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Growth and Mating of *Cryptococcus neoformans* var. *grubii* on Woody Debris

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Abstract A total of 36 *Cryptococcus neoformans* strains originating from South Africa were screened for wood degrading enzymes. All strains tested positive for cellulase activity while none where capable of xylan degradation. Three *C. neoformans* var. *grubii* strains, originating from clinical and environmental samples, representing the same genotype (VNI/AFLP1—*C. neoformans* var. *grubii*) and MATα, were evaluated for growth on debris of two common tree species in South Africa: *Acacia mearnsii* and *Eucalyptus camaldulensis*. The mating capability of all the *C. neoformans* strains was evaluated on similar debris. Strains grown on *A. mearnsii* yielded *substantially* greater yeast populations. A total of 26%, 6%, 46%, and 80% of the 36 *C. neoformans* strains tested were either able to mate or develop filaments when crossed on *A. mearnsii* and *E. camaldulensis* debris, V8 juice, and yeast carbon base (YCB) agar, respectively. Filamentation and monokaryotic fruiting was observed in 3% of strains when *C. neoformans* was cultured on either *A. mearnsii*, *E. camaldulensis* debris, or YCB. The results indicate that this fungus is capable of completing its life cycle and can produce basidiospores on woody debris. In the future, these findings should be considered when studying the epidemiology, microbial ecology, and proposed infection process of this global pathogen.

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Introduction

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic fungal pathogen responsible for causing meningitis in immunocompromised individuals [9, 19, 34], particularly those infected with the HIV and suffering from AIDS. The incidence of infection among these individuals is estimated at approximately 10% [10] with the majority of these cases being attributed to *C. neoformans* var. *grubii* [26, 34].

The pathogen belongs to the basidiomycetous order *Tremellales* and occurs primarily as haploid unicellular yeasts [4]. The fungus, however, is capable of maintaining a hyphal state that forms characteristic *Tremella*-like haustorial branches. Basidiospores are formed apically on the basidium either via dikaryotic, monokaryotic fruiting, or same sex cell fusions [20, 24, 25, 29, 46, 47]. The last of these has been observed in *C. neoformans* and the closely related species *Cryptococcus gattii*, where naturally occurring populations of both these pathogens display levels of sexual recombination, despite being comprised solely of a single mating type, MATα [8, 23, 41]. This would provide further means of basidiospore production that along with desiccated yeast cells [15, 36, 43] may represent infectious propagules.

The exact environmental origin of the infectious propagules remains unclear. Recently, mating of *C. neoformans* strains, as well as basidiospore production, were observed

when the fungus was cultured on pigeon guano as well as certain plant species, both of which are suspected habitats of this pathogen [38, 48]. In addition, similar to most *Tremellales* species, *C. neoformans* has been frequently isolated from habitats rich in lignocellulosic material, including fir trees, almond trees, eucalyptus trees, woody debris, and decaying wood [14, 21, 27, 39, 45]. The fungus produces laccase [50], an enzyme that is known to be implicated in lignin degradation, as it catalyzes oxidation of the phenolic components of this complex monomer [33]. Also, a cellulase gene was detected within the genome of *C. neoformans* [31], pointing to decaying wood as the pathogens' possible natural habitat. Although current research has shown that certain plant products are capable of enhancing the mating efficiency of *Cryptococcus* [48], it remains unclear if *C. neoformans* displays any ecological association with a particular tree species. Also, it is unknown whether *C. neoformans* is capable of producing and maintaining all of its developmental stages on a woody substrate devoid of other microbes or whether similar to other members of the *Tremellales*, it requires various microbial interactions to do so [5].

The objectives of this study were to firstly test whether clinical and environmental isolates of *C. neoformans* var. *grubii* are capable of growth when cultured on woody debris, derived from tree species common in South Africa and, if so, whether these isolates are capable of producing and maintaining a hyphal phase and completing their life cycle under these conditions.

Materials and Methods

Strains and Culture Conditions

The reference strains—*C. neoformans* var. *neoformans* CBS132, CBS10511 (JEC20), and CBS10513 (JEC21); *C. neoformans* var. *grubii* CBS10515 (H99) and CBS9172—were obtained from the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. *C. neoformans* var. *grubii* isolates, CBS10571–CBS10574, were obtained from environmental sources in South Africa and deposited into the culture collection of the CBS, Utrecht, The Netherlands. Clinical *C. neoformans* var. *grubii* strains MRC8855–MRC8868, MRC8878–MRC8884, and MRC8887–MRC8891 and *C. neoformans* var. *neoformans* MRC8856 were obtained from the culture collection of the Medical Research Council (MRC), PROMEC Unit, Tygerberg, South Africa. Three more clinical *C. neoformans* var. *grubii* strains—ABO-C13, ABO-C14, and ABO-CF—were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa. The reference strains *Aspergillus niger* ABO-AN1, *Cryptococcus laurentii* ABO-1A, *Cryptococcus podzolicus* ABO-5A, *Pycnoporus* sp. ABO-P1, *Saccharomyces cerevisiae* ABO-SC1, and *Trichoderma reesei* ABO-TR1 were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa. All strains were maintained by periodic transfer to yeast peptone glucose (pH 5.5) agar supplemented with 200 mg/L chloramphenicol (Sigma Aldrich, Germany) [49] and incubated at 22°C.

Cellulase Activity

All the *C. neoformans* strains, excluding the reference strains *C. neoformans* var. *neoformans* JEC20 and JEC21 and *C. neoformans* var. *grubii* CBS9172, were inoculated onto carboxymethylcellulose agar (CMC; Sigma Aldrich, Germany) plates and incubated at 22°C for 1 week [12]. Plates were stained using 0.1% Congo Red solution (B&M Scientific, South Africa) for 15 min and destained with 1 M sodium chloride (NaCl, Merck Biolab, South Africa) solution for 15 min. Plates were examined for the production of clear zones indicative of enzyme activity. *Trichoderma reesei* ABO-TR1 served as positive control, while *S. cerevisiae* ABO-SC1 served as negative control. All plates were inoculated in triplicate.

Xylanase Activity

All the *C. neoformans* strains, excluding the reference strains *C. neoformans* var. *neoformans* JEC20 and JEC21 and *C. neoformans* var. *grubii* CBS 9172, were inoculated on Remazol Brilliant Blue-Xylan (Sigma Aldrich, Germany) covalently linked to beechwood 4-*O*-methyl-D-glucurono-D-xylan (Sigma Aldrich, Germany) and incubated at 22°C for 1 week [16]. Plates were examined for the production of clear zones indicative of xylanase activity. *A. niger* ABO-AP1 served as positive control, while *S. cerevisiae* ABO-SC1 served as negative control. All plates were inoculated in triplicate.

Components Used to Construct the Woody Substrate

Acacia mearnsii and *Eucalyptus camaldulensis* trees from the Stellenbosch area in the Western Cape, South Africa, were felled. Each tree was split into four components: the main stem, branches, twigs, and leaves. The components were then reduced in size by means of chipping and milling and dried for 24 h at 100°C. Components were further reduced in size, with a Retsch (ZM-1) ultra centrifugal mill with a 6.0-mm sieve, resulting in roughly the same particle size distribution for each of the four components. To obtain the woody debris, the four components were subsequently mixed in a ratio of 50% (*w*/*w*) main stem,

16% (*w*/*w*) branches, 16% (*w*/*w*) twigs, and 16% (*w*/*w*) leaves.

Red clay, donated by Corobrik Brick Works (Pty) Ltd., Stellenbosch, Western Cape, South Africa, was dried in an oven at 100°C for 48 h, ground using a pestle and mortar, and sieved using a 2.0-mm sieve. Silica sand was washed using a 1-M hydrochloric acid solution, rinsed twice using distilled water, and dried at 100°C for 48 h.

Physicochemical Analysis of the Woody Substrate

The substrates used during the experimentation were either woody debris of *A. mearnsii* or *E. camaldulensis*, or a woody substrate comprising of woody debris and red clay or silica sand. Where two components were combined to produce the woody substrate, a ratio of 1:1 (*w*/*w*) was applied. Field capacity, defined as the water content when all free water has been drained from soil through gravity, was gravimetrically determined for both the woody debris and woody substrates according to the methods described in literature [1, 7].

The organic carbon of the woody debris and each woody substrate was determined using the Walkey–Black method [37], while the total nitrogen content of each substrate was determined by digesting it in a LECO FP-528 nitrogen analyzer. Bray-2 extract [44] was used to determine the phosphate, while di-ammonium ethylenediaminetetraacetic acid extract [2] was used to determine copper, zinc, and manganese content of each woody substrate. The pH was determined in a solution of KCl, while boron was determined in a hot water extract [17]. The exchangeable cations (calcium, magnesium, potassium, and sodium) were determined using a 1-M ammonium acetate extract, and the cation exchange capacity of each woody substrate was calculated [13].

Preparation of Woody Debris Solid State Cultures Containing *C. neoformans* var. *grubii*

Screw capped glass jars (50 mL) were used to house the solid state cultures. A total of 10 g of either woody debris or premixed woody substrate was added to each glass jar, autoclaved (121°C, 15 min), and dried in an oven at 50°C for 48 h.

Each series of woody debris and woody substrates were subsequently inoculated with a single *C. neoformans* var. *grubii* strain, namely H99, CBS10571, or MRC8890, obtained from pre-inoculum cultures, resulting in a final concentration of 7.5×10^5 yeast cells per g of woody debris or woody substrate. The moisture content of each of the resulting microcosms was adjusted to field capacity using sterile distilled water; jars were vortexed and incubated in the dark at 26°C in a sealed Tupperware container (100×600×300 mm). Sterile distilled water was added to the latter one resulting in a moist chamber.

After an hour, a total of 1-g solid state culture was sampled from each jar and yeasts were enumerated using dilution plates with malt extract agar (Merck Biolab, South Africa) supplemented with 200 mg/L chloramphenicol (Sigma Aldrich, Germany). Plates were incubated at 30°C for 48 h before the colonies were counted. Enumeration of yeasts was repeated on days 4, 7, 10, 14, 20, and 30. Data obtained were plotted on a log graph using Microsoft Office Excel 2003.

Fruiting of *C. neoformans* on Woody Debris, V8 Juice, and Yeast Carbon Base Agar

Four different mating media were used to evaluate the mating capacity of the *C. neoformans* strains on a solid medium. The first two comprised of *A. mearnsii* or *E. camaldulensis* debris (200 g/L) suspended in 2% water agar (*w*/*v*; Merck Biolab, South Africa) and autoclaved (121°C; 15 min). V8 juice agar was prepared as previously described [11], while yeast carbon base (YCB) (Difco, USA) solution was prepared as per the manufacturer's instructions; however, no additional nitrogen source was included. The YCB suspension was subsequently added to autoclaved (121°C; 15 min) 2% (*w*/*v*) water agar [49]. Agar plates were prepared by dispensing approximately 20-mL cooled molten agar media into Petri dishes (90 mm diameter).

All environmental and clinical *C. neoformans* strains, as well as the type strains *C. neoformans* var. *neoformans* CBS132 and *C. neoformans* var. *grubii* H99, were each inoculated onto a series of 16 individual agar plates, comprising of four *A. mearnsii*, four *E. camaldulensis*, four V8 juice, and four YCB plates. The cultures on three of the four plates of a particular medium were then crossed with one of three *C. neoformans* reference strains namely, JEC20 (*a*/D), JEC21 (α/D), and CBS9172 (*a*/A). The fourth plate remained as a monoculture in order to detect monokaryotic fruiting. All plates were sealed using Parafilm "M" (Pechiney Plastic Packaging, Chicago, IL, USA) and cultured at 25°C in an incubator (Function Line, Heraeus Instruments) for 3–5 weeks in the dark. Each week, the plates were inspected macroscopically and microscopically for the formation of hyphae, clamp connections, and basidiospores [11, 25, 49].

Results

Screening for Cellulase and Xylanase Activity

Although the clear zones on the Congo red stained CMC plates were limited to the immediate vicinity of the

colonies, all the *C. neoformans* strains tested positive for cellulase activity. None of the strains tested positive for xylanase production.

Physicochemical Analysis of the Woody Substrate

Woody debris displayed a higher field capacity than the woody substrates comprising of woody debris in combination with either clay or silica sand (Table 1). Chemical analysis revealed that *A. mearnsii* debris displayed significantly lower levels of the trace elements magnesium, manganese, and zinc than *E. camaldulensis* debris (Table 1); however, it contained more than twice the amount of nitrogen than the latter debris. From Table 1, it is also obvious that the addition of either clay or sand to the woody debris resulted in dilution of the nutrients.

Survival of *C. neoformans* on Woody Substrate

All three strains representing *C. neoformans* var. *grubii* were able to grow and survive within the woody substrates up until the end of the incubation period (Fig. 1). Of the different woody substrates used in the study, woody debris and red clay supported the smallest yeast population, while the growth curve obtained with the latter substrate (Fig. 1B) was more erratic than on those obtained with the other substrates (Fig. 1A,C). The substrate comprising of woody debris and silica sand supported greater yeast populations than those containing clay (Fig. 1B,C).

Fruiting of *C. neoformans* on Woody Debris, V8 Juice, and Yeast Carbon Base Agar

Thirty-six *C. neoformans* strains were evaluated with regard to their mating ability when mated on woody debris, V8 juice, and YCB agar. A total of 26% and 6% of the *C. neoformans* strains displayed dikaryotic fruiting or filamentation when crossed on *A. mearnsii* (Table 2) and *E. camaldulensis* (Table 3) debris, respectively. Hyphal development originated from the edges of the colony and spread across the media. In some cases, hyphal patches developed in the center of the yeast colony, although lateral growth remained limited. Interestingly, older hyphae developed a brown pigmentation possibly as a result of melanin formation or other pigments originating from the woody debris. The formation of basidia and basidiospores was confirmed using a compound microscope (Fig. 2).

Three percent of the *C. neoformans* strains displayed limited filamentation when cultured on *A. mearnsii* (Table 2) and *E. camaldulensis* (Table 3) debris, respectively; however, the formation of either fused or unfused clamp connections, basidia, and basidiospores was not evident. Those strains showing no fruiting structures when mated on

Table 1 Chemical properties, as well as field capacity, determined for six combinations of woody debris used during the experimentation

Substrate	Field capacity (%)	pH	P (mg/kg)	CEC (cmol/kg)	Exchangeable cations (cmol/kg)				Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	B (mg/kg)	N (%)	C (%)
					Na	K	Ca	Mg						
Eucalyptus[a]	65.2	4.3 (0.0)	160 (10.8)	35.1 (1.7)	3.2 (0.2)	8.6 (0.3)	5.4 (0.6)	12.3 (0.9)	1.3 (0.3)	8.5 (0.3)	305.8 (10.5)	7.4 (1.3)	0.4 (0.04)	53.0 (0.2)
EC	51.1	4.3 (0.0)	90.3 (11.2)	22.9 (0.2)	2.1 (0.04)	5.1 (0.1)	4.3 (0.2)	8.2 (0.1)	1.9 (0.6)	6.1 (0.4)	226.6 (14.6)	3.8 (0.5)	0.2 (0.02)	27.0 (3.8)
ES	40.2	4.4 (0.06)	92.7 (11)	19.1 (0.6)	1.6 (0.2)	4.5 (0.4)	3.5 (0.4)	6.1 (0.5)	2.9 (0.3)	7.3 (0.6)	164.3 (11.7)	4.6 (0.5)	0.2 (0.02)	30.4 (8.3)
Acacia[b]	62.1	4.5 (0.0)	86.7 (20.4)	27.6 (1.9)	3.0 (0.2)	8.2 (0.4)	6.0 (1)	6.5 (0.5)	1.8 (0.5)	5.0 (0.4)	11.3 (1.7)	8.5 (0.9)	1.0 (0.1)	52.9 (0.5)
AC	51.4	4.6 (0.0)	38 (6.2)	17.4 (1.3)	2.0 (0.1)	4.7 (0.3)	3.7 (0.3)	4.8 (0.3)	1.4 (0.3)	3.4 (0.1)	32.7 (0.5)	5.6 (0.4)	0.5 (0.05)	29.9 (0.9)
AS	41.4	4.7 (0.06)	56 (4)	15.7 (1.4)	1.8 (0.2)	4.7 (0.5)	3.0 (0.05)	3.7 (0.5)	2.4 (0.3)	5.2 (0.4)	6.5 (0.2)	5.4 (0.7)	0.6 (0.1)	24.7 (1.3)

Values represent the mean of three repetitions while values in parentheses represent standard deviations
EC-*E. camaldulensis* and red clay, ES-*E. camaldulensis* and silica sand, AC-*A. mearnsii* and red clay, AS-*A. mearnsii* and silica sand, *CEC* cation exchange capacity
[a] *E. camaldulensis* debris
[b] *A. mearnsii* debris

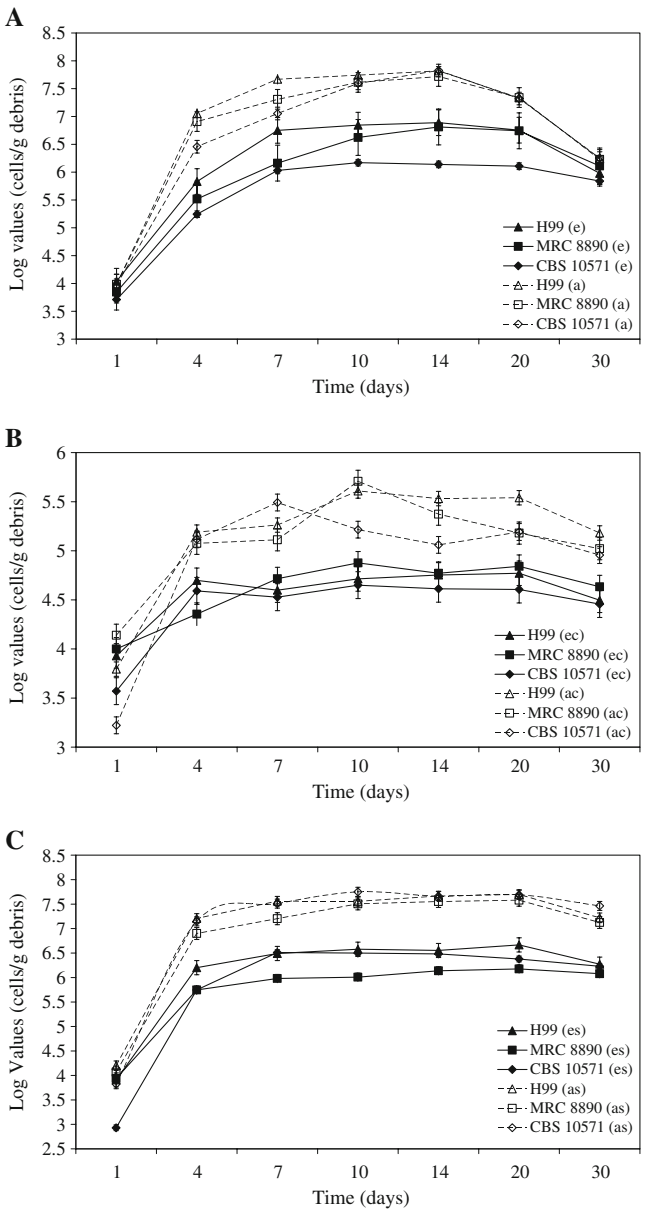

Figure 1 Growth curves of *C. neoformans* var. *grubii* H99, MRC8890 and CBS10571, in solid state cultures containing different substrates, all with a moisture content equal to 100% field capacity. The substrates were **A** the woody debris of *E. camaldulensis* (*e*) or *A. mearnsii* (*a*), **B** clay and either *E. camaldulensis* (*ec*) or *A. mearnsii* (*ac*) debris, and **C** silica sand and either *E. camaldulensis* (*es*) or *A. mearnsii* (*as*) debris. *Values* represent the mean of three repetitions while the *bars* denote standard deviations

woody debris were capable of yeast-like growth, displaying brown, "jelly-like" colonies with a texture similar to the fruiting bodies characteristic of the *Tremellales*.

A total of 46% and 80% of all *C. neoformans* strains displayed dikaryotic fruiting or filamentation when crossed on V8 juice (Table 4) and YCB (Table 5) agar, respectively. Only 3% of the *C. neoformans* strains displayed monokaryotic fruiting when cultured on YCB (Table 5). None of *C. neoformans* strains displayed fruiting when mated on any medium with *C. neoformans* var. *neoformans* JEC21.

Discussion

The repeated isolation of *C. neoformans* strains from woody habitats would indicate that this fungal pathogen is capable of utilizing the components of lignocellulose that consist primarily of cellulose, hemicellulose, and lignin. Prompted by the identification of a cellulase gene within the *C. neoformans* genome [31], as well as the ability of *C. gattii* to survive on wood agar [40], our research indicates that although *C. neoformans* is incapable of degrading xylan, a component of hemicellulose, this pathogen does posses cellulase activity. However, cellulose was only degraded within the immediate vicinity of the *C. neoformans* colonies. This may suggest that lignin and cellulose, which are large polymers unable of traversing the cell membrane [33], are both degraded by enzymes covalently bound to the cell wall, namely laccase [50] and cellulase.

Interestingly, although species of the order *Tremelalles* are commonly associated with decaying wood, a large number are also known to be mycoparasitic, producing haustorial branches, and are often found in association with other wood degrading fungi [5]. *C. neoformans* is capable of producing haustorial branches [25], and it has already been found to interact parasitically with certain plant species [48]. Our own research complements these findings since we determined that *C. neoformans* var. *grubii*,

Table 2 Fertile *C. neoformans* strains when crossed with suitable reference strains on media containing the woody debris of *A. mearnsii*

Accession Number	Serotype	Mating type	Crossing on A. mearnsii debris		
			CBS9172 (a/A)	JEC20 (a/D)	Haploid fruiting
H99	A	MATα	+	+	–
CBS[a] 10571	A	MATα	–	+	–
CBS 10573	A	MATα	–	+	–
CBS 10574	A	MATα	+	+	+[c]
MRC[b] 8891	A	MATα	–	+	–
MRC 8883	A	MATα	–	+	–
MRC 8857	A	MATα	–	+	–
MRC 8858	A	MATα	–	+	–
MRC 8865	A	MATα	–	+	–

+ Positive for mating as both basidiospores and fused clamp connections were observed under the microscope (Nikon Eclipse E2000, Japan)

[a] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

[b] Medical Research Council, Tygerberg, South Africa

[c] Limited hyphal growth observed under the microscope (Nikon SMZ-10A, Japan)

Table 3 Fertile *C. neoformans* strains when crossed with suitable reference strains on media containing the woody debris of *E. camaldulensis*

Accession number	Serotype	Mating type	Crossing on *E. camaldulensis* debris		
			CBS9172 (a/A)	JEC20 (a/D)	Haploid fruiting
CBS[a] 10574	A	MATα	–	+[c]	+[c]
MRC[b] 8865	A	MATα	–	+[c]	–

[a] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
[b] Medical Research Council, Tygerberg, South Africa
[c] Limited hyphal growth observed under the microscope (Nikon SMZ-10A, Japan)

originating from both environmental and clinical sources, was also capable of saprophytic growth on both *A. mearnsii* and *E. camaldulensis* debris. This growth occurred in the absence of additional nutrients and other microbes. Thus, these findings confirm that depending on the natural substrate available to the yeast, it may either act as a parasite or saprophyte.

Although all three *C. neoformans* strains showed similar growth curves on the same substrate, the overall yeast numbers reached were substantially higher on substrates containing *A. mearnsii* debris than on those containing *E. camaldulensis* debris (Fig. 1A). This may be as a result of the higher nitrogen levels present in the *A. mearnsii* woody material (Table 1). From an ecological perspective, nitrogen is considered to be an important limiting factor for many ecosystems [3]. The ability of *A. mearnsii* to fix nitrogen may implicate this tree species as an important natural nitrogen reservoir [18].

Interestingly, the combination of woody debris and silica sand was capable of maintaining and supporting relatively high yeast populations, despite its lower nutrient content when compared to the woody debris as sole nutrient source. The fact that the yeasts appeared to remain viable for a longer period in this combined substrate may be due to the release of toxic components, such as tannins, from the woody debris [35, 42]. Also, since the field capacity of the woody debris and silica sand combinations was lower than that of the other substrates (Table 1) and *C. neoformans* is known to be an obligate aerobic fungus [25], growth in the woody debris and clay containing substrates could have been impaired as a result of oxygen limitation.

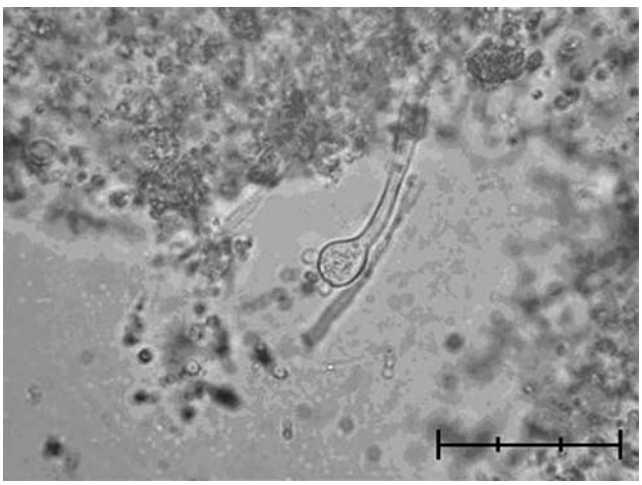

Figure 2 The production of basidiospores and basidia during mating of *C. neoformans* var. *grubii* CBS10573 and *C. neoformans* var. *neoformans* JEC20 when crossed on *A. mearnsii* viewed at ×1,000 magnification (Nikon Eclipse E2000, Japan). The *scale bar* represents a total length of 30 μM

The ability of *C. neoformans* to reproduce asexually when cultured solely on woody substrate raised questions as to whether or not this yeast is capable of completing its life cycle when cultured on woody debris. In the case of *C. gattii*, both mating types have been isolated from a single tree. These isolates, however, showed no or little genetic diversity, indicating no or limited recombination [22]. Although similar observations have been made with regards to natural *C. neoformans* populations [30], the capability of *C. neoformans* to form a hybrid diploid between variety *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* [6, 11, 28] would serve as an additional pathway with regards to basidiospore development, as well as increasing the overall genetic diversity of this pathogen.

Our research indicated that *C. neoformans* is indeed capable of reproducing sexually when cultured on woody debris as sole nutrient source. The majority of mating on woody debris occurred on media containing *A. mearnsii* as nutrient source (Table 2; Fig. 2) while notably less mating occurred on the media containing *E. camaldulensis* (Table 3), indicating that the tree species may impact on the mating frequency of *C. neoformans*. Similarly, Xue and coworkers were able to demonstrate that certain plant products were able to enhance the mating efficiency of *Cryptococcus* [48]. In our study, the frequency of mating among the cryptococcal strains increased notably under standard laboratory conditions (Tables 4 and 5), when compared to mating on woody debris (Tables 2 and 3).

It should be noted that all fertile strains observed on both the woody debris as well as the V8 juice agar represented *C. neoformans* var. *grubii*. On woody debris, however, only two of these strains mated with the reference strain of the

Table 4 Fertile *C. neoformans* strains when crossed with suitable reference strains on V8 juice agar

Accession number	Serotype	Mating type	Crossing on V8 juice agar		
			CBS 9172 (a/A)	JEC20 (a/D)	Haploid fruiting
H99	A	MATα	–	+	–
CBS[a] 10571	A	MATα	+	+	–
CBS 10572	A	MATα	–	+	–
CBS 10573	A	MATα	+	+	–
MRC[b] 8888	A	MATα	+	+	–
MRC 8890	A	MATα	–	+	–
MRC 8879	A	MATα	+	+	–
MRC 8880	A	MATα	–	+	–
MRC 8883	A	MATα	+	–	–
MRC 8857	A	MATα	–	+	–
MRC 8861	A	MATα	–	+	–
MRC 8864	A	MATα	–	+	–
MRC 8865	A	MATα	–	+	–
MRC 8866	A	MATα	–	+	–
MRC 8867	A	MATα	+	–	–
MRC 8868	A	MATα	–	+	–

+ Positive for mating as both basidiospores and fused clamp connections were observed under the microscope (Nikon Eclipse E2000, Japan)
[a] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
[b] Medical Research Council, Tygerberg, South Africa

same serotype, namely *C. neoformans* var. *grubii* CBS9172 (Tables 2 and 3). This inability or delayed mating on woody debris could be a result of continual culturing of the reference strain *C. neoformans* var. *grubii* CBS9172. Alternatively, the impaired mating between *C. neoformans* var. *grubii* strains of opposite mating types may give an indication as to the mating type bias seen among environmental isolates. Interestingly, compared to mating on

Table 5 Fertile *C. neoformans* strains when crossed with suitable reference strains on YCB agar

Accession number	Serotype	Mating type	Crossing on YCB media		
			CBS 9172 (a/A)	JEC20 (a/D)	Haploid fruiting
H99	A	MATα	+	+	–
CBS[a] 132	A	MATα	+[c]	–	–
CBS 10571	A	MATα	+	+	–
CBS 10572	A	MATα	–	+	–
CBS 10573	A	MATα	+	+	–
CBS 10574	A	MATα	–	+	–
MRC[b] 8887	A	MATα	+[c]	+	–
MRC 8888	A	MATα	–	+	–
MRC 8890	A	MATα	+	+	–
MRC 8891	A	MATα	–	+	–
MRC 8879	A	MATα	+	+	–
MRC 8880	A	MATα	+[c]	+	–
MRC 8882	A	MATα	+[c]	–	–
MRC 8884	A	MATα	+[c]	+	–
MRC 8885	A	MATα	+[c]	–	–
MRC 8855	A	MATα	+[c]	–	–
MRC 8856	D	MATα	+	+	+[d]
MRC 8857	A	MATα	+	+	–
MRC 8858	A	MATα	+[c]	–	–
MRC 8859	A	MATα	+[c]	–	–
MRC 8860	A	MATα	+[c]	–	–
MRC 8861	A	MATα	+[c]	–	–
MRC 8863	A	MATα	+[c]	–	–
MRC 8864	A	MATα	+[c]	–	–
MRC 8865	A	MATα	–	+	–
MRC 8866	A	MATα	+[c]	–	–
MRC 8867	A	MATα	+[c]	–	–
MRC 8868	A	MATα	+	–	–

+ Positive for mating as both basidiospores and fused clamp connections were observed under the microscope (Nikon Eclipse E2000, Japan)
[a] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
[b] Medical Research Council, Tygerberg, South Africa
[c] Limited hyphal growth observed under the microscope (Nikon SMZ-10A, Japan)
[d] Positive for haploid fruiting as basidiospores but no complete clamp connections were observed under the microscope (Nikon Eclipse E2000, Japan)

woody debris (Tables 2 and 3), an increase in mating frequency between strains of the same serotype (serotype A) was noted when strains were crossed on YCB agar (Table 5). This increase in mating frequency may be as a result of nutrient limitations, particularly nitrogen, as the YCB contained no nitrogen source.

Although monokaryotic fruiting is proposed to occur under certain stress conditions, particularly nutrient starvation and more specifically, nitrogen limitation [32, 46, 47], our research revealed the presence of filamentation when *C. neoformans* var. *grubii* was cultured on *A. mearnsii* (Table 3), despite its relatively high nitrogen content (Table 1). Only one *C. neoformans* var. *neoformans* strain (MRC 8856) displayed monokaryotic fruiting when cultured on YCB (Table 5); however, it should be noted that this development pathway has already been well characterized in this serotype [46]. This low frequency of monokaryotic fruiting among the isolates could be a result of high tannin levels [42] within the woody debris. Alternatively, it should also be noted that unlike mating, monokaryotic fruiting is regarded as being rather inefficient, often requiring longer periods of incubation and may produce erratic filamentation patterns [29]

Conclusions

All strains of the opportunistic human pathogen, *C. neoformans* var. *grubii*, showed cellulase activity in plate assays. The latter one is an essential cellulolytic enzyme required for growth within a woody environment. In addition, representative strains of *C. neoformans* var. *grubii* were capable of growth and survival when cultured on woody debris as a substrate. Some strains were able to mate on a medium containing the woody debris as sole nutrient source. The frequency of this mating among the strains, however, seemed to depend on the tree species from which the woody debris originated. Nevertheless, it is obvious that *C. neoformans* var. *grubii* is capable of producing all its ontogenic stages when cultured on woody debris.

This is the first reported observation of the occurrence of the hyphal phase of *C. neoformans* when cultured on woody debris, a suspected natural habitat of this yeast pathogen. This observation may yet prove to be a vital link with regards to the extensive cryptococcosis outbreak observed on Vancouver Island [20] as a result of high levels of *C. gattii* within the natural reserves in that area.

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References

1. Abbott TS (ed) (1985) In: Soil testing service: methods and interpretation. NSW Department of Agriculture, Sydney
2. Beyers CP, Coetzer FJ (1971) Effect of concentration, pH and time on the properties of di-ammonium EDTA as a multiple soil extractant. Agrochemophysica 3:49–54
3. Blackburn TH (1983) The microbial nitrogen cycle. In: Krumbein CWE (ed) Microbial geochemistry. Blackwell, Oxford, UK, pp 63–89
4. Boekhout T, van Belkum A, Leenders ACAP, Verbrugh H, Mukamurangwa P, Swinne D, Scheffers WA (1997) Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects. Int J Syst Bacteriol 47:432–442
5. Boekhout T, Bandoni RJ, Fell JW, Kwon-Chung KJ (1998) Discussion of teleomorphic and anamorphic genera of the heterobasidiomycetous yeasts. In: Kurtzman CP, Fell JW (eds) The yeasts, a taxonomic study. Elsevier, Amsterdam, The Netherlands, pp 609–625
6. Boekhout T, Theelen B, Diaz M, Fell JW, Hop WCJ, Abeln ECA, Dromer F, Meyer W (2001) Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiol 147:891–907
7. British Columbia, Ministry of Agriculture, Food and Fisheries. Resource Management Branch (2002) Soil water storage capacity and available soil moisture. Water Conservation Factsheet (http://www.agf.gov.bc.ca/resmgmt/publist/600series/619000–1.pdf)
8. Bui T, Lin X, Malik R, Heitman J, Carter D (2008) Isolates of *Cryptococcus neoformans* from infected animals reveal genetic exchange in unisexual, α-mating type populations. Eukaryot Cell (in press)
9. Casadevall A, Steenbergen J, Nosanchuk J (2003) 'Ready made' virulence and 'dual use' virulence factors in pathogenic environment—the *Cryptococcus neoformans* paradigm. Curr Opinion Microbiol 6:332–337
10. Chuck SL, Sande MA (1989) Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. N Engl J Med 321:794–799
11. Cogliati M, Esposto MC, Clarke DL, Wickes BL, Viviani MA (2001) Origin of *Cryptococcus neoformans* var. *neoformans* diploid strains. J Clin Microbiol 39:3889–3894
12. De Koker TH, Zhao J, Allsop SF, Janse BJH (2000) Isolation and enzymatic characterization of South African white rot fungi. Mycol Res 104:820–824
13. Doll EC, Lucas RE (1973) Testing soils for potassium, calcium and magnesium. In: Walsh LM, Beatons JD (eds) Soil testing and plant analysis. Soil Science Society of America, Madison, WI, USA, pp 133–152
14. Ellis DH, Pfeiffer TJ (1990) Natural habitat of *Cryptococcus neoformans* var. *gattii*. J Clin Microbiol 28:1642–1644
15. Ellis DH, Pfeiffer TJ (1990) Ecology, life cycle and infectious propagule of *Cryptococcus neoformans*. Lancet 336:923–925
16. Farkas VM, Liskova M, Bielly P (1985) Novel media for detection of microbial producers of cellulase and xylanase. FEMS Microbiol Lett 28:137–140
17. Fertilizer Society of South Africa. (1974) Manual of soil analysis methods. FSSA publication no. 37, Fertilizer Society of South Africa, Lynnewood Ridge, Pretoria, South Africa
18. Forrester DI, Schortemeyer M, Stock WD, Bauhus J, Khanna PK, Cowie AL (2007) Assessing nitrogen fixation in mixed- and single-species plantations of *Eucalyptus globulus* and *Acacia mearnsii*. Tree Physiol 27:1319–1328
19. Franzot SP, Fries BC, Cleare W, Casadevall A (1998) Genetic relationship between *Cryptococcus neoformans* var. *neoformans* strains of serotypes A and D. J Clin Microbiol 36:2200–2204
20. Fraser JA, Giles SS, Wenink EC, Geunnes-Boyer SG, Wright JR, Diezmann S, Allen A, Stajichl JE, Dietrichl FS, Perfect JR, Heitman

J (2005) Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. Nature 437:1360–1364
21. Halliday C, Bui T, Krockenberger M, Malik R, Ellis D, Carter D (1999) Presence of α and *a* mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gatti* strains from Australia. J Clin Microbiol 37:2920–2926
22. Halliday CL, Carter DA (2003) Clonal reproduction and limited dispersal in an environmental population of *Cryptococcus neoformans* var. *gattii* isolates from Australia. J Clin Microbiol 41:703–711
23. Hiremath SS, Chowdhary A, Kowshik T, Randhawa HS, Sun S, Xu J (2008) Long-distance dispersal and recombination in environmental populations of *Cryptococcus neoformans* var. *grubii* from India. Microbiol 154:1513–1524
24. Kwon-Chung KJ (1975) A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. Mycologia 61:1197–1200
25. Kwon-Chung KJ (1998) *Filobasidiella* Kwon-Chung. In: Kurtzman CP, Fell JW (eds) The yeasts, a taxonomic study. Elsevier, Amsterdam, The Netherlands, pp 656–662
26. Kwon-Chung KJ, Bennett JE (1984) Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. Am J Epidemiol 120:123–130
27. Lazéra MS, Pires FDA, Camillo-Coura L, Nishikawa MM, Bezerra CCF, Trilles L, Wanke B (1996) Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. J Med Vet Mycol 34:127–131
28. Lengeler KB, Cox GM, Heitman J (2001) Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. Infect Immun 69:115–122
29. Lin X, Hull CM, Heitman J (2005) Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans*. Nature 434:1017–1021
30. Litvinseva AP, Thakur R, Vilgalys R, Mitchell TG (2006) Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (Serotype A), including a unique population in Botswana. Genetics 172:2223–2238
31. Loftus BJ, Fung E, Roncaglia P, Rowley D, Amedeo P, Bruno D, Vamathevan J, Miranda M, Anderson IJ, Fraser JA, Allen JE, Bosdet IE, Brent MR, Chiu R, Doering TL, Donlin MJ, D'Souza CA, Fox DS, Grinberg V, Fu J, Fukushima M, Haas BJ, Huang JC, Janbon G, Jones SJ, Koo HL, Krzywinski MI, Kwon-Chung JK, Lengeler KB, Maiti R, Marra MA, Marra RE, Mathewson CA, Mitchell TG, Pertea M, Riggs FR, Salzberg SL, Schein JE, Shvartsbeyn A, Shin H, Shumway M, Specht CA, Suh BB, Tenney A, Utterback TR, Wickes BL, Wortman JR, Wye NH, Kronstad JW, Lodge JK, Heitman J, Davis RW, Fraser CM, Hyman RW (2005) The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. Science 307:1321–1324
32. McClelland CM, Chang YC, Varma A, Kwon-Chung KJ (2003) Uniqueness of the mating system in *Cryptococcus neoformans*. Trends in Microbiol 12:208–212
33. Min KL, Kim YH, Kim YW, Jung HS, Hah YC (2001) Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis*. Arch Biochem Biophys 392:279–286
34. Mitchell TG, Perfect JR (1995) Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev 8:515–548
35. Nathan SS (2006) The use of *Eucalyptus tereticornis* Sm. (Myrtaceae) oil (leaf extract) as a natural larvicidal agent against the malaria vector *Anopheles stephensi* Liston (Diptera: Culicidae). Bioresour Tech 98:1856–1860
36. Neilson JB, Fromtling RA, Bulmer GS (1977) *Cryptococcus neoformans*: size range of infectious particles from aerosolized soil. Infect Immun 17:634–638
37. Nelson DW, Sommers LE (1982) Total carbon, organic carbon and organic matter. In: Page AL (ed) Methods of soil analysis, part 2. American Society of Agronomy, Madison, WI, USA, p 199
38. Nielsen K, De Obaldia AL, Heitman J (2007) *Cryptococcus neoformans* mates on pigeon guano: implications for the realized ecological niche and globalization. Eukaryot Cell 6:949–959
39. Randhawa HS, Mussa AY, Khan ZU (2000) Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other yeast-like fungi of clinical importance. Mycopathologia 151:63–69
40. Ren P, Springer DJ, Behr MJ, Samsonoff WA, Chaturvedi S, Chaturvedi V (2006) Transcription factor STE12α has distinct roles in morphogenesis, virulence, and ecological fitness of the primary pathogenic yeast *Cryptococcus gattii*. Eukaryot Cell 6:1065–1080
41. Saul N, Krockenberger M, Carter D (2008) Evidence of recombination in mixed-mating-type and α-only populations of *Cryptococcus gattii* sourced from single eucalyptus tree hollows. Eukaryot Cell 7:727–734
42. Smith AH, Imlay JA, Mackie RI (2003) Increasing the oxidative stress response allows *Escherichia coli* to overcome inhibitory effects of condensed tannins. Appl Environ Microbiol 69:3406–3411
43. Sorrell T, Ellis D (1997) Ecology of *Cryptococcus neoformans*. Rev Iberoam Microbiol 14:42–43
44. Thomas GW, Peaslee DE (1973) Testing for soil phosphorus. In: Walsh LM, Beatons JD (eds) Soil testing and plant analysis. Soil Science Society of America, Madison, WI, USA, pp 15–122
45. Trilles L, Lazéra M, Wanke B, Theelen B, Boekhout T (2003) Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. Med Mycol 41:383–390
46. Tscharke RL, Lazéra M, Chang YC, Wickes BL, Kwon-Chung KJ (2003) Haploid fruiting in *Cryptococcus neoformans* is not mating type α-specific. Fung Gen Biol 39:230–237
47. Wickes BL, Mayorga ME, Edman U, Edman JC (1996) Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the α-mating type. Prot Natl Acad Sci U S A 93:7327–7331
48. Xue C, Tada Y, Dong X, Heitman J (2007) The human fungal pathogen *Cryptococcus* can complete its sexual cycle during a pathogenic association with plants. Cell Host Microbe 1:246–248
49. Yarrow D (1998) Methods for isolation, maintenance and identification of yeasts. In: Kurtzman CP, Fell JW (eds) The yeasts, a taxonomic study. Elsevier, Amsterdam, The Netherlands, pp 77–98
50. Zhu X, Gibbons J, Garcia-Rivera J, Casadevall A, Williamson PR (2001) Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. Infect Immun 69:5589–5596