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# Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees

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*Cryptococcus neoformans* var. *neoformans* was repeatedly isolated from decaying wood forming hollows in living trees growing in urban areas of Rio de Janeiro, Brazil. A new natural habitat for *C. neoformans* var. *neoformans* has been found that is not associated with specific trees.

## Introduction

Decomposing pigeon droppings and soil contaminated with avian faecal material are the most commonly reported natural substrates for *C. neoformans* var. *neoformans* [1]. The relationship of *C. neoformans* and its teleomorph *Filobasidiella neoformans* to wood and other plant material has been the subject of several recent investigations. Dried samples of leaves and stems are suitable for growth of the fungus under laboratory conditions [2]. *C. neoformans* has been isolated from wood collected from both outside and inside a hollow tree trunk within an aviary at the Antwerp zoological garden [3].

*C. neoformans* var. *neoformans* was isolated from the sawdust of the tropical tree *Entandophragma* spp. in Kinshasa, Zaire [3]. *C. neoformans* var. *gattii* was isolated from a German patient who was exposed to high levels of wood dust while working as a ventilation mechanic in sawmills and woodworking factories [4].

Based upon the hypothesis that wood could be a natural habitat for both varieties of *C. neoformans*, 477 samples of tropical wood were examined. None of the samples was positive for this yeast [5]. The frequent recovery of var. *neoformans* in house dust in Bujumbura, raises the question of what is the source of *C. neoformans* [6]. The authors for this study suggest that the possible existence of the teleomorphic phase is related to a natural niche associated with wood in the environment.

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*Filobasidium floriforme*, the teleomorph for the anamorphic yeast *Cryptococcus albidus*, which is closely related to *Filobasidiella neoformans*, has been described from dead florets of the plume grass *Erianthus gigantus* [7]. Moreover, *F. neoformans* var. *neoformans* has been obtained by streaking sexually compatible isolates of *C. neoformans* onto the inner surface of autoclaved pieces of bark from *Eucalyptus leucoxilon* [8].

The isolation of *C. neoformans* var. *gattii* from environmental sources in 1990, showed an association of this yeast with *Eucalyptus camaldulensis*. The fungus was isolated from debris (wood, bark, leaves, flowers) collected under the canopies of *E. camaldulensis* growing in Australia [9,10]. Unfortunately, the specific association of *C. neoformans* var. *neoformans* with specific types of plant material under natural conditions has not yet been established.

Based on the previous isolation of *C. neoformans* var. *neoformans* from plant debris contained within a rotten core of the Java plum tree (*Syzygium jambolana*) [11], we present our investigations regarding the isolation of *C. neoformans* var. *neoformans* from decomposing wood contained within hollows of living trees.

## Materials and methods

### Wood sampling

Thirty-one different species of living trees in urban areas of Rio de Janeiro, Brazil, having hollows with decomposing wood, were chosen for investigation. Fourteen trees were located on the campus of the Oswaldo Cruz

Foundation and the remaining 17 in the northern areas of the city. Sixty-two samples were collected by scraping the inner decaying wood of the hollows and the outer bark of the trunks of each tree.

### Wood processing

After homogenization, 1 g of each sample was suspended in 50 ml of sterile physiological saline with 0·2 g $l^{-1}$ chloramphenicol, vigorously shaken for 5 min and then allowed to settle for 30 min. The supernatant was aspirated, inoculated onto niger seed agar (NSA) medium, incubated at room temperature and observed for 5 days; 0·1 ml of supernatant was placed on each plate, 10 plates for each sample were prepared. Using 1 ml of supernatant, 28 samples obtained from 14 of the trees were simultaneously inoculated intraperitoneally into five albino Swiss mice weighing 18–20 g. One control animal was used for each sample. After 4 weeks of incubation, the animals were killed by ether inhalation and submitted to necropsy. Liver, spleen and brain samples were inoculated onto Sabouraud glucose agar medium (Difco, USA) with chloramphenicol (0·5 g $l^{-1}$), incubated at room temperature and observed for 10 weeks.

### Air sampling

The Biotest RCS centrifugal air sampler (Biotest AG, Frankfurt, Germany) with plastic strips containing NSA medium was used to detect airborne propagules of *C. neoformans* on the campus of the Oswaldo Cruz Foundation.

### Identification of C. neoformans isolates

Brown colonies on NSA medium were identified by the use of thermotolerance at 35 °C, cycloheximide sensitivity, urease production, fermentation tests (glucose, galactose, sucrose, maltose, lactose and raffinose) and assimilation tests (galactose, sucrose, maltose, trehalose, lactose, xylose, mannitol, inositol and potassium nitrate) performed by the Wickerham technique [12]. Canavanine-glycine-bromthymol blue medium (CGB) was used to determine the variety of the isolates [13].

## Results

Eight of 31 tree hollows yielded colonies of *C. neoformans* var. *neoformans*: five pink shower trees (*Cassia grandis*), November shower tree (*Senna multijuga*) and two fig trees (*Ficus microcarpa*). All positive samples were obtained from decaying wood on the inner surface of the hollows; no other samples studied were positive (Table 1).

**Table 1** *C. neoformans* var. *neoformans* isolated from decaying wood in cavities in hollow trunks in Rio de Janeiro, Brazil

| Tree identification | Hollows studied/ positive | First isolation on NSA plates ($10^3$ CFU) |
|---|---|---|
| Pink shower tree/*Cassia grandis* | 7/5[a] | 2/0·1/0·3/3·4 |
| November shower/*Senna multijuga* | 2/1 | 0·1 |
| Fig tree/*Ficus microcarpa* | 12/2 | 0·3/0·5 |
| Java plum/*Syzygium jambolana* | 5/0 | – |
| Munguba/*Bombax munguba* | 2/0 | – |
| Coconut palm/*Cocos nucifera* | 1/0 | – |
| Mango tree/*Mangifera indica* | 2/0 | – |
| Total | 31/8 | |

[a]One was positive only by animal inoculation.

The intraperitoneal inoculation experiments using mice yielded identical results, except for one experiment involving a *Cassia* tree hollow, which was positive using animal inoculation and negative by the direct plating method.

On the campus of the Oswaldo Cruz Foundation, 11 hollows were negative, but three hollows in *C. grandis* trees adjoining each other along a road were positive. One of the trees was blown laying on the ground and exhibited advanced trunk destruction. Serial scrapings performed on the inner surfaces of the remaining two hollows in *C. grandis* (A and B) yielded *C. neoformans* var. *neoformans* from January 1992 until December 1993. In hollow A (Fig. 1), 16 of 22 samples were positive (Fig. 2). Inside the hollow we observed a continuous process of wood decay, resulting in a progressive enlargement of the hollow. The concentration of *C. neoformans* cells observed in these samples ranged from 0·05 to 3·5 × $10^3$ CFU $g^{-1}$. A peak of 7·5 to 8 × $10^4$ CFU $g^{-1}$, observed during September–November 1993, was followed by a partial loss of the upper wall cavity with a lowering of the CFU $g^{-1}$ in subsequent samples. Moreover, 83 *C. neoformans* strains obtained from hollow A were tested using the CGB test, all being of the var. *neoformans*. In hollow B, a partially sheltered cavity at the base of the trunk, 11 of 22 samples were positive (Fig. 2). The concentration range of CFU $g^{-1}$ of *C. neoformans* was lower than that observed within hollow A. Ten strains obtained from hollow B were CGB-negative, being recognized as the var. *neoformans*.

Using the Biotest RCS centrifugal air sampler and NSA strips we conducted 53 air sampling experiments. Air (4240 l) under and around the two positive trees was sampled during their flowering period. Sixty air sampling experiments (4800 l) were also conducted during the flowering of other *Cassia* trees in the same area. All 113

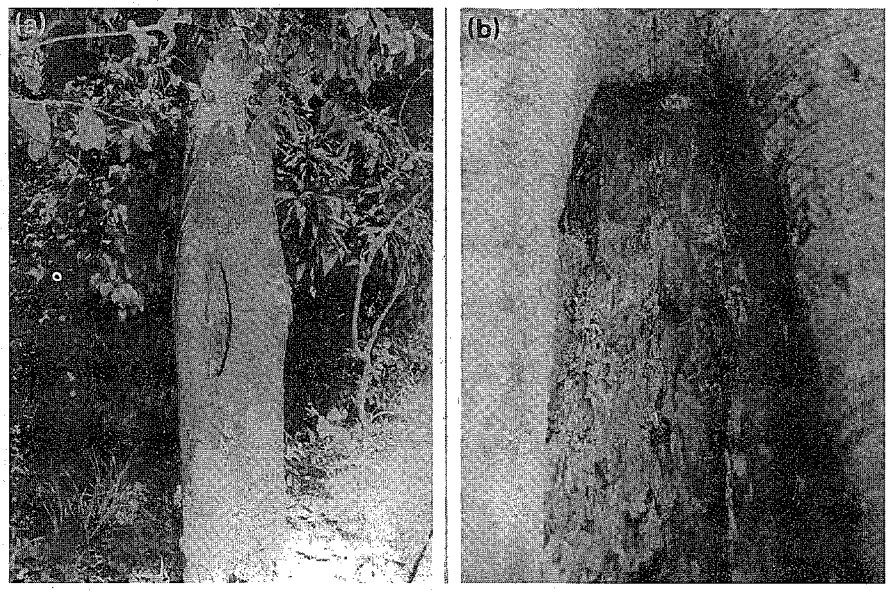



**Fig. 1** (a) Hollow trunk of a pink-shower tree (*Cassia grandis*). (b) Inside view of the same hollow showing decaying wood positive for *C. neoformans* var. *neoformans*.

air samplings were negative, except for the one obtained at the entrance of hollow A.

During the study no pigeons were observed nesting nor roosting in the positive hollows. Some birds, such as sparrows and tyrant fly-catchers, were observed roosting on tree branches. However, inside the two positive hollows and other negative hollows located on the same road, most contained *Polistes canadensis* wasp nests.

One year after the first samplings, the 11 negative hollows remained negative using both the direct plating method and animal inoculation.

## Discussion

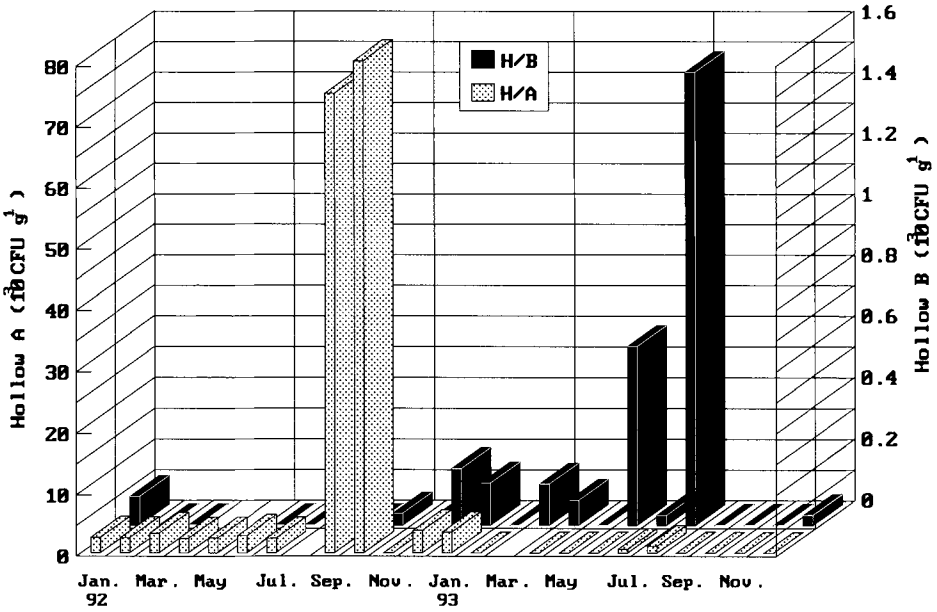
*C. neoformans* var. *neoformans* was isolated from decaying wood present on the inner surface of hollows occurring in the trunks of several different living trees: pink shower trees (*Cassia grandis*), November shower tree (*Senna multijuga*) and fig trees (*Ficus microcarpa*). The long period of recovery of the yeast from hollows of *Cassia* trees suggests colonization of these microenvironments. The presence of airborne propagules of the fungus were detected only inside the hollow. The negative results from the air sampling experiments does not exclude the presence of a small number of *C. neoformans* propagules that may not have been detected by this method. Sampling times and locations may not have been optimal for the recovery of the yeast. Our findings differ from the isolation of *C. neoformans* var. *gattii* from *E. camaldulensis* in Australia, where a large number of airborne propagules of the fungus were observed during the flowering period for the trees sampled.

All positive samples were fibrous, discoloured wood, suggesting partial lignin and xilan degradation and advanced stages of decay. Byproducts of wood decomposition may support saprophytic growth of yeasts, including *C. neoformans*. The isolation of the yeast from partially delignified wood [14] tends to support this possible hypothesis as well as the fact that *C. neoformans* has phenoloxidase activity. The ligninolytic system is largely oxidative and non-specific. The evidence for the role of phenoloxidases in lignin degradation has been studied in wood rot fungi, especially white rot [15]. Further studies are necessary to evaluate *C. neoformans* as a potential degrading agent of lignin polymers.

Hollows of living trees provide environments that are sheltered, damp and probably less exposed to changes in climatic conditions. Recently, *C. neoformans* var. *gattii* was isolated from bark and wood debris collected within a hollow of *Eucalyptus tereticornis*. Three other isolates of the var. *gattii* were obtained from sheltered, damp areas associated with humus in natural stands of *E. tereticornis*, a species closely related to *E. camaldulensis* [16].

We demonstrated a new natural habitat of *C. neoformans* var. *neoformans*. It appears that *C. neoformans* var. *neoformans* is not associated with a particular tree, but rather with a specialized niche resulting from the natural biodegradation of wood that provides a favourable substratum for its growth. Moreover, it is possible that the teleomorphic form *F. neoformans* may occur in this type of habitat.

All the hollows described in this study were formed in the heartwood of the trunks. They did not contain slime flux, which can support the growth of many

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H/B | 0.1 | 0 | 0 | - | 0.2 | 0 | 0 | 0 | - | 0.05 | 0 | 0.2 | 0.15 | 0 | 0.15 | 0.1 | 0 | 0.6 | 0.05 | 1.5 | 0 | 0 | 0 | 0.05 |
| H/A | 2 | 2 | 2.5 | 1.8 | 2 | 2.5 | 2 | - | 75 | 80 | 0.05 | 3.5 | 2.5 | 0 | - | 0 | 0 | 0 | 0.2 | 0.9 | 0 | 0 | 0.05 | 0.1 |

**Fig. 2** *Cryptococcus neoformans* var. *neoformans* growth ($10^3$ CFU $g^{-1}$) on decomposing wood scraped inside two positive hollows of *Cassia grandis* trees.

microorganisms such as yeasts. Additional studies are necessary before we will have a clear understanding of the dynamics associated with this habitat.

More recently, the saprophytic growth on *Eucalyptus* wood was suggested as the principal source of *C. neoformans* var. *gattii* in nature, particularly *Eucalyptus* containing large amounts of lignin and polyphenols such as that found in the wood of *E. camaldulensis* [17]. The endophytic relation of var. *gattii* to a specific host tree deserves more study.

Repeated examination of avian droppings in a zoo led to the conclusion that parrots and budgerigars may provide through their faecal material important natural sources of *C. neoformans* var. *neoformans* [3]. Moreover, psittacine birds have a close relationship with wood through their habit of nesting in hollow trunks and nibbling on wood.

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