Evaluation of biodeterioration control methods for fungal damage on paper based cultural heritage materials

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Abstract: Fungal deterioration is one of the main threats to the conservation of paper documents, compromising both their physical and aesthetic integrity. Despite this, many institutions lack specific research evaluating the effectiveness of methods used to control fungal biodeterioration. This study assessed the effectiveness of ethanol at concentrations of 70%, 75%, and 80%, as well as mechanical vacuum cleaning, in controlling *Eurotium sp*. and *Penicillium sp*. isolated from historical manuscripts. The treatments were analyzed both independiently and in combination. The results challenge traditional recommendations, by demonstrating that 70% ethanol concentration is not fully effective against *Eurotium sp*. and *Penicillium sp*., two fungal genera commonly found in archives and libraries. Notably, the combined treatment -vacuum cleaning followed by the application of 80% ethanol-proved particularly effective, highlighting its potential as a viable strategy for controlling biodeterioration in historical documents.

Keywords: Biocide, Ethanol, Eurotium sp., Penicillium sp., Vacuum cleaning

Evaluación de métodos de control de biodeterioro causado por hongos en obras del patrimonio cultural en papel

Resumen: El deterioro fúngico es una de las principales amenazas para la conservación de documentos en papel, afectando tanto su integridad física como estética. A pesar de ello, muchas instituciones carecen de investigaciones específicas que evalúen la efectividad de los métodos empleados para controlar el biodeterioro fúngico. En este estudio se evaluó la efectividad del etanol (70%, 75% y 80%), así como de la limpieza mecánica con aspiradora, en el control de *Eurotium sp.* y *Penicillium sp.* aislados de manuscritos históricos. Se analizaron los efectos del etanol y el aspirado como tratamientos independientes y combinados. Los resultados desafían las recomendaciones tradicionales, al demostrar que la concentración de etanol al 70% no es totalmente eficaz contra *Eurotium sp.* y *Penicillium sp*., dos géneros fúngicos comúnmente reportados en archivos y bibliotecas. En particular, el tratamiento combinado -limpieza con aspiradora seguido de la aplicación de etanol al 80%- resultó especialmente eficaz, lo que pone de relieve su potencial como estrategia viable para controlar el biodeterioro de los documentos históricos.

Palabras clave: Biocida, Etanol, *Eurotium sp., Penicillium sp*.,Aspiradora.

Avaliação de métodos de controlo da biodeterioração para danos fúngicos em materiais de património cultural em papel

Resumo: A deterioração fúngica é uma das principais ameaças à conservação de documentos em papel, comprometendo tanto a sua integridade física como estética. Apesar disso, muitas instituições carecem de investigação específica que avalie a eficácia dos métodos utilizados para controlar a biodeterioração fúngica. Este estudo avaliou a eficácia do etanol em concentrações de 70%, 75% e 80%, bem como da limpeza mecânica por aspiração, no controlo de *Eurotium sp*. e *Penicillium sp*., isolados de manuscritos históricos. Os tratamentos foram analisados tanto de forma independente como em combinação. Os resultados põem em causa recomendações tradicionais, ao demonstrar que a concentração de etanol a 70% não é totalmente eficaz contra *Eurotium sp*. e *Penicillium sp*., dois géneros fúngicos frequentemente encontrados em arquivos e bibliotecas. Destaca-se que o tratamento combinado, limpeza por aspiração seguida da aplicação de etanol a 80%, revelou uma eficácia particularmente elevada, evidenciando o seu potencial como estratégia viável para o controlo da biodeterioração em documentos históricos.

Palavras-chave: biocida, etanol, Eurotium sp., Penicillium sp., limpeza por aspiração

Introduction

The conservation of documentary heritage, especially paper documents, faces considerable challenges due to various agents of deterioration, among which fungal colonization stands out as one of the main causes of loss of information and aesthetic features of the documents. The term biodeterioration has been widely adopted in the scientific field to refer to the biological degradation processes of materials. Biodeterioration refers to the harmful modifications that a material undergoes as a result of the biological activity of living organisms. This phenomenon often occurs alongside physicochemical deterioration processes, whose progression is influenced by environmental factors such as relative humidity, temperature, and exposure to light (Sameño,2018). In the case of paper, fungal biodeterioration follows a four-phase cycle: contamination (via air, contact with contaminated materials, or vectors), spore germination, hyphal and colony development, and finally, the decomposition of the work (Gallo *et al.* 2003). During this process, fungi produce enzymes such as cellulases, amylases, xylanases, and gelatinases, which break down the main structural components of paper, including cellulose, adhesives, and glues (Abdel-Maksoud *et al.* 2022; Vaillant 2013). Consequently, hyphal growth and enzymatic action trigger a loss of mechanical resistance in the paper, weakening it and making it more susceptible to degradation (Kirtzel *et al.* 2019).

This general mechanism of fungal deterioration has been corroborated by numerous case studies. For instance, research by Shamsian *et al.* (2006) and Zotti *et al.* (2008) has highlighted the high prevalence and diversity of *Aspergillus* and *Penicillium* species in historical manuscripts, emphasizing their ability to degrade cellulosic materials and thrive even under low humidity conditions. In a collection of ancient manuscripts, Shamsian *et al.* (2006) reported that *Aspergillus spp*. and *Penicillium spp.* accounted for approximately 64% of fungal contaminations, with a high frequency of both surface and internal colonization. Similarly, Zotti *et al.* (2008) identified 14 species of filamentous fungi in samples from historical paper, with *Penicillium* being the most represented genus. These findings are not isolated; other studies worldwide support this trend, reporting up to 61 species of *Penicillium* and 44 of *Aspergillus* isolated from deteriorated paper materials, as well as from surfaces and air in libraries and archives (Pinheiro *et al.* 2019; Oetari *et al.* 2016; Teixeira *et al.* 2018). Species such as *A. flavus, A. fumigatus*, A. *versicolor, P. citrinum, P. spinulosum*, and *P. commune* have been consistently isolated from deteriorated documents in various regions and environmental conditions (Zotti *et al.* 2008; Okpalanozie *et al.* 2018; Kraková *et al.* 2018). This widespread distribution, along with their enzymatic potential and adaptability, explains why these two genera are recurrently associated with the biodeterioration of paper worldwide.

Recent studies have shown that fungi like *Aspergillus niger* and *Penicillium chrysogenium* possess a remarkable ability to secrete extracellular hydrolytic enzymes that weaken and alter the paper structure, significantly contributing to biodeterioration (Fouda *et al.* 2022). Additionally, these organisms produce acidic metabolites and pigments that cause aesthetic deterioration and stains. These include naphthoquinones, anthraquinones, and azaphilones, responsible for reddish, yellow, and violet discolorations on paper (Pavlović *et al.* 2022). The restoration and conservation of paper-based works previously affected by fungal contamination aim to halt the degradation process and preserve the original characteristics of the documents (Cubero 2007).

Control methods can be classified into indirect and direct approaches. Direct methods involve physical, chemical, and biological treatments designed to eliminate the microorganisms responsible for deterioration (Sameño 2018). Among the chemical approaches, biocides, also known as chemical agents, are frequently used. Ethanol, for example, is a widely applied antiseptic effective in the inactivation of bacteria, viruses, and fungi (Vargas 2011). Its mechanism of action involves the denaturation of microbial proteins and disruption of cell membranes, compromising the structural integrity and viability of the organisms (Droguett 2023). On the other hand, mechanical methods focus on the physical removal of microbial structures from the document surface. This procedure typically involves the use of tools such as brushes, spatulas, and vacuum cleaners to remove hyphae, spores, and other accumulated contaminants from affected substrates (Cappitelli *et al*. 2020). While these techniques are effective at reducing visible fungal load, their action is limited as they do not ensure complete eradication of fungal spores, which may remain dormant and reactivatable under favorable environmental conditions.

The preservation of documentary heritage becomes even more critical when considering that historical documents are not merely physical artifacts but also carriers of knowledge. Their deterioration represents a direct threat to the transmission of information to future generations. A particularly concerning case involves manuscripts preserved by the Turkish Manuscripts Institution in Istanbul, where restoration professionals face significant challenges due to the lack of specific studies evaluating the effectiveness of current biodeterioration control methods. Of particular interest is the limited understanding of whether vacuum cleaning alone is sufficient or if it should be complemented by chemical treatments such as ethanol application. Therefore, the aim of this study is to identify the most effective method for controlling fungal biodeterioration in paper-based materials. To achieve this, the efficacy of various ethanol concentrations will be assessed, both independently and in combination with mechanical cleaning technique, to determine the optimal strategy for the conservation of contaminated documentary heritage.

Materials and Methods

—*Isolation of Fungal strains*

Two fungal strains (F1 and F2) isolated from historic callighraphy paper sheets mounted on wooden panel were employed. Isolation has been carried out in laboratories of Directorate of the Turkish Institution for Manuscripts in

Istanbul. Callighraphy sheets which were in bad condition were found in Bursa Manuscripts Library [Figure 1]. There were stains, lost parts and decomposition on paper through activity of microorganisms.

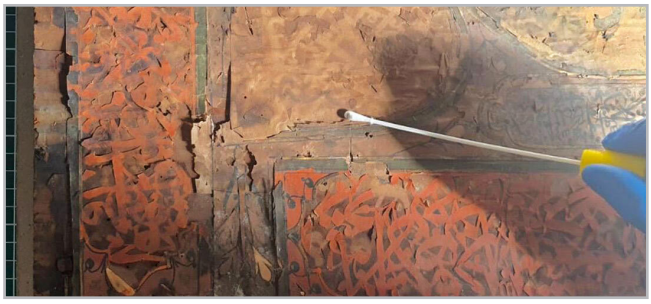

Figura 1.- Callighraphy sheets. Bursa Manuscripts Library. *Copywright*: authors.

The samples were taken with cotton sterile swabs from the biodeteriorated areas. Swabs were inoculated on PDA medium petri dishes with spread plate method. Plates were incubated at 26±1ºC for 7 days. Pure fungal colonies were obtained by subculturing from media containing all the isolated fungi of mixed colonies. Morphological characterization of each strain was carried out using lactophenol blue staining to observe fungal reproductive structures under a microscope.

Evaluation of antifungal activity *in vitro*

The effectiveness of ethanol as biocide was evaluated using ethanol concentrations of 70%, 75%, and 80%. The procedure involved extracting an agar "plug" with fungal growth, which was submerged in an Eppendorf tube containing the respective ethanol concentration for an exposure time of 8 minutes. This exposure time was selected based on the results obtained by Lucas *et al.* (2017). Subsequently, the agar plug was placed on PDA plates and incubated at 25 °C for 8 days. This procedure was performed in triplicate for each strain, using 0.85% w/v sterile saline solution as a control.

Fungal growth following exposure to different ethanol concentrations was evaluated by measuring the diameter of the fungal colonies and comparing it to growth without ethanol exposure. The percentage inhibition of fungal growth was calculated using Equation 1.

$$\% \text{ of inhibition} = \frac{(\text{Control plate diameter} - \text{Biocide plate diameter})}{(\text{Control plate diameter})} \times 100$$

Evaluation of the effect of ethanol on conidial germination

From the pure cultures of the two selected fungal strains, a 5 mm agar plug was transferred into a tube containing 10 ml of 0.85% w/v sterile saline solution. After shaking to detach conidia from the agar, a 10 µl aliquot of the conidial suspension was transferred to a Neubauer chamber. The concentration was adjusted by diluting the suspension with sterile saline to reach a final concentration of 1×10^6 conidia/ml for inoculation (Sequeira *et al.* 2017). A microculture chamber was set up by placing a coverslip elevated on two swabs in a Petri dish, which was sterilized in an autoclave. After sterilization, a 1 x 1 cm fragment of solid PDA was placed in the center of the coverslip within the Petri dish.

Inoculation and ethanol treatment

Following Sequeira *et al.* (2017), 3 µl of the conidial suspension were inoculated onto the agar fragment, and 10 µl of the respective ethanol solution (70%, 75%, or 80%) were applied to the inoculated samples. A flame-sterilized coverslip was placed over the sample. For the control, 0.85% w/v saline solution was used instead of ethanol. To maintain humidity, 1 ml of sterile glycerinated water was added to the bottom of the Petri dish. The microcultures were incubated at room temperature (19°C approx.) and daily microscopic observations were performed for 5 days to assess conidial germination. For each ethanol treatment and control, five replicates were prepared for each strain (F1 and F2). Conidial development was classified as follows: "-" for no germination, "+" for conidial germination, "++" for hyphal development, and "+++" for intense mycelial growth (Sequeira et al., 2017).

Evaluation of control methods on paper samples

— Preparation of paper samples with fungal growth

Following Sequeira *et al.* (2017), discs of Whatman No. 1 filter paper (90 mm diameter) were sterilized in autoclave. The discs were placed in individual Petri dishes containing PDA media, inoculated with 10 µl of a conidial suspension (1×10^6 conidia/ml) prepared from a mixture of the two fungal species (F1 and F2), and incubated at 25 °C for 3 days. The paper discs were then transferred to sterile Petri dishes and air-dried for 10 days. Subsequently, the discs were cut into triangular shapes to facilitate treatment application.

Application of treatments

Biodeterioratin control methods were applied as follows:

Treatment 1: Ethanol application. Ethanol was evenly applied to both sides of the paper discs using a swab. A second application ensured thorough impregnation. Two ethanol concentrations were tested: 80% identified as the most effective in in vitro tests, and 70%, a concentration commonly in conservation practices.

Treatment 2: Vacuum cleaning + Ethanol application. Paper discs were cleaned using a vacuum cleaner employed by restorers, applying suction for 60 seconds on each side. Ethanol was then applied at the two tested concentrations (80% and 70%).

Treatment 3: Vacuum cleaning only. Discs were cleaned using the vacuum cleaner for 60 seconds with no ethanol applied.

Each treatment was performed in triplicate, with untreated samples serving as controls. After treatment, the paper discs were placed in fresh Petri dishes containing PDA and incubated at 19-20 °C for seven days. Fungal recolonization on the paper samples was documented through photographs.

Assessment of treatment effectiveness

A qualitative scale was used to classify fungal growth:

"-": No visible fungal growth, indicating the treatment was effective.
"+": Minimal growth, suggesting partial effectiveness.
"++": Moderate growth, indicating limited treatment effectiveness.
"+++": Extensive and dense fungal growth, signifying ineffective treatment.

Results and Analysis

The macroscopic and microscopic observations revealed distinct morphological features in the two isolated fungal strains. Strain F1 displayed oval to elliptical greenish ascospores, while strain F2 exhibited round yellow-green conidiospores [Figure 2]. These characteristics led to identification of F1 as a species within the subgenus *Eurotium*, and F2 within the genus Penicillium.

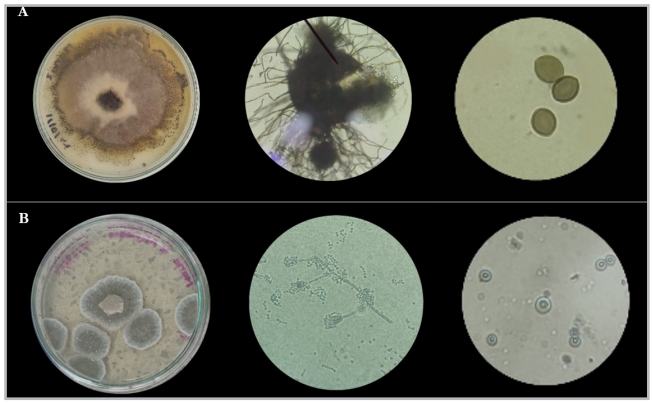

Figura 2.- Microscopic characteristics observed at 40x of strains F1 and F2. (A) Cleistothecium and ascospores of strain F1 (*Eurotium sp.*); (B) Conidioma and conidiospores of strain F2 (*Penicillium sp.*). Copywright: photographs taken by the authors.

The identification of F1 as a potential member of *Eurotium* is supported by the presence of cleistothecia, a hallmark of this subgenus. These sexual reproductive structures are consistent with descriptions by Benjamin (1955), who noted the presence of yellow, globose ascocarps ranging from 50 to 175 µm in diameter, embedded in a dense hyphal matrix. This observation aligns with the findings of Visagie *et al*. (2017), who described *Eurotium* species as the teleomorphic (sexual) state of certain *Aspergillus* species. The morphological features observed here are therefore indicative of this group and suggest a possible link to xerophilic fungi often associated with biodeterioration in archival materials. In contrast, strain F2 exhibited a typical *Penicillium* morphology, with conidiophores arising from the hyphae and branching into terminal clusters. This structure is characteristic of the asexual reproductive phase of the genus and is commonly found in indoor environments and on cellulose-based degraded substrates (Borrego *et al.* 2012).

In vitro antifungal activity

In vitro assays demonstrated fungal growth for both strains (F1 and F2) at all ethanol concentrations tested (70%, 75%, and 80%) [Figure 3].

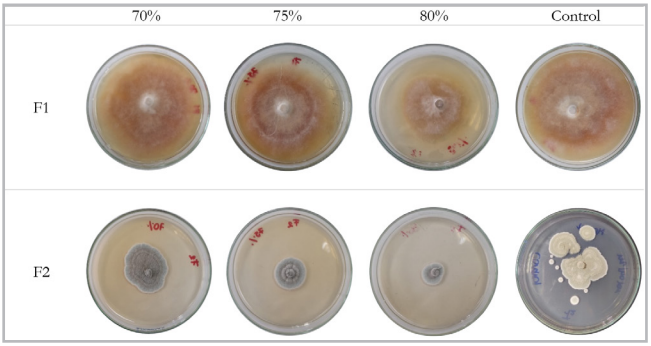

Figura 3.- Microscopic characteristics observed at 40x of strains F1 and F2. (A) Cleistothecium and ascospores of strain F1 (*Eurotium sp.*); (B) Conidioma and conidiospores of strain F2 (*Penicillium sp.*). Copywright: photographs taken by the authors.

However, a progressive reduction in colony diameter was observed with increasing ethanol concentration, suggesting a dose-dependent inhibitory effect. The largest colony areas were recorded at 70% ethanol, while the smallest were observed at 80% ethanol. These results provide initial evidence of the tolerance mechanisms exhibited by both *Eurotium sp*. and *Penicillium sp*., as growth was maintained even at higher ethanol concentrations. Such behavior suggests that these strains possess adaptive strategies that allow survival under stress conditions imposed by ethanol exposure.

Results presented in Figure 4 highlight a marked difference in sensitivity between the two strains. F2 (*Penicillium sp*.) exhibited higher inhibition percentages compared to F1

(*Eurotium sp.*) at all ethanol concentrations [Figure 4 A]. At 80% ethanol, *Penicillium sp*. achieved 63% inhibition, contrasting with *Eurotium sp.*, which only reached 31%. This trend persisted at lower ethanol concentrations: 49.99% and 19.01% inhibition for *Penicillium sp*. at 75% and 70% ethanol, respectively, compared to 7.17% and 1.6% for *Eurotium sp*. Statistical analysis of colony diameters confirmed the significance of these findings [Figure 4 B].

At 70% ethanol, *Eurotium sp*. exhibited significantly larger growth (82.33 mm average diameter) compared to *Penicillium sp.* (49.67 mm). Increasing ethanol concentration to 75% further reduced colony diameters for both strains, with *Penicillium sp.* showing a more pronounced decrease (30.67 mm). At 80% ethanol, both strains exhibited further reductions, though *Eurotium sp*. maintained significantly larger growth compared to *Penicillium sp.* The observed

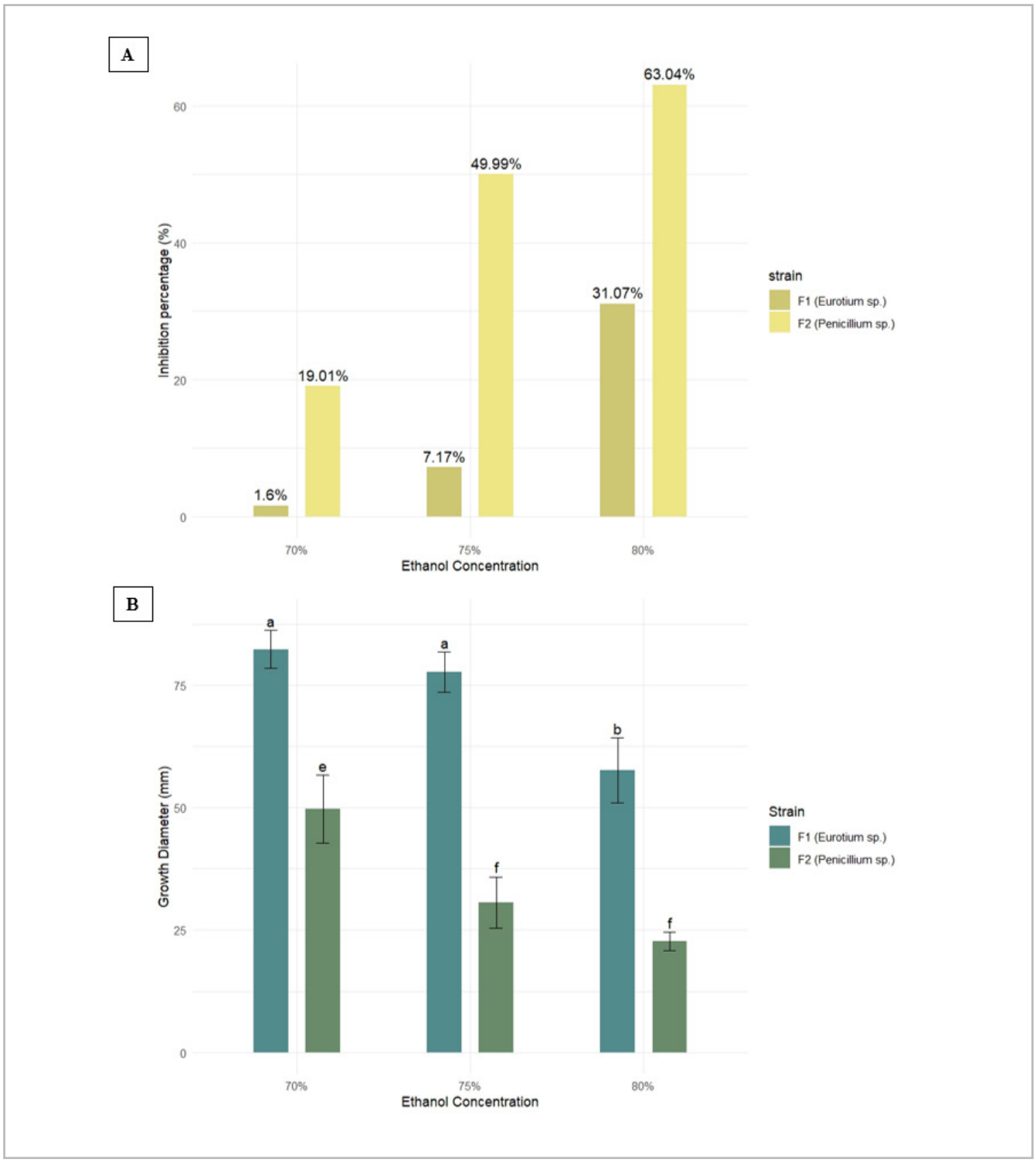

Figura 4.- A. Evaluation of the antifungal activity of 70%, 75% and 80% ethanol. B. Growth diameters of strains F1 and F2 in response to ethanol concentrations based on Tukey's test.

resistance may primarily be associated with metabolic stress response mechanisms. Certain fungal strains can activate biochemical pathways to repair cellular damage or modify their metabolism to better tolerate environmental stress (Pontón & Quindós 2006). The tolerance of *Eurotium sp*. and *Penicillium sp*. to high ethanol concentrations indicates the presence of detoxification mechanisms or antioxidant systems that mitigate ethanol's toxic effects. For instance, in *Penicillium purpurogenum*, ethanol can affect biofilm formation and biomass, generating oxidative stress that some filamentous fungi can counteract using antioxidant systems to neutralize the toxic effects of this solvent (Husseiny *et al.* 2014).

Additionally, ethanol's quick evaporation implies that its concentration decreases over time, removing the stress factor on treated surfaces. Once the unfavorable conditions cease due to ethanol evaporation, these fungi may exit their dormant state and resume their normal metabolic processes, promoting growth. This phenomenon could explain the fungal development observed in both strains after ethanol exposure during the contact method. These results suggest that ethanol primarily acts as a fungistatic agent, temporarily limiting the growth of *Eurotium sp.* (strain F1) and *Penicillium sp*. (strain F2) but not fully eliminating fungal colonies.

Evaluation effect on conidial germination

The results obtained from the microcultures show a differential response in the germination of conidia from *Eurotium sp.* (F1) and *Penicillium sp.* (F2) under increasing ethanol concentrations, reflecting their distinct structural and metabolic strategies for stress resistance. Figure 5 and table 1 show the results for *Eurotium sp*.

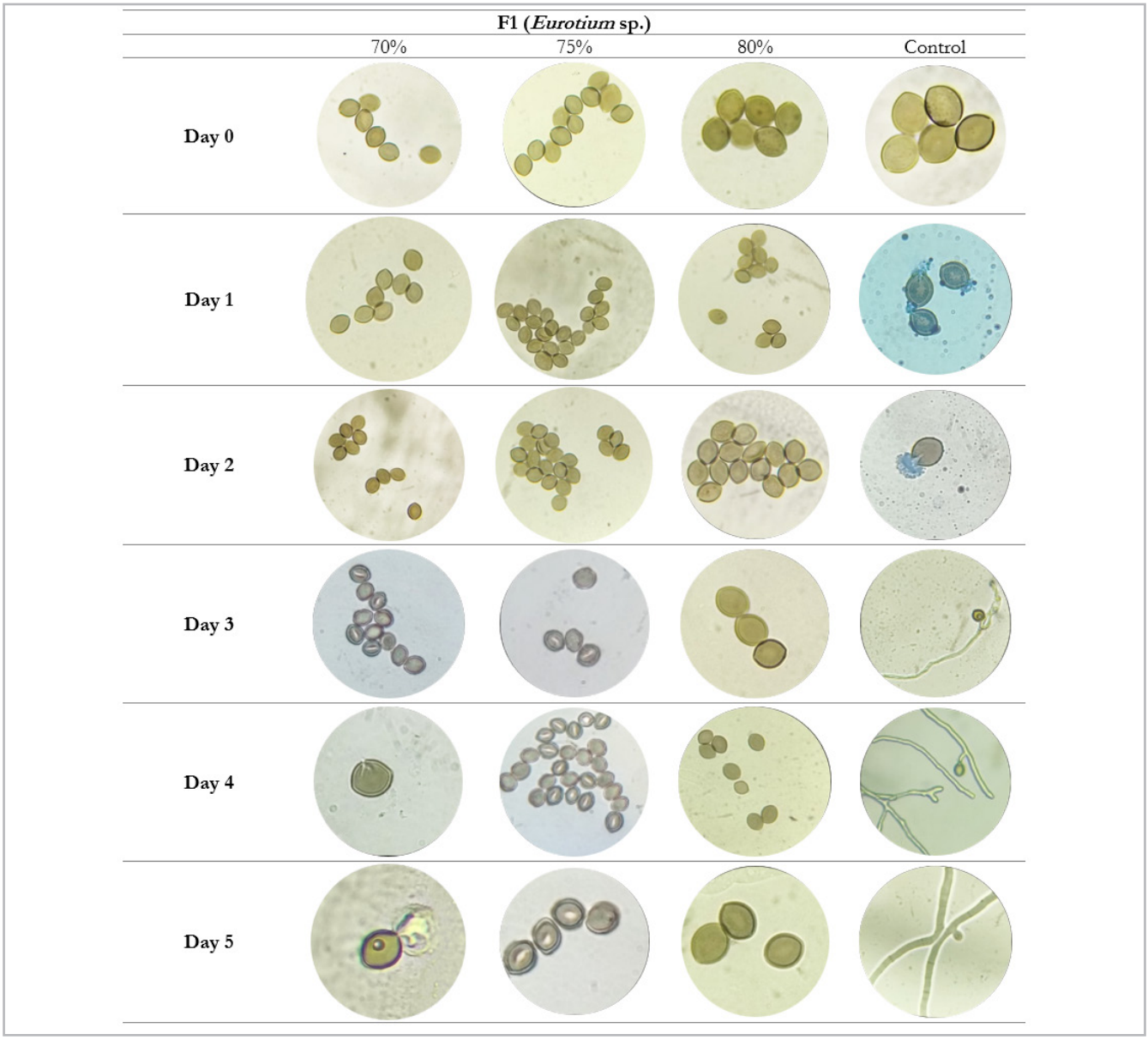

Figura 5.- Microscopic observations of F1 ascospore germination under ethanol effect (100x).

F1						
						Days
Ethanol concentration	**0**	**1**	**2**	**3**	**4**	**5**
70%	-	-	-	-	+	+
75%	-	-	-	-	-	-
80%	-	-	-	-	-	-
Control	-	+	+	++	+++	+++

Table 1.- Qualitative analysis of F1 ascospore germination over 5 days. -: Absence of germination, +: Conidia germination, ++: Hyphae development, +++: Intense mycelium development.

At 70% ethanol, germination began on the first day, reaching "+" classification by day 5. At 75% and 80%, no germination was observed during the evaluation period. In the ethanol-free control, conidial germination occurred from day 1, with intense mycelial development classified as "+++" by days 4 and 5. Figure 6 and table 2 show the results for *Penicillium sp*.

F2 conidia germinated across all ethanol concentrations. Germination began on day 2 at 70% ethanol, and on day 3 at 75% and 80% ethanol. Moderate mycelial development ("++") was observed in all treatments. This behavior may be related to physiological differences between

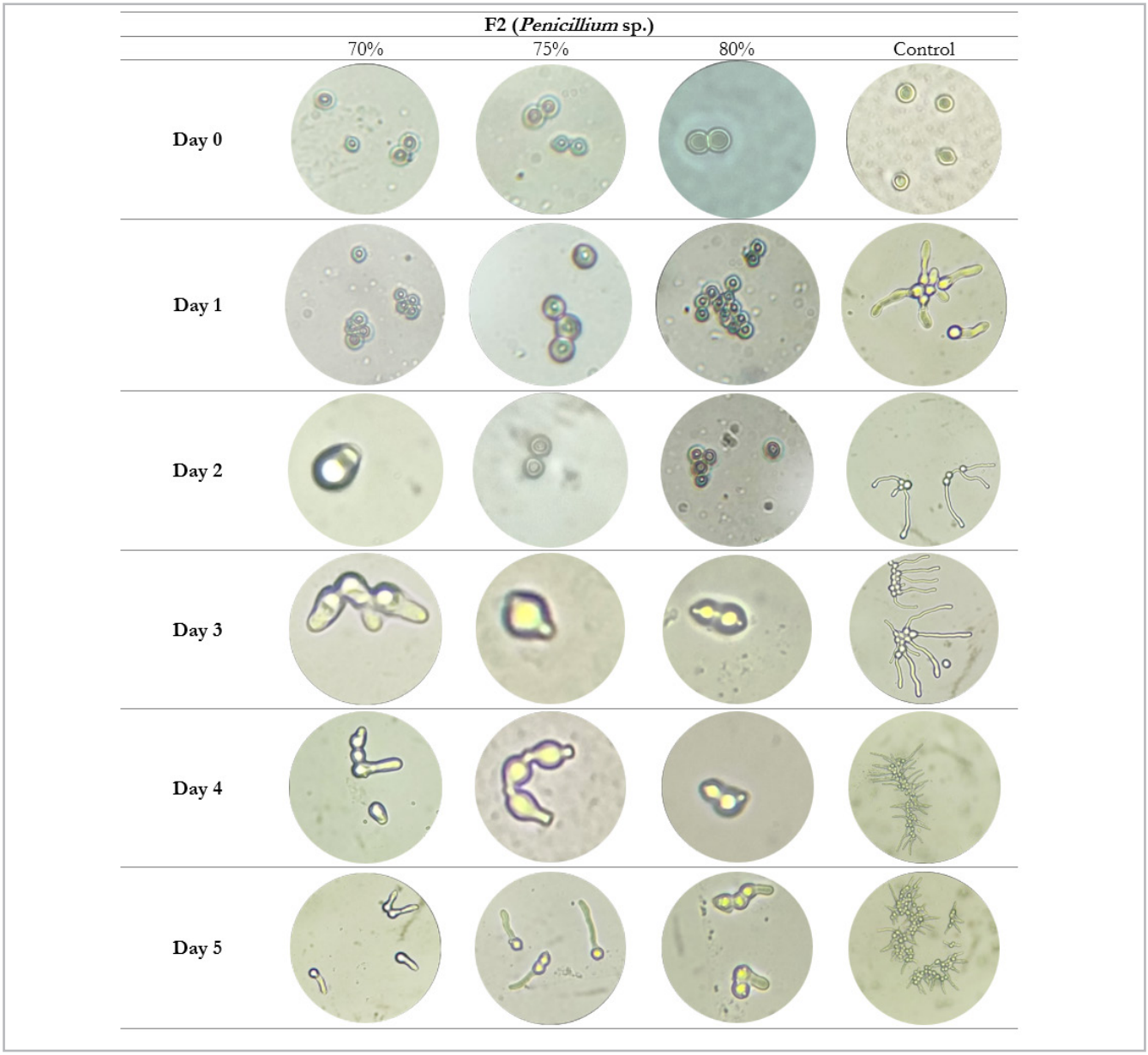

Figura 6.- Microscopic observations of F2 conidiospore germination (100x).

F2						
						Days
Ethanol concentration	**0**	**1**	**2**	**3**	**4**	**5**
70%	-	-	+	+	+	+
75%	-	-	-	+	+	+
80%	-	-	-	+	+	+
Control	-	+	++	++	++	++

Table 2.- Qualitative analysis of F2 conidiospore germination over 5 days. -: Absence of germination, +: Conidia germination, ++: Hyphae development, +++: Intense mycelium development.

ascospores and conidia. Ascospores are widely recognized for their high resistance to extreme conditions such as heat, dehydration, UV radiation, pressure, and oxidative stress, compared to asexual spores (Wyatt *et al.* 2014a). This resistance is due to a series of adaptations, including a thick cell wall, low water content, high cytoplasmic viscosity, and the accumulation of compatible solutes such as trehalose, mannitol, and trehalose-derived oligosaccharides (Dijksterhuis *et al.* 2007; Dijksterhuis *et al.* 2002). These characteristics enable ascospores to survive even dry heat treatments above 60 to 85 °C (Dijksterhuis & Teunissen 2004; Reyns *et al.* 2003; Butz *et al.* 1996). For example, ascospores of *Talaromyces macrosporus* survive more than 30 minutes at 85 °C, with germination rates above 90% after exposure (Wyatt *et al.* 2015), while those of *Neosartorya fischeri* (teleomorph of *Aspergillus*) survive for more than 7 days at 60 °C with a relative humidity below 0.5% (Wyatt *et al.* 2014b).

From this perspective, the absence of germination in F1 at 75% and 80% ethanol may be due to a mechanism of induced dormancy, through which ascospores avoid initiating metabolic processes under adverse conditions that could compromise their viability. This behavior has been associated with high cytoplasmic viscosity, a characteristic observed in ascospores of *Neosartorya fischeri* and *Talaromyces macrosporus*, which limits intracellular mobility and delays metabolism, thereby reducing the production of reactive metabolites (Wyatt *et al*. 2014c). In fact, comparative studies have shown that cytoplasmic viscosity in these ascospores can reach values close to 15 cP, significantly higher than those recorded in conidia (3.5–4.8 cP), supporting their ability to remain dormant until conditions become favorable for germination (Dijksterhuis *et al.* 2007). In this context, the behavior of F1 may be interpreted as an adaptive response that favors long-term survival at the cost of immediate germination.

In contrast, the conidia of F2 showed a greater tendency to germinate even under ethanolic stress conditions. Although conidia are generally less resistant than ascospores, a wide variability in stress tolerance has been documented. For example, conidia of *Aspergillus niger* can survive at 50 °C for an hour and retain their germination capacity (Ruijter *et al.* 2003). This tolerance has been broadly associated with structural properties common to fungal conidia, whose cell wall is composed of multiple layers, including an outer layer rich in melanins and hydrophobins, which are responsible for resistance to adverse conditions such as desiccation and UV radiation (Pihet *et al*. 2009; Yang *et al.* 2022; Ball *et al.* 2019).

In the case of the genus *Penicillium*, it has been shown that hydrated conidia of *P. chrysogenum*, *P. italicum*, and *P. digitatum* can be inactivated after prolonged ethanol treatments (Dao *et al*. 2008). However, *P. chrysogenum* exhibits lower susceptibility, which has been attributed to its greater physiological tolerance and its ability to grow under low water activity conditions (Dantigny *et al.* 2005; Wyatt *et al.* 2014a). This suggests that strain F2 may share similar adaptations, enabling it to maintain germination processes even in the presence of ethanol. Although germination was slower at higher concentrations, the ability to activate under these conditions reflects significant physiological plasticity.

Moreover, germination processes are also conditioned by the environmental conditions of the incubation setting. In this case, conditions were used to simulate heritage environments such as archives and libraries, with temperatures around 19 °C. This factor may have contributed to slowing the germination process in both strains. The results suggest that F1 ascospores, although highly resistant, tend toward dormancy in ethanol-containing environments, while F2 conidia exhibit a greater, though limited, active tolerance that allows them to germinate even under ethanolic stress. Understanding these mechanisms is crucial for developing more effective control strategies against fungal biodeterioration, particularly in contexts where alcohols are used as biocides.

Effectiveness of control methods on paper samples

The treatments application on paper discs showed Ethanol at 80% effectively reduced fungal colonization and growth time. Combined treatment of vacuum cleaning followed by ethanol application proved particularly effective, resulting in complete inhibition of F2 colonies and minimal development of F1 colonies [Figure 7], [Table 3].

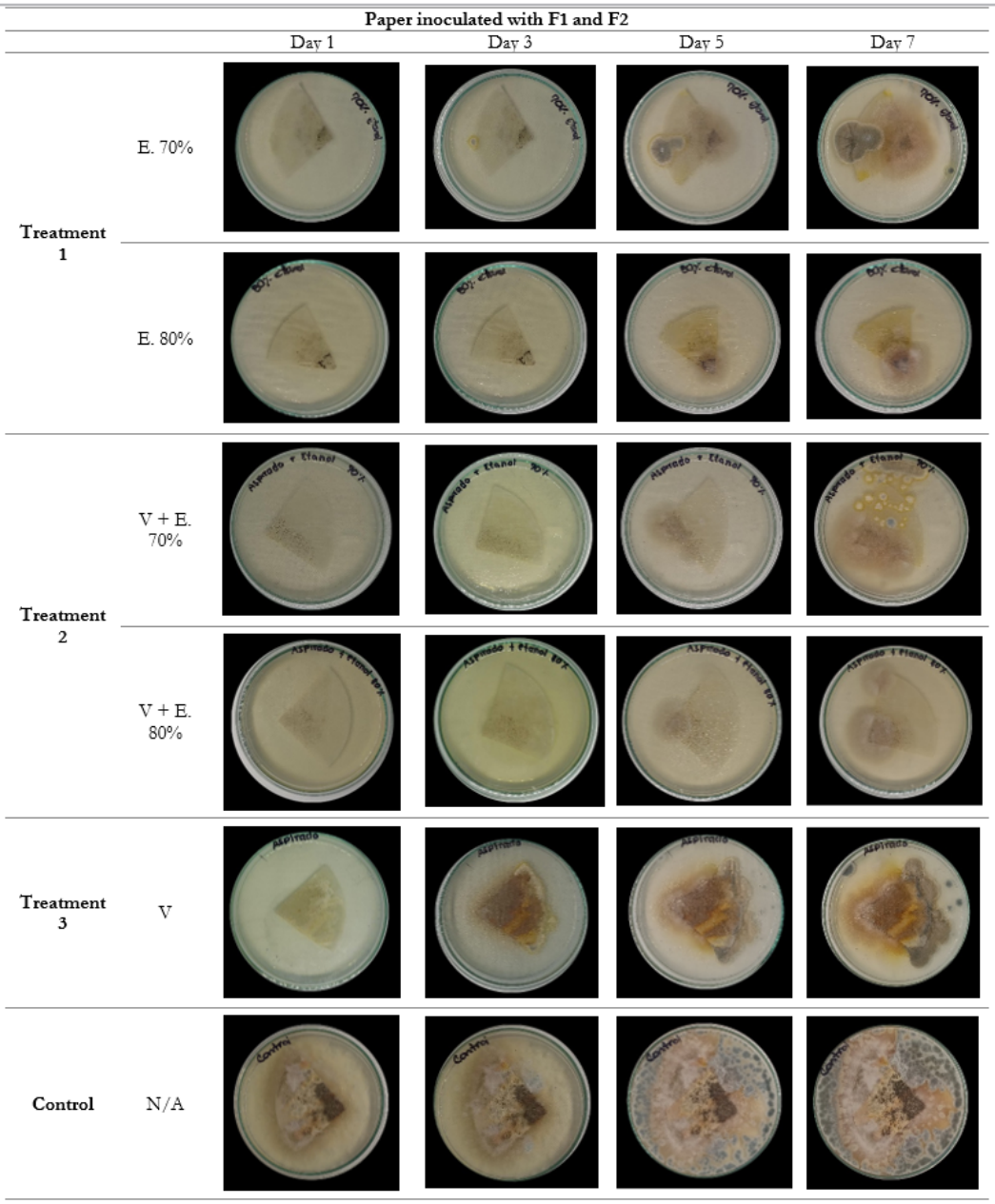

Figura 7.- Photographic evidence of fungal development on paper inoculated with F1 and F2 strains under various control treatments: ethanol (E), vacuum cleaning (A), vacuum cleaning followed by ethanol application (A+E), and no treatment (N/A).

	Development of F1 colonies on paper				Development of F2 colonies on paper			
	Days				Days			
	1	3	5	7	1	3	5	7
E. 70%	-	-	++	+++	-	+	++	+++
E. 80%	-	-	+	++	-	-	-	-
V + E. 70%	-	-	++	+++	-	-	+	++
V + E. 80%	-	-	+	++	-	-	-	-
V	-	+	+++	+++	-	+	+++	+++
Control	+	++	+++	+++	-	+	+++	+++

Table 3.- Intensity of fungal colony development on paper samples. +: Minimal growth with limited paper coverage; ++: Visible growth covering a significant portion of the paper; +++: Extensive and dense fungal growth covering most of the paper.

In most conservation centers, libraries and museums, the use of 70% ethanol has been considered one of the most effective techniques for restoring and preserving artifacts. A water-ethanol mixture in a 30:70 (v/v) ratio is a recommended practice (Lucas *et al.* 2017). However, the results of this study challenge this recommendation, showing that 70% ethanol concentration is not completely effective against *Eurotium sp*. and *Penicillium sp*., common fungal genera found in archives and libraries.

Vacuum cleaning, a common method for conserving paper materials, also presented limitations. Observations revealed that fungal colony development in vacuum-treated areas was comparable to the control group, which received no treatment. Although vacuuming effectively removes a significant portion of surface spores and hyphae, previous studies (Cappitelli & Villa 2020) have highlighted its drawbacks, such as the risk of spore dispersion and limited surface cleaning efficacy. Spores may adhere to vacuum nozzles and disperse during cleaning movements, increasing the risk of contamination to other objects and the workspace. Furthermore, this method may be insufficient to remove hyphae deeply embedded in paper fibers, allowing fungal re-emergence and potential recolonization of the material.

Although treatment with 80% ethanol alone reduced the growth of strain F1 and no colony formation was observed for strain F2 [Table 3], the paper exhibited intense yellowing, suggesting aesthetic damage to the material [Figure 7]. Fungi produce various pigments, mainly polyketides and carotenoids, which stain paper surfaces (Pavlović *et al.* 2022). When active fungi are present on the paper surface, they produce secondary metabolites, including pigments like melanin (Pavlović *et al.* 2022; Nitiu *et al.* 2020). These pigments, along with acids and enzymes secreted by fungi, can significantly alter the physical and chemical properties of paper (Borrego *et al.* 2018). When ethanol is applied directly to these active fungi, the metabolites, such as pigments, can dissolve and diffuse on the paper surface, causing yellow discoloration. However, as observed in Figure 7, the combined treatment of vacuuming followed by 80% ethanol application did not result in prominent discoloration. This suggests that vacuuming helps partially remove spores, mycelium, and other fungal structures present on the paper surface. Consequently, the amount of pigmented or acidic substances reacting with ethanol is significantly reduced. When 80% ethanol was applied after vacuuming, a significant reduction in strain F1 growth diameter was observed, while strain F2 showed no signs of development. This combination increases the success rate of fungal colony elimination while maintaining paper integrity.

Conclusion

Although 70% ethanol is commonly used in conservation treatments in archives, libraries and museums, this study questions its effectiveness against strains of *Eurotium sp*. and *Penicillium sp*. isolated from paper artifacts. The in vitro procedures, including contact method and microcultures, suggest that 80% ethanol concentrations are more effective in inhibiting the growth of these strains. In this context, 80% ethanol primarily acts as a fungistatic agent, providing a high inhibition percentage but not eliminating fungal colonies.

The research suggests a combined method in which vacuum cleaning is first incorporated to remove surface fungal growth, followed by an 80% ethanol application, which is more efficient in inhibiting fungal growth and conidia germination, as well as preventing recolonization on paper. This protocol significantly reduces the growth of *Eurotium sp*. (strain F1) and eliminates the development of *Penicillium sp.* (strain F2), achieving greater effectiveness in combating fungal biodeterioration in paper artifacts.

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