Biodeterioration of Paper: A SEM Study of Fungal Spoilage Reproduced Under Controlled Conditions

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Summary: Biodeterioration phenomena represent a complex of physical and chemical alteration processes in various materials, such as those constituting the objects that represent our cultural heritage. The biodegradation of paper is conditioned by several variables such as the materials from which cellulose is obtained, the manufacturing processes employed, the occurrence of other affecting substances such as lignin or metallic compounds, and by the environmental conditions in which papers are conserved. In this study, biodeterioration of paper was artificially induced in order to evaluate the role of a range of chemical and physical variables on damage caused by cellulolytic fungi. A variable pressure SEM instrument was used to characterise paper samples with different fibre origins, and alterations obtained *in vitro*. Two fungal strains, *Aspergillus terreus* Thom and *Chaetomium globosum* Kunze, which are cellulolytic species frequently associated with paper spoilage, were used to produce stains with characteristics close to those observable on art objects made from paper. The stains obtained on the different samples of paper were compared at both low and high magnification, in order to visualize the macro- and microscopic characteristics of paper fibres, inorganic constituents, impurities, and the deteriorating agents related to the spoiled areas. During this survey it was observed that single paper characteristics can strongly influence the intensity and the results of the fungal action. For example, the activity of a fungal strain on paper grades containing fibres of the same origin, but with different sizing, led to the formation of profoundly different stains and alterations. Moreover fungal structures, analysed by low vacuum SEM, in areas on paper corresponding to the stains appeared in different physiological states suggesting an important effect of paper constituents on fungal growth and their sporulating ability.

Keywords: cellulose; degradation; fungi; paper; SEM

Introduction

Biodeterioration phenomena represent a complex of natural physical and chemical spoilage processes in various materials, such as those composing most of the objects currently considered cultural heritage, and are caused by the growth of very different organisms. These are generically called biodeteriogens,[1] but they are all characterised by the saprotrophic ability of using substrata to sustain their growth and reproduction. In paper-supported works of art (documents, books, prints, etc.) many factors occur together and contribute to the formation of different forms of biological alteration.[2–4] At a relative humidity (RH) level higher than 65%, and a temperature higher than 20 °C, moisture content of paper can reach 8–10%, with consequent water activity (a_w), namely the ratio of the

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vapour pressure of the water in the substrate and the vapour pressure of pure water at the same temperature and RH,[5–7] higher than 0.65: in these conditions microbial spores can germinate and develop by using paper as a growth medium, thus affecting the object of cultural value.[2–8] Paper, before being the constituent of ancient books and precious manuscripts, is an organic material, and therefore a source of nourishment for many organisms. It is chiefly made of cellulose of various origin (cotton, linen, hardwood, softwood, hemp), but in addition paper usually contains sizes, fillers, traces of metallic elements (such as iron, copper) and other substances (such as inks, pigments, etc.) which make it a complex and heterogeneous medium. Cellulose, from the chemical point of view, is a linear polymer of glucose, a hygroscopic compound affected by many chemical and biological agents. However, microbial activity on paper is possible only in the presence of unbound water in the substrate which is available for the growth of the mould. Bound water is the fraction of water that forms a monomolecular layer covalently bonded to molecules in the materials, and it has an a_w lower than 0.2; water weakly bonded to cellulose molecules and forming a multilayered film located in capillaries less than 30 μm in diameter has an a_w still lower than 0.65–0.70; only the free water that is present in capillaries grater than 30 μm in diameter has an a_w above 0.65–0.70 and can actually brings about cellulose degradation processes.[7–11]

Fungal species (over 200) are the main cause of damage to objects of cultural heritage made of or supported on paper,[2–11] and many of them have, in fact, at least a partial cellulolytic action. In paper-supported works of art (documents, books, prints, etc.) the colonisation of cellulose substrata by specific groups of microorganisms often poses more than a simple aesthetic problem. In fact, the microbial activity affects not only the appearance of the objects causing stains, patinas, etc., but often consists of a deep modification of their chemical and physical structure.

In this study paper samples with artificially induced damage were studied using a variable pressure SEM instrument. The variable pressure SEM technique proved to be particularly useful because it allowed for the direct observation of a non-conductive material such as paper, and its chemical characterisation without the need of surface metallization. With this equipment the spots and stains occurring on naturally aged and artificially biodeteriorated papers, could be observed at the right magnification in order to study, in the spoiled areas of the samples, the microscopic characteristics of cellulose fibres, inorganic constituents, impurities, and deteriorating agents. A set of samples with experimentally induced alterations was obtained as reported in the materials and methods section. The set up of the experimental protocol was governed by the aim of studying paper biodeterioration phenomena in simplified models in which some of the variables could be controlled. Paper samples with artificially induced stains and spots were then analysed macro- and microscopically and the behaviours of the different components in the built-up model-systems were evaluated. The results of this paper were obtained in the framework of the CNR Project ''Progetto Finalizzato Beni Culturali, Sottoprogetto 3'' of the Consiglio Nazionale delle Ricerche (CNR- Rome) with the cooperation of ICB-CNR Rome, with the cooperation of the ICPL, and ING - Ministero per i Beni e le Attività Culturali.

Materials and Methods

Paper Types

Six types of paper of different compositions (Table 1), on account of fiber origins, chemical treatments undergone during the manufacturing processes, and sizing and filling materials, were used in this study. As a control-paper Whatman 1CHR (for chromatography) Cat. N° 3001 917 made of pure linter and not subjected to any sizing procedure was used. The paper samples were cut into 2×6 cm strips. Before being

Table 1.
Caption of paper samples and their characteristics

Sample N°	Origin of the fibres	Filling material	Sizing	Pulp pH
1	Chemical pulp of hemp	kaolin and alum	rosin	4.5–5.0
2	Chemical pulp of hemp	calcium carbonate	alkyl ketene dimer	7.5–8.0
3	Chemical pulp of linen	kaolin and alum	rosin	4.5–5.0
4	Chemical pulp of linen	calcium carbonate	alkyl ketene dimer	7.5–8.0
5	Chemical pulp from softwood	kaolin and alum	rosin	4.5–5.0
6	Chemical pulp from softwood	calcium carbonate	alkyl ketene dimer	7.5–8.0
0	Cotton (Whatman)	none	none	6.5–7.0

inoculated with fungal spores, both sides of the strips were exposed to sterilising UV light for 45 minutes, using a tubular lamp with light emission at 254 nm collocated parallel to samples at 10 cm of distance from them, corresponding in a dose of approximately 40 mW.s $\cdot$ cm^{-2}, in order to decontaminate the surface of samples from airborne fungal and bacterial cells which could result, during the incubation of paper, in undesired growth and side effects.

Fungal Strains used for the Test

Two fungal strains were utilised to inoculate the paper strips. *Aspergillus terreus* Thom and *Chaetomium globosum* Kunze both obtained by the ATCC culture collection. Both these species are considered frequently associated with library materials biodeterioration and are cellulolytic.[12–13] The two strains differ in their behaviour on the substrata and also in their reproductive traits. *A. terreus* is a deuteromycetes producing only mitosporic conidia because it has lost the sexual part of its biological cycle. *C. globosum* is an ascomycete, producing meiotic fruiting bodies which contain spores that result from sexual reproduction. The ability of the two species in the spoilage of paper differs in intensity and quality.

Mycelial Cultures and Inoculum of Fungi on Paper

Fungal cultures were inoculated on MEA (Malt Extract Agar)[13] and incubated at 25 °C. *C. globosum* strain was cultered for 14 days to obtain the production of perithecia which were then harvested using a loop and then squashed in a watch glass in order to effect the release of the spores. The spores from *A. terreus* were obtained by scraping the surface of 7-day-old cultures with a swab. Spores were then suspended in 30 ml of sterile, distilled water containing 0.02% Tween 80 (Merck-Schuchardt - Germany). The spore suspensions were filtered through sterile gauze to remove impurities. The spore density was defined for each strain by counting the elements in a Thoma Chamber to obtain a spore concentration of $4 \times 10 \cdot \mu l^{-2}$. A defined volume of each spore suspension was diluted with nutritive broth (Sabouraud Broth by Difco, Becton Dickinson, USA) in order to inoculate the paper strips. All the inoculations were performed under a laminar flow hood to assure sterility in the procedures. We used 100μl of broth for every paper strip, depositing 3–4 drops of this on to each strip. Control samples consist of strips of each type of paper inoculated with 100μl of broth not containing fungal spores. Four replicates for each treatment were considered. Each inoculated paper strip was placed in a 15 cm polystyrene Petri dish. Two levels of relative humidity (RH) were used during the test: 100% and 75%, and the temperature was maintained at 25 °C. The conditions were created in double-bottomed glass containers; the 100% RH was obtained using distilled water; the 75% RH level was obtained using a saturated solution of NaCl.[4.5] The Petri dishes containing the samples were placed in the glass containers that were kept in a thermostatic cell at T = 27 °C for 7 days. A sensor to register the internal RH (Hygrolog-D Rotronic AY- Swiss) was kept in each container throughout the incubation time.

SEM Analysis

The analysis of paper samples before and after fungal growth was conducted with a variable pressure SEM instrument (LEO 1450 VP, Carl-Zeiss Electron Microscopy Group) that allows the direct observation of non-conductive materials. This SEM technique permitted the description of paper surfaces without preparation by means of metallization. The possibility of observing uncoated specimens, with a not conductive surface, allowed appreciating the signals given by backscattered electrons that are the result of electron-nucleus interaction. The characteristics of a backscattered electron image are dependent primarily upon the nature of the specimen. The intensity of elastically scattered electrons in the signal significantly influences the image contrast, and this intensity, in turn, depends upon the average atomic number of the specimen and the incident angle of the primary beam on the specimen.[14] Differences in atomic number of the specimen give rise to appreciable contrast in the image. The differences in chemical composition, and therefore in average atomic number of the organic (vegetable fibres and fungal mycelium) and inorganic materials allowed for well contrasted observation of filling materials and ions distribution on paper surfaces, and the fungal structures growing on them.[14]

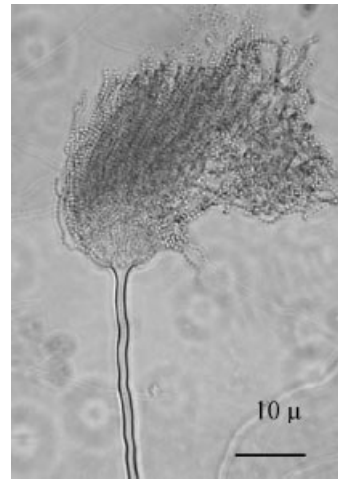

Figure 1.
A conidiophore of *Aspergillus terreus* with chains of conidia. Picture obtained with an Olympus AX60 optical microscope.

Results and Discussion

The two fungal strains (Figure 1 and 2) showed marked differences in their growth and sensitivity to limiting factors (relative humidity), and to substrata (paper/sizing qualities).

C. globosum showed little or no growth at 75% RH while at 100% RH its spoiling effect on substrata was vigorous. The pattern of growth of *C. globosum* on the paper samples clearly differed between paper qualities with different types of sizing, while smaller differences were noted between different types of fibres. Figure 3 shows the paper strips with *Chaetomium*'s perithecia which, being of large dimensions (they can reach 0.5 mm in diameter), and darkly coloured, can be visualised by the naked eye. Two well defined growth patterns could be perceived in samples carrying the *Chaetomium* inoculum: A) development of fungal perithecia only in correspondence to the inoculum points and B) diffuse development of fungal perithecia all over the paper strips. Development type A was described in all the paper samples

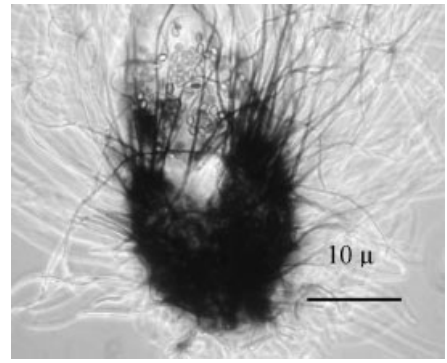

Figure 2.
An open perithecia (fruit-body) of *Chaetomium globosum*; meiotic spores can be seen among the perithecial hairs (sterile hyphae). Picture obtained with an Olympus AX60 optical microscope.

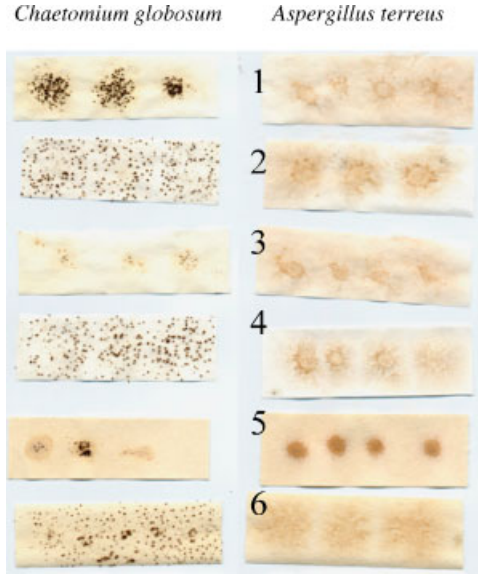

Figure 3.
Paper samples obtained inoculating fungal spores of *Chaetomium globosum* (samples on the left) and *Aspergillus terreus* (samples on the right) on the paper grades 1 to 6, as listed in Table 1. Picture obtained with a Photo Stylus Epson Scanner.

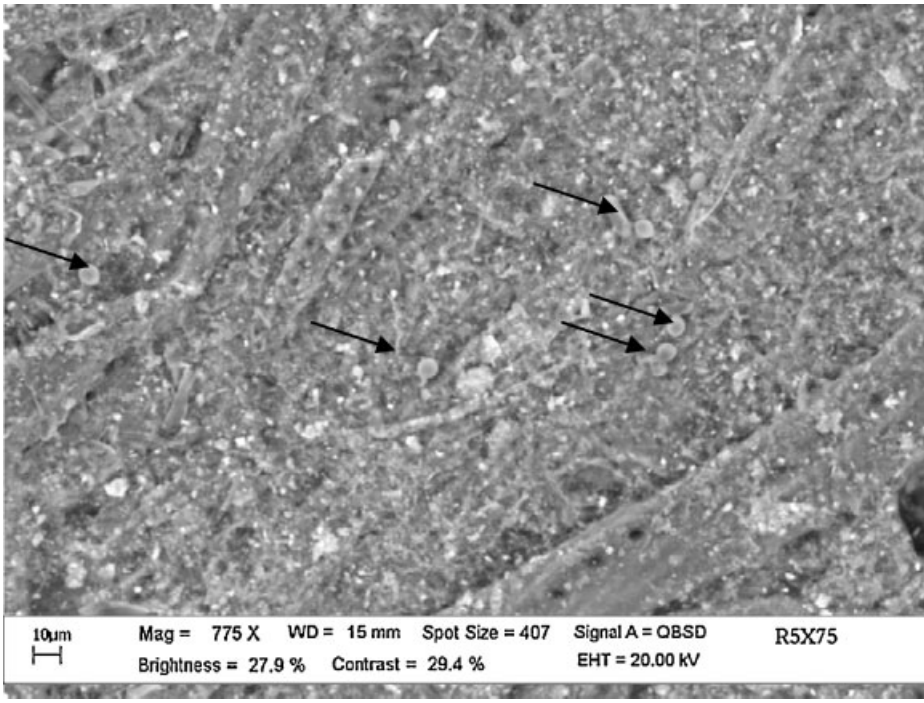

Figure 4.
SEM picture at 775×. Paper sample n. 5 made of softwood and sized with rosin, kaolin and alum. Arrows indicate the not germinated spores of *C. globosum* deposited on paper samples and incubated at 75% of RH.

characterised by an acidic pH and sizing made with rosin, alum and kaolin. Among these samples, despite the same growth pattern, some quantitative differences in the *Chaetomium*'s perithecia growth were observed, namely: the hemp>linen>softwood>controls. Development type B was typical of all the three samples containing alkyl ketene dimer (AKD) and calcium carbonate as sizing material and a neutral pH. Among these samples a gradient was observed in the development of perithecia, the hemp sample being the most palatable to the fungal strain and the control less so. Additionally, quantitative differences between neutral samples were less marked than between acidic samples.

A. terreus showed mycelial growth and conidial production at both 75% RH and 100% RH, although its spread and conidial production was more vigorous with higher RH. The pattern of growth of *A. terreus*on the paper samples (Figure 3) differed between paper qualities with different types of sizing, especially in the case of softwood samples, while smaller differences could be seen in hemp and linen samples. In common with samples inoculated with *Chaetomium,* the growth pattern of *A. terreus*on acidic samples could be described as ''more localised'' with respect to the inoculation points.

SEM observations were performed on all the samples and were aimed at the description of mycelial growth in the different areas of the samples. With SEM imaging it was possible to define the occurrence of spores in the *Chaetomium*'s perithecia, the degree of maturation of conidial heads in *Aspergillus*, the sporulation of inoculated spores in samples where no growth was recorded by the naked eye or at reflected light stereomicroscopy, the presence and degree of development of fungal hyphae in correspondence to the inner area of the inoculation points on samples, at the boundaries of

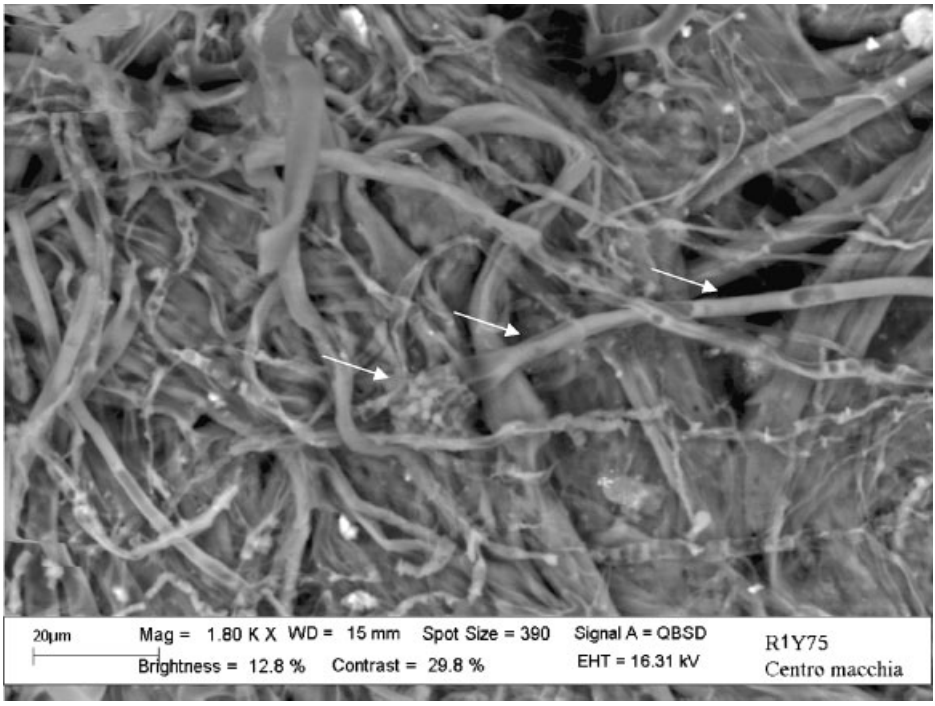

Figure 5.
SEM picture at 1.800×. Paper sample n. 1 made of hemp and sized with rosin, kaolin and alum. Arrows indicate a conidiophora of *A. terreus* with short chains of conidia, developed at 75% RH.

visible colonies and in the area outside the inoculation points.

According to our SEM observations the spores of *C. globosum* deposited on paper samples and incubated at 75% of RH did not germinate at all (Figure 4), indicating that for this set of samples the limiting factor for fungal activity as a paper spoiler was water availability. A different situation was disclosed by SEM images for *A. terreus*, because this species grew at 75% RH (Figure 5) and in most samples produced conidiophora with conidia, mainly in the area of inoculum, independent of the reaction of the sample. The main difference shown by *A. terreus* in its growth on acidic and neutral paper samples at 75% RH was the tendency in the first case of producing a dense mycelium with scarce hyphae exploring the sample, with an overgrowth in the inoculum point, whilst in neutral paper samples a more dispersed and aerial mycelium was observed.

The presence of mature conida of *A. terreus* was noted in both acidic and neutral samples, but the conidiophores were completely developed only in samples incubated at 100% RH (Figure 6), and almost exclusively limited to the inoculum area, this suggesting that water and nutrients availability played an important role in fungal production of mitotic spores.

The production of perithecia by *C. globosum* according to our microscopic observations appeared limited to the inoculum point only in samples with an acidic sizing, indicating that the formation of reproductive structures requires nutrients, but also, the absence of influential elements such as the acidity of the medium. *C. globosum* on neutral paper samples and with a RH of 100% (Figure 7) produced fruiting bodies all over the paper stripes, independent of the presence of the nutritive microelements that were deposited on paper with the broth at the inoculum point.

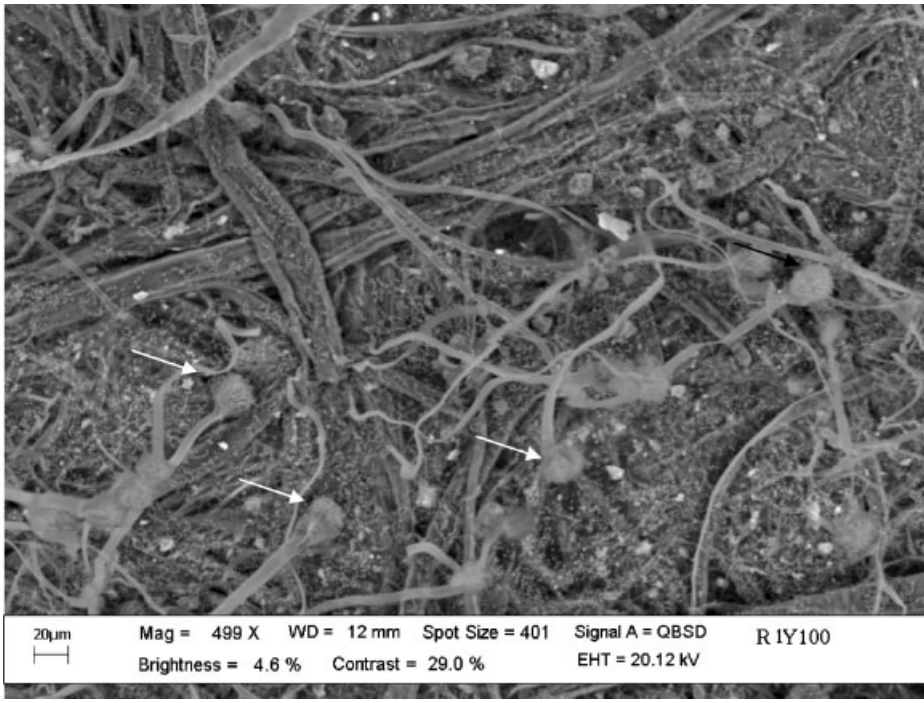

Figure 6.
SEM picture at 499×. Paper sample n. 1 made of hemp and sized with rosin, kaolin and alum. Arrows indicate conidiophores of *A. terreus* with conidia, developed at 100% RH.

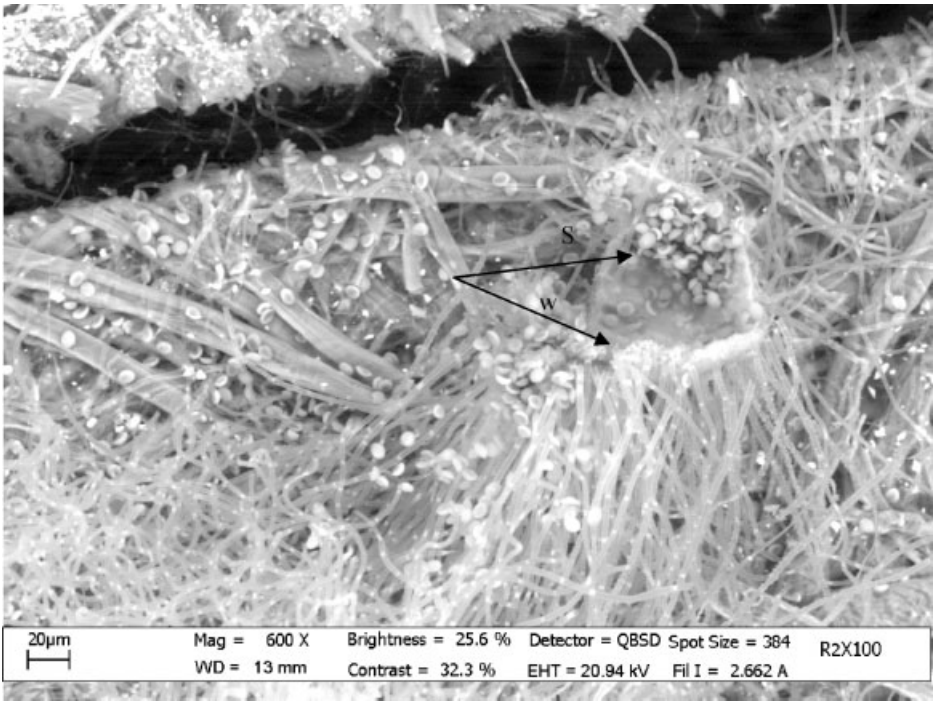

Figure 7.
SEM picture at 600×. Paper sample n. 2 made of hemp and sized with AKD and calcium carbonate. Arrows indicate the walls (w) of a broken perithecia of *C. globosum* with spores (s) developed at 100% RH.

A possible explanation of the different growth pattern in acidic and neutral paper samples is the possibility of the fungal strains performing a nutrient translocation from the inoculum point to other areas of the sample, where the only nutritive matter consists of the paper itself. Acidic reaction or some other chemical factors limited, in the acidic samples, the growth of aerial hyphae capable of translocation of nutrients.

The remarkable differences observed between paper samples with different sizing and filling materials suggest that these play a major role in promoting or inhibiting fungal growth. Deeper insight into the nature of these materials could help in the interpretation of results.

Sizing is necessary to give paper the appropriate properties for writing and printing.[15] Generally they are classified in reactive (synthetic materials such as alkyl ketene dimer) and non-reactive (rosin) depending on the presence of covalent bonding with cellulose molecules.[15,16] AKD is the main sizing agent extensively used in the pH range of 7–10.[16] Commercially available AKDs consist of waxy materials insoluble in water with a variable chain length.[17] They are prepared from natural fatty acid sources and stearic acid is mainly used for this purpose. During paper sizing processes AKD reacts with cellulose fibre and forms a beta-keto ester bond that makes paper hydrophobic.[17]

Rosin (also called colophony) is a translucent resin, soluble in alcohol, ether, turpentine, and several other organic solvents, and in solutions of various metal hydroxides. Rosin is a mixture of several compounds, chiefly abietic acid. It is used for internal sizing of paper and paperboard to enhance its ability to repel moisture.[18] Alum is commonly used for precipitating rosin size on to the pulp fibres to impart water resistant properties (when water

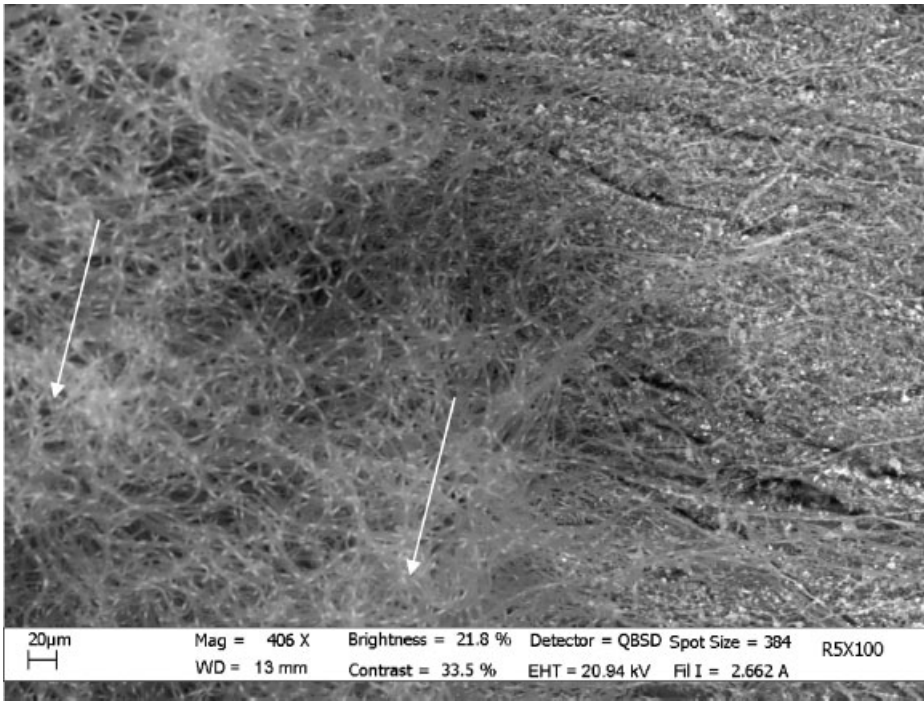

Figure 8.
SEM picture at 406x. Paper sample n. 5 made of chemical pulp of softwood, sized with rosin, kaolin and alum and incubated at 100% RH. Arrows indicate perithecia of *C. globosum* with well developed sterile hyphae, but no visible spores.

colours, inks, etc. are used) to the paper made using it. Kaolin is a naturally occurring white form of anhydrous aluminium silicate clay mineral that is added to paper pulp, increasing the opacity and other characteristics of the sheet. It is also used to make a paper coating mixture.[19–21] Calcium carbonate generally gives opacity and bulking characteristics. As well as filler calcium carbonate is used to buffer acidity due to several causes (i.e. pollution, acid inks, etc.) and it is also used in permanent papermaking to provide an alkaline reserve.[16,17] As shown in Table 1 paper grades sized by means of rosin and alum are acidic, while those sized with alkyl ketene dimer are rather alkaline. Acidic environments promote biodeterioration by means of fungal growth[22] mainly deuteromycetes (*A. terreus*), while alkaline environments can stimulate specific groups of ascomycetes such as *C. globosum,*whose growth is optimal at the pH interval 7.1–10.4. [13,22,23] The positive effect of neutral to alkaline reaction in paper on its spoilage by *C. globosum* was confirmed by this study. According to SEM observations the neutral to alkaline pH also promoted the maturation of the perithecia and the production of ascospores.

In addition, the chemical activity of rosin's aromatic compounds can have fungi static effects that can explain the growth pattern of *C. globosum* on all paper samples sized with rosin and of *A. terreus* in some of the samples sized with rosin. According to SEM observations, perithecia of *C. globosum* cultured on acidic paper samples could barely produce ascospores (Figure 8), even though their sterile hyphae appeared well developed suggesting a good usage of substrata by the fungus.

The exploitation of the substrata was much localised when rosin and kaolin were

present, while AKD and calcium carbonate supported a better growth of the fungi, especially the *C. globosum*, independent of the origin of the fibres.

The sample of paper made with a chemical pulp of softwood and sized with rosin and kaolin appeared less suitable for fungal growth and development, probably on account of the co-occurrence of both an acidic and chemical (aromatic) obstacle to hyphal exploitation of surfaces, and the presence of lignin and therpenic compounds due to the origin of the pulp.

Conclusions

In this study we have observed that not only, and not always, fibres in paper influence the intensity and the results of the biodeteriorating action of fungi, but other factors also profoundly and intrinsically act to define the event of degradation. For example, fungal activity on paper containing different types of sizing led to very different stains and damage. Moreover, SEM observation of fungal structures in corresponding stained areas showed that despite an apparent homogeneous growth of the fungal strain, different physiological situations could be found, suggesting a sharp and varied effect of paper constituents on the different phases of the fungal life cycle. Further studies are needed in order to explain some of the observed phenomena, but there is plenty of evidence to suggest that natural alterations occurring in paper should be studied, considering the large number of variables that interact in causing these processes.

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