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Ethanol as an antifungal treatment for paper: short-term and long-term effects

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In paper conservation ethanol is used as an antifungal agent. However, information on the antifungal efficacy of this alcohol is scarce and often inconsistent. In this study, we clarify if ethanol is effective and safe to use in paper conservation in the short as well as in the long term. None of the tested ethanol concentrations (5–100%) promoted conidia germination, but rather delayed or entirely inhibited it, depending on alcohol concentration and contact time. In a simulation of an interventive treatment of samples colonized by fungi, all the tested ethanolic solutions (30, 70, and 100%) revealed antifungal activity. The best results were obtained with 70% ethanol, showing fungicidal properties on four of the five-tested fungal species (*Aspergillus niger*, *Cladosporium cladosporioides*, *Penicillium chrysogenum*, and *Penicillium corylophilum*). No deleterious effects of 70% ethanol on the tested paper were observed either in the short or in the long term.

Keywords: Ethanol, Paper conservation, Fungi

Introduction

Ethanol is a universally acknowledged disinfectant, antiseptic, and preservative. Owing to its multiplicity of toxic effects, ethanol is generally considered to be a non-specific antimicrobial, ranked among the membrane-active agents (Block, 2001: 231; Paulus, 2004: 444). Although the specific nature or site of action of ethanol is not fully known, the most widely adopted theory is that ethanol acts through coagulation/denaturation of proteins and membrane damage, interfering with metabolism and causing cell lysis (McDonnell & Russell, 1999; Block, 2001: 231).

In the field of heritage conservation, among other applications, ethanol is often used by paper and book conservators as an antifungal (Sequeira *et al.*, 2014). However, the literature on the actual efficiency of ethanol as an antifungal is scarce and often inconsistent. While some authors recommend such treatment of affected paper (Florian *et al.*, 1994: 20; Brokerhof *et al.*, 2007; Child, 2011) and others confirm the fungicidal activity of ethanol vapours (Bacílková, 2006), or demonstrate complete inhibition of fungal development on samples treated with ethanol at 70% (Valentin, 1986), still other researchers indicate that ethanol may act as a fungal spore activator (Florian, 2002: 37; Guild & MacDonald, 2007), or that 70% ethanol does not have sporicidal properties (Nittérus, 2000). Consequently, it could be questioned if this treatment should continue to be used.

In this study, we intended to clarify if ethanol is suitable for use as an antifungal in paper conservation practice. The main questions to answer were whether ethanol enhances or inhibits the germination of fungal spores, which water/ethanol concentration is the most efficient, what are the preventive and interventive antifungal effects of ethanol, and the effect of ethanol on the chemical and physical properties of paper in the short and long term.

First, experiments were made using *Penicillium chrysogenum*, as it is one of the most commonly isolated fungi from paper/book materials worldwide (Mesquita *et al.*, 2009; Bergadi *et al.*, 2014; Sato *et al.*, 2014). It is also highly cellulolytic (Chinedu *et al.*, 2011) and its colonies grow fast and sporulate in standard media (Pitt & Hocking, 2009: 235). The ethanol concentration that provided best antifungal results with *P. chrysogenum* was then tested as an interventive treatment with a mixture of fungal species

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composed of five of the most commonly identified fungal species on paper collections: *Aspergillus niger*, *P. chrysogenum*, *Penicillium corylophlum*, *Chaetomium globosum,* and *Cladosporium cladosporioides* (Di Bonaventura *et al*., 2003; Lourenço *et al*., 2005; Mesquita *et al*., 2009; Bergadi *et al*., 2014). The mixed inoculum was used in order to simulate a real-case scenario where paper collections are exposed to contamination from several species and competition and suppression strategies can occur, and only the most well-adapted organism(s) will develop.

Materials and methods

Paper

Whatman® filter paper #1 was selected as the model paper as it is additive-free and has a high-cellulose content (98% w/w), which reduces the number of variables in the results, and also due to its frequent use in paper conservation and biodeterioration research (Michaelsen *et al*., 2006; Zervos & Moropoulou, 2006), which makes it comparable to other studies. The paper has an average thickness of 180 μm, grammage of 88 g/m^2, and 0.06% ash content.

Fungal species

Penicillium chrysogenum Thom was selected for tests with a single species. It was obtained from the mycological collection of Universidade do Minho (Braga, Portugal).

The mixed inoculum was composed of *Aspergillus niger* Tiegh., *Chaetomium globosum* Kunze, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Penicillium corylophilum* Berk. and M. A. Curtis and also *Penicillium chrysogenum*. C. *cladosporioides* and *C. globosum* were obtained from the mycological collection of Universidade do Minho.

Inoculum preparation

Fungal strains were plated on potato dextrose agar (PDA, Oxoid, UK) and incubated at 22°C for 15 (*A. niger*, *P. chrysogenum*, *P. corylophilum*, and *C. cladosporioides*) and 30 days (*C. globosum*). For inoculum preparation, spores were harvested by pipetting sterile 0.05% Tween 80 (Panreac) on the surface of colonies and collecting the suspension. For *C. globosum*, the suspension was gently crushed with a sterile glass rod to release ascospores from perithecia and asci. The concentration of spore suspension for each fungal species was determined with a haemocytometer and adjusted to 1×10^6 spores/ml.

When the mixed inoculum was used to inoculate the samples (for treatments with EtOH 70%), aliquots of the inoculum from each single species (with the same concentration as above) were added to a flask followed by vortexing. To determine the viability of the single species, each individual inoculum was also plated on PDA.

Inhibition effect of ethanol on *P. chrysogenum* in liquid medium

Malt extract medium (Scharlau, Spain) solutions (50 ml each) at 1.5% were prepared in 100 ml Erlenmeyer flasks closed with cotton plugs with four different ethanol (99.5%, Carlo Erba, Spain) concentrations if 0, 5, 10, and 20% (v/v). These concentrations (lower than the ones tested in the following assays) were selected in order to assess the lowest concentration of ethanol that could inhibit fungal growth by constant contact with ethanol, which was expected to be inferior to temporary contact.

Each malt extract solution was sterilized by autoclaving and the respective volume of filter-sterilized (0.2-μm cellulose acetate membrane, VWR) ethanol was added to the cooled medium to achieve the required ethanol concentration. The flasks were prepared in triplicate.

Each sample of liquid growth medium was inoculated with 100 μl of a *P. chrysogenum* conidial suspension (1×10^6 conidia/ml) and incubated statically with diurnal periodicity of light at 22 ± 2°C. Three blank control samples of uninoculated growth medium were also prepared.

After 3, 7, and 14 days of incubation, fungal inhibition was qualitatively observed and the results recorded as: + (growth), − (no growth).

Microscopic observations of the effect of ethanol on the germination of *P. chrysogenum* conidia

Eight customized slides were prepared consisting of two overlaid microscope glass slides attached with autoclave tape. From the top slide a 1 cm^2 section was previously cut from the centre, creating a cavity (Fig. 1). After sterilization of the slides, 50 μl of PDA was added to the cavity of each slide and allowed to solidify; 3 μl of a suspension of 1×10^6 conidia/ml was poured over the PDA and allow to dry; 10 μl of each ethanol solution was pipetted on two of the samples over the inoculum. The tested treatment solutions were 100/0%; 70/30%; 30/70%; 0%/100% of water (distilled and sterilized)/ethanol (99.5%, Carlo Erba, Spain). These will be referred to as 0% EtOH, 30% EtOH, 70% EtOH, and 100% EtOH, respectively. On one half of the tested samples (one for each ethanol concentration), the treatment solutions were left to evaporate for 1.5 hours in a laminar flow chamber before covering with a glass coverslip and sealing with autoclave tape, in order to study the effect of a temporary contact between ethanol and fungal spores. These are referred as Group A: evaporated specimens. The other half were immediately covered and sealed to

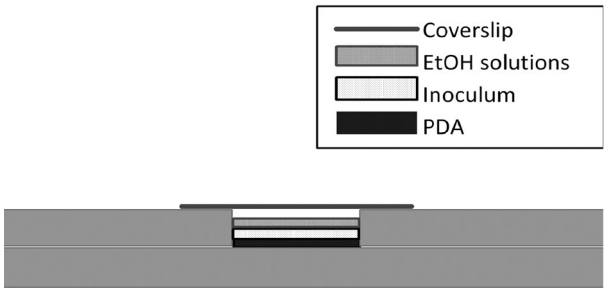

Figure 1 Scheme of experimental set used for microscopic observation of the effect of ethanol on the germination of *P. chrysogenum* conidia.

observe the effect of ethanol in a continuous contact with fungal spores. These are the Group B: non-evaporated specimens. In the process of evaporation of ethanolic solutions (Group A), the PDA medium also dried out, which impeded the development of fungi. To these samples, 10 µl of liquid malt extract medium (Scharlau, Spain) was added. All the slides were kept in individual petri dishes lined with wet filter paper and sealed with paraffin film (Parafilm M®, Bemis NA, USA) to help maintain the moisture content in samples.

The samples were observed under a Leica DMR microscope and photographed with a Leica DFC320 camera using Nomarski differential interference contrast optics. Three random areas of each sample were studied. Observations were made directly after preparation of the slides (0 days of incubation) and after 1, 2, and 5 days of incubation.

Effect of ethanol on the development of fungi

Whatman® #1 paper discs (45-mm diameter) were numbered, weighed, and sterilized by autoclaving.

Preventive treatment

Each paper sample was placed in an individual sterile 60-mm diameter Petri dish, and treated by pipetting 300 µl of the treatment solution (the quantity determined empirically as the one required to soak the entire sample) along the periphery of the sample and allowing the solution to migrate to the centre. This method was chosen in order to replicate the one used in the interventive assay, where the pipetting in the centre of the sample (location of the fungal colonies) would cause a dispersal of the spores. The tested treatment solutions were the same as used in the previous assay: 0, 30, 70, and 100% EtOH. Treated samples were kept in individual Petri dishes and dried under continuous vacuum in a desiccator, at 15 ± 5 mBar for two hours. Petri dishes (60-mm diameter) with PDA medium were prepared. In order to facilitate the extraction of the paper samples for biomass measurement and make sure that the colonies were growing only on the paper and not immersed in the PDA, sterile nitrocellulose membranes (0.45-µm porosity, 47-mm diameter, GVS, Spain) were placed over the PDA and the paper samples on top of them. Each paper sample was inoculated with 10 µl of a 1×10^6 conidia/ml suspension and incubated at 22 ± 2°C, in Petri dishes closed with Parafilm®. Three control samples for each experiment variation were kept un-inoculated.

Interventive treatment

Each paper sample was firstly put in individual Petri dishes with PDA, inoculated (10 µl of a 1×10^6 conidia/ml suspension) and incubated for three days. They were then removed from the PDA, placed in clean Petri dishes and dried under vacuum (15 ± 5 mBar) for 20 hours. Prior to treatment, each sample was cleaned with vacuum cleaner (MUNTZ 555-MU-E with PHU-10 smaller brush) for 60 seconds and weighed afterwards. This intended to simulate a generally used paper conservation practice where the application of ethanol as an antifungal is preceded by drying and superficial cleaning of the fungi (Nittérus, 2000; Brokerhof *et al.*, 2007; Sequeira *et al.*, 2014). The ethanol solutions were applied in the same way as in the preventive assay. The samples were then transferred to new Petri dishes with fresh PDA, over sterile nitrocellulose membranes and re-incubated at 22 ± 2°C, in Petri dishes closed with Parafilm®. Three control samples for each experiment variation were kept un-inoculated.

Fungal growth evaluation

Colony growth and biomass were determined after 3, 7, 14, and 30 days of incubation (in the preventive assay, the experiment was stopped at 14 days of incubation as all the samples were completely colonized at that stage). Since the measurement of biomass is a destructive analysis, different samples (in triplicate) from each treatment variable were prepared for analysis at each incubation period and selected randomly. Colony growth was determined in the same samples used for biomass quantification at each incubation period.

To quantify colony growth, Petri dishes were opened and the samples photographed inside a laminar flow chamber. Two pictures were taken of each sample, one with direct lighting (to register the colour of colonies) and another with oblique lighting (to better discern the contours and dimension of the colonies). The area of the colonies was measured digitally using the software ImageJ (version 1.46r; W. S. Rasband, U.S. National Institutes of Health, Bethesda, MD [http://rsb.info.nih.gov/ij/]). Only the growth occurring on paper samples was quantified, having as a maximum the total area of paper samples.

Paper samples inoculated with the mixed inoculum were also observed with a binocular microscope (Leica MZ16) and recorded with a digital camera (Leica ICD) to identify which species were developing.

For fungal biomass quantification, the paper samples were detached from nitrocellulose membranes, dried under vacuum for 20 hours, and kept inside the laminar flow chamber for one hour to stabilize the weight at the environmental relative humidity (RH). A Sartorius LE623S precision scale was used to weigh the samples. Biomass was calculated by subtracting the weight of the samples after incubation from their initial weight – for the preventive assay, this initial weight is the one prior to treatment and inoculation; for the interventive assay the initial weight is the weight after vacuum cleaning and before treatment.

Evaluation of physical and chemical alterations caused by 70% EtOH treatment on paper samples

Whatman® #1 paper samples treated with 70% EtOH were analysed before and after treatment and after accelerated degradation. Non-treated samples were used as controls in all analyses.

Accelerated degradation was performed at 80 ± 0.5°C and 65 ± 2% RH (ISO5630/3, 1986), for 329 hours in a FITOCLIMA 150 EDTU climate chamber. Before and after accelerated degradation, the samples were kept at 22 ± 2°C, 50 ± 1% RH in a desiccator.

pH determination

pH measurements were performed using the cold extraction method, according to Tappi 509 (TAPPI, 2011), using a Docu-pH Meter, Sartorius, with a Py-P22 electrode.

Colourimetry

Colour measurements were carried out with a hand-held colorimeter Data Color International®. The colorimetric coordinates CIE L*a*b* were calculated with a D65 Standard Illuminant and 10° Standard Observer. Reported values are the average of three samples from which three distinct areas were analysed and each area was measured in triplicate.

Folding endurance

Folding endurance (log of number of double folds) was determined according to ISO 5626:1993 (ISO 5626, 1993) with a Köhler-Molin instrument, Lorentzen & Wettre. The applied tension was 0.25 kg. Ten samples were analyzed for each type of paper treatment.

Weight change

Weight change was calculated from three replicates for each treatment. The samples were weighed with a Sartorius LE623S precision scale before treatment, one day after treatment and one day after removal from the climate chamber. Prior to weighing, the samples were removed from the desiccator at 50% RH and left to reach equilibrium with the ambient RH in the scale room for two hours. The RH in the scale room on the three different days of analysis was 35, 38, and 36% RH, respectively.

Statistical analysis

Data were analysed using a *t*-test when only two treatments were being compared and one-way analysis of variance (ANOVA) when comparing more than two treatments. When significant differences were detected, Fisher's least significant difference (LDS) *post hoc* test was used for multiple comparisons (Massart *et al*., 1998). A significance level of 0.05 was applied using Statistica software v12 (StatSoft, Inc., 1984–2013, Tulsa, OK, USA).

Results and discussion

Inhibiting effect of ethanol on P. chrysogenum *in light medium*

As shown in Table 1, all tested concentrations of ethanol in malt extract liquid medium completely inhibited the development of *P. chrysogenum* during the 14-day period. In control samples, without ethanol, fungal development was already observed after three days of incubation. These results show that for a direct continuous contact with *P. chrysogenum* spores, percentages of ethanol as low as 5% are enough to inhibit fungal development.

Similar results were obtained for this species by Dantigny *et al*. (2005b), where the constant exposure of *P. chrysogenum* conidia to ethanol vapours at 4% (w/w) concentrations (~5.02% v/v) resulted in a total inhibition of germination during three weeks of incubation.

Dantigny *et al*. (2005a) determined the minimum inhibitory concentration (MIC) for liquid treatments with ethanol on 12 fungal species, and for all of them the estimated MIC was close to the one obtained

Table 1 ***P. chrysogenum*** **development on liquid medium with ethanol concentration (v/v).**

	Incubation (days)		
[EtOH]	3	7	14
0%	+	++	++
5%	–	–	–
10%	–	–	–
20%	–	–	–

Evaluation: –, no growth; +, growth; ++, intense growth.

for *P. chrysogenum*, in the range 2.14–6.43% (w/w) (~2.75–7.82% v/v).

Microscopic observation of the effect of ethanol on the germination of P. chrysogenum *spores*

Table 2 summarizes the results from the observation of the germination of *P. chrysogenum* spores under the influence of ethanol. The captured images are presented in Supplementary Table S1.

In the samples where the contact with ethanol was only temporary (evaporated samples), initially fewer conidia germinated and mycelial development was less intense in samples treated with 70% EtOH and 100% EtOH than with 0 and 30% EtOH. Nevertheless, the hyphae ultimately colonized the entire samples.

When conidia were in constant contact with ethanol (non-evaporated samples), germination was delayed in the 30% EtOH samples (occurring only after five days of incubation and in only a few conidia) and was totally inhibited by 70 and 100% EtOH.

In both cases, there was no promotion of germination caused by ethanol but instead the opposite: conidia germination was either delayed or totally inhibited, depending on the percentage and contact time with ethanol. According to a previous study, only very diluted levels of ethanol (0.5–1.5% ethanol) showed a stimulatory effect on *Trichoderma reesei* and acted as a conidial activator, while in ethanol concentrations greater than 2%, germination was completely inhibited (Sharma, 1992).

The fact that low levels of ethanol are required for complete inhibition of fungal development (see section: Inhibiting effect of ethanol on *P. chrysogenum* in liquid medium) in addition to the reversibility of inhibition suggests that the alcohol may be functioning by inhibiting the enzyme(s) required for germination (Trujillo & Laible, 1970).

Gurtovenko & Anwar (2009) studied the interaction of ethanol with biological membranes, and at concentrations below 30.5% (v/v) ethanol induces the expansion of the membranes together with a reduction of their thickness, as well as causing disorders and enhancement of the interdigitation of lipid acyl chains. However, the bilayer structure of the membranes is maintained.

Table 2 Effect of different concentrations of ethanol on the germination and development of *P. chrysogenum.*

Sample		Incubation (days)			
		0	1	2	5
A: Evaporated	0%	−	++	+++	+++
	30%	−	++	+++	+++
	70%	−	+	+++	+++
	100%	−	+	+++	+++
B: Non-evaporated	0%	−	++	+++	+++
	30%	−	−	−	+
	70%	−	−	−	−
	100%	−	−	−	−

Evaluation: −, no germination; + germination; ++, hyphae development; +++, intense mycelium development.

Preventive antifungal effect of different ethanol concentrations on paper inoculated with P. chrysogenum

The same ethanol concentrations and fungal species used in the previous experiment were tested on paper samples, where the paper was firstly treated and then contaminated, simulating a preventive paper conservation treatment.

Fig. 2 shows that the treatment of paper samples with ethanol did not have a significant effect on *P. chrysogenum* development.

The only significant differences in fungal growth were obtained after three days of incubation in the samples treated with 0% EtOH, which show a significantly higher ($P < 0.05$) colonized area than all the other treatments.

This greater area of growth in samples treated with 0% EtOH was not accompanied by a significantly higher biomass value. It was likely due to a spreading of the spores in the more hydrated and swollen paper fibres structure caused by this aqueous treatment.

After 7 and 14 days of incubation no significant differences between treatments could be detected in colonized area and biomass results ($P > 0.05$). At 14 days of incubation all samples were completely colonized as shown in Fig. 3.

Ethanol under appropriate conditions can form complexes with cellulose, which can remain stable for long periods in anhydrous conditions or under a high vacuum (Arney & Pollack, 1980). However, these complexes are not stable when moist, and merely the moisture in the air can completely displace the solvent (Arney & Pollack, 1980). The process of drying the samples under vacuum after treatment, together with the elevated moisture from the PDA, must have been displaced the ethanol molecules from the paper and no significant antifungal effect is retained.

Interventive antifungal effect of different ethanol concentrations on paper colonized by P. chrysogenum

The results obtained from the simulation of an interventive treatment, where paper samples were firstly colonized by fungi and subsequently treated with ethanol, are shown in Fig. 4.

While in the control samples without treatment (WT) and in the samples treated with 0% EtOH, *P. chrysogenum* quickly resumed growth, the samples

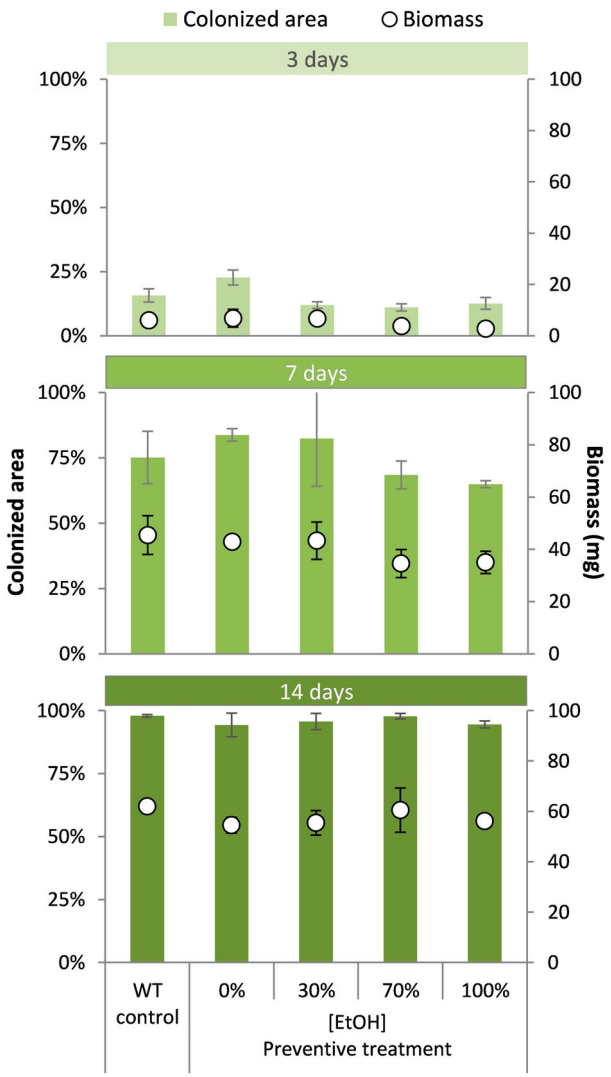

Figure 2 Biomass (circles) and colonized area (bars) measurements at 3, 7, and 14 days of incubation for *P. chrysogenum* growing on paper-treated preventively with different ethanol concentrations. WT stands for WT control. Values are the average of three replicates per treatment ± SD.

treated with the different ethanol concentrations showed a delay or a total absence of fungal development (Fig. 4). This is most likely due to an irreversible inactivation of vegetative cells caused by the alcohol (Trujillo & Laible, 1970), whereas the subsequent growth probably originates from spores.

The best antifungal effect was obtained with 70% EtOH where no fungal growth was observed along the duration of this assay (30 days). As the spores of *P. chrysogenum* were not able to develop at all when returned to conducive conditions (Fig. 3), this points to a sporicidal effect of 70% EtOH on this particular species.

The antifungal effect of 30% EtOH was similar to the one obtained with 100% EtOH. The differences in colonized area and biomass obtained from these two treatments were not statistically significant at any incubation period ($P > 0.05$). The antifungal effect of both these treatments was only partial, as there were surviving fungal structures that were able to subsequently colonize the samples.

These results are in accordance with the literature, where it is reported that the greatest microbiocidal effect of ethanol is reached at concentrations of 60–70% (Block, 2001: 236; Paulus, 2004: 445; Fraise *et al.*, 2013: 37). This optimal concentration is likely related to the mechanism of action of ethanol as an antimicrobial, which is believed to derive mainly from protein coagulation/denaturation, resulting in disruptions of cytoplasmic integrity, denaturation of essential proteins, interference with metabolism, and cell lysis (Block, 2001: 231; Fraise *et al.*, 2013: 145). Protein coagulation/denaturation occurs only within certain concentration limits around an optimal alcohol level. In the absence of water, proteins are not as readily denaturated (Block, 2001: 231; Fraise *et al.*, 2013: 145). These factors may afford an explanation why absolute ethanol, a dehydrating agent, is not as effective as water–ethanol mixtures. Accordingly, 70% concentration must be within the mentioned optimal alcohol level range for microbial protein denaturation.

When comparing the interventive and the preventive treatments, we can conclude that after an initial colonization, even if the samples are thoroughly cleaned, the re-growth is much faster and intense than the initial colonization. This observation is clear when we compare the fungal growth after three days of incubation in the WT samples in Fig. 2 (preventive) and in Fig. 4 (interventive), where the colonized area and biomass are five times higher in this last one. Several factors can justify this effect: a higher number of spores in the paper matrix after colonization than the one present in the initial inoculum; a spreading of the spores with the cleaning process; the resumption of growth of already established mycelium; and/or physical–chemical alterations in the substrate rendering it more susceptible to fungal growth.

Also, as shown in Fig. 3, the colonized surface of the samples is more heterogeneous after the interventive treatment, likely due to the development of more individual colonies with different phenotypes, and/or antagonizing interaction between individual colonies.

70%-EtOH treatment of paper inoculated with a mixture of five fungal species

The ethanol concentration that led to optimal antifungal results with *P. chrysogenum* (ethanol at 70%), was tested with a mixed inoculum consisting of five fungal species (*A. niger, P. chrysogenum, P. corylophilum, C. Globosum*, and *C. cladosporioides*).

Before the treatment, paper samples were incubated for three days after inoculation with the mixed

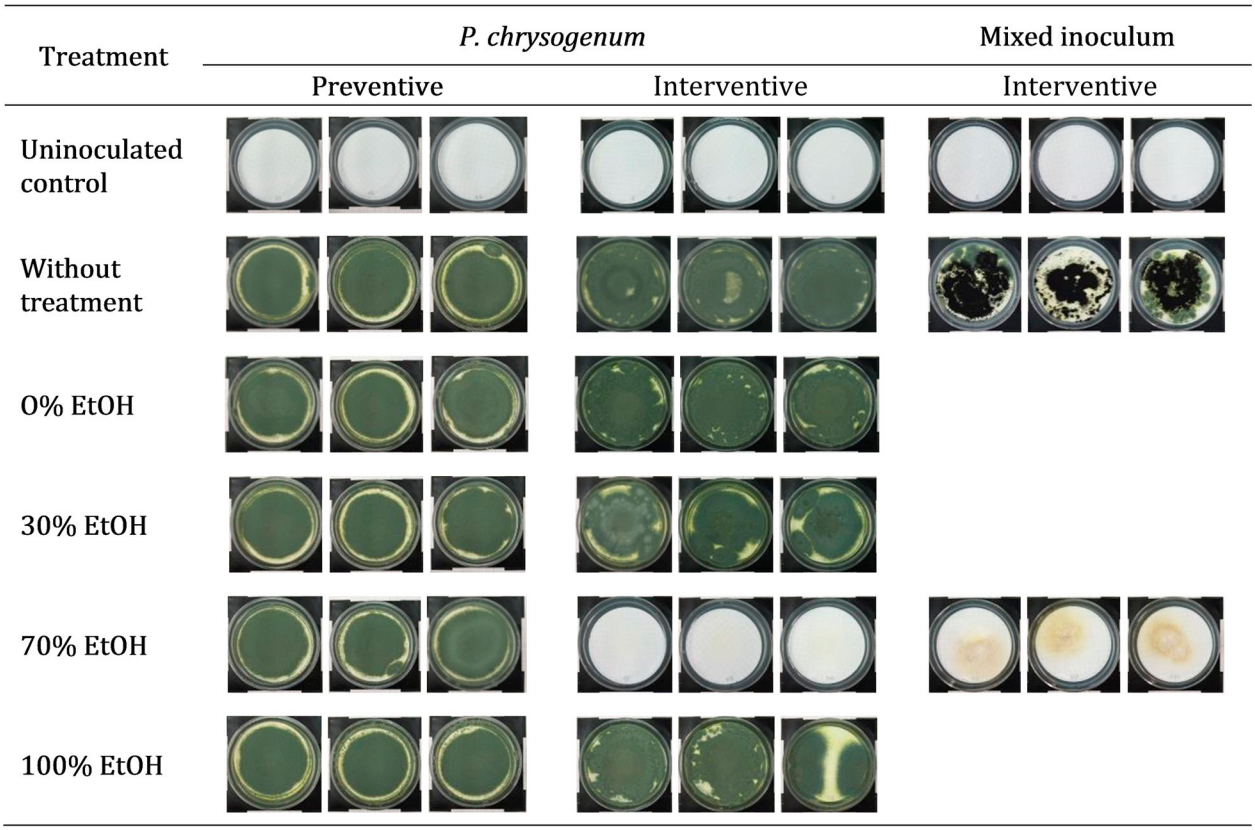

Figure 3 Photographic comparison between the preventive and interventive treatments of paper samples inoculated with *P. chrysogenum* and mixed inoculum at 14 days of incubation.

inoculum. The only species that were distinctly growing at that stage were Penicillium species (*P. corylophilum* and *P. chrysogenum* were not visually distinguishable at this stage) and occasionally *A. niger*. These species grow faster than *C. globosum* and *C. cladosporioides* (as observed in our viability control sets, where each individual inoculum was plated before mixing) and through the faster colonization and/or production of inhibiting metabolites, they outgrew the other species.

After vacuum cleaning and treatment, *Penicillium* and *Aspergillus* rapidly resumed growth on the WT control samples, and *A. niger* in due course outgrew Penicillium (Fig. 3). No fungal development was observed on the samples treated with EtOH 70% after three days of incubation (Fig. 5), as in the previous assay with only *P. chrysogenum*. Nevertheless, after seven days of incubation, fungal growth was already visible (Fig. 5) although the only fungal species that was developing on the ethanol-treated samples was *C. globosum*.

Ch. globosum initially grows more slowly than *A. niger*, *P. chrysogenum*, or *P. corylophilum*. Additionally the samples have not been colonized by it before treatment. This could explain why the control WT samples were already thoroughly colonized by Aspergillus and Penicillium after three days of incubation and no fungal growth was detected on the samples treated with EtOH 70%.

Nittérus (2000) also observed the survival of *C. globosum* and a total inactivation of *A. niger* after a 70% ethanol immersion treatment on paper samples. Bacílková (2006) also reported the inactivation of *A. niger* on paper samples treated with ethanol vapour at concentrations between 30 and 90%, and no regrowth was observed for at least 14 days after the evaporation of the alcohol.

C. globosum is the only fungus from the tested mixed inoculum that has ascospores instead of conidia, and ascospores are often resistant to heat, pressure, and chemicals (Pitt & Hocking, 2009:14).

C. cladosporioides did not grow on these samples, either before or after treatment, although its viability was positive in the viability control plates. It was probably inhibited by the other more competitive colonizing species.

The effect of 70% ethanol on paper stability

Colour, pH, folding endurance, and weight were measured to evaluate the influence of the 70%-EtOH treatment on the chemical and physical properties of paper. The obtained results before and after accelerated degradation are presented in Table 3.

Colour measurements (Table 3) reveal that accelerated degradation caused darkening and yellowing of all the samples. However, no significant differences ($P > 0.05$) could be detected between the colour of

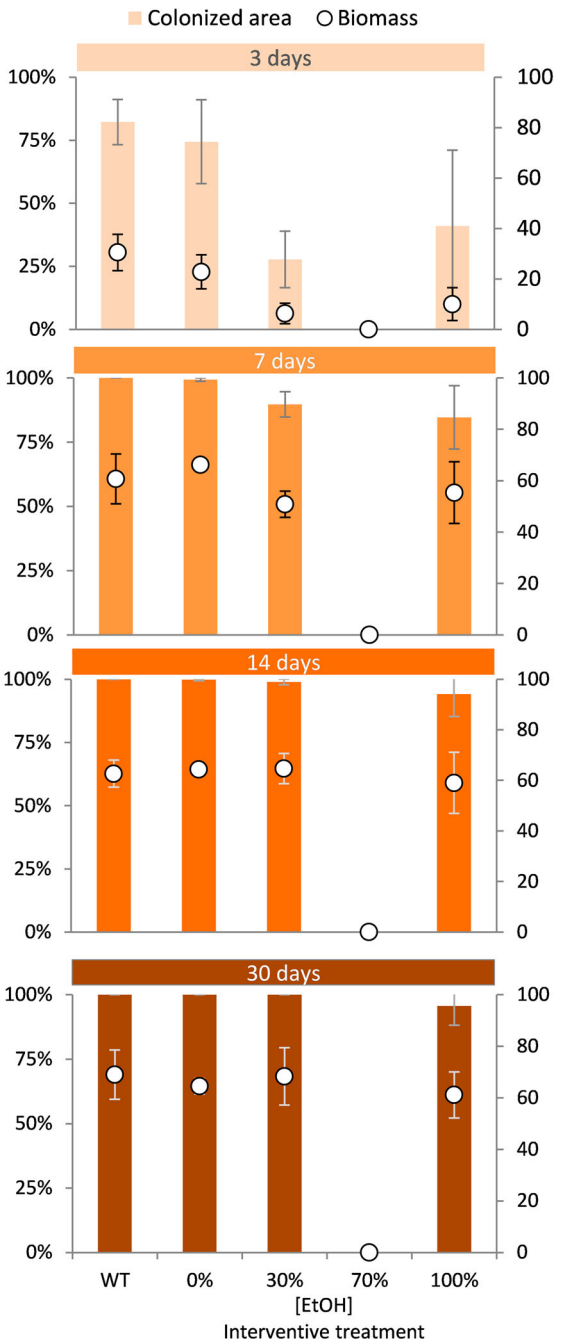

Figure 4 Results from the interventive ethanol treatment on paper colonized by *P. chrysogenum* at 3, 7, 14, and 30 days of incubation. Colonized area measurements represented in bars and biomass measurements in circles. Values are the average of three replicates per treatment ± SD.

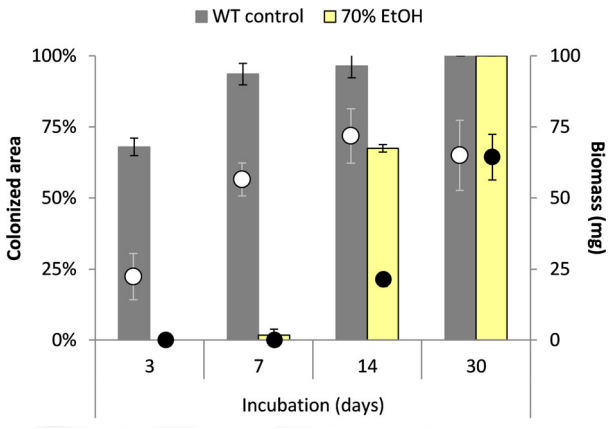

Figure 5 Results from the interventive treatment with 70% ethanol on paper inoculated with a mixed inoculum composed by *A. niger*, *P. chrysogenum*, *P. corylophilum*, *C. Globosum,* and *C. cladosporioides*, at 3, 7, 14, and 30 days of incubation. WT stands for control without treatment. Colonized area is represented in bars and biomass values in circles. Values are the average of three replicates per treatment ± SD.

the non-treated samples and the samples treated with ethanol, either before or after the experiment.

The differences in weight presented in Table 3 are the result of the subtraction of the weight after treatment or after degradation from the initial weight. The 70%-EtOH samples showed a significantly higher weight gain than the WT ones after treatment – the slight weight gain in the WT samples after treatment may be related to the higher RH in the weighing laboratory (see section 'Materials and methods' – weight variation). Taking into account that according to the literature (Arney & Pollack, 1980), after 10 hours at 50% RH ethanol is completely evaporated from this kind of paper, and in this case, the paper was at that RH for ca. 20 hours; two hypotheses may be presented to justify these results. Firstly, the fact that an ethanol–water mixture was used instead of pure ethanol could alter the evaporation rate from paper; or the water present in the ethanol solution could have reacted with cellulose, creating thermodynamically stable structures that slow down evaporation. Secondly, cellulose, due the hydroxyl groups in its structure, has a strong affinity for materials containing hydroxyl groups, such as water. As a result of the interaction between cellulose and water, the volume of the polymer increases, due to growth of amorphous regions and expansion of crystalline regions, which is associated with weight gain (Khazraji & Robert, 2013). After degradation, the weight of samples returned to initial values, meaning that the changes caused by the treatment were reversed.

The pH values of the WT and 70% EtOH samples were identical before accelerated degradation (Table 3). With degradation both samples acidified, although the pH of the WT samples (pH 5.05) was slightly lower than that of the 70% EtOH samples (pH 5.25).

The folding endurance results before degradation for the WT and 70% EtOH samples were analogous. The degradation process caused a mechanical resistance decrease in both types of samples, although the ethanol treated samples show a slightly smaller decline than the non-treated samples (Table 3).

Table 3 Results of analyses of paper samples without treatment (WT) and treated with 70% ethanol (70% EtOH), before and after accelerated degradation

	Before accelerated degradation			After accelerated degradation		
	WT	70%EtOH	*P*-value	WT	70%EtOH	*P*-value
CIE L*a*b* colourimetry	L* = 97.37 ± 0.04 a* = −0.07 ± 0.01 b* = 1.21 ± 0.04	L* = 97.35 ± 0.02 a*=−0.06 ± 0.00 b* = 1.17 ± 0.06	0.519 0.190 0.462	L* = 95.69 ± 0.09 a*=−0.29 ± 0.03 b* = 4.23 ± 0.23	L* = 95.92 ± 0.24 a*=−0.20 ± 0.06 b* = 3.93 ± 0.40	0.193 0.064 0.317
Δ Weight (%)	0.27 ± 0.25	1.24 ± 0.18	*0.005*	0.05 ± 0.19	0.16 ± 0.33	0.641
pH	6.31 ± 0.02	6.31 ± 0.05	1.00	5.05 ± 0.02	5.25 ± 0.02	*0.0014*
Folding endurance	2.58 ± 0.18	2.57 ± 0.16	0.968	1.82 ± 0.09	1.98 ± 0.15	*0.0077*

The values represent the average of three replicates in the case of colour, pH and weight determination and 10 replicates in the case of determination of folding endurance ± SD. *P*-values lower than the confidence level of 0.05 are in italics.

Conclusions

The present work was undertaken to clarify whether the use of ethanol as an antifungal treatment for paper is appropriate.

According to our results, *P. chrysogenum* was totally inhibited when in continuous contact with concentrations of ethanol as low as 5%. All of the tested ethanolic solutions had a negative effect on conidia germination of *P. chrysogenum*, depending on ethanol concentration and contact time.

The treatments of paper samples with different ethanol concentrations simulating a real paper conservation scenario led to distinct results. While the preventive treatment revealed no significant antifungal effect on treated paper samples, the interventive treatment was very effective in impeding fungal development.

All the tested ethanol solutions (30, 70, and 100%) showed antifungal properties when used as an interventive treatment for paper colonized by *P. chrysogenum*. Antifungal activity of the 30 and 100% EtOH solutions was similar. Spore germination was initially reduced but not completely inhibited. In the samples treated with 70% EtOH, the antifungal effect was total and no growth of *P. chrysogenum* was observed throughout the 30 days of re-incubation. This indicates a fungicidal activity of the 70% solution as the fungus did not grow after returning to suitable conditions.

When tested as an intervention on paper inoculated with a mixture of five of the most common fungal species found on paper-based collections, 70% EtOH totally prevented the development of four of the five-tested species, although *C. globosum* was able to resume growth after the treatment.

Ethanol at a concentration of 70% caused no deleterious effects on Whatman® #1 paper either prior to or after accelerated degradation, compared to non-treated samples. High purity ethanol (99.5%) was used in these tests. When using commercial ethanol solutions, conservators need to be aware of its purity, as undesired residues may be introduced to the paper matrix.

The presented analyses were performed on standard paper without additives and no writing or painting media were tested. In a real paper document or work of art different materials may be present and undesirable reactions may occur, such as dissolution. Therefore, each material should be thoroughly tested before treatment.

Ethanol at 70% has advantageous properties compared to other antifungal treatments generally used in paper conservation. It is readily available in any conservation studio and evaporates quickly leaving no residues that could negatively affect the treated material or human health. The development of resistance of fungi to ethanol is not a significant issue, especially at concentrations used for disinfection (Block, 2001: 234). Nonetheless, inhalation of ethanol can cause respiratory tract irritation, and direct contact may cause skin irritation and dehydration, therefore, it must be handled using protective clothing and masks or in a fume hood.

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Supplemental Material

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