénico com ação secundária que apenas se manifesta em árvores adultas já debilitadas por outros agentes integrados no complexo processo de declínio dos montados. *B. mediterranea* tem um comportamento endofítico, colonizando assintomaticamente tecidos e órgãos de toda a parte aérea das árvores, e assumindo um desenvolvimento patogénico quando os hospedeiros são sujeitos a fatores de stresse, como períodos de seca. No entanto, nos últimos anos, tem vindo a observar-se um aumento acentuado da doença, nomeadamente em árvores jovens que exibem sintomas atípicos: a camada de cortiça fende surgindo massas castanhas pulverulentas abundantes sobre o entrecasco, a copa da árvore seca completamente e a árvore morre em poucos meses. Estas ocorrências sugerem uma alteração de comportamento do fungo, do hospedeiro ou de ambos: poderão estar envolvidas espécies afins a *B. mediterranea*, diferentes variedades do patogénio, ou novos fatores estão a influenciar a epidemiologia da doença e/ou a resistência do hospedeiro.

Assim, pretende-se com este trabalho esclarecer a situação do carvão do entrecasco nos montados de sobro no país, caracterizando o(s) agente(s) envolvido(s), em particular nos casos dos novos ataques descritos, avaliando a diversidade do fungo na natureza em diferentes condições. É ainda essencial conhecer os mecanismos desta ampla dispersão do agente causal e as suas perspetivas de evolução enquadrada no atual contexto de alterações climáticas.

A análise morfológica e molecular do fungo isolado a partir das árvores jovens infetadas revelou tratar-se de *Nodulisporium* sp., o estado anamórfico de *B. mediterranea* que, apesar de ter sido descrita em hospedeiros em Espanha e Itália, não ocorria normalmente de forma visível nas condições portuguesas, nos hospedeiros adultos já degradados em que habitualmente a doença se manifesta.

De modo a esclarecer a dispersão do fungo nos hospedeiros, em especial a contaminação das árvores jovens, foram realizados vários ensaios. Foi instalado um equipamento capta-esporos tipo Hirst e uma estação meteorológica no montado no concelho de Grândola que permitiu registar a dispersão de ascósporos como principal meio de disseminação da doença. A sua libertação ocorre ao longo do ano, variando de acordo com as condições meteorológicas: a precipitação é o principal fator, sendo períodos de três dias consecutivos com precipitação

superior a 0.5 mm determinantes para o aumento significativo de esporos presentes no ar; a temperatura apresenta uma relação negativa com a libertação dos ascósporos e a velocidade do vento está relacionada de forma positiva, constituindo o veículo de dispersão entre os hospedeiros. A transmissão direta do fungo de um hospedeiro para a sua descendência através do desenvolvimento sistémico de tecidos infetados foi avaliada considerando a via seminal e o aproveitamento da rebentação de toiça de árvores afetadas para o estabelecimento de novas plantas. Verificou-se que, apesar de o fungo estar presente nos tecidos das bolotas provenientes tanto de árvores degradadas como assintomáticas, não ocorre a sua transferência para os tecidos das plântulas germinadas, assim como nos rebentamentos de toiça o fungo não foi detetado. Deste modo, estas práticas silvícolas não constituem um risco acrescido para a disseminação da doença. A dispersão aérea generalizada pelo montado é então o principal meio de dispersão de *B. mediterranea*, podendo ocorrer a infeção precoce das plantas, o que se verificou pelo isolamento do fungo a partir de regeneração natural de sobreiro com menos de três anos, pelo que é necessária a adoção de medidas que favoreçam o bom estado fisiológico e sanitário das árvores na gestão da doença.

Para determinar a variabilidade do agente associado à doença nas diferentes situações de expressão da doença, foi reunida uma coleção de isolados obtidos a partir de sobreiros com diferentes idades e sintomatologias (incluindo tecidos assintomáticos), e de outros hospedeiros em diversas localidades em Portugal, de sobreiros dos países mediterrânicos produtores e um isolado de referência de *B. mediterranea* da coleção Centraalbureau voor Schimmelcultures (CBS). A identificação de todos os isolados foi confirmada por amplificação com os *primers* específicos MED1/ MED2.

Numa primeira tentativa para estabelecer uma relação entre os isolados aplicou-se a técnica *microsatellite-primed* PCR (MSP-PCR) utilizando cinco *primers*, de cuja análise conjunta resultou um dendrograma que demonstrou uma elevada variabilidade entre todos os isolados, que no geral apresentaram uma percentagem de similaridade superior a 46 %, agrupando-se sucessivamente em conjuntos mais restritos mas que não representam qualquer correspondência com os fatores de origem dos isolados. Apenas o isolado de referência, obtido de *Q. robur* na Holanda, se diferenciou do grupo de isolados da bacia mediterrânica. Do mesmo modo, a análise das características culturais da coleção de isolados juntamente com as taxas de crescimento a 25 ^{0}C revelou uma elevada variabilidade entre os isolados, com uma percentagem de similaridade total de 55 %.

Como as abordagens globais à coleção de isolados com recurso à técnica MSP-PCR e características culturais não possibilitaram fundamentar a seleção coerente de alguns para a continuação do estudo mais aprofundado foram escolhidos 36 exemplares de acordo com a sua origem geográfica, hospedeiro e sintomatologia exibida. Foi realizado o estudo filogenético do conjunto de isolados selecionados para a região ITS-rDNA e a análise conjunta das sequências codificantes TEF, TUB1 e TUB2, tendo-se demonstrado, mais uma vez, a diversidade indiscriminada entre isolados, no entanto, foi possível concluir que toda esta variabilidade é de fato intraespecífica uma vez que todas as sequências se agruparam em relação às espécies *outgroup* mais próximas (*B. atropunctata* e *B. nummularia*). A análise conjunta das sequências obtidas para a região ITS-rDNA com as sequências de *B. mediterranea* disponíveis no GenBank permitiu ainda visualizar a diferenciação dos isolados da região mediterrânica que se agruparam à parte dos isolados das restantes partes do mundo (Europa a norte dos Pirinéus e América do Norte).

A comparação de carateres morfológicos e fisiológicos dos isolados selecionados, que incluiu a caracterização cultural, as dimensões dos conídios e os crescimentos a diferentes temperaturas, corroborou as conclusões da análise genética uma vez que as descrições dos isolados estão de acordo com as de outros autores para a espécie *B. mediterranea* mas revelaram um elevado nível de variabilidade não relacionável com os dados de proveniência dos isolados.

Com o intuito de esclarecer a origem de uma diversidade intraespecífica tão elevada foi realizado um ensaio adicional em que se compararam por MSP-PCR isolados de cultura monoascospórica obtidos do mesmo estroma de hospedeiros de diferentes localizações. Concluiu-se que, mesmo a este nível, a diversidade é tal que não possibilita sequer a distinção da origem dos isolados. O fato de *B. mediterranea* ser uma espécie heterotálica e com elevada taxa de reprodução sexuada contribui para esta variabilidade genética da espécie, conferindo-lhe flexibilidade e capacidade de sobrevivência e adaptação às condições ambientais.

Na realidade, na região mediterrânica pode considerar-se a evolução de *B. mediterranea* no sentido do agravamento da doença particularmente para o sobreiro. Nesta região o fungo reúne as condições necessárias para o seu desenvolvimento e, de acordo com as previsões das alterações climáticas, será cada vez mais favorecido: o comportamento termófilo do fungo acompanha o aumento global da temperatura e os períodos de seca condicionam o hospedeiro acelerando a transição do fungo da fase latente para a patogénica.

Assim, este trabalho contribuiu para o esclarecimento do aparecimento e dispersão de novos surtos da doença do carvão do entrecasco, nomeadamente nas árvores jovens, e constitui um alerta para a situação de declínio do sobreiro que tende a agravar-se nas atuais perspetivas. É essencial dar continuidade a esta linha de trabalho no sentido de compreender melhor os mecanismos que desencadeiam a doença e desenvolver meios de controlo para este fungo.

1. INTRODUCTION

The cork oak (*Quercus suber* L.) is an emblematic tree from the Mediterranean Basin, particularly South-western Europe and Northern Africa. It is an essential component of a combination of semi-natural ecosystems, of which the Portuguese *montados* are a paradigm. Multifunctional systems of land use, they integrate cultural landscapes of high ecological, historic and socioeconomic value. In addition to cork and products such as hunting or pastures, cork oak forests perform important tasks in regulating the water cycle and in soil conservation, being important in combating desertification, housing high levels of biodiversity and functioning like carbon sinks, being able to contribute to the mitigating of greenhouse gas effects. Cork stoppers are natural products whose extraction, besides not affecting the ecosystem processes, allow cork oak forests to be managed as multiple use systems able to perform essential ecosystem services. Careful management and adequate added value of the services rendered by these systems are essential for the sustainability and benefit maintenance generated for the society (Pereira et al. 2008).

Large areas of cork oak tree mortality have been observed since the beginning of the XX century, raising concerns on the sustainability of such systems. Decline is not restricted to cork oak as mortality of the genus *Quercus* has been observed in different countries of Europe, United States of America and Japan (Brasier et al. 1992; Degreef 1992; Oak et al. 1996; Osazko, 2000; Matsuda et al. 2010; Sonesson & Drobyshev 2010; Haavik et al. 2011). This weakening process that has been taking place on the cork oak is a phenomenon whose comprehension is of high complexity and should be, above all, considered as a result of the interaction of multiple biotic and abiotic causes occurring simultaneously or in succession (Sousa et al. 2007).

The fungus *Biscogniauxia mediterranea* (de Not.) Kuntze. is a pathogen responsible for necrosis on stems and branches of hardwood species, known as charcoal canker, being its incidence particularly alarming on *Q. suber* (Torres 1985). Susceptibility to the fungus is associated with periods of severe drought or fire. As the host is subjected to water stress, the pathogen can easily spread through large cavity vessels, and colonize bark and woody tissues, being able to kill the host in a single growing season (Vannini & Valentini 1994). It spends part of its life-cycle as an endophyte living in different host tissues and organs, including bark, wood, buds, twigs, and leaves (Anselmi et al. 2000). The ability to survive in a latent phase in healthy tissues could represent an important strategy of this fungus. In fact, being already inside the host, the fungus can rapidly spread from several infection points during its pathogenic phase (Mazzaglia et al. 2001).

In Portugal, the charcoal canker appears with high frequency in degraded cork oak stands occurring systematically in weakened or dead trees and plant material lying on the ground (Santos 2003). However, recently, its focus have been increasing on young and adult trees without other symptoms (Figure 1), suggesting a change in behavior of the fungus, host or both: we can be in the presence of a related species or infra-specific varieties of *B. mediterranea*, or new factors are influencing the epidemiology of disease and/ or the resistance of the host (Sousa et al. 2007).

As it has been considered a secondary pathogen that only attacks weakened hosts, charcoal canker has been neglected until its recent conspicuous outbreaks and there are no updated studies on the occurrence, dispersion and characterization of *B. mediterranea* in Portugal.

Figure 1. Young plantation of cork oak with high mortality incidence; trees exhibiting atypical charcoal canker symptoms (Montemor-o-Novo, Portugal).

OBJECTIVES

The main objectives of the present research were to clarify the situation of the charcoal canker associated to cork oak in Portugal, framing it in the issue of the cork oak decline, under the context of global change.

It was essential to clarify the occurrence of the *B. mediterranea* in the stands, in particular the increasingly frequent appearance of atypical symptoms especially in young cork oak plantations in Portugal, linking them with the development of disease. It was also a goal to

determine dispersion of the fungus in the stand through different ways: to ascertain the direct transmission of the fungus both through seeds and in stump sprouting regeneration from infected trees that are used to replace declined trees and to evaluate airborne dispersal of the fungi, determining meteorological conditions and favourable periods for dispersal and establishment of the fungus in new hosts in natural conditions.

To understand the factors involved in the increase of the disease in Portugal, particularly if any changes have occurred at the level of the pathogen, it is essential to assess the occurrence of the disease along the major areas of *montado* and cork oak forests in different conditions of disease expression, compared to other hosts species and in other Mediterranean countries. In this sense, it is necessary to clarify the pathogen involved in the different situations, assessing the diversity of the agent by morphological, physiological and molecular methods.

Discuss the prospects of the charcoal canker evolution in cork oak, based on current knowledge and considering the climatic changes predictions for the Mediterranean region, is a mandatory step towards the development of effective measures to prevent and control the disease progression.

THESIS STRUCTURE

The current research comprises five chapters, with the results presented in the form of four scientific papers, either already published (three) or submitted for publication (one) to peer-reviewed journals, and one additional non published sub-chapter. 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, with particular focus on the role of *B. mediterranea* in the phenomenon, characterization of the fungus, development of the disease and its endophytic behavior, and the favoring factors, along with the presentation of the main objectives of this thesis and its structure.

Chapter 2 presents studies on the outbreak of disease in the cork oak stands and is divided into two sub-chapters:

- Sub-chapter 2.1 describes the increasing number of young vigorous trees exhibiting charcoal canker symptoms in Portugal, in particular the unusual anamorphic stage.

- Sub-chapter 2.2 investigates the dispersion of the fungus in the field. Airborne spreading of ascospores was evaluated using a spore trapping apparatus and related with meteorological conditions. Transmission of the fungus by infected tissues was also evaluated in acorns and seedlings from declined and asymptomatic trees, in natural regeneration and in stump sprouting regeneration from infected trees.

Chapter 3 focuses on the evaluation of *B. mediterranea* diversity associated to cork oak and is divided in three sub-chapters:

- Sub-chapter 3.1 assesses the fungi diversity within the host tree.

- Sub-chapter 3.2 analyzes the genetic diversity and phylogenetic relationships of *B. mediterranea* isolates associated to cork oak using molecular tools: a collection of isolates from different countries and locations, different hosts species and disease expression were compared by microsatellite-primed PCR profiles and individual and multigene phylogenies.

- Sub-chapter 3.3 evaluates the diversity of the same collection of *B. mediterranea* isolates in a morpho-cultural and physiological perspective.

Finally, in chapters 4, the results of the five publications/ manuscripts are summarized and reviewed in a discussion. In chapter 5 the main conclusions are presented suggesting future lines of research. By the end, all literature cited is listed, excluding the references of the papers which are integrated in each publication.

1.1. CORK OAK - DISTRIBUTION, SOCIO-ECONOMIC AND ENVIRONMENTAL IMPACTS

Q. suber is an evergreen oak that belongs to the order of Fagales and the family of Fagaceae, characterized by the presence of a conspicuous thick and furrowed bark with a continuous layer of cork in its outer part. It is this cork bark that gave the cork oak its notoriety and economic importance as a cork producer. It mostly integrates multifunctional agro-forestry systems (called *Montado* in Portugal and *Dehesa* in Spain), usually combining the production of cork with cattle grazing, hunting and other non-wood productions (Pereira 2007).

The cork oak is a typical species of the Western Mediterranean region, occurring spontaneously in Portugal and Spain, but, also, in Morocco, in Northern Algeria and in Tunisia. In addition, it is found in more restricted areas in the south of France and on the west coast of Italy, including Sicily, Corsica and Sardinia (Pereira et al. 2008). In Portugal the highest concentration is found south of the river Tagus in the regions of Alentejo and Tagus valley (districts of Setúbal, Évora, Beja, Portalegre e Santarém) (Pereira 2007).

In the first half of the XIX century the cork oak forests, as we know them today, began to be established. These are privately owned and manmade agro-forestry systems which emerged as a response to market demand for cork stoppers. This product not only was the origin of the establishment of this kind of forests, but it remained until the present time as its fundamental economic support (Mendes 2009). However, there are several other fields in which cork is used. This can be possible due to the foray of newer technological innovations which has enabled cork to be used in automobile, military and space industries, building, architecture, furniture, footwear, fashion accessories or decorative objects, etc (APCOR 2014).

Cork oak is currently the second Portuguese forest species, occupying 23 % of forest area (ICNF 2013). Portugal is the country with the largest cork oak area, with 34 % of the world production area, followed by Spain and Morocco. The world's cork production was in 2013 201428 tons, being Portugal the main producer, with 49.6 % of world production and also the world leader in exports of cork, with a share of 63.9 %. With a total value of exports in 2013 of 834.3 million €, the cork industry has a high economic and social importance in Portugal: cork oak *montados* and forest originate 12 thousand direct job posts in the industry, 6500 job in forest exploitation and, indirectly, thousands of employment positions related to other cork oak forest products (livestock farming, restaurants, tourism, etc.), contributing to 2.3 % of the total annual national export and 30 % of the combined Portuguese forestry exportations

(APCOR 2014). The economic value of cork oak is also related to other incomes associated with the cork forest: hunting, honey, mushrooms and livestock farming (Pereira et al. 2008).

Currently, cork oak savannas cover approximately 1.5 million ha in Europe and 1 million ha in North Africa. They have a sparse tree cover of cork oak (30–60 *Q. suber* trees per hectare), at times mixed with Holm oak (*Quercus rotundifolia* Lam.) and, more rarely, with other tree species (eg. *Pinus pinaster* Ainton, *Pinus pinea* L.). The understory is composed of a heterogeneous mix of shrub formations interspersed with grasslands, fallows, and, less often, cereal crops (Bugalho et al. 2011).

Mediterranean ecosystems are particularly rich in species of fauna and flora, constituting biodiversity hotspot. Mediterranean Basin has between 15 to 25 thousand plant species, a number of species much higher than found in the rest of Europe. More than half of these species are endemic to the Mediterranean. Cork oak is one of these endemics. In addition *montados* and cork oak forests are important reservoirs of biological diversity. The Natura 2000 network, a pan-European network of classified nature conservation areas, classifies *montados* (habitat 6310) and cork oak forests (habitat 9330) as very important for the conservation of biodiversity (Pereira et al. 2008). They also play an important role in the global carbon budget. As with old-growth forests (Luyssaert et al. 2008), cork oak *montados* accumulate and maintain carbon stocks for long periods (Bugalho et al. 2011).

Cork oak trees show a high ecological plasticity, occurring in a wide range of environmental conditions (Pausas et al. 2009). This species is well adapted to Mediterranean type climate, with mild, wet winters and dry, hot summers, occurring from more continental regions to coastal areas with Mediterranean and Atlantic influence. It grows well with mean annual precipitation of 600–1000 mm, but stands up to 2000 mm, 500 mm being the minimum usually considered for a balanced tree development (Natividade 1950; Pereira 2007). The optimum mean annual temperature is in the range 13–16 ^{o}C, although the species can also occur in environments with up to 19 ^{o}C. In general, the temperatures of -5 and 40 ^{o}C may be considered as the limits for cork oak growth (Pereira 2007). Cork oak grows from sea level to 2000 m of altitude, but optimum growth occurs below 600 m. The species is tolerant to a variety of soils with the exception of calcareous and limestone substrates. It may grow on poor and shallow soils, with low nitrogen and organic matter content and it allows a pH range between 4.8 and 7.0. However, cork oak occurs preferentially in siliceous and sandy soils, preferring deep well aerated and drained soils, being very sensitive to compaction and water logging (Pereira 2007; Catry et al. 2012).

The main characteristics of cork oak that contribute to the species' long—term ecological success are well-developed root system, in depth to tap water in underneath aquifers during drought periods and in surface to ensure enough nutrient supply, leaf lifespan and seasonality that allow maintenance of photosynthesis in the beginning of the growth period until full canopy renewal, long flowering period and annual and biennal acorn production, morphological and phenotypical diversity, protective cork layer in the outer bark that imparts considerable fire resistance (Pereira 2007).

1.1.1. CORK OAK DECLINE

Cork oak decline has been reported in south-western Portugal since the 1980s (Cabral et al. 1992). It is a major concern regarding the sustainability of these forests and for ensuring the raw-material supply for the cork industry. Cork oak decline has been described as a widespread and complex phenomenon, triggered by decrease of tree vigor and physiological stress (e.g. drought) and subsequent attack by pathogens, similarly to what occurs with oaks and other broadleaf trees in Europe (Brasier 1996; Ferreira 2000; Oszako 2000; Thomas et al. 2002), North America (Oak et al. 1996; Fortin et al. 2008; Kabrick et al. 2008) and Asia (Kubono & Ito 2002).

In the Mediterranean, since the 1970s the frequency of droughts has increased significantly, and a long-term process of aridification seems to be under way as a part of the generalized trend of global warming (Pereira et al. 2009). Climate change scenarios suggest an aggravation of environmental conditions for cork oak in the Mediterranean, namely through increasing temperatures and decreasing precipitation (Pausas 2004; Giannakopoulos et al. 2009; Pereira et al. 2009). In general, these factors are likely to increase the severity of plant water stress and increase the rate of nutrient losses from the soil (Pereira et al. 2009). More frequent and longer term droughts may negatively affect cork oak ecosystems in the future, by decreasing tree health and increasing the conditions conductive to the spread of some pests and diseases. Additionally, climate change is also expected to affect the current fire regimes in many regions, including the Mediterranean, by extending the fire season and increasing fire danger (Pausas 2004; Westerling et al. 2006, Flannigan et al. 2009; Catry et al. 2012).

Cork oak decline can affect both young and adult plants and can be either acute or chronic. In the first instance there is a sudden and uniform drying out of the foliage and the plant can even die during that same vegetative season. In the chronic form trees grow with difficulty

even for years with worsening symptoms. Firstly, the leaves turn yellow, then they dry out prematurely, but cases in which phylloptosis occurs directly are not rare. The plant reacts by growing new sprouts, generally microphyllics. In the more advanced stages, cortical necrotic areas in the trunk and biggest branches appear. They sometimes evolve into cankers which are often wet due to the emission of blackish exudates. The progressive drying out of the branches follows, starting with those at the highest part of the crown and those situated more internally. The tree produces new epicormic sprouts, often numerous and in bundles, which in turn die. In time, entire branches and eventually the whole plant dry out (Franceschini & Luciano 2009).

According to Costa et al. (2010), in the Mediterranean region, cork oak mortality has a higher probability of occurrence in areas with land use disturbances mainly due to land use extensification (e.g. shrub encroachment in cork oak woodlands, or agriculture abandonment) and in xeric conditions, that is, in shallow soils in flat and southerly slope landforms. Overall, the site attributes consistently associated with cork oak mortality were those which contribute to tree water stress, by inhibiting either a deep root development or the access to groundwater which is the main reliable water source for evergreen oaks in Mediterranean woodlands. The agroforestry management system and landscape features should be used with logistic regression procedures to predict major mortality incidence and therefore to identify “red signs” for cork oak mortality, which can be used for preventive or mitigation planning.

The decay of cork oak stands is therefore a complex phenomenon, that result of the interaction of multiple causes (Cabral & Sardinha 1992; Silva 2002; Sousa et al. 2007). Several factors influencing the decline are summarized into three groups, according to (i) predisposing factors, if they act during the life of the tree, weakening it; (ii) induction factors, if they act independently of tree vigor, but with more serious impact on weaker trees; (iii) accelerating factors, that only act on previously weakened trees (Table 1).

Table 1. Factors influencing cork oak decline (adapted from Cabral & Sardinha 1992; Sousa 1995)

Predisposing factors	Inducing factors	Accelerating factors
• overexploration 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

CORK OAK PESTS AND DISEASES

The occurrence of outbreaks of pests and diseases is often associated with states of decline of forest stands. Indeed, in addition to primary parasites, many species whose behavior is usually secondary parasite may become highly hazardous if habitat and food conditions are favorable (Cabral et al. 1993).

According to Ferreira & Ferreira (1986), 92 species of insects that can damage the cork oak but not all having economic repercussions are known in Portugal. It is generally considered that the defoliators such as the lepidopterans *Lymantria dispar* L., *Euproctis chrysorrhoea* L. and *Tortrix viridana* L., and hymenopteran *Periclista* spp. act as primary factors, while xylophages are considered as secondary agents (Sousa 1995). Nevertheless, since the 1980's, coinciding with the progressive degradation of the cork oak, an increase of beetle species attacks such as *Coroebus florentinus* (Herbst.), *C. undatus* (Fab) and xylomycetophagous *Platypus cylindrus* Fab. has been observed (Sousa 1995; Cabral & Ferreira 1999). The main pests that occur currently in Portugal, its damages, symptoms and favorable factors are summarized in Table 2.

P. cylindrus severe infestations have been observed in apparently healthy cork oaks causing widespread tree death within three months to one year and a half after the attack, depending on the host vigor and resistance. This insect establishes symbioses with ambrosia fungi essential to its nourishment and host colonization, mainly Opthiostomatales including several species that are pathogenic to cork oak. The combined action of extensive boring into the heartwood and the inoculation of ambrosia fungi leads to an increase in tree mortality over the past few years (Sousa & Inácio 2005; Henriques et al. 2006; Inácio et al. 2012).

A large number of fungi has been mentioned associated with the cork oak trees: pathogenics, saprophytics and symbionts. Santos et al. (1999) compiled all the information concerning fungi associated with different tree organs in a reference article for the mycoflora of cork oak in Portugal. According to Sousa et al. (2007) pathogens can be distinguished as primary pathogens, *Phytophthora cinnamomi* Rands and *Botryosphaeria* spp., and as secondary pathogens *Armillaria mellea* (Vahl:Fr.) Kumm., *Botryosphaeria* spp., *B. mediterranea*, *Coryneum modonium* (Sacc.) Griff. & Maubl. and *Endothiella gyrosa* Sacc.. The main diseases that occur currently in Portugal, its damages, symptoms and favorable factors are summarized in Table 2.

P. cinnamomi is a fungus-like organism (Oomycota/ Chromista), a soil-borne pathogen that causes primary infections in the root system, being often pointed out as a determining factor for sudden death of cork oak (Brasier et al. 1992; Moreira 2002; Tuset et al. 2002). The involvement of *Diplodia corticola* A.J.L. Phillips, A. Alves & J. Luque (a member of the Botryosphaeriaceae) on the decline of cork oak forests is also well known, causing symptoms like dieback, canker and vascular necrosis in oak trees (Alves et al. 2004; Linaldeddu et al. 2009; Fernandes et al. 2014).

Table 2 – The most important pests and diseases of cork oak in Portugal (adapted from Henriques 2007)

Pests			
Scientific Name	**Order / Family**	**Symptoms**	**Favorable factors**
Defoliators			
Euproctis chrysorrhoea	Lepidoptera / Lymantriidae	nests of leaves and silk; postures on leaf underside, skeletonized leaves, defoliation	poor soils and dry and very hot summer
Lymantria dispar	Lepidoptera / Lymantriidae	cream-colored postures, in the trunk, defoliation	weakened trees, with cracks in the bark of the trunk
Tortrix viridana	Lepidoptera / Tortricidae	rolled leaves with silk webs, buds destruction, defoliation	weakened and isolated trees
Periclista spp.	Hymenoptera / Tenthredinidae	chewed leaves, stratified defoliation	unfavorable climate and soils
Xylophagous and bark beetles			
Coroebus florentinus	Coleoptera / Buprestidae	reddish leaves and dry branches, bark blistering	isolated trees and exposed to sunlight
Coroebus undatus	Coleoptera / Buprestidae	larval galleries in the inner bark, yellowish exudates in the cork	weakened trees with thin bark, sandy, poor and acid soils, drought, excessive pruning and debarking
Platypus cylindrus	Coleoptera / Platypodidae	brownish leaves that then fall, circular holes and orange sawdust in the trunk	stressed, recently debarked trees
Diseases			
Scientific name	**Phylum / Order**	**Symptoms**	**Favorable factors**
Armillaria mellea	Basidiomycota / Agaricales	Crown progressive dieback; mycelium, carpophores and rhizomorphs	Weakened trees
Biscogniauxia mediterranea	Ascomycota / Xylariales	Transparency of the crown, cracks and carbonaceous stroma	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)/ Peronosporales	Tree decline, crown transparency, black exudations, stem rot	Mild winters, flooded soils

1.2. Charcoal canker, a cork oak disease caused by *Biscogniauxia mediterranea*

1.2.1. The species *Biscogniauxia mediterranea*

The fungus *B. mediterranea* is classified in the genus *Biscogniauxia*, family Xylariaceae, order Xylariales, sub-class Xylariomycetidae, class Sordariomycetes, division Pezizomycotina, phylum Ascomycota and kingdom Fungi. The actual preferred name of this species was revised by Kuntze in 1891 (Rev. Gen. Plant. 2: 398. 1891), but the synonym *Hypoxylon mediterraneum* was widely used, nevertheless the classification of this fungus has taken several changes being homotypic synonyms (Ju et al. 1998):

- *Sphaeria mediterranea* De Not. 1853
- *Nummularia mediterranea* (De Not.) Sacc. 1882
- *Nummularia regia* (De Not.) Sacc. var. *mediterranea* (De Not.) Traverso 1906
- *Hypoxylon mediterraneum* (De Not.) J.H Miller, 1941
- *Numulariola mediterranea* (De Not) P. Martin, 1969

Based on morphological features of the stroma and ascospores dimensions, three varieties of this species were described (Ju et al. 1998):

- *B. mediterranea* var. *mediterranea* (De Not.) Kuntze 1891
- *B. mediterranea* var. *macrospora* (J. H. Mill.) Y. M. Ju & J. D. Rogers 1998
- *B. mediterranea* var. *microspora* (J. H. Mill.) Y. M. Ju & J. D. Rogers 1998

The Xylariaceae

The Xylariaceae is one of the largest and most diverse families of Ascomycota, currently comprising over 1300 accepted species, and many more remain to be discovered. They are cosmopolitan, ubiquitous wood-degraders, but some genera are typically encountered on dung or associated with insect nests (Whalley 1996) and belong to the most prolific producers of bioactive secondary metabolites within the fungal kingdom, including antibiotics and phytotoxic compounds (Stadler 2011). They clearly exhibit their highest diversity in the tropics, but even in the temperate climate zones, new species are continuously being discovered.

Xylariaceous endophytes have been found in all investigated major groups of plants including conifers, monocots, dicots, ferns, and lycopsids plus liverworts/ hepatics (Davis et al. 2003).

Although are not usually considered to be important as plant pathogens, a growing number of species are now acknowledged to cause considerable economic loss in natural ecosystems or under agricultural conditions. Representatives of the family cause canker in trees, root rots in a wide range of plant species, and needle blights in conifers (Edwards et al. 2003) but often only when the host is water stressed (Whalley 1996).

Main characters used to delimitate genera within the family Xylariaceae include the morphology of asci, ascospores and stromata (unipartite, bipartite, uniperitheciate, ultiperitheciate, etc.), the presence of extractable stromatal pigments in KOH and the different anamorphs exhibited. These have been traditionally classified as *Nodulisporium*-like (including *Xylocladium*, *Periconiella*, *Virgariella* and more simple anamorphs with *Sporothrix*-like branching patterns), *Geniculosporium*-like (including *Acanthodochium*, *Dematophora*, *Dicyma* and *Xylocoremium*), *Libertella* and *Lindquistia*. Although there is no universally accepted system of classification for these teleomorphic genera, due to overlap in morphology and poorly resolved phylogeny, the view most widely accepted in recent times distributes most of the genera of the family in two large groups, the Hypoxyloideae, characterized by the production of stromatal pigments in KOH and *Nodulisporium*-like anamorphs (e.g. *Hypoxylon*, *Biscogniauxia*, *Camillea*, *Daldinia*), and the Xylarioideae, which do not yield stromatal pigments in KOH and produce *Geniculosporium*-like anamorphs (e.g. *Xylaria*, *Rosellinia*, *Nemania*) (Hsieh et al. 2005; Peláez et al. 2008; Tang et al. 2009).

The genus *Biscogniauxia* has a worldwide distribution with over 50 recognized taxa. Besides *B. mediterranea* other species cause canker under specific conditions. There is good evidence that these species also occur in healthy living trees as endophytes and then become invasive under water stress conditions. The most common examples are the *B. nummularia* (Bull.) Kuntze, *B. nothofagi* Whalley, Laessoe & Kile and *B. uniapiculata* (Penz. & Sacc.) Whalley & Laessoe that cause cankers in *Fagus*, *Nothofagus* and *Eucalyptus* (Nugent et al. 2005).

THE TELEOMORPH

Biscogniauxia taxa are exclusively parasites of dicotyledonous angiosperms having evolved with them on seasonally dry sites. Its species develop within the bark of hardwood parasites and forces produced between the inner outer stromatal layers along with the raised rims break the overlaying bark (Ju et al. 1998). Therefore, teleomorph is observed directly in the host. Moreover, in *B. mediterranea* the stroma results by the coexistence of compatible

mycelia in the host since the fungus has a heterothallic mating system, which represents an important internal source of genetic variability of the population, providing the fungus with the genetic flexibility for long-term survival and adaptation to the environment (Vannini et al. 1999).

Teleomorph of *B. mediterranea* is described as:

> Stromata applanate to slightly convex; surface dull black to shiny black, carbonaceous, with a dark brown to blackish outer layer long persisting at margin, 2-3 mm thick. Perithecia obovoid to tubular with black coarsely papillate ostioles. Short-stipitate, amyloid asci with dark brown ascospores, unicellular, ellipsoid with narrowly rounded ends, 13-17 x 5-7 µm, with straight germ slit spore-length, corresponding to *B. mediterranea* var. *microspora* (according to Ju et al. 1998).

This description is refered to current samples from cork oak from the region of Grândola (Portugal), that were observed, including by histological sections (Figure 2a-e). Studies of this species in Portugal, made by Barbosa in 1958, also indicate the variety *microspora* associated with charcoal canker in cork oak. According to Ju et al. (1998), the teleomorph of the typical variety presents ascospores with 15.5-21 x (6.5-)7-10 µm and the variety *macrospora* presents larger ascospores with 20-26 x 11.5-13 µm.

THE ANAMORPH

In natural conditions, the anamorphic stage although rare is refered as preceding perithecial development. It takes place under the outer bark of the host, leading to visible swellings on thin branches due to the accumulation of large amounts of conidia. The outer bark then cracks exposing on the surface a dry conidia mass of powdery aspect. In thick branches conidia remain under the outer bark not getting exposed (Jiménez et al. 2005a). Conidia of many, if not all, *Biscogniauxia* species germinate so they are undoubtedly, along with ascospores, effective inocula (Callan & Rogers 1986).

The shape of the conidia produced in culture and in infected branches is different. While in *in vitro* culture dominates the formation of obovoid conidia, matching the description given by Ju et al. (1998), conidia produced in the branches are always ellipsoidal, as described by Oliva & Molinas (1984). These differences in the morphology of conidia might have influence on their infectivity (Jiménez et al. 2005a). Anamorphs in culture are a variation of *Nodulisporium* Preuss, usually considered to be *Periconiella*-like (Jong & Rogers 1972; Callan & Rogers 1986;

Ju & Rogers 1996), however this species shows enormous variation in size, shape and structure of the conidiophores, ranging all the way from a simple *Sporothrix* type through *Acrostaphylus* to the extremely complex *Basidiobotrys* (Martin 1969).

Conidiophores are coarse, usually becoming warty and brown at maturity. Conidia are produced holoblastically from conidiogenous areas that become swollen from repeated conidial production in a limited area. Conidiogenous cell proliferation is essentially sympodial. Branching is from a main axis with apparent "apical dominance". This type of conidial apparatus is found in some *Hypoxylon* species and is illustrated by Ju & Rogers (1996). Conidia are mostly ovoid to obovoid with a flattened secession scar (Figure 2f).

The study of the anamorph stage of *B. mediterranea* is conducted in a set of isolates in order to evaluate the diversity of species associated with *Q. suber* in sub-chapter 3.3., therefore its further description will be accomplished in that section.

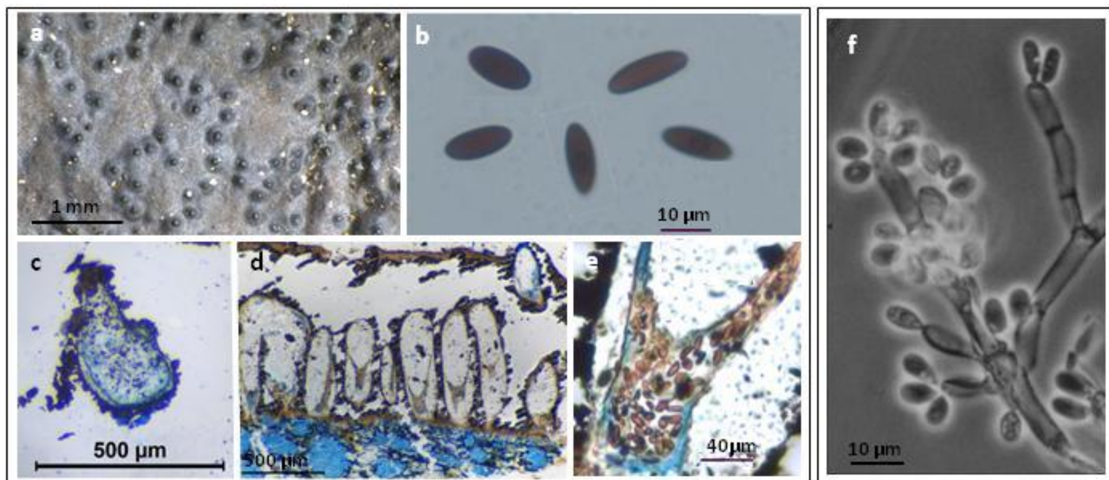

Figure 2. *Biscogniauxia mediterranea* in cork oak: a) aspect of the surface of the carbonaceous stroma; b) ascospores; c-e) aspects of the perithecia with ascospores; f) conidiophores with conidia in axenic culture.

1.2.2. The development of the disease

Distribution and incidence

B. mediterranea is widely dispersed in Europe, North America, Africa, New Zealand and Asia (China and India) (Jurc & Ogris et al. 2006, Mirabolfathy et al. 2011). It has been associated with the decay of oaks, being particularly alarming on *Q. suber* (Mazzaglia et al. 2001), however it has also been reported on *Q. alba* L., *Q. castaneifolia* Mey., *Q. cerris* L., *Q. coccifera* L., *Q. faginea* Lam., *Q. frainetto* Ten., *Q. imbricaria* Michx., *Q. lusitanica* Lam., *Q. palustris* Münchh., *Q. pubescens* Willd., *Q. pyrenaica* Willd., *Q. robur* L., *Q. rotundifolia*, *Q. virgiliana* Ten. (Collado et al. 2001; Mazzaglia et al. 2001; Jiménez et al. 2005a, Giambra et al. 2009, Mirabolfathy et al. 2011). The disease can occur as well on *Acer* sp., *Alnus* sp., *Castanea* sp., *Ceanothus* sp., *Eucalyptus* sp., *Fagus* sp., *Juglans* sp., *Lithocarpus* sp., *Platanus* sp., *Populus* sp. and *Rubus* sp. (Ju & Rogers 1996; Nugent et al. 2005).

In the Mediterranean basin, following the geographical distribution of cork oak, *B. mediterranea* is associated with its decline in Portugal (Santos 2003), Spain (Jiménez et al. 2005a), Italy (Franceschini et al. 2005), Morocco (Assali & Falki 2006), Algeria (Khouja et al. 2010), Tunisia (Linaldeddu et al. 2010).

In Portugal, charcoal canker was described in cork oak in 1930 (Câmara 1930). In a 1974 survey of cork oak stands in Portugal, 41.5 % of trees were recorded as diseased, with *B. mediterranea* being the principal pathogen (Macara 1975). Also Santos & Martins (1992) observed that the fungus was associated with ca. 40 % of the trees in an advanced stage of decline, with defoliation degree between 80-100 %. Because of its high frequency of occurrence in highly degraded stands focusing systematically on trees in advanced phase of decline or dead and plant material (trunk and branches) lying on the ground, *B. mediterranea* has been considered as a secondary pathogen (Santos 2003). Nevertheless, more recent observations recorded an increased incidence of the disease especially in young trees with atypical disease symptoms (Sousa et al. 2007).

For the generality of hosts this fungus was classified as a facultative, nonaggressive parasite that produces disease only on stressed hosts (Vannini & Mugnozza 1991). However, besides *Q. suber*, the aggravation of the disease has also been reported in *Q. cerris* in Italy (Vannini & Mugnozza 1991). Collado et al. (2001) registered different frequencies of colonization of *B. mediterranea* for different hosts, suggesting some level of host specificity or “expression specificity”. According to this last concept, some fungi are able to live endophytically within

the internal tissues of a variety of hosts, but they are only able to complete their life cycle and develop the teleomorph in a more restricted list of hosts (some times only in one species).

DISEASE EFFECTS

B. mediterranea infection causes the drying up of the woody organs and the appearance of lesions commonly called charcoal cankers. These appear following the laceration of the bark due to the growth pressure from the blackish stroma which the fungus forms in the colonized cortical tissues. In this stroma the sexual reproductive organs are clearly differentiated and, occasionally, the agamic ones (Franceschini & Luciano 2009).

The fungus can infect all aerial organs of the plants, in particular, the buds and, with a limited frequency, the roots (Linaldeddu et al. 2005a), however the attack is expressed by the production of carbonaceous stroma in the trunks and branches of infected hosts (Santos 2003) (Figure 3a, b). The foliage becomes discolorated and dry, and a dark exudation with a strong tannin odor may appear very soon after its colonization of plants (Vannini & Valentini 1994) (Figure 3c). In young affected trees is frequent to observe a brown powdery mass in the trunk and branches, under the cracked cork (Figure 3d).

Figure 3. Symptoms and signs of charcoal canker in cork oak: a) tree with dead branch with evident carbonaceous stroma; b) cork oak branch with the cork cracked and stroma underneath; c) dark exudation in the trunk, this symptom might be associated to multiple decline agents; d) branch of a young cork oak with brown powdery mass.

The fungus induces discoloration of the woody tissues. The spread of *B. mediterranea* within the xylem has been confirmed by microscopic observation of mycelia in the vessels by Vannini & Valentini (1994) who observed increased fungal growth in the xylem of seedlings in response

to water stress. There was a close relationship between the extent of discoloration and the loss of hydraulic conductivity. Because hydraulic conductivity losses parallel an increase in the number of embolized vessels, the formation of empty spaces in the vascular system could provide an effective way for the fungus to spread by mycelial elongation. After penetration of the vascular system, *B. mediterranea* increases the extent of xylem embolism, also contributing to the decline of infected trees which may be caused, wholly or in part, by a lack of xylem functionality and, hence, transport of water to the crown (Vannini & Valentini 1994). However, according to Collado et al. (2001), in *Q. rotundifolia*, the fungus, clearly more frequent in woody tissues, was found to colonize bark exclusively, not being recovered from xylem samples. Therefore, the authors argue that this fungus should be considered a phellophyte (a fungus colonizing only the outer bark, as opposite to true endophytes, inhabiting xylem). The preference of *B. mediterranea* to colonize bark, compared to decorticated wood, has been previously been reported (Whalley 1985).

Q. suber has a characteristic that singularize this species and is the basis for a feasible and sustainable exploitation of the tree as a cork producer. Cork is the outer tissue of the trunk and branches of trees, consisting of cells in which suberin is the main component. It is part of the periderm. The periderm is the result of the activity of a meristem, the cork cambium or phellogen, and is a system with a three-part layered structure: (a) phellogen, (b) phellem or cork, is formed by the phellogen to the outside, (c) phelloderm is divided by the phellogen to the interior (Figure 4). The removal of the cork layer exposes the phellogen to the atmosphere and it dies and dries out as well as the underneath cells. The exposure of the living tissues of the phloem induces the formation of new periderm (Pereira 2007).

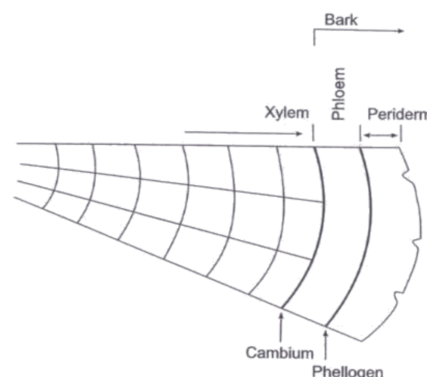

Figure 4. Schematic drawing of a cross-section of a tree stem showing the wood, the phloem and the periderm and the location of the lateral meristems (cambium and phellogen) (Pereira 2007).

The infection is found during all months of the year and the host can be killed in a single growing season. A large number of black stromata erupt simultaneously from the dead bark tissues harboring perithecia with ascospores that are considered the most important inoculum units. They are discharged through the year and germinate in a wide range of environmental conditions (Vannini et al. 1996a).

Infection by different genotypes can occur at different periods of the host life cycle, however, colonization of the tissues and reproduction probably occur at the same time from all the infection points. Such behavior could explain how *B. mediterranea*, although considered a weakness parasite, is able to kill large trees in a short time (Vannini et al. 1999).

In addition, three phytotoxic compounds were obtained from *B. mediterranea* by liquid culture filtrates: biscopyran, phenylacetic acid and 5-methylmellein. Phenylacetic acid, assayed at concentrations ranging from 0.26 to 0.026 mM, was toxic to *Q. suber*. Necrotic lesions appeared on the leaves within 5 days after absorption of the toxic solution. Cork oak cuttings wilted within 10 days at 0.26 mM. When byscopyran was assayed at concentration ranging from 0.26 to 0.026 mM on cork oak cuttings, epinasty was observed. The compound 5-methylmellein plays a role in disease induced on the host plant (Evidente et al. 2005).

ENDOPHYTIC FUNGI

The most common definition of endophytes is “all organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host” (Hyde & Soytong 2008). ‘Fungal’ endophytes are a taxonomically and ecologically heterogeneous group of ubiquitous intercellular ‘fungi’ belonging to the Ascomycota (Petrini et al. 1992), Basidiomycota and Oomycota (Saikkonen et al. 1998) that seem to make up a large fraction of the fungal biodiversity (Arnold et al. 2000; Arnold et al. 2001). They have been found in every studied plant species (algae, mosses, ferns, conifers, grasses, palms, shrubs and monocotyledonous and dicotyledonous angiosperms) from agricultural land to natural forests (Arnold 2007; Hyde & Soytong 2008).

In general, two major groups of endophytic fungi have been recognized reflecting differences in evolutionary relatedness, taxonomy, plant hosts, and ecological functions: clavicipitalean and non-clavicipitalean endophytes (Petrini 1991; Stone & Petrini 1997; Stone et al. 2004; Schulz & Boyle 2005). Species of the Clavicipitaceae form symbioses almost exclusively with grass hosts. Grass endophytes colonize their hosts systemically (except the roots) and several

species are transmitted vertically by seeds to the next host generation. In contrast, host colonization by non-clavicipitalean endophytes is mainly non-systemic and is restricted to disjunctive, endophytic microthalli which may consist of only a few cells (Stone 1987). Non-clavicipitalean endophytes represent a broad range of species from several families of ascomycetes and can be recovered from asymptomatic tissues of nonvascular plants, ferns and allies, conifers, and angiosperms (Sieber et al. 1988).

Continuous forests can also be fragmented byr the endophytic fungi in several ways. First, discontinuous distribution of suitable host species may constrain distribution of specialist endophyte in a continuous mixed forest. Second, fungal assemblage may vary along the forest succession, and the same fungal species may occupy different micro-habitats or host species even within the lifespan of the fungus. Third, endophyte diversity may be fine-tuned by genetic structure of otherwise seemingly continuous and homogenous forests or hybrid zones of the host species (Saikkonen 2007).

Endophytic fungi, although accumulate as tissue ages, may infect living organisms but symptoms do not develop, because, once inside the tissue, they assume a quiescent (latent) state either for the whole lifetime of the infected plant tissue or for an extended period of time, i.e. until environmental conditions are favorable for the fungus or the phase disposition of the host changes to the advantage of the fungus (Sieber 2007). A position of an endophytic fungus in an ecosystem, and how it responds to resources and enemies, may change because a fungus continuously affect, and is affected by both abiotic and biotic environment within the limits of its genotype and phenotypic plasticity. Consequently, the length of extended latency period of the fungus may vary in ecological time (Saikkonen 2007).

Much have been written on the roles of endophytes, they have been implicated in mutualism, decreased herbivory, increased drought resistance, increased disease resistance and enhancement of plant growth, nevertheless, there are numerous examples of endophytes that become pathogens (Hyde & Soytong 2008). Sieber (2007) states that endophytic “pathogens” have co-evolved with their hosts and are thus not highly virulent. Apart from genetic factors, most switches from a mutualistic to a parasitic interaction are characterized by an imbalance in nutrient exchange (Kogel et al. 2006) or by environmental variations (Hendry et al. 2002; Moricca & Ragazzi 2008). Furthermore, the type of interaction between an endophyte and a host plant is modulated if the plant is subjected to physiological stress and/or senescence. The plant under stress is then attacked by the endophyte and is invariably killed by it. It is precisely the weakness parasites living as endophytes that may become pathogenic as the physiological

state of the trees deteriorates, and thus break the state of endemic equilibrium (Ragazzi et al. 2001).

A high number of bioactive compounds have been found in endophytes particularly from higher plants, which have proved to be a rich source of bioactive and chemically novel compounds (Cheng et al. 2012), they produce secondary metabolites with antimicrobial, insecticidal and many other bioactivities, which are becoming valuable sources for the discovery of new pharmaceuticals, agrochemicals and lead compounds (Van Veen et al. 1997).

FAVORABLE FACTORS

Throughout the different stages of development of the disease, both *B. mediterranea* and susceptible hosts are conditioned by abiotic and biotic factors. Environmental stresses such as drought, low or high temperatures, high irradiance and lack of some soil nutrients may lead to physiological plant modifications and influence the plant susceptibility to fungal pathogens (Linaldeddu et al. 2011).

Water stress is the main predisposing factor in charcoal canker development. Under conditions of water stress, alterations in cell metabolism result in both qualitative and quantitative changes in carbohydrate and amino acid composition that can improve the availability of nutrients to pathogens. Another possible effect of water stress on plant-pathogen interaction is a decrease in the plant's ability to develop a physical barrier against pathogens, because cell enlargement and division are affected by changes in water relations (Vannini & Valentini 1994). In addition, the change of water content in the host tissues is an interacting factor that can trigger the activity of *B. mediterranea*. Studies carried out mainly in *Q. cerris* but also in *Q. suber*, confirmed that the fungus accumulates in the host tissues and it is during the dry growing seasons that it has a better chance to proliferate in asymptomatic tissues (Vannini & Mugnozza 1991; Collado et al. 2001).

Artificial inoculation of the fungus in bark tissues in *Q. cerris* and monitoring fungus colonization in natural conditions submitted to different water regimes revealed that mass colonization of bark and woody tissues and symptoms of charcoal disease only occurred on hosts with predawn leaf water potential (PWP) values below -3.0 MPa, which correspond to water stress conditions associated with severe loss in xylem conductivity because of embolism (Vannini & Mugnozza 1991; Vannini & Valentini 1994; Vannini et al. 2009; Linaldeddu et al. 2011). Pathogenicity tests preformed in cut branches or seedlings of *Q. suber, Q. cerris*, *Q.*

rotundifolia and *Q. coccifera* showed that *B. mediterranea* has a prevailing ability to develop on dead tissues and although the fungus is able to colonize the plant endophytically, cortical necrosis only develops when plants suffer severe water stress, showing that cork oak is the more susceptible and that high temperatures favor the development of cortical necrosis and the formation of carbonaceous stroma (Vannini & Valentini 1994; Santos 1999; Luque et al. 2000; Jiménez et al. 2005c; Vannini et al. 2009).

Water stress, besides favoring the endophytic infections of some opportunistic fungal pathogens, and causing a reduction in cork oak endophytic microbial biodiversity, seems to negatively influence the endophytic proliferation of some antagonistic fungal species, such as *Trichoderma citrinoviride* Bissett (Linaldeddu et al. 2011).

In the opposite, the discharge and germination of ascospores depend on the presence of water or high relative air humidity. Ascospores are ejected from stromata in forest during most of the year following periods of high precipitation (Vannini et al. 1996b; Jiminéz et al. 2005b; Linaldeddu et al. 2005a). In Italy, ascospores release may occur in all seasons, while conidia are usually released at the end of the summer (Linaldeddu et al. 2005a).

The optimal temperature for ascospores germination is 35 ^{o}C, but 25 and 30 ^{o}C are still favorable, while 20 and 40 ^{o}C reduced germination. Ascospores kept at 5 ^{o}C were able to germinate after short exposure to temperatures ranging from 20 to 35 ^{o}C (Vannini et al. 1996b). According to Linaldeddu et al. (2005a) the infection can be found at temperatures ranging from 5 to 40 ^{o}C. The optimum temperature for growth of *B. mediterranea* is 30 ^{o}C (Vannini & Valentini 1994). This wide temperatures range undoubtedly contributes to the wide diffusion of *B. mediterranea* in Mediterranean environments, where the winters are becoming milder and the rainfall distribution more irregular, even in the hottest season. The cork oak is extracted from the trees during the summer, and this exposes large areas of life tissues of the trunk, making it particularly susceptible to infection of this fungus (Franceschini & Luciano 2009).

The plant decay consequent to the fire stimulates the latent infection of *B. mediterranea*. Its endophytic incidence in different plant tissues appears to be related to the intensity of the damage. Probably the loss of water in the tissues could be the main factor that induced symptomatic attacks (Anselmi & Mazzaglia 2005).

Atmospheric pollution is an abiotic stress factor affecting increasingly Mediterranean forests, namely ozone deposition (Lindner et al. 2010). Paoletti et al. (2007) showed that pre-exposure

to ozone increases susceptibility of *Q. cerris* leaves to successful fungal attacks by *B. mediterranea*. The slight ozone-induced reduction in the amount of epicuticular waxes and increase in leaf wettability may favor fungal penetration of the cuticle. However, the main cause of the increased leaf injury in ozone-exposed seedlings appeared to be the higher germination rate of spores than in control leaves. Ozone may alter the chemical composition of epicuticular waxes, thus changing the environment for spore attachment and germination, and alter content and quality of leaf nitrogen and carbohydrates, thus rendering the leaves a better substrate for fungi.

The presence of susceptible host and its physiological condition interfere both in colonization by the fungus and in its development in endophytic and pathogenic stages. The host tissues apparently stimulate ascospores germination. A higher availability of nutrients for the spores or the presence of germination stimulators could be involved. The stimulation of germ-tube formation in the presence of wounds could account for the better chance this fungus has of penetrating the host through small discontinuities. Minor discontinuities, more than major wounds, are considered potential ways of entry for fungi (Vannini et al. 1996a).

Changes in plant susceptibility to disease can be observed as the result of a change in the nutrition of the host. The nitrogen supply of the host plant has been known to exert a considerable effect on both host and pathogen. The amino acids can stimulate or inhibit fungal parasitic development, when their balance in host tissues is switched from normal, changes in nutritional conditions for the fungus might result. The presence of nutritive basal medium of L-amino acids (alanine, aspargine, glycine and proline) influenced the behavior of *B. mediterranea in vitro*: larger colony growth and dry weight of mycelium was observed on aspargine-enriched medium, while limited performance was shown by glycine-enriched medium; in contrast, a variable behavior of the isolates was reported when alanine and proline were the only nitrogen source (Turco et al. 2005). The fungus tolerates a wide range of pH (Vannini et al. 1996b).

In addition to the action of primary pathogens that weaken the host, such as *Botryosphaeria* spp. and *P. cinnamomi* (Branco et al. 2014), there are several insects that may be involved in different ways. Outbreaks of *L. dispar* on *Q. cerris* are known from southern Europe and are often claimed as factors predisposing the trees to colonization by weak plant pathogens, such as *B. mediterranea*. Insect defoliation seems to promote fungal development in stressed trees, whereas its effect is negligible in watered plants (Capretti & Battisti 2007). On the other hand, insects act as fungus vectors between hosts: the ambrosia beetle *P. cylindrus* was reported to

specifically transport *B. mediterranea* in *Q. suber* (Inácio et al. 2011), and *Agrilus graminis* Gory & Laporte and *Tropideres* sp. transport *B. mediterranea* to *Q. cerris* in Italy (Vannini et al. 1996a). There is also a strong correlation between the presence of holes made by the insects *Cerambyx* spp. and *B. mediterranea* infection in cork oak (Martin et al. 2005).

As part of <u>global climate change</u>, according to Intergovernmental Panel on Climate Change (IPCC), until the end of the XXI century, for the Mediterranean zone annual mean temperatures are projected to increase in the order of 3-4 ºC (4-5 ºC in summer and 2-3 ºC in winter). Yearly rainfall is expected to drop by up to 20 % of current annual precipitation (up to 50 % less in the summer), whereas winter precipitation is expected to increase. Changes in frequency, intensity, and duration of extreme events are likely to result in more hot days, heat waves, heavy precipitation events and fewer cold days (Linder et al. 2010).

Forests are particularly sensitive to climate change, because the long life-span of trees does not allow for rapid adaptation to environmental changes. Associated with climate change there are several factors affecting forest ecosystems, which can act independently or in combination. Changes in the chemical atmospheric environment such as CO_2, ozone and nitrogen concentrations affect tree physiology, carbon allocation and plant interactions, resulting in complex interactions with other climatic impact factors (Keenan et al. 2011). The changes in climate will also have associated consequences for biotic (frequency and consequences of pest and disease outbreaks) and abiotic disturbances (changes in fire occurrence, changes in wind storm frequency and intensity) with strong implications for forest ecosystems (Linder et al. 2010).

Theoretically, three main types of disease-host effects can be expected in response to climate changes: (1) direct effect on pathogens; (2) indirect effects on pathogens through other community interactions; (3) interaction effects through host physiology; here, two types of interactions can be distinguished: an effect on tree susceptibility to disease - the predisposition concept; or combined effects of infection and change on tree physiology: the multiple stress concept (Desprez-Loustau et al. 2006).

The evolution of climate in temperate regions may specifically favor thermophilic fungi able to persist in trees until the water regime is disrupted. Pathogens of Mediterranean or tropical origin extending their geographic range in response to climate change represent a serious threat (Allen et al. 2010). Climatic changes will, as a consequence, favor the impact of charcoal

disease in *Q. suber* forests, which is already predicted by several authors (Desprez-Loustau et al. 2006; La Porta et al. 2008).

CONTROL MEASURES

So far, there are no effective curative treatments for charcoal canker in cork oak, therefore it is essential to adopt preventive measures to control the disease. As the development of *B. mediterranea* is associated with tree weakness, the best control lies in correct cultural practices to improve tree vigor, such as avoid the over explotation of the stand, avoid deep soil mobilization and correct macro- and micronutrients and pH balance in the soil (IEFC 2002).

The application of good sanitary practices in the stand is essential for the maintenance of trees, including more targeted measures for this disease. It is important bearing in mind both the high polyphagy of *B. mediterranea* and the almost uninterrupted release and diffusion of its spores throughout the whole year (Linaldeddu et al. 2005a). To prevent the spread of the fungus of through its spores from carbonaceous stromas, dried branches and trunks of cork oaks and other hosts, should immediately be pruned, and dead trees should be cut. There is no need to remove the root systems of died trees because it is a fungal infection that does not attack the roots (Juan 1985). All the dead material cut or remaining in the soil must be removed out of the stand otherwise the fungus continues to develop in the saprophytic state and releasing spores. Pruning wounds in live branches might be one of the entrances used by the fungus to introduce itself as an endophyte in the host trees. To prevent this, pruning wounds of living branches must be protected with a disinfecting sealant (Juan 1985).

The cork extraction is carried out during the summer, exposing large areas of live trunk tissue, making it particularly susceptible to infection of the fungus. Thus, it would be important that the trunks would be treated with suitable fungicides immediately after cork extraction, above all in wet summers (Linaldeddu et al. 2005a). All tools used for pruning and cork extraction must be disinfected between trees to prevent direct contamination of subsequent intervened trees (IEFC 2002).

The use of antagonistic microorganisms for controlling charcoal canker is currently being explored. In vitro, *Trichoderma viride* Pers.:Fr. proved to be effective against *B. mediterranea*, it did not show any at distance inhibition but after the contact it was able to rapidly parasitize the mycelium of *B. mediterranea* and to actively reduce its growth, limiting it to a very small portion of the plate or destroying it completely (Mazzaglia et al. 2005). Also *T. citrinoviride* is

characterized by a strong antagonistic activity against the main pathogens involved in oak decline (Linaldeddu et al. 2005b). This species isolated from cork oak has been shown to produce a mixture of polypeptide antibiotics (peptaibols) in liquid culture that showed a strong antifungal activity against seven dangerous forest tree pathogens (Maddau et al. 2009).

Laboratorial Detection Methods

Xylareaceous fungi recovered as endophytes are difficult to identify to the species level based on morphology in pure culture due to the lack of cultural descriptions and diagnostic characteristics for many species (Collado et al. 2001). Moreover, in order to study the location of *B. mediterranea* in the host tissue during the latent phase, traditional approaches such as isolation from tissues, have several limitations and difficulties. Different parameters affect the reliability of the results and the possibility of detecting the fungus, for example the type of tissue, size of the sample, and competition with other organisms (Mazzaglia et al. 2001).

Using molecular approaches, sequence analysis of the internal transcribed spacer (ITS) region of the rDNA (including 5,8S rRNA gene) may be used for the identification of *B. mediterranea* (Collado et al. 2001). Mazzaglia et al. (2001) developed the primers MED1 and MED2, comprised between ITS1 and ITS4 generic primers for the rDNA, that are highly specific for *B. mediterranea*, being able to amplify the target sequences both *in vitro* and *in vivo*, which enhanced the possibility to study the presence and localization of the fungus during its latent phase in *Quercus* species. Luchi et al. (2005) developed a real-time quantitative PCR method for the detection of *B. mediterranea* in symptomless oaks tissues using specific primers and probe, and a TaqMan assay procedure. Real-time PCR is a sensitive and fast technique able to specifically detect and quantify the DNA of *B. mediterranea* in oak tissue. This diagnostic method is a precise tool to localize fungi in symptomless plant tissues and promises to advance our understanding of fungal infection during their latent phase.

2. *Biscogniauxia mediterranea* in cork oak stands

2.1. New outbreaks of charcoal canker on young cork oak trees in Portugal

Henriques J, Inácio ML, Lima A, Sousa E. 2012. IOBC/wprs Bulletin 76: 85-88

2.2. Factors affecting the dispersion of *Biscogniauxia mediterranea* in Portuguese cork oak stands

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New outbreaks of charcoal canker on young cork oak trees in Portugal

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Abstract: *Biscogniauxia mediterranea*, the causal agent of charcoal canker, is widely spread in Mediterranean region affecting mainly *Quercus suber*. It has been considered a secondary pathogen that only attacks weakened hosts but, in the last years, we have been witnessing an increasing number of young vigorous trees exhibiting charcoal canker symptoms in Portugal. Beyond the fungus teleomorphic stage usually present in the infected hosts, it has been frequently observed in young trees its anamorphic stage. The impact of climatic changes on fungal development and forest vulnerability is discussed, particularly in the Mediterranean scenario.

Key words: *Quercus suber; Biscogniauxia mediterranea;* anamorph; climatic change

Introduction

Biscogniauxia mediterranea (de Not.) Kuntze has long been recognized as the causal agent of a necrotic disease on stems and branches of hardwoods, known as charcoal canker (Vannini et al. 1999). The fungus occurs on a wide range of hosts, particularly of the genus *Quercus*, and has been regarded as an important contributing factor in the decline of cork oak (*Quercus suber* L.) in Europe and North Africa (Nugent et al. 2005). In spite of this close association with cork oak stand decline, several discordant opinions have been put forward regarding the relationships between this fungus and its preferred host. It has been considered an opportunistic parasite that attacks trees already damaged by other biotic or abiotic factors. Host susceptibility to the fungus has been associated to periods of severe drought, insect defoliation or fire. As the host is subjected to water stress, the pathogen can easily spread through large cavity vessels, and colonize bark and woody tissues, being able to kill the host in a single growing season (Vannini & Valentini 1994). It can spend part of its life-cycle as an endophyte living in different symptomless host tissues and organs which represents an important strategy: being already inside the host, the fungus can rapidly spread from several infection points during its pathogenic phase (Mazzaglia et al. 2001). A large number of black stromata erupt simultaneously from the dead bark tissues harbouring perithecia with ascospores that are considered the most important inoculum units of *B. mediterranea* (Vannini et al. 1999). In natural conditions, conidia are produced on a forming stromata under the cork that becomes thicker and harder as the sexual stage of the fungus develops. Conidia only become visible and are released on thin branches; in thick branches their presence has only been recorded after removing the cork, never being released to the environment (Jiménez et al. 2005).

In Portugal, poor attention has been paid to cork oak charcoal canker as it used to develop only in mature trees already weakened by other agents (Santos 2003). Nevertheless, recently, numerous young cork oak trees have been suffering a sudden decline process exhibiting an atypical expansion of this disease. This work reports the preliminary results of

investigations carried out to clarify the *B. mediterranea* involvement in these affected young trees.

Material and methods

Young cork oak plantations of *ca.* 15 years old with severe mortality were observed in the main Portuguese cork oak productive areas (Alentejo and Ribatejo). Declined trees were cut and the logs and infected branches were analysed in the laboratory. Infected branches were cut in transversal sections to observe the area colonized by fungus.

Fungi were isolated and identified based on cultural and morphological features assessed on cultures grown on potato-dextrose agar (Difco PDA, USA) after five days, in the darkness at 25±1°C. Macroscopic characters of colonies were described after 5 days of growth; colour names are from Saccardo (1891).

The molecular analysis of the isolates was based on the amplification and sequencing of the ITS rDNA region, according to Collado et al. (2001) using ITS4/ITS5 primers. For a preliminary taxonomical placement, each sequence was used as a query for blastn of BLAST (Basic Local Alignment Search Tool) searches against the NCBI (National Center for Biotechnology Information) nucleotide databases.

Results and discussion

Young cork oak trees begin their decline process as the foliage becomes discolorated and dry, the trunk exhibits viscous liquid exudates and the cracking of the cork. In the liber is evident the presence of a brown powdery fungi. The observation of transversal sections of infected branches showed the liber completely colonized by the fungi. This description coincides with Jiménez et al. (2005) report of the anamorphic stage of *B. mediterranea* on thin branches.

The characteristics of the isolated fungi are in accordance with the genera *Nodulisporium* Preuss: mycelium is partly immersed, conidiophores macronematous and mononematous, arising laterally from the brownish vegetative hyphae, with principal axis erect, septate, branched, hyaline to light brown, slightly rugouse, conidiogenic cells are poliblastic and sympodial, slender or short and thick and verticillated. Conidia are sympodulosporic, acropleurogenous, unicellular, hyaline or brown to olive in mass, ellipsoidal or obovoid, smooth or roughened, with a small frill when detached. Cultural aspect is effuse, cotton-like, with high density, white to grey with green emergences in the colony center and no zonation, the color of the lower surface is fuliginous.

Anamorphs of *Biscogniauxia* genus are referable to the form-genus *Nodulisporium* (Hsieh & Rogers 2005). Collado et al. (2001) and Giambra et al. (2009) also characterize *Nodulisporium* sp. as the anamorphic form of *B. mediterranea*. Ju & Rogers (1996) and Ju et al. (1998) described it based on branching patterns of its conidiogenous structure, detailing it as *Periconiella*-like.

The nucleotide sequences of the ITS4/ITS5 primed products were homologous in length among isolates with ca. 600bp. This analysis confirmed the relation of the isolated fungus with *B. mediterranea*: BLAST search of the NCBI database showed that the sequences closest to those of *Nodulisporium* sp. were from *B. mediterranea*. The highest similarities (with a maximum identity 100% and E value 0) found were with *B. mediterranea* from *Quercus ilex* in Central Spain (NCBI accession numbers AF280625.1 and AF280624.1) and from *Quercus cerris* in Tuscany, Italy (NCBI accession number AJ246222.1).

Charcoal canker is one of the most frequent disorders on cork oak in Portugal. *B. mediterranea* has been described as endophyte in oak tissues that only attacks weakened hosts being thus considered a secondary pathogen (Santos, 2003). It has always been reported in perithecial form; this is the first Portuguese report of the occurrence and characterisation of its mitosporic form in natural conditions.

Moreover, *B. mediterranea* incidence in young cork oak trees without other decline symptoms suggests a possible behaviour disturbance of this pathogen that may be becoming more aggressive. Such observations might be related with the actual global climate changes scenario, particularly severe in the Mediterranean region (Desprez-Loustau et al. 2006; Lindner et al. 2010).

Climatic changes appear to favor the impact of charcoal canker in *Q. suber* forests. Periods of drought have greatly increased susceptibility of trees to stress-induced pathogens. Abundance of *B. mediterranea* in tissues and its ability to shift from the endophytic to the pathogenic phase is mediated by water stress conditions of the host (Desprez-Loustau et al. 2006). The whole pathosystem favors the disease, the host tree became more debilitated and the fungus finds suitable conditions for its development: *B mediterranea* is a highly thermophilic species with the capacity to develop under very low water potential (Vannini et al. 1996).

Further studies are needed on the fungus biology and on the young trees physiological and sanitary state. It is necessary to clarify the epidemiology of the charcoal canker on Portuguese *montados* conditions.

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Factors Affecting the Dispersion of *Biscogniauxia mediterranea* in Portuguese Cork Oak Stands

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Abstract. Charcoal canker is a frequent *Quercus suber* disease that nowadays contributes to its decline in Portugal. It is caused by *Biscogniauxia mediterranea*, a wide dispersed endophytic fungus that may be present in symptomless trees, becoming more aggressive in susceptible stressed hosts. This work aims to clarify the fungus dispersal by airborne dispersion and transmission from a tree to its progeny through infected tissues. Thus a Hirst spore-trap and meteorological station were installed in the forest. The results confirmed that ascospores spread as airborne inoculum in natural conditions varying throughout the year. Precipitation is the main factor for ascospores release and wind the way of dispersal. The transmission of the fungus by infected tissues was evaluated in acorns and seedlings from declined and asymptomatic trees, in natural regeneration and in stump sprouting regeneration from infected trees. Although *B. mediterranea* is present in the seeds, it was not found in seedlings, indicating there is no vertical transmission of the disease. It was also found in natural regeneration in a reduced rate, not due to seed contamination but by early aerial infection. In stump sprouting the fungus was not detected, so it can be used to replace trees.

Key word: acorn, charcoal canker disease, meteorological conditions, natural regeneration, *Quercus suber*, stump sprout

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Fatores que afetam a dispersão de *Biscogniauxia mediterranea* nos montados em Portugal

Resumo. O carvão do entrecasco é uma doença frequente de *Quercus suber* que atualmente contribui para o seu declínio em Portugal. Esta doença é provocada por *Biscogniauxia mediterranea*, um fungo endófito que pode estar presente em árvores assintomáticas, tornando-se agressivo em hospedeiros sob stress. Este trabalho tem como objetivo esclarecer a dispersão do fungo por via aérea e a transmissão através de tecidos infetados da árvore para a descendência. Assim, foram instalados no montado um aparelho capta-esporos tipo Hirst e uma estação meteorológica. Os resultados confirmaram que ascósporos dispersam-se no ar em condições naturais variando ao longo do ano. A precipitação é o principal fator para a libertação dos ascósporos e o vento o meio de dispersão. A transmissão do fungo por tecidos infetados foi avaliada em bolotas e plântulas de árvores degradadas e assintomáticas, em regeneração natural e em rebentamentos de toiça de árvores infetadas. Apesar de *B. mediterranea* estar presente nas sementes, não foi detetada nas plântulas, indicando que não há transmissão vertical da doença. Foi encontrado em taxa reduzida na regeneração natural, não pela contaminação de sementes, mas por infeção aérea precoce. Nos rebentamentos de toiça o fungo não foi detetado, pelo que poderão ser utilizados para substituição de árvores degradadas.

Palavras-chave: bolota, carvão do entrecasco, condições meteorológicas, *Quercus suber*, rebentamentos de toiça, regeneração natural

Facteurs affectant la dispersion de *Biscogniauxia mediterranea* en peuplements de chêne-liège au Portugal

Résumé. Le charbon-de-la-mère est une maladie fréquente du *Quercus suber* entrainant actuellement son déclin au Portugal. Cette maladie est causée par *Biscogniauxia mediterranea*, un champignon endophyte qui peut être présent dans les arbres asymptomatiques. Il devient plus agressif chez les hôtes sous stress. Ce travail vise à clarifier la dispersion du champignon dans l'air et la transmission à travers des tissus infectés de l'arbre à la descendance. Ainsi, un piège à spores Hirst et une station météorologique ont été installés dans la subéraie. Les résultats ont confirmé que les ascospores sont dispersées dans l'air dans les conditions naturelles variant tout au long de l'année. La précipitation est le principal facteur pour la libération des ascospores et le vent le milieu de la dispersion. La transmission du champignon par les tissus infectés a été évaluée dans les glands et les semis d'arbres dépérissants et asymptomatiques, dans la régénération naturelle et des rejets de souche des arbres morts contaminés. Bien que *B. mediterranea* soit présente dans les glands, il n'a pas été trouvé dans les semis, indiquant absence de transmission verticale de la maladie. Il a été trouvé en taux réduit dans la régénération naturelle, pas à cause de la contamination des glands, mais par une infection aérienne prématurée. Dans les rejets de souche le champignon n'a pas été détecté, de sorte qu'ils peuvent être utilisés pour remplacer les arbres dépérissants.

Mots-clés: charbon-de-la-mère, conditions météorologiques, gland, *Quercus suber,* régénération naturelle, rejets de souche

Introduction

Biscogniauxia mediterranea (De Not.) O. Kuntze is an endophytic fungus, widespread in Portuguese cork oak (*Quercus suber* L.) forests causing charcoal canker disease, presently considered one of the interveners on cork oak decline. This fungus was described in cork oak in Portugal in 1931, as secondary pathogen, associated with trees in an advanced state of decline. However, recently, numerous younger and not degraded cork oak trees have been suffering a sudden decline exhibiting an atypical expansion of this disease (HENRIQUES *et al.*, 2012).

The fungus can live as an endophyte in all of the aerial organs of the plants and act as a pathogen when the oaks suffer prolonged periods of stress. It induces discoloration of the woody tissues, dieback and stem and branch cankers, progressing to the appearance of characteristic black carbonaceous stromata on dead tissues (SANTOS, 2003). In the stromal surface, mature small papillae shaped protrusions are observed corresponding to perithecial necks. Within perithecia are produced ascospores that are considered the most important propagation units of *B. mediterranea*. Also conidia and mycelia fragments constitute effective inoculum for fungus dissemination (JIMÉNEZ *et al.*, 2005). A great abundance of propagules is produced on colonized parts of the tree.

The dispersal of inoculum units is essential for plant pathogens to infect new hosts and complete their life cycles, for gene flow and diversification of pathogen populations. It is important to understand dispersal mechanisms in order to devise better methods to detect plant pathogens and control the disease they cause (WEST, 2014). In general, inoculum are transported to susceptible hosts by abiotic and biotic vectors: plant pathogens can be dispersed by air, rain, water or soil, and by vectors such as animals, pollen, various microbes, people and machinery and on infected plant material including seeds (WEST, 2014).

The most relevant factor for *B. mediterranea* dispersion in the field is related with meteorological conditions. In fact, both discharge and germination of ascospores are influenced by them: the ejection of ascospores is associated with high precipitation or high relative humidity and is greatly reduced during dry period (VANNINI *et al.*, 1996b).

Insects are the main biotic vectors, not only transporting the inoculum but also causing wounds that act as an infection point (JIMÉNEZ *et al.*, 2005). They spread the pathogens in short distances within the fields but also over long distances depending on insects' bioecology (WEST, 2014). Several insects have

been identified as *B. mediterranea* vectors, such as *Platypus cylindrus* associated to cork oak in Portugal (INÁCIO *et al.*, 2011) and *Agrilus* spp. and *Tropideres* spp. associated to *Quercus cerris* in Italy (VANNINI *et al.*, 1996a). In Spain and Morocco, the presence of *Cerambyx* spp. was associated with the incidence of the disease in *Q. suber*, although no evidence proved their action as fungi vectors (MARTÍN *et al.*, 2005).

Other pathway for *B. mediterranea* spread is manmade operations in managed forests: the fungal spores transported by pruning or cork removal forestry equipment can infect other trees. Also, a bad cork removal practice impedes the natural healing of injuries and facilitates the development of diseases (MONTOYA OLIVER, 1988).

Direct transmission of plant diseases might occur through infected plant tissues including vertical transmission when the pathogen infects the seed progeny of an infected plant (ZABALGOGEAZCOA, 2008) and through root and stump sprouting. *B. mediterranea* has been found as endophyte in different host tissues and organs including leaves, buds, twigs, branches and trunk, wood and bark (FRANCESCHINI *et al.*, 2002). Nevertheless, there aren't references to studies searching this fungus endophytically on acorns of *Quercus* spp. questioning the possibility of seed borne transmission, nor in stump sprouts, commonly used in Portuguese managed stands when the stump sprouts in apparently good vegetative and phytosanitary conditions are used to replace felled trees.

The objective of this work is to clarify the dispersal of *B. mediterranea* in Portuguese cork oak stands conditions, namely by aerial dispersal of ascospores associated with meteorological conditions and the dispersion of the fungus from a tree to its progeny through infected tissues: seeds and stump sprout regeneration.

Methods

Aerial dispersion of ascospores of *Biscogniauxia mediterranea* associated with meteorological conditions: To monitor *B. mediterranea* ascospores dispersion in field conditions, a 7 day recording volumetric spore trap (Burkard), described by HIRST (1952), and a meteorological station (HOBOware®Pro) were installed in Herdade das Barradas da Serra, Grândola (Alentejo, Portugal), from 5th July 2011 to 13th December 2012. The data of wind velocity were provided by Escola Profissional de Desenvolvimento Rural de Grândola. The stand is located at 38º11'29.35''N 8º37'12.38''O at 150 m altitude with steep slope relief and

southeast exposure. It has a density of 147 trees/ha and an agrosilvopastoral land use management.

Based on logging interval of 30 min, daily and weekly average air temperature, relative humidity and daily and weekly total precipitation were obtained. Daily wind velocity was obtained from 10 min intervals average. Associations between the weekly total numbers of ascospores and the meteorological variables were verified through regression analysis, for the period of October 2011 to October 2012. Association between daily total numbers of ascospores and the daily wind velocity was also verified through regression analysis for the period of September to October 2011. The relation between the number of ascospores and meteorological variables were tested by Pearson's coefficient for linear correlations, or Spearman coefficient for non linear correlations. The effect of temperature on spores release was further studied for the period of October 2011 to October 2012: three classes of weekly average temperature were considered (6–12 ºC, 12–18 ºC and 18–24ºC) that were compared using an one way factor ANOVA and a LSD test for determination of homogeneous groups. In order to clarify the relation of ascospores dispersion with precipitation, two classes were considered: days with precipitation above 0.5 mm (1) and days below (0). Non-parametric Kruskall-Wallis test were used to compare the number of captured ascospores between the number of consecutive days with precipitation above 0.5 mm in three days periods. When significant differences were found, post-hoc paired differences were evaluated with Mann-Whitney U tests. Statistical analyses were performed using STATISTICA 6 software.

Dispersion of *Biscogniauxia mediterranea* through infected tissues: seeds and stump sprout regeneration: 200 acorns were collected from three asymptomatic trees and 200 from three declined trees in the region of Grândola in November 2012. One hundred acorns of each lot were used for immediate fungi isolation and the other hundred were germinated for further isolation of fungi from the seedlings.

Acorns were cut into pericarp and seed coat together (protection tissues) and inner seed (embryo and endosperm), using a sample of about 5 mm^2 of each part for fungus isolation. All samples were superficially sterilized through immersion in 9% H_2O_2 for 12 min in the case of protection tissues and 6 min for inner seed, subsequently rinsed five times in sterile water and left to dry on sterile filter paper under aseptic conditions (GONTHIER *et al.*, 2006; LINALDEDDU *et al.*, 2011). All samples were placed in Petri dishes containing Potato Dextrose Agar (PDA, Difco, USA) acidified with lactic acid (1 ml lactic

acid 85% / l PDA, PDAA) to avoid bacterial growth and incubated at 25±1°C in darkness. The development of *B. mediterranea* was assessed after 5 and 10 days of growth.

A second batch of 80 acorns from undifferentiated origin were collected and cut into protection tissues (pericarp and seed coat), embryo and endosperm to clarify the presence of the fungi in the different parts of the inner seed. Samples preparation was as described above with the following times of sterilization in 9% H_2O_2: 10 min for external tissues, 2 min for embryo and 6 min for endosperm (GONTHIER *et al.*, 2006; LINALDEDDU *et al.*, 2011).

The preparation of the acorns for germination was made in accordance to the International Seed Testing Association protocol (ISTA, 1996). The acorns were previously superficially disinfected with sodium hypochlorite 1.5% for 5 min. The trays were kept in a control climatic chamber at 20ºC, with a photoperiod of 16 l/8 d for 80 days. The portion of the stem between the cotyledon leaves and next leaves of each seedling was sectioned and used for fungi isolation. Samples preparation was as described above with superficial sterilization in 9% H_2O_2 for 12 min (GONTHIER *et al.*, 2006; LINALDEDDU *et al.*, 2011).

100 samples of asymptomatic natural regeneration of cork oak less than three years old were collected in stands in the region of Grândola. The portion of the stem between the first and second leaf node was sectioned and used for fungi isolation. Samples preparation was as described above with superficial sterilization in 9% H_2O_2 for 15 min (GONTHIER *et al.*, 2006; LINALDEDDU *et al.*, 2011).

To search for *B. mediterranea* in stump sprouts data from two study plots installed in a cork oak plantation stand, in Herdade de Corta-Rabos in the municipality of Montemor-o-Novo (Alentejo, Portugal) were used. The plantation was made in 1995; the two plots of 150 trees were installed in 2010 and monitored until 2013. All the trees were characterized, and the sanitary conditions were observed every year, in particular for the presence of charcoal canker disease. 100 samples of asymptomatic stump sprouts less than three years old of cork oak trees that had charcoal canker were collected. The portion of the stem between the first and second leaf node was sectioned and used for fungi isolation. Samples preparation was as described above with superficial sterilization in 9% H_2O_2 for 15 min (GONTHIER *et al.*, 2006; LINALDEDDU *et al.*, 2011).

Results

Aerial dispersion of ascospores of *Biscogniauxia mediterranea* associated with meteorological conditions: Ascospores of *B. mediterranea* were captured by the spore-trap device, over the period of study, with varying amounts throughout the year. Figure 1 represents the data of the daily captures of *B. mediterranea* ascospores from 5th July 2011 to 13th December 2012 and the daily average temperature, average relative humidity, average wind velocity and total precipitation for the same period (data collection was interrupted due to equipment failure for some periods).

A linear regression model adjusted between the number of ascospores weekly captured and the weekly total precipitation revealed an accuracy of 54%. Pearson's correlation coefficient indicated a positive significant relationship between the two variables ($r=0.74$, $p<0.05$, $n=43$). It was not possible to adjust any model to the relation between the weekly ascospores capture and weekly medium temperature and relative humidity and also to daily ascospores capture and daily wind velocity. However, the Spearman's correlation coefficients obtained indicated a negative significant relation between weekly ascospores capture and average temperature ($r_s=-0.62$, $p<0.05$, $n=43$), a positive but not significant relation with relative humidity ($r_s=0.21$, $p>0.05$, $n=43$) and a positive significant correlation between daily ascospores capture and wind velocity ($r_s=0.38$, $p<0.05$, $n=43$).

The distribution of weekly captures of ascospores in accordance with average temperatures (Figure 2) allowed to distinguish three classes of temperature: 6–12 ºC, 12–18 ºC and 18–24ºC. The result of one way factor ANOVA demonstrated that temperature interval has a significant effect on the release of ascospores ($F_{(2,40)}=10.1164$; $p=0.0003$) and LSD test showed that the class of temperature 12-18°C induced the highest release of ascospores ($\bar{x}$ of ascospores = 116.3333) differing significantly from classes 6–12 ºC ($\bar{x}$ of ascospores = 34.5000) and 18–24 ºC ($\bar{x}$ of ascospores = 6.7273).

The results of the analysis of relation of the number of daily ascospores captures and number of consecutive days with precipitation above 0.5 mm in three days periods prior to the captures is showed in Table 1. Significant differences were only found between three consecutive days with precipitation below 0.5 mm and three consecutive days with precipitation above it.

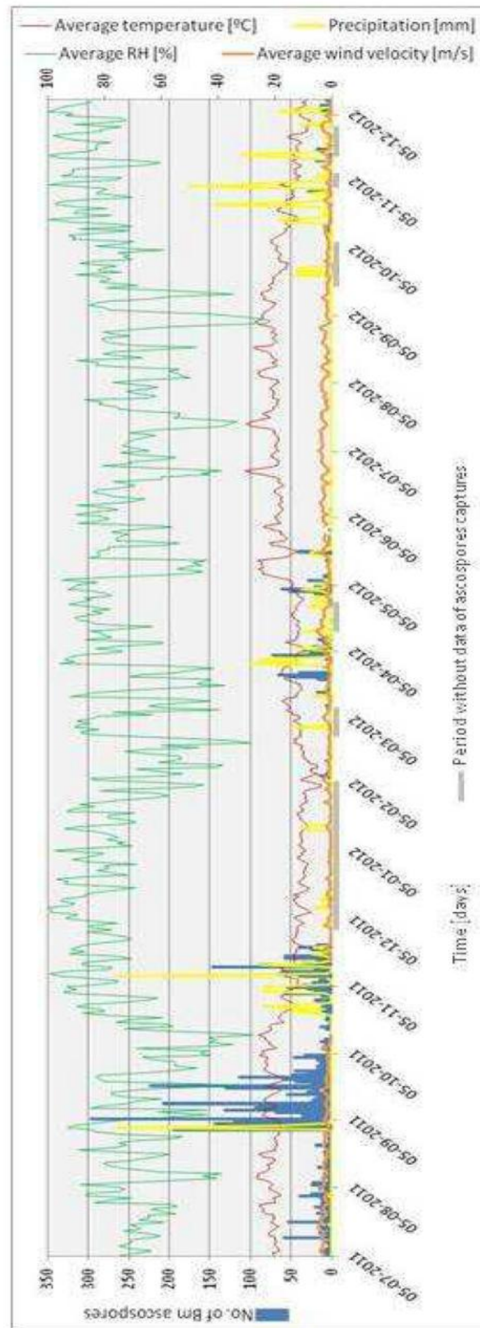

Figure 1 *- Daily captures of ascospores of Biscogniauxia mediterranea and meteorological variables from 5th July 2011 to 13th December 2012*

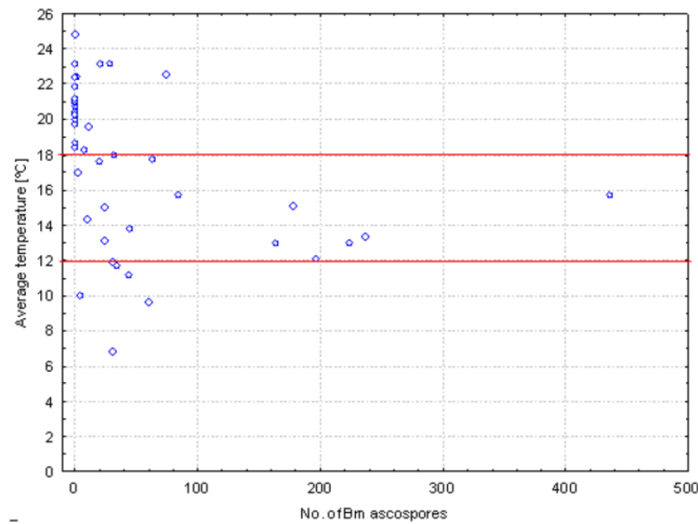

Figure 2 *- Distribution of weekly captures of ascospores according to weekly average temperatures for the period of October 2011 to October 2012*

Table 1 *– Results of the Mann-Whitney U tests comparing the number of daily ascospores captures and the number of consecutive days with precipitation above 0.5mm (Nº days P>0.5 mm) in three and four days periods prior to captures*

Period	Nº days P>0.5 mm	N	Ascospores $\bar{x}$±SD	Nº days P>0.5 mm		
				0	1	2
3 days	0	114	24.63±40.23			
	1	12	29.75±31.04	U=489.50 p=0.1060 n.s.		
	2	8	28±23.83	U=299.00 p=0.1044 n.s.	U=46.00 p=0.8774 n.s.	
	3	10	43.6±42.50	U=326.50 p=0.0254*	U=48,00 p=0.4288n.s	U=33.50 p=0.5636n.s.

n.s. no statistical significance
*Statistically significant (Mann-Whitney U test, p<0.05)

<u>Dispersion of *Biscogniauxia mediterranea* through infected tissues: seeds and stump sprout regeneration:</u> *B. mediterranea* was isolated both from acorns of declined and asymptomatic trees (Figure 3a). In both cases the fungus was isolated from protection tissues, but it was found in a slightly higher quantity in

declined trees: 6% of protection tissues of acorns from declined trees and 4% from asymptomatic trees. In the inner seed tissues the fungus was only found in acorns from declined trees and only in 1% of the samples. However, the fungus was not isolated from any seedling germinated from the acorns batches of asymptomatic and declining trees.

To clarify the presence of *B. mediterranea* within the acorns and the non-transmission to the seedling, the second batch of acorns from a different origin was evaluated showing that 6% of the samples were infected in the protection tissues and, in the inner seed, 14% were infected in the endosperm but none in the embryo (Figure 3b). In this second batch the rate of infection of the protection tissues was equivalent to acorns from declined trees of the first batch, however, the level of contamination of inner tissues was higher. Nevertheless, the fungus was not detected in the embryo.

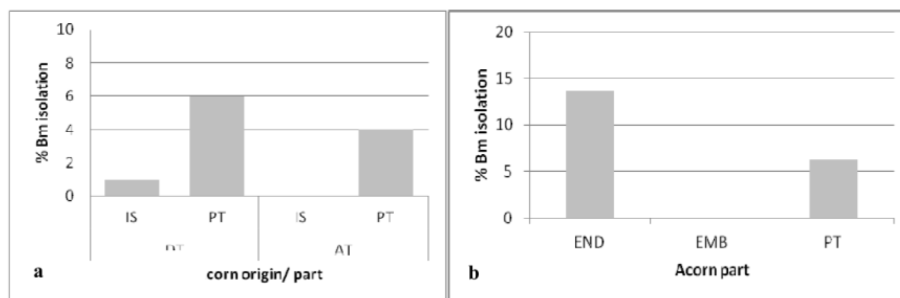

Figure 3 *– Isolation of Biscogniauxia mediterranea from (a) acorns collected from declined trees (DT) and asymptomatic trees (AT), divided in inner seed (IS), which includes embryo and endosperm, and protection tissues (PT), which includes pericarp and seed coat; (b) acorns parts: endosperm (END), embryo (EMB) and protection tissues*

From cork oak natural regeneration with less than three years old, *B. mediterranea* was only isolated in 1% of the samples.

B. mediterranea was not isolated from the samples of asymptomatic sprouts collected from stumps of cork oak trees that had charcoal canker disease.

Discussion

Spore-trapping studies showed that *Biscogniauxia mediterranea* ascospores spread as airborne inoculum in natural cork oak stand conditions. The dispersal of ascospores occurs throughout the year with a large variation in the amount of spores present in the air, ceasing almost completely during the warm and dry season. Precipitation is the main meteorological variable that influences ascospores dispersion being positively related with it. However, the results demonstrate that significant differences in ascospores discharge are found after a period of three consecutive days with precipitation above 0.5 mm. The consecutive days with precipitation ensure the humidity needed for the ejection of ascospores and to prevent their desiccation until the accommodation in the host and germination.

Our results are in agreement with other authors in the theory that precipitation is the determining factor for the dispersal of ascospores of *B. mediterranea*. According to VANNINI *et al.* (1996b), ejection of ascospores is associated with high precipitation or high relative humidity and is greatly reduced during dry periods. JIMÉNEZ *et al.* (2005) found a positive significant correlation of the amount of sporulation of *B. mediterranea* with monthly precipitation (mm/day), days with precipitation (regardless of the quantity) and relative humidity. However, the daily and hour level approaches demonstrate that only days with precipitation above 0.5 mm significantly influence the discharge of spores with no direct relationship with the quantity of precipitation. This relationship probably reflects the fact that in the months with more capture of ascospores there were longer periods of time with precipitation, more than the total amount of rainfall.

The temperature is negatively related with ascospores dispersion as was also demonstrated by JIMÉNEZ *et al.* (2005) although VANNINI *et al.* (1996b) argued that average monthly temperatures do not affect *B. mediterranea* ascospores discharge. In fact, during the warmer season of 2012 the ascospores release ceased. The summer of 2011 was particularly rainy in the Alentejo region (IPMA, 2011a, 2011b) so that despite high temperatures there was dispersal of ascospores. These observations allow us to conclude that although both the temperature and precipitation significantly influence the dispersion of spores, precipitation is the most determining factor for the effect.

Although the results have not shown a significant correlation of ascospores discharge and relative humidity, it revealed a positive tendency which was expected as the humidity increases with precipitation. JIMÉNEZ *et al.* (2005)

confirmed the relation of *B. mediterranea* ascospores captures and average relative humidity.

The wind velocity is also associated with ascospores dispersion as assures their aerial transport. JIMÉNEZ *et al.* (2005) found a divergence between the hours with precipitation and the hours with spores captures in trap, which shows that although precipitation is needed to cause the discharge of ascospores, the primary vehicle for dispersion of ascospores is the wind, not water, because in the hours with more precipitation the dispersal of ascospores decreased, being significantly higher in subsequent hours.

Transmission of *B. mediterranea* to new plants from infected *Q. suber* tissues, seeds and sprouts was not observed in this work. The isolation of *B. mediterranea* in cork oak acorns was low in asymptomatic and declined trees. In both cases the fungus was found in protection tissues even when it wasn't present in inner tissues which may suggest a superficial infection of the seed due to aerial dispersion of fungus inoculum. Only in acorns from declined trees the fungus was found in inner seed, suggesting the systemic contamination of the seed, nevertheless in neither situation the fungus was found in seedlings of *Q. suber* germinated from seeds of the same origin. Although the contamination rate of the seeds is low, apparently the fungus does not pass from seed to the plant, which is supported by the results showing that within the seed the fungus was only present in the cotyledons but not in the embryo.

Seed-borne transmission of endophytes is intensively studied on clavicipitaceous endophytes and their grass hosts (SCHARLD *et al.*, 2004), but in tree hosts it is poorly known. Nevertheless, the results presented in this work are in line with others. According to ZABALGOGEAZCOA (2008), horizontal transmission seems to be the predominant mechanism of dispersion among endophytic species. Using RT-PCR techniques, *B. mediterranea* DNA was not detected on oak epicotyls (*Quercus cerris* and *Quercus ilex*) (LUCHI *et al.*, 2005), neither from two weeks old seedlings of *Q. cerris* generated from surface sterilized acorns in growth chamber, using species specific primers PCR (MAZZAGLIA *et al.*, 2001). Infection studies of *Discula quercina*, an endophyte of *Quercus garryana*, showed that despite the presence of the endophyte in the acorn seed coat and cotyledons, it does not appear to grow systemically throughout the plant (WILSON and CARROLL, 1994). Studies in tropical trees showed that seeds and seedlings are virtually endophyte-free, and the incidence of fungal endophytes increases as leaves or seeds grow older, indicating that this dynamics must be driven by horizontal transmission (ARNOLD *et al.*, 2003; GALLERY *et al.*, 2007).

The fact that plants of natural regeneration under three years old might be contaminated by *B. mediterranea*, even in a very low rate, should also be a result of air-borne dissemination of the fungus in the field. Plant age has an effect upon endophyte diversity and density. As time of exposure to endophyte inoculum increases, plants seem to accumulate an increasing number of endophytes in their tissues. Because of this, older plant parts may harbor more endophytes than younger ones (ZABALGOGEAZCOA, 2008). Nevertheless, it is important to consider that plants might contain *B. mediterranea* growing endophytically.

All the analyzed stump sprouts were free of *B. mediterranea* which suggests that the fungus doesn't grow systemically from the base of the trunk or roots to the sprouts. The fungus colonizes bark and woody tissues and of all tree aerial organs but there aren't any references to its presence in the roots in *Q. suber* or other hosts (FRANCESCHINI *et al*., 2002). Therefore, with respect to the charcoal canker disease, the use of stump sprouts seems to be a safe practice for the development of the tree which will then be exposed to possible contamination like other trees.

Overall, the primary means of dispersal of *B. mediterranea* in cork oak stands is by air. The determining factor for the significant release of ascospores is the precipitation, being particularly aggravated in short periods with consecutive days of precipitation above 0.5 mm and mild temperature. The wind velocity is also decisive since it is the vehicle for the dispersal of ascospores. The airborne dispersal of ascospores constitutes an indiscriminate source of inoculum for the trees that can infect all aerial organs including seeds. However, seeds do not constitute a source of contamination since the fungus does not develop systemically to the seedlings. Also the stump sprouts being originally exempted from the fungus can be exploited for the regeneration of trees. In any case, the early contamination of plants/ trees occurs thus being essential the permanent management of the stand aiming a good health and physiological status of the tree.

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3. DIVERSITY OF *BISCOGNIAUXIA MEDITERRANEA* ASSOCIATED TO CORK OAK

3.1. DIVERSITY OF BISCOGNIAUXIA MEDITERRANEA WITHIN SINGLE STROMATA ON CORK OAK

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3.2. ANALYSIS OF THE GENETIC DIVERSITY AND PHYLOGENETIC RELATIONSHIPS OF *BISCOGNIAUXIA MEDITERRANEA* ISOLATES ASSOCIATED WITH CORK OAK

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3.3. MORPHO-CULTURAL AND PHYSIOLOGICAL FEATURES OF *BISCOGNIAUXIA MEDITERRANEA* ISOLATES ASSOCIATED WITH CORK OAK

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Research Article

Diversity of *Biscogniauxia mediterranea* within Single Stromata on Cork Oak

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Charcoal canker, caused by the fungus *Biscogniauxia mediterranea*, is one of the most frequent diseases of cork oak in Portugal. The pathogen has been considered a secondary invader that attacks only stressed hosts; however, in recent years, an increasing number of young trees exhibiting the disease symptoms have been recorded. A collection of monoascosporic cultures isolated from single stromata of *B. mediterranea* in cork oak from different locations was analyzed by means of microsatellite—Primed Polymerase Chain Reaction—using three microsatellite primers, in order to detect the genetic variation of the population thus discussing its plasticity and ability to adapt to different conditions. The results showed a high level of genetic variability among isolates obtained from the same stroma, being impossible to distinguish isolates from individual stromata neither from different geographical location.

1. Introduction

Quercus suber L., cork oak, is the most emblematic tree of Portugal due to its high environmental, social, and economical value. *Biscogniauxia mediterranea* (De Not.) O. Kuntze (Xylariaceae, Xylariales) is well known as the causal agent of charcoal canker in cork oak [1]. This fungus can live as an endophyte in all of the aerial organs of the oak plants and can act as an opportunistic pathogen when the hosts suffer prolonged periods of stress. In those conditions, *B. mediterranea* is able to rapidly colonize the xylem and bark tissues, induce necrosis and canker formation, and accelerate tree decline and eventually death [2–4]. The great abundance of inoculum produced on colonized parts of the tree and the dispersal of fungal ascospores, airborne and by insects, is important factors accounting for fungal spread in the forests [5–7].

Recent observations on Portuguese cork oak stands revealed the increased incidence of charcoal canker and the presence of atypical symptoms, especially in young trees, which questioned whether some alteration occurred on the disease epidemiology [8, 9]. The evidence of high genetic variability and the heterothallic mating system can support the adaptive strategy of the fungus and its epidemiology [10], particularly facing actual conditions of climate change which appear to favor the impact of charcoal disease in *Q. suber* forests [11].

With this work we intended to evaluate the diversity of *B. mediterranea* within individual hosts in Portugal, through the analysis of Microsatellite—Primed Polymerase Chain Reaction (MSP-PCR) profiles of monoascosporic cultures isolated from single stromas in cork oak.

2. Material and Methods

A collection of 16 isolates of *B. mediterranea* was analyzed, eight monoascosporic isolates obtained from two stromata from adult declined *Q. suber* sampled in Comporta (A) and Grândola (B) (Alentejo, Portugal). Monoascosporic cultures

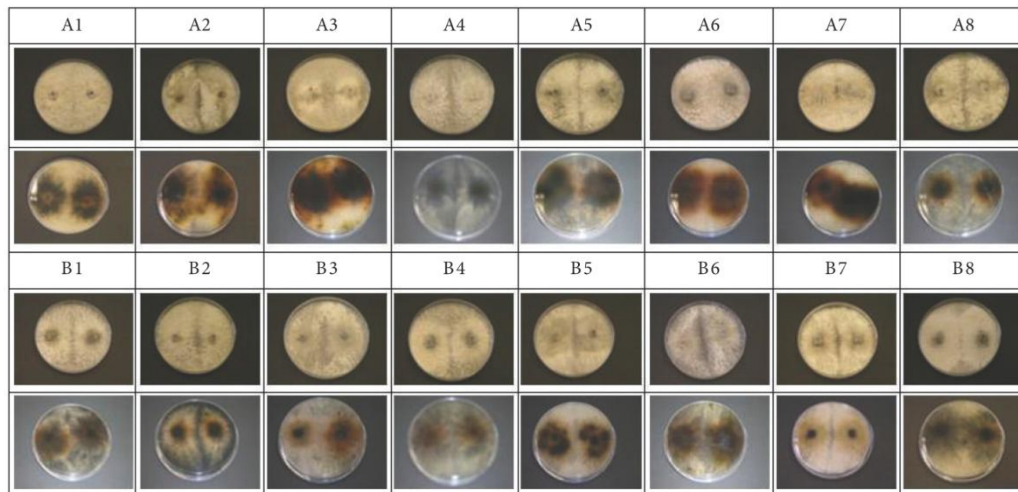

Figure 1: Seven days colonies on PDA of monoascospores isolates of *Biscogniauxia mediterranea* from the stromas A and B (surface and reverse).

were started by incubating stromata in Petri dishes at 22°C and 100% relative humidity. After 12 h the ascospores discharges on the dishes were collected, and single ascospores were individualized with a needle and plated on Potato Dextrose Agar (PDA, Difco, USA) acidified with lactic acid (1 mL lactic acid 85%/L PDA, PDAA) and incubated at 25 ± 1°C in darkness [12]. Seven-day growth cultures on PDA were evaluated for the general aspect of the colony, density, and surface and reverse colors (according to Rayner color chart [13]). Voucher specimens of each isolate were deposited in the fungal collection at Micoteca da Estação Agronómica Nacional (MEAN) at INIAV (Oeiras, Portugal).

DNA was isolated from mycelia scraped from the surface of a PDA plate and extracted with the DNeasy Plant Mini Kit (Qiagen, USA), following the manufacturer's instructions. Instead of using ground lyophilized mycelia, fresh mycelium was used and disrupted by adding approximately 50 μL of glass beads (425–600 μm diameter) to the extraction buffer and vortexing for 2 min before and after RNase A incubation [14].

The collection of 16 isolates was analyzed by means of MSP-PCR. The profiles were generated following the protocol of Uddin and Stevenson [15] using the primers (CAG)5, (GACA)4, and (GTG)5. PCR reactions were carried out in a total reaction volume of 25 μL containing approximately 10 ng of genomic DNA (quantified by Nanodrop 2000 Spectrophotometer, Thermo Scientific, USA), 1 μM of either oligonucleotide primer, 1x Dream Taq buffer (DreamTaq PCR Master Mix, Fermentas, Germany) which includes Taq polymerase (unknown concentration), 0.2 mM dNTPs, and 3 mM $MgCl_2$. Thermal cycling was performed on a Tgradient Thermocycler (Biometra, Germany) using the following parameters: an initial incubation at 94°C for 2 min, followed by 40 cycles of 30 s at 93°C, 1 min at 53°C, 30 s at 72°C, and a final 72°C extension period of 10 min. Amplicons were separated by electrophoresis at 7 V cm^{-1} in agarose gel (1.5%) containing 0.5 μg/mL ethidium bromide and 1x TBE running buffer. Data analysis was visualized by Versa Doc Gel Imaging System (BioRad, USA). The isolates were clustered on the basis of their profiles in consensus dendrogram built with NTSYSpc2 (Numerical Taxonomy and Multivariate Analysis System, version 2.1) using DICE coefficient and UPGMA.

3. Results

The isolates of *B. mediterranea* presented high variability in culture, especially in pigmentation and presence of aerial mycelium. Monoascosporic isolates from the same stroma showed evident differences among cultures (Figure 1). Cultural aspects of the seven days colonies from both stromas varied from velvety to wholly with mycelial tufts dispersed in the culture to velvety with sectors (according to the density) and tufts, density media to high. Colors differ from white with vinaceous buff aerial mycelium, grayish sepia to smoke grey, or butt margin with olivaceous center. In some colonies dark brown exudates are frequent. The reverse of the colonies varies from buff margin with umber to olivaceous center, buff to honey margin, saffron to sienna center, or pale mouse grey to mouse grey, with darker spots dispersed in the culture or strong diffusible pigment.

The molecular analysis of monoascosporic isolates by MSP-PCR resolved distinct amplification banding patterns between 0,25 and 1,2 kb for the primer (CAG)5, between 0,25 kb and 0,7 kb for (GACA)4, and between 0,5 and 1,3 kb for (GTG)5, resulting in a total of 20 different band positions. The three primers generated different amplification patterns among isolates even from the same stroma. One consensus dendrogram was obtained from combined analysis of the profiles generated by the three primers for isolates from a single stroma (Figure 2) and for the all set of isolates (Figure 3).

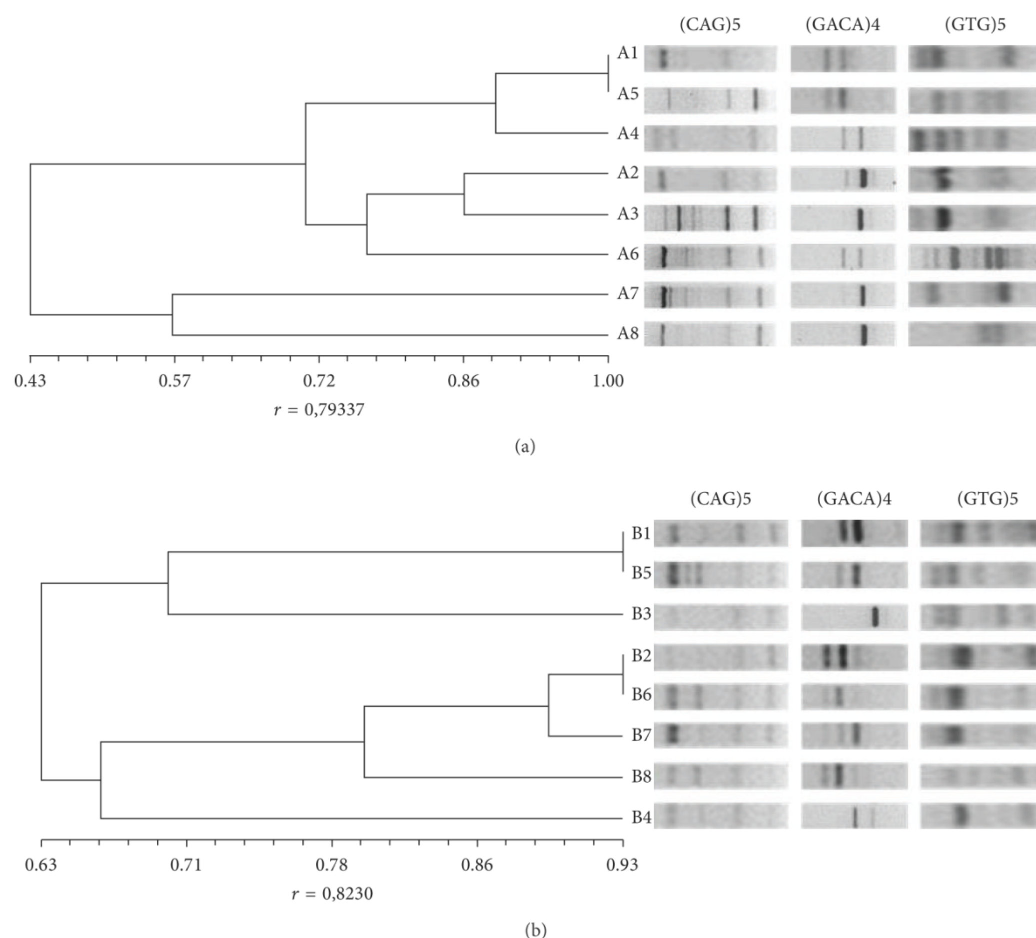

Figure 2: Consensus dendrograms from (CAG)5, (GACA)4, and (GTG)5 MSP-PCR profiles performed in NTSYSpc2 using DICE's correlation coefficient and UPGMA. Scale bar represents percentage of similarity. (a) Analysis of *Biscogniauxia mediterranea* isolates obtained from stroma A (Comporta); (b) analysis of *B. mediterranea* isolates obtained from stroma B (Grândola).

Among the monoascosporic cultures originated from the stroma collected both in Comporta and in Grândola, the isolates exhibited a high level of variability within each stroma, clustering in two main groups. In Comporta, a cluster with two isolates and another with six isolates were formed with 43% similarity. In the first group the two isolates were 57% similar and in the second group the isolates clustered at different levels higher than 70% similarity, with only two 100% similar isolates (Figure 2). In Grândola, the two main groups were 63% similar, presenting three isolates in a cluster in which one is segregated with 70% similarity and two were up to 90% similar, and the other five isolates clustered at different levels of similarity higher than 65%, with also two isolates up to 90% similar (Figure 2). The joint analysis of isolates from the two sites showed a high variability among all, and the isolates were grouped in increasing levels of similarity above 46%, with no distinction between the isolates of each local (Figure 3).

4. Discussion

In the present study, a high genetic variability of *B. mediterranea* was detected within populations of monoascosporic cultures isolated from single stromata and from different hosts/localities. Individually, the MSP-PCR primers profiles showed a high degree of diversity among isolates from each sampled stroma, and a joint analysis of all isolates did not reveal clustering according to the different stroma. The use of combined MSP-PCR profiles has strengthened these observations, highlighting the vast genetic diversity among isolates. The occurrence of high genetic variability of *B. mediterranea* in a single stroma is in line with the results presented by Vannini et al. [10] in which the variability of the fungus was assessed by random amplified polymorphic DNA (RAPD); however, this approach allowed the discrimination of monoascosporic isolates within a single ascus, a single stroma, and among stromata. Also Schiaffino et al. [16], using

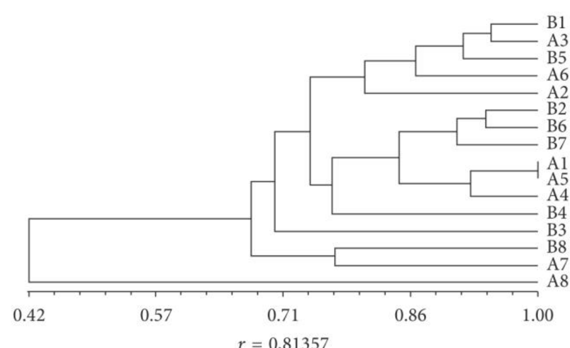

FIGURE 3: Analysis of *Biscogniauxia mediterranea* total isolates. Similarity consensus dendrogram from (CAG)5, (GACA)4, and (GTG)5 MSP-PCR profiles performed in NTSYSpc2 using DICE's correlation coefficient and UPGMA.

the same technique, showed high level of genetic variability among isolates from a restricted area of Sardinia as well as among isolates from different localities.

The application of neutral genetic markers to plant pathogens has enabled the finding that a lesion is often colonized by several genetically distinct individuals, suggesting that host coinfection is relatively common. Examples include *Phaeosphaeria nodorum* [17] and *Mycosphaerella graminicola* on wheat [18], *Alternaria* sp. on pear leaves [19], *Ascochyta rabiei* on chickpea [20], *Aspergillus flavus* on cotton [21], *Rhynchosporium secalis* on barley [22], and *Leptosphaeria maculans* on oilseed rape [23].

The presence of high genetic variability also in small populations could be partially explained by the consideration that ascospores are the most important dispersal and inoculums units in *B. mediterranea*, as in other Xylariaceae, and those new genotypes can spread over long distances [24]. The high rate of sexual reproduction and the heterothallic mating system of this fungus represent an important internal source of genetic variability of the population [10]. Being a heterothallic fungus, the occurrence of multiple genotypes in the same lesion allows isolates of opposite mating types to come together and reproduce sexually. Frequent sexual reproduction in turn will ensure frequent recombination and increased evolutionary adaptability [18].

In addition, infection by different genotypes can occur at different times of the host life cycle, keeping the fungus as endophyte. However, colonization of the tissues and reproduction occur at the same time from all the infection points, when the host is subjected to stress. Such behavior could explain how this fungus, though being considered a weakness parasite, is able to kill large trees in a short period [10].

Large variability of *B. mediterranea* population is extremely important for its epidemiology since it provides the fungus with genetic flexibility for long-term survival and adaptation to the environment. Coexistence of pathogen clones within the same host plant has manifold biological implications beyond the increased opportunities for sexual reproduction. For example, coexistence can affect host health or infectiousness and affect the transmission success of individual clones, thus shaping the evolution of traits such as virulence/aggressiveness or fungicide resistance [18].

It is important to consider this high adaptive capacity of *B. mediterranea*, notably in the scenario of climate change. The predictions that comprise fungus' physiological features already indicate that the impact of charcoal disease in *Q. suber* forests will be favored under the aggravated climate conditions for the Mediterranean basin [2, 25].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Analysis of the genetic diversity and phylogenetic relationships of *Biscogniauxia mediterranea* isolates associated with cork oak

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Summary

Biscogniauxia mediterranea is one of the most frequent fungal pathogens involved in cork oak decline in the Mediterranean Basin, causing charcoal canker. In Portugal, this disease is widespread on adult declining trees but nowadays it increasingly affects young trees and exhibits atypical symptoms, leading to the hypothesis that some change in the fungus may have occurred. In order to evaluate the genetic diversity and phylogenetic relationship of *B. mediterranea* associated with cork oak, 102 isolates were obtained from young and adult trees of *Quercus suber* and other hosts species with different disease expression and under distinct conditions, from several Mediterranean countries. The collection of isolates was analyzed by microsatellite-primed PCR profiles and individual and multigene phylogenies using Maximum-Likelihood approach based on nucleotide sequences of the internal transcribed spacers of nuclear ribosomal DNA, translation elongation factor 1-α and the β-tubulin. The results showed a high level of intraspecific polymorphism and genetic diversity among Mediterranean isolates that were detached from wider world isolates grouping themselves on a separate cluster, but didn't allow any other relationship with the surveyed factors. Considering this adaptive capacity of the fungus in the Mediterranean-climate ecosystems and under the present climatic change scenario, all conditions are gathered to favor aggravation of the disease in cork oak stands.

1 Introduction

Biscogniauxia mediterranea (De Not.) O. Kuntze is a xylariaceous fungus responsible for a necrotic disease on stems and branches of hardwoods, known as charcoal canker. The fungus has a worldwide distribution and occurs in a wide range of hosts in particular on the genus *Quercus*, attacking individuals weakened by environmental stresses such as drought and fire (Vannini *et al.* 1999). This fungus is known to have a latent phase during which it lives asymptomatically as an endophyte within the host (Mazzaglia *et al.* 2001a). When the tree is

subjected to stress it spreads rapidly and a large number of black stromata harboring perithecia erupt simultaneously from the dead bark (Collado *et al.* 2001).

B. mediterranea was detected in cork oak (*Quercus suber* L.) in Portugal in 1930 (Câmara 1930). It has had a high frequency of occurrence in highly degraded stands focusing systematically on trees in advanced phase of decline or dead, and wood material lying on the ground, being thus considered as a secondary pathogen (Santos 2003). Nevertheless, recent observations describe the increased incidence of the disease and the presence of atypical symptoms especially in young trees that begin their decline process as the foliage becomes discolorated and dry, the trunk exhibits viscous liquid exudates and the cracking of cork, but in the liber, the presence of a brown powdery fungi becomes evident (Henriques *et al.* 2012). These observations led to question whether some change may have occurred on the epidemiology of the disease (Sousa *et al.* 2007).

Reinforcing this idea, Vannini *et al.* (1999) and Schiaffino *et al.* (2002) using RAPD-PCR (Random Amplified Polymorphic DNA – Polymerase Chain Reaction) methods confirmed a large genetic variability in *B. mediterranea* at different sampling levels: stromata and geographical areas. This high polymorphism can be explained, according to Vannini *et al.* (1999), by the coexistence of compatible mycelia, since the fungus is heterothallic, features of the biological cycle and its ecological adaptations, which may indicate the existence of infra-specific varieties (Sousa *et al.* 2007).

The present work aimed to evaluate the genetic diversity and phylogenetic relationships of *B. mediterranea* associated with *Q. suber* in Portugal, from hosts with different disease expression and in distinct conditions, isolates from different host species and from the other Mediterranean countries, according to the geographical distribution of cork oak.

2 Material and methods

2.1 Isolate collection

Sampling and culture

Sixty samples of *Q. suber* exhibiting signs of charcoal canker were collected, following the geographical distribution of cork oak in Portugal, more intensely in the main production areas, from which 13 were from young trees, 5 with brown powdery mass in the trunk. Samples from the same host were also obtained in other countries of the Mediterranean basin: 3 from Spain,

4 from continental France and 5 from Corsica, 2 from Italy – Sardinia, 1 from Morocco, 6 from Algeria and 2 from Tunisia. From the insect *Platypus cylindrus* Fab. associated to cork oak were collected 7 samples in Portugal and 2 in Morocco. Samples from other hosts in Portugal were also included: 2 from *Quercus faginea* Lam., 3 from *Quercus robur* L., 1 from *Quercus rotundifolia* Lam., 2 from *Castanea sativa* Mill. and 1 from *Eucaliptus globolus* Labill.. As a reference isolate one strain of *B. mediterranea* from *Q. robur* was used, from the Netherlands (CBS101016). A total of 102 isolates were included in the study (Table 1).

Axenic fungal isolates were obtained directly from the carbonaceous stromata, from the mitosporic structures erupting in the branches, symptomless branches and acorns. Samples were removed with a scalpel and surface-sterilized for 1 min in sodium hypochlorite 1.5 % and rinsed in sterile dH_2O. Cultures were established in Potato Dextrose Agar (PDA, Difco, USA) acidified with lactic acid (1 ml lactic acid 85 %/ l PDA, PDAA) to avoid bacterial growth and incubated at 25±1 °C in darkness. Cultures were then transferred to a water agar medium and established pure hyphal tip cultures. Voucher specimens of representative isolates were deposited in the fungal collection at the "Micoteca da Estação Agronómica Nacional" (MEAN), INIAV (Oeiras, Portugal) (Table 1).

DNA extraction

For all 102 isolates, DNA was extracted from mycelia scraped from the surface of a PDA plate and extracted with the DNeasy Plant Mini Kit (Qiagen, USA), following the manufacturer's instructions. Fresh mycelium was used, disrupted by adding approx. 50 µl of glass beads (425 – 600 µm diam.) to the extraction buffer and vortexing for 2 min before and after RNase A incubation (Diogo *et al.* 2010).

The identity of all *B. mediterranea* isolates was confirmed by PCR amplification with specific primers MED1/ MED2 (Mazzaglia *et al.* 2001a). PCR amplification was executed with DreamTaq PCR Master Mix (2X) (Thermo Scientific, Germany). The reaction mixture contained 1x Dream Taq buffer which includes Taq DNA Polymerase (unknown concentration), 2 mM $MgCl_2$ and 0.2 mM of each dNTP, 1 µM of each primer and 1 µl of template DNA, in a final volume of 25 µl. Thermal cycling was performed on a Tgradient Thermocycler (Biometra, Germany) using the following cycling conditions: initial denaturation at 95 ºC for 2 min 30 s, followed by 35 cycles consisting of denaturation at 95 ºC for 30 s, annealing 62 ºC for the first two cycles, 61 ºC for the following two and 60 ºC for the remaining for 30 s and extension at 72 ºC for 32 s, a final

extension at 72 ºC for 5 min. The products were resolved by electrophoresis at 5 $V.cm^{-1}$ in agarose gel (1.5 %) containing 0.5 µg/ml ethidium bromide and 1x TBE running buffer. Data analysis was visualized by VersaDoc Gel Imaging System (BioRad, USA).

2.2 Molecular diversity analysis

The total collection of isolates was analyzed by means of microsatellite-primed PCR (MSP-PCR) fingerprinting profiles. In addition, for a set of representative isolates, individual and multilocus phylogenies were constructed using nucleotide sequences of the total internal transcribed spacers (ITS) of ribosomal RNA gene, translation elongation factor 1-α (TEF) and the β-tubulin (TUB).

MSP-PCR

As a preliminary clustering step for the assessment of the genetic variability among the set of 102 isolates, MSP-PCR fingerprinting profiles were generated following the protocol of Uddin and Stevenson (1997) using the primers $(CAG)_5$, $(GACA)_4$, $(GTG)_5$, $(ACAC)_5$ or phage M13 core sequence (GAGGGTGGNGGNTCT). PCR reactions were carried out in a total reaction volume of 25 µl containing approximately 10 ng of genomic DNA (quantified by Nanodrop 2000 Spectrophotometer, Thermo Scientific, USA), 1 µM of each oligonucleotide primer, 1x Dream Taq buffer which includes Taq polymerase (unknown concentration), 0.2 mM dNTPs and 3 mM $MgCl_2$. Thermal cycling was performed on a Tgradient Thermocycler using the following parameters: an initial incubation at 94 °C for 2 min, followed by 40 cycles of 30 s at 93 °C, 1 min at 53 °C, 30 s at 72 °C, and a final 72 °C extension period of 10 min. To ensure reproducibility of the amplified DNA fragments, all PCRs were performed in duplicate for each isolate and reactions without DNA were performed to determine if contaminant DNA was present. Amplicons were analyzed by electrophoresis under a constant voltage of 7 $V.cm^{-1}$ on a 1.5 % agarose gel in 1x TBE running buffer containing 0.5 µg/ml ethidium bromide staining. The DNA fragments were visualized using the VersaDoc Gel Imaging System. For data analysis, each band with a different electrophoretic mobility was assigned a position number and a mark of 1 or 0 based upon the presence or absence of the band. Only reproducible bands were considered for analysis. Bands common to all isolates were excluded from the analysis. Isolates were clustered on the basis of their profiles in a consensus dendrogram built with NTSYSpc

(Numerical Taxonomy and Multivariate Analysis System - Version 2.02h) using DICE coefficient and Unweighted Pair Group Method (UPGMA).

Sequence Analysis

For phylogenetic analysis, 36 representative isolates of *B. mediterranea* were selected according to host species, geographic location, age and host symptoms, along with the reference isolate (CBS101016). ITS region of ribosomal DNA (rDNA) and two nuclear protein coding genes, TEF and two regions of TUB (TUB1 and TUB2), were sequenced for all selected samples and deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (accession numbers are included in Table 1).

The ITS rDNA region was amplified using the DreamTaq PCR Master Mix (2X) and the universal primers ITS4/ ITS5 which target conserved regions in the 18S and 28S rDNA genes (White *et al.* 1990; Collado *et al.* 2001). The reaction mixture contained 1x Dream Taq buffer, which includes Taq DNA Polymerase, 2 mM $MgCl_2$ and 0.2 mM of each dNTP, 0.2 µM of each primer and 1 µl of template DNA (diluted 1:10), in a final volume of 25 µl. Thermal cycling was performed on a Tgradient Thermocycler using the following parameters: initial denaturation at 94 ºC for 2 min, 30 cycles at 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 1 min, and a final elongation at 72 ºC for 7 min. For PCR amplification of the TEF gene region, the reaction mixture was the same as described before but using 1 µM of each primer EF1-728F/ EF1-986R (Carbone & Kohn 1999; Cohen 2005). The thermal cycling conditions were: initial denaturation at 95 ºC for 8 min, 35 cycles at 95 ºC for 15 s, 55 ºC for 20 s and 72 ºC for 1 min, and a final elongation at 72 ºC for 5 min. For the TUB1 and TUB2 genes, the reaction mixture was the same as described before using primers A1/ B1 for TUB1 and A2/ B2 for TUB2 (Glass & Donaldson 1995; Hsieh *et al.* 2005). The thermal cycling conditions were: initial denaturation at 94 ºC for 1 min, 30 cycles at 94 ºC for 1 min, 48 ºC for 1 min for TUB1 and 60 ºC for 1 min for TUB2 and 72 ºC for 1 min, and a final elongation at 72 ºC for 5 min. PCR products were viewed under UV illumination on a 1 % agarose gel electrophoresis in the VersaDoc Gel Imaging System. Amplification products were purified with the GeneJET™ PCR Purification Kit (Fermentas, Germany) following the manufacturer's instructions. Sequencing was performed at STABVIDA facilities (Caparica, Portugal) on a DNA analyzer ABI PRISM 3730xl (Applied Biosystems). Chromatograms were edited and consensus sequences generated with FinchTV Version 1.4.0 (Geospiza Inc.). Consensus sequences for all isolates were compiled into a single

file (Fasta format) and aligned using Clustal W (Thompson *et al.* 1994). When necessary, subsequent manual adjustments were made.

For phylogenetic analyses two datasets were compiled. The analysis of the ITS region of ribosomal DNA (rDNA) was performed separately with the 36 generated sequences and a set of additional *B. mediterranea* sequences available in GenBank for the same DNA region (Table 1). The analysis of the three nuclear protein coding genes was performed in a combined dataset for the 36 isolates. TEF, TUB1 and TUB2 sequences were concatenated using Concatenator V 1.0.1. All the outgroup sequences were retrieved from the GenBank and were selected based on the genetic distance within species of the genus *Biscogniauxia* and family Xylariaceae (Mazzaglia *et al.* 2001b; Hsieh *et al.* 2005; Peláez *et al.* 2008; Tang *et al.* 2009). The chosen outgroups were *Biscogniauxia atropunctata*, *Biscogniauxia nummularia* and *Xylaria hypoxylon* (Table 1).

Phylogenetic trees were estimated under Maximum Likelihood (ML) using MEGA version 6 (Tamura *et al.* 2013). The best nucleotide substitution patterns were selected based on the analyses of best-fit models, using the lowest BIC scores (Bayesian Information Criterion) to describe the substitution pattern. Node support was calculated using 1000 bootstrap replicates.

Table 1. Isolates and DNA sequences of *Biscogniauxia mediterranea* and outgroups included in the analysis

Biscogniauxia mediterranea								
Isolate[a]	**Year**	**Collected/ Isolated by**	**Isolated from**	**Location**	**GenBank accession numbers[b]**			
					ITS	**TEF**	**TUB1**	**TUB2**
Bm04.001/ MEAN960	2004	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Montemor-o-Novo	**KM216752**	**KM216788**	**KM267164**	**KM267202**
Bm05.001	2005	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Chamusca				
Bm05.002	2005	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Sardoal				
Bm05.003	2005	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Sines				
Bm05.004	2005	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Montemor-o-Novo				
Bm06.001	2006	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Coruche				
Bm06.002	2006	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Coruche				
Bm06.003/ MEAN961	2006	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Morocco, Mamora	**KM216753**	**KM216789**	**KM267165**	**KM267200**
Bm06.004	2006	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Sesimbra				
Bm06.005	2006	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Sines				
Bm07.001	2007	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Grândola				
Bm07.003/ MEAN962	2007	M.L. Inácio & J. Henriques	*Quercus suber*, young undecorked tree with brown powdery mass	Portugal, Grândola	**KM216754**	**KM216790**	**KM267166**	**KM267203**
Bm08.001	2008	M.L. Inácio	*Quercus suber*, adult tree branch with carbonaceous stroma	Portugal, Benavente				
Bm08.002	2008	M.L. Inácio	*Quercus suber*, young undecorked tree with carbonaceous stroma	Portugal, Grândola				
Bm08.003	2008	M.L. Inácio	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Chamusca				
Bm09.001/ MEAN963	2009	J. Henriques	*Quercus suber*, adult with carbonaceous stroma	Tunisia, Kroufa	**KM216755**	**KM216791**	**KM267167**	**KM267204**
Bm10.001/ MEAN964	2010	J. Henriques	*Quercus suber*, young undecorked tree with brown powdery mass	Portugal, Montemor-o-Novo	**KM216756**	**KM216792**	**KM267168**	**KM267205**
Bm10.002	2010	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Viana do Alentejo				
Bm10.003	2010	J. Henriques	*Quercus suber*, young undecorked tree with carbonaceous stroma	Portugal, Viana do Alentejo				
Bm10.004	2010	J. Henriques	*Quercus suber*, young undecorked tree with carbonaceous stroma	Portugal, Viana do Alentejo				
Bm10.005	2010	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Viana do Alentejo				
Bm10.006/ MEAN965	2010	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Alenquer	**KM216757**	**KM216793**	**KM267169**	**KM267206**
Bm10.007	2010	J. Henriques	*Quercus suber*, adult tree branch with carbonaceous stroma	Portugal, Alenquer				
Bm10.008	2010	A. Matos/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Abrantes				
Bm10.009	2010	A. Matos/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Abrantes				
Bm10.010	2010	A. Matos/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Abrantes				
Bm10.012/ MEAN966	2010	J. Henriques	*Quercus rotundifolia*, adult tree with carbonaceous stroma	Portugal, Serpa	**KM216758**	**KM216794**	**KM267170**	**KM267207**
Bm10.014	2010	L. Caparica/ J. Henriques	*Quercus suber*, young undecorked tree with carbonaceous stroma	Portugal, Grândola				
Bm10.015	2010	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Italy, Sardinia				
Bm10.016/ MEAN967	2010	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Italy, Sardinia	**KM216759**	**KM216795**	**KM267171**	**KM267208**
Bm10.017	2010	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Viana do Alentejo				
Bm10.018/ MEAN968	2010	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Chamusca	**KM216760**	**KM216796**	**KM267172**	**KM267209**
Bm10.019/ MEAN969	2010	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Grândola	**KM216761**	**KM216797**	**KM267173**	**KM267210**
Bm10.020	2010	H. Bragança/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Nisa				
Bm10.021	2010	H. Bragança/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Marvão				
Bm10.022	2010	J. Rosendo/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Silves				
Bm10.023/ MEAN970	2010	J. Rosendo/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Silves	**KM216762**	**KM216798**	**KM267174**	**KM267201**
Bm10.024/ MEAN971	2010	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Grândola	**KM216763**	**KM216799**	**KM267175**	**KM267211**
Bm11.001	2011	P. Naves/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Almodôvar				
Bm11.002	2011	P. Pacheco Marques/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Ponte de Sôr				
Bm11.003/ MEAN972	2011	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Grândola	**KM216764**	**KM216800**	**KM267176**	**KM267212**
Bm12.001	2012	A. Costa/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Benavente				
Bm12.002	2012	L. Martins/ J. Henriques	*Quercus suber*, adult tree branch with carbonaceous stroma	Portugal, Mirandela				
Bm12.003	2012	L. Martins/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Mirandela				
Bm12.004/ MEAN973	2012	L. Martins/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Mirandela	**KM216765**	**KM216801**	**KM267177**	**KM267213**
Bm12.005/ MEAN974	2012	APFC/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Coruche	**KM216766**	**KM216802**	**KM267178**	**KM267214**
Bm12.006	2012	APFC/ J. Henriques	*Quercus suber*, young undecorked tree with carbonaceous stroma	Portugal, Coruche				
Bm12.009	2012	APFC/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Coruche				
Bm12.010	2012	APFC/ J. Henriques	*Quercus suber*, young undecorked tree with carbonaceous stroma	Portugal, Coruche				
Bm12.011	2012	APFC/ J. Henriques	*Quercus suber*, young undecorked tree with carbonaceous stroma	Portugal, Coruche				

Isolate[a]	Year	Collected/ Isolated by	Isolated from	Location	GenBank accession numbers[b]			
					ITS	TEF	TUB1	TUB2
Bm12.012	2012	APFC/ J. Henriques	*Quercus suber*, young tree with carbonaceous stroma	Portugal, Coruche				
Bm12.013/ MEAN975	2012	APFC/ J. Henriques	*Quercus suber*, young undecorked tree with brown powdery mass	Portugal, Coruche	**KM216767**	**KM216803**	**KM267179**	**KM267215**
Bm12.014/ MEAN976	2012	J. Henriques	*Quercus suber*, young undecorked tree with carbonaceous stroma	Portugal, Grândola	**KM216768**	**KM216804**	**KM267180**	**KM267216**
Bm12.015/ MEAN977	2009	M. Z. Boutiti	*Quercus suber*, adult tree symptomless branch	Tunisia, Kroufa	**KM216769**	**KM216805**	**KM267181**	**KM267217**
Bm12.016	2012	A. Matos/ J. Henriques	*Quercus suber*, young tree with carbonaceous stroma	Portugal, Setúbal				
Bm12.017/ MEAN978	2012	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Grândola	**KM216770**	**KM216806**	**KM267182**	**KM267218**
Bm12.018	2012	L. Santos/ J. Henriques	*Quercus suber*, symptomless acorn	Portugal, Montijo				
Bm12.019	2012	L. Santos/ J. Henriques	*Quercus suber*, symptomless acorn	Portugal, Montijo				
Bm12.020	2012	L. Santos/ J. Henriques	*Quercus suber*, symptomless acorn	Portugal, Montijo				
Bm12.021	2012	L. Santos/ J. Henriques	*Quercus suber*, symptomless acorn	Portugal, Alcácer do Sal				
Bm12.022/ MEAN979	2012	P. Naves/ J. Henriques	*Quercus suber*, young undecorked tree with carbonaceous stroma	Portugal, Tróia	**KM216771**	**KM216807**	**KM267183**	**KM267219**
Bm12.023/ MEAN980	2012	H. Bragança & E. Diogo/ J. Henriques	*Eucalyptus globulus*, adult tree with carbonaceous stroma	Portugal, Odemira	**KM216772**	**KM216808**	**KM267184**	**KM267220**
Bm12.024/ MEAN981	2012	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Terras de Bouro	**KM216773**	**KM216809**	**KM267185**	**KM267221**
Bm12.025/ MEAN982	2012	J. Henriques	*Quercus suber*, young undecorked tree with brown powdery mass	Portugal, Terras de Bouro	**KM216774**	**KM216810**	**KM267186**	**KM267222**
Bm12.026	2012	A. Matos/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Penha Garcia				
Bm12.027/ MEAN983	2012	J. Henriques	*Quercus robur*, adult tree with carbonaceous stroma	Portugal, Terras de Bouro	**KM216775**	**KM216811**	**KM267187**	**KM267223**
Bm12.028	2012	J. Henriques	*Quercus robur*, adult tree with carbonaceous stroma	Portugal, Terras de Bouro				
Bm12.029	2012	J. Henriques	*Quercus robur*, adult tree with carbonaceous stroma	Portugal, Terras de Bouro				
Bm12.031/ MEAN984	2012	J. Henriques	*Castanea sativa*, adult tree with carbonaceous stroma	Portugal, Terras de Bouro	**KM216776**	**KM216812**	**KM267188**	**KM267224**
Bm12.032/ MEAN985	2012	J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Mafra	**KM216777**	**KM216813**	**KM267189**	**KM267225**
Bm12.033/ MEAN986	2012	J. Henriques	*Quercus faginea*, adult tree with carbonaceous stroma	Portugal, Mafra	**KM216778**	**KM216814**	**KM267190**	**KM267226**
Bm12.035/ MEAN987	2012	J. Henriques	*Castanea sativa*, adult tree with carbonaceous stroma	Portugal, Mafra	**KM216779**	**KM216815**	**KM267191**	**KM267227**
Bm12.036	2012	J. Henriques	*Quercus faginea*, adult tree with carbonaceous stroma	Portugal, Mafra				
Bm12.038	2012	P. Naves/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Portugal, Palmela				
Bm12.039/ MEAN988	2012	R. Piazzeta/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	France, Vivès	**KM216780**	**KM216816**	**KM267192**	**KM267228**
Bm12.040	2012	R. Piazzeta/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	France, Vivès				
Bm12.041	2012	R. Piazzeta/ J. Henriques	*Quercus suber*, dead adult tree with carbonaceous stroma	France, Vivès				
Bm12.042	2012	R. Piazzeta/ J. Henriques	*Quercus suber*, dead adult tree with carbonaceous stroma	France, Vivès				
Bm13.001	2013	H. Baudriller-Cacaud/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	France, Corse du Sud				
Bm13.002	2013	H. Baudriller-Cacaud/ J. Henriques	*Quercus suber*, dead adult tree with carbonaceous stroma	France, Corse du Sud				
Bm13.003	2013	H. Baudriller-Cacaud/ J. Henriques	*Quercus suber*, dead adult tree with carbonaceous stroma	France, Corse du Sud				
Bm13.004/ MEAN989	2013	H. Baudriller-Cacaud/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	France, Corse du Sud	**KM216781**	**KM216817**	**KM267193**	**KM267229**
Bm13.005	2013	H. Baudriller-Cacaud/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	France, Corse du Sud				
Bm13.006	2013	A. Mounia/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Algeria, Anaba				
Bm13.007/ MEAN990	2013	A. Mounia/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Algeria, Anaba	**KM216782**	**KM216818**	**KM267194**	**KM267230**
Bm13.008/ MEAN991	2013	L. Belhoucine/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Algeria, Tlemcen	**KM216783**	**KM216819**	**KM267195**	**KM267231**
Bm13.009	2013	L. Belhoucine/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Algeria, Tlemcen				
Bm13.010	2013	L. Belhoucine/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Algeria, Tlemcen				
Bm13.011	2013	L. Belhoucine/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Algeria, Tlemcen				
Bm13.012	2013	A. Soto Sánchez/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Spain, Cordoba				
Bm13.013/ MEAN992	2013	A. Soto Sánchez/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Spain, Cordoba	**KM216784**	**KM216820**	**KM267196**	**KM267232**
Bm13.014	2013	A. Soto Sánchez/ J. Henriques	*Quercus suber*, adult tree with carbonaceous stroma	Spain, Cordoba				
Pc96.009/ MEAN993	1996	E. Sousa	*Platypus cylindrus* associated to *Quercus suber*	Portugal, Coruche	**KM216785**	**KM216821**	**KM267197**	**KM267233**
Pc04.002	2004	M.L. Inácio	*Platypus cylindrus* associated to *Quercus suber*	Morocco, Mamora				
Pc05.008	2005	M.L. Inácio	*Platypus cylindrus* associated to *Quercus suber*	Portugal, Coruche	FR734186			
Pc06.049	2006	M.L. Inácio & J. Henriques	*Platypus cylindrus* associated to *Quercus suber*	Portugal, Coruche				
Pc06.050	2006	M.L. Inácio & J. Henriques	*Platypus cylindrus* associated to *Quercus suber*	Portugal, Coruche				
Pc06.052	2006	M.L. Inácio & J. Henriques	*Platypus cylindrus* associated to *Quercus suber*	Portugal, Coruche				
Pc06.054	2006	M.L. Inácio & J. Henriques	*Platypus cylindrus* associated to *Quercus suber*	Morocco, Mamora				
Pc07.002	2007	M.L. Inácio & J. Henriques	*Platypus cylindrus* associated to *Quercus suber*	Portugal, Grândola				
Pc08.002/ MEAN994	2008	M.L. Inácio & J. Henriques	*Platypus cylindrus* associated to *Quercus suber*	Portugal, Montemor-o-Novo	**KM216786**	**KM216822**	**KM267198**	**KM267234**
CBS101016/ MEAN995	1998		*Quercus robur*, dead branch	Netherlands, Bremmert-Kootwijk	**KM216787**	**KM216823**	**KM267199**	**KM267235**

Isolate[a]	Year	Collected/ Isolated by	Isolated from	Location	GenBank accession numbers[b]			
					ITS	TEF	TUB1	TUB2
	1994		*Quercus rotundifolia*, twigs	Spain, Monte del Prado, Madrid	AF280625			
	1994		*Quercus rotundifolia*, twigs	Spain, Monte del Prado, Madrid	AF280626			
	1994		*Quercus rotundifolia*, twigs	Spain, Monte del Prado, Madrid	AF326479			
	1995		*Quercus rotundifolia,* trunk bark	Spain, Ontígola, Madrid	AF326480			
	1995		corticated wood	France, Oloron, Foret de Bugangue	EF026134			
	1996		*Quercus rotundifolia*, twigs	Spain, La Matilla, Guadalajara	AF326481			
	1996		*Quercus rotundifolia*, trunk bark	Spain, La Matilla, Guadalajara	AF326482			
	1996		*Quercus cerris*	Italy, Latium, Monte Rufeno	AJ246222			
	1996		*Quercus cerris*	Italy, Monte Rufeno	AJ246224			
	1997		*Quercus suber*	Morocco, Mamora	AJ246220			
	1997		*Quercus cerris*	Italy, Latium, Tolfa	AJ246221			
	1997		*Quercus cerris*	Italy, Latium, Rome, Tormancina	AJ246227			
	1998		*Quercus suber,* ascomata	Spain, Sierra de Aracena, Huelva	AF280624			
	2010		*Fraxinus excelsior*	Italy, Chiusi della Verna, Arezzo	JX262798			
	2011		*Quercus castaneifolia*	Iran	JF295127			
	2011		*Quercus castaneifolia*	Iran	JF295128			
	2011		*Quercus castaneifolia*	Iran	JF295129			
	2012		*Opuntia humifusa*, stem tissue	USA	KF850388			
	unknown		unknown	Italy, Apulia, Foggia,Cagnano	AJ246223			
	unknown		unknown	Italy, Apulia, Bari, Putignano	AJ246225			
	unknown		unknown	Italy, Apulia, Bari, Gravina	AJ246226			
	unknown		*Quercus cerris*	Spain, Barcelona, B. Girona	AJ246228			
	unknown		unknown	USA	AJ390413			
	unknown		*Quercus rubra*	USA, New Jersey	AJ390414			
	unknown		*Holcus lanatus*	Spain	FN394711			
	unknown		*Populus x euramericana*	Spain, Palencia	FR648379			
	unknown		*Taxus globosa*, branch	Mexico	GQ377479			
	unknown		*Taxus globosa*, bark	Mexico	GQ377490			
	unknown		Non identified arthropod	Portugal, Coimbra	JQ781705			
	unknown		Non identified arthropod	Portugal, Coimbra	JQ781706			
	unknown		Non identified arthropod	Portugal, Coimbra	JQ781707			
	unknown		Non identified arthropod	Portugal, Coimbra	JQ781708			
	unknown		Non identified arthropod	Portugal, Coimbra	JQ781709			
	unknown		Non identified arthropod	Portugal, Coimbra	JQ781799			
	unknown		*Pinus sylvestris*	Spain	JX421711			
	unknown		*Pinus sylvestris*	Spain	JX421730			
Outgroups								
Biscogniauxia atropunctata	unknown		*Quercus* sp.	Costa Rica	AJ390411			
Biscogniauxia nummularia	unknown		*Fagus sylvatica*	Italy, Mongiana, Florence	AJ246230			
	unknown		*Salix alba*	Slovakia			GQ428324	GQ428324
Xylaria hypoxylon	2005		*Fagus sylvatica,* old trunk	Germany	AM993138			
	2006		wood	Taiwan			GQ487703	GQ487703
	unknown		soil	Mexico		AY327490		

[a] Bm, Pc: work collections; MEAN: Micoteca da Estação Agronómica Nacional, INIAV, Oeiras, Portugal; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
[b] Accession numbers of sequences newly produced (bold) or downloaded from GenBank
[Adérito Matos, Augusta Costa, Edmundo Sousa, Eugénio Diogo, Helena Bragança, Lourdes Santos, M. Lurdes Inácio, Pedro Naves - INIAV, Oeiras, Portugal; Amoura Mounia - Université d'El Tarf, Département de Biologie, Faculté des Sciences, Algeria; Antonia Soto Sánchez - Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain; Associação de Produtores Florestais do Concelho de Coruche e Limítrofes (APFC), Coruche - Portugal; Haïmad Baudriller-Cacaud, Centre Régional de la Propriété Forestière de Corse, France; Jorge Dias – Instituto da Conservação da Natureza e das Florestas, I. P. (ICNF) - Parque Nacional da Peneda-Gerês, Portugal; José Rosendo - ICNF – Departm. Conservação da Natureza e Florestas do Algarve, Olhão, Portugal; Latifa Belhoucine - Laboratory 3: Conservation Management of water, soil and forests - University of Tlemcen, Algeria; Luís Caparica – ICNF – Departm. Conservação da Natureza e Florestas do Alentejo, Alcácer do Sal, Portugal; Luís Martins - Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal; Meriem Zouaoui Boutiti - Institut National de Recherche en Génie Rural, Eaux et Forêts, Tunisia; Pedro Carrilho- ICNF - Tapada Nacional de Mafra, Portugal; Pedro Pacheco Marques – Fundação João Lopes Fernandes, Montargil, Portugal; Renaud Piazzeta - Institut Mediterranéen du Liège, Vivès, France.]

3 Results

All the isolates were confirmed as *B. mediterranea* using the amplification with the specific primers MED1/ MED2 that resolved, as expected, only one PCR product of approximately 380 bp. An example is presented in Figure 1.

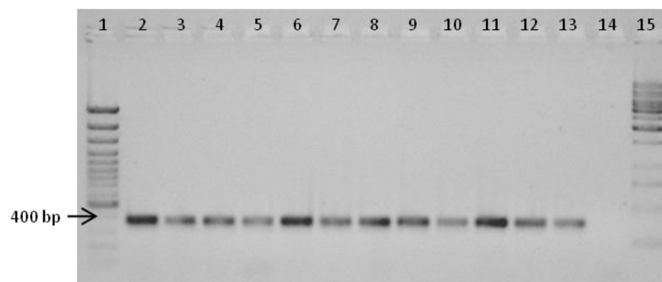

Figure 1. Ethidium bromide-stained agarose gel of PCR amplification products using MED1 and MED2 primers for different samples of *Biscogniauxia mediterranea*. Lanes as follows: (1) GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, Germany), (2) Bm04.001, (3) Bm05.001, (4) Bm05.002, (5) Bm05.003, (6) Bm05.004, (7) Bm06.001, (8) Bm06.002, (9) Bm06.003, (10) Bm06.004, (11) Bm06.005, (12) Bm07.001, (13) CBS101016, (14) Negative control, (15) GeneRuler™ 1kb DNA Ladder (Fermentas, Germany).

3.1 MSP-PCR

The variability analysis assessed genetic diversity within the collection of 102 isolates of *B. mediterranea* using the five primers. All primers generated complex fingerprints, with band sizes ranging from 0.4 to 1.5 kb for $(CAG)_5$, $(GACA)_4$ and $(GTG)_5$, from 0.5 to 1.2 kb for $(ACAC)_5$ and from 0.25 to 1.2 kb for M13, resulting in a total of 74 different band positions. As a first approach, individual cluster analyses were performed with the data matrix generated by each primer and no strict correlation was obtained. A consensus dendrogram using UPGMA analysis was obtained from the combined analysis of profiles generated with the five primers for the complete set of isolates (Figure 2). The cophenetic correlation coefficient of this UPGMA analysis was 0.81, indicating that the dendrogram was an excellent fit representation of the original data. All isolates presented a similarity of 46 %. However, this combined analysis did not allow a coherent clustering of the *B. mediterranea* isolates according to the main parameters under study. The dendrogram shows a large group of clustered isolates collected from different hosts, from trees of varying ages and disease symptoms, throughout the Mediterranean basin, which is separated from the CBS101016 isolate, originated from *Q. robur* from the Netherlands. Within the large group, the samples are successively clustered with different percentages of similarity higher than ca. 63 %.

Figure 2. MSP-PCR analysis of *Biscogniauxia mediterranea* total isolates. Consensus dendrogram from $(CAG)_5$, $(GACA)_4$, $(GTG)_5$, $(ACAC)_5$ and phage M13 core sequence MSP-PCR profiles performed in NTSYSpc2 using DICE's correlation coefficient and UPGMA. Scale bar represents percentage of similarity.

3.2 Sequence analysis

For the representative isolates of *B. mediterranea* amplification products of approximately 580 bp for ITS, 380 bp for TEF, 230 bp for TUB1 and 530 bp for TUB2 were obtained.

The dataset used for ITS analysis, consisted of 73 ingroup taxa and three outgroup taxa and the alignment contained 545 characters including alignment gaps, 342 were conserved sites and 55 were parsimony informative. The ML trees were computed using the Kimura 2-parameter substitution model with a bootstrap test (1000 replicates) and the rate variation model was allowed with a discrete gamma distribution (5 categories). Analysis of the tree with the highest log likelihood (Figure 3) resolved two main groups, with a bootstrap value of 99 %: samples deriving from the Mediterranean region, from different hosts, and samples originated from the American continent (USA and Mexico) and two sequences from northern Europe (France - Pyrenees and the Netherlands), also from different hosts. Within the Mediterranean group all samples are very close phylogenetically, clustering in groups that have less consistent bootstrap values and not allowing the distinction of any group or tendency related with the factors under study: isolate origin, host species, tree conditions and expression of the fungus.

For the protein coding genes (TEF, TUB1 and TUB2), the individual trees obtained were ineffective in resolving *B. mediterranea* isolates according to any referred factor (data not shown). TUB1 analysis, distinguishes the sample Bm09.001 from the remaining samples with high bootstrap value. The three gene combined data sets (Figure 4) consisted of 36 ingroup taxa and two outgroup taxa and the alignment contained 1197 characters including alignment gaps, 657 were conserved sites and 279 were parsimony informative. The ML trees were computed using the Hasegawa-Kishino-Yano substitution model with a bootstrap test (1000 replicates) and the rate variation model was allowed with a discrete gamma distribution (5 categories). This dataset didn’t also enable the separation of particular clusters for the resolution of isolates from different origins and conditions, encompassing all isolates of the species with only the discrimination of Bm09.001, isolated from a stroma on *Q. suber* from Tunisia. All the *B. mediterranea* isolates are phylogenetically close remaining distinct from the outgroup species.

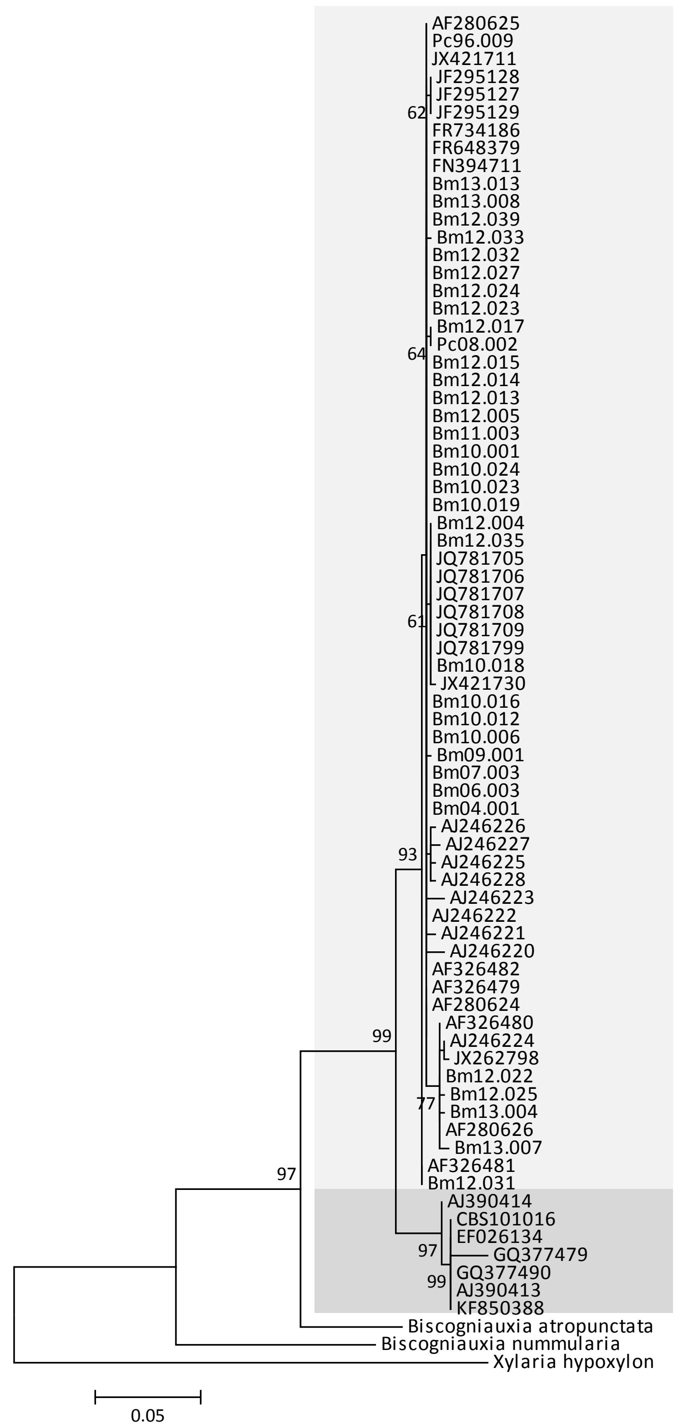

Figure 3. Molecular phylogenetic analysis of ITS rDNA sequences of *Biscogniauxia mediterranea* by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-1903.5196) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.6916)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 76 nucleotide sequences. There were a total of 545 positions in the final dataset. Bootstrap values below 60 not shown. Evolutionary analyses were conducted in MEGA6.

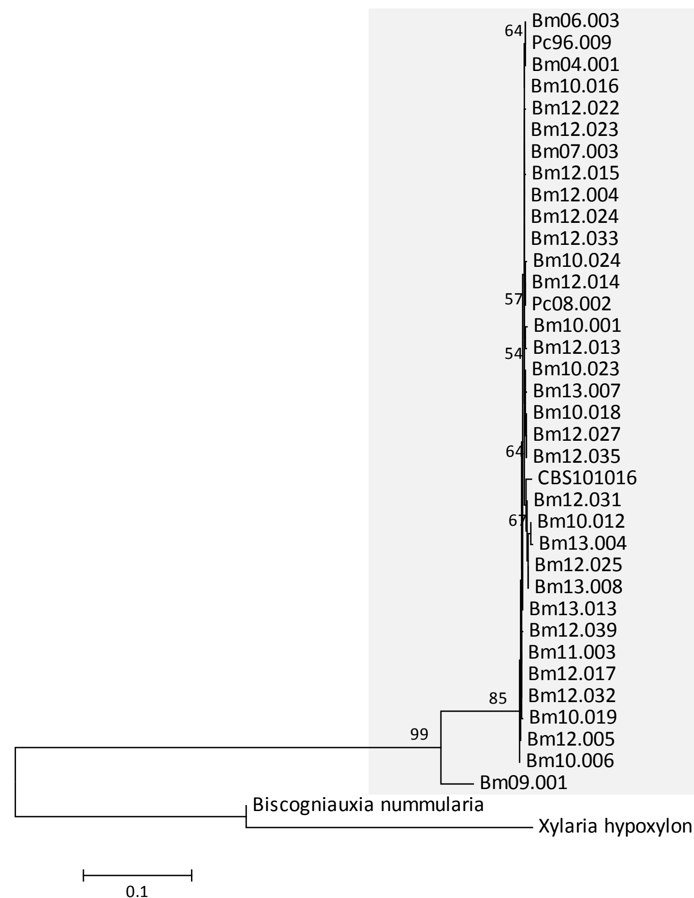

Figure 4. Molecular Phylogenetic analysis of concatenated TEF, TUB1 and TUB2 sequences of *Biscogniauxia mediterranea* by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-4229.7853) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.0023)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 38 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1197 positions in the final dataset. Bootstrap values below 50 not shown. Evolutionary analyses were conducted in MEGA6.

4 Discussion

This study represents a significance progress for the evaluation of genetic variability of *B. mediterranea* under the approach of MSP-PCR and multigene phylogenetic relationship among a representative set of isolates. It is an attempt to relate the species diversity with the geographical origin, host species, tree conditions and expression of the fungus as carbonaceous stroma (teleomorphic stage) or brown powdery mass (mitosporic stage). It

includes a set of isolates obtained mostly from *Q. suber* and other hosts like *Q. faginea*, *Q. robur*, *Q. rotundifolia*, *C. sativa* and *E. globolus* collected from Portugal and other Mediterranean countries (Spain, France, Italy, Morocco, Algeria and Tunisia) and isolates obtained from *P. cylindrus* associated to *Q. suber* in Portugal and Morocco.

All the performed analyzes have provided concordant results in order to disclose a high diversity among isolates of *B. mediterranea*. The MSP-PCR analyses of the complete set of isolates along with a reference isolate CBS101016 revealed an extremely high genetic diversity among the Mediterranean isolates that successively clustered in groups of few isolates, not being possible to correlate this arrangement with any factor. The five used primers [$(CAG)_5$, $(GACA)_4$, $(GTG)_5$, $(ACAC)_5$ and phage M13 core sequence] were effective in detecting polymorphisms, thus strengthening this analysis. Schiaffino *et al.* (2002) using RAPD-PCR also detected a high level of genetic variability within a population from a single area of Sardinia as well as among isolates from different localities in this island and Spain. In Portugal, this variability has been reported among isolates from individual stromata and from different geographical locations (Henriques *et al.* 2014b). Phylogenetic analyses of ITS rDNA sequences didn't also resolve the isolates in order to relate them with any of the studied factors but the joint analysis with all *B. mediterranea* sequences from GenBank placed Mediterranean isolates (including Iran) in a separate cluster from isolates originated from Central Europe (The Netherlands and France - Pyrenees) and North America (USA and Mexico), suggesting the existence of some degree of divergence between the specimens of the Mediterranean in relation to the other regions. Collado *et al.* (2001) discussed the precision of identification of some of these American specimens, confirming the accuracy of these conclusions.

The protein coding genes combined analysis (TEF, TUB1 and TUB2) was performed only among the sequences generated in this study and outgroup species because for these regions there weren't additional sequences of *B. mediterranea* available in GenBank (only one sequence for β-tubulin gene - AY951684), therefore, this collection of sequences associated with *Q. suber* and covering its entire distribution constitutes a significant contribution to further research on the pathogen. Once more, the results showed the clustering of the isolates without any relationship to the surveyed factors and low rate of substitutions per site. The isolate Bm09.001, originated from a stroma in *Q. suber* from Tunisia, formed an isolated branch within the group as a consequence of the dissimilar TUB1 sequence, probably be due to occasional mutations in this gene region.

B. mediterranea has been described as an endophyte and weak pathogen in a broad host range (Jiménez *et al.* 2005a). In Portugal, its incidence has been increasing in an atypical way, especially in young cork oaks, which led to suggest the existence of related species or infra-specific varieties intervening in charcoal canker development (Sousa *et al.* 2007). Nevertheless, both ITS rDNA and protein-coding gene phylogenetic approaches proved that the isolates are phylogenetically close, presenting low nucleotide substitution rates among them. ITS rDNA analysis confirmed that the genetic diversity found among isolates is within the species *B. mediterranea*. All isolates as well as the sequences of *B. mediterranea* taken from GenBank evolved from a common ancestor, forming a very distinct cluster from the close outgroup species, namely *B. atropuntacta*, which according to phylogenetic analysis of *Biscogniauxia* species is the closest specie to *B. mediterranea* (Hsieh *et al.* 2005; Peláez *et al.* 2008). The low bootstrap values in the inner nodes of the ML trees may be due to the overall intraspecific variability.

Vannini *et al.* (1999) explained the high genetic variability of *B. mediterranea*, even in small populations, based on the heterothallic mating system of this species which represents an important internal source of genetic variability of the population. In addition, this fungus has a high rate of sexual reproduction, even in the pathogenic stage (Jiménez *et al.* 2005a), producing a large amount of ascospores. They constitute the most important dispersal and inoculum units and can be spread over long distances, airborne and by insects, contributing for fungal spread in the forests (Jiménez *et al.* 2005b; Inácio *et al.* 2011; Henriques *et al.* 2014a). Long-distance air dispersal is especially relevant for phytopathogenic fungi, because wind dispersal of their spores for hundreds or thousands of kilometers has caused the spread of several important diseases on a continental or global scale and allows the regular reestablishment of diseases (Brown & Hovmaller 2002), as well as human activities and international transport of infected plants and wood products. The strongly stochastic nature of long-distance dispersal causes founder effects in pathogen populations, such that genotypes that cause epidemics in new territories or on hosts may be atypical (Brown & Hovmaller 2002). Also, as plants have been redistributed from their centers of origin, fungi have followed, resulting in outbreaks of such diseases like chestnut blight (*Cryphonectria parasitica* (Murrill) M.E. Barr) (Brown & Hovmaller 2002). The range of pathogens found on different hosts shows considerable diversity that may be associated with the evolutionary history of their hosts or ecological criteria like the host's architectural complexity or the extent of the pathogen's natural range (Burdon & Silk 1997).

Evolution involves additional mechanisms such as physical isolation, which can lead to non-selective or neutral means that advance and promote population divergences (Papke & Ward 2004). Subsequent to an isolation event, the restriction of gene flow between the two resulting populations allows a nascent population to independently accumulate mutations and/or lose allelic diversity through genetic drift (Papke & Ward 2004). Ecological factors like climate constitute a driver for evolutionary change (Erwin 2009). Mediterranean-climate ecosystems, with their characteristic and unique climatic regimes of mild, wet winters and warm, dry summers, are home to remarkable and globally significant levels of biodiversity and endemism (Cowling *et al.* 1996). All mechanisms that allowed the evolution of *B. mediterranea* in the Mediterranean region, coupled with the particular susceptibility of cork oak to this fungus, led to the high incidence of the disease in this region and its increasing severity.

Overall, *B. mediterranea*, presented a high level of intraspecific polymorphism and genetic diversity, but the analysis didn't allow to associate it with any factor related with the host species or age, geographic position or charcoal canker expression. Large variability of the population is extremely important for the epidemiology of the fungus since it provides the genetic flexibility for long-term survival and adaptation to the environment (Vannini *et al.* 1999). Thus, it is possible to conclude that the expression of atypical symptoms and abundance of charcoal canker disease in cork oak stands in Portugal may not be due to a change in the fungus but a result from its ability to adapt and favoritism by the changes that have taken place in the climate (La Porta *et al.* 2008), in particular in the Mediterranean region.

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Morpho-cultural and physiological features of *Biscogniauxia mediterranea* isolates associated with cork oak

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ABSTRACT

Biscogniauxia mediterranea is a widespread fungus that causes charcoal disease on *Quercus suber* and other hardwood hosts. It had been considered a secondary pathogen causing the disease only in stressed hosts. However, its frequency and severity in cork oak stands has been increasing, inclusive in young trees without other decline signs and developing atypical symptoms. The present work aims to characterize morphological and cultural characters of 102 isolates of *B. mediterranea* mainly originated from cork oak in Portugal and other Mediterranean countries, and also from other hardwood hosts, in order to evaluate the intraspecific variability in relation to disease expression. The mitosporic state of the fungus was described and the identity of all isolates was confirmed by PCR amplification with specific primers MED1/ MED2. The analyzed features were cultural characteristics, conidial dimensions and growth rates at different temperatures. Clustering UPGMA analyses combining the different parameters were preformed. Morphological characterization of the isolates revealed a high variability among isolates and the cardinal temperatures showed the high plasticity of the tendentiously thermophilic fungus that in most cases presented growth between 5 and 45 ºC. It was not possible to relate the disease development data with any of the analyzed features. Nevertheless, the results highlighted the variability and adaptation ability of this fungus in the present worrying scenario that is already favoring the increase of the disease.

INTRODUCTION

Biscogniauxia mediterranea (De Not.) O. Kuntze (syn. *Hypoxylon mediterraneum* (De Not.) Mill.) is a xylareaceous fungus that exists for part of its life as an endophyte in the host tissues including twigs, bark, leaves, and, to a lesser extent, wood. In hosts subjected to environmental stress, it is able to rapid colonize the xylem and bark tissues, to induce necrosis and canker formation, and to accelerate tree decline and eventually death. This fungus occurs on a wide range of hardwood hosts and particularly on the genus *Quercus* (Vannini et al. 2009).

In Portugal the charcoal canker caused by *B. mediterranea* is very frequent in cork oak stands being one of the agents involved in its present weakening process, as well as in other countries of the Mediterranean Basin (Bouhraoua 2002; Franceschini et al. 2005; Jiménez et al. 2005a; Assali & Falki 2006; Sousa et al. 2007; Khouja et al. 2010). This fungus was diagnosed in Portugal since 1930 (Câmara, 1930), being considered as a secondary pathogen (Santos 2003), however its incidence has been increasing also in younger trees without other symptoms of decline (Sousa et al. 2007). In addition, the development of the mitosporic stage in the host instead of the typical perithecial stroma has become more frequent in the stands (Henriques et al. 2012). All this changes in the fungus progress led to the hypothesis that something new could have arisen like the presence of other related species or the existence of infra-specific varieties (Sousa et al. 2007).

Several studies focusing the diversity of *B. mediterranea*, using different molecular methods detected a large genetic variability in this species (Vannini et al. 1999; Schiaffino et al. 2002; Henriques et al. 2014b; Henriques et al. submitted) although didn´t related it to any factor of disease expression or different hosts and geographic origin (Henriques et al. submitted). Morphological and cultural studies of *B. mediterranea* are purely descriptive contributing to the assessment of the fungus and disease (Ju et al. 1998; Collado et al. 2001; Jiménez et al. 2005a; Giambra et al. 2009) but with no comparison of these parameters among multiple isolates of this species.

The objective of this work is to evaluate the intraspecific diversity of *B. mediterranea* associated to cork oak using morphological and cultural characters, attempting to relate it with the atypical development of the disease.

MATERIAL AND METHODS

Isolates collection and culture

60 samples of *Q. suber* exhibiting signs of charcoal canker were collected following the geographical distribution of cork oak in Portugal, more intensely in the main production areas, from which 13 were from young trees, 5 with brown powdery mass. Samples from the same host were also obtained in other countries of the Mediterranean basin: 3 from Spain, 4 from continental France and 5 from Corse, 2 from Italy – Sardinia, 1 from Morocco, 6 from Algeria and 2 from Tunisia. From the insect *Platypus cylindrus* Fab. associated to cork oak were collected 7 samples in Portugal and 2 in Morocco. Were also included samples from other

hosts in Portugal: 2 from *Quercus faginea* Lam., 3 from *Quercus robur* L., 1 from *Quercus rotundifolia* Lam., 2 from *Castanea sativa* Mill. and 1 from *Eucalyptus globulus* Labill.. As a reference 1 strain of *B. mediterranea* from *Q. robur*, Netherlands (CBS101016), was used. A total of 102 isolates were included in the study (Table 1).

Fungal isolates were obtained directly from the carbonaceous stromata, from the mitosporic structures erupting in the branches and from symptomless branches and acorns. Samples were removed with a sterile scalpel and surface-sterilized for 1 min in sodium hypochlorite 1.5 % and rinsed in sterile dH_2O. Cultures were established in Potato Dextrose Agar (PDA, Difco, USA) acidified with lactic acid (1 ml lactic acid 85 %/ l PDA, PDAA) to avoid bacterial growth and incubated at 25±1 °C in darkness. Cultures were then transferred to water agar medium and established pure hyphal tip cultures. Voucher specimens of representative isolates were deposited in the fungal collection at Micoteca of Estação Agronómica Nacional (MEAN), INIAV (Oeiras, Portugal).

Confirmation of the isolates identification

The identity of all *B. mediterranea* isolates was confirmed by PCR amplification with specific primers MED1/ MED2 (Mazzaglia et al. 2001). DNA was isolated from mycelia scraped from the surface of a PDA plate and extracted with the DNeasy Plant Mini Kit (Qiagen, USA), following the manufacturer's instructions. Fresh mycelium was used, disrupted by adding approx. 50 µl of glass beads (425 – 600 µm diam.) to the extraction buffer and vortexing for 2 min before and after RNase A incubation (Diogo et al. 2010). PCR amplification was performed with DreamTaq PCR Master Mix (2x) (Thermo Scientific, Germany). The reaction mixture contained 1x Dream Taq buffer which includes Taq DNA Polymerase (unknown concentration), 2 mM $MgCl_2$ and 0.2 mM of each dNTP, 1 µM of each primer and 1 µl of template DNA (diluted 1:10), in a final volume of 25 µl. Thermal cycling was performed on a Tgradient Thermocycler (Biometra, Germany) using the following cycling conditions: initial denaturation at 95 ^{0}C for 2 min 30 s, followed by 35 cycles consisting of denaturation at 95 ^{0}C for 30 s, annealing 62 ^{0}C for the first two cycles, 61 ^{0}C for the following two and 60 ^{0}C for the remaining for 30 s and extension at 72 ^{0}C for 32 s, a final extension at 72 ^{0}C for 5 min. The products were resolved by electrophoresis at 5 $V.cm^{-1}$ in agarose gel (1,5 %) containing 0,5 µg/ml ethidium bromide and 1x TBE running buffer. Data analysis was visualized by VersaDoc Gel Imaging System (BioRad, USA).

Morphological and growth temperature characterization

The culture characterization was assessed for the complete collection of isolates. Cultures were grown on PDA supplemented with yeast extract (Liofilchem, Italy) 5g/l (PDYA) in 90 mm diam dishes at 25±1 °C in darkness (Jiménez et al. 2005a). Growth rates were determined by measuring colony diameters in two orthogonal directions after 3 and 7 days of culture, with six replicate plates per isolate. Morphological characters were observed on the seventh day: colony color (surface and reverse), texture, density, zonation, presence of exudates and other structures. Color was described using the color chart of Rayner (1970).

For the mitosporic characterization and cardinal temperatures assessment, 36 representative isolates of *B. mediterranea* were selected according to the host species, geographic location, age and host symptoms.

For the observation of mitosporic structures and conidial measurements isolates were incubated in PDA at 25±1 ºC under 12:12 h periods of near-UV light (NUV; 400-315 nm; Philips TL-D 15W Actinic BL, The Netherlands). Microscopic preparations were assembled in lactoglycerol 80 %. For each isolate, 30 conidia were measured at 600x magnification using an Olympus BX-41 TF microscope. Measurements are given as (minimum-) lower limit of a 95 % confidence interval - average - upper limit of a 95 % confidence interval (-maximum). Statistical analyses were performed using STATISTICA 6 software. Digital images were obtained with a ProgRes® CapturePro 2.8 - JENOPTIK Optical Systems coupled to the microscope.

Minimum and maximum growth temperatures were determined after a preliminary assay described in Henriques (2007). Growth rates were assessed by inoculating 90 mm diam PDA dishes with a 7 mm diam plug cut from the edge of an actively growing colony. Trials were conducted at decreasing and increasing temperatures from 7.5 ºC and 37.5 ºC, respectively, at five-degree intervals (7.5 ºC, 5 ºC, 0 ºC; 37.5 ºC, 40 ºC, 45 ºC, 50 ºC) until the cessation of the growths. Colony diameters were measured in two orthogonal directions after 3, 7 and 14 days of incubation in the dark, with three replicate plates per isolate at each temperature.

The isolates were clustered on the basis of their morphological and growth rates characteristics in a dendrogram built with NTSYSpc2 (Numerical Taxonomy and Multivariate Analysis System - Version 2.02h) using Simple Matching coefficient (SM) and Unweighted Pair Group Method (UPGMA). Two separate clustering analyses were preformed: (1) analysis of the complete set of isolates based on cultural characteristic (transformed in a quantitative scale by classes) and growth rates at 25±1 ºC and (2) analysis of the 36 selected isolates based on

cultural characteristics, conidia dimensions, and growth rates at 25±1 ^{0}C and at minimum and maximum tested temperatures.

Table 1. Isolates of *Biscogniauxia mediterranea* included in the analysis

Isolate[a]	Collected/ Isolated by, Year	Isolated from	Location
Bm04.001	M.L. Inácio, 2004	*Quercus suber*, adult tree with carbonaceous stroma	Montemor-o-Novo, Portugal
Bm05.001	M.L. Inácio, 2005	*Quercus suber*, adult tree with carbonaceous stroma	Chamusca, Portugal
Bm05.002	M.L. Inácio, 2005	*Quercus suber*, adult tree with carbonaceous stroma	Sardoal, Portugal
Bm05.003	M.L. Inácio, 2005	*Quercus suber*, adult tree with carbonaceous stroma	Sines, Portugal
Bm05.004	M.L. Inácio, 2005	*Quercus suber*, adult tree with carbonaceous stroma	Montemor-o-Novo, Portugal
Bm06.001	M.L. Inácio, 2006	*Quercus suber*, adult tree with carbonaceous stroma	Coruche, Portugal
Bm06.002	M.L. Inácio, 2006	*Quercus suber*, adult tree with carbonaceous stroma	Coruche, Portugal
Bm06.003	M.L. Inácio, 2006	*Quercus suber*, adult tree with carbonaceous stroma	Mamora, Morocco
Bm06.004	M.L. Inácio, 2006	*Quercus suber*, adult tree with carbonaceous stroma	Sesimbra, Portugal
Bm06.005	M.L. Inácio, 2006	*Quercus suber*, adult tree with carbonaceous stroma	Sines, Portugal
Bm07.001	M.L. Inácio, 2007	*Quercus suber*, adult tree with carbonaceous stroma	Grândola, Portugal
Bm07.003	M.L. Inácio & J. Henriques, 2007	*Quercus suber*, young undecorked tree with brown powdery mass	Grândola, Portugal
Bm08.001	M.L. Inácio, 2008	*Quercus suber*, adult tree with carbonaceous stroma	Benavente, Portugal
Bm08.002	M.L. Inácio, 2008	*Quercus suber*, young undecorked tree with carbonaceous stroma	Grândola, Portugal
Bm08.003	M.L. Inácio, 2008	*Quercus suber*, adult tree with carbonaceous stroma	Chamusca, Portugal
Bm09.001	J. Henriques, 2009	*Quercus suber*, adult tree with carbonaceous stroma	Kroufa, Tunisia
Bm10.001	J. Henriques, 2010	*Quercus suber*, young undecorked tree with brown powdery mass	Montemor-o-Novo, Portugal
Bm10.002	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Viana do Alentejo, Portugal
Bm10.003	J. Henriques, 2010	*Quercus suber*, young undecorked tree with carbonaceous stroma	Viana do Alentejo, Portugal
Bm10.004	J. Henriques, 2010	*Quercus suber*, young undecorked tree with carbonaceous stroma	Viana do Alentejo, Portugal
Bm10.005	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Viana do Alentejo, Portugal
Bm10.006	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Alenquer, Portugal
Bm10.007	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Alenquer, Portugal
Bm10.008	A. Matos/ J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Abrantes, Portugal
Bm10.009	A. Matos/ J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Abrantes, Portugal
Bm10.010	A. Matos/ J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Abrantes, Portugal
Bm10.012	J. Henriques, 2010	*Quercus rotundifolia*, adult tree with carbonaceous stroma	Serpa, Portugal
Bm10.014	L. Caparica/ J. Henriques, 2010	*Quercus suber*, young undecorked tree with carbonaceous stroma	Grândola, Portugal
Bm10.015	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Sardinia, Italy
Bm10.016	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Sardinia, Italy
Bm10.017	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Viana do Alentejo, Portugal
Bm10.018	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Chamusca, Portugal
Bm10.019	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Grândola, Portugal
Bm10.020	H. Bragança/ J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Nisa, Portugal
Bm10.021	H. Bragança/ J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Marvão, Portugal
Bm10.022	J. Rosendo/ J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Silves, Portugal
Bm10.023	J. Rosendo/ J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Silves, Portugal
Bm10.024	J. Henriques, 2010	*Quercus suber*, adult tree with carbonaceous stroma	Grândola, Portugal
Bm11.001	P. Naves/ J. Henriques, 2011	*Quercus suber*, adult tree with carbonaceous stroma	Almodôvar, Portugal
Bm11.002	P. Pacheco Marques/ J. Henriques, 2011	*Quercus suber*, adult tree with carbonaceous stroma	Ponte de Sôr, Portugal
Bm11.003	J. Henriques, 2011	*Quercus suber*, adult tree with carbonaceous stroma	Grândola, Portugal
Bm12.001	A. Costa/ J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Benavente, Portugal
Bm12.002	L. Martins/ J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Mirandela, Portugal
Bm12.003	L. Martins/ J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Mirandela, Portugal
Bm12.004	L. Martins/ J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Mirandela, Portugal
Bm12.005	APFC/ J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Coruche, Portugal
Bm12.006	APFC/ J. Henriques, 2012	*Quercus suber*, young undecorked tree with carbonaceous stroma	Coruche, Portugal
Bm12.009	APFC/ J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Coruche, Portugal
Bm12.010	APFC/ J. Henriques, 2012	*Quercus suber*, young undecorked tree with carbonaceous stroma	Coruche, Portugal
Bm12.011	APFC/ J. Henriques, 2012	*Quercus suber*, young undecorked tree with carbonaceous stroma	Coruche, Portugal
Bm12.012	APFC/ J. Henriques, 2012	*Quercus suber*, young tree with carbonaceous stroma	Coruche, Portugal
Bm12.013	APFC/ J. Henriques, 2012	*Quercus suber*, young undecorked tree with brown powdery mass	Coruche, Portugal
Bm12.014	J. Henriques, 2012	*Quercus suber*, young undecorked tree with carbonaceous stroma	Grândola, Portugal
Bm12.015	M. Z. Boutiti, 2009	*Quercus suber*, adult tree symptomless	Kroufa, Tunisia
Bm12.016	A. Matos/ J. Henriques, 2012	*Quercus suber*, young tree with carbonaceous stroma	Setúbal, Portugal
Bm12.017	J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Grândola, Portugal

Isolate[a]	Collected/ Isolated by, Year	Isolated from	Location
Bm12.018	L. Santos/ J. Henriques, 2012	*Quercus suber*, symptomless acorn	Montijo, Portugal
Bm12.019	L. Santos/ J. Henriques, 2012	*Quercus suber*, symptomless acorn	Montijo, Portugal
Bm12.020	L. Santos/ J. Henriques, 2012	*Quercus suber*, symptomless acorn	Montijo, Portugal
Bm12.021	L. Santos/ J. Henriques, 2012	*Quercus suber*, symptomless acorn	Alcácer do Sal, Portugal
Bm12.022	P. Naves/ J. Henriques, 2012	*Quercus suber*, young undecorked tree with carbonaceous stroma	Tróia, Portugal
Bm12.023	H. Bragança & E. Diogo/ J. Henriques, 2012	*Eucalyptus globulus*, adult tree with carbonaceous stroma	Odemira, Portugal
Bm12.024	J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Terras de Bouro, Portugal
Bm12.025	J. Henriques, 2012	*Quercus suber*, young undecorked tree with brown powdery mass	Terras de Bouro, Portugal
Bm12.026	A. Matos/ J. Henriques, 2012	*Quercus suber*, adult tree carbonaceous stroma	Penha Garcia, Portugal
Bm12.027	J. Henriques, 2012	*Quercus robur*, adult tree with carbonaceous stroma	Terras de Bouro, Portugal
Bm12.028	J. Henriques, 2012	*Quercus robur*, adult tree with carbonaceous stroma	Terras de Bouro, Portugal
Bm12.029	J. Henriques, 2012	*Quercus robur*, adult tree with carbonaceous stroma	Terras de Bouro, Portugal
Bm12.031	J. Henriques, 2012	*Castanea sativa*, adult tree with carbonaceous stroma	Terras de Bouro, Portugal
Bm12.032	J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Mafra, Portugal
Bm12.033	J. Henriques, 2012	*Quercus faginea*, adult tree with carbonaceous stroma	Mafra, Portugal
Bm12.035	J. Henriques, 2012	*Castanea sativa*, adult tree with carbonaceous stroma	Mafra, Portugal
Bm12.036	J. Henriques, 2012	*Quercus faginea*, adult tree with carbonaceous stroma	Mafra, Portugal
Bm12.038	P. Naves/ J. Henriques 2012	*Quercus suber*, adult tree with carbonaceous stroma	Palmela, Portugal
Bm12.039	R. Piazzeta/ J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Vivès, France
Bm12.040	R. Piazzeta/ J. Henriques, 2012	*Quercus suber*, adult tree with carbonaceous stroma	Vivès, France
Bm12.041	R. Piazzeta/ J. Henriques, 2012	*Quercus suber*, dead adult tree with carbonaceous stroma	Vivès, France
Bm12.042	R. Piazzeta/ J. Henriques, 2012	*Quercus suber*, dead adult tree with carbonaceous stroma	Vivès, France
Bm13.001	H. Baudriller-Cacaud/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Corse du Sud, France
Bm13.002	H. Baudriller-Cacaud/ J. Henriques, 2013	*Quercus suber*, dead adult tree with carbonaceous stroma	Corse du Sud, France
Bm13.003	H. Baudriller-Cacaud/ J. Henriques, 2013	*Quercus suber*, dead adult tree with carbonaceous stroma	Corse du Sud, France
Bm13.004	H. Baudriller-Cacaud/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Corse du Sud, France
Bm13.005	H. Baudriller-Cacaud/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Corse du Sud, France
Bm13.006	A. Mounia/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Anaba, Algeria
Bm13.007	A. Mounia/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Anaba, Algeria
Bm13.008	L. Belhoucine/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Tlemcen, Algeria
Bm13.009	L. Belhoucine/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Tlemcen, Algeria
Bm13.010	L. Belhoucine/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Tlemcen, Algeria
Bm13.011	L. Belhoucine/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Tlemcen, Algeria
Bm13.012	A. Soto Sánchez/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Cordoba, Spain
Bm13.013	A. Soto Sánchez/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Cordoba, Spain
Bm13.014	A. Soto Sánchez/ J. Henriques, 2013	*Quercus suber*, adult tree with carbonaceous stroma	Cordoba, Spain
Pc96.009	E. Sousa, 1996	*Platypus cylindrus* associated to *Quercus suber*	Coruche, Portugal
Pc04.002	M.L. Inácio, 2004	*Platypus cylindrus* associated to *Quercus suber*	Mamora, Morocco
Pc05.008	M.L. Inácio, 2005	*Platypus cylindrus* associated to *Quercus suber*	Coruche, Portugal
Pc06.049	M.L. Inácio & J. Henriques, 2006	*Platypus cylindrus* associated to *Quercus suber*	Coruche, Portugal
Pc06.050	M.L. Inácio & J. Henriques, 2006	*Platypus cylindrus* associated to *Quercus suber*	Coruche, Portugal
Pc06.052	M.L. Inácio & J. Henriques, 2006	*Platypus cylindrus* associated to *Quercus suber*	Coruche, Portugal
Pc06.054	M.L. Inácio & J. Henriques, 2006	*Platypus cylindrus* associated to *Quercus suber*	Mamora, Morocco
Pc07.002	M.L. Inácio & J. Henriques, 2007	*Platypus cylindrus* associated to *Quercus suber*	Grândola, Portugal
Pc08.002	M.L. Inácio & J. Henriques, 2008	*Platypus cylindrus* associated to *Quercus suber*	Montemor-o-Novo, Portugal
CBS101016	1998	*Quercus robur*, dead branch	Bremmert-Kootwijk, Netherlands

[a]Bm, Pc: work collections; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

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RESULTS

All the isolates were confirmed as *B. mediterranea* species by the amplification with the specific primers MED1/ MED2 that generated, as expected, only one PCR product of approximately 380 bp.

The complete set of isolates was characterized according to the main cultural features grown in PDYA at 25±1 °C (Table 2, Figure 1). The isolates showed high variability in all observed parameters (cultural aspects, density, color and zonation), particularly in color both in the surface and in the reverse of the culture. In general cultures aspects varied from velvety to wholly with mycelial tufts dispersed in the culture to velvety with sectors (according to the density) and tufts, density media to high. Colors differ from white with vinaceous buff aerial mycelium, grayish sepia to smoke grey, or butt margin with olivaceous center. In some colonies dark brown exudates are frequent. The reverse of the colonies varies from buff margin with umber to olivaceous center, buff to honey margin, saffron to sienna center, or pale mouse grey to mouse grey, with darker spots dispersed in the culture or strong diffusible pigment. There were some particular cases in which the cultures were more variable. The growth of the isolates measured at the third and seventh day of culture was also very variable: all isolates grew but with different rates, at the third day of growth some isolates had reached a whole range of dimensions from 16 mm to the maximum diameter of the petri dish. At the seventh days of growth most isolates have reached the maximum diameter of the culture, not allowing the determination of its actual growth rate capacity (Table 2).

Table 2. Cultural characteristics of the *Biscogniauxia mediterranea* isolates grown on PDYA at 25±1 ºC and growth rates at the 3rd and 7th day

Isolate	Surface				Reverse	Growth rate [mm]	
	Cultural aspect	Density	Color	Zonation		3 d	7 d
Bm04.001	velvety	media/ high	white with vinaceous buff aerial mycelium	absent	idem upper face, except in color buff margin with umber to olivaceous center	76.2	- (a)
Bm05.001	velvety	media/ high	white with vinaceous buff aerial mycelium	absent	idem upper face, except in color buff margin with umber to olivaceous center	27.7	77.2
Bm05.002	velvety	media/ high	white with vinaceous buff aerial mycelium	absent	idem upper face, except in color buff margin with umber to olivaceous center	27.6	77.2
Bm05.003	velvety	media/ high	white with vinaceous buff aerial mycelium	absent	idem upper face, except in color buff	28.5	77.1
Bm05.004	velvety to wholly with mycelial tufts dispersed in the culture	high	buff to vinaceous buff	absent	idem upper face, except in color buff	28.8	75.2
Bm06.001	velvety	media	buff margin with olivaceous center	absent	idem upper face, except in color buff	20.2	66.8
Bm06.002	velvety	media/ high	saffron with brown vinaceous aerial mycelium	absent	idem upper face, except in color saffron to sienna	28.2	-
Bm06.003	velvety	media	buff	absent	idem upper face, except in color buff	75.5	-
Bm06.004	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff to hazel	absent	idem upper face, except in color buff to honey	34.7	-
Bm06.005	velvety to wholly with mycelial tufts dispersed in the culture	high	buff	absent	idem upper face, except in color luteous	26.8	-
Bm07.001	velvety	media/ high	white with vinaceous buff aerial mycelium	absent	idem upper face, except in color buff	28.0	72.4
Bm07.003	velvety	media/ high	hazel margin with dark mouse grey center	absent	idem upper face, except in color buff to honey margin and brown vinaceous center	61.8	-
Bm08.001	velvety	media/ high	buff	absent	idem upper face, except in color buff	45.1	-
Bm08.002	velvety	media/ high	white with vinaceous buff aerial mycelium	absent	idem upper face, except in color buff margin with umber to olivaceous center	28.2	76.5
Bm08.003	velvety	media/ high	white with vinaceous buff aerial mycelium	absent	idem upper face, except in color buff margin with umber to olivaceous center	27.2	77.1
Bm09.001	velvety to wholly with mycelial tufts dispersed in the culture	high	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	73.8	-
Bm10.001	velvety	media/ high	buff	absent	idem upper face, except in color buff	71.4	-
Bm10.002	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia	absent	idem upper face, except in color pale mouse grey to mouse grey with apricot center	24.7	63.2
Bm10.003	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff to greyish sepia	absent	idem upper face, except in color vinaceous buff to umber	28.8	75. 3
Bm10.004	velvety	media	buff to vinaceous buff	absent	idem upper face, except in color buff with fawn zoning	31.1	-
Bm10.005	velvety to wholly with mycelial tufts dispersed in the culture	high	hazel	absent	idem upper face, except in blood color	56.8	-
Bm10.006	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia to smoke grey	absent	idem upper face, except in color fuscous black to dark mouse grey	74.8	-
Bm10.007	velvety	high	dark brick to brown vinaceous	absent	idem upper face, except in color brown vinaceous	49.5	-
Bm10.008	velvety	media	honey margin with vinaceous buff center	absent	idem upper face, except in color dark brick	36.5	-

Isolate	Surface				Reverse	Growth rate [mm]	
	Cultural aspect	Density	Color	Zonation		3 d	7 d
Bm10.009	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia to smoke grey	absent	idem upper face, except in color fuscous black to dark mouse grey	78.0 (a)	-
Bm10.010	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color brown vinaceous	48.2	-
Bm10.012	velvety to wholly with mycelial tufts dispersed in the culture	high	color non uniform between isabelline and buff, exudate frequent dark brown	absent	idem upper face, except in color sienna with darker spots in the center	73.4	-
Bm10.014	velvety to wholly with mycelial tufts dispersed in the culture	high	color non uniform between isabelline and buff, exudate frequent dark brown	absent	idem upper face, except in color sienna with darker spots in the center	65.2	-
Bm10.015	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia	absent	idem upper face, except in color fuscous black	60.9	-
Bm10.016	velvety with sectors	media/ high	white with vinaceous buff sectors	absent	idem upper face, except in color buff with hazel sectors	78.0	-
Bm10.017	velvety with sectors	media	white with pale purplish grey sectors	absent	idem upper face, except in color buff	32.7	-
Bm10.018	velvety	media	buff to honey	concentric and radially striate	idem upper face, except in color buff with dark brick zoning	28.5	69.9
Bm10.019	velvety	media	white	absent	idem upper face, except in color buff	45.7	-
Bm10.020	velvety to wholly with mycelial tufts dispersed in the culture	high	color non uniform between isabelline and buff, exudate frequent dark brown	absent	idem upper face, except in color sienna with darker spots in the center	54.0	-
Bm10.021	velvety to wholly with mycelial tufts dispersed in the culture	high	color non uniform between isabelline and buff, exudate frequent dark brown	absent	idem upper face, except in color sienna with darker spots in the center	49.0	-
Bm10.022	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color brown vinaceous	68.2	-
Bm10.023	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna to fuscous black	71.4	-
Bm10.024	velvety to wholly with mycelial tufts dispersed in the culture	high	hazel	absent	idem upper face, except in color dark brick	78.0	-
Bm11.001	velvety	media/ high	olivaceous to greyish sepia	absent	idem upper face, except in color hazel to umber	76.9	-
Bm11.002	velvety to wholly with mycelial tufts dispersed in the culture	high	color non uniform between isabelline and buff, exudate frequent dark brown	absent	idem upper face, except in color sienna with darker spots in the center	63.9	-
Bm11.003	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna to fuscous black	73.7	-
Bm12.001	velvety to wholly with mycelial tufts dispersed in the culture	high	fuscous black	absent	idem upper face, except in color violaceous black	78.0	-
Bm12.002	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia	absent	idem upper face, except in color pale mouse grey to mouse grey margin with apricot center	38.5	74.3
Bm12.003	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna to fuscous black	60.8	-
Bm12.004	velvety to wholly with mycelial tufts dispersed in the culture	high	fuscous black	absent	idem upper face, except in color violaceous black	30.2	-
Bm12.005	velvety with mycelial strands	media	buff	absent	idem upper face, except in color buff margin and umber to brown vinaceous center	66.1	-
Bm12.006	velvety to wholly with mycelial tufts dispersed in the culture	high	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	45.4	-

Isolate	Surface				Reverse	Growth rate [mm]	
	Cultural aspect	Density	Color	Zonation		3 d	7 d
Bm12.009	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna with darker spots disperse in the culture	71.2	-
Bm12.010	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff to hazel	absent	idem upper face, except in color buff to honey margin and brown vinaceous center	67.3	-
Bm12.011	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia to fuscous	absent	idem upper face, except in color olivaceous with fuscous black center	39.2	-
Bm12.012	velvety to wholly with mycelial tufts dispersed in the culture	high	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	20.7	56.7
Bm12.013	velvety to wholly with mycelial tufts dispersed in the culture	media	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	75.8	-
Bm12.014	velvety	media	white	absent	idem upper face, except in color buff margin with umber to olivaceous center	77.6	-
Bm12.015	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna with darker spots disperse in the culture	68.0	-
Bm12.016	velvety to wholly with mycelial tufts dispersed in the culture	high	hazel	absent	idem upper face, except in color dark brick	56.4	-
Bm12.017	velvety to wholly with mycelial tufts dispersed in the culture	high	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	63.2	-
Bm12.018	velvety to wholly with mycelial tufts dispersed in the culture	media	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	73.9	-
Bm12.019	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia to fuscous	absent	idem upper face, except in color olivaceous margin with fuscous black center	70.8	-
Bm12.020	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color buff margin with umber to olivaceous center	52.2	-
Bm12.021	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia to smoke grey	absent	idem upper face, except in color fuscous black to dark mouse grey	74.2	-
Bm12.022	velvety	high	buff	absent	idem upper face, except in color dark brick	61.2	-
Bm12.023	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna to fuscous black	70.8	-
Bm12.024	velvety to wholly with mycelial tufts dispersed in the culture	high	color non uniform between isabelline and buff, exudate frequent dark brown	absent	idem upper face, except in color sienna with darker spots in the center	64.6	-
Bm12.025	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia to smoke grey	absent	idem upper face, except in color fuscous black to dark mouse grey	57.7	-
Bm12.026	velvety	media	dark mouse grey to greyish sepia	absent	idem upper face, except in color buff to brown vinaceous	75.2	-
Bm12.027	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia to smoke grey	absent	idem upper face, except in color fuscous black to dark mouse grey	73.8	-
Bm12.028	velvety	media	white	absent	idem upper face, except in color buff margin with umber to olivaceous center	78.0	-
Bm12.029	powdery with dendritic growth	media	greyish sepia	absent	idem upper face, except in color sienna to dark brick	25.5	-
Bm12.031	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia	absent	idem upper face, except in color fuscous black	78.0	-
Bm12.032	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color buff	64.8	-

Isolate	Surface				Reverse	Growth rate [mm]	
	Cultural aspect	Density	Color	Zonation		3 d	7 d
Bm12.033	velvety to wholly with mycelial tufts dispersed in the culture	high	color non uniform between isabelline and buff, exudate frequent dark brown	absent	idem upper face, except in color sienna with darker spots in the center	72.1	-
Bm12.035	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna to fuscous black	70.4	-
Bm12.036	velvety	media	honey margin with vinaceous buff center	absent	idem upper face, except in color honey margin and brown vinaceous center	52.2	-
Bm12.038	velvety to wholly with mycelial tufts dispersed in the culture	high	color non uniform between isabelline and buff, exudate frequent dark brown	absent	idem upper face, except in color sienna with darker spots in the center	78.0	-
Bm12.039	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna to fuscous black	62.8	-
Bm12.040	velvety to wholly with mycelial tufts dispersed in the culture	high	buff to vinaceous buff	absent	idem upper face, except in color ochreous	56.9	-
Bm12.041	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia to smoke grey	absent	idem upper face, except in color fuscous black to dark mouse grey	65.2	-
Bm12.042	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna with darker spots disperse in the culture	78.0	-
Bm13.001	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia to smoke grey	absent	idem upper face, except in color fuscous black to dark mouse grey	74.6	-
Bm13.002	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna to fuscous black	77.6	-
Bm13.003	velvety to wholly with mycelial tufts dispersed in the culture	high	fuscous black	absent	idem upper face, except in color violaceous black	69.4	-
Bm13.004	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color brown vinaceous	78.0	-
Bm13.005	velvety	media	white	absent	idem upper face, except in color buff margin with umber to olivaceous center	78.0	-
Bm13.006	velvety to wholly with mycelial tufts dispersed in the culture	high	rosy buff to cinnamon	absent	idem upper face, except in blood color	78.0	-
Bm13.007	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna to fuscous black	78.0	-
Bm13.008	velvety	media/ high	white	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	74.1	-
Bm13.009	velvety	media/ high	white	absent	idem upper face, except in color buff	74.6	-
Bm13.010	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna to fuscous black	74.4	-
Bm13.011	velvety to wholly with mycelial tufts dispersed in the culture	high	vinaceous buff	absent	idem upper face, except in color sienna with darker spots disperse in the culture	77.1	-
Bm13.012	velvety to wholly with mycelial tufts dispersed in the culture	high	greyish sepia	absent	idem upper face, except in color pale mouse grey to mouse grey with apricot center	62.4	-
Bm13.013	velvety to wholly with mycelial tufts dispersed in the culture	media	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	77.0	-

Isolate	Surface				Reverse	Growth rate [mm]	
	Cultural aspect	Density	Color	Zonation		3 d	7 d
Bm13.014	velvety with sectors (according to the density) and tufts	media/low in sectors	buff with sulphur yellow and olivaceous tufts	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	69.9	-
Pc04.002	velvety to wholly with mycelial tufts dispersed in the culture	high	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	30.4	-
Pc05.008	velvety	media/ high	buff	absent	idem upper face, except in color buff margin and dark brick center	28.3	74.1
Pc06.049	velvety	media	buff margin with olivaceous center	concentric	idem upper face, except in color buff margin and brown vinaceous center	28.1	-
Pc06.050	velvety	media	buff	absent	idem upper face, except in color buff	21.2	69.0
Pc06.052	velvety	media	white	absent	idem upper face, except in color buff margin with umber to olivaceous center	27.5	62.9
Pc06.054	velvety	media/ high	buff	absent	idem upper face, except in color buff	15.9	67.7
Pc07.002	velvety	media/ high	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	27.0	-
Pc08.002	velvety	media	white	absent	idem upper face, except in color buff margin with umber to olivaceous center	22.8	61.3
Pc96.009	velvety to wholly with mycelial tufts dispersed in the culture	high	buff margin with olivaceous center	absent	idem upper face, except in color sulphur yellow margin and greenish black center, with strong diffusible pigment	27.7	74.5
CBS101016	velvety to wholly with mycelial tufts dispersed in the culture	high	buff	absent	idem upper face, except in color buff margin and brown vinaceous center	72.7	-

(a) - growth has reached the maximum diameter of the culture, 78.00 mm, not representing the real potential growth of the isolate

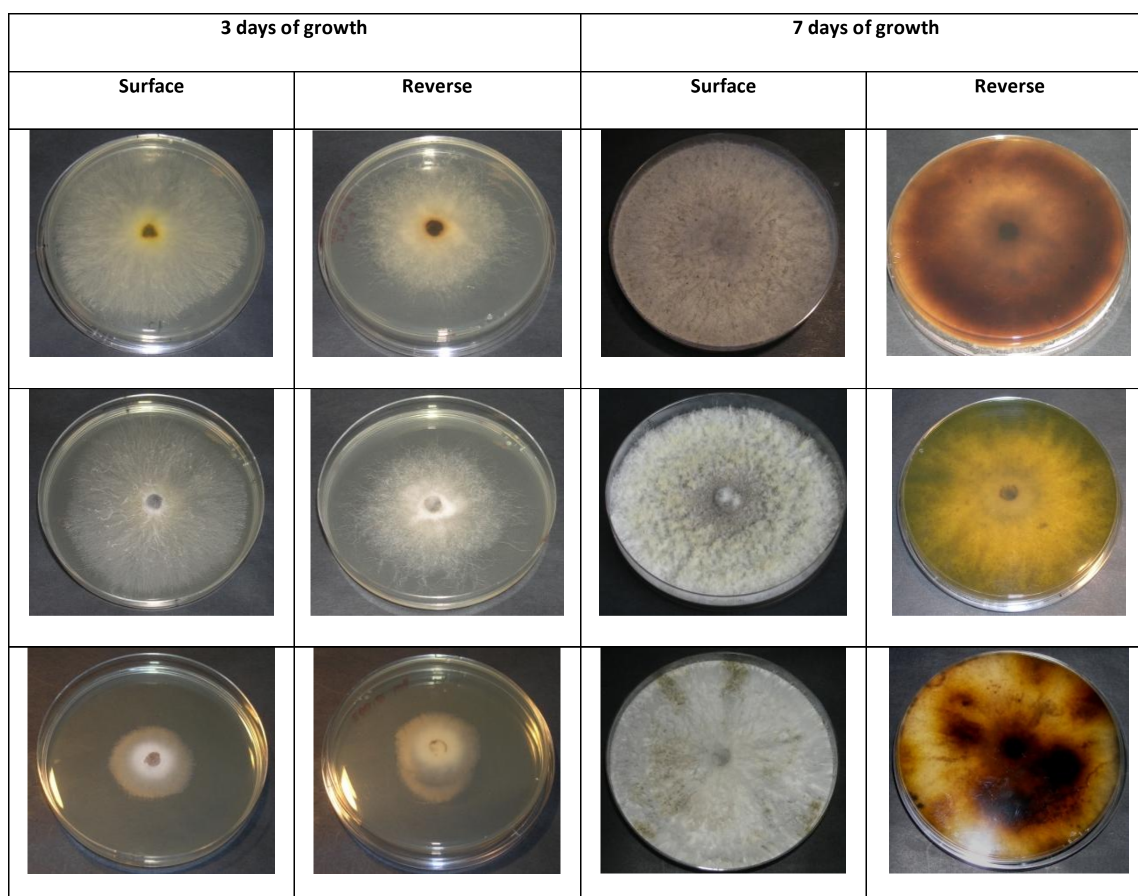

Figure 1. Examples of the different aspects of *Biscogniauxia mediterranea* cultures after 3 and 7 days of growth on PDYA at 25±1 ºC (Bm12.022, Bm12.006, Bm10.018).

The combined clustering analysis of cultural characteristics and growth rates at 25 ºC is represented in Figure 2. It did not allow consistent grouping of isolates according to the study variables (isolate origin, host species, tree conditions and expression of the fungus) revealing the high level of variability among isolates of *B. mediterranea*. The all set of isolates present a similarity rate of 55 %, forming two major groups that include 30 and 72 isolates, respectively, with a subsequent arrangement of successive clustering of isolates with crescent similarity reaching the 100 % in a few cases. Nevertheless, it was not possible to relate any grouping level of the isolates with the source of the isolates. The cophenetic correlation coefficient of this UPGMA analysis was 0.75, indicating the dendrogram was a reasonable fit representation of the original data.

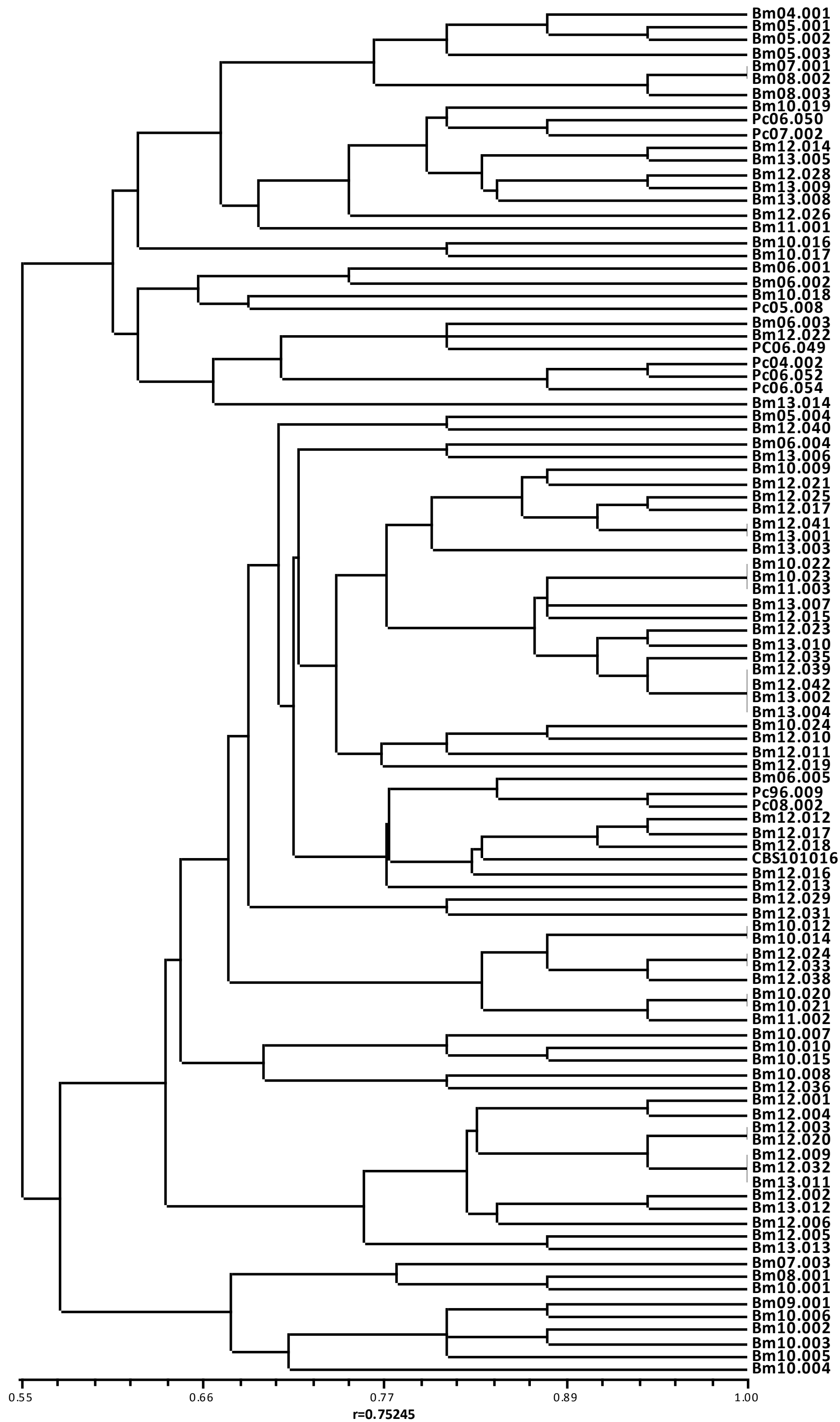

Figure 2. Dendrogram of the isolates clustering according to cultural and growth rates parameters performed in NTSYSpc2 using SM correlation coefficient and UPGMA. Scale bar represents percentage of similarity.

Microscopic observation of the selected isolates of *B. mediterranea* allowed the characterization of the conidiogenous structures. In general, for all observed isolates, the mitosporic form was described as: mycelium partly immersed; conidiophores macronematous and mononematous, arising laterally from the brownish vegetative hyphae, with principal axis erect, septate, branched, hyaline to light brown, slightly rugouse; conidiogenic cells poliblastic and sympodial, integrated and terminal becoming intercalary, slender or short and thick and verticillated, cylindrical to clavate. Conidia are sympodulosporic, acropleurogenous, unicellular, 0-septate, hyaline or brown to olive in mass, ellipsoidal or obovoid, smooth or roughened, with a small frill when detached. The dimensions of conidia are variable, presented in Table 3. No correlation was found between length and width of conidia ($r=-0.03$, $p<0.05$, $N=390$). Despite showing variability between replicates, no significant differences were found in the dimensions of conidia of each isolate ($F_{(870,930)}=0.0983$, $p=1.0$). However, among isolates there were significant differences in conidia dimensions ($F_{(30,1829)}=3.0596$, $p<0.0001$). In Figure 3 are presented examples of observed conidiophores and conidia. Five of the 36 selected isolates did not produce asexual reproductive structures.

Figure 3. Examples of conidiophores and conidia of *Biscogniauxia mediterranea* (Bm09.001, Bm10.012, Bm12.013, Bm12.015, Bm12.017, Bm12.032, Bm13.007, Bm13.008, Bm13.014).

Growth rates for maximum and minimum temperatures of *B. mediterranea* selected isolates at the 3rd, 7th and 14th day of culture, as well as for intermediate tested temperatures, are presented in Table 3. For most isolates the minimum growth temperature was at 5 ºC except for Bm12.015 (from *Q. suber*, Tunisia), Bm12.033 (from *Q. faginea*, Mafra, Portugal) and CBS101016 (*Q. robur*, Netherlands) that was at 7.5 ºC. For maximum growth temperatures, most isolates reached the 45 ºC but seven isolates grew only until 40 ºC: Bm07.003 (from

young *Q. suber* with mitosporic stage of the fungus, Grândola, Portugal), Bm10.016 (from *Q. suber*, Sardinia), Bm10.018 (*Q. suber*, Chamusca, Portugal), Bm12.032 (*Q. suber*, Mafra, Portugal), Bm12.033, Bm12.035 (*C. sativa*, Mafra, Portugal) and CBS101016. All selected isolates from the region of Mafra (Portugal) presented a similar behavior with regard to this parameter, to the other isolates there was no pattern related to its origin (geographical, host or syntomatologycal).

Combining all of the studied parameters (cultural characteristics and growth rates at 25±1 ^{0}C, conidial dimensions and growth rates at maximum, minimum and close temperatures) on a common clustering analysis for the 36 selected isolates was generated dendrogram shown in Figure 4. In this approach all isolates present 39 % of similarity, subdivided into groups of increasing resemblance to a maximum of 83 % of similarity, however, once more, these groups do not represent any connection with the source parameters of the isolates. The cophenetic correlation coefficient of this UPGMA analysis was 0.71, indicating the dendrogram was a reasonable fit representation of the original data.

Tabela 3. Conidial dimensions of selected isolates of *Biscogniauxia mediterranea* in PDA and growth rates at minimum and maximum tested temperatures at the 3rd and 7th day (growths at limit temperatures in bold)

Isolate	Conidial dimension [µm]		Growth [mm]																				
	Length	Width	0 ºC			5 ºC			7.5 ºC			37.5 ºC			40 ºC			45 ºC			50 ºC		
			3 d	7 d	14 d	3 d	7 d	14 d	3 d	7 d	14 d	3 d	7 d	14 d	3 d	7 d	14 d	3 d	7 d	14 d	3 d	7 d	14 d
Bm04.001	(3.30-) 4.00 - 4.27 - 4.54 (-5.81)	(2.15-) 2.51 - 2.63 - 2.74 (-3.37)	0.0	0.0	0.0	0.0	0.0	**2.7**	0.0	1.5	10.8	43.3	- (a)	-	20.0	29.2	31.8	0.0	0.0	**24.3**	0.0	0.0	0.0
Bm06.003	(2.55-) 4.22 - 4.53 - 4.85 (-6.16)	(1.78-) 2.22 - 2.36 - 2.51 (-3.23)	0.0	0.0	0.0	0.0	0.0	**2.2**	0.0	4.5	12.3	39.5	-	-	5.0	6.3	6.5	0.0	0.0	**12.3**	0.0	0.0	0.0
Bm07.003	not sporulated	not sporulated	0.0	0.0	0.0	0.0	0.0	**0.2**	0.0	1.7	6.2	39.2	-	-	5.7	12.0	**12.0**	0.0	0.0	0.0	---	---	---
Bm09.001	(3.04-) 4.82 - 5.40 - 5.98 (-11.45)	(1.51-) 1.83 - 1.93 - 2.02 (-2.49)	0.0	0.0	0.0	0.0	0.0	**0.7**	0.0	1.2	5.8	66.8	-	-	1.5	9.5	17.8	0.0	0.0	**0.3**	0.0	0.0	0.0
Bm10.001	(3.75-) 4.64 - 5.02 - 5.40 (-8.29)	(1.32-) 1.94 - 2.06 - 2.18 (-2.74)	0.0	0.0	0.0	0.0	0.0	**3.0**	0.0	2.2	9.5	63.7	-	-	14.5	19.2	22.8	0.0	0.0	**24.5**	0.0	0.0	0.0
Bm10.006	(4.34-) 5.27 - 5.51 - 5.75 (-6.98)	(1.81-) 2.20 - 2.31 - 2.42 (-2.92)	0.0	0.0	0.0	0.0	0.0	**2.7**	0.0	1.0	6.2	60.5	-	-	2.8	17.7	23.8	0.0	0.0	**13.0**	0.0	0.0	0.0
Bm10.012	(4.40-) 6.06 - 6.47 - 6.87 (-8.52)	(1.50-) 1.79 - 1.85 - 1.92 (-2.22)	0.0	0.0	0.0	0.0	0.0	**2.3**	0.0	2.0	4.5	37.3	-	-	5.0	12.5	15.0	0.0	0.0	**10.8**	0.0	0.0	0.0
Bm10.016	(3.62-) 5.01 - 5.40 - 5.79 (-7.69)	(1.55-) 1.97 - 2.08 - 2.19 (-2.58)	0.0	0.0	0.0	0.0	0.0	**2.0**	0.0	1.2	5.0	40.7	-	-	8.7	11.8	**18.0**	0.0	0.0	0.0	---	---	---
Bm10.018	not sporulated	not sporulated	0.0	0.0	0.0	0.0	0.0	**3.7**	2.2	4.0	14.3	29.0	64.8	-	4.2	5.2	**5.9**	0.0	0.0	0.0	---	---	---
Bm10.019	(4.09-) 5.09 - 5.64 - 6.19 (-9.69)	(0.91-) 1.85 - 1.95 - 2.06 (-2.35)	0.0	0.0	0.0	0.0	0.0	**10.2**	0.3	5.0	11.7	23.0	38.7	54.3	5.5	12.3	12.7	0.0	0.0	**10.2**	0.0	0.0	0.0
Bm10.023	(3.43-) 4.51 - 4.79 - 5.06 (-6.59)	(1.78-) 2.07 - 2.17 - 2.28 (-2.84)	0.0	0.0	0.0	0.0	0.0	**18.0**	0.0	1.0	3.7	73.0	-	-	11.5	26.5	31.5	0.0	0.0	**18.0**	0.0	0.0	0.0
Bm10.024	not sporulated	not sporulated	0.0	0.0	0.0	0.0	0.0	**2.5**	0.0	2.3	12.2	54.2	-	-	9.0	19.0	32.2	0.0	0.0	**21.3**	0.0	0.0	0.0
Bm11.003	(3.43-) 5.26 - 5.78 - 6.29 (-9.70)	(1.27-) 1.92 - 2.04 - 2.16 (-2.86)	0.0	0.0	0.0	0.0	0.0	**2.3**	0.0	4.0	14.8	69.0	-	-	13.2	30.2	46.0	0.0	0.0	**23.0**	0.0	0.0	0.0
Bm12.004	(3.14-) 3.90 - 4.14 - 4.38 (-5.79)	(1.62-) 2.25 - 2.35 - 2.46 (-2.94)	0.0	0.0	0.0	0.0	0.0	**1.7**	0.0	1.3	6.5	69.0	-	-	13.8	44.3	57.8	0.0	0.0	**39.8**	0.0	0.0	0.0
Bm12.005	(2.51-) 3.90 - 4.11 - 4.33 (-4.98)	(1.85-) 2.24 - 2.36 - 2.49 (-3.17)	0.0	0.0	0.0	0.0	0.0	**1.5**	1.8	2.8	6.0	54.2	-	-	6.7	16.2	33.0	0.0	0.0	**30.8**	0.0	0.0	0.0
Bm12.013	(2.78-) 3.86 - 4.07 - 4.27 (-5.03)	(1.55-) 1.97 - 2.04 - 2.12 (-2.44)	0.0	0.0	0.0	0.0	0.0	**8.3**	0.0	0.7	3.2	40.0	-	-	4.3	12.3	27.2	0.0	0.0	**8.3**	0.0	0.0	0.0
Bm12.014	(5.61-) 6.62 - 7.03 - 7.44 (-10.90)	(1.72-) 2.17 - 2.32 - 2.46 (-3.08)	0.0	0.0	0.0	0.0	0.0	**15.0**	0.0	1.3	6.0	26.7	66.0	-	3.0	4.5	10.0	0.0	0.0	**16.0**	0.0	0.0	0.0
Bm12.015	(4.41-) 5.42 - 5.70 - 5.97 (-7.11)	(1.42-) 1.80 - 1.95 - 2.11 (-3.08)	---	---	---	0.0	0.0	0.0	0.0	1.7	**7.8**	30.8	62.0	-	4.4	9.2	39.5	0.0	0.0	**3.7**	0.0	0.0	0.0
Bm12.017	(3.29-) 4.25 - 4.54 - 4.83 (-6.05)	(1.51-) 2.05 - 2.15 - 2.26 (-2.82)	0.0	0.0	0.0	0.0	0.0	**2.0**	1.7	3.8	15.3	60.2	-	-	9.8	11.3	11.3	0.0	0.0	**17.8**	0.0	0.0	0.0
Bm12.022	(3.79-) 4.92 - 5.17 - 5.42 (-6.34)	(1.63-) 2.04 - 2.15 - 2.27 (-2.85)	0.0	0.0	0.0	0.0	0.0	**1.0**	0.0	2.2	19.0	73.8	-	-	9.0	27.0	30.0	0.0	0.0	**34.8**	0.0	0.0	0.0
Bm12.023	not sporulated	not sporulated	0.0	0.0	0.0	0.0	0.0	**4.3**	0.0	1.3	1.7	40.3	73.5	-	6.5	18.4	56.3	0.0	0.0	**16.0**	0.0	0.0	0.0
Bm12.024	(4.11-) 4.91 - 5.21 - 5.51 (-7.37)	(1.43-) 1.75 - 1.84 - 1.94 (-2.33)	0.0	0.0	0.0	0.0	0.0	**0.7**	0.0	0.7	2.2	67.2	-	-	5.2	28.0	48.8	0.0	0.0	**17.0**	0.0	0.0	0.0
Bm12.025	(3.36-) 4.72 - 5.04 - 5.35 (-7.10)	(1.88-) 2.46 - 2.58 - 2.71 (-3.27)	0.0	0.0	0.0	0.0	0.0	**1.3**	0.0	1.5	11.0	51.0	-	-	6.8	16.7	27.0	0.0	0.0	**37.2**	0.0	0.0	0.0
Bm12.027	(3.88-) 5.04 - 5.46 - 5.89 (-8.47)	(1.24-) 1.63 - 1.73 - 1.84 (-2.40)	0.0	0.0	0.0	0.0	0.0	**1.3**	0.0	2.7	17.7	55.8	-	-	5.8	15.3	30.0	0.0	0.0	**31.3**	0.0	0.0	0.0
Bm12.031	(3.21-) 4.00 - 4.21 - 4.41 (-5.53)	(0.93-) 1.34 - 1.43 - 1.52 (-2.00)	0.0	0.0	0.0	0.0	0.0	**5.3**	0.0	2.8	13.3	63.0	-	-	10.3	21.0	43.7	0.0	0.0	**28.0**	0.0	0.0	0.0
Bm12.032	(3.29-) 4.27 - 4.49 - 4.71 (-5.79)	(1.58-) 1.88 - 1.95 - 2.03 (-2.30)	0.0	0.0	0.0	0.0	0.0	**6.2**	0.0	1.5	6.7	62.7	-	-	5.7	12.2	**32.0**	0.0	0.0	0.0	---	---	---
Bm12.033	not sporulated	not sporulated	---	---	---	0.0	0.0	0.0	5.7	16.3	**32.0**	37.0	65.3	-	7.0	7.0	**23.2**	0.0	0.0	0.0	---	---	---
Bm12.035	(3.15-) 4.40 - 4.71 - 5.02 (-6.91)	(1.34-) 1.68 - 1.77 - 1.87 (-2.48)	0.0	0.0	0.0	0.0	0.0	**0.3**	0.0	0.7	5.3	48.7	-	-	2.8	4.8	**26.0**	0.0	0.0	0.0	---	---	---
Bm12.039	(3.41-) 4.68 - 4.98 - 5.28 (-6.31)	(1.57-) 1.80 - 1.89 - 1.98 (-2.49)	0.0	0.0	0.0	0.0	0.0	**0.3**	0.0	1.8	5.5	71.0	-	-	9.3	27.7	55.3	0.0	0.0	**23.7**	0.0	0.0	0.0
Bm13.004	(2.74-) 3.78 - 4.07 - 4.36 (-6.67)	(1.45-) 1.89 - 2.02 - 2.15 (-2.86)	0.0	0.0	0.0	0.0	0.0	**1.8**	0.0	3.5	17.7	49.2	-	-	9.2	31.0	34.5	0.0	0.0	**54.2**	0.0	0.0	0.0
Bm13.007	(3.66-) 4.35 - 4.55 - 4.76 (-5.93)	(1.70-) 2.15 - 2.27 - 2.38 (-2.87)	0.0	0.0	0.0	0.0	0.0	**3.2**	0.0	2.8	9.3	56.7	-	-	11.0	19.7	28.2	0.0	0.0	**32.5**	0.0	0.0	0.0
Bm13.008	(4.21-) 6.06 - 6.83 - 7.60 (-14.27)	(1.52-) 1.84 - 1.94 - 2.05 (-2.56)	0.0	0.0	0.0	0.0	0.0	**5.5**	2.2	5.3	13.8	34.0	63.0	-	8.3	13.2	19.8	0.0	0.0	**7.0**	0.0	0.0	0.0
Bm13.013	(3.14-) 4.25 - 4.50 - 4.76 (-6.01)	(1.57-) 1.94 - 2.06 - 2.18 (-2.95)	0.0	0.0	0.0	0.0	0.0	**2.2**	2.0	5.2	20.5	51.5	-	-	6.5	19.5	33.3	0.0	0.0	**11.0**	0.0	0.0	0.0
Pc08.002	(3.62-) 5.81 - 6.22 - 6.63 (-8.78)	(1.12-) 1.59 - 1.70 - 1.82 (-2.33)	0.0	0.0	0.0	0.0	0.0	**2.0**	1.2	1.8	9.8	29.5	68.2	-	8.0	8.0	9.0	0.0	0.0	**14.0**	0.0	0.0	0.0
Pc96.009	(3.60-) 4.69 - 4.91 - 5.13 (-6.17)	(1.45-) 1.92 - 2.05 - 2.17 (-2.76)	0.0	0.0	0.0	0.0	0.0	**1.5**	0.0	0.0	0.0	54.2	-	-	11.3	18.3	15.2	0.0	0.0	**23.0**	0.0	0.0	0.0
CBS101016	(3.31-) 4.45 - 4.71 - 4.97 (-5.85)	(1.59-) 2.04 - 2.13 - 2.23 (-2.65)	---	---	---	0.0	0.0	0.0	0.0	2.2	**8.7**	74.5	-	-	18.8	44.5	**45.2**	0.0	0.0	0.0	---	---	---

(a) - growth has reached the maximum diameter of the culture, 78.00 mm, not representing the real potential growth of the isolate

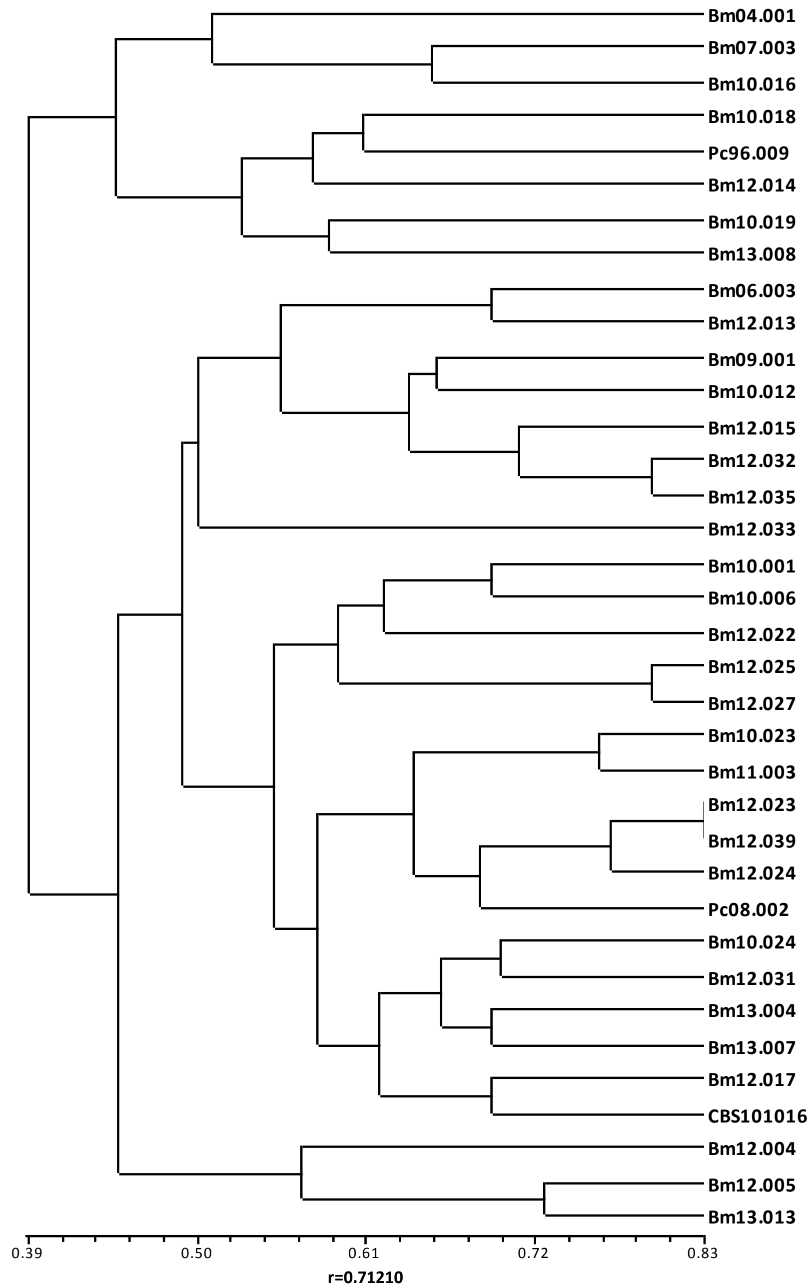

Figure 4. Dendrogram of the isolates clustering according to conidial measures and growth rates at different temperatures, performed in NTSYSpc2 using SM correlation coefficient and UPGMA. Scale bar represents percentage of similarity.

DISCUSSION

The morphological characters described for the isolates of *B. mediterranea* outlined in this study revealed a marked variability among isolates for all the studied parameters. All analyzes involved the study of mitosporic phase of the fungus that is classified, as described by other authors, in the genus *Nodulisporium* Preuss. (Collado et al. 2001; Giambra et al. 2009), nevertheless some authors suggest its classification within the *Periconiella*-like subgroup based

on the apparent apical dominance of the main axis in the branching pattern of the conidiophores (Ju et al. 1998; Jiménez et al. 2005a). The lack of diagnostic morphological characters in culture needed to identify the fungus at the species level prompted the use of molecular techniques to assess its identity.

The results presented here are in line with the work of Jiménez et al. (2005a), they described several isolates of *B. mediterranea* obtained in Spain from different locations and hosts (*Q. suber*, *Q. faginea*, *Q. rotundifolia* and *C. sativa*) and observed high variability among provenances in terms of colony morphology, growth rate and reproductive structures. A great variability was observed between isolates, in the coloration of the colonies, the distribution of pigment and evolution with incubation time. Also, the analysis of variance showed significant differences in the maximum rate of growth of the different isolates, but could not establish homogeneous groups according to their origin. The conidial dimensions study showed that the conidia developed in the hosts had variable dimension from the ones in culture; although analysis of variance demonstrated significant differences between the isolates, were unable to discriminate different groups depending on the length of conidia, but depending on its width, being significantly wider conidia produced in the branches of *Q. suber* and *Q. rotundifolia*. Giambra et al. (2009) also proved the macro/ microscopic morphological variability of *B. mediterranea* isolates obtained from *Quercus* sp. in Italy in relation to the host and season (Table 4).

Table 4. Conidial dimensions of *Biscogniauxia mediterranea* presented by several authors

Authors	Conidial dimensions [µm]
Malençon & Marion, 1952	5.6-6.5 x 3
Barbosa 1958	2.75-6.25 x 2.25-3.25
Jong & Rogers, 1972 (in Collado et al. 2001)	4-5 x 2-3 (var. *mediterraneum*)
	5-8 x 2-3 (var. *microspora*)
Collado et al. 2001	4.0-7.5 x 2-3.5
Jiménez et al. 2005a	(2.6)-4.1-(5.2) x (2.2)-2.9-(4.2) (produced in infected branches)
	(1.9)-3.9-(6.6) x (1.2)-2.1-(3.7) (produced in culture)
Giambra et al. 2009	6.13 x 3.33 (February 2006)
	4.97 x 3.34 (April 2006)
	5.64 x 2.97 (June 2006)
	5.14 x 2.87 (June 2006)
	5.64 x 3.30 (June 2006)
	5.54 x 3.61 (January 2007)
This study	(2.51)-5.08-(14.27) x (0.91)-2.07-(3.37)

The dimensions of conidia of different isolates showed variability both in length and in width. Results reported by other authors, summarized in Table 4, have confirmed this observation. The mean values of the two determined parameters fall under the dimensions presented by

other authors, however, the extreme values denote a large range of values obtained in this study, particularly for the length of conidia, whose values might correspond to outliers (Table 3 and Table 4). Jong and Rogers (1972) described two isolates of *H. mediterraneum* ascribed to different varieties, namely var. *mediterraneum* and var. *microspora*, which differ primarily in the size of ascospores but also in morphological features of the anamorph formed in culture by both strains (Collado et al. 2001), nevertheless, the range of variation of the conidial states formed by this species in culture can extend far beyond those limits, not allowing the consistent application of this classification.

B. mediterranea is considered a thermophilic fungus, presenting an optimal growth temperature of 35 ⁰C (Henriques 2007). The maximum and minimum temperatures analysis for selected isolates revealed that, in general, the isolates maintained the development between 5 and 45 ⁰C, although some present their growth in the minimum temperature of 7.5 ⁰C and others at maximum of 40 ⁰C. Vannini et al. (1996) tested the temperature range for ascospores germination and concluded that the optimal was also 35 ⁰C, but 25 and 30 ⁰C were still favorable, while 20 and 40 ⁰C reduced germination. Ascospores kept at 5 ⁰C were able to germinate after short exposure to temperatures ranging from 20 to 35 ⁰C.

This ability of *B. mediterranea* to develop in a wide range of temperatures points out its plasticity to adapt to environmental conditions, particularly in the Mediterranean basin. In this region, presently, average daily maximum temperatures in the winter months are about 15-16 °C and reach 30-31 °C in the summer but climate change scenario projections suggests that by 2100 temperatures will increase between 4-5 ⁰C (Lindner et al. 2010). The evolution of climate in temperate regions may specifically favor endophytic microflora inhabiting trees species, especially thermophilic fungi, which are able to persist in trees until the water regime is disrupted and develop rapidly causing sudden forest dieback (Desprez-Loustau et al. 2006; Lindner et al. 2010).

The clustering analysis that combined both temperature growth conditions and morphological features of the isolates of *B. mediterranea* contributed to display the existing diversity among them regardless of the parameter in question. The numerous groups of isolates formed with different grades of similarity, do not represent consistent and coherent clusters that allow discuss charcoal canker expression in different hosts or relate to its origin. This variability is in agreement with the results obtained by MSP-PCR and multigenic phylogenetic analysis applied to this same set of isolates (Henriques et al. submitted) and also with the results of other authors (Vannini et al. 1999; Schiaffino et al. 2002). The explanation of this fact may be based

on the wide spread of this fungus that is able to colonize numerous hardwood hosts; their dispersion is very efficient, occurring mainly through airborne ascospores or transported by insects (Jiménez et al. 2005b; Inácio et al. 2011; Henriques et al. 2014a); the high rate of sexual reproduction and the heterothallic mating system of this fungus that represent an essential internal source of genetic variability of the population (Vannini et al. 1999). Given the variability and adaptability of this fungus, especially in conditions that are favoring its expansion, it is crucial to develop direct and indirect measures for control of the disease.

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4. General discussion

The fungus *Biscogniauxia mediterranea* is known in Portugal since 1930 (Câmara 1930) as responsible for a disease in *Quercus suber*, the charcoal canker, though it inhabits in other hardwood hosts and has a worldwide distribution. *B. mediterranea* has an endophytic behavior, colonizing the healthy tissues of the hosts without expressing any symptoms. Whenever infected trees are subjected to stress that triggers its weakening, the fungus finds conditions to enhance its development, acting as a secondary pathogen integrated in the complex process of cork oak decline (Vannini et al. 2009). The evident symptom of this disease is the formation of carbonaceous stroma under the fractured cork, which is very common in the branches of elder declined trees or in dead branches lying on the ground (Santos 2003). However, in the last years, atypical symptoms have been reported, mainly on young cork oak trees in which the leaves dry and a brown powdery mass emerges in the trunks. Sousa et al. (2007) suggested that some new factors could be involved in the disease such as a species close to *B. mediterranea* or new infraspecific varieties. Therefore, the main aim of this thesis was to clarify the pathogenic agent(s) involved in charcoal canker in cork oak, in particular in those new outbreaks, ascertaining the fungus diversity available in the nature. Also, it was essential to understand the mechanisms of this wide disease spread in the stands and its prospective of evolution.

To begin with, the brown powdery mass present in the dying young cork oak trees was characterized and identified as a species of *Nodulisporium* sp., the mitosporic stage of *B. mediterranea* (Henriques et al. 2012). This form of the fungus had already been described in *Quercus* spp. in Spain (Jiménez et al. 2005a) and Italy (Linaldeddu et al. 2005a). According to Jiménez et al. (2005a) the fungus develops under the outer bark of the host, leading to visible swellings on thin branches due to the accumulation of large amounts of conidia, the bark then cracks exposing the dry conidia mass of powdery aspect. The Spanish observations also revealed that, in natural conditions, the anamorphic stage appears first, preceding perithecial development. In Portugal, this stage is not usually observed in the stands in mature declining trees affected with charcoal canker, possibly for being a temporary phase of short duration preceding the formation of the carbonaceous stroma or, as also states Jiménez et al. (2005a), it occur in thick branches where conidia remain under the outer bark not getting uncovered.

To understand the way of contamination of those cork oaks by *B. mediterranea* in the stands several assays were performed to access the possibility of direct transmission of the fungus through colonized tissues of a tree to its progeny, as well as the evaluation of airborne dispersion of ascospores in natural conditions. It was demonstrated that no transmission of the fungus occurs through the seed. The fungus may be present at the level of outer layers of

the acorns both from asymptomatic and healthy trees, however, was only detected internally in the cotyledons of acorns of declining trees. Anyway, in neither situation the fungus was detected in seedlings germinated from acorns of the same analyzed batches (Henriques et al. 2014a).

Seed transmission of endophytic fungi is well known in the case of clavicipitaceous endophytes and their grass hosts (Scharld et al. 2004), but in tree hosts it is poorly studied. According to Saikkonen (2007), in woody plants, its size, complex architecture and long age of sexual maturity would be constrains for systemic growth of the fungi and its vertical transmission, nevertheless, most of those studies are based on observations of foliar endophytes with a different behavior from *B. mediterranea* that is able to colonize all aerial organs of the hosts (Franceschini et al. 2002), presenting a systemic growth through the woody tissues, particularly heightened in hosts under stress (Vannini & Valentini 1994, Linaldeddu et al. 2011). In fact it was observed that the fungus could grow even in the acorn but does not have the ability to pass to the newly plant. Those results were essential because, given the observations of high mortality of cork oaks in new plantations, was necessary to ensure that nurseries would not be a source of contamination of charcoal canker. It was also eliminated the hypothesis of fungus transmission by stump sprouts of dead trees infected by the fungus, which might be justified by the absence of the fungus at the roots (Franceschini et al. 2002). It was then clarified that this frequent practice in the stands does not constitute an increased risk for the development of disease (Henriques et al. 2014a).

Not occurring fungus transmission directly between hosts tissues, the airborne dispersal takes the lead role in the spreading of the disease in stands and over long distances, despite the transport of the fungus by insects (Vannini et al. 1996a, Martín et al. 2005, Inácio et al. 2011) and through the trees management tools (Oliver 1988). In the field, all aerial organs of the plants are exposed to fungi inocula that are transported through the air. In this study, it was shown that plants can get infected by *B. mediterranea* from an early age, as the fungi was isolated from asymptomatic seedlings less than 3 years old, moreover (Henriques et al. 2014a), the density and diversity of endophytic fungi accumulate as plants ages (Zabalgogeazcoa 2008).

It was confirmed that *B. mediterranea* ascospores spread as airborne inoculum in natural conditions varying throughout the year and ceasing almost completely during the warm and dry season. Precipitation is the main factor for ascospores release being positively related with it. A period of three consecutive days with precipitation above 0.5 mm is determinant for a

significant increase in ascospores discharge. With relative humidity wasn´t found a significant correlation with ascospores discharge although it revealed a positive tendency (Henriques et al. 2014a). Other authors confirmed this relation of precipitation and relative humidity with ascospores release, even though their investigations had not elapsed under natural conditions (Vannini et al. 1996b, Jiménez et al. 2005b). These results were therefore expectable as the consecutive days with precipitation ensure the necessary humidity for the ejection of ascospores and to prevent their desiccation until the accommodation in the host and germination. The temperature might influence also ascospores dispersion being negatively related with it, which is in agreement with Jiménez et al. (2005b) results. The wind velocity is important as well since it is the vehicle for the dispersal of ascospores. In fact, Jiménez et al. (2005b) found a divergence between the hours with precipitation and the hours with spores captures in trap, showing that although precipitation is needed to cause the discharge of ascospores, the primary vehicle for dispersion of ascospores is the wind, not water.

The analysis of these results from a practical perspective highlights the importance of reducing the inoculum available in stands, by pruning and felling infected branches or trees and removing the remains out of the stand. It is also important to avoid the execution of forestry practices in the most favorable meteorological periods for dispersal of ascospores.

Another major component of this work consists in comparing *B. mediterranea* isolates from different sources in the perspective of evaluating the variable expressions of the fungus in the host. The identification of all the studied isolates was confirmed with specific primers according to Mazzaglia et al. (2001). *B. mediterranea* isolates obtained from young cork oak trees, isolates from older declined trees with the carbonaceous stroma and from asymptomatic tissues, covering the cork oak distribution in Portugal and the Mediterranean basin, along with isolates from other host species and a reference culture from CBS collection were compared by molecular, morphological and physiological approaches in order to search for differences at any level that could be related with the disease expression. MSP-PCR profiles of the whole set of isolates revealed an extremely high variability among isolates that successively clustered in groups with increasing similarity rate but without any perceptible coherence related with isolate source. Only the reference isolate that was obtained from *Q. robur* from the north of Europe was consistently apart from the rest of the group (Henriques et al. submitted). Also the combined clustering analysis of cultural characteristics of the isolates and growth rates at 25 ^{0}C in PDYA did not allow consistent grouping of isolates according to the study variables (Henriques et al. unpublished). Although the configuration of the

dendrogram obtained from the two approaches presented a different relation among the isolates, they corroborate the high level of variability among isolates of *B. mediterranea*.

Once the general approaches for the analyses of the collection didn't allow their coherent clustering in order to select isolates for detailed study, 36 representative isolates of *B. mediterranea* were chosen according to the host species, geographic location, age and host symptoms, along with the reference isolate. This group of isolates was submitted to phylogenetic sequence analysis for the ITS-rDNA region, protein coding genes combined analysis (TEF, TUB1 and TUB2), morphological characterization of the cultures, conidial dimensions and growth rates at different extreme temperatures. Once more, all the results indicated a high level of variability among isolates and didn't provide any relation with the origin of the isolates (Henriques et al submitted; unpublished). Morphological and cultural observations although variable were in agreement with other authors descriptions of *B. mediterranea* but not enabled to define the isolates varieties as its biometric characteristics ranged and overlapped the described values (Ju et al. 1998; Collado et al. 2001; Jiménez et al. 2005a; Giambra et al. 2009; Henriques et al. unpublished).

With the purpose of understanding all that genetic diversity, a restricted group of monoascosporic isolates of *B. mediterranea* obtained from individual stromata from host trees at distinct locations were also analyzed using the method MSP-PCR. A high genetic variability was detected within those populations, also expressed in the morphological variability of the cultures. Considering separately each stromata, the observations are identical, leading to the conclusion that even a single stroma in a host tree constitutes an extremely high source of variability of *B. mediterranea* in the nature (Henriques et al. 2014b). Schiaffino et al. (2002) using RAPD obtained concordant results in the assessment of the variability of this species in a restricted area of Sardinia as well as among isolates from different localities in the island and Spain. This variability is partially justified by the high rate of sexual reproduction and the heterothallic mating system of *B. mediterranea* that represents an important internal source of genetic variability of the population (Vannini et al. 1999). Fertile perithecia are largely produced in natural conditions suggesting that, during the pathogenic phase, characterized by mass colonization of woody and bark tissues, opposite mating types must meet and outcross. Moreover, the presence of a high number of different genotypes on the same host can occur, due to multiple infections at different times, even when the fungus is living as endophyte (Vannini et al. 1999). The fact that ascospores constitute the most important spread and inoculum units, its abundance and ample range of dispersal conditions also contribute to the existence of vast variability. The phylogenetic analysis, particularly for the ITS region, has held

to clarify that all the ascertained variability is indeed intraspecific, as all *B. mediterranea* isolates were clustered in a separate group of the closer species *B. atropunctata* and *B. nummularia* (Hsieh et al. 2005; Peláez et al. 2008).

Another interesting conclusion drawn from ITS-rDNA phylogenetic analysis of all sequences obtained in this work along with the available sequences in GenBank until date was the position of all the sequences obtained from isolates from the Mediterranean region in a separate cluster in relation to the isolates from North America and the rest of Europe (France - Pyrenees and the Netherlands) (Henriques et al. submitted). In the Mediterranean, biological diversity (understood as the sum of the diversity of genes, species and ecosystems) in forests, *garrigues* and *maquis* has, compared to other ecoregions, a number of singular features in terms of high species number and endemism rate, and extraordinary genetic diversity both between and within populations (Fady-Welterlen 2005). This biological richness results from evolutionary processes acting in the context of a distinctive geographical and topographical diversity, with high mountain ranges, peninsulas, and one of the largest archipelagos in the world (the Mediterranean sea, including several hundred islands) resulting in a wide range of local climates; it is also related to paleo-history and anthropogenic influences such as land uses and creation of mosaic-like landscapes (Palahi et al. 2008). However, the Mediterranean region has a genuine warm/dry summer climate type in a transitional zone between two markedly different zones (the humid/cold temperate zone and the hot desert zone) and, consequently, is highly sensitive to environmental changes. Indeed, shifts in the climate band of only few degrees of latitude towards the north or south may result in dramatic changes, such as desertification in areas which previously had a humid climate (Regato 2008). Drought is a natural phenomenon in the Mediterranean region, mainly due to its irregular climate with the periodic extension of the typical lower summer precipitation over longer periods. However, drought frequency and intensity have increased in recent decades and it is projected that they will become even worse in the light of global climate change, rising temperatures and decreasing precipitation (Lindner et al. 2010).

Facing these perspectives, the impact of charcoal disease in *Q. suber* forests will be favored along the Mediterranean basin. Periods of drought greatly increase susceptibility of trees to stress-induced pathogens, the abundance of *B. mediterranea* in tissues and its ability to shift from the endophytic to the pathogenic phase which is mediated by water stress conditions of the host (Desprez-Loustau et al. 2006). Being a thermophilic fungus that tolerates a wide range of temperatures, from 5 to 45 ^{o}C, and with the capacity to develop under very low water

potential (Vannini et al. 1996b), the whole pathosystem favors the disease: the host tree became more debilitated and the fungus finds suitable conditions for its development.

5. CONCLUDING REMARKS

The aim of this work was to study the actual situation of charcoal canker on cork oak, which is becoming more frequent in Portuguese stands, particularly aggravated in young trees exhibiting atypical symptoms. This disease is caused by the fungus *Biscogniauxia mediterranea* that has an endophytic behavior in a wide range of hardwood hosts, becoming pathogenic under stress situations, namely under drought conditions. It used to develop its carbonaceous stroma only in mature trees already suffering a decline process being considered a secondary pathogen but these recent observations lead to question even the etiology of the disease, raising the possibility that other related species or infraspecific taxa could be involved.

With this purpose, several work lines were undertaken. The atypical, brown powdery mass that develops on young cork oak trees was analyzed and described as the mitosporic stage of *B. mediterranea* being established the unequivocal relationship of these symptoms with the disease. Different ways of infection of the trees were ascertained through seeds transmission and use of stump sprouts of contaminated trees to grow new trees. It was concluded that the vertical infection of new hosts, due to systemic fungal growth in the hosts' tissues, doesn't occur. On the other hand, airborne transmission of the fungus in the stands natural conditions was accessed, confirming that ascospores are the main inocula that are spread through the year according to meteorological conditions: significant ascospores discharge occurs after a period of three consecutive days with precipitation above 0.5 mm and in mild temperatures, the wind velocity is associated with the effective ascospores transport. This kind of dissemination constitutes an indiscriminate source of inocula in the stand, infecting all aerial organs of trees of all ages and conditions. Also insect mediated contamination and manmade operations both in forestry management and global plant and woody products trades contribute for the dispersion of *B. mediterranea* at the stand and wide world levels.

The diversity among *B. mediterranea* isolates associated to *Quercus suber* with different ages and disease expression, collected in all the producer countries of the Mediterranean basin and from other hardwood hosts species was analyzed by molecular, morphological and physiological approaches. All results were consistent towards the evidence that all of the isolates obtained in different situations belong to the species *B. mediterranea*, although an extremely high intraspecific variability was revealed, not being possible to relate any cluster disposition of the isolates with their sources. Even the comparison of isolates obtained from single stromata in individual hosts showed such a variability that didn't allow identification of the isolates from the same provenance. The heterothallic nature of the fungus and its high rate of sexual reproduction contribute to this high intraspecific variability that provides genetic flexibility for long-term survival and adaptation to the environment.

Considering the worldwide distribution of *B. mediterranea* is verified that the population of the fungus existing in the Mediterranean region is distinct from other locations such as North America and the rest of Europe. Probably having evolved towards a better adaptation in this region, the effects of this fungus on hosts are more aggravated in this region, in particular in *Q. suber* and *Q. cerris*, in Portugal and Italy, respectively. In fact, the Mediterranean climate is suitable for *B. mediterranea* growth and the predicted climate change scenarios for the coming years will even favor its development, once this fungus survives in a wide range of growth temperatures, between 5 and 45 °C, and takes profit of low water availability.

Overall, all results obtained in this work points out for an aggravation of charcoal canker in cork oak, which we are already witnessing. Further work is necessary to better understand this disease, namely the mechanisms that trigger the transition of the fungus from the latent phase to become pathogenic to the host. Also, the development of effective means of control for this disease is urgently needed. The use of biological tools such as antagonistic fungi, e.g. *Trichoderma* spp., is a possibility that must be clarified and developed in a practical sense. The fragile state of the *montados* and all its ecological and socio-economic importance deserve all our investment.

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