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FUNGI INVOLVED IN BIODETERIORATION OF DOCUMENTS IN PAPER AND EFFECT ON SUBSTRATE

S. Borrego[a]*, P. Guiamet[b,c], I. Vivar[a], P. Battistoni[b]

[a] Laboratorio de Conservación Preventiva. Archivo Nacional de la República de Cuba. Compostela 906 esq a San Isidro, CP: 10100, Habana Vieja, La Habana, Cuba.

[b] Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), Departamento de Química, Facultad de Ciencias Exactas, UNLP, CCT La Plata- CONICET. C.C. 16, Suc.4 (1900), La Plata, Argentina

[c] Facultad de Ciencias Veterinarias, UNLP, La Plata, Argentina

*Corresponding author, E-mail: sofy.borrego@gmail.com, sborrego62@aol.com, Phone: (53)78629436, Fax: (53)78668080

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ABSTRACT

Fungi damage valuable documents mechanically, chemically and aesthetically because they form hyphae, excrete pigments and organic acids, generating particular local conditions that modify the physical-chemical properties of the different documentary supports. The aims of this research were to determine the fungal genera associated with the paper documents biodeterioration and to demonstrate the biofilm formation and the damages. Sampling was done from documents with signs of biodeterioration using sterile cotton swabs. After serial dilutions from the samples, plates with Extract Malt Agar were inoculated and incubated at 30 °C for 7 days. Additional little fragments of the damage zone from each analyzed document were observed using stereomicroscopy, environmental scanning electron microscopy (ESEM) and scanning electron microscopy (SEM). To evaluate the biodeterioration potential risk the strains were grown on cellulose, the acids production and pigments secretion were evaluated too. The genera *Aspergillus, Cladosporium, Fusarium* and *Scopulariopsis* were predominant. Isolated strains excreted acids into the culture medium; most of them grew well on cellulose and a few excreted pigments. The formation of a mature biofilm and the production of extracellular polymeric substances by fungi, as well as a dense biofouling mainly formed by dust mites, were evidenced by the SEM and ESEM observations. Also the observations showed that these strains were able to attach to paper fibre causing damage on them. The observation under optical microscopy of dead insect found inside the book showed fungal adhesion on the insect body and SEM support that this adhesion was formed by *Aspergillus* sp.

Keywords: biodeterioration by fungi, fungal biofilm, paper documents, fungi on Coleoptera body.

HONGOS INVOLUCRADOS EN EL BIODETERIORO DE DOCUMENTOS EN PAPEL Y SU EFECTO EN EL SUSTRATO.

RESUMEN

Los hongos dañan documentos valiosos mecánica, química y estéticamente porque forman hifas, excretan pigmentos y ácidos orgánicos, generando condiciones locales particulares que modifican las propiedades físico-químicas de los diferentes soportes documentales. Los objetivos fueron determinar los géneros fúngicos asociados con el biodeterioro de documentos en papel y demostrar el biofilm formado. Documentos con signos de biodeterioro se muestrearon utilizando hisopos estériles. Después de diluciones seriadas de las muestras, placas con Extracto de Agar de Malta se inocularon e incubaron a 30°C durante 7 días. Fragmentos pequeños adicionales de la zona afectada en cada documento analizado se observó mediante estereomicroscopía, microscopía electrónica de barrido ambiental (ESEM) y microscopía electrónica de barrido (SEM). Para evaluar el riesgo potencial de biodeterioro, las cepas se cultivaron en celulosa, también se evaluó la producción de ácidos y la secreción de pigmentos. Los géneros *Aspergillus, Cladosporium, Fusarium* y *Scopulariopsis* fueron predominantes. Todos los aislados excretaron ácidos al medio de cultivo, la mayoría de ellos crecieron bien en celulosa y algunos excretaron pigmentos. La formación de un biofilm maduro y la producción de sustancias poliméricas extracelulares por hongos, así como un biofouling denso formado principalmente por ácaros del polvo, se evidenciaron en las observaciones SEM y ESEM. También las observaciones mostraron que estas cepas fueron capaces de adherirse al papel

causando daño en sus fibras. La observación bajo microscopía óptica de insectos muertos encontrados dentro de un libro mostró la adhesión de hongos en su exoesqueleto y SEM respaldó que esa adhesión era del hongo *Aspergillus* sp.

Palabras claves: biodeterioro fúngico, biofilm fúngico, documentos en papel, hongo en cuerpo de coleóptero.

INTRODUCTION

Since ancient times, paper has been one of the most used materials to record human knowledge. Although the use of electronic copies is currently widely spread [1], most people still use and value the manuscripts and printed materials. Therefore, preserving and maintaining printed materials is crucial for librarians and specialists in libraries and information centers [2]. Biodeterioration of paper made materials is a worldwide problem that causes great damage to unique manuscripts and books stored in archives, museums and libraries.

The basic component of paper is cellulose; although, other constituents like starch, sugar, other carbohydrates and lignin are present [3]. Thus, paper is susceptible to a wide range of biological agents, including fungi, which have a remarkable capacity of degrading cellulose as it provides a satisfactory medium for mold growth [4]. On the other hand, library and archives indoor environments provide microorganisms with nutritional requirements which contribute to their growth and dispersion under conditions of appropriate temperature and relative humidity [5, 6].

Filamentous fungi are organisms with an important role in the degradation of organic waste, such as wood and paper [7]. The fungal ability to produce extracellular enzymes is well established. They can produce hydrolytic enzymes such as cellulase, xylanase, pectinase, etc. Also, they spoil valuable documents mechanically, chemically and aesthetically because they form hyphae, produce and excrete pigments and organic acids [8].

The majority of fungi need a high relative humidity and temperature to grow and develop, its development is enhanced in microclimatic environments caused by condensation, but some fungal species are able to live at low water activities for that are classified as xerophilic fungi; they are perfectly adapted to indoor environments and thrive in dusty environments, lack of ventilation or water retention by hygroscopic materials for these materials with a very low water activity can be colonized by xerophilic species [9]. They can be found in the indoor air of archives, libraries and museums where much paper exists. Dust is a good source for these fungi to feed and grow; these conditions intensify fungal contamination [10]. Fungal degradation and the documentary materials deterioration is a worldwide problem that causes great damage to especially paper documents stored in the archives, libraries and museums [11,12].

The aims of this work were to determine the fungal genera associated with the visual signs of biodeterioration and to demonstrate the biofilm formed.

MATERIALS AND METHODS

Selection of heritage documents. A total of twenty-five documents in paper of 19th and 20th centuries were analyzed. Eighteen of them are stored in the National Archive of the Republic of Cuba (NARC) whilst seven in different Argentinean archives.

Isolation of fungi from paper documents surfaces. Sample collections were performed from a 2 cm^2 surface of each graphic material with sterile cotton swabs [10,13]. 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 (0.1ml) on Petri dishes containing Malt Extract Agar supplemented with NaCl (7.5%) [14]. After, the plates were incubated at 30°C for 7 days. Fungal concentration was reported in CFU/cm^2.

Analysis of biofilms and biofouling formed on paper documents. Small fragments extracted from damaged areas in some analyzed document were observed using

stereomicroscopy (Olympus at 20X), scanning electron microscopy (ESEM) and scanning electron microscopy (SEM). Also a dead insect that was found inside a book was observed with a stereomicroscopic image (20X).

In the observations made by ESEM an INSPECT, QUANTA 200 scanning electron microscope operated at an accelerating voltage of 20 - 25 kV was used.

Microscopic procedure for SEM observations. The samples of biofilm formation artificially on paper and the insect were observed by scanning electron microscopy (SEM). Bioadhesion assays with selected strains of *Scopulariopsis* sp. from documents were monitored. Bioadhesion to paper and biodeterioration were observed by SEM (Jeol 6,360 LV). These samples were not fixed and were metalized with Au/Pd. In the case of the biofilm of *Scopulariopsis* sp. samples were kept in a closed chamber with pure ethanol for 24 h and metalized with Au/Pd prior to observation.

Identification of the fungi isolated. Cultural and morphological characteristics of fungal colonies as well as conidiophores and conidia fungal structures of all isolated strains were observed and the identification was performed according to different manuals [15,16].

Determination of the relative frequency (RF) of the fungal genera isolated from documents and their ecological categories. The RF determination was made according to Esquivel *et al*., (2003) [17] to determine the ecological category of the fungi general isolated. It was necessary to use the following formula:

RF = (Times a genus is detected/Total number of sampling realized) x 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%.

Qualitative determination of the cellulolytic activity and the production of pigments by fungi. The isolated fungi were seeded in slants on a saline culture medium and followed procedure was reported previously [14].

Determination of the production of acid. A suspension of spores from each isolated fungus was seeded in a minimal liquid medium and followed procedure was reported previously [14].

RESULTS AND DISCUSSION

Fungal diversity detected on the studied documentary supports

Table 1 shows the concentrations of fungi isolated from the different documents stored at NARC which are preserved in wrappers appropriate. However, figure 1 shows the book that was sampled in an Argentine archive.

The obtained fungal concentrations on photos were the highest and ranged between 100 - 6100 CFU/cm^2 whilst on maps the concentrations were the lowest and ranged from 5 - 45 CFU/cm^2. However the obtained concentrations on manuscripts were more variable and range from 10 - 300 CFU/cm^2.

Table 1. Fungal concentration detected on the documents conserved in NARC with archival quality wrappings**.**

Code	Material analyzed	Fungal concentration (CFU/cm^2)
B1*	Book in paper	0.1 x 10
D1	Manuscripts (XVIII – XIX centuries)	1.0 x 10
D2		3.0 x 10^2
D3		2.0 x 10
D4		2.0 x 10
D5		1.0 x 10^2
D6		1.0 x 10
P1	Photo on paper with albumen emulsion	5.0 x 10^2
P2	Photo on paper with gelatin emulsion	1.2 x 10^2
P3		5.0 x 10^2
P4		3.2 x 10^2
P5		1.0 x 10^2
P6		6.1 x 10^2
P7*		1.0 x 10^2
P8*		0
M1	Map on paper	0.8 x 10
M2		0.5 x 10
M3		1.8 x 10
M4		4.5 x 10
M5		1.5 x 10
M6		0.5 x 10
M7*		0.3 x 10
NA1*	Notarial acts	1.4 x 10^3
NA2*		5.0 x 10^2
NA3*		2.0 x 10^4

*: Indicative that these documents are stored in Argentine archives.

The fact that the photographs have a higher fungal concentration could be due to the presence of the emulsion both of albumin and gelatin. The photographic emulsions are more hygroscopic than paper and moreover they protean nature makes it highly receptive to the fungal agents facilitating their viability for a long time [18]. This contributes significantly to the maintenance of viable fungal propagules. It is a fact that fungal propagules are in all ecosystems, such as is the case of the indoor air of document repositories, dust and on the collections materials. Although it is possible to maintain the propagules in low concentrations (through regular cleaning and establishing physical barriers that help eliminate or prevent the arrival of dust) it is impossible to eliminate them entirely [14].

Eight different fungal genera, two yeast genera and one white non-sporing septate mycelia were detected in the Cuban documents (Table 2). This result demonstrates the great fungal diversity that is on the documents analyzed in spite of being clean and protected by appropriate wrappings.

From the Argentine book which was very contaminated were isolated the species *A. niger, A. flavus, Cladosporium* sp., *Penicillium* sp., *Talaromyces helicus, Scopulariopsis* sp., *Fusarium* sp. and white non-sporing septate mycelia.

Each document or book evaluated represents one microbial ecosystem composed by a fungal community which is formed by one or more fungal species. For this reason, different genera could be detected in one only material. *Aspergillus, Penicillium* and *Cladosporium* were the predominant genera and were classified ecologically as abundant, frequent and common, respectively. It is highlight that a teleomorph of *Aspergillus* genus and two yeast genera (*Candida* spp. and *Rhodotorula* spp.) were isolated from photos and they were classified as rare genera; as well as *Scopulariopsis* spp. was found on Argentine document and similarly was classified as rare genus [17]. Some of these genera have been isolated from paper documents and have been reported previously [5,7,11,14,18-23].

Degradation of paper cellulose by the fungal isolates

Physiological characterization of isolated filamentous fungal revealed that 98% of them grew at the expense of filter paper as sole carbon source (this paper is composed by pure cellulose, but is a mixture of α and β cellulose), indicative that they are capable of degrading the cellulose of paper with varying intensity (Fig. 2 and 3). In addition, it was shown that most of the isolated fungal strains could adhere to the paper and formed mature biofilms (Fig. 1). Also, 95% of the strains produced organic acids that were excreted into the culture medium according previously reported [9,14,18,19].

SEM and ESEM revealed signs of the rupture of paper fibers as a consequence of the *Scopulariopsis* sp. growth and biodegradation activity (Fig. 3). It is known that *Scopulariopsis* is a saprophytic fungus and has been isolated from soils, plant material, insects and dung [24]. Also some species of this genus are big cellulase and xylanase producers [25]. Similar result was reported previously [26]. It also shows evident biofouling and the presence of biofilm in some documents stored both in the NARC and in the Argentine archives, although with different magnitudes. Also, much of the material stored in three archives had fox-like reddish-brown color spots, type 'foxing' (Fig.1) [19,27].

A biofouling formed by dust mite's exoskeletons fundamentally was observed on one documents preserved in an Argentine archive although some pollen particles were observed in other document too (Fig. 4). Similar results were reported previously [28,29]. This is indicative that the wrapped documents that are conserved in repositories with natural ventilation are always penetrated by small amounts of dust that serves as biofouling; therefore it is imperative to carry out special systematic cleanings.

To assess the degree of degradation in historical document collection one can also apply modern instrumental methods such us: UV-Vis spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), ESEM, X- ray diffraction (XRD), atomic absorption spectroscopy (AAS), hermogravimetriy (TG), atomic force microscopy (AFM), etc. Depending on the analysis techniques used, the amount of sample taken for analysis can be reduced or be measured *in situ* [30].

Table 2. Fungal genera detected in the paper documents conserved in the Cuban and Argentine archives.

Genera	*Aspergillus*	*Penicillium*	*Cladosporium*	*Alternaria*	*Emericella* *	*Eurotium* *	*Gliomastix*	*Paecilomyces*	*Scopulariopsis*	*Trichophyton*	*Candida*	*Rhodotorula*	WNSM
B1§	+	-	-	-	-	-	-	-	-	-	-	-	-
D1	+	+	+	-	-	-	-	+	-	-	-	-	-
D2	+	-	-	-	-	-	+	+	-	-	-	-	+
D3	+	-	+	-	-	-	-	-	-	-	-	-	+
D4	+	+	-	-	-	-	-	-	-	+	-	-	-
D5	+	+	-	-	-	-	-	-	-	-	-	-	-
D6	+	-	+	-	-	-	-	-	-	-	-	-	-
P1	+	+	-	-	-	-	-	-	-	-	-	-	-
P2	+	-	-	-	-	-	-	-	-	-	+	-	-
P3	+	+	-	-	-	+	-	-	-	-	-	-	-
P4	+	+	-	-	-	-	-	-	-	-	-	-	-
P5	+	+	-	-	-	-	-	-	-	-	+	+	+
P6	+	-	+	-	-	+	-	-	-	-	-	-	-
P7§	+	+	-	-	-	-	-	-	-	-	-	-	-
M1	+	+	+	-	-	-	-	-	-	-	-	-	-
M2	+	+	-	-	-	-	-	-	-	-	-	-	-
M3	+	-	-	-	-	-	-	-	-	-	-	-	-
M4	+	-	+	-	-	-	-	-	-	-	-	-	-
M5	-	+	-	-	-	-	-	-	-	-	+	-	-
M6	+	-	-	-	+	-	-	-	-	-	-	-	-
M7§	+	-	-	+	-	-	-	-	+	-	-	-	-
NA 1§	+	+	-	-	-	-	-	-	-	-	-	-	-
NA 2§	+	+	-	-	-	-	-	-	-	-	-	-	-
NA 3§	+	-	+	-	-	-	-	-	-	-	-	-	-
RF (%)	96	52	24	4	4	8	4	8	4	4	12	4	12
EC	A	F	O	R	R	R	R	R	R	R	R	R	R

B: Indicate Book. D: Indicate Document. P: Indicate Photo. M: Indicate Map. NA: Indicate Notarial Act. §: Indicate that these documents are stored in Argentine archives. *: Indicates a teleomorph of *Aspergillus* genus. WNSM: White Non-sporing Septate Mycelia. According to Esquivel *et al*. (2003) [17] when the RF is between: 100 - 81% the genus is considered ecologically as Abundant (A); 80 - 61% as Common (C); 60 - 41% as Frequent (F); 40 - 21 % as Occasional (O); 20 - 0.01% as Rare (R).

potential source of biodeterioration. Colonization or microbial growth on a material always produces their biodegradation.

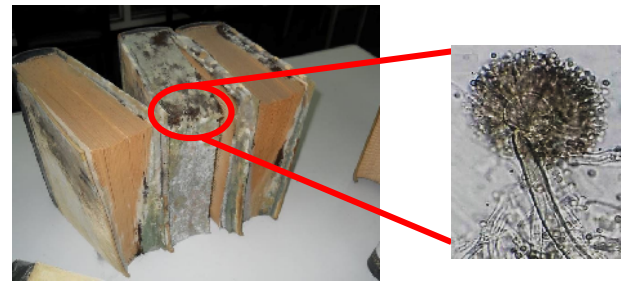

Fig. 1. Book that was sampled in an Argentine archive. Micrograph of optical microscopy (400X) of *Aspergillus niger*, one of the species detected on the book analyzed.

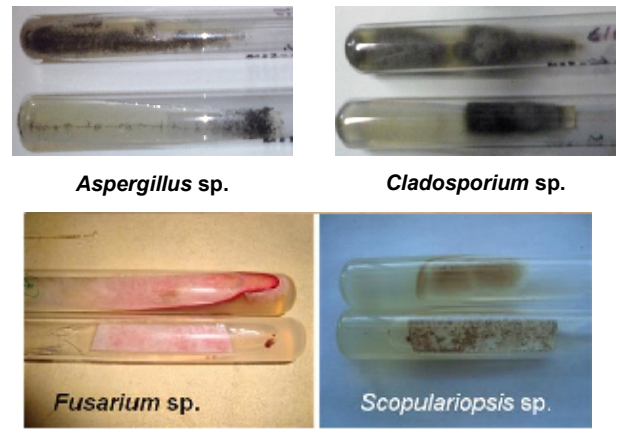

Fig. 2. Abundant growth on the filter paper of the studied fungal strains and mature biofilm formed in the paper strips that are indicative of a high fungal degradation; they have also excreted dark pigments to the paper.

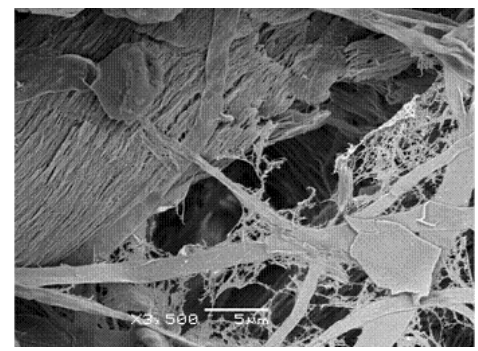

Fig. 3. SEM image of deteriorated cellulose fibers by *Scopulariopsis* sp.

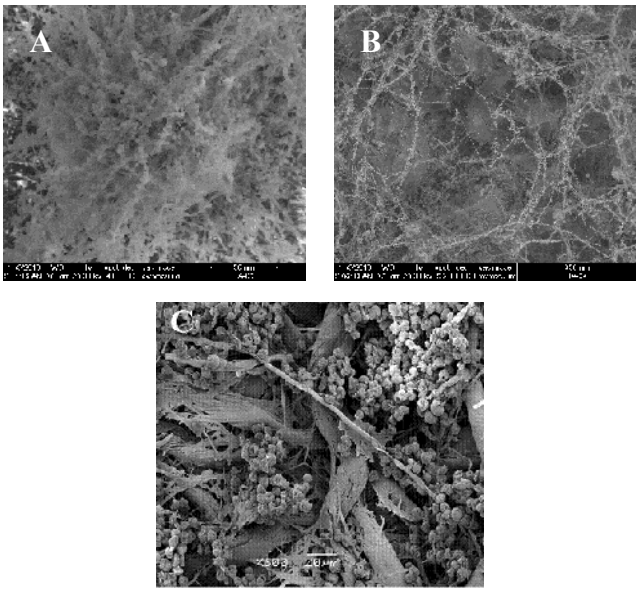

Fig. 4. A: ESEM image of dense fungal biofilm formed by different fungal isolates observed on document in paper. **B:** ESEM observations show a biofouling formed by dust mite's exoskeletons fundamentally observed on document in paper. **C:** SEM image of biofilm formation artificially on paper after 7 days by *Scopulariopsis* sp.

Dead insect detected in a book

By carefully reviewing some documents as part of the systematic reviews we carry out within the Integrated Pest Management Program we detect in a book one insect belonging to the Fam. Dermestidae (Coleoptera) usually present in archives and libraries. The microscopic observations revealed that the insect had its exoskeleton colonized by fungi and a detailed observation of these structures evidenced that they belonged to the genus *Aspergillus* because the conidiophores present were characteristic of this genus. The exoskeleton colonization of the beetle found was confirmed using SEM and is part of the biofouling existing in that book (Fig. 5).

In relation to the insects Trovão *et al*. (2013) [31] showed that the insects are vectors that facilitate the dispersion of fungal spores and propagules. Therefore our results corroborate what these authors previously reported.

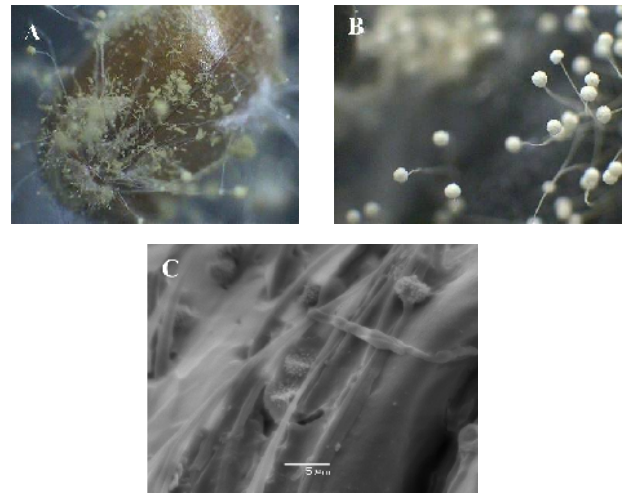

Fig. 5. A: Optic microscopy image with Coleoptera colonized by fungi (20X at stereomicroscopic). **B:** Stereomicroscopic image (20X) that evidence the colonization by *Aspergillus* sp. because the typical conidiophores were shown. **C:** SEM image detail of Coleoptera and growth of conidiophore of *Aspergillus* sp.

CONCLUSION

It was possible to determine the fungal concentrations on different documents and books stored in Cuban and Argentine archives. It is demonstrated evidence through different microscopic techniques the impact of these fungi in the biofilms formation and the biodeterioration in paper.

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