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TECHNICAL NOTE

PATHOLOGY/BIOLOGY

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Soil Fungi: Their Potential use as a Forensic Tool*

ABSTRACT: As a grave is an anomalous environment and differs from its surroundings, criminal investigators employ different techniques for locating, recovering, and analyzing clandestine graves. In this study were identified the fungi found in the soil under corpses in decomposition with an aim at relating the copresence of human remains and different fungal species. Were isolated the fungi in three ways: soil washing, serial dilutions, and moist chamber growth. *Dichotomomyces cejpii, Talaromyces trachyspermus, Talaromyces flavus*, and *Talaromyces* sp. were the representative species found—with those belonging to the ammonia group, whose fungi are the first in the succession of cadaver decomposition directly in the ground. The mycobiota found at the present study area clearly differs to mycobiota identified in control sample and from previously described species for other areas of Buenos Aires Province, Argentina. Further forensic examples of this type are needed to develop fully the detailed use of mycology as a forensic tool.

KEYWORDS: forensic science, forensic mycology, forensic Ascomycota, soil fungi, forensic taphonomy, cadaver decomposition, fungal succession

Forensic mycology is a relatively new term used for a description of the fungal species present in the vicinity of human cadavers as well as those fungal groups potentially useful in establishing a time of death (1,2). Taphonomy is the subdiscipline of paleontology that studies the organic and fossilized postmortem remains in the records from different geologic periods. Forensic taphonomy aims at understanding the conditions influencing the decomposition process to estimate the postmortem interval and determine the cause and manner of death. This branch of forensics—incorporating many techniques from a wide range of disciplines such as archeology (3), entomology (4), soil microbiology (5), and botany (6)—has been used to locate, uncover, and analyze clandestine graves.

In recent decades, only few case studies involving both mycology and taphonomy based on fungi have been used as a basis for forensic mycology in the United States (1,2,7), Japan (8,9), and Brazil (10) as well as more recently in Argentina. This new subdiscipline of the forensic sciences is increasing worldwide as certain groups of fungi have generated a substantial body of data concerning the decomposition of cadavers and their related environment. This information has demonstrated that different fungal groups—referred to as ammonia or postputrefaction fungi—can serve as aboveground indicators of the presence of graves in forest ecosystems (2).

The burial of human cadavers under natural or seminatural conditions is sometimes performed in an attempt to hide the evidence of a crime. The ability to locate clandestine graves by means of fungi can thus constitute a useful tool in the investigative process. Although forensic entomology—involving a detection of the insects present on human corpses—is the most frequently applied science in criminal investigations and has allowed the resolution of complex criminal cases, techniques employing fungi could also constitute relevant tools for such forensic purposes.

The aim of this work was to isolate and identify fungi in association with human cadavers in advanced states of decomposition.

Materials and Methods

Human remains were found in December 2009 in a field near Pergamino city, Buenos Aires Province (−34° 21′ 41.6874″S; 60° 5′ 22.9914″W), Argentina. The site contained temporary puddles from a previous rain. The predominant vegetation was herbaceous with sorghum as the most common plant. Climatologic data obtained from a government weather station located at a distance of 60 km from the site showed that the mean ambient temperature from the time of disappearance (November 14, 2009) to the discovery of the remains (December 8, 2009) had ranged from 15.3 and 24.1°C, while the average precipitation had ranged between 0 and 41 mm. The cadaver was found 24 days after death with the skull already reduced to a skeleton and with the arms and legs in a state of advanced decomposition (Fig. 1*A*).

Samples of soil in contact with the remains (Fig. 1*B*) and 15 m away of body (control sample) were taken with a sterile

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FIG. 1—*Photograph taken at the discovery of human remains moment. (A) Human remains in an advanced stage of decomposition. (B) Site where the soil sample was taken for this study. In the picture can be observed the luxuriant vegetation of the site.*

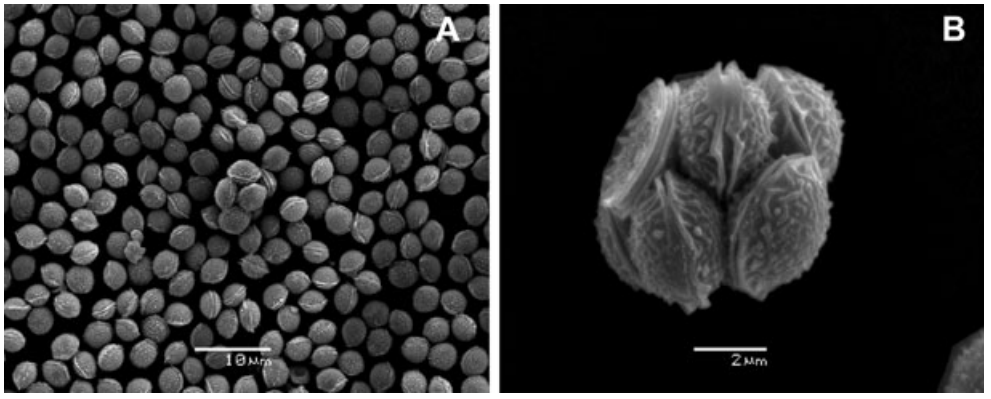

FIG. 2—*(A) Ascospores of* Dichotomomyces cejpii *showing the wall ornamentation under scanning electron microscopy (2500×). (B) Globose asco with ascospores (7500×).*

spoon at the time of the discovery, carried to the laboratory in hermetically sealed plastic bags, and processed according to the following methods: (i) A washing was carried out as described by Parkinson and Williams (11). The soil particles were washed at least 10 times with sterile water, with the water being thoroughly drained after each wash. The soil particles were then dried for 24 h on filter paper. Next, 50 particles each were plated into potato dextrose agar (PDA) and malt yeast extract Agar (MYA) containing streptomycin (100 μg/mL) and chloramphenicol (50 μg/mL) to inhibit bacterial growth. The relative frequency is the number of particles bearing a specific fungus/total number of particles for each culture media (12); (ii) Serial dilutions were made as described by Warcup (13). Of each dilution, 100 μL was plated in triplicate in each one of following culture media: PDA, MYA, and water agar. The mean colony-forming units (CFUs) and standard deviations were registered; (iii) Fifty soil particles were incubated in 10 90-mm Petri dishes containing moistened filter paper (to maintain humidity) at five particles per dish at 25°C. After Elíades et al. (14), we will refer to this approach as the moist chamber method. With this technique, every individual soil particle was considered a distinct sampling unit, and the frequency of occurrence was calculated as: the number of particles bearing a specific fungus/total number of particles analyzed (50).

The morphologic structures of fungi were observed by optical microscopy, through the use of cotton blue staining. The ultrastructure of the fungal isolate was observed under a Jeol JSM-6360 scanning electron microscope (JSM-6390LV, Jeol, Akishima, Tokyo, Japan) in the Electronic Microscope Service in the Museum of Natural Sciences of La Plata. Original taxonomical papers and specific works as Sotolk et al. (15) and Domsch et al. (16) were used for identifying sporulating fungi.

Results

Through the moist chamber technique, we identified *Dichotomomyces cejpii* (Milko) Scott (Ascomycota, Eurotiomycetes; Fig. 2) in the samples of soil particles at a relative frequency of 0.94, whereas *Talaromyces trachyspermus* (Shear) Sotolk & Samson (Fig. 3) and *Talaromyces* sp. were observed at a relative frequency of only 0.04 and 0.02, respectively. In the control sample were identified *Mucor hiemalis* (Hagem) Schipper and *Mortierella* sp. (Zygomycota), and anamorphic stages of Ascomycota as *Penicillium frequentans* (Wehmer) Westling,

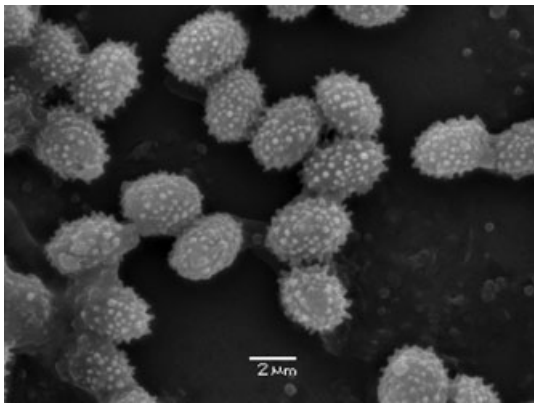

FIG. 3—*Ascospores of* Talaromyces trachyspermu *showing the wall ornamentation with crowded warts under scanning electron microscopy (5500×).*

Aspergillus sp., and *Penicillium* sp. *Penicillium* sp. was the most frequent species found in the control sample analyzed by this technique (relative frequency 0.14) (Table 1).

By the soil washing method, the most common species found was *D. cejpii*, it assaying at a higher relative frequency in the MYA than in the PDA culture medium. *Talaromyces* sp., *T. trachyspermus*, *Talaromyces flavus* (Klocker) Sotolk & Samson (Fig. 4), *P. frequentans*, *Penicillium restrictum* Gilman & Abbott, and *Penicillium purpurescens* (Sopp) Raper & Thom were observed by this method as well, but at a low frequency (Table 1). On the other hand, the zygomycetes *Mortierella* sp. and *M. hiemalis* (Table 1), and *Absidia* sp. (relative frequency 0.02) were recorded in the control sample. The relative frequency of anamorphic stages of Ascomycota fungi *P. frequentans*, *Penicillum* sp., *Aspergillus* sp, and *Cladosporium cladosporioides* (Fries.) de Vries are shown in Table 1. Others anamorphic stages of Ascomycota as *Purpureocillium lilacinum* (Thom) Luannsa-ard, Houbraken; Hywel-Jones & Samson, *Trichoderma koningii* Oudem, *Fusarium oxysporum* Schlecht, *Clonostachys reosea* Fries were also observed.

Fungal quantification by the serial dilution method gave the following abundance values: $2.5 \pm 0.6 \times 10^6$ and $1.9 \pm 0.6 \times 10^6$ CFUs for *D. cejpii* in MYA and PDA medium, respectively, as opposed to $1.1 \pm 0.8 \times 10^5$ CFU for *T. trachyspermus* and 20 ± 8 CFU for *Talaromyces* sp., both in PDA medium (Table 1). In the control sample were recorded CFUs of anamorphic stages of Ascomycota: *Aspergillus* sp., *Penicillium* sp, and *P. frequentans* (Table 1). CFUs of *T. koningii, F. oxysporum,* and *Aspergillus terreus* Thom were also observed. *Penicillium frequentans* was the most often quantified Ascomycete species ($4.3 \pm 1.5 \times 10^4$) in PDA (Table 1). *Mucor hiemalis* was the only zygomycetes found through this technique (Table 1).

Dichotomomyces cejpii, *T. trachyspermus*, *T. flavus*, and *Talaromyces* sp., the most representative species of this work, were deposited in the collection of Botanical Institute C. Spegazzini, National University of La Plata, under the accession numbers LPSC 1160, 1161, 1162, and 1163, respectively.

Discussion

In recent decades, a number of field experiments and case studies in mycology have given useful data for detecting sites

TABLE 1—*Fungal species found in soil samples taken under decaying human remains and 15 m away from it (control). The table shows only common species found using the three techniques employed and all culture media for sample and control, and the fungi common between samples soil treated and control.*

	Soil samples taken under human rest						Control					
	Method						Method					
	Soil wash Relative frequency ($N = 50$)		Serial dilution (1×104) (mean ± SD) ($N = 3$)			Moist chamber Relative frequency ($N = 50$)	Soil wash Relative frequency ($N = 50$)		Serial dilution (1×104) (mean ± SD) ($N = 3$)			Moist chamber Relative frequency ($N = 50$)
Fungal species	MYA	PDA	MYA	PDA	WA		MYA	PDA	MYA	PDA	WA	
Dichotomomyces cejpii	0.52	0.33	$253 \pm 59.8 \times 10^4$	$192 \pm 54.9 \times 10^4$	$15.6 \pm 4.1 \times 10^4$	0.94	0	0	NF	NF	NF	0
Talaromyces trachyspermus	0.04	1.24	$2 \pm 1 \times 10^4$	$10.6 \pm 8.3 \times 10^4$	$0.6 \pm 0.5 \times 10^4$	0.04	0	0	NF	NF	NF	0
Talaromyces sp.	0.02	0.02	$4.3 \pm 1.5 \times 10^4$	$20 \pm 8.0 \times 10^4$	$2.3 \pm 1.5 \times 10^4$	0.02	0	0	NF	NF	NF	0
Cladosporium cladosporioides	0.02	0	NF	NF	NF	0	0.02	0	$1.3 \pm 1.5 \times 10^4$	NF	NF	0
Penicillium sp.	0.02	0.33	$3.1 \pm 1 \times 10^4$	$5.6 \pm 1.6 \times 10^4$	NF	0	0.06	0	$2.3 \pm 0.6 \times 10^4$	$1.3 \pm 1.5 \times 10^4$	NF	0.08
Penicillium frequentans	0	0.02	NF	NF	NF	NF	0	0.08	$2.6 \pm 1.5 \times 10^4$	$4.3 \pm 1.5 \times 10^4$	$3.3 \pm 2.6 \times 10^4$	0.04
Aspergillus sp.	0	0	NF	NF	NF	0	0.02	0.08	$0.51 \pm 1.3 \times 10^4$	$0.4 \pm 1.6 \times 10^4$	NF	0.02
Mortierella sp.	0	0	NF	NF	NF	0	0.3	0.25	$1.3 \pm 1.5 \times 10^4$	$1.6 \pm 0.6 \times 10^4$	$0.3 \pm 0.6 \times 10^4$	0.04
Mucor hiemalis	0	0	NF	NF	NF	0	0.04	0.06	$1 \pm 1 \times 10^4$	$0.6 \pm 0.5 \times 10^4$	$0.4 \pm 0.6 \times 10^4$	0.02

MYA, malt extract yeast agar; PDA, potato dextrose agar; WA, water agar; NF, not found.

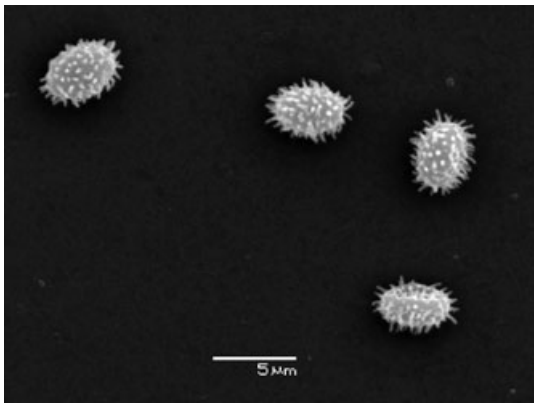

FIG. 4—Talaromyces flavus *ascospores showing their spinulose wall under scanning electron microscopy (4000×).*

where cadaver decomposition has taken place. Those data have demonstrated that certain chemoecologic groups of fungi can act as aboveground grave markers (1,2,17,18). According to Carter and Tibbett (2), varieties of fungi that can potentially act as clandestine grave markers are known as ammonia and postputrefaction fungi and can serve as a tool for the estimation of the postburial interval as well.

The mycobiota found at the present study area clearly differs from the mycobiota identified in the control sample. The zygomycota fungi such as *M. hiemalis*, *Absidia* sp., and *Mortierella* sp are usually found in soil, manure, organic matter in decomposition, and stored grain, and some species are known as sugar fungi by the lack of enzymes to degrade complex carbohydrates (19,20). The authors note the absence of these species in the soil sample taken under the decomposing human body. Crater and Tibbet (2) refer to zygomycetes as postputrefaction fungi, but in our study were present only in the control soil sample, being part of fungal biota of Buenos Aires Province's soils, as mentioned by Cabello et al. (21) and Eliades et al. (12,14,22). This difference could be related to the nitrogen-containing compounds entering the soil from the decomposition of the human remains present. In the particular case presented here, the discovery of geofungal Eurotiomycetes would be consistent with a time of death at about 25 days before the discovery of the body (2).

The ammonia fungi in experimental forest soil treated with urea, ammonium, or other nitrogenous compounds that release ammonia upon decomposition can form fruiting structures (i.e., mushrooms) more quickly than would otherwise naturally occur (i.e., at sites that have not undergone experimental chemical treatment (2)). The ammonia and postputrefaction fungi are part of the same succession of biota that occurs during the natural decomposition process, the ammonia fungi structures constituting the first identifiable ones in this succession. These early fungi can bear fruiting bodies from 1 to 10 months after soil fertilization with nitrogenous compounds and consist in the ascomycetes (anamorphs and teleomorphs) along with the saprotrophic basidiomycetes (23,24). According to Sagara (23) and Fukiharu and Hongo (24)—although their work dealt with forest soils—*D. cejpii*, *T. trachyspermus*, *T. flavus*, and *Talaromyces* sp., of the Ascomycota phylum, would correspond to early phase fungi: thus in our case, these structures were found 25 days after the individual's death. The contribution of nitrogen compounds including amino acids from the proteins of the decomposing remains could have had a significant influence on the occurrence of these fungal species under the cadaver.

Many examples of fungi associated with animal remains have been documented, but only few involving human postmortem decomposition (1,2). The present study is the first record, for Argentina and for South America, of soil fungi occurring at the site of a decaying human body under natural conditions.

Dichotomomyces cejpii was the dominant species in the soil samples. This fungus has been mentioned by Godeas (25) in Buenos Aires, Argentina, in association with leaf litter, but the present findings constitute the first record of the involvement of *D. cejpii* in the decomposition process of a cadaver buried in grassland soil. In the soil samples cultured *in vitro* in the laboratory, we observed both the teleomorph *D. cejpii* and its anamorph *Polypaecilum* spp. *T. trachyspermus* was described by Elíades et al. (22) as being present in soils in the Buenos Aires Province.

A systematic survey of fungal fruiting structures on grassland soil could be used to designate potential grave sites, thereby reducing the amount of time required to screen a large area. Such surveys would be appropriate where burial over months or years was suspected as a cadaver-related fruiting would not occur immediately after burial.

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