Ambrosia fungi in the insect-fungi symbiosis in relation to cork oak decline

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Summary Ambrosia fungi live associated with beetles (Scolytidae and Platypodidae) in host trees and act as a food source for the insects. The symbiotic relation is important to the colonizing strategies of host trees by beetles. Ambrosia fungi are dimorphic: they grow as ambrosial form and as mycelium. The fungi are highly specialized, adapted to a specific beetle and to the biotope where they both live. In addition other fungi have been found such as tree pathogenic fungi that may play a role in insects' host colonization success. Saprophytic fungi are also present in insects' galleries. These may decompose cellulose and/or be antagonistic to other less beneficial fungi. This paper summarizes the importance of ambrosia fungi and the interaction with insects and hosts. The possibility of the transport of pathogenic fungi by *Platypus cylindrus* to cork oak thus contributing to its decline is discussed.

Key words *Raffaelea*, *Platypus cylindrus*, *Quercus suber,* Ambrosia fungi

Ambrosia fungi form a symbiosis with ambrosia beetles (Scolytidae and Platypodidae) and host trees. Ambrosia beetles cause serious damages to agricultural crops and forests trees [9]. However, many questions remain unanswered. The ambrosia beetle problem is extremely complex because the host plant, beetles and fungi are all involved.

This interaction between beetles and fungi is a result of evolution which optimises the capacity of the insects to utilise the plant as a habitat [25]. The insects-fungi relation is expressed in an ectosymbiosis in which the fungi live outside the body of the insects but are temporarily stored in special organs of ectodermal origin for purposes of dissemination [18]. Insects carry viable inoculum in sac-like structures, called mycangia, located at particular sites which are characteristic of the species [6]. The inoculum is protected from desiccation during the entire life of the beetle and is disseminated into new breeding sites at the time of excavation of the tunnels [6].

This symbiotic relation seems to have a special importance in host trees colonizing strategies and offer several advantages for insects and fungi. Ambrosia fungi (i) constitute a source of nourishment for ambrosia beetles as they cannot feed from wood, (ii) increase tree decline and wood tissues decomposition that allow gallery building, (iii) create a favourable environment for good development of insect descendants and (iv) induce pheromones production [14,35]. The fungi have developed efficient mechanisms of dissemination and a procedure for direct inoculation in favourable hosts [10,35,36].

Ambrosia fungi were grouped by Batra [9] as primary and auxiliary. Primary ambrosia fungi are highly insect species specific, and their distribution corresponds with those of insect symbionts. They are present and dominant in the tunnels where they are eaten by the larvae and isolated regularly from the mycangia of the beetles in the flight stage or when excavating tunnels. These obligate mutualistic fungi are extremely susceptible to drought and are generally not found outside the mycangia or the galleries of the beetles [7]. 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 [9]. Many such opportunistic fungi have mucilaginous spores that can be transmitted externally by diverse invertebrates including insects. Most auxiliary fungi are easy to culture and some have been mistaken for primary ambrosia fungi [25].

Typically, ambrosia fungi are dimorphic: they grow as the ambrosial (yeast-like) form or as mycelium. The larvae and adults secretions may be responsible for the ambrosia form that can be observed after physical contact between the insect and the fungus. Ambrosial cells, under appropriate conditions, give rise to vegetative mycelium and vice-versa [8]. This phenomenon is referred to as pleomorphism [9].

Although fungi associated and carried by insects present common morphological adaptations [11], cladistic

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analysis of characters derived from nuclear encoded small subunit (18S) rDNA sequences show that at least two lineages of fungi have become associated as primary symbionts of mycetophagous beetles: thus not all ambrosia fungi share a common history [12,31].

The taxonomy of ambrosia fungi is somewhat confused and, although most may be placed within four mitosporic genera, *Ambrosiella*, *Raffaelea*, *Monacrosporium* and *Phialophoropsis*, it is clear that many more genera are involved including *Fusarium*, *Acremonium*, *Candida* and *Graphium*. Yeasts such as *Ascoidea* and *Endomycopsis* may also be of some importance [4,6,8,18].

In addition to fungi directly involved in insect nourishment, others have been found associated with them, such as host pathogenic fungi that may play a role in insect selection and host colonization. These are *Botryodiplodia*, *Ceratocystis*, *Graphium*, *Leptographium* and *Ophiostoma* [2,36]. Cladistic studies show that ambrosia fungi, such as species of *Ambrosiella* are closely related to species of either *Ophiostoma* or *Ceratocystis* based on rDNA sequences and supported by patterns of cycloheximide sensitivity [12].

Several saprobes are also frequently present in insects' galleries, such as *Aspergillus* spp., *Penicillium* spp., *Mucor* spp., *Trichoderma* spp., *Gliocladium* spp., *Paecilomyces* sp., *Trichothecium roseum* (Pers:Fr) Link, *Scytalidium* sp., and these have a commensal relationship [13,20,35]. The permanent presence of these fungi associated with the insects indicates their possible importance in the symbiosis, such as decomposers of cellulose and/or antagonism that inhibits the growth of other fungi.

The increase of Scolytidae and Platypodidae infestations in forests indicates that certain alterations have arisen to enable these outbreaks [25,26,34]. *Platypus cylindrus* Fab. is a cork oak (*Quercus suber* L.) xylomycetophagous insect that has come to assume an increasing importance in Portugal and Mediterranean basin countries [5,30]. It is associated with oak decline [32,33]. Until recently damage produced by this insect was limited to dead or weakened trees. The understanding of recent population outbreaks can be based on three assumptions: (i) gradual changes to cork oak dynamics, (ii) changes to the insects and their natural enemies populations dynamics and (iii) development of more specific host colonization mechanisms [33]. Novel fungal symbiosis may contribute to the weakness of the host and create improved conditions for the establishment of insects [35].

The main goal of this work is to define the fungi associated with the insect *P. cylindrus* in relation to *Q. suber* disease and the implications on cork oak decline.

Materials and methods

Infested logs of cork oak trees that exhibit decline symptoms were collected and associated insects captured in fabric traps. Insects' mycangia were observed under a binocular magnifier and compared in both sexes.

The insects were aseptically dissected and their mycangia, gut and parts of the exoskeleton were plated in Malt Extract Agar (MEA, Difco, USA), or MEA added with streptomycin (0.5 g/l) or cycloheximide (0.5 g/l) and incubated at 24±1 °C in the dark [9,13]. Fragments of wood (25 mm^2) from galleries were treated similarly. Pure cultures of each fungi isolate were obtained and identified based on morphological features. Identification at species level was performed at Centraalbureau voor Schimmelcultures (CBS, The Netherlands).

Results

The observation of *P. cylindrus* mycangia showed spheroidal cavities symmetrically laid out as ovoid shape on the insects' prothorax in both sexes. However, these are more numerous in the female than in the male (Figure 1).

Several fungi were isolated from female and male mycangia, guts and exoskeleton and also from cork oak galleries (Table 1). Particular attention was paid to *Raffaelea* isolates, as primary ambrosia fungi of *P. cylindrus* [1,28]. Two different isolates of this genus were obtained: one has obovoid-claviform conidia (Figure 2a) and the other has obovoid-triangular conidia (Figure 2b). Most *Raffaelea* isolates were obtained from MEA plus cycloheximide being the only fungus able to grow on that medium.

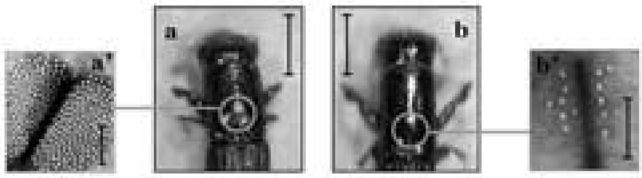

Figure 1. *Platypus cylindrus* mycangia. a) female, b) male (Bars a, b: 1 mm; a', b': 0.1 mm).

Table 1. Fungi isolated from *Platypus cylindrus* mycangia, gut and exoskeleton and cork oak galleries.

Fungi	Gallery	Insect		
		mycangia	gut	exoskeleton
Acremonium sp.	X	X		X
Aspergillus spp.	X	X	X	X
Fusarium sp.	X			
Gliocladium spp.	X	X	X	X
Mucorales	X			X
Nodulisporium sp.	X			X
Paecilomyces sp.	X			X
Penicillium spp.	X	X	X	X
Raffaelea spp.	X	X	X	X
Trichoderma spp.	X	X	X	X

X = detected.

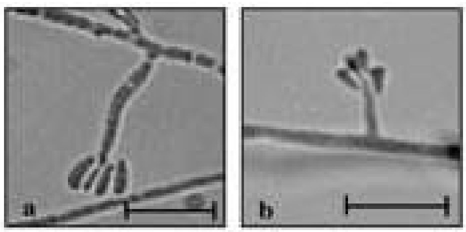

Figure 2. Conidiophores with conidia (a: obovoid-claviform, b: obovoid-triangular) of *Raffaelea* spp. associated with *Platypus cylindrus* (Bars: 10 µm).

Discussion

The higher number of mycangia observed in *P. cylindrus* females is in agreement with earlier works [13,35] and supports the notion that most fungi found in the galleries are transported in the female's mycangia [3].

The relative importance of all fungi isolated is difficult to ascertain. Some are potentially pathogenic to the host tree as others seem to be antagonistic or simply saprobes. Attention needs to be given to those fungi directly involved in host trees colonization in particular.

Raffaelea ambrosiae and *Raffaelea montetyi* are primary ambrosia fungi specific to *P. cylindrus* and associated with cork oak and other Fagaceae [1,28]. So far, twelve species have been described in the genus *Raffaelea*, mostly associated with ambrosia beetles tunnels in the wood and with mycangia of the beetle [24]. Although the sexual state is lacking, observations of conidial development support the placement of the genus *Raffaelea* within the Ophiostomatales [19]. When *Raffaelea hennebertii* Scott & du Toit is excluded from consideration, *Raffaelea* resolves as a monophyletic lineage which forms a sister group to species of the genus *Ophiostoma*, from 18S ribosomal DNA sequences analysis [21]. This assignment also corresponds with data about the dispersal and vector relationship with these fungi [19]. The Ophiostomatales are well-known and economically important sapstaining fungus occurring world-wide on hardwoods and commercially produced pines. Recently, studies of oak decline in Europe showed that the complex *Ophiostoma* / *Ceratocystis* was pathogenic to *Quercus* trees [2,16,17]. *Raffaelea quercivora* Kubono & Ito has proven pathogenicity, being associated with mass mortality of fagaceous trees in Japan, especially *Quercus serrata* and *Quercus mongolia* [23].

Nodulisporium sp. is another potentially important fungus because comparison and phylogenetic analysis of sequences of nucleic acid from several representatives of this genus and a group of xylariaceous fungi with Nodulisporium-like anamorphs revealed that *Nodulisporium* sp. was conspecific with the phytopathogenic species *Biscogniauxia mediterranea* [15]. This fungus is considered to be an opportunistic parasite responsible for necrosis on stems and branches of *Quercus* species and particularly *Q. suber* [27].

The isolated fungi of the genus *Acremonium* and *Gliocladium* can be considered ambrosia fungi as they were isolated from the insect gut and mycangia. These two genera may represent tree pathogenic fungi as they may be relaters to a *Nectria* sp. anamorph [22]. *Acremonium* sp., *Gliocladium* sp., *Trichoderma* sp., *Penicillium* sp. and *Paecilomyces* sp. are all potentially antagonistic fungi that may play a role in fungi control in the insects' galleries. Equally they may be saprobes [20,22].

In order to clarify the insect-fungi-host interaction we intent to continue searching for *P. cylindrus* associated mycoflora and the contribution of each predominant fungus to cork oak decline.

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