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

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

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ABSTRACT

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

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

RESUMO

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

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

INTRODUCTION

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

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

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

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

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

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

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

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

MATERIAL AND METHODS

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

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

Morphological characterisation

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

Cycloheximide sensitivity

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

Molecular analysis

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

RESULTS AND DISCUSSION

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

Morphological characterisation

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

Table 1 – Macroscopic description of the Raffaelea cultures on PDA

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

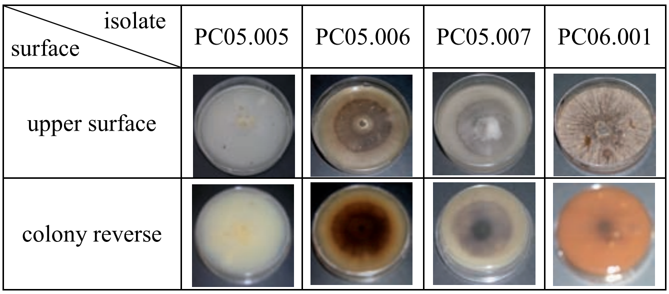


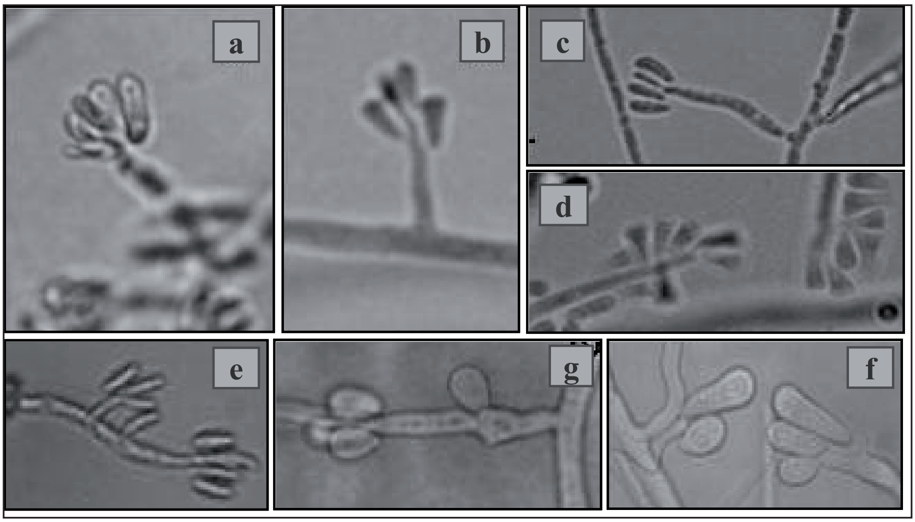
Figure 1 – Typical cultural aspect (surface, reverse) of *Raffaelea* isolates.

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

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

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

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



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

Cycloheximide sensitivity

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

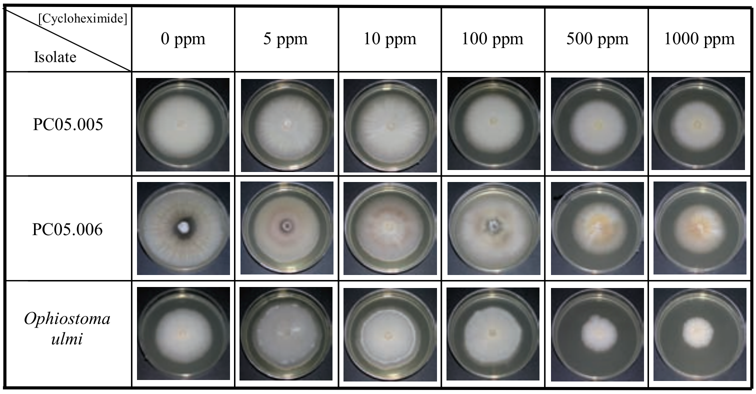
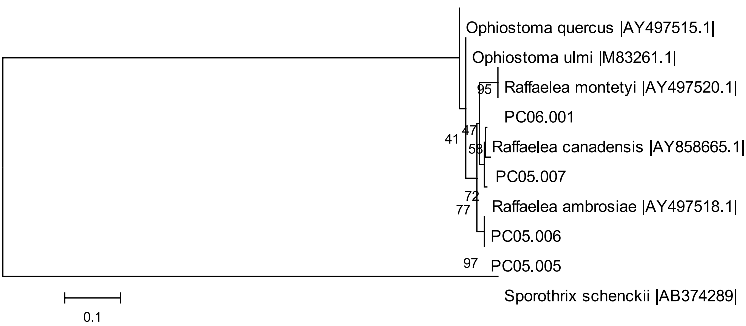


Figure 3 – Results of the cycloheximide sensitivity test for two *Raffaelea* isolates in comparison with an *Ophiostoma ulmi* isolate.

Molecular analysis

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



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

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

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

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

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

CONCLUSION

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

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

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

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

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

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