FUNGI ASSOCIATED TO *PLATYPUS CYLINDRUS* FAB. (COLEOPTERA: PLATYPODIDAE) IN CORK OAK

FUNGOS ASSOCIADOS AO INSECTO *PLATYPUS CYLINDRUS* FAB. (COLEOPTERA: PLATYPODIDAE) EM SOBREIRO

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ABSTRACT

Platypus cylindrus is a pest that since the 80's of the last century has been considered a cork oak mortality agent in Portugal. It is an ambrosia beetle that establishes complex symbioses with fungi whose role in the insect-fungus-host interaction has not been completely clarified. In order to characterize *P. cylindrus* associated micoflora in Portugal, fungi were isolated from different beetle organs and from its galleries in cork oak trees. Fungi of the genera *Acremonium*, *Aspergillus*, *Beauveria*, *Botrytis*, *Chaetomium*, *Fusarium*, *Geotrichum*, *Gliocladium*, *Nodulisporium*, *Paecilomyces*, *Penicillium*, *Raffaelea*, *Scytalidium*, *Trichoderma* and of the order Mucorales were identified. An actinomycete of the genus *Streptomyces* was also identified. Some of these genera were related for the first time to this interaction. In the present work the isolated fungi are characterized and their contribution for beetle population establishment and tree weakness is discussed.

Key-words: ambrosia beetle, decline, interaction, mycoflora, *Quercus suber*.

RESUMO

Platypus cylindrus é uma praga que desde os anos 80 do século passado tem sido referida como agente de mortalidade do sobreiro em Portugal. É um insecto ambrósia que estabelece simbioses complexas com fungos cujo papel não está completamente esclarecido na interacção insecto-fungo-sobreiro. Com o objectivo de caracterizar a micoflora associada a *P. cylindrus* em Portugal foram efectuados isolamentos a partir de diferentes órgãos do insecto e suas galerias em sobreiro. Identificaram-se fungos dos géneros *Acremonium*, *Aspergillus*, *Beauveria*, *Botrytis*, *Chaetomium*, *Fusarium*, *Geotrichum*, *Gliocladium*, *Nodulisporium*, *Paecilomyces*, *Penicillium*, *Raffaelea*, *Scytalidium*, *Trichoderma* e da ordem Mucorales. Foi igualmente identificado um actinomiceta do género *Streptomyces*. Alguns destes géneros são referidos pela primeira vez nesta interacção. No presente trabalho caracterizam-se os fungos isolados e discute-se a sua contribuição para o estabelecimento das populações do insecto e enfraquecimento das árvores.

Palavras-chave: insecto ambrósia, declínio, interacção, micoflora, *Quercus suber*.

INTRODUÇÃO

Scolytidae and Platypodidae are among the most successful wood-inhabiting beetles causing damage of economic significance to trees and timber (Cassier *et al.*, 1996). *Platypus cylindrus* Fab. is a cork oak (*Quercus suber* L.) pest that has come to assume an increasing importance in Portugal and Mediterranean basin

countries (Ferreira & Ferreira, 1989; Chakali *et al.*, 2002; Riziero *et al.*, 2002; Sousa *et al.*, 2005). Until recently, damages produced by this insect were limited to dead or weakened trees. The understanding of recent population outbreaks, mainly in Portuguese cork oak stands, can be based on three assumptions: (i) gradual changes of the cork oak stand dynamics, (ii) development of more specific host colonization mechanisms and (iii) changes on the insects and their natural enemies' population dynamics (Sousa & Inácio, 2005). Within the host colonization strategies, it's essential to consider the fungal symbiosis that may contribute to the host weakness and create better conditions for the establishment of insects (Henriques *et al.*, 2006).

In deed, *P. cylindrus*, as almost all members of the Platypodidae, is denominated an ambrosia beetle because larvae and adults feed mainly on fungi (ambrosia fungi) that cover the gallery walls (Batra, 1963). This insect-fungi relation is expressed in an ectosymbiosis in which the fungi live outside the insects' body but are temporarily stored in special ectodermical organs for dissemination purposes (Francke-Grosmann, 1967). Insects carry viable inoculum in sac-like structures, called mycangia, located in the prothorax; the inoculum is protected from desiccation during the entire life of the beetle and is disseminated into new breeding sites at the time of tunnel excavation (Batra, 1963; Sousa & Inácio, 2005).

Ambrosia fungi definition includes a set of concepts whose interception allows the classification of several fungi as ambrosia: i) direct participation in insect feeding; ii) presence inside insect galleries in the host; iii) dimorphism, meaning ability to grow both as yeast and mycelia; iv) possible specificity in the insect-fungi-host relationship (Batra, 1963; Beaver, 1989).

Batra (1985) grouped ambrosia fungi as primary and auxiliary. Primary ambrosia fungi are highly insect species specific and their distribution correspond to those of insect symbionts. They are present and dominant in the tunnels and isolated regularly from the mycangia of the beetles in the flight stage or when excavating tunnels. Auxiliary ambrosia fungi are transitory, non-specific with respect to symbiont insect and may appear after insect development. They may not be present in larval cradles or in adult beetles; and their habitat and distribution range are unrestricted and unrelated to that of the ambrosia beetles.

Several fungi have already been isolated from *P. cylindrus* and from galleries in *Quercus* spp. (Baker, 1963; Cassier *et al.*, 1996; Sousa *et al.*, 1997; Morelet, 1998; Henriques *et al.*, 2006). Their constant presence associated to the insect allows us to presume that they play an important role in the symbiosis. Besides the implication in insect feeding, ambrosia fungi might also be involved in other processes such as host weakness, through pathogenic action; decomposition of lignocellulolitic compounds, which helps gallery construction and enables fungi colonization; and/or antagonism that controls the growth of other fungi (Sousa & Inácio, 2005; Henriques *et al.*, 2006).

The aim of the present work is to characterize the micoflora associated to *P. cylindrus* and discuss its contribution for beetle populations' establishment and host weakness.

MATERIAL AND METHODS

Four infested logs of cork oak trees exhibiting decline symptoms were collected from three of the main Portuguese cork oak productive regions: Chamusca (Ribatejo province), Montemor and Grândola (Alentejo province). The logs were maintained in the laboratory in order to capture 100 *P. cylindrus* insects, males and females, as they emerged. Samplings were repeated during 2005, 2006 and 2007. The insects were aseptically dissected in mycangia, intestine and parts of the exoskeleton (elytra). The logs were cut in order to identify the different insect gallery sections: cork, inner-bark, pre-parental section, larval section and gallery end. One complete gallery was observed from each log and six samples (fragments with 1 cm^2) of each section were collected.

The samples were surface sterilized with a sodium hypochlorite solution (1%) for 1 min, rinsed with distilled sterilized water and then plated in malt extract agar (MEA, Difco, USA) added with streptomycin (500 mg/l) and MEA added with cycloheximide (500 mg/l). The cultures were incubated at 24±1ºC in darkness. Pure cultures of each fungi isolate were obtained and identified as genus based on morphological features according to Ellis (1971, 1976), Lanier et al. (1978), Kiffer & Morelet (1997) and Barnett & Hunter (1998). Cultural description of colonies was made on potato dextrose agar (PDA, Difco, USA).

RESULTS

Fifteen genera were isolated from the different insect parts and from all cork oak gallery sections (Table 1). The identified fungi are classified in very distinct orders including the Ascomycota (Eurotiales, Heliotioales, Hypocreales, Ophiostomatales, Saccharomycetales, Sordiales and a genus not assigned to any order) and Basidiomycota (Xylariales) although all the obtained genera, except *Chaetomium*, were isolated in the mitosporic state. Also, an actinomycete was isolated from the insect (Streptomycetales).

Table 1 – Genera isolated from *Platypus cylindrus* intestine, exoskeleton and mycangia and from the different sections of insects galleries on cork oak.

Isolates		Insect			Gallery				
Order	Genus	Intestine	Exoskeleton	Mycangia	Cork	Inner-bark	Pre-parental section	Larval section	Gallery end
Eurotiales	*Aspergillus*	•	•	•	•	•	•	•	•
	Paecilomyces		•		•	•	•	•	•
	Penicillium	•	•	•	•	•	•	•	•
Helotiales	*Botrytis*				•				
Hypocreales	*Acremonium*	•	•	•	•	•	•	•	•
	Beauveria		•						
	Fusarium				•		•		
	Gliocladium	•	•	•	•	•	•		•
	Trichoderma	•	•	•	•	•	•	•	•
Ophiostomatales	*Raffaelea*	•	•	•	•	•	•	•	•
Saccharomycetales	*Geotrichum*				•	•	•		
Sordiales	*Chaetomium*						•	•	
incertae sedis	*Scytalidium*		•	•	•	•	•	•	•
Xylariales	*Nodulisporium*	•	•	•	•	•	•	•	•
Actinomycetales*	*Streptomyces**	•	•	•					

* Streptomyces (order Actinomycetales) is an actinomycete, the other genera described belong to Fungi.

The isolated genera were described according to the morphological features based on optical microscopic observations and literature guides. In table 2 the main cultural features for all isolated genera are described.

From the genus *Aspergillus* several individuals with different cultural characteristics were isolated, probably belonging to different species. Their microscopic features are: hyphae septate and branched, conidiophores macronematous, mononematous, erect, simple, often with a foot cell, with a terminal vesicle bearing short branches or phialides radiating from the entire surface. When present, the branches are in one or several series and the terminal ones

Table 2 – Cultural characteristics on PDA of the isolated genera.

Genera	Upper surface				Lower surface	Observations
	Cultural aspect	Density	Color	Zonation		
Aspergillus	effuse, powdery	high	white, yellow, several green tonalities or black	weak or absent	idem to upper face except the color (yellowish)	
Paecilomyces	effuse, powdery	high	green-brownish	absent	idem upper face	
Penicillium	effuse, powdery	high	green to brown	radial	idem upper face except the color (yellowish)	produces coloured oozing
Botrytis	effuse, felted	light	grey	absent	idem upper face	
Acremonium	effuse, felted	high	white	absent	idem upper face	
Beauveria	effuse, cotton-like to powdery	high	white	absent	idem upper face	
Fusarium	effuse, cotton-like	high	white	absent	idem to upper face except the color (carmine to yellow)	carmine pigmentation of the media
Gliocladium	felted	high	sulfur yellow to straw yellow	light concentric	idem to upper face except the color (citric yellow)	
Trichoderma	effuse, floccose	media	green-yellowish with white to grey flakes	media concentric	idem upper face	green-yellowish pigmentation of the media
Raffaelea	effuse, yeast-like, some with a felted mycelium in the colony center	light to media	cream-colored, light olive-green or fuliginous	light-concentric or absent	idem upper face	
Geotrichum	effuse, powdery	light	white	absent	idem upper face	
Chaetomium	effuse, felted	high	initially white turning grey to olive and reddish with maturation	absent	idem upper face except the color (dark brown)	
Streptomyces	powdery	high	grey	radial	idem upper face except the color (yellowish)	
Nodulisporium	effuse, cotton-like	high	white to grey with green emergences in the colony center	absent	idem upper face except the color (fuliginous)	
Scytalidium	effuse, felted to farinaceous in the colony edge	light	hazelly	radial	idem upper face	

always bear phialides. Conidiogenic cells monophialidic, discrete, several arising together at the end of terminal branches or over the surface of the vesicles, mostly determinate, rarely percurrent, ampulliform or lageniform, collarettes sometimes present. Conidia are phialosporic, unicellular, dry, smooth, rugose, echinulate, globose and hyaline but colored in mass, disposed in long basiptal chains (Figure 1a).

Penicillium produces conidiophores macronematous, mononematous, simple or branched, penicilated, ending in a group of phialides. The walls of conidiophores may be smooth and thin or variously roughened, with aerial portions appearing delicately echinulate, granular or asperulate. The penicillus covers all the branching system and can be monoverticillate or biverticillate, symmetrical

or asymmetrical. Conidia are phialosporic, unicellular, dry, globose or ovoid, hyaline to green (Figure 1b).

Paecilomyces produces conidiophores macronematous, mononematous, arising as upright branches from hyphal ropes or aerial mycelium, smooth-walled, usually with several stages of irregular branching, and frequently bearing secondary branches, with divergent peniciled phialides, cylindrical to ellipsoidal in the lower part, usually narrowing abruptly into a long cylindrical neck. Conidia phialosporic, unicellular, hyaline, ovoid, smooth-walled, highly variable in size produced in long, strongly divergent chains (Figure 1c).

Several *Raffaelea* isolates were obtained showing a great macro and microscopic variability, probably corresponding to different species. This genus produces hyphae hyaline and septate that bound together forming compact hyphae ropes. Superficial sporodochia, effuse, white to brownish. Conidiophores are macronematous and mononematous, erect, septate, slender with a tapered apex, producing simpodulosporic conidiae that leave cicatricial scars in the conidiogenous cells. Conidiae are unicellular and hyaline, with variable forms (triangular, oval, allantoid, fusiform or truncated claviform) and dimensions (Figure 1d).

Fusarium produces conidiophores macronematous and mononematous, variable, slender, and simple or stout, short, septate and branched that originates phialosporic conidia, hyaline, canoe-shaped, with 5 to 6 transversal septa and collected in a slimy drop. No microconidia were observed (Figure 1e).

Trichoderma conidiophores macronematous and mononematous, hyaline and highly ramified, disposed in pyramidal structures with inserted phialides in 90º angles. Conidia are phialosporic, enteroblastic and monoblastic, unicellular, green, globose to subglobose and remain grouped in the top of the phialides (Figure 1f).

Beauveria produces conidiophores micronematous and mononematous, simple, irre-

gularly grouped, inflated in the base and tapered in the apical fertile portion that appears sinusoidal after conidia production. Conidia are simpodulosporic, unicellular, dry, hyalines and ovoid with small denticles, giving the conidiogenous cells a spiny appearance (Figure 1g).

Gliocladium presents conidiophores macronematous and mononematous, erect, septate and branched, ending in a branched system of phialides disposed in tight penicilate structure with three phialides per metulae. Phialides narrowly cylindrical to sub-ulate, taper slightly towards the tip, smooth-walled. Conidia are phialosporic, unicellular, hyaline, ovoid or cylindrical and aggregated in conidial masses, slimy to watery, whitish or light-yellow, never forming imbricate chains (Figure 1i).

Acremonium produces hyphae hyaline septate that sometimes bound together by anastomoses. Conidiophores are macronematous and mononematous, erect, solitary to weakly branched, straight, tapered to the apex, with basal septum to separate the conidiophore from the vegetative hyphae. Conidia are phialosporic, unicellular and ovoid, aggregated in a slimy drop (Figure 1k).

Geotrichum produces conidiophores micronematous and mononematous producing arthrosporic conidiae, hyaline, unicellular, dry, smooth, short cylindrical with truncated bases, resulting from fragmentation of undifferentiated hyphae by fission through double septum, (Figure 1l).

Botrytis has conidiophores macronematous, mononematous, straight or flexuous, smooth, brown, slender, irregularly branched with enlarged apical polyblastic cells where botryoblatosporic conidia are produced simultaneously; conidia solitary, simple, smooth, hyaline to grey in mass, unicellular, ovoid with a denticule. Mycelium immersed or superficial, with brownish course hyphae. Sclerotia frequently present (Figure 1h).

Chaetomium produces superficial perithecia, brown, single, globose, covered with different sized brown filaments; asci clavate,

pedunculate with evanescent walls. Ascospores unicellular, light olive-brown and lemoniform, smooth, often pushed out of ostiole in a cirrhus (Figure 1j).

Streptomyces produces filaments that originate very small spores by fragmentation that remain disposed in helical chains (Figure 1m).

Nodulisporium produces mycelium partly immersed and partly superficial, 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, slender or short and thick, verticilated. Conidiae sympodulosporic, acropleurogenous, unicellular, hyaline or brown to olive in mass, ellipsoidal or obovoid, smooth or roughened, with a small frill when detached (Figure 1n).

Scytalidium produces sparse thick hyphae, septate, hardly branched, hyaline or light-brown, disposed in parallel; conidiophores micronematous and mononematous with terminal conidiogenic cell originating arthroconidiae by holothalic fragmentation. Conidia are catenulated, schizogenous, uni or bicellular, hyaline, rectangular. It also forms terminal or interpolate chlamydospores, with thick wall, brown and ellipsoidal (Figure 1o).
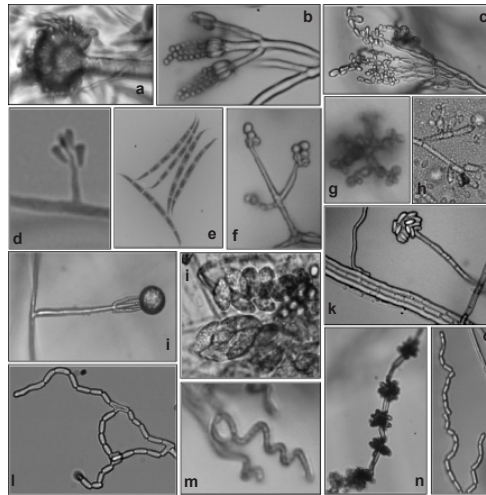
Figure 1 – Reproductive structures of isolated genus: a) *Aspergillus* (x1000); b) *Penicillium* (x1000); c) *Paecilomyces* (x600); d) *Raffaelea* (x600); e) *Fusarium* (x600); f) *Trichoderma* (x600); g) *Beauveria* (x1000); h) *Botrytis* (x600); i) *Gliocladium* (x400); j) *Chaetomium* (x1000); k) *Acremonium* (x600); l) *Geotrichum*; m) *Streptomyces* (x1000); n) *Nodulisporium* (x600); o) *Scytalidium* (x600).

In *P. cylindrus* galleries on cork oak, greater fungi variety were presented than in the insects' body. In both cases were isolated cosmopolite fungi and others more specific for insect-fungi relation. Concerning isolates from insects, it was in the exoskeleton that was found the major diversity of genus, followed by mycangia and then intestine. Among sexes, no relevant differences were observed. *Penicillium*, *Raffaelea* and *Aspergillus* were the most frequent genus in the three sampled organs and for both sexes. *Beauveria* and *Streptomyces* were only isolated from the insect, the former only from the exoskeleton.

Along *P. cylindrus* galleries in cork oak, the majority of the isolated genera were found in all sections and, in general, in si-

milar proportions, highlighting cosmopolite and saprophytic fungi such as *Penicillium*, *Trichoderma*, *Gliocladium* and *Scytalidium*, but also *Raffaelea* was isolated in a considerable percentage in all sections. *Botrytis*, *Fusarium*, *Geotrichum* and *Chaetomium* were isolated only from the galleries but in very low percentages and distribution.

In Figures 2 and 3 are represented the distribution of the isolated fungi both in *P. cylindrus* body and along its galleries in cork oak, respectively.
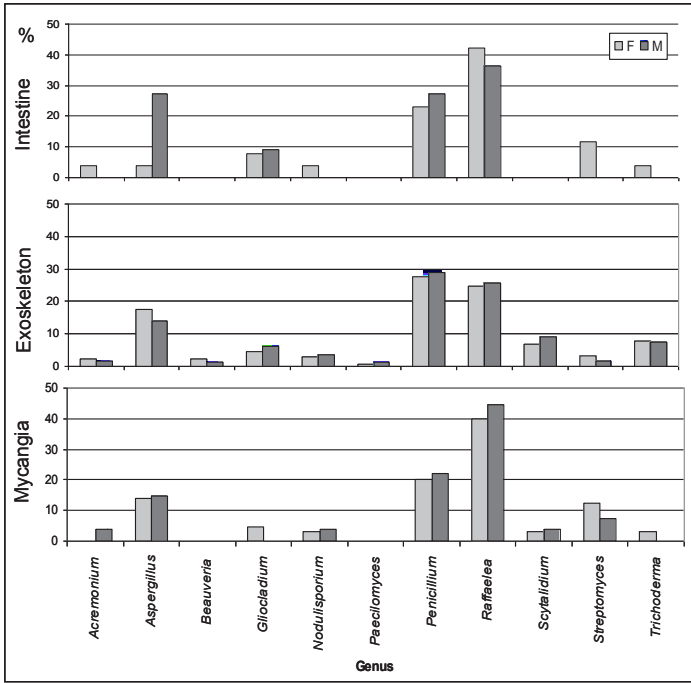

Figure 2 – Percentage of isolated genera in the different body parts (intestine, exoskeleton and mycangia) of *Platypus cylindrus* females (F) and males (M).
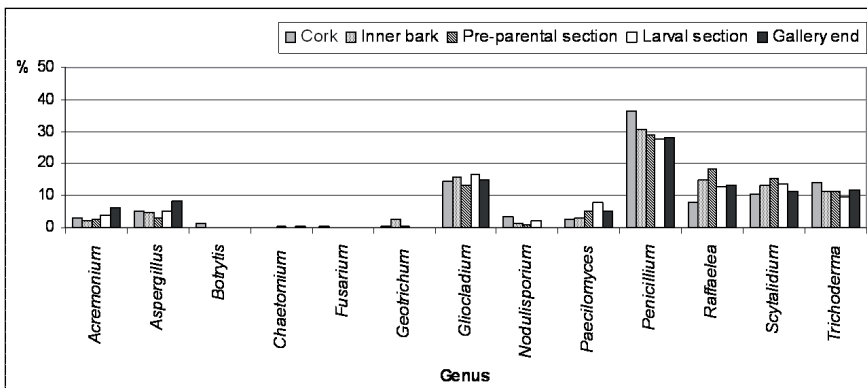

Figure 3 – Percentage of isolated genera from the different *Platypus cylindrus* galleries on cork oak (cork, inner bark, pre-parental section, larval section and gallery end).

DISCUSSION

From all isolated genera, *Raffaelea* is highlighted because of its known importance as ambrosia fungi and both its frequency and distribution (Beaver, 1989). This genus includes twelve species, the majority associated with ambrosia beetles (Kubono & Ito, 2002; Bisby *et al.*, 2006). Two species (*R. ambrosiae* and *R. montetyi*) are identified as *P. cylindrus* primary ambrosia fungi (Arx & Hennebert, 1965; Morelet, 1998) and have been isolated both from the insect organs and from its galleries in the host (Sousa *et al.*, 1997; Morelet, 1998). Although *Raffaelea* sexual state is lacking, observations of conidial development support its placement within the Ophiostomatales (Gebhardt & Oberwinkler, 2005) and, 18S ribosomal DNA sequence analysis, shows a monophyletic lineage which forms a sister group to species of the genus *Ophiostoma* (Jones & Blackwell, 1998). The Ophiostomatales are economically important sapstaining fungi occurring world-wide on hardwoods and commercially produced pines, and are in some cases, already known as pathogenic to oaks (Degreef, 1992). The effect of *Raffaelea* spp. on cork oak remains unknown, but in Japan, *R. quercivora* involved in an interaction with *P. quercivorus* has proven pathogenicity to several oak trees (Kubono & Ito, 2002; Kinnura & Kobayashi, 2006).

Other isolated fungi were already related to *P. cylindrus*. Sousa *et al.* (1997) isolated *A. carbonarius* from mycangia and cork oak galleries. *Pa. variotii* has been isolated from galleries in cork oak and other oaks (Baker, 1963). Also *Penicillium* was found both on the insect and in oak galleries (Baker, 1963; Cassier *et al.*, 1996; Sousa *et al.*, 1997). *Botrytis* was isolated from galleries in oak, as well as *Acremonium* and *Fusarium* (Baker, 1963). *Acremonium* sp. and *F. solani, Gliocladium roseum* and *G. solani* has also been isolated by Sousa *et al.* (1997) from *P. cylindrus* organs and galleries in cork oak. Cassier *et al.* (1996) isolated *Trichoderma* from the insect and *T. viride* was isolated from galleries on cork oak and other oaks (Baker, 1963; Sousa *et al.*, 1997). *Nodulisporium* and *Scytalidium* were found in mycangia and galleries on cork oak, the last one only in the host (Sousa *et al.*, 1997).

Four genera were newly associated with this interaction: *Beauveria*, *Geotrichum*, *Chaetomium* and *Streptomyces*. *Beauveria* is an entomopathogenic fungus and two species (*B. bassiana* and *B. brongniartii*) are already known as harmful to *Platypus* spp. (Glare *et al.*, 2002). Several species of *Geotrichum* have been identified in association with insects (Suh & Blackwell, 2006) and the genus *Streptomyces* act as an antimicrobial defense in the termite-fungi association (Mueller & Gerardo, 2002). *Chaetomium* is a very common genus in soil and plant debris, in particular in wood (Hawksworth, 1995) and it might be a *Scytalidium* teleomorph (Halin, 1997).

The role of each isolated genus in the insect-fungi-host interaction is discussed. Most of them are considered cosmopolite and saprophytic fungi (Hawksworth *et al.*, 1995; Kiffer & Morelet, 1997) which can be involved just in a commensalistic relation or might play some other action due to their isolation frequency and distribution among samples.

Following *P. cylindrus* host colonization process, symbiotic fungi may start to act as wood degrading, thus facilitating galleries' excavation. *Trichoderma,* as a great extrolite producer such as lignocellulolictic enzymes, might be an active intervenient in that process (Samuels, 1996). Then, to overcome host defense reaction, the insect might inoculate phytopathogenic fungi. *Nodulisporium* might be relevant in this part as the ITS rDNA analysis proved that it is a *Biscogniauxia mediterranea* anamorph, which is responsible for cork oak charcoal disease (Collado *et al.*, 2001). Other isolated genera might also interfere in this phase: *Acremonium* which might be *Nectria* anamorph, thus being a potential pathogenic to several woody plants and *Fusarium* also pathogenic to several plant organs (Kiffer & Morelet, 1997). Within the galleries, fungi that

will be nourishment for larvae develop in a controlled manner by the permanent care of parental insects but probably also due to the antagonistic action of fungi over others. *Gliocladium* and *Trichoderma* are known by their antagonistic activity being used as biocontrol agents (Papavizas, 1985). *Streptomyces* and *Scytalidium* might act in the fungi colonies management within the galleries. Also in the insect body this management might be essential to achieve ambrosia fungi transport. The final role of the isolated fungi, which is the base of this insect-fungi interaction, is the larvae nourishment. According to definition, ambrosia fungi are eaten by the insects and thus are the ones found in their intestine. Nevertheless, whereas larvae eat exclusively ambrosia fungi, the adults might also eat wood, which explains all the cosmopolite and saprophytic fungi found in their intestine that were already contaminating tree wood. Also, in mycangia, where specific fungi for this interaction are transported, ambrosia fungi are expected to be present. In both cases, intestine and mycangia, *Raffaelea* was the most frequent genus, which supports the fact that some species of this genus are *P. cylindrus* primary ambrosia fungi. The other isolated fungi might be considered auxiliary ambrosia fungi as sustained by Batra (1985) or simply be worldwide saprobes that are frequently present in host tissues.

In conclusion, there is a vast diversity of fungi associated to *P. cylindrus*-cork oak interaction. Some could be determinant to the success of the insect colonization, mainly *Raffaelea* which is its principal ambrosia fungi and also might be pathogenic to host trees.

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