SCAP
SOCIEDADE DE CIÊNCIAS AGRÁRIAS DE PORTUGAL

Morphological and genetic diversity of *Biscogniauxia mediterranea* associated to *Quercus suber* in the Mediterranean Basin

Diversidade morfológica e genética de *Biscogniauxia mediterranea* associada a *Quercus suber* na bacia mediterrânica

Joana Henriques[1*], Filomena Nóbrega[1], Edmundo Sousa[1] and Arlindo Lima[2]

[1] *Instituto Nacional de Investigação Agrária e Veterinária, I.P. – Unidade Estratégica de Sistemas Agrários e Florestais e Sanidade Vegetal, Quinta do Marquês, 2780-159 Oeiras, Portugal. E-mail: *joana.henriques@iniav.pt, author for correspondence.*
[2] *Centro de Engenharia dos Biossistemas, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal*

Received/Recebido: 2015.03.05
Accepted/Aceite: 2015.05.04

ABSTRACT

Biscogniauxia mediterranea is a widespread fungus that causes charcoal disease on cork oak and other hardwood hosts. It had been considered a secondary pathogen causing the disease only in stressed hosts. However, its frequency and severity have been increasing, inclusive in young trees without other decline signs and developing atypical symptoms. The present work aims to assess the fungus' variability in the Mediterranean basin, following cork oak geographical distribution. A collection of 36 isolates originated from cork oak in Portugal and other Mediterranean countries, from other hosts and from trees with different ages and disease expression were analyzed by cultural characteristics, conidial dimensions, and growth rates at different temperatures and by microsatellite-primed PCR profiles. Clustering UPGMA analyses combining different parameters were preformed. All the approaches revealed high level of intraspecific polymorphism among Mediterranean isolates, not allowing relating the disease development with any analyzed features. The results highlighted the variability of this fungus that induces its adaptation ability in the present worrying scenario of climatic change. All the conditions are gathered to favor the aggravation of the disease in cork oak stands.

Keywords: charcoal canker, cork oak, intraspecific variability, *Nodulisporium* sp

RESUMO

Biscogniauxia mediterranea é o fungo responsável pela doença do carvão do entrecasco em sobreiro e outros hospedeiros lenhosos. Tem sido considerado um patogénio secundário que causa doença apenas em hospedeiros debilitados. No entanto, a sua frequência e nocividade têm vindo a aumentar, inclusive em árvores jovens sem outros sinais de declínio e desenvolvendo sintomas atípicos. Este trabalho tem como objetivo avaliar a variabilidade do fungo na bacia mediterrânica, seguindo a distribuição geográfica sobreiro. Uma coleção de 36 isolados provenientes do sobreiro em Portugal e noutros países do Mediterrâneo, de outros hospedeiros e de árvores com diferentes idades e expressão da doença foram analisadas de acordo com as suas características culturais, dimensões de conídios, taxas de crescimento em diferentes temperaturas e por perfis de *microsatellite-primed* PCR. Foram realizadas análises de clusters UPGMA com diferentes parâmetros. Todas as abordagens revelaram elevado grau de polimorfismo intraespecífico entre os isolados, não permitindo relacionar o desenvolvimento da doença com as características analisadas. Estes resultados evidenciaram a variabilidade do fungo que induz a sua capacidade de adaptação no atual e preocupante cenário de alterações climáticas. Todas as condições estão reunidas para favorecer o agravamento da doença no montado.

Palavras-chave: carvão do entrecasco, *Nodulisporium* sp., sobreiro, variabilidade intraespecífica

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, 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 in 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). 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 molecular, morphological and cultural characters, attempting to relate it with the atypical development of the disease.

Material and Methods

Isolates collection and culture

A collection of isolates of *B. mediterranea* from *Q. suber* exhibiting signs of charcoal canker were obtained following the geographical distribution of cork oak in Portugal, more intensely in the main production areas, and including young undecorked trees. Samples from the same host were also obtained in other countries of the Mediterranean basin (Spain, continental France and Corse, Italy – Sardinia, Morocco, Algeria and Tunisia), from the insect *Platypus cylindrus* Fab. associated to cork oak and from other hosts in Portugal: *Quercus faginea* Lam., *Quercus robur* L., *Quercus rotundifolia* Lam., *Castanea sativa* Mill. and *Eucaliptus globolus* Labill.. As a reference, a isolate of *B. mediterranea* from *Q. robur*, Netherlands (CBS101016), was used. A total of 36 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, from symptomless branches and from insects' body. Samples were removed with a sterile scalpel, 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) and incubated at 25±1 °C in darkness. Cultures were then transferred to water agar medium and established pure hyphal tip cultures. Voucher specimens were deposited in the fungal collection at Micoteca of Estação Agronómica Nacional (MEAN), INIAV (Oeiras, Portugal).

The identity of all *B. mediterranea* isolates was confirmed by PCR amplification with specific primers MED1/ MED2 according to 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. Thermal cycling was performed on a Tgradient Thermocycler (Biometra, Germany). 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).

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

Isolate[a]	Year	Host	Source	Location	Collected/ Isolated by
Bm04.001	2004	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Montemor-o-Novo	M.L. Inácio
Bm06.003	2006	*Quercus suber*	adult tree with carbonaceous stroma	Morocco, Mamora	M.L. Inácio
Bm07.003	2007	*Quercus suber*	young undecorked tree with brown powdery mass	Portugal, Grândola	M.L. Inácio
Bm09.001	2009	*Quercus suber*	adult tree with carbonaceous stroma	Tunisia, Kroufa	J. Henriques
Bm10.001	2010	*Quercus suber*	young undecorked tree with brown powdery mass	Portugal, Montemor-o-Novo	J. Henriques
Bm10.006	2010	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Alenquer	J. Henriques
Bm10.012	2010	*Quercus rotundifolia*	adult tree with carbonaceous stroma	Portugal, Serpa	J. Henriques
Bm10.016	2010	*Quercus suber*	adult tree with carbonaceous stroma	Italy, Sardinia	J. Henriques
Bm10.018	2010	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Chamusca	J. Henriques
Bm10.019	2010	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Grândola	J. Henriques
Bm10.023	2010	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Silves	J. Rosendo/ J. Henriques
Bm10.024	2010	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Grândola	J. Henriques
Bm11.003	2011	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Grândola	J. Henriques
Bm12.004	2012	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Mirandela	L. Martins/ J. Henriques
Bm12.005	2012	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Coruche	APFC/ J. Henriques
Bm12.013	2012	*Quercus suber*	young undecorked tree with brown powdery mass	Portugal, Coruche	APFC/ J. Henriques
Bm12.014	2012	*Quercus suber*	young undecorked tree with carbonaceous stroma	Portugal, Grândola	J. Henriques
Bm12.015	2009	*Quercus suber*	adult tree symptomless branch	Tunisia, Kroufa	M. Z. Boutiti
Bm12.017	2012	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Grândola	J. Henriques
Bm12.022	2012	*Quercus suber*	young undecorked tree with carbonaceous stroma	Portugal, Tróia	P. Naves/ J. Henriques
Bm12.023	2012	*Eucalyptus globolus*	adult tree with carbonaceous stroma	Portugal, Odemira	H. Bragança & E. Diogo/ J. Henriques
Bm12.024	2012	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Terras de Bouro	J. Henriques
Bm12.025	2012	*Quercus suber*	young undecorked tree with brown powdery mass	Portugal, Terras de Bouro	J. Henriques
Bm12.027	2012	*Quercus robur*	adult tree with carbonaceous stroma	Portugal, Terras de Bouro	J. Henriques
Bm12.031	2012	*Castanea sativa*	adult tree with carbonaceous stroma	Portugal, Terras de Bouro	J. Henriques
Bm12.032	2012	*Quercus suber*	adult tree with carbonaceous stroma	Portugal, Mafra	J. Henriques
Bm12.033	2012	*Quercus faginea*	adult tree with carbonaceous stroma	Portugal, Mafra	J. Henriques

Isolate[a]	Year	Host	Source	Location	Collected/ Isolated by
Bm12.035	2012	*Castanea sativa*	adult tree with carbonaceous stroma	Portugal, Mafra	J. Henriques
Bm12.039	2012	*Quercus suber*	adult tree with carbonaceous stroma	France, Vivès	R. Piazzeta/ J. Henriques
Bm13.004	2013	*Quercus suber*	adult tree with carbonaceous stroma	France, Corse du Sud	H. Baudriller-Cacaud/ J. Henriques
Bm13.007	2013	*Quercus suber*	adult tree with carbonaceous stroma	Algeria, Anaba	A. Mounia/ J. Henriques
Bm13.008	2013	*Quercus suber*	adult tree with carbonaceous stroma	Algeria, Tlemcen	L. Belhoucine/ J. Henriques
Bm13.013	2013	*Quercus suber*	adult tree with carbonaceous stroma	Spain, Cordoba	A. Soto Sánchez/ J. Henriques
Pc96.009	1996	*Quercus suber*	*Platypus cylindrus*	Portugal, Coruche	E. Sousa
Pc08.002	2008	*Quercus suber*	*Platypus cylindrus*	Portugal, Montemor-o-Novo	M.L. Inácio & J. Henriques
CBS101016	1998	*Quercus robur*	dead branch	Netherlands, Bremmert-Kootwijk	

[a]Bm, Pc: work collections; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
[Edmundo Sousa, Eugénio Diogo, Helena Bragança, Maria de 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 – Dep.Conservação da Natureza e Florestas do Algarve, Olhão, Portugal; Latifa Belhoucine - Laboratory 31: Conservation Management of water, soil and forests - University of Tlemcen, Algeria; 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; Renaud Piazzeta - Institut Mediterranéen du Liège, Vivès, France.]

Morphological and growth temperature characterization

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 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 and the ProgResSpeed XT core 5 – Jenoptik image software. 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.

Minimum and maximum growth temperatures were determined after a preliminary essay 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 ^{0}C and 37.5 ^{0}C, respectively, at five-degree intervals (7.5 ^{0}C, 5 ^{0}C, 0 ^{0}C; 37.5 ^{0}C, 40 ^{0}C, 45 ^{0}C, 50 ^{0}C) until the cessation of the growth. 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).

MSP-PCR

MSP-PCR fingerprinting profiles were generated following the protocol of Uddin & Stevenson (1997) using the primers $(CAG)_5$, $(GACA)_4$, $(GTG)_5$, $(ACAC)_5$ or phage M13 core sequence (GAGGGTGGNGGNTCT). Thermal cycling was performed on a Tgradient Thermocycler. 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 an agarose gel as described above. 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 on the presence or absence of the band. Only reproducible bands were considered for analysis. Bands common to all isolates were excluded from the analysis. The isolates were clustered on the basis of their profiles in a consensus dendrogram built with NTSYSpc2 using DICE coefficient and UPGMA.

Results and Discussion

All the collected isolates were confirmed as *B. mediterranea* species by the amplification with the specific primers MED1/ MED2 (Mazzaglia *et al*., 2001) that generated, as expected, only one PCR product of approximately 380 bp. All analyzes were performed in the mitosporic phase of the fungus that is classified 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 isolates were characterized according to the main cultural features grown in PDYA at 25±1 °C, showing high variability in all observed parameters, and 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 with mycelial strands, density media to high. Colors differ from white, buff to vinaceous buff, grayish sepia to smoke grey, or buff margin with olivaceous center. In some colonies dark brown exudates are frequent. The reverse of the colonies varies from buff to buff margin with umber to olivaceous center, sienna with darker spots, sienna to fuscous black, fuscous black to dark mouse grey or sulphur yellow margin and greenish black center, with strong diffusible pigment. The growth of the isolates measured at the third and seventh day of culture was also variable: the isolates grew with different rates, at the third day of growth some isolates had reached a range of dimensions from 28 mm to the maximum diameter of the culture. At the seventh day of growth most isolates have reached the maximum diameter of the culture, not allowing the determination of its actual growth rate capacity.

Microscopic observation of the isolates of *B. mediterranea* allowed the characterization of the conidiogenous structures. In general 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, with an average of (2.51)-5.08-(14.27) x (0.91)-2.07-(3.37) μm. No correlation was found between length and width of conidia (r=-0.03, N=930). 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). Five isolates did not produce asexual reproductive structures. Results of conidial dimensions reported by other authors, summarized in Table 2, confirmed these observations. 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. 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.

Table 2 - 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	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)

For most isolates the minimum growth temperature was at 5 ºC except for Bm12.015, Bm12.033 and CBS101016 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, Bm10.016, Bm10.018, Bm12.032, Bm12.033, Bm12.035 and CBS101016. All selected isolates from the region of Mafra (Portugal) presented a similar behavior with regard to this parameter, for the other isolates there was no pattern related to its origin (geographical, host or syntomatologycal). *B. mediterranea* is considered a thermophilic fungus, presenting an optimal growth temperature of 35 ºC (Henriques, 2007). Vannini *et al.* (1996) tested the temperature range for ascospores germination and concluded that the optimal was also 35 ºC, but 25 and 30 ºC were still favorable, while 20 and 40 ºC reduced germination. Ascospores kept at 5 ºC were able to germinate after short exposure to temperatures ranging from 20 to 35 ºC. 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 ºC (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). Other biotic or abiotic factors that might respond to climate change also might contribute to stress the host tree thus increasing its susceptibility to fungi development.

Individual descriptions of culture and conidial morphology and growth rates at different temperatures are detailed in Henriques (2014). The combined clustering analysis of these parameters generated the dendrogram shown in Figure 1A. All isolates present 39 % of similarity, subdivided into groups of increasing resemblance to a maximum of 83 % of similarity, however, 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.

The MSP-PCR analysis assessed genetic diversity within the collection 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 70 different band positions. A consensus dendrogram using UPGMA analysis was obtained from combined analysis of the profiles generated with the five primers for the complete set of isolates (Figure 1B). The cophenetic correlation coefficient of this UPGMA analysis was 0.86, indicating the dendrogram was an excellent fit representation of the original data. All the isolates present a similarity of 42 %. However, this combined analysis did not allow a coherent clustering of the 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, that 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. 60 %.

The clustering analysis of morpho-physiological features and MSP-PCR of the isolates of *B. mediterranea* contributed to display the existing diversity among them. In both analysis, the numerous groups of isolates formed with different grades of similarity are not equivalents and do not represent coherent clusters that allow relating them with charcoal canker expression, different host species or geographical origin. The results presented here are in line with the work of Jiménez *et al.* (2005a), who described several isolates of *B. mediterranea* obtained in Spain from different locations and hosts, observing high variability among provenances in colony morphology, growth rate and reproductive structures, but couldn't establish homogeneous groups according to their origin. Also Vannini *et al.* (1999) and Schiaffino *et al.* (2002) assessed the high variability of this species using other molecular markers. The explanation of this intraspecific variability may be based on the wide spread of *B. mediterranea* that is able to colonize numerous hardwood species and disperse efficiently, 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), and even within the same host (Henriques *et al.,* 2014b).

Conclusions

The morphological and molecular analysis of *B. mediterranea* revealed a high variability among isolates, not allowing relating them with charcoal canker symptoms, host or geographic origin. Although the clustering arrangement is not coincident, both approaches emphasize the intraspecific diversity that provides the fungus with genetic flexibility for long-term survival and adaptation to the environment. In fact, the Mediterranean climate is suitable for *B. mediterranea* growth and the predicted climate change scenarios even favor its development and the aggravation of charcoal canker on *Q. suber*.

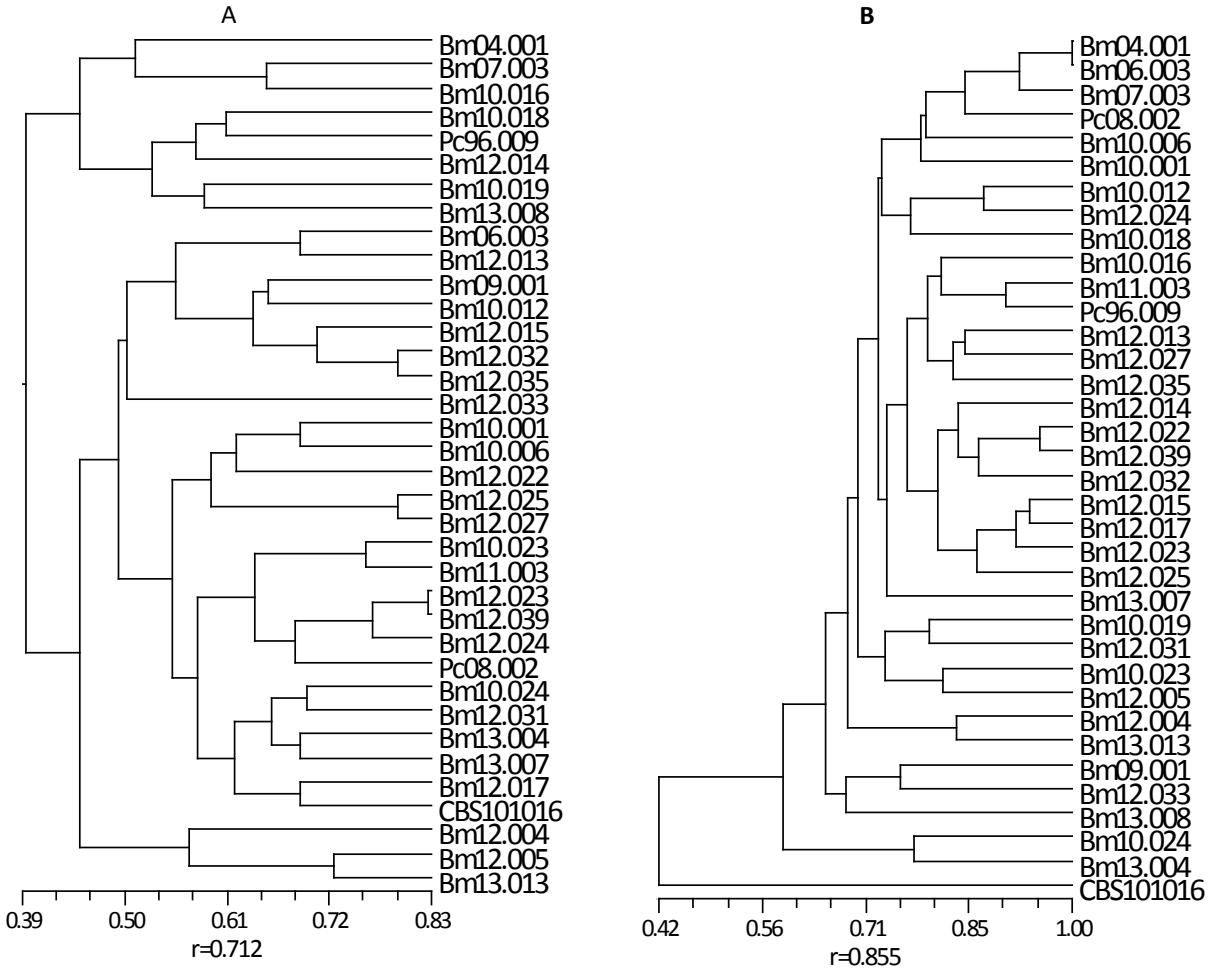

Figure 1 - A - Dendrogram of *Biscogniauxia mediterranea* isolates clustering according to cultural morphology, conidial measures and growth rates at different temperatures, performed in NTSYSpc2 using SM correlation coefficient and UPGMA. Scale bar represents percentage of similarity; **B** - MSP-PCR analysis of *B. mediterranea* 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.

Acknowledgements

Authors are grateful to everyone who contributed to the sample collection, to Eng. Filomena Caetano, Doctor Paula Ramos and Doctor Maria de Lurdes Inácio for the participation on cultural characterization of the fungal isolates and to Doctor Luís Bonifácio for the help in statistical analysis. This research was partially supported by Fundação para a Ciência e a Tecnologia, Portugal (grant number SFRH/BD/46787/2008).

References

Assali, F. and Falki, K. (2006) - Aperçu sur l'état de vitalité des subéraies et des peuplements de chêne vert au Maroc. *In*: *Seminar "The vitality of cork oak and holm oak stands – current situation, state of knowledge and actions to take*". 25-26 Octobre 2006, Évora, Portugal. 12 pp.

Bouhraoua, R.T.; Villemant, C.; Khelil, M.A. and Bouchaour, S. (2002) - Situation sanitaire de quelques subéraies de l`Ouest algérien: impact dês xylophages. *IOBC/wprs Bulletin*, vol. 25, p. 85-92.

Câmara, MS. (1930) - Contributions ad Mycoflorum Lusitaniae. Centuriae VIII et IX. *Anais de Instituto Superior de Agronomia*, vol. 3, 84 p.

Collado, J.; Platas, G. and Peláez, F. (2001) - Identification of an endophytic *Nodulisporium* sp. from

Quercus ilex in central Spain as the anamorph of *Biscogniauxia mediterranea* by rDNA sequence analysis and effect of different ecological factors on distribution of the fungus. *Mycologia*, vol. 93, n. 5, p. 875-886.

Desprez-Loustau, M.; Marçais, B.; Nageleisen, L.M.; Piou, D. and Vannini, A. (2006) - Interactive effects of drought and pathogens in forest trees. *Annals of Forest Science*, vol. 63, n. 6, p. 597-612.

Franceschini, A.; Linaldeddu, B.T. and Marras, F. (2005) - Présence et répartition des champignons endophytes dans les subéraies de Sardaigne (Italie). *IOBC/wprs Bulletin*, vol. 28, p. 67-74.

Giambra, S.; Torta, L.; Scopel, C.; Causin, R. and Burruano, S. (2009) - Primi studi su *Biscogniauxia mediterranea* in Sicilia Occidentale. *In: Atti del Terzo Congresso Nazionale di Selvicoltura.* Taormina (ME), Accademia Italiana di Scienze Forestali, Firenzi, Italy. p. 1394-1396.

Henriques, J. (2007) - *Fungos associados a Platypus cylindrus Fab. (Coleoptera: Platypodidae) e sua relação com o declínio do sobreiro em Portugal*. Dissertação de Mestrado em Biologia de Pragas e Doenças de Plantas, Universidade de Évora, Évora, 118 p.

Henriques, J. (2014) – *Charcoal canker (Biscogniauxia mediterranea) in cork oak decline in Portugal.* Tese apresentada para obtenção de grau de Doutor em Engenharia Florestal e dos Recursos Naturais, Instituto Superior de Agronomia, Universidade de Lisboa, 124 p.

Henriques, J.; Barrento, M.J.; Bonifácio, L.; Gomes, A.A.; Lima, A. and Sousa, E. (2014a) - Factors affecting the dispersion of *Biscogniauxia mediterranea* in Portuguese cork oak stands. *Silva Lusitana*, vol. 22, n. 1, p. 83-97.

Henriques, J.; Inácio, M.L.; Lima, A. and Sousa, E. (2012) - New outbreaks of charcoal canker on young cork oak trees in Portugal. *IOBC/wprs Bulletin*, vol. 76, p. 85-88.

Henriques, J.; Nóbrega, F.; Sousa, E. and Lima, A. (2014b) - Diversity of *Biscogniauxia mediterranea* within single stromata on cork oak. *Journal of Mycology*, article ID 324349, 5p.

Inácio, M.L.; Henriques, J.; Guerra-Guimarães, L.; Gil-Azinheira, H.; Lima, A. and Sousa, E. (2011) - *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) transports *Biscogniauxia mediterranea*, agent of cork oak charcoal canker. *Boletín de Sanidad Vegetal. Plagas*, vol. 37, n. 2, p. 181-186.

Jiménez, J.J.; Sánchez, M.E. and Trapero, A. (2005a) - El chancro carbonoso de *Quercus* I: distribución y caracterización del agente causal. *Boletín de Sanidad Vegetal. Plagas*, vol. 31, n. 4, p. 549-562.

Jiménez, J.J.; Sánchez, M.E. and Trapero, A. (2005b) - El chancro carbonoso de *Quercus* III: Dispersión de ascosporas del agente causal. *Boletín de Sanidad Vegetal. Plagas*, vol. 31, n. 4, p. 577-585.

Jong, S.C. and Rogers, J.D. (1972) - Illustrations and descriptions of conidial states of some *Hypohylon* species. Washington Agricultural Experiment Station. *Technical Bulletin*, vol. 71, p. 1-51.

Ju, Y.-M.; Rogers, J.D.; Martin, F.S. and Granmo, A. (1998) - The genus *Biscogniauxia*. *Mycotaxon*, vol. 66, p. 1-98.

Khouja, M.L.; Ben Jamaa, M.L.; Franceschini, A.; Khaldi, A.; Nouri, N.; Sellemi, H. and Hamrouni, L. (2010) - Observations on tree decline of different cork oak (*Quercus suber* L.) provenances in the experimental site of Tebaba, North-western Tunisia. *IOBC/wprs Bulletin*, vol.57, p. 53-59.

Lindner, M.; Maroschek, M.; Netherer, S.; Kremer, A.; Barbati, A.; Garcia-Gonzalo, J.; Seidl, R.; Delzon, S.; Corona, P.; Kolström, M.; Lexter, M.J. and Marchetti, M. (2010) - Climate change impacts, adaptative capacity, and vulnerability of European forest ecosystems. *Forest Ecology and Management*, vol. 259, n. 4, p. 698-709.

Mazzaglia, A.; Anselmi, N.; Gasbarri, A. and Vannini, A. (2001) - Development of Polymerase Chain Reaction (PCR) assay for the specific detection of *Biscogniauxia mediterranea* living as an endophyte in oak tissues. *Mycological Research*, vol.105, n.8, p. 952-956.

Rayner, R.W. (1970) - *A Mycological Colour Chart*. British Mycological Society and CAB International Mycological Institute, Kew.

Santos, M.N.S. (2003) - Contribuição para o conhecimento das relações *Quercus suber - Biscogniauxia mediterranea* (syn. *Hypoxylon mediterraneum*). *Silva Lusitana*, vol. 11, n. 1, p. 21-29.

Schiaffino, A.; Francheschini, A.; Maddau, L. and Serra, S. (2002) - Molecular characterization of *Biscogniauxia mediterranea* (De Not.) strains isolated from the declining trees of *Quercus suber* L. in Sardinia. *IOBC/wprs Bulletin*, vol. 25, p. 37-40.

Sousa, E.; Santos, M.N.; Varela, M.C. and Henriques, J. (2007) - *Perda do vigor dos montados de sobro e azinho: análise da situação e perspectivas* (Documento síntese). MADRP; DGRF; INRB, I.P., 80pp.

Uddin, W. and Stevenson, K.L. (1997) - Pathogenicity of a species of *Phomopsis* causing a shoot blight on peach in Georgia and evaluation of possible infection courts. *Plant Disease*, vol. 81, n. 9, p. 983-989.

Vannini, A.; Paganini, R. and Anselmi, N. (1996) - Factors affecting discharge and germination of ascospores of *Hypoxylon mediterraneum* (De Not.) Mill.. *European Journal of Forest Pathology*, vol. 26, n. 1, p. 12–24.

Vannini, A.; Mazzaglia, A. and Anselmi, N. (1999) - Use of random amplified polymorphic DNA (RAPD) for detection of genetic variation and proof of the heterothallic mating system in *Hypoxylon mediterraneum*. *European Journal of Forest Pathology*, vol. 29, n. 3, p. 209-218.

Vannini, A.; Lucero, G.; Anselmi, N. and Vettraino, A.M. (2009) - Response of endophytic *Biscogniauxia mediterranea* to variation in leaf water potential of *Quercus cerris*. *Forest Pathology*, vol. 39, n. 1, p. 8-14.