ORIGINAL PAPER

Fungal bioleaching of mineral components in a twentieth-century illuminated parchment

F. Pinzari · P. Colaizzi · O. Maggi · A. M. Persiani ·
R. Schütz · I. Rabin

Abstract In this work, we applied scanning electron microscopy (SEM), microanalysis and Raman spectroscopy to study the fungi inhabiting a richly illuminated parchment document and the damage induced by their activity. To that aim, we collected samples of fungal mycelium from the deteriorated areas on a removable adhesive tape specifically intended for lifting fungi without damaging the support. SEM analysis of the adhesive tape samples showed the co-occurrence of several species of fungi. One strain closely resembling *Acremonium* species was observed only in the tape micrographs but no agar cultures were obtained. Its fungal structures showed the production of abundant oxalates with an outstanding leaching of the calcium-based materials of parchment (typically manufactured with gypsum and lime). Needle-like crystals of calcium oxalate produced by the fungus forming a uniform and quite regular grid around conidial slimy heads were documented.

F. Pinzari (✉) · P. Colaizzi
ICRCPAL Biology Department,
Via Milano 76,
00184 Rome, Italy
e-mail: flavia.pinzari@beniculturali.it

O. Maggi · A. M. Persiani
Department of Environmental Biology, Sapienza University,
00185 Rome, Italy

R. Schütz
Max Planck Institute of Colloids and Interfaces,
14476 Golm, Germany

I. Rabin
(BAM) Federal Institute for Materials Research and Testing,
12205 Berlin, Germany

As a result, the areas affected by moulds were weakened, stained and characterised by a powdery patina rich in calcium. Confocal μ-Raman confirmed the presence of oxalates while EDS showed the presence of calcium in crystals. We conclude that the defacement of the parchment was due to both collagenolytic activity, and to the biotransformation of calcium-based minerals by fungi.

Keywords Parchment · Biodeterioration · SEM-EDS · Raman · Oxalates · Fungi

Introduction

The subject of this study is a document written on parchment, dating back to the twentieth century (Fig. 1) and finely illuminated. The work of art belongs to the Memorial Museum of the Armed Forces in Rome and consists of the kind of document which used to accompany official flags on warships. A diffuse and defacing biological damage affected both the bare surface and the decorated areas [1]. At first sight, the object appeared affected by moulds. A white powdery efflorescence disfigured both the back side of the document, and some front parts where the pigments were cracked, whitish and powdery (Fig. 2). Besides the microbiological diagnostic, scanning electron microscopy (SEM), microanalysis and Raman spectroscopy were applied to study the fungi inhabiting this valuable work of art and to describe the spoiling mechanisms of the different constitutive materials by the fungi. A deep understanding of the physical and chemical changes to the parchment caused by the attack of fungi and bacteria enable conservators and restorers to provide specific answers to the problems caused by deterioration. A radically different type of damage, in fact, could result from the attack of different

Fig. 1 Warships flag document: Ariete Torpediniere Ettore Fieramosca

microbial species, according to the enzymes they have, and to the possibility of the co-occurrence of different species like in natural successions of decomposers on organic substrates.
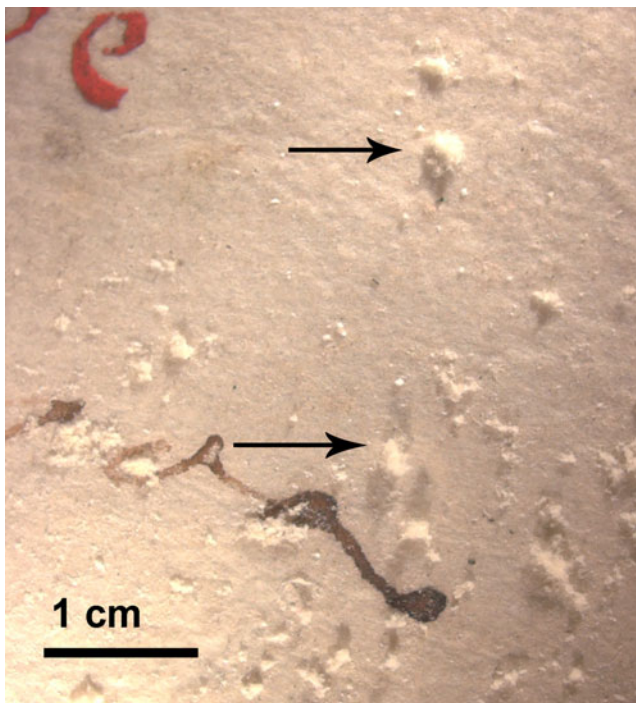

Fig. 2 Detail of the parchment surface with fungal white efflorescence (arrows). The bar indicates 1 cm

Parchment was used as the common writing material from the second century BC to the end of the Middle Ages, when it was joined by paper [2]. However, it has continued to be used for special purposes, such as bookbinding or official documents like the case study here presented. Parchment is derived from untanned animal skins from sheep, goats, lambs, pigs, and calves. The chemical composition of parchment is based on collagen, a natural biopolymer with relative molecular mass of 350 kDa, as an average mass value, that can vary according to the origin of the raw material and its state of repair. The collagen is constructed from a triple helix with random coil telo-peptides [2].

Works of art supported on parchment represent highly composite objects because from the animal parchment, in fact, involves the cleaning and removal of hair mechanically by scraping the skin. This is followed by its desiccation using sodium or potassium chloride, adjustment of the pH by treatment with ammonium chloride or sulphate with lime and a fluid application of potash alum often with flour and egg yolk to give a final suppleness in preparation for the artistic decoration or literary work. As a consequence, the degradation of ancient parchment is a complex process which involves several processes other than the hydrolytic cleavage of the peptide backbone [3].

Degradation of ancient parchment or vellum is a complex process: microorganisms can hydrolyze collagen fibres and other proteinaceous structures of parchment, but can also act modifying its inorganic components, or can produce pigments and organic acids which discolour parchment and cause indirect damage. According to Karbowska-Berent and Strzelczyk [4], *Streptomycetes* is the bacterial group that plays a large part in the deterioration of many kinds of historic documents and books supported on parchment. These filamentous bacteria are known to produce many types of enzymes, especially collagenases (proteases) that are capable of destroying collagen by their hydrolytic activity. They are alkaliphiles and therefore develop on parchments (prepared by rubbing in chalk). Historic parchment provides good conditions also for the development of proteolytic fungi, among which numerous representatives of Zygomycetes prevail, e.g. *Mucor*, Ascomycetes such as *Chaetomium* and *Gymnoascus*, as well as imperfect (mitosporic) fungi in the genera *Aureobasidium*, *Epicoccum*, *Trichoderma*, *Verticillium*, etc. The microbiota of objects made of leather comprises members of these fungal genera, with the addition of representatives of the genus *Paecilomyces*, which is highly lipolytic [4–8].

Microbial investigations are usually based on cultivation strategies which cannot be regarded as reliable in terms of reflecting the microbial diversity present in art samples. The culturing techniques can be assisted by a comparison with

more modern approaches. The use of scanning electron microscopy for examination of biodeteriorated parchment is, for example, considered to be a valuable approach to the problems of ancient manuscripts conservation. In particular, variable pressure SEM has recently proved to represent an excellent tool for demonstrating spatial relationships between microorganisms and substrata because hydrated, non conducting samples can be viewed with a minimum of manipulation. In this case study, a metabolic approach to the evaluation of microbial role in cultural heritage defacement is for the first time coupled to the analysis of chemical changes in the support by means of VP-SEM, microanalysis and Raman spectroscopy. The study of the causes of degradation is of great relevance in order to obtain a better understanding of the mechanisms that result in leaving the cultural object in a bad state of conservation, and to better address the conservation treatments.

Materials and methods

Sampling procedures

Samples of fungal structures from damaged areas were carried out using *swab sampling*. Sterile cotton swabs were wiped across spots showing visible damage on several areas of the document and then used to inoculate Petri plates containing Malt Extract Agar, Czapeck agar and dichloran glycerol Agar [9] to obtain cultures suitable for fungal identification. Plates were incubated for 7 to 14 days at 26 °C in the dark for fungal growth and isolation in pure culture. Most common fungal isolates, obtained from agar plates, were identified at the genus level by examination of biometric and microscopic features [10–12].

Moreover, *removable transparent adhesive tapes* (Fungi-Tape, Scientific Device Lab., Inc Glenview, IL) were gently pressed over the fungal spots, to collect mycelia and fruiting structures [13].

Functional profiling

Biolog[tm] FF microplates (Hayward, CA, USA) were used to provide a detailed profile of the functional capabilities of each of the fungal isolates on 95+1 low molecular weight carbon sources (1 is only water). The microtiter plate by Biolog contains 96 wells with different carbon substrates. The plates rely on a redox dye (iodo-nitro-phenyltetrazolium) to detect respiration (NADH formation) of sole carbon sources. The content of the wells is supplied in a dry state; the medium is reconstituted when the wet sample is added. The inoculations of the pure fungal cultures were performed as described by [14]. Optical densities of the wells were measured at 490 nm using a microtitre plate

reader (Vmax, Molecular Devices, CA, USA). Measurements were effected immediately following inoculation (time zero), and subsequently at 24, 48, 72, 96, 168 and 240 h intervals, in order to obtain a maximum colour development curve for each well. Raw data were transferred to an Excel (Microsoft) sheet according to sample, replicate (three replicates each), and reading time (ten reading points, one every 24 h). The 96 substrates (95+ water) were divided into 15 guilds: heptose, hexoses, pentoses, sugar acids, hexosamines, polyols, polysaccharides, oligosaccharides, glucosides, peptides, L-amino acids, biogene and heterocyclic amines, TCA-cycle intermediates, aliphatic organic acids, others, and the average absorbance for all wells within each category calculated.

Optical microscope examination

Fragments of tape with fungal samples were stained with a drop of fluorescein diacetate solution (20 μg of FDA in 1 ml of phosphate buffer solution pH 7.5) for qualitative observations of active structures using an epifluorescent microscope (Leica DM 5000) equipped with a FITC filter (blue excitation wave length, 495 nm). Active structures (positive staining) were assessed by the presence of a greenish fluorescence emanating from the cytoplasm of spores and hyphae, due to the liberation of fluorescein by enzymatic (hydrolytic) cleavage [15]. Samples stained with FDA were observed after 20 min of incubation in the dark at 20 °C.

Scanning electron microscope observations

SEM analysis of adhesive tapes used to capture fungal structures was made using an EVO 50 Scanning Electron Microscope produced by the Carl-Zeiss Electron Microscopy Group (Oxford, UK). Tape fragments measuring 5–10 mm in diameter bearing the fungal structures were cut and mounted on to a 12-mm metal stub (Agar Scientific, Essex, England, pin *stubs* cod. G301F), using double-sided carbon adhesive tape (Agar Scientific, Essex, England, G3347). Only after having observed samples with SEM in variable pressure mode, at 20 keV, were some of the samples covered with gold with a Baltec Sputter Coater for a further analysis in high vacuum mode. The sputtering was performed under an argon gas flow, at 50 mm working distance with 0.05 mbar of pressure and a current of 40 mA, for 60 s to obtain a film of gold of about 15 nm [16].
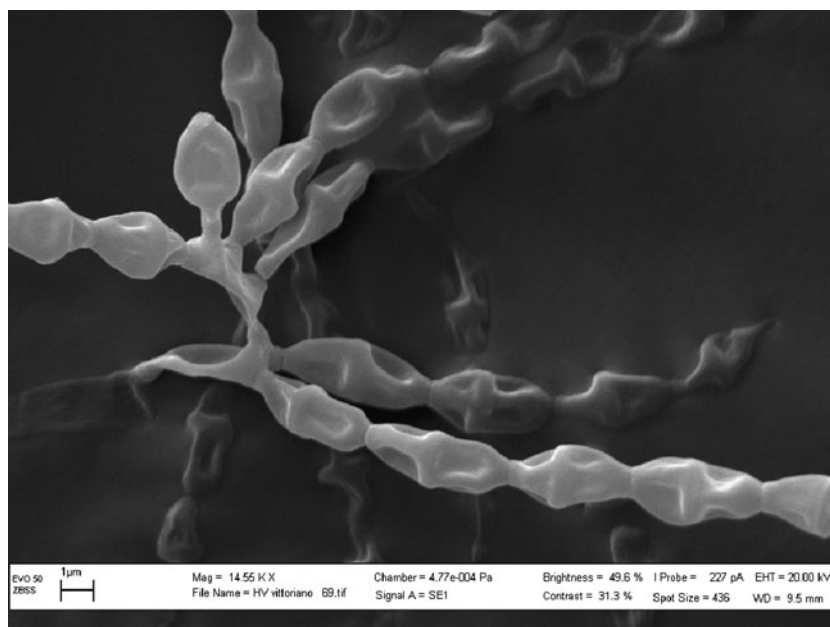
EDS analysis

A chemical characterisation of the inorganic components of the adhesive tape samples was performed by means

of electronic dispersion spectroscopy (EDS INCA Energy 250), which allows for an X-ray area scanning of what is brought into focus in SEM images, thereby creating a compositional map of the sample's surface [16]. Reference elemental intensities acquired from pure compounds (standards) are commonly utilized for calibrating SEM-EDX systems. In the case study showed in this paper, conventional ZAF correction [16] integrated into Oxford INCA 250 microanalysis package was applied to the spectrum dataset (Oxford Instruments) [17]. EDS analysis was made at 20 kV accelerating voltage with a tungsten filament.

Raman spectroscopy

For further characterisation of chemical changes in bio-deteriorated areas of the document adhesive tapes with samples from the damaged areas were studied with a confocal Raman microscope (CRM200, WITec, Ulm, Germany) equipped with piezo-scanner (P-500, Physik Instrumente, Karlsruhe, Germany). The diode pumped 785 nm near infra-red (NIR) laser excitation (Toptica Photonics AG, Graefelfing, Germany) was used in combination with 100× NIR (Olympus Mplan IR, NA=0.95) microscope objective. The spectra were acquired using a cooled CCD (PI-MAX, Princeton Instruments Inc., Trenton, NJ, USA) behind a grating (300 gmm^{-1}) spectrograph (Acton, Princeton Instruments Inc., Trenton, NJ, USA) in the spectral range 100–3,250 cm^{-1} with a spectral resolution of 6 cm^{-1}. To obtain full profiles of the samples on the tapes we conducted depth scans with 5 μm step. The spectra were recorded with acquisition time of 2 s and 30 acquisitions per scan.

Statistics

The data obtained with Biolog FF microplates, and the microanalytical data (EDS) were analyzed using factorial analysis of variance (ANOVA) implemented in the XLStat 9.0 (Addinsoft, Paris) software package. Post hoc comparisons were done using Tukey's honestly significant difference test.

Results

Two species of fungi were significantly associated with the discoloured and weakened areas of the illuminated parchment: *Diploöspora rosea* Grove (Fig. 3) and *Aspergillus versicolor* (Vuill.) Tirab. (Fig. 4). *A. versicolor* has been previously isolated from both paper and parchment affected by discoloration and structural damage [18] and as a species exhibits a high amlylolytic and gelatinolytic activity, and it is only moderately cellulolytic. It is a species that can deteriorate also polymeric materials, grow on building materials, and cinematographic films [19] indicating that it has a great plasticity and physiological versatility. *A. versicolor* is generally xerophilic, meaning that it can grow at low water activity (<0.80). The minimum and maximum growth temperatures for *A. versicolor* are 4 °C and 40 °C with an optimum at 30 °C. Its optimal water activity is 0.95 with a minimum of 0.75, in fact, *A. versicolor* is often encountered in environments with increased salinity. *A. versicolor* is widespread in terrestrial ecosystems from polar to southern latitudes, and it is considered to be an important causative agent of secondary aspergilloses in humans.

Fig. 3 The filamentous fungus *Diploöspora rosea* Grove sampled by means of adhesive tape and observed at SEM in high vacuum mode, after metallization with gold. Secondary electrons detector, 20,000 kV. The fungus appears naturally dehydrated. Details of conidia production and branching can be observed
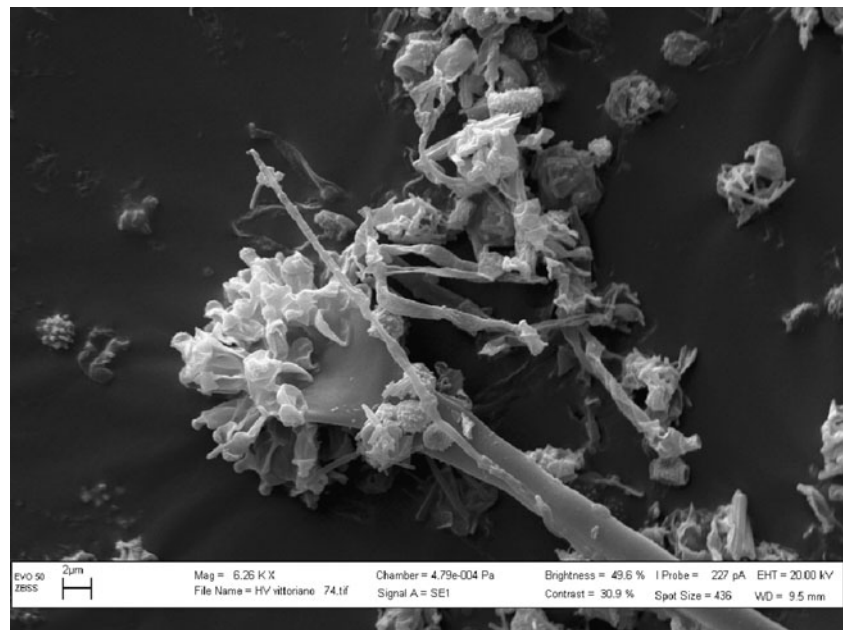
D. rosea is an anamorphic Pezizomycotina fungus with two celled conidia produced in chains [20]. This species was described the first time on wet cellulose paper in 1913 as reported in its first description by Grove in 1916 [21]. The colony on malt extract agar showed a very slow growth, reaching 1 cm in diameter after 2 weeks of incubation. When seen dry, the chains looked as if the spores are united by isthmuses, but this is due to the apiculate ends. Singly the spores appear colourless, but they form masses which have a distinct rosy tint. The conidiophores are conspicuously but irregularly branched with primary, secondary and tertiary strongly divergent branches. The conidia are blastospores and develop in long acropetal chains.

Saprotrophic fungi could potentially be divided into nutrient-related functional groups based on the type and quantity of extracellular enzymes secreted in each functional guild, as measured with the substrate microarrays. The metabolic phenotyping (Fig. 5) showed marked differences in the use of substrate between these two fungi. A significantly higher (alpha=0.05, $p < 0.001$) proteolytic activity was observed in *A. versicolor*, while a more carbohydrate-oriented metabolism was addressed to *D. rosea*. Not all the "functional guilds" are showed in the histogram, but only those where a difference of behaviour between the two strains was observed (Fig. 5).

SEM observation of the adhesive tape samples showed the co-occurrence on parchment of the two species of fungi. A third fungal strain (Figs. 6 and 7) was observed only in the tape micrographs but no agar cultures were obtained. The observation of its structures, as captured with the adhesive tape, with optical microscope equipped for epifluorescence after the FDA staining

revealed its viability. In fact, the presence of a greenish fluorescence in its conidia (Fig. 8) indicates the viability of the fungus. Its structures can be attributed to *Acremonium*, a filamentous conidial fungus. Species of *Acremonium* lack known sexual stages, and their identifications are based on morphology, notably the type of conidiogenesis and conidiophore morphology. *Acremonium* is a genus of fungi in the Hypocreaceae family. *Acremonium* hyphae are fine and hyaline and produce mostly simple phialides. Their conidia are usually one-celled, hyaline or pigmented, globose to cylindrical, and mostly aggregated in slimy
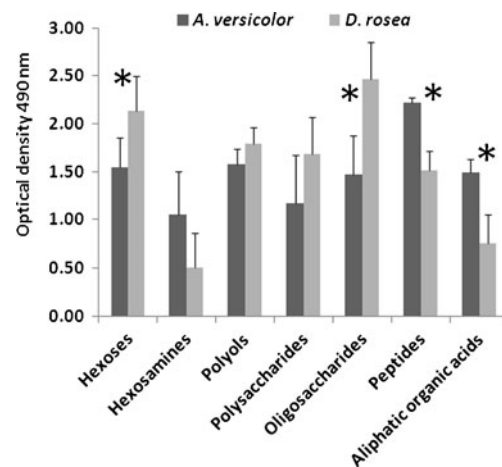

Fig. 5 Summary of results of the metabolic profiling of the two co-occurring fungi, *Diploöspora rosea* and *Aspergillus versicolor*. The average absorbance for all wells within each metabolic category calculated. Only categories where there is a statistically significant difference between the two fungi are showed. Strong differences with $p < 0.001$ (Tukey test, alpha<0.05) are indicated with an *asterisk*. Categories without the *asterisk* are significantly different in the two fungi for $0.05 > p > 0.001$

SEM micrograph obtained in high vacuum mode on gold-sputtered samples. Samples of adhesive tape with attached the *Acremonium* hyphal structures. Secondary electrons detector, 20,000 kV. The micrograph was acquired at a low magnification to show the huge amount of the mycelium with associated biogenic crystals that could be recovered in a small area of the parchment
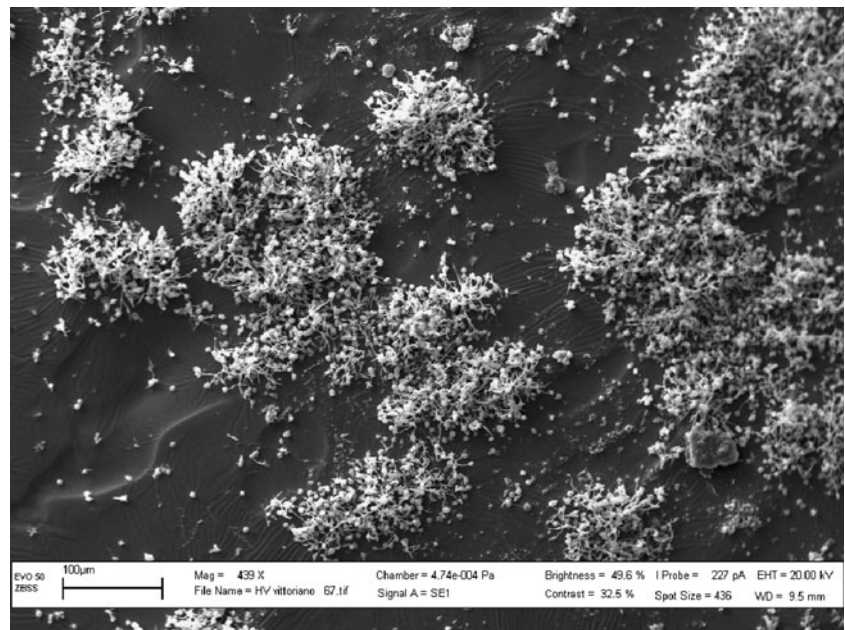
heads at the apex of each phialide. The genus *Acremonium* currently contains approximately 100 species, of which most are saprophytic, being isolated from dead plant material and soil. Many species of *Acremonium* are recognized as opportunistic pathogens of man and animals, causing mycetoma and onychomycosis. The cephalosporins, a class of β-lactam antibiotics, were originally derived from *Acremonium* (which was previously known as *Cephalosporium*).

This organism showed the production of abundant biogenic crystals. Two different types of structures were observed, more columnar, forming crossing solids, and needle-like raphidic structures that formed a uniform and

quite regular grid around conidial slimy heads. EDS analysis (Figs. 9a and b) showed the presence of calcium and potassium in both types of crystals, although some significant differences between the two could be observed by repeating up to 15 measurements pointing in the SEM micrographs the different types of crystals selectively by means of EDS topographical analysis (Table 1). The needle-like crystals were associated to lower amounts of Ca, and a higher content of S, P and K. These last three elements can be reasonably associated to the presence of fungal conidia, than to the composition of the needle-like crystals.

The formation of crystals by the fungus coincided the leaching of the calcium-based materials of the underlying

Fig. 7 SEM micrograph obtained in high vacuum mode on gold-sputtered samples. *Acremonium* fruiting structures. Secondary electrons detector, 20,000 kV. Two different types of biogenic mineral structures were observed, more columnar, forming crossing solids, and needle-like raphidic structures that formed a uniform and quite regular grid around conidial slimy heads
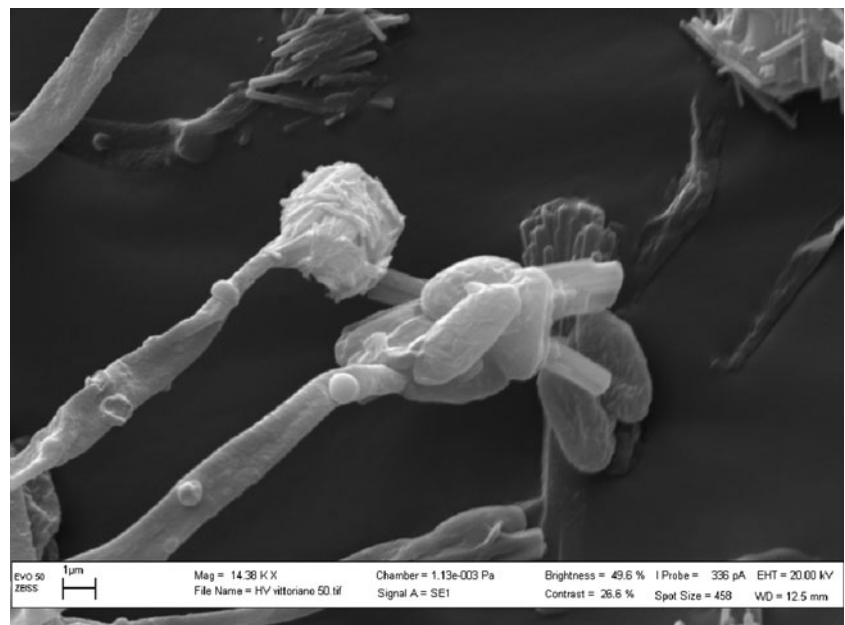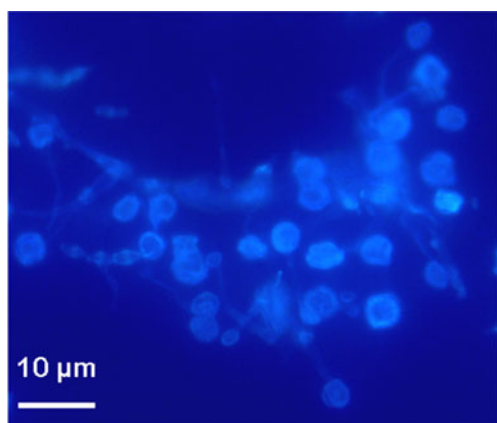
Fig. 8 Observation of *Acremonium* sampled by means of adhesive tape: the fungus observed with optical microscope equipped for epifluorescence after FDA staining. The presence of a *blue fluorescence* indicates the viability of the fungus. The *bars* in the pictures indicate 10 μm

parchment (typically manufactured with gypsum). As a result, the areas of the parchment affected by these moulds were weakened, slightly stained and characterised by the presence of a powdery raised patina rich in calcium.

Confocal μ-Raman profile of the adhesive tape sample clearly shows that the crystals associated to the fungus were oxalates. In Fig. 10a, the upper and the lower curves serve as the reference spectra of the adhesive tape and weddelite crystals, respectively. The middle portion of the graphics presents the relevant spectra from the depth scan whereby the contribution from the adhesive tape diminishes from top to bottom. The central spectrum (bold solid black) has the least contamination from the tape and presents almost pure calcium oxalate dihydrate (weddelite) spectrum with prominent peaks at 1,476 cm^{-1} (ν CO_2 sym.), 910 cm^{-1} (ν CC) and 504 cm^{-1} (δ CO_2 sym.) [22]. It is noteworthy that neither calcareous substrate nor calcium oxalate monohydrate could be detected in this sample. Exclusive presence of weddelite in the presence of excessive humidity has been reported for biodegradation of medieval mortar specimen from Stibbard Church [23]. Analysis of the sample from the less degraded area (Fig. 10b) shows, however, considerable presence of the both calcareous substrate (gypsum) and calcium oxalate monohydrate. Despite the fact that gypsum with its bands at 414, 493, 619, 670, 1,007, and 1,132 cm^{-1}, respectively, clearly dominates the picture, we can identify whewellite by the characteristic doublet at 1,475/1,490 cm^{-1} and a band at 900 cm^{-1} [22].

Discussion

The defacement of the parchment was due to both collagenolytic activity and to the biotransformation of calcium-based minerals by fungi. Mycolyths production is
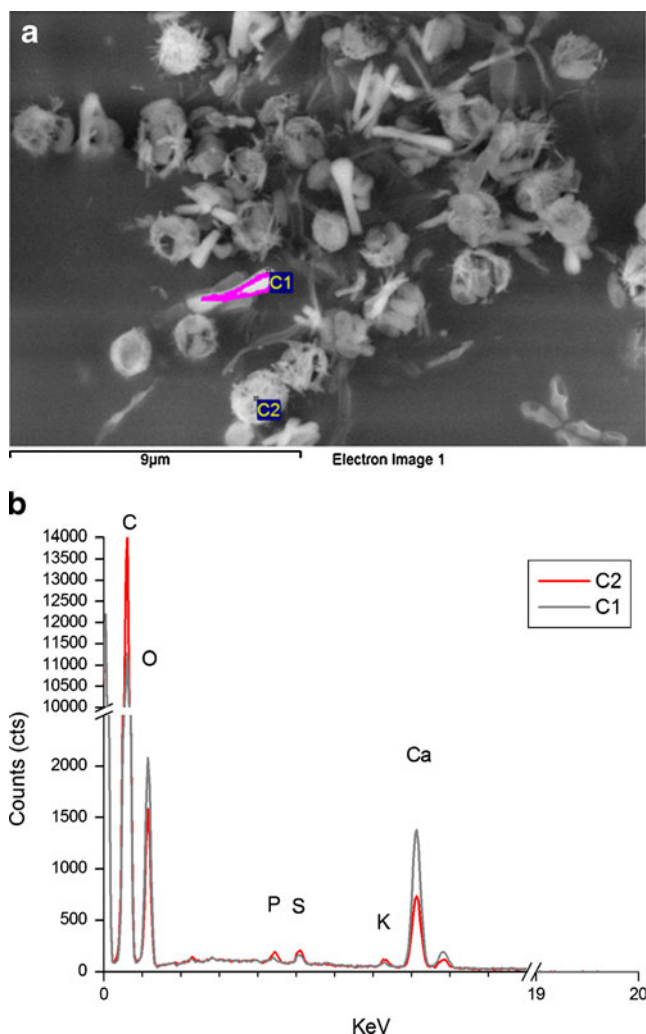

Fig. 9 a SEM micrograph obtained in variable pressure mode (50 Pa) of adhesive tape samples. *Acremonium* fruiting structures showing the associated crystals. Backscattered electrons detector, 20,000 kV. Two different types of biogenic mineral structures were observed, more columnar, forming crossing solids, and needle-like raphidic structures around conidial slimy heads. The backscatter coefficient increases with increasing atomic number and so higher atomic number elements (calcium with respect to carbon and oxygen) appear brighter in the image **b** elemental dispersive spectroscopy (EDS—INCA Energy 250) was used to obtain a chemical characterisation of the two forms of biogenic crystals (*C1* and *C2*) associated to the conidia of the fungus. The main component is Ca, but the needle-like crystals (*C2*) showed also a significantly higher presence of P and K (cfr Table 1)

caused by the metal-binding capacity of the organic acids generated by the fungi. These, together with the presence of hydrogen ions, cause substrate mineral hydrolysis and mineral neoformation through metal-acid complexation. The action of these acids is combined with the action of fungal respiratory CO_2, which generates carbonic acid in the growth environment: it can be hypothesized that the combined action of these two acid groups produce the dissolution pattern observed on the calcium materials, and that a subsequent spontaneous re-crystallization of fungal

Table 1 Summary of results of the energy dispersive X-ray spectrometry (SEM-EDS topographical analysis) performed on the two types of biogenic crystals

	C1	C2
C	73.5±2.4	79.2±4.4
O[a]	22.7±1.4	16.3±2.3
P[a]	0.0±0.0	1.1±0.5
S	0.2±0.1	0.7±0.1
K[a]	0.1±0.0	1.2±0.2
Ca[a]	3.6±0.2	1.8±0.4
Total	100	100

The measurements were performed on ten different points for each type of crystal. The table reports average values of weight% of the detectable elements, and standard deviation values

[a] Significant differences in elemental content of the two types of crystals

oxalic acid with the Ca^{++} ions on chitin nucleation sites brought about the formation of the new crystals close to fungal spores. Fungal-derived calcium oxalate can be monohydrate (whewellite) or dihydrate (weddelite). A suggested function or consequence of calcium oxalate formation is the detoxification of calcium, which can be highly toxic within cells [24]. Nevertheless, according to the aspect assumed by the slimy heads of the conidia in *Acremonium*, the uniform and quite regular grid of needles of oxalates resembles a protective structure, possibly evolved as a protection against the grazing activity of micro-arthropods like mites or mycetophagous insects. The extraction of the *Acremonium* DNA from adhesive tape was performed but samples were too small and affected by inorganic salts, and no sequences useful for further identification [25] could unfortunately be obtained.

The microbial species that can damage materials in indoor environments are chiefly primary colonisers capable of rapid growth even when water activity is low (i.e. aw< 0.8). When a substrate is attacked by a fungus, its water activity changes sufficiently to support the growth of other species (fungal and bacterial), as in natural successions [26]. Secondary colonisers are species that have a high resistance to stress; these species develop thanks to unstable microenvironments whose existences are linked to many variables, like small changes in air temperature or humidity due to night/day alternation. Poor ventilation and surface temperature homogeneity could have produced water condensation points and local micro-climates with higher water availability than in the rest of the document. These circumstances could have promoted one or more of the fungal species isolated from the object; as a result, these were able to proliferate in places where the overall environmental conditions would otherwise appear to be hostile to microbial life. The differences in the metabolic
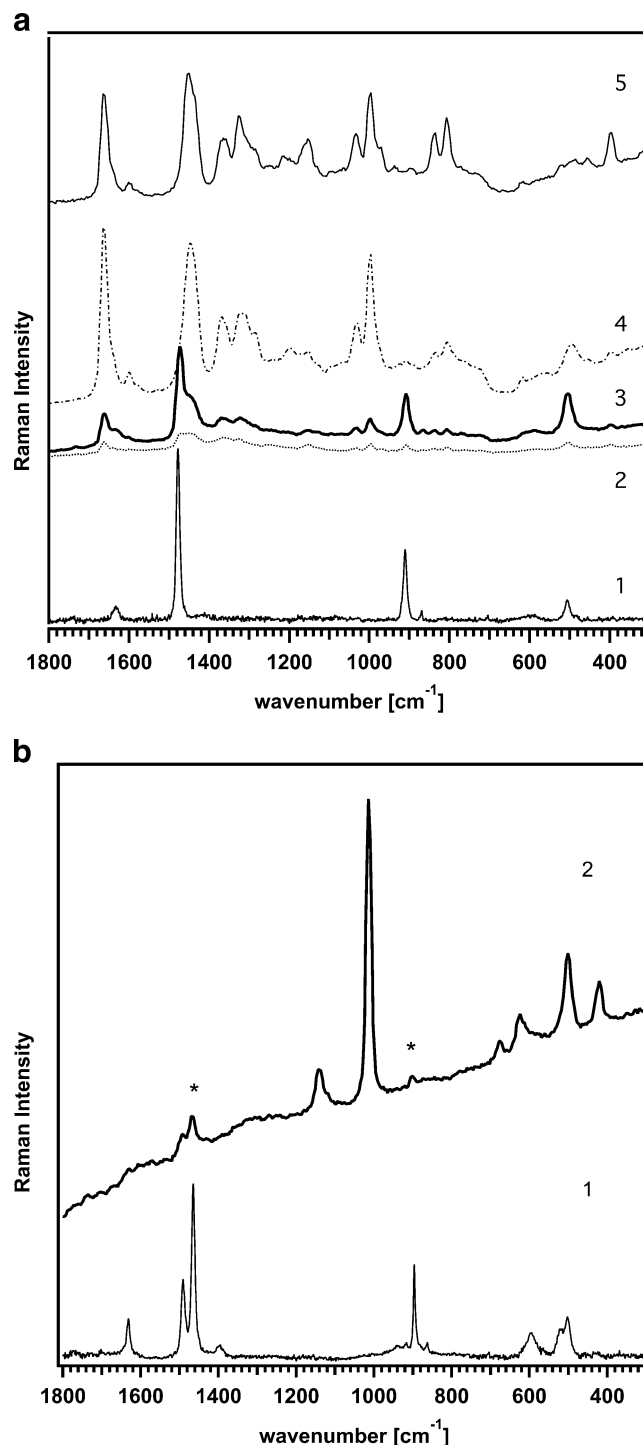
Fig 10 a Raman depth scan (*curves 2–4—stacked*) with a step of 5 μm as compared to the reference spectra of calcium oxalate dihydrate (*1*) and adhesive tape used for the sample collection (*5*). The spectrum *3* contains the least contamination from the adhesive tape and attests for a considerable presence of weddelite. The intensity on Y-axis is expressed as Raman Arbitrary Units **b** Raman spectrum from the depth-scan series of the less degraded area (*2*) presented together (*stacked*) with a reference spectrum of calcium oxalate monohydrate (*1*). *Asterisks* mark fingerprint peaks of whewellite. The Intensity on Y-axis is expressed as Raman Arbitrary Units

profile of the prevailing fungal species suggest that one species among *Acremonium*, *Diploöspora* or *Aspergillus* acted as primary coloniser of the document, while the growth of the other two species could be a consequence of the changes at the microenvironment operated by the pioneer species. The metabolic profile of the *Acremonium* species could not be obtained due to the difficulties in its in vitro cultivation. Therefore, its role in the fungal succession that brought to the defacement of the parchment substrate can only be guessed, while we knew that *Aspergillus* acted mainly in the hydrolytic cleavage of the peptide backbone, and that the *Diploöspora* was a slow-growing sugar consumer.

Conclusions

A better understanding of the natural mechanisms that support the establishment of different species assemblages on manmade objects could be of help in defining a new approach to assuring proper conservation of cultural assets. The niche concept implicitly assumes that neighbouring species have negative impacts (or a niche-shrinking affect) on one another [27] and is firmly bound to the principle of competitive exclusion (that no two species can occupy the same niche).

Indoor environments, although artificial, represent niches for microbes and fungi to inhabit. In these places materials are stored and assembled to form simplified "ecosystems" where microbial species can grow and reproduce. Ecological insight into materials spoilage and indoor niche colonisation by bacteria, insects and fungi could represent a successful approach for modelling and predicting microbial dynamics that can occur on objects of cultural value. In this paper, we demonstrated that the co-occurrence of two fungi on the same support is due to different carbon sources degradation ability, namely a partitioning of the "niche" that the object of cultural value represented for the saprotrophic fungi.

Although the object presented interesting damage, from the point of view of scientists studying cultural heritage degradation, its study presented some limitations, mainly due to sampling restrictions. Nevertheless, the goal of defining the cause and degree of damage was reached. In this case study, we showed that the methodology required for a conservative approach to the restoration of valuable objects of art made from parchment includes a wide range of sampling techniques and instruments that cannot easily be standardised. Classic cultivation and sampling methods and innovative techniques must be specially adapted on a case by case basis to best suit the particular situation that materials encountered in cultural heritage of value present. The integrated techniques applied in this study showed the advantage of resulting in an exhaustive series of tests performed on very small samples.

In this case study, a metabolic approach to the evaluation of microbial role in cultural heritage defacement is for the first time coupled to the analysis of chemical changes in the support by means of VP-SEM, microanalysis and Raman spectroscopy. To the best of our knowledge, biogenic calcium oxalate formation and bioleaching of inorganic salts on historical parchment has never been documented up till now. Moreover, this is the first time that a functional profiling (Biolog approach) was used to characterise the fungal community defacing a historical parchment. The success in addressing a different role of each fungal species found on the object represents a new and promising result that can be very important in the set up of more efficient conservation treatments for objects of cultural value. Targeted disinfecting treatments could be particularly useful especially when several microbial species are present at once. A nonspecific treatment could result, in fact, in the selection of particularly aggressive microbial strains [28] that can overgrow after the removal of the competing species.

References

1. Pinzari F, Colaizzi P, Lunghini D, Maggi O (2010) Cultured fungi: fungal assemblages that affect cultural heritage in archives and libraries. Proceedings of the 9th International Mycological Congress, IMC9 The Biology of Fungi. 1–6 August, Edinburgh, UK
2. Reed R (1972) Ancient Skins Parchments and Leathers. Department of Food and Leather Science, University of Leeds. Seminar, London, England
3. Florian M-L E (2007) Protein facts. Fibrous proteins in cultural and natural history artifacts. Archetype, London
4. Karbowska-Berent J, Strzelczyk AB (2000) The role of *Streptomycetes* in the biodeterioration of historic parchment. Nicholas Copernicus University, Torun, Poland
5. Polacheck I (1989) Damage to an ancient parchment document by *Aspergillus* Mycopathologia. Kluwer, Belgium, 106:89–93
6. Petushkova JP, Koestler RJ (1996) Biodeterioration studies on parchment and leather attacked by bacteria in the Commonwealth of Socialist States. International Conference on Conservation and Restoration of Archival and Library Materials, Erice, I: 195–211. 22–29 April
7. Rebrikova NL, Dmitrieva MB (1996) Experimental Investigation of Parchment Manuscripts with Traces of Microbiological Damage International Conference on Conservation and Restoration of Archival and Library Materials. Erice 1:275–283, 22–29 April
8. Kowalik R (1980) Microbiodeterioration of library materials Part 2. Microbiodecomposition of basic organic library materials. Chapter 4. Denmark. Restaurator 4:200–208
9. Samson RA, Varga J (2007) *Aspergillus* sytematics in the genomic era. Stud Mycol 59:206
10. Domsch KH, Gams W, Anderson TH (1980) *Acremonium* Link. Ex Fr 1821 pp 16 compendium of soil fungi. Academic, London
11. Fincher RM, Fisher JF, Lovell RD, Newman CL, Espinel AI, Shadomy HJ (1991) Infection due to the fungus *Acremonium* (*Cephalosporium*). Med (Baltimore) 70:398–409

12. Pitt JI, Hocking AD (1997) Fungi and food spoilage, 2nd edn. Blackie, London

13. Pinzari F, Montanari M, Michaelsen A, and Pinar G (2010) Analytical protocols for the assessment of biological damage in historical documents. Coalition Newsletters 19:6–12 (ISSN 1579–8410 5, www.rtphc.csic.es/boletin.htm)

14. Druzhinina IS, Schmoll M, Seiboth B, Kubicek CP (2006) Global carbon utilization profiles of wild-type, mutant, and transformant strains of Hypocrea jecorina. Appl Environ Microbiol 72:2126–2132

15. Lundgren B (1981) Fluorescein diacetate as a stain of metabolically active bacteria in soil 36. Blackwell, Oikos, pp 17–22

16. Goldstein JI, Newbury DE, Echlin P, Joy DC, Lyman CE, Lifshin E, Sawyer L, Michael JR (2003) Scanning electron microscopy and X-ray microanalysis. Kluwer, New York, 689 pp

17. Hall TA, Gupta BL (1984) The application of EDXS to the biological sciences. J Microsc 136:193–208

18. Zyska B (1997) Fungi isolated from library materials: a review of the literature. Int Biodet & Biodeg 40:43–51

19. Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B (2010) Food and Indoor Fungi, CBS Laboratory Manual Series, CBS-KNAW Fungal Diversity Centre, ISBN 978-90-70315-82-3, 390 pp

20. Hughes SG (1968) Diploospora. Can J of Botany, (Notes) 46 (9):1159–1160

21. Grove WB (1916) New or noteworthy fungi. J Botany, London 54:217–223

22. Edwards HGM, Farwell DW, Jenkins R, Seaward MRD (1992) Vibrational Raman spectroscopic studies of calcium oxalate monohydrate and dihydrate in lichen encrustations on renaissance frescoes. J Raman Spectrosc 23:185–189

23. Edwards HGM, Farwell DW (2008) The conservational heritage of wall paintings and buildings: an FT-Raman spectroscopic study of prehistoric, Roman, mediaeval and Renaissance lime substrates and mortars. J Raman Spectrosc 39:985–992

24. Gadd GM (2007) Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycol Res 111:3–49

25. Michaelsen A, Pinzari F, Ripka K, Lubitz W, Piñar G (2006) Application of molecular techniques for identification of fungal communities colonising paper material. Int Biodet & Biodeg 58:133–141

26. McGill WB, Cole CV (1981) Comparative aspects of cycling of organic C, N, S, and P through soil organic matter. Geoderma 26:267–286

27. Higashi M (1993) An extension of niche theory for complex interactions. In Mutualism and Community Organization: Behavioural, Theoretical, and Food-Web Approaches. Kawanabe H et al (eds) Oxford University Press, pp 311–322

28. Strzelczyk A (2001) Adaptation to fungicides of fungi damaging paper. Int Biodet & Biodeg 48:255–262