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Mold on Stored Photographs and Maps: A Case Study

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Abstract

The aims of this research were to isolate fungi from different archival objects, identify the principal fungal species, and determine their metabolic characteristics. Three photographic processes, three carved plant leaves, and four maps, all without evidence of mold, were analysed. Samples were taken from a 1 cm^2 area of each object with sterile, wet swabs. The swabs were immersed in 1 mL of sterile physiological solution and various dilutions seeded on Extract Malt Agar Petri dishes. After fungal strains were isolated and identified, their different metabolic activities were determined and related to possible biodeteriogenic and pathogenic risks. Though the objects analysed were clean, appropriately housed, and stored at a low temperature and relative humidity, their fungal concentration ranged between 10^1 and 10^3 CFU/cm^2. The predominant fungal genera on all objects were *Aspergillus*, *Penicillium* and *Cladosporium*. 100% of the strains isolated were capable of degrading cellulose and excreted acids. 70% of the species degraded protein and starch, and 50% excreted pigments. Two virulence factors related to the fungal pathogenicity were also analysed: up to 23.8% of the species isolated were positive for both attributes (growth at 37°C; production of hemolysine). These results demonstrate that fungi are present on archive materials, even if the objects appear to be clean and are housed appropriately, if the temperature and relative humidity rapidly increase and remain high for several days, biodeterioration could start. Although the results of virulence factors analysis do not confirm the pathogenicity of these species, it is indicative of the risk that they could represent for the health of library and archives staff.

INTRODUCTION

Paper has long been the dominant support for recording human knowledge, and its deterioration is a serious risk for libraries and archives. The importance of preserving and maintaining printed materials is crucial for librarians, archivists, and information specialists. Also, studies have shown that majority of people still use printed materials despite the existence and currency of electronic copies (Bankole, 2010).

Filamentous fungi – molds – are organisms that play a major role in destroying and degrading the carbon and nitrogen found in materials such as wood and paper (Simon-Nobbe *et al*., 2008). The ability of fungi to produce extracellular enzymes is well established: they can produce hydrolytic enzymes such as cellulase, xylanase, and pectinase. They may also damage valuable documents mechanically, chemically, and aesthetically (Valentín, 2010; Chadeganipour *et al*., 2013).

The majority of fungi need high relative humidity and temperature to grow and develop, but some fungal species are able to live at low water activities. Classified as xerophilic fungi (liking dry supports), they are perfectly adapted to indoor environments and thrive in the microclimatic

niches caused by condensation, lack of ventilation, or water retention of hygroscopic materials (Micheluz *et al*., 2015). They can be found in the indoor air of archives, libraries, and museums, where large collections of paper are kept. Dust is a major food source for these fungi, and dusty conditions intensify fungal contamination (Bankole, 2010). Though biodeterioration of documentary materials is a worldwide problem, causing great damage to the unique collections in the archives, libraries and museums, there are few studies about fungal contaminations in these institutions (Arai, 2000; Szczepanowska and Cavaliere, 2000; Florian and Manning, 2000; Villalba *et al*., 2004; Mateus *et al*., 2004; Cappitelli and Sorlini, 2005; Rakotonirainy *et al*., 2007; Mesquita *et al*., 2009; Michaelsen *et al*., 2010; Borrego *et al*., 2010; 2012; Guiamet *et al*., 2011; Araujo Reis-Menezes *et al*., 2011; Guiamet *et al*., 2011; Zotti *et al*., 2011; Kraková *et al*., 2012; Chadeganipour *et al*., 2013; Vivar *et al*., 2013; Micheluz *et al*., 2015).

The environments within archives and libraries, particularly older institutions, provide the nutritional requirements for the growth of fungi. Based on their geographical location and weather conditions, archives and libraries contain more fungi qualitatively and quantitatively than other enclosed spaces. Protecting cellulosic materials from mold is necessary for libraries and archives, as manuscripts, books, magazines, watercolors, and photographs are made primarly of paper. The pathogenicity of fungi is important for workers involved in collection maintenance (Florian, 2003; Borrego and Perdomo, 2012). Virulence factors are the complex molecules produced by a pathogenic microorganism that enable them to cause diseases. The types of virulence factors produced vary depending on the fungi's taxonomic group or strain (Molina *et al*., 2014).

For these reasons, the aim of this research was to isolate the fungi present on different types of archival documents and photographs from the 19th and 20th centuries, identify the principal fungal genera and species, and determine the metabolic characteristics of these fungal strains.

MATERIALS AND METHODS

Materials Analyzed. Ten nineteenth and twentieth century archival objects were selected for analysis: two carved tobacco leaves, one carved poplar leaf, one tintype, one ambrotype, one albumen print, and four maps. Two of the maps have a paper primary support and two have textile primary supports. These ten objects are considered special collection materials and are held in a repository with appropriate housings and storage furniture, at 17°C and 55% RH.

Fig. 1. The two tobacco leaves, carved with images of Cuban personalities from the middle of the 20th century; sample numbers F1 *(left)* and F2 *(right)*

Isolation of fungi. Samples were taken from a 1 cm^2 area of each object with sterile cotton swabs. The swabs were then immersed in 1 ml of sterile physiological solution. The samples were thoroughly shaken and serial dilutions were made. Each dilution was inoculated (0.1 ml) on Petri dishes containing Extract Malt Agar (BIOCEN, Cuba) supplemented with NaCl (7.5%) (Rojas *et al*., 2002). Afterwards, the plates were incubated at 28°C for 7 days. Fungal concentration was reported in CFU/cm^2.

Identification of fungal isolates. The cultural and morphological characteristics of fungal colonies were observed and identification done according to Barnett and Hunter (1987), Klich and Pitt (1994), Pitt (2000) and *Aspergillus* Image Bank (2006). Morphological (macroscopic and microscopic) features and nutrient assimilation were taken into account for the identification of yeast (Carrillo, 2003).

Determination of the relative frequency (RF) of the fungi genera isolated and their ecological categories for each ecosystem.

The relative frequency (RF) determination was made according to Esquivel *et al*. (2003) and used to determine the ecological category of the fungi isolated. The following formula was used:

$$\text{relative frequency (\%)} = \left[\frac{\text{number of samples positive for a genera or species}}{\text{total samples analysed}} \right] \times 100$$

The ecological categories are: Abundant (A) with RF = 100 – 81%; Common (C) with RF = 80 – 61%; Frequent (F) with RF = 60 – 41%; Occasional (O) with RF = 40 – 21%; Rare (R) with RF = 20 – 0.1%.

Determination of biodeteriogenic potential of the isolated fungal species.

a. **Qualitative determination of the cellulolytic activity and the production of pigments.** The isolated fungi were seeded in slants with a saline culture medium of the following composition per liter: 2g sodium nitrate; 1 g dipotassium phosphate; 0.5 g magnesium sulphate; 0.5 g potassium chloride; 0.01 g ferrous sulphate: 20 g agar; at pH 5.5. In one case, a strip of filter paper (4.8 cm x 1 cm, 50 mg of filter paper) was used as a sole carbon source, and in the other, crystalline cellulose (1%) was used. The cultures were incubated at 28°C during 21 days (Borrego *et al*., 2010; Borrego and Perdomo, 2012; Borrego *et al*., 2012).

b. **Qualitative determination of the production of acid.** A suspension of spores from each isolated fungus was seeded in a minimal liquid medium of identical composition to the one above, but with 1% glucose as the carbon source, 0.03% phenol red, at pH 7. The cultures were incubated at 28°C for 3 days. A change of color from red to yellow is indicative of the production of acids, and the pH of the culture medium was measured using a pH meter (Borrego *et al*., 2010).

c. **Qualitative determination of the proteolytic activity.** Proteolytic activity was determined using the gelatin hydrolysis assay in a tube test. Each isolate was puncture inoculated inside gelatin medium in a test tube. The medium composition was identical to the previous assays,

but with 120 g/L gelatin added as the carbon source. The inoculated tubes were incubated for 7 days at 28°C. Afterwards they were stored at 4°C. A gelatin hydrolysis reaction was evidenced by the degree of medium liquefaction observed when the tubes were inverted (Borrego *et al*., 2010; Borrego and Perdomo, 2012; Borrego *et al*., 2012).

d. **Qualitative determination of the amylolytic activity.** Each isolated fungal strain was seeded in a Petri dish with a saline composition similar to the one previously used with 5 g/L starch employed as the carbon source. After 7 days of incubation at 28°C, 5mL of Lugol's reagent were added to each culture plate. The presence of a colorless zone around the fungal colonies a positive indication of hydrolysis (Borrego *et al*., 2010; Borrego and Perdomo, 2012; Borrego *et al*., 2012).

Determination of some virulence factors related to the fungal pathogenicity.

a. **Growth at 37°C.** The species were seeded in Petri dishes with Extract Malt agar medium and were incubated for 7 - 10 days at 37ºC (Llop *et al*., 2001). A growth of the species was considered as a positive result.

b. **Qualitative determination of hemolytic activity at 37°C.** 5 mL of defibrinated sheep blood was aseptically added to each 95 mL Czapek agar plates at 45ºC with 0.5 g/L chloramphenicol added to prevent bacterial growth. Each strain was puncture inoculated in Petri dishes and incubated at 37°C for 10 to 14 days. A zone of clarification surrounding the colonies was considered a positive result (Bogomolova and Kirtsideli, 2009).

RESULTS AND DISCUSSION

Table 1 shows the concentration of fungi isolated from the different archival objects. The concentration of fungi on the photographs and carved plant leaves ranged between 10 - 10^3 CFU/cm^2, and for the maps the concentration ranged between 0 and 10^2 CFU/cm^2. This indicates that the maps are less soiled than the photographs and carved plant leaves. This is a logical observation, as some objects are composed of organic materials with higher biological receptivity for fungi. For instance, plant leaves provide conditions that support viable fungal spores. Because of its delicacy, leaves are unusual mediums for artistic works. The preparation for carving does not subject the leaf any to invasive chemical processes, so fungal spores already present remain on the object and, under the appropriate conditions, may proliferate. The nature of the process brings along its own microbial biota. The albumen photograph is similar: its proteinaceous nature makes it highly receptive to fungal agents

Table 1. Fungi concentration detected

Code	Object	Fungal concentration (CFU/cm^2)
F1	Tobacco leaf	2 x 10^2
F2	Tobacco leaf	5 x 10^2
F3	Poplar leaf	1 x 10^3
F4	Tintype	5 x 10
F5	Ambrotype	3 x 10
F6	Albumen print	5 x 10^2
M1	Map, paper support	8
M2	Map, paper support	5
M3	Map, textile support	8 x 10^2
M4	Map, textile support	0

(Bucková *et al*., 2014) and contributes significantly to the maintenance of viable fungal propagules.

Fungal propagules are in every ecosystem or microecosystem, including document repositories and archival collections. Although it is possible to maintain the propagules at low concentrations, through regular housekeeping policies and procedures, it is impossible to eliminate them entirely. All microorganisms have certain nutritional and environmental requirements - maximum, minimum and optimal - that favor growth and colonization. As the environments within libraries and archives can be controlled, attention should be paid to what environmental conditions foster the development of microorganisms. Fungi are mostly mesophilic (prefering temperatures between 22-30°C), acidophilus (pH 4-6), and grow well at relative humidities above 70% (Valentín, 2010). Only if the temperature, humidity, and acidity of the substrate is favorable can the fungal spores germinate and grow abundantly. For photographs, the main limiting factor that determines the development of fungi is water, although some xerophilic/halophilic actinomycetes and fungi have been associated with these materials (Bucková *et al*., 2014).

The fungal genera identified and their ecological frequencies appear in table 2. Each archival object evaluated represents one microbial ecosystem. It was observed that the predominant genera were *Aspergillus* and *Penicillium*, with relative frequencies of 100% (an ecological category of abundant) for the photographs and carved plant leaves. For the maps these genera were frequent (RF = 50%) and common (RF = 50%). *Eurotium* (a teleomorph of *Aspergillus*) and *Cladosporium* genera only were detected in two photographic processes (F3 and F5) and are therefore considered rare genera. Yeast of genus *Candida* was detected on both a tobacco leaf (F1) and a map (M3) making the genus ecologically rare for the leaves and occasional for the maps. The detected fungal genera are in good agreement with other authors who have studied fungal genera on gelatin photographs (Abrusci, 2005; Bogomolova *et al*., 2007; Bucková *et al*., 2014). Borrego *et al*. (2010) and Borrego and Perdomo (2014) previously sampled carved plant leaves and silver gelatin photographs, detecting *Candida* spp. and members of the genus *Penicillium*, both with high RF values. *Penicillium* and *Aspergillus* are major constituents of indoor fungal aerobiota, which explains its prevalence on objects. The genus *Candida* could have been introduced from the outside environment or have come from contaminated materials after production: certain species of *Candida* are typically body biota (Bonifaz, 2012), which suggests anthropogenic pollution by users handling the collection.

Table 2. Fungal genera detected and their relative frequency (RF) and ecological category (EC)

Genera	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**RF (%)**	**EC**	**M1**	**M2**	**M3**	**M4**	**RF (%)**	**EC**
Aspergillus	X	X	X	X	X	X	100	A	X	X	-	-	50	F
Penicillium	X	-	X	X	-	X	66.7	C	X	X	X	-	75	C
Eurotium	-	-	X	-	X	-	16.7	R	-	-	-	-	0	-
Cladosporium	-	-	-	-	X	-	16.7	R	-	-	-	-	0	-
Candida	-	X	-	-	-	-	16.7	R	-	-	X	-	25	O
According to Esquivel *et al*. (2003): RF 100 – 81% is considered ecologically Abundant (A); 80 – 61% as Common (C); 60 – 41% as Frequent (F); 40 – 21% as Occasional (O); 20 – 0.01% as Rare (R).														

Physiological characterization of filamentous fungal isolates revealed that all of them grew with filter paper (α and β cellulose) and crystalline cellulose (α-cellulose, more difficult to degrade) as the sole carbon source, and that all species are capable of degrading cellulose (Table 3). Likewise, all of them degraded starch and produced acids. Almost 86% of them degraded the gelatin, but only a few species excreted pigments on paper. Also, evidence of different enzymatic activities was obtained: this was observed when the two *Aspergillus niger* strains are compared. It is generally evidenced that the isolated fungi have an important biodeteriorante potential.

The enzymatic degradation of cellulose and starch by all tested strains would endanger all paper supports and cellulosic textiles. Cellulose is crucial for the stability of both and degradation causes breaking and mechanical fatigue of these materials. Cellulolytic fungal strains can also affect collodion (nitrocellulose) photographic binders. Starch, as a sizing agent and adhesive, is also at risk. *Aspergillus flavus, A. candidus, Penicillium citrinum, P. jantinellum and P. janczewskii* showed a higher capacity to secrete the cellulases and amylases so damaging to cellulosic materials. Acidification of the medium through the excretion of organic acids also favors acid hydrolysis and promotes further secondary colonization of the material by other fungal species. Among the various acids secreted by fungi are acetic, fumaric, citric, and oxalic acids (Vivar *et al*., 2013).

Table 3. Qualitative cellulolytic, proteolytic and amylolytic activities, production of pigments and acids by the isolated fungal strains

		Cellulolytic activity		**Proteolytic activity**	**Amylolytic activity**	
Materials	**Species**	**Filter Paper Degradation**	**Crystalline Cellulose Degradation**	**Gelatin Degradation**	**Starch Degradation**	**Acid Production (pH)**
PHOTOGRAPHS/LEAVES	*Aspergillus flavus* 1	+ + +	+	+	+	+
	Aspergillus flavus 2	+ + +	+ +	+	+	+
	Aspergillus niger 1	+	+ +	+	+	+
	Aspergillus niger 2	+	+ + +	+	+	+
	Eurotium chevalieri	+/-	+	+	+	+
	Cladosporium cladosporioides	+ + [a]	+ + +	+	+	+
	Penicillium citrinum 1	+ + + [b]	+ +	+	+	+
	Penicillium citrinum 2	+ + +	+	-	+	+
	Penicillium chrysogenum	+ +	+ +	+	+	+
	Penicillium janczewskii	+ + [b]	+ +	+	+	+
	Candida sp.	-	-	+	-	+
MAPS	*Aspergillus candidus*	+ + +	+ +	+	+	+
	Penicillium jantinellum	+ + + [b]	+ + +	+	+	+
	Penicillium janczewskii	+ + +	+ +	+	+	+
	Penicillium restrictus	+ + +	+ +	-	+	+
	+ + +: Indicates abundant growth, + +: Indicates moderate growth, +: Indicates poor growth, it is also indicative of the proteolytic and amylolytic activities, +/-: Indicates very poor growth, -: Indicates NO cellulolytic, proteolytic and amylolytic activities. [a]: Indicates presence of brown color on the filter paper, [b]: Indicates presence of yellow color on the filter paper.					

86% of the isolates showed proteolytic activity, an aspect worth noting in relation to photographs with proteinaceous components. The albumen or gelatin layer is where the image is located. Proteolytic activity is therefore a main biodeteriogenic agent for this type of material. The test performed to determine gelatinases was qualitative, and a positive result was observed with *Candida* sp.

In this study, fungal growth at 37°C and hemolysine production at 37°C were evaluated using common solid test media as these tests are standard methods to determine the virulence factors of many human pathogenicity of fungi (table 4). Most fungi are not able to grow at temperatures above 30°C, which is why the ability of certain species (or strains) to grow at the body temperatures of warm blooded animals is advantageous: hosts can be colonized and nutrients obtained without competition from other microorganisms. Of course, this is also dependent on the immune status of the host. The use of pharmaceuticals such as antibiotics or other stressful conditions can cause immunosuppressed states; this facilitates infection by opportunistic pathogens such as environmental fungi. In this study *Aspergillus* spp. species grew well at 37°C, suggesting its ability to colonize tissues (table 4).

Table 4. Results of sample growth at 37°C and hemolytic activity, both of which define some virulence factors related to the fungal pathogenicity in humans

Materials	Species	Growth at 37°C	Hemolytic activity at 37°C
PHOTOGRAPHS/LEAVES	*Aspergillus flavus* 1	+	-
	Aspergillus flavus 2	+	+
	Aspergillus niger 1	+	-
	Aspergillus niger 2	+	-
	Eurotium chevalieri	-	-
	Cladosporium cladosporioides	-	-
	Penicillium citrinum 1	+	+
	Penicillium citrinum 2	+	+
	Penicillium chrysogenum	+	+
	Penicillium janczewskii	+	-
	Candida sp.	+	+
MAPS	*Aspergillus candidus*	+	-
	Penicillium jantinellum	+	-
	Penicillium janczewskii	+	-
	Penicillium restrictus	+	+
	Candida sp.	-	-

Hemolysins, toxins that destroy red blood cells, are produced by certain microorganisms (Nayak et al., 2013). This phenomenon compromises the transport of oxygen to tissues, triggering episodes of anemia and facilitating invasion of blood vessels by the pathogen. 37% of the isolates in this study produced this type of toxins, especially those of the genus *Penicillium* (table 4). The results show that some fungal species growth at 37°C or excrete hemolysine, while others show both capabilities. It is also possible to note that different strains of the same fungal species have dissimilar behaviors, as shown by the two strains of *Aspergillus flavus* and *Candida* sp. These hemolysin producing isolates should be considered the most dangerous to the health of archives/library staff and should be paid special attention. Figure 2 shows the behavior of the virulence factors of the isolated species. As saprophytic fungal species can have a similar

behavior, their occurrence in the isolated fungi can only provide a rough assessment of the potential fungal health risk. Only specific (e.g., animal) tests may unequivocally prove the pathogenicity of strains.

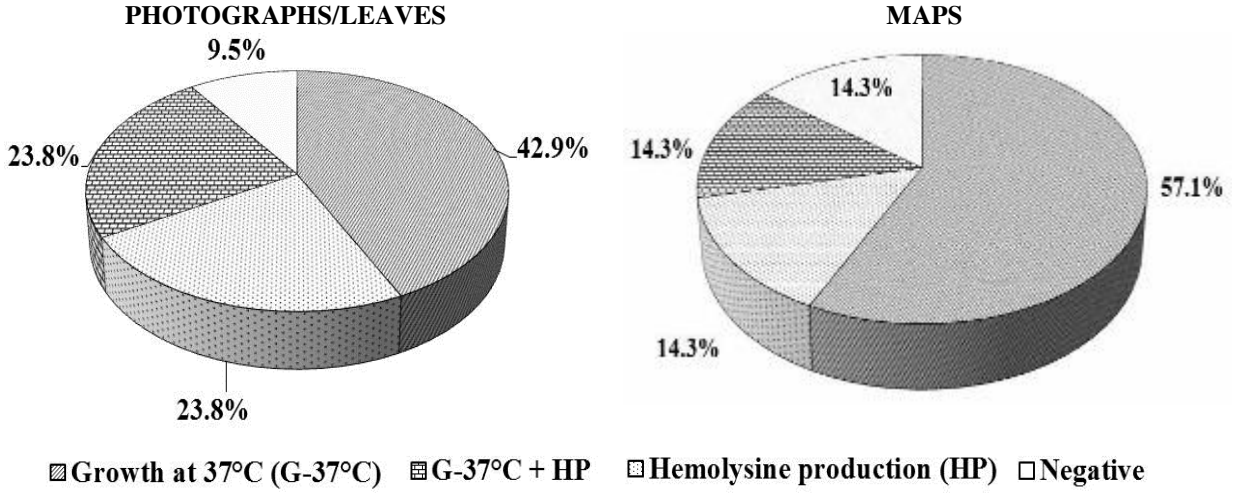

Fig. 2. Behavior of two virulence factors related to the pathogenicity in isolated fungal strains

CONCLUSIONS

- The documentary materials analyzed were contaminated by fungi in spite of a clean appearance and appropriate housings and storage furniture.

- The higher concentrations of fungi were detected on the carved plant leaves.

- The predominant fungal genera were *Aspergillus* and *Penicillium*, with an ecological category of abundant for the photographs and carved plant leaves and frequent (*Aspergillus*) and common (*Pencillium*) for the maps.

- It was demonstrated that all of the isolated species are capable of degrading cellulose and excreting acids. Most are also capable of degrading proteins and producing pigments. For these reasons, the presence of these fungal species represents a potential risk for biodeterioration if temperature and relative humidity increase and remain high for several days: it is important that appropriate climatic conditions be maintained year-round.

- Two virulence factors related to the fungal pathogenicity in humans were analyzed. Only 23.8% of the species isolated from the photographs and 14.3% of those isolated from the maps showed both attributes (growth at 37°C and production of hemolysine). Although these results are not a complete confirmation of the pathogenicity of these species, it is a sign of the potential risk they post for the staff of library and archives.

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