Silva Lusitana, nº especial: 89 - 99, 2011

Contribution of Symbiotic Fungi to Cork Oak Colonization by *Platypus cylindrus* (Coleoptera: Platypodidae)

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

Contribuição dos Fungos Simbiontes na Colonização do Sobreiro por *Platypus cylindrus* (Coleoptera: Platypodidae)

Palavras-chave: *Platypus cylindrus* Fab. (Coleoptera: Platypodidae), considerado um inseto secundário, é atualmente tido como agente primário desempenhando um papel determinante no declínio do montado. Para além do ataque massivo, *P. cylindrus* associa-se a fungos dos quais depende para a sua sobrevivência e colonização do hospedeiro. O isolamento de fungos a partir do incesto permitiu a identificação de sete géneros potencialmente envolvidos no seu estabelecimento: *Acremonium, Biscogniauxia, Botryosphaeria, Gliocladium, Raffaelea, Scytalidium* e *Trichoderma*. Os fungos mais frequentes foram espécies do género *Raffaelea* (fungos ambrósia) principalmente associadas aos micângios e conteúdo intestinal, confirmando o seu papel como simbiontes primários, e possivelmente com capacidade para enfraquecer o hospedeiro. Do mesmo modo, os géneros *Biscogniauxia* e *Botryosphaeria* poderão atuar de forma a ultrapassar as defesas das árvores. Os géneros *Scytalidium, Gliocladium* e *Trichoderma*, conhecidos pela sua capacidade degradadora da madeira, desempenham um papel pioneiro na colonização do hospedeiro. Estes resultados demonstram a estreita associação entre *P. cylindrus* e os seus fungos ambrósia, principalmente do género *Raffaelea*, e fungos ambrósia auxiliares, cuja presença faz parte da estratégia do inseto para a colonização do hospedeiro.
Palavras-chave: Inseto ambrósia; fungos ambrósia; *Quercus suber*; *Raffaelea* spp.; Ophiostomatales

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Introduction

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

P. cylindrus attack is usually limited to dead or weakened trees (SEABRA 1939; BAETA-NEVES, 1950; ESPAÑOL, 1964). Sporadic attacks were also described on apparently healthy trees (BALACHOWSKY, 1949) and in Morocco this beetle is considered an important pest of cork oak (*Quercus suber* L.) (VILLEMENT and FRAVAL, 1993; SOUSA *et al.*, 2005). In Portugal, since the 1980's, severe infestations were observed in apparently healthy cork oaks (SOUSA, 1992; SOUSA and DÉBOUZIE, 2002) causing widespread tree death within three months to one year and a half after the attack, depending on the host vigour and resistance (SOUSA and INÁCIO, 2005).

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

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

Material and methods

Collection

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

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

Fungal isolation and identification

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

Statistical analysis

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

Results

Specialized organs for transporting fungi

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

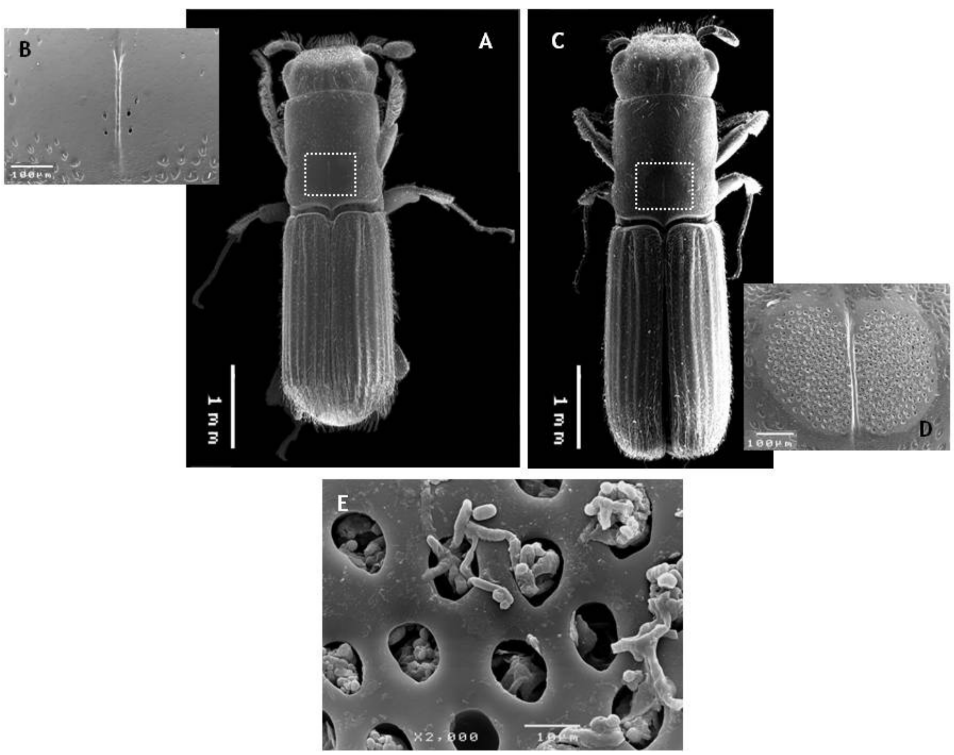

Figure 1 - Scanning electron micrographs of *Platypus cylindrus* adults. A. Male. B. Male mycangia. C. Female. D. Female mycangia. E. Growing mycelium and spores on female mycangia cavities

Fungal isolation and identification

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

The mycobiota obtained from individual *P. cylindrus* did not significantly differ in the three years of fungal isolation ($F_{2.123}$ = 0.2255; p = 0.7985).

Although females may transport large amounts of fungal propagules, in terms of frequency of vectored fungi there were no statistically significant differences between males and females ($F_{1.124}$ = 0.0708; p = 0.7906). Therefore, results for both sexes were pooled.

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

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

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

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

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

The proportions of fungi found in mycangia and in the intestine versus phoretic on the exoskeleton were significantly different ($F_{2.123} = 16.5784$; $p < 0.001$). Besides *Botryosphaeria* sp. several non-ophiostomatoid fungi were isolated mainly from exoskeleton surfaces. In the mycangia these fungi were the rarest and the insects' gut showed lower diversity of fungi. Species of *Biscogniauxia*, *Gliocladium* and *Trichoderma* genera were found in all the insect organs. *Scytalidium* sp. and *Acremonium* sp. were not found in the intestinal content.

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

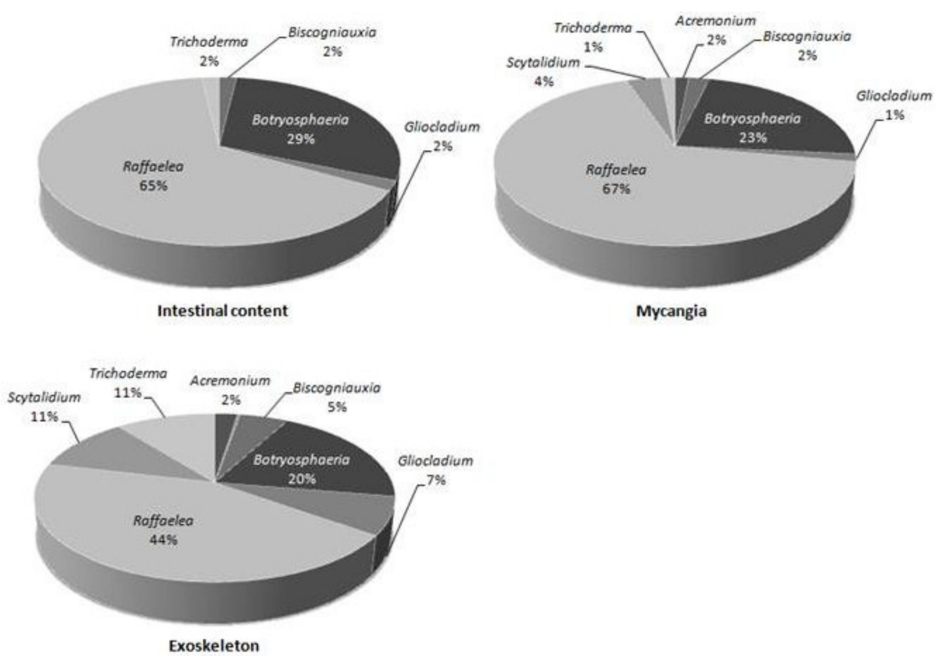

Figure 2 - Genera of fungi (%) isolated from the intestine, mycangia and exoskeleton of *Platypus cylindrus*

Discussion

In this study *Platypus cylindrus* was found to transport several fungi, out of which the most frequent was the ophiostomatoid species of *Raffaelea*. *Raffaelea ambrosiae* was considered the main ambrosia fungi and several authors reported this species as the principal symbiont of the oak pinhole borer (BAKER, 1963; ARX and HENNEBERT, 1965; SOUSA *et al.,* 1995). However, and according to more recent work, *P. cylindrus* is associated with other *Raffaelea* species namely *R. montetyi,* an ambrosia fungus that decays wood (MORELET, 1998; INÁCIO *et al.*, 2008), and *R. canadensis* (INÁCIO *et al.,* 2008). The Ophiostomatales are economically important sapstaining fungi that occur worldwide on hardwoods. Moreover, some species of *Raffaelea* that are closely associated with ambrosia insects cause serious outbreaks in healthy trees (KUBONO and ITO, 2002; MURATA *et al.*, 2005; FRAEDRICH *et al.,* 2008; KIM *et al.*, 2009; HARRINGTON *et al.,* 2010).

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

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

The association of *P. cylindrus* with cosmopolitan fungi is well documented by others (BAKER, 1963; CASSIER *et al.*, 1996; SOUSA *et al.,* 1997). In the present study, they were consistently isolated from the exoskeleton and mycangia, even after an accurate and thorough disinfection by fractional sterilization (FRANCKE-GROSMANN, 1956) to avoid saprobes growth (data not shown). It is possible that they might play a role in the insect-fungi interaction and thus in the establishment of the insect. It has been emphasized that these secondary symbionts may act as wood degrading agents to facilitate galleries excavation. *Gliocladium* sp., *Trichoderma* sp. and *Scytalidium* sp., as producers of lignocellulolytic enzymes might have this pioneer role (KIFFER and MORELET, 1997; SZAKACS and TENGERDY, 1997;

MADDAU *et al.*, 2009; INÁCIO *et al.*, 2010b). Moreover, species of *Trichoderma* and *Gliocladium* are known for their antagonistic activity and may possibly control fungal growth inside the galleries (HENRIQUES, 2007).

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

Acknowledgements

We thank Doctor Maria Costa Ferreira (INRB, I.P.) for reviewing the manuscript and Octávio Chaveiro for the technical assistance in SEM photos. This research was supported in part by a grant from the Fundação para a Ciência e a Tecnologia BD/26033/2005.

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