Microbial Ecology

Isolation and Characterization of *Cryptococcus neoformans* Varieties Recovered from Natural Sources in Bogotá, Colombia, and Study of Ecological Conditions in the Area

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Abstract

Cryptococcus neoformans, the etiological agent of cryptococcosis, has been associated with avian droppings and certain trees in different countries, including Colombia. *C neoformans* environmental isolates were obtained in urban areas in Bogotá, Colombia, and the strains recovered were phenotypically characterized. Attempts to determine the ecological conditions (micro- and macroclimatic) possibly related to their habitat were also undertaken. Four hundred and eighty samples from bark, soil around trunk bases, and detritus inside hollows of 32 trees were collected in three urban areas during a 5-month period, as well as 89 avian droppings samples from different places. Of plant samples, 6.7% collected from nine tree species yielded *C. neoformans* var. *gattii*, serotype B strains in 99% of the cases, and *C. neoformans* var. *grubii*, serotype A in 1%. The yeast was more frequently recovered from bark than from soil or detritus inside hollows, and from trees with hollows or rotted wood rather than from trees in which birds nest. *C. neoformans* was present with higher frequency and density in the rainy season than in the dry season; we found that slightly higher temperature and humidity values of the microhabitat, as compared to those of the environment, favored fungal occurrence, but the phenological state of the tree did not. Of dropping samples, 7.9% yielded *C. neoformans* strains, all of them *C. neoformans* var. *grubii*, serotype A. The yeast was obtained more frequently from dry droppings than from moist ones, but neither the sunlight exposure nor the site of collection of samples was correlated with this occurrence. Population density was significantly higher in droppings than in tree samples. Under laboratory conditions, isolates of different serotype showed similar capsular sizes. Water content and pH ranges were wide and did not show any significant difference between positive and negative samples.

Introduction

The basidiomycetous yeast *Cryptococcus neoformans* is the etiological agent of cryptococcosis, an opportunistic systemic mycosis that affects the central nervous system. Human infection is thought to be acquired by inhalation of airborne propagules (<3 μm) from an environmental source [5]. Three varieties of the yeast are recognized: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), *C. neoformans* var. *gattii* (serotypes B and C), and one hybrid corresponding to the serotype AD [12]. The varieties differ in phenotype, genotype and epidemiology, as well as in their geographic distribution and natural habitat [30].

The varieties *grubii* and *neoformans* have a global distribution and affect mainly immunocompromised patients [2]. The incidence of cryptococcosis caused by variety *grubii* has increased markedly worldwide in recent years because of its occurrence in HIV-infected patients [23]. On the other hand, variety *gattii*, occurring predominantly in nonimmunocompromised individuals, is restricted to tropical and subtropical regions, although this variety was recently reported in the nontropical area of Vancouver Island, Canada [5, 10, 30, 33; Fyfe M et al. Unprecedent outbreak of *Cryptococcus neoformans* var. *gattii* infections in British Columbia, Canada P1.1, 5th International Conference on *Cryptococcus* and cryptococcosis. Adelaide, Australia, 2002]. Avian droppings, mainly from pigeons *(Columba livia)* [2, 3, 5, 8, 28, 31], and various tree species have been reported as the main saprophytic source of *C. neoformans* var. *grubii* and var. *neoformans* in nature [19, 21, 24, 27]. On the other hand,

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C. neoformans var. *gattii* has been associated with eucalyptus [10, 15] and other tree species [4, 15, 20, 21, 27, 30, 34].

In Colombia, *C. neoformans* var. *grubii* (serotype A), has been isolated from pigeon droppings [3, 6, 8, 22], from vegetal material of eucalyptus trees in Bogotá [9], and from detritus of *Cassia* sp. and seeds of *Moquilea tomentosa* in Cúcuta [4, 6], where *C. neoformans* var. *gattii* (serotype C), was also recovered from almond trees *(Terminalia catappa)* detritus [4, 6]. *C. neoformans* var. *gattii* (serotype B), was recently found in association with *Eucalyptus* sp. in a small town near Bogotá (E. Quintero, personal communication).

It has been proposed that the primary natural habitat of *C. neoformans* results from natural biodegradation of wood [19–21]. However, the possible relation between environmental strains of *C neoformans* isolated from plant material and the contamination of this material with avian droppings has not been explored yet. In addition, *C. neoformans* survival in saprophytic sources may be affected by factors such as pH, humidity, temperature, and sunlight [5]. In this regard, the aim of this survey was to recover *C. neoformans* environmental isolates from avian droppings and plant material in different urban areas in Bogotá, Colombia, and characterize them phenotypically. Attempts to determine some ecological conditions (micro- and macroclimatic) possibly related to the habitat of this clinically relevant yeast were also undertaken.

Methods

Study Area. Bogotá, the capital of Colombia, located 2,630 m above sea level, has a temperate subhumid climate characterized by a bimodal thermal regime, consisting of two rainy seasons (December–March and June–September) and two dry seasons (March–June and September–December); the annual rainfall is 952 mm and the average temperature is 14°C with small variations (<5°C) over the year [26]. Three big urban areas of vegetation and high environmental importance were selected for plant sample collection: the Botanical Garden ''Jose Celestino Mutis'' (JB) (4°41′ N, 74°06′ W), the metropolitan park ''El Lago'' (PEL) (4°40′ N, 74°03′ W), and the ''Universidad Nacional de Colombia'' campus (UN) (4°38′ N, 74°06′ W).

Droppings samples were collected in 12 places around the city, where a high concentration of pigeons was observed: two parks, three schools, some dwellings, an aviary inside a park, a building, an abandoned carpenter's workshop, and a pigeon loft.

Sample Collection. Plant sample collection was done monthly during a 5-month period (January to May 2003) from 32 trees located in the three areas mentioned above. The trees were divided into two groups (16 trees in each): group 1, trees used by birds to nest, and group 2, trees with hollows or rotted wood. The distinction made was based on physical characteristics observed. Trees belonging to group 1 showed an evident association with birds: contamination by their droppings, nests, birds' eggs, and the direct observation of birds nesting in the tree. Moreover, the trees had very dense canopies with broad leaves; providing considerable shade; no *Eucalyptus* species were found near these trees. On the other hand, trees belonging to group 2 had no such an association with birds compared with trees of group 1. These trees were taller (> 10 m) and less leafy, and the leaves were more narrow. Hollows and rotted wood in the trunk were common characteristics of trees belonging to group 2. The two groups were mutually exclusive and contained a completely different range of species.

A total of 480 samples were collected: 270 from 18 trees located in JB, 120 from eight trees in UN, and 90 from six trees in PEL. During each collection, three samples from each tree were taken: one from the bark, one from soil around the trunk base, and one from detritus inside hollows in the trunk. This last one was collected using a sterile swab soaked in saline solution 0.85% with which the inner hollow surfaces were rubbed; the swab was then transported in Stuart transport medium (Difco Laboratories, Maryland, USA) [34]. Approximately 5 g from bark and soil samples was collected and transported in hermetic plastic bags to the laboratory.

Five to 40 droppings samples were collected per location, taking 10 g of substrate, and transported in hermetic plastic bags to the laboratory. During the collection period some sample characteristics were registered: sunlight exposure (exposed, intermediate, sheltered), humidity (moist or dry), and collection place (pigeons nest, soil, wood, etc.).

Ecological Conditions in the Area. During collection periods relative humidity and temperature were measured using a portable Hygrotest 6400 thermohygrometer (Testoterm, Germany) and sunlight intensity using a photometer (Canon EOS 3000); these instruments were placed directly on the substrate (soil and bark) where samples were collected. In addition, these variables were measured beneath the tree canopy, 2 m from the trunk and at 1 m height above the soil in each of the trees sampled during each date of collection to calculate differences in temperature, humidity, and sunlight intensity between the environment and the substratum (microclimatic variables) [Lazera M, PhD Thesis. Universidade Federal do Rio de Janeiro, Brazil, 1995].

Climatic data (rainfall, relative humidity, temperature, and sunlight hours) of Bogotá's environment during the study period, as reported by the National Hydrology,

Meteorology and Environmental Studies Institute "IDEAM," were taken into account for the analysis (macroclimatic variables). Additionally, we registered the phenological state of the trees, checking in which of four stages (bud, flower, fruit, and sterile) the trees were.

Sample Processing and Strain Isolation and Identification. Droppings, bark, and soil samples were processed as follows: 5 g of the sample were suspended in 25 ml of phosphate-buffered saline (PBS) [9] and allowed to settle for 30 min then the sample was filtered and 50 μL of antibiotic solution, (20 U/mL streptomycin and 40 U/mL penicillin) was added. Then 100 μL was cultured on Niger-seed agar (*Guizotia abyssinica*) of the following composition: Niger seeds 50 g; glucose, creatinine, and potassium phosphate, 1 g; Bacto Agar 15 g; penicillin G, 20 U/mL; streptomycin, 40 U/mL; biphenyl 0.1% (1 g/20 mL ethanol) and distilled water, 1000 mL [31]. All plates were incubated at 27°C for 2 weeks with daily observation. The material collected with the swabs was directly plated on the same medium.

Colony-forming units (CFU) were counted and expressed per g of sample, except for those collected with the swab method. Suggestive dark brown colonies (phenol oxidase activity) were subcultured on Sabouraud glucose agar plates for confirmation tests [18]: capsule presence [18], urease production [17], lack of nitrate reductase enzyme [18], carbohydrate assimilation [1] and ability to grow at 37°C for 72 h. The variety was determined using canavanine–glycine–bromothymol blue agar [16], and the serotype was established using a slide agglutination test with specific antisera (Crypto-check, Iatron Lab, Tokio, Japan).

The total diameter (cell and capsule), cellular diameter, and capsular size of 20 yeast cells of each isolate were also measured [11].

Chemical Properties of Samples. We evaluated pH of all positive and the same number of negative samples (used as controls) collected from droppings and plant material, suspending 1 g of the sample in 10 mL of $CaCl_2 \cdot H_2O$ 0.01 M solution, then incubating it for 24 h at room temperature and measuring the pH with a potentiometer [29]. We also determined the water content of samples as follows: fresh sample was weighed, then dried at 80°C for 72 h in a stove, and weighed again; the water content was calculated in terms of percentage based on fresh and dry weight.

Statistical Analysis. Positive samples and trees were studied by analysis of variance (ANOVA) based on chi-square test; ANOVA based on an F test was applied to population densities, to capsular size, and to total and cellular diameters [32]. Yeast occurrence over time was analyzed by analysis of multiple variance (MANOVA) based on the repeated measures model [14].

A canonical discriminant analysis was carried out to detect which of the microclimatic variables (differences in temperature, humidity, and sunlight intensity) were maximally correlated with yeast presence/absence in each substrate (soil and bark). Using the variables selected by this analysis, two canonical variables were constructed to reduce the dimensions of the analysis and facilitate the graphical representation of the results. Canonical variables are linear combinations of the selected variables weighed by canonical coefficients that show the ratio of importance of each variable. The order of the canonical variable shows its discriminant capacity. The first canonical variable generates a new coordinate system, which maximizes the separation between the groups (presence/absence); the second canonical variable maximizes the separation discounting the data of the first variable, and so forth [14].

Using the Pearson correlation coefficient, the relation between yeast presence in pigeon droppings and the variables registered for these samples was assessed, as well as between yeast occurrence in plant samples and the phenological state of the trees [32]. This same coefficient was used to detect a possible relationship between population densities of the yeast, pH, and water content of samples. Student's t-test was used to compare pH and water content in positive and negative samples [32]. All analyses were made using the statistical analysis system (SAS) for Windows v. 8.02.

Results

Monthly Study of Trees. Out of 480 samples processed, 38 (7.9%) were positive for *C. neoformans:* 11/270 (4.1%) collected at the JB, 11/120 (9.2%) at the UN, and 16/90 (17.8%) at the PEL. Of the trees belonging to 7 families and 9 species (gymnosperms as well as angiosperms) 65.6% (21/32) were positive for *C. neoformans* on at least one occasion (Table 1). The frequency of isolation from trees with hollows or rotted wood (group 2) for all types of samples was significantly higher than from trees in which birds nest (group 1) ($P = 0.001$) (Table 1). The result based on the proportion of positive trees showed that 43.8% (7/16) of group 1 trees were positive, in contrast with 87.5% (14/16) of group 2, indicating a significantly higher isolation frequency of the yeast from trees with hollows or rotted wood than from trees used as perches by birds ($P = 0.008$) (Table 1). However, population densities (CFU/g) of *C. neoformans* were similar in both groups, 157 CFU/g in group 1 and 175 CFU/g in group 2 ($P = 0.3$). *C. neoformans* was isolated more frequently from *Eucalyptus* spp. than from the other tree species (Table 1). Positive trees yielded

Table 1. Species studied and proportion of positive trees and samples

			Trees			*Samples*		
Group	*Tree species*	*Family*	+	*n*	%	+	*n*	%
1	*Ficus soatensis*[a]	Moraceae	4	11	36.4	5	165	3.1
	Ficus tequendamae	Moraceae	0	2	0	0	30	0
	Croton bogotanus[a]	Euphorbiaceae	1	1	100	1	15	6.7
	Croton funckianus[a]	Euphorbiaceae	1	1	100	1	15	6.7
	Coussapoa sp.[a]	Cecropiaceae	1	1	100	1	15	6.7
	Total		7	16	43.8	8	240	3.3
2	*Eucalyptus camaldulensis*	Myrtaceae	7	7	100	14	105	13.3
	Eucalyptus. golobulus[a]	Myrtaceae	2	2	100	6	30	20.0
	Eucalyptus sp. (dead)	Myrtaceae	1	1	100	3	15	20.0
	Pinus radiata[a]	Pinaceae	2	2	100	4	30	13.3
	Cupressus lusitanica[a]	Cupressaceae	1	1	100	2	15	13.3
	Acacia decurrens[a]	Fabaceae	1	3	33.3	1	45	2.2
	Total		14	16	87.5	30	240	12.5

[a]First report in the literature.

isolates in one, two, or three occasions, but not in all 5 months (data not shown).

Considering all 32 trees studied, yeast was more frequently isolated from bark (14.4%, 23/160) than from soil (7.5%, 12/160), and from detritus inside hollows (1.9%, 3/160) ($P = 0.01$), although the average population density of the yeast in positive samples was significantly higher in soil (275 CFU/g) than in bark samples (130 CFU/g) ($P = 0.03$). Such a trend was also found within each group of trees.

During January, February, and March, isolation frequency was low in both groups of trees compared to April and May, when it drastically increased ($P < 0.0001$) (Fig. 1). The average population density of *C. neoformans* for each month ranged from 50 to 230 CFU/g; the maximum value observed in April (Fig. 1). Average densities calculated for January, February and March are partially representative since we obtained only one or two positive samples in these months.

In a similar way, rainfall and relative humidity values increased over time, with maximum values in April (Fig. 2), contrary to sunlight hours (Table 2). Although the variation in average temperature was small, variation in ranges of maximum and minimum temperatures was important, decreasing gradually over time, showing the narrowest range in April (Table 2).

Canonical discriminant analysis determined that differences in temperature and humidity, but not in sunlight intensity, between the environment and the substrate were correlated with *C. neoformans* occurrence both in bark and soil. Only the first canonical variable tends to separate the two clouds representing presence and absence (Fig. 3). When this variable takes negative values or values near zero, *C. neoformans* presence tends to be favored, which takes place when substrata temperature and humidity were higher than the environmental ones (Canonical 1 equation). Canonical analysis suggested that although these are determinant factors, there must be other ones, since the two clouds were not completely separated.

Regarding trees' phenological state, those positive for *C. neoformans* colonization on one or more occasions were observed to be in flower, fruit, and sterile stages, but not in the bud stage. However, we did not find any correlation between yeast occurrence and the phenological stage of the tree (bud $P = 0.31$; flower $P = 0.84$; fruit $P = 0.94$; sterile $P = 0.60$).

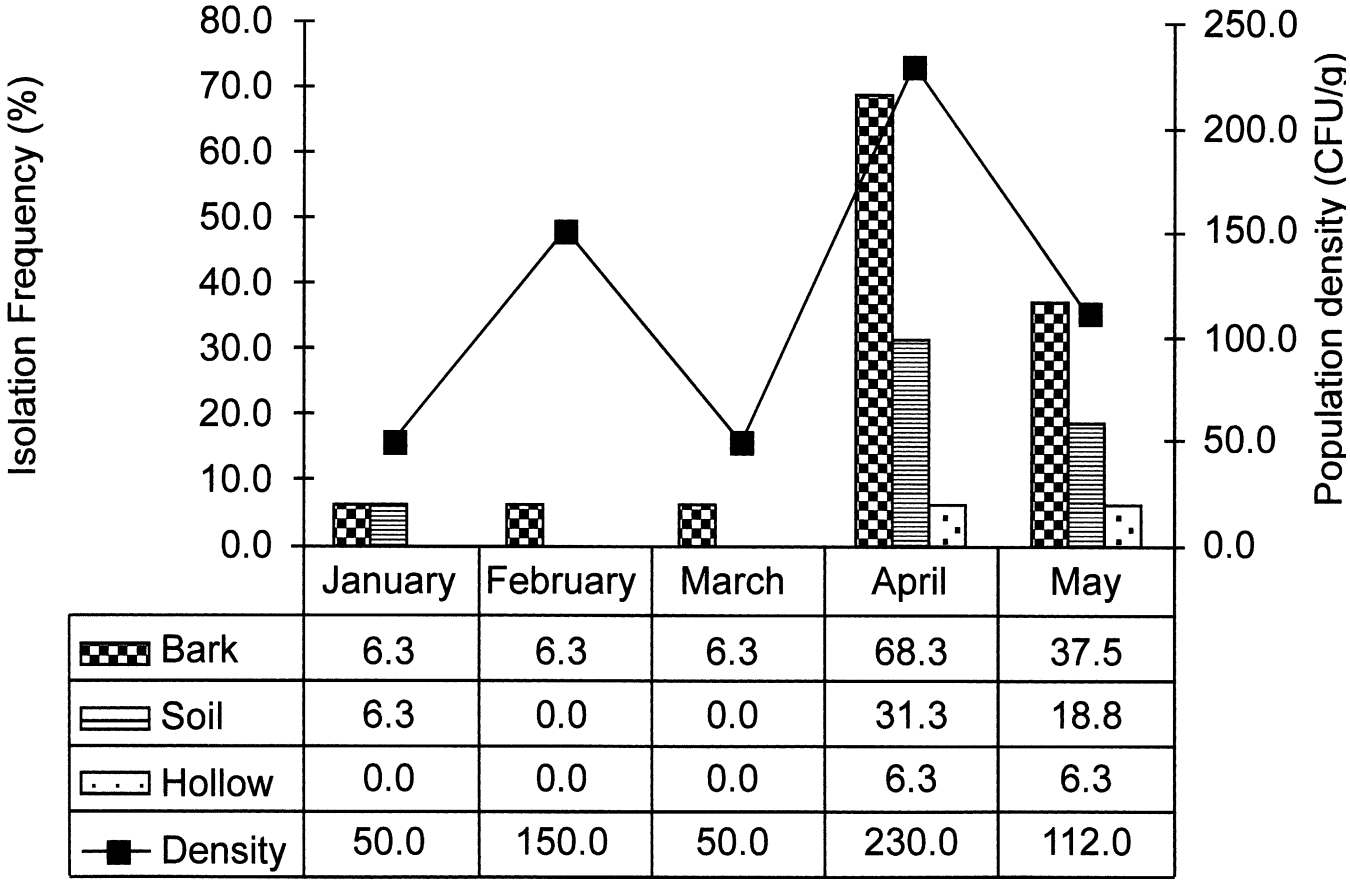
Out of 98 strains recovered, 97 (99%) corresponded to C. *neoformans*, serotype B, and only 1%, obtained in January from *E. camaldulensis* bark, was *C. neoformans*, serotype A. In April and May, *C. neoformans* var. *gattii*, serotype B, was recovered from bark and soil samples of the same eucalyptus tree.

Avian Droppings Samples. Isolation frequency in droppings samples was 6.7% (6/89), ranging from 0 to 40% in the different locations under study. Yeast isolation from dry droppings was significantly more frequent (14.2%) than from moist droppings (1.8%) ($P = 0.05$). No significant differences in the isolation frequency were observed when sunlight exposure or collection site was analyzed ($P = 0.49$, $P = 0.27$, respectively). All isolates recovered from this type of sample were *C. neoformans*, serotype A.

Population densities in excreta samples ranged between 400 and 4,300 CFU/g with an average value of 1,525 CFU/g, significantly higher than in plant samples, where the average population density was 168 CFU/g ranging from 50 to 1050 CFU/g ($P < 0.0001$).

Isolates Characterization. All isolates exhibited capsule, had phenol oxidase, lacked nitrate reductase, and did not assimilate lactose and melibiose. All but two grew at 37°C, hidrolized urea, and assimilated inositol.

Serotype A isolates showed a significantly higher total (5.2 μm) and cellular (3.7 μm) diameter than serotype B



	January	February	March	April	May
Bark	6.3	6.3	6.3	68.3	37.5
Soil	6.3	0.0	0.0	31.3	18.8
Hollow	0.0	0.0	0.0	6.3	6.3
Density	50.0	150.0	50.0	230.0	112.0

Figure 1. Variation in isolation frequency (%) and population density (CFU/g) of *Cryptococcus neoformans* in different kinds of samples during the study period.

strains (4.8 and 3.4 μm, respectively) ($P = 0.0005$, $P = 0.002$, respectively), even though capsular size was similar in both kinds of isolates (0.7 μm) ($P = 0.08$). No significant differences in total, cellular, and capsular size were found when comparing strains of different origin (excreta and trees) ($P = 0.86$, $P = 0.70$, $P = 0.69$, respectively).

Chemical Properties of Samples. The pH and water content values did not show any significant differences when comparing positive and negative samples ($P = 0.22$; $P = 0.34$). In positive samples average pH was 5.55, ranging from 2.69 to 8.65, whereas water content was 23.69% on average, ranging from 4.08 to 75.14%. The average pH of droppings samples was more alkaline

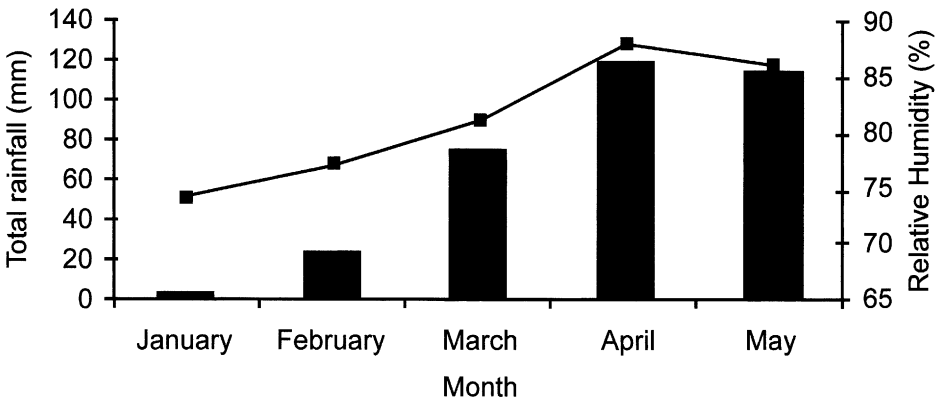


Figure 2. Total rainfall and relative humidity variation during the study.

Table 2. Macroclimatic conditions

Variable	*January*	*February*	*March*	*April*	*May*
Sunlight (hours/month)	230	130	120	60	70
Average temperature (°C)	14.0	14.6	14.3	14.4	14.8
Minimum average temperature (°C)	5.3	8.9	8.7	9.7	9.0
Maximum average temperature (°C)	21.0	20.8	20.3	19.4	19.6

than that of soil and plant samples (8.01 ± 0.6 and 5.53 ± 2.1, respectively).

Discussion

This survey succeeded in isolating *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *gattii* (serotype B) from environmental sources in Bogotá. These are the most prevalent varieties in clinical cases in our country [6, 25]. We achieved the isolation of *C. neoformans* var. *gattii*, serotype B environmental strains, whose natural source in Colombia remained unknown until the beginning of 2003, in spite of previous exhaustive search carried out in a region endemic for this serotype [4, 6].

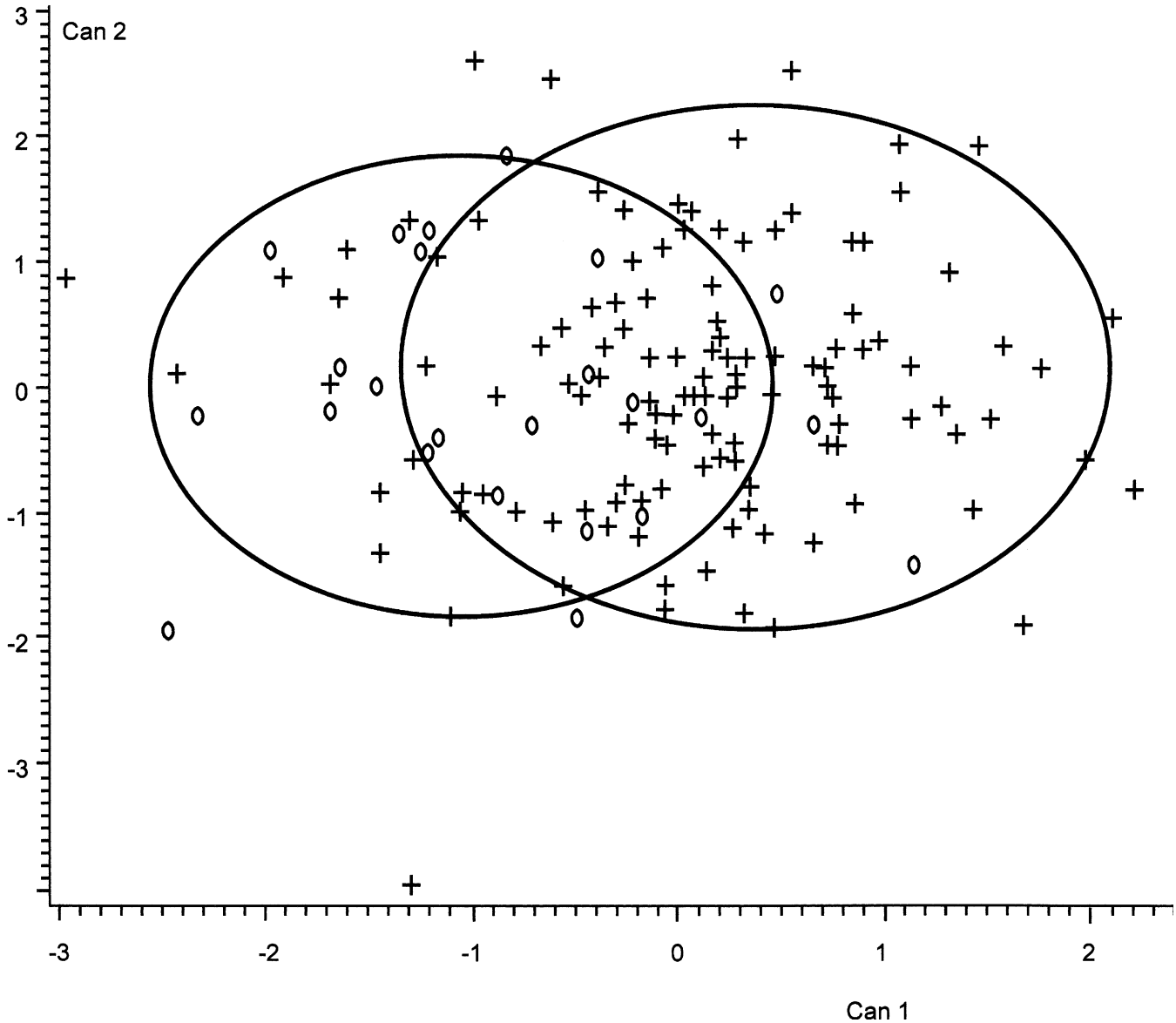


Figure 3. Graphical representation of canonical variables. 0: presence; +: absence; Can1 (first canonical variable) = 0.5 D_t + 0.1 D_h; Can2 (second canonical variable) = 0.07 D_t + 0.02 D_h; D_t, difference in temperature between the environment and the substrate; D_h, difference in humidity between the environment and the substrate.

The presence of this variety in the environment has not shown a regular pattern [30]. Moreover, this study represents the first report of the association of *C. neoformans*, serotype B, with eight new species of trees: *Eucalyptus globulus*, *Ficus soatensis*, *Croton bogotanus*, *Croton funckianus*, *Coussapoa* sp., *Cupressus lusitanica*, *Pinus radiata*, and *Acacia decurrens*, which corroborates the existence of an unspecific association between this fungus and a host tree, as proposed by Lazera and reinforced by other authors [15, 19, 27]. On the other hand, according to previous findings [10, 15, 24], we demonstrated the preference of this variety for eucalyptus, even in a dead tree, possibly due to specific properties of the wood.

Our results suggest that it is more probable to find *C. neoformans* associated with trees with hollows or rotted wood than with trees in which birds nest, even though this yeast could reach similar densities in both kinds of substrata. Contrary to expectations, all but one isolate, recovered from plant samples of trees used as perches by birds, were *C. neoformans* var. *gattii*, serotype B. Variety *gattii* has never been found in association with avian excrement, so we can discard that *C. neoformans* occurrence in this kind of trees might have been the result of excrement contamination, as we initially expected.

In this survey, the fungus showed more frequent bark than soil colonization, as expected, supporting the concept that the primary niche of variety *gattii* seems to be that resulting from wood biodegradation [20, 21]. The low isolation frequencies observed in samples from detritus inside hollows are probably due to the small quantity of samples collected with the swab sampling method as compared to the higher quantity collected with the other technique. However, population density of *C. neoformans* in this substrata is probably high because in some cases we found the same number of colonies, one or two, per plate for swab and nonswab samples, in spite of the significantly lower quantity of sample collected with the swab.

Both varieties *(grubii* and *gattii)* were recovered from the same *E. camaldulensis* tree, although not simultaneously, which shows that both populations could be occupying the same niche. In a similar way, studies in Brazil have reported this observation from *Eucalyptus* sp. [24] and *Cassia grandis* [21]. The only *C. neoformans* var. *grubii* isolate obtained in this study was recovered in a dry month (January), a fact that can be related to its higher thermotolerance level and desiccation resistance [5, 18].

As in previous studies in Australia, Brazil, and India [15, 20, 27], we proved the permanence of this yeast in nature during various months, even though none of the trees was positive in all five samplings carried out. A previous study in Australia did not find any indication of seasonal variation in environmental presence [15]. In contrast, the study of Connolly et al. remarks an apparent seasonal variation in nasal colonization of koalas in Australia, being high from February to September and less in December [7]. Similar results were found in India [27], where population densities of *C. neoformans* var. *neoformans* in two trees showed a seven- to eightfold decline in the extremely hot summer of June compared with levels seen in March (mild spring). Likewise, density of *C. neoformans* var. *gattii* in a tree fell more than threefold in summer compared with levels seen in winter. However, the small sample size of the study prevented the delineation of definite trends. Our results reveal an interesting changing pattern of occurrence and density related to climatic and environmental conditions. Rainy months (April and May), characterized by high rainfall and humidity, few hours of sunlight, less extreme temperatures and a slightly higher average temperature, favored the occurrence and propagation of *C. neoformans* in our environment more than dry months.

Similarly to macroclimatic conditions, substratum temperature and humidity values slightly higher than those in the environment appeared to be favorable for the habitat of the yeast. Climatic variations in microhabitats (bark, soil, and hollows) could be less drastic than in the environment, providing the yeast a more protected habitat. Furthermore, the wood degradation process requires high temperature and humidity values. Other influencing factors suggested by the canonical discriminant analysis could be biotic (e.g., other organisms) or abiotic (e.g., chemical composition of substrate) [5]. Additional studies are required to elucidate the role of wind in *C. neoformans* propagation.

As in a previous study [20], we did not find any correlation between *C. neoformans* occurrence and the phenological sate of trees, in contrast to an Australian survey [10] which reported *C. neoformans* presence associated with flowering eucalyptus. It is noteworthy that in Australia the flowering season coincides with the rainy period [10], which agrees with our results.

Isolation frequency from avian excrements was low compared to other studies [8, 22, 24] and to the frequency obtained in this study from vegetal material, possibly because of an insufficient sampling. In contrast, average population density of *C. neoformans* was higher in excrement than in tree samples, as already observed in a study in Brazil [24], even though the densities found by us were much lower. This suggests that avian droppings offer suitable conditions and possibly less competition for growth, and, thus, would be a potentially more dangerous source of human infection.

Other studies had already reported a more frequent isolation of the yeast from dry than from moist excrement [28]. Dry excrement is a favorable substratum since it has fewer bacteria and therefore less competition, which could help explain the higher population density

found in this substratum [28]. Even though the sensitivity of *C. neoformans* to UV radiation has been demonstrated *in vitro* [5], as well as an inverse relation between sunlight exposure of excrement and *C. neoformans* occurrence [3, 24], in this survey sunlight exposure of samples did not appear to be a decisive factor.

Our study shows how a pathogenic agent such as *C. neoformans* can colonize various zones in a city, and how its population densities can vary within zones. It is possible that this particular yeast colonizes several places by means of its transportation in avian droppings and the dispersion of propagules in the wind. Nonetheless, population density in Bogotá was lower than in other countries such as Brazil [19–21], Australia [34], and India [27].

The capsule has been considered both an important virulence factor and a protecting one against environmental factors [13]. The yeast cells were not smaller than 3 μm, which is the necessary size to penetrate inside pulmonary alveoli [5], and isolates of different serotypes appear to have similar capsules. However, it is important to note that these measurements were made under laboratory conditions, which could have modified the cellular and capsular sizes. It would be appropriate to measure *C. neoformans* yeast cells in their natural environment in order to assess this phenotypic characteristic with more certainty.

Similarly to a previous Colombian study [3], the wide pH and water content range found in this study suggests that the habitat of *C. neoformans* could be less demanding regarding these two factors. The higher pH value observed in excrement samples as compared to vegetal ones could partially explain why variety *gattii* is absent in excrements, while variety *grubii* is not. These data should be taken into account in future production of antifungal agents for *C. neoformans* control in urban environments. In fact, alkalinization with lime solution had been recommended as useful tool in eradicating *C. neoformans* from contaminated sites such as pigeon coops [5].

Ecological studies are relevant in the epidemiology of a pathogenic agent. Many questions about the ecology of *C. neoformans* still remain unanswered, such as the actual role of birds. Future surveys seeking to understand its life cycle under *in vivo* conditions would be of great importance.

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