

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

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

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

### **INTRODUCTION**

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

*P. cylindrus* bore galleries and lay eggs in the nutrient-poor sapwood of selected hosts

and introduce into their tunnels ambrosia fungi which grow on the tunnel walls and serve as the main source of food for the adults and their offspring. The principal ambrosia fungi are *Raffaelea* spp. and the inoculation of some *Raffaelea* species caused wilting and/or death of cork oak seedlings and thus would be a pathogenic fungi (INÁCIO *et al.*, 2008; INÁCIO *et al.*, 2011). Nevertheless, the recent modification of insects' behavior leads us to suppose that new associations may have arisen. Taking into consideration that charcoal canker caused by *Biscogniauxia mediterranea* (de Not.) Kuntze is increasing in Portuguese cork oak stands, namely in young trees which exhibit a sudden decline process (SOUSA *et al.*, 2007), *P. cylindrus* could be hypothesized as a disease vector

spreading the inoculum in oak stands. Thus, the goal of this work was to ascertain the transportation of *B. mediterranea* by *P. cylindrus* and to verify its pathogenicity towards cork oak and the effect of phytotoxic compounds on disease development.

## MATERIAL AND METHODS

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

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

Inoculation experiments were conducted in May 2008 on 1 year-old seedlings and repeated in October 2008. 16 seedlings were wound inoculated with a mycelial plug and the same number for the wound control plants, in both seasons. Symptoms (wilting

or death) were assessed daily for 4 months after inoculation. The length of stem lesions was measured after removal of the outer bark. Fungal isolations of the discolored sapwood of each newly dead or wilted seedling were carried out.

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

## RESULTS

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

The comparisons with the published sequences of the GenBank nucleotide sequences databases confirmed the relation of the isolates under study with *B. mediterranea*: the highest similarity (maximum identity-100%) was found with *B. mediterranea* isolated from *Quercus ilex* in Spain (AF280625.1 and AF280624.1) and *Q. cerris*, in Italy (AJ246222.1). No significant

length variation was observed in ITS amplicons, whose average length was 560 nucleotides. The sequence data that were suc-

cessfully sequenced were submitted to the GenBank nucleotide sequences databases as accessions FR734186 and FR734187.

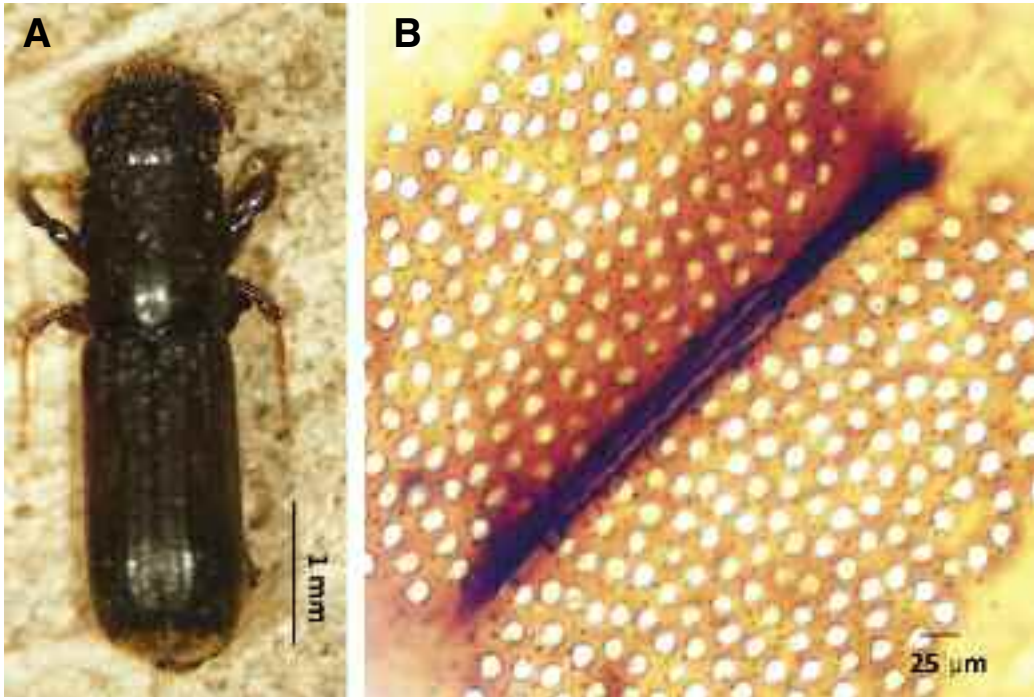


Figure 1. *Platypus cylindrus*: A. adult female, B. female mycangia with pits filled with fungal spores



Figure 2. *Biscogniauxia mediterranea* anamorph: A. colony morphology after 10 days on malt extract agar in a 90 mm diameter plate; B-C. Light micrographs; B. verticillate conidiogenous cells (x400); C. conidia and conidiophore (x400).

Control seedlings remained asymptomatic during the trial. No statistically significant differences between season inoculation and symptoms appearance were found ( $F_{1,28}=0,0039$ ;  $p=0,9506$ ), and therefore the results for both seasons were pooled. All but two seedlings inoculated with the pathogen showed bark and xylem lesion at the end of the experiment (average length lesion= $1,8\pm0,3$  cm) (Fig. 3). These results agree with the assays of LINALDEDDU *et al.* (2009). The fungus was re-isolated from the stems of all symptomatic plants.

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

## DISCUSSION

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

Charcoal canker is one of the most frequent disorders of Portuguese cork oak stands, *B. mediterranea* being considered an endophyte in oak tissues (SANTOS, 2003). However, its presence has increased in the last years especially in young cork oak trees without other symptoms of decline. The en-

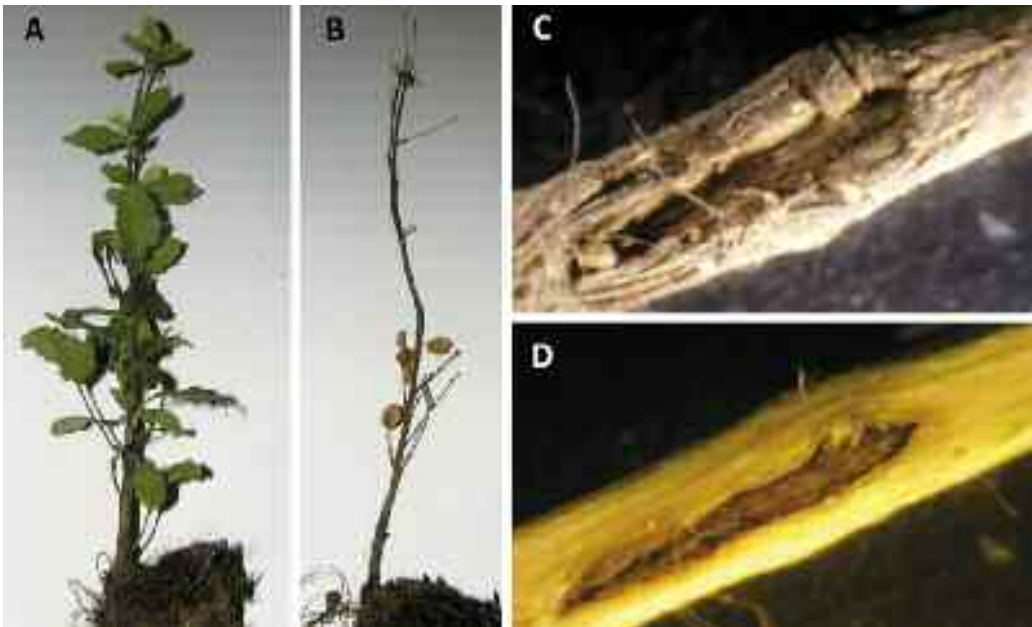


Figure 3. Pathogenicity tests on 1-year-old cork oak seedlings: A. control seedling; B-D. symptoms four months after inoculation with *Biscogniauxia mediterranea*; B. inoculated seedling; C. canker in the stem; D. bark and xylem necrosis.

tomochoric nature of its spores and the close association with *P. cylindrus* lead us to conclude that the insect outbreaks might contribute to disease spread, as stated by others (VANNINI & VALENTINI 1994). Being already inside the host, the fungus can rapidly

spread from several infection points (MAZZAGLIA *et al.* 2001). So, the direct inoculation of the pathogen into new hosts may increase disease incidence in cork oak stands, even when the attacks of the beetles yielding the fungus are aborted.

#### RESUMEN

INÁCIO, J. M. L., HENRIQUES, L. GUERRA-GUIMARÃES, H. GIL AZINHEIRA, A. LIMA, E. SOUSA. 2011. *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) transporta *Biscogniauxia mediterranea*, el agente del chancro carbonoso del alcornoque. *Bol. San. Veg. Plagas*, **37**: 181-186.

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

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

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