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Synthetic Consolidants Attacked by Melanin-Producing Fungi: Case Study of the Biodeterioration of Milan (Italy) Cathedral Marble Treated with Acrylics▽

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Monuments and artistic stone surfaces are often consolidated and protected with synthetic polymers, in particular, acrylics. Although it is generally thought that acrylic polymers are resistant to biodeterioration, we report for the first time the systematic occurrence of dematiaceous meristematic fungi on many marble samples of the cathedral in Milan (Italy) previously treated with this material. Fourier transform infrared spectroscopy applied to the Milan cathedral stone samples revealed characteristic features of biodeteriorated synthetic resins that differentiated them from the aged but nonbiodeteriorated samples. Samples showing biological colonization were analyzed for the presence of fungi. Cultivation and morphological characterization and methods independent from cultivation, such as denaturing gradient gel electrophoresis coupled with partial 18S rRNA gene sequencing and immunofluorescence staining with melanin-binding antibodies, showed that melanin-producing species are heavily present on stone surfaces protected with acrylic resins. This observation raises the question of the effectiveness of acrylics in protecting stone artworks.

The protection and consolidation of stone materials is a critical step for the conservation of outdoor architectural monuments. Over the past decades a range of synthetic adhesives, consolidants, and protectives have been applied to monuments to attempt to enhance their long-term preservation. Polyacrylates and polymethacrylates are among those more frequently used. Superficial treatments made with them are meant to have both protective and consolidating properties (19). In this respect, the Milan cathedral is not an exception: since an intervention in 1972, its marble surfaces have been protected with acrylic resins (poly-isobutylmethacrylate). Before applying such resins, learning the durability of the treatments in outdoor conditions is of crucial importance for conservation.

Over the past 40 years the chemical and physical stability of acrylic homo- and copolymers has been extensively investigated, and acrylics have appeared to be a suitable solution for use in cultural heritage conservation (18). While natural polymers are highly prone to biodeterioration, synthetic resins differ with respect to their susceptibilities to fungal attack, depending on their chemical nature, the environmental conditions, and the way they are applied (5, 7). Freshly dried acrylic resins are among the resins most resistant to biological damage (6). However, little is known about the susceptibility to biological degradation of naturally aged acrylic resins, the only exception being the facade of Tempio Malatestiano (Rimini, Italy) treated with acrylic resins that presented black fungal growth in cracks and fissures (22).

The Milan cathedral is currently under conservation treatment, as it appears to be seriously damaged by surface erosion, microfractures, detachments, and thick crusts as well as biological growth. In particular, at the first inspection, the Milan cathedral presented an extensive blackening in the areas previously consolidated or protected with synthetic products. The blackening of stone surfaces may be caused by a variety of mechanisms, including air pollution, fly ash, oxidation of metal, and biological pigments such as melanin (11, 15, 32). Numerous studies have established that most of the blackening on artistic marbles and limestones exposed to outdoor environments is caused by dematiaceous fungi and, in particular, by those manifesting meristematic and sometimes yeast-like growth with budding cells (26). The pigmentation of these fungi is largely due to deposition of melanins in the cell wall (reviewed in reference 20). Meristematic fungi form black clump-like cauliflower-like colonies consisting of isodiametrically dividing cells that colonize the rock surface and penetrate into the rock. It is well known that meristematic fungi, many of which have their natural ecological niche on rocks, physically attack the rock and cause esthetic and structural damage on artistic stone. The cause of damage is not acid formation and dissolution of the mineral compounds but rather intercrystalline growth causing physical disruption of the weakest structural components of the crystals and resulting in biopitting and formation of cracks and fissures (11, 27, 38). In addition, the growth of black fungi on white or light-colored rocks causes selective absorption of solar radiation that can lead to local extension of crystals and, as a consequence, crystal decohesion

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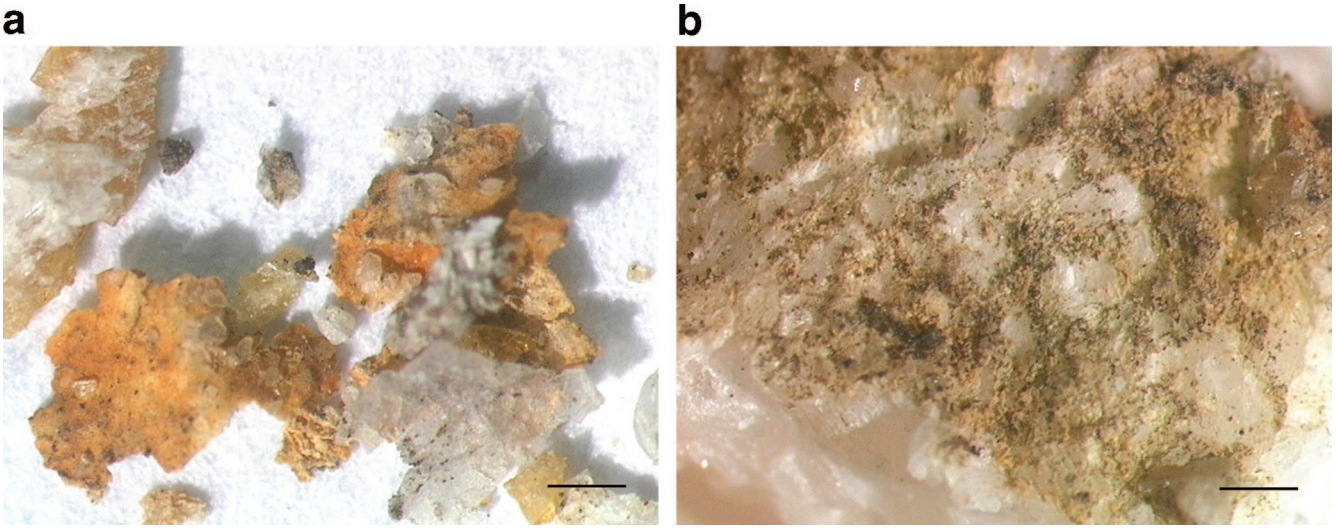

FIG. 1. (a) Sample 14F033: FTIR spectrum image of a small quantity of orange film residue. (b) Sample 14F034: FTIR spectrum image of a pink-beige patina over calcite crystals. Scale bars, 500 μm.

(12). Differences in solar radiation adsorption by nonaffected rock and rock affected by black fungi can result in temperature differences and thermal stresses that may promote rock cracking and degradation.

The aim of this work was to study the fungal microflora present on the synthetic resins used to consolidate the Milan cathedral in order to inform conservators of the possible detrimental effects of the use of synthetic polymers for the consolidation and protection of stone surfaces.

MATERIALS AND METHODS

Sampling. Marble fragments were generally collected from the facade of the Cathedral in Milan where biological patinas were visually evinced on consolidated and protected marble. These patinas were always blackish in color. Sample 14F033 was taken in an apparently nonbiodeteriorated area. Sampling was performed using a sterile lancet and scalpel, and fragments were stored in sterile tubes at room temperature.

Sample codes are connected to the identification of the area of the facade from which they were obtained. The sample codes are presented in the form XXYZZZ, where XX indicates the number of floors, Y represents the longitudinal sector (lettered from A to X from left to right across the facade), and ZZZ represents the sequential sample number.

Mycological analyses of marble samples. Preliminary identification was based on the macroscopic features of colonies growing on agar plates and the micromorphology of the reproductive structures.

(i) Culture techniques. The presence of fungal colonization on the biodeteriorated Candoglia marble of the Milan cathedral was evaluated using 2% malt extract agar and dichloran rose bengal medium from Fluka (32, 38). Marble chips were incubated in the cultural medium at 25°C for 1 month to allow for the detection of slowly growing fungi.

(ii) Microscopic observations. Touch preparations with adhesive tape (Fungi Tape; DID s.p.a., Milan, Italy) were used for direct microscopic observation according to the methodology described by Urzì and De Leo (36). The samples were analyzed using a digital epifluorescence microscope (Leica DM4000B) equipped with a CoolSnap CF camera (Photometrics, Roper Scientific). Digital images were acquired by RS Image, version 1.7.3 (Roper Scientific, Inc.).

FTIR. Fourier transform infrared analyses, used to detect the acrylic resin in the samples, were carried out by a Nicolet Nexus spectrophotometer coupled with a Nicolet Continuum Fourier transform infrared spectroscopy (FTIR) microscope equipped with a HgCdTe detector cooled with liquid N_2; spectra were recorded by a Graseby-Specac diamond cell accessory in transmission mode between 4,000 and 700 cm^{-1}. To avoid contamination by the carbonatic substrate, the samples were carefully collected under an optical microscope by means of a needle sampler.

Immunostaining technique. The immunostaining was employed to detect melanin—and therefore melanin-producing fungi—on the samples. Marble chips were immersed for 30 to 40 min in phosphate-buffered saline solution (pH 7.0). The debris of the marble surface was attached to a freshly coated poly-L-lysine slide (Sigma Chemical Corporation, St. Louis, MO). Slides were incubated in Superblock (Pierce, Rockford, IL) blocking buffer for 4 h followed by incubation with 10 μg/ml of the melanin-binding monoclonal antibody immunoglobulin M 6D2 (μκ) (23) overnight at 4°C. After a wash, the slides were incubated with a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin M (Southern Biotechnologies Associates, Inc., Birmingham, AL) for 1 h at 37°C. The slides were washed, mounted using a 50% glycerol–50% phosphate-buffered saline–0.1 M *N*-propyl gallate solution, and viewed with an Olympus (Melville, NY) AX70 microscope equipped with an FITC filter. Negative controls consisted of slides incubated with the monoclonal antibody 5C11 (μκ), which binds mycobacterial lipoarabinomannan (14), as the primary antibody or with FITC-labeled antibody alone.

DGGE to study fungal communities on marble samples and sequencing and phylogenetic analysis of DGGE bands. Fungi growing on marble facade of the cathedral in Milan were characterized using denaturing gradient gel electrophoresis (DGGE), a method independent from cultivation. Total DNA was extracted from samples pulverized in a mortar following a method previously described (24). DGGE fingerprint analysis using the 18S rRNA gene was performed as described by Kowalchuk et al. (17) with the primers NS1-GC and NS2 except that the primer annealing temperature of the thermal protocol was reduced to 50°C to improve fragment amplification. PCR amplicons were separated in a 7% polyacrylamide gel with a denaturing gradient of urea and formamide of 40% (top) and 60% (bottom), where 100% denaturation is considered to represent 7 M urea and 40% formamide. The electrophoresis was run at 110 V for 14 h at 58°C in a D-Code apparatus (Bio-Rad). The gel was stained in a 1× solution of SYBR green (Molecular Probes, Leiden, The Netherlands) for 30 min and its image captured in UV transillumination with a digital camera supported by a Gel Doc 2000 apparatus (Bio-Rad). Bands of interest were cut from the gel with a sterile scalpel; the DNA was extracted by incubating the gel fragments for 12 h in 100 μl of sterile distilled water at 37°C under agitation. A 10-μl volume of the solution was then used as a template with the same DGGE primers without the GC clamp and the same PCR conditions applied to the original stone DNA to reamplify the fragment. The obtained amplicons were then purified using a QIAquick PCR purification kit (QIAGEN, Milan, Italy) according to the manufacturer's instructions. Purified products were then sequenced using the NS1 primer, a DYEnamic ET terminator cycle sequencing kit (Pharmacia), and an ABI 310 automated sequencer (Applied Biosystems). The resulting sequences were compared with the sequence database at the National Center for Biotechnology Information using BLASTN facilities (1). Alignment

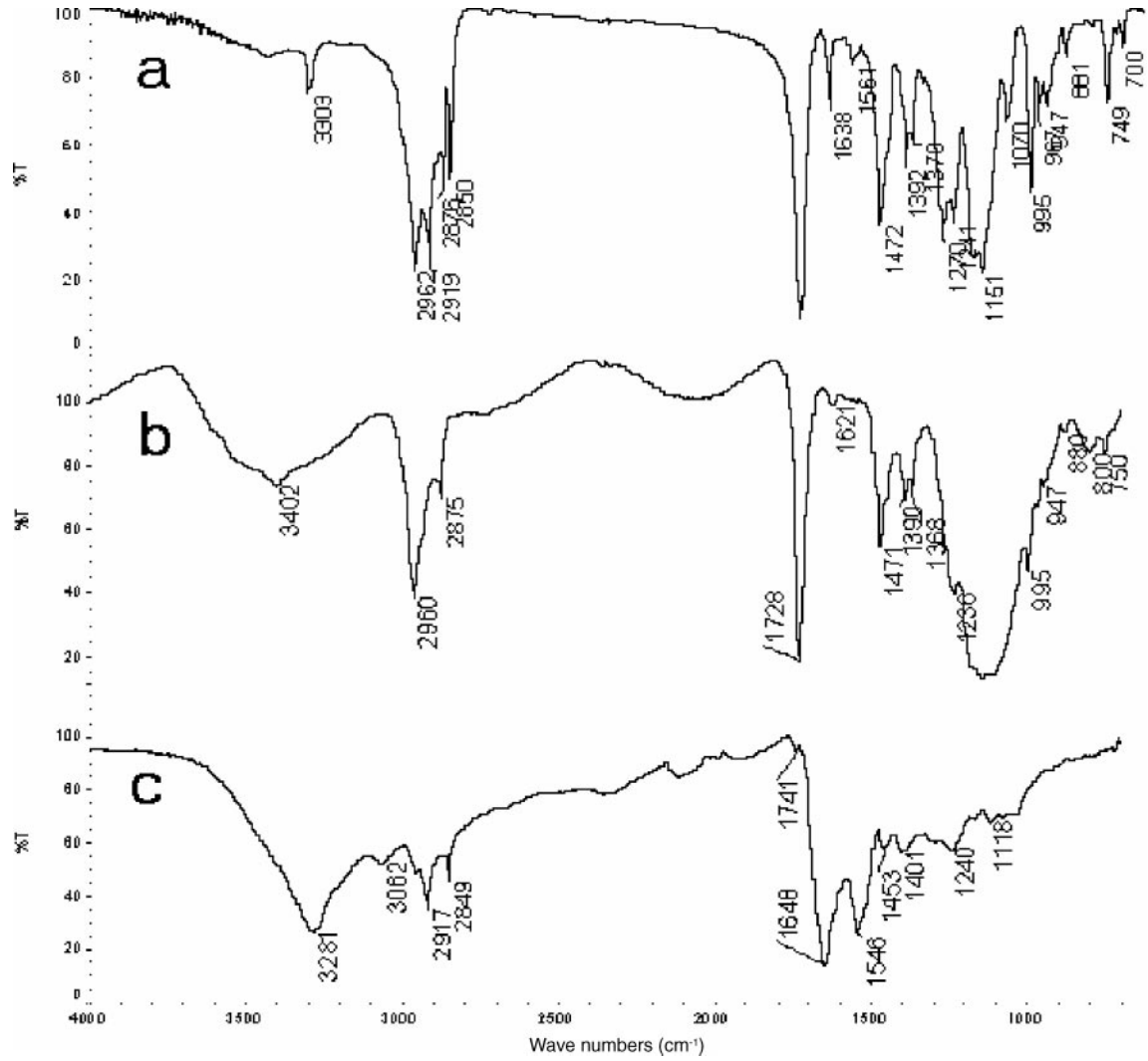

FIG. 2. FTIR spectrum of (a) a film of "Surface Clear Preserving Opaco" freshly cast on a sodium chloride window; (b) sample 14F033, representing marble microfragments with calcite crystals and residues of acrylic polymer seen as an orange thin film; and (c) sample 14F034, representing a biological patina on calcite crystals where the polymeric film is almost completely deteriorated.

with the corresponding 18S rRNA genes was performed by using software available at the Ribosomal Database Project website (9); the secondary structure was taken into account when this was done. Phylogenetic analyses were performed by using Jukes and Cantor distance estimation with a TREECON 1.3b package (37). A 50% majority rule bootstrap consensus tree (1,000 replicates) was generated. Gaps were treated as a fifth base.

Nucleotide sequence accession numbers. The nucleotide sequences of 18S rRNA genes were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the accession numbers AM236865 to AM236873.

RESULTS

The investigation of synthetic resins. The acrylic resin applied on the Milan cathedral facade in the 1970s was a formulated product called "Surface Clear Preserving Opaco" (supplied by ARD Raccanello, Padova, Italy); the resin is polyisobutylmethacrylate charged with an additive that gives a mat aspect to the final coating. Figure 1 shows the surface deterioration of sample 14F033, with an orange film (Fig. 1a), and sample 14F034, with a pink-beige noncontinuous patina (Fig. 1b). The orange film is an aged residue of the acrylic protective coating, still present in some areas, and the pink-beige patina with blackish biological growth is present in adjacent surface areas which were definitely treated in the 1970s with the same polymeric coating.

As FTIR is commonly used to identify synthetic polymeric products, this technique was also employed on our samples (Fig. 2). Figure 2 shows the freshly cast acrylic "Surface Clear Preserving Opaco" (Fig. 2a) and the FTIR spectra corresponding to samples 14F033 and 14F034 (see Fig. 2b and 2c, respectively). In Fig. 2, the peaks at 3,303, 2,919, 2,850, 1,638, 1,561, and 700 cm^{-1} should be ascribed to the additive. In the case of the orange thin film of the protective coating on 14F033, FTIR analysis identified the acrylic polymer together with a large amount of gypsum (see peaks at 1,621 and 1,147 cm^{-1}), as shown in Fig. 2b. In contrast, the acrylic polymer was no longer evident on fragment 14F034 showing the biological patina (Fig. 2c). Indeed, the 995 cm^{-1} peak, related to the isobutyl group,

TABLE 1. List of fungal taxa identified on the basis of the macroscopic features of colonies and the micromorphology of the reproductive structures detected on the 10 marble specimens of the Milan cathedral

Taxon or sample	Presence of growth on indicated sample[a]									
	21HO25	I4F033	14F035	14F034	I3E036	20S028	I0M023	10T02I	20I026	19O029
Alternaria spp.				×				×		
Aspergillus sp.				×						
Cladosporium spp.				×		×		×	×	
Epicoccum nigrum						×				
Pink yeast	×									
Black yeast	×	×	×	×	×					
Black microcolony			×	×			×		×	×
Mycelia sterilia					×			×		

[a] A cross indicates that fungal growth was present on the sample.

and the 2,962 and 1,392 cm^{-1} peaks, related to the methyl groups, are drastically reduced; at the same time, the 1,730 cm^{-1} absorption band related to the stretching vibration of carbonyl group is broadened toward higher frequencies. In addition, FTIR analysis of sample 14F034 revealed the presence of a peptidic bond (see the peaks at 1,648 and 1,546 cm^{-1}) that was ascribed to some proteinaceous material related to the fungal growth as previously reported (6).

Thus the biodeterioration pattern of the acrylic resin in the presence of microorganisms is definitely different from that obtained after environmental aging.

The phenotypic identification of fungi. The list of cultivable fungi identified on the basis of the macroscopic features of colonies and the micromorphology of the reproductive structures found on the marble is listed in Table 1. Dematiaceous species belonging to the genera *Alternaria*, *Cladosporium*, and *Epicoccum* were found together with species belonging to the genus *Aspergillus*. Black and pink yeast cells were also found in some samples. All the microorganisms isolated have been reported in literature as common stone taxa (26, 35). On each sample at least one organism that produces melanin was found. In all the samples, the presence of biological structures showing meristematic growth was also determined directly on the surface by using the adhesive tape procedure without any cultivation step.

DGGE and sequencing. Traditional microbiological techniques are not always useful in investigating multicellular and sporulating organisms, as not all fungal species can be easily isolated by the currently available methods and, in addition, slow-growing fungi are often overgrown by fast-growing ubiquitous species of minor ecological importance in culture (29). To better characterize the fungal microflora, a DGGE analysis coupled with partial sequencing of 18S rRNA gene fragments was performed. Although the primer annealing temperature was reduced to 50°C to better address the amplification of fungal DNA, successful amplification was possible for 6 samples out of 10. Even though the efficiencies of amplification differed among samples, nine bands were clearly visible and the corresponding sequences were determined (Table 2). Fungi belonging to *Talaromyces flavus*, *Glyphium elatum*, *Cenococcum geophilum*, *Eladia saccula*, and *Phoma herbarum* were identified. Similarities of the closest relatives found in BLASTN searches were between 96% and 100%. Less than 100% homology on the 18S rRNA gene is not sufficient to identify a fungal species. However, for our purposes, it is sufficient information to evaluate the presence of black fungi on the investigated samples. Bands F2 and F5 attributed to *Talaromyces flavus* and *Glyphium elatum* were dominant in DGGE patterns, and they could have originated from fungal species dominant in the population (data not shown). Three bands were attributed to algae belonging to *Trebouxia jamesii* of the order *Microthamniales*.

Immunostaining technique. In order to obtain rapid identification and localization of melanin-producing fungi with a method that is accurate and relatively easy to perform, the immunostaining technique was applied to the 10 stone specimens. The immunostaining procedure proved that the control (a sample from intact, freshly quarried Candoglia marble) was not fluorescent under the microscope whereas all 10 samples from the Milan cathedral showed fungal struc-

TABLE 2. Identification of partial 18S rRNA gene sequences isolated from DGGE profiles

Band	Closest relative	Accession no.	Taxon	% Similarity	Sequencing result for indicated sample[a]					
					21HO25	14F035	14F034	20S028	I0M023	10T02I
F1	*Trebouxia jamesii*	Z68700	*Chlorophyta*	98				×		××
F2	*Talaromyces flavus*	M83262	*Ascomycota*	96				×		××
F3	*Trebouxia jamesii*	Z68705	*Chlorophyta*	99	×			×	×	×
F4	*Trebouxia jamesii*	Z68700	*Chlorophyta*	99	×				×	×
F5	*Glyphium elatum*	AF346419	*Ascomycota*	100	××				×	×
F7	*Cenococcum geophilum*	L76615	*Ascomycota*	98				×		
F8	*Glyphium elatum*	AF346419	*Ascomycota*	100				×		
F14	*Eladia saccula*	AB031391	*Ascomycota*	98			××			
F15	*Phoma herbarum*	AY293775	*Ascomycota*	98		××				

[a] Lightface crosses indicate weak bands in DGGE profiles; bold crosses indicate DGGE bands sequenced; double crosses indicate bands of strong intensity.

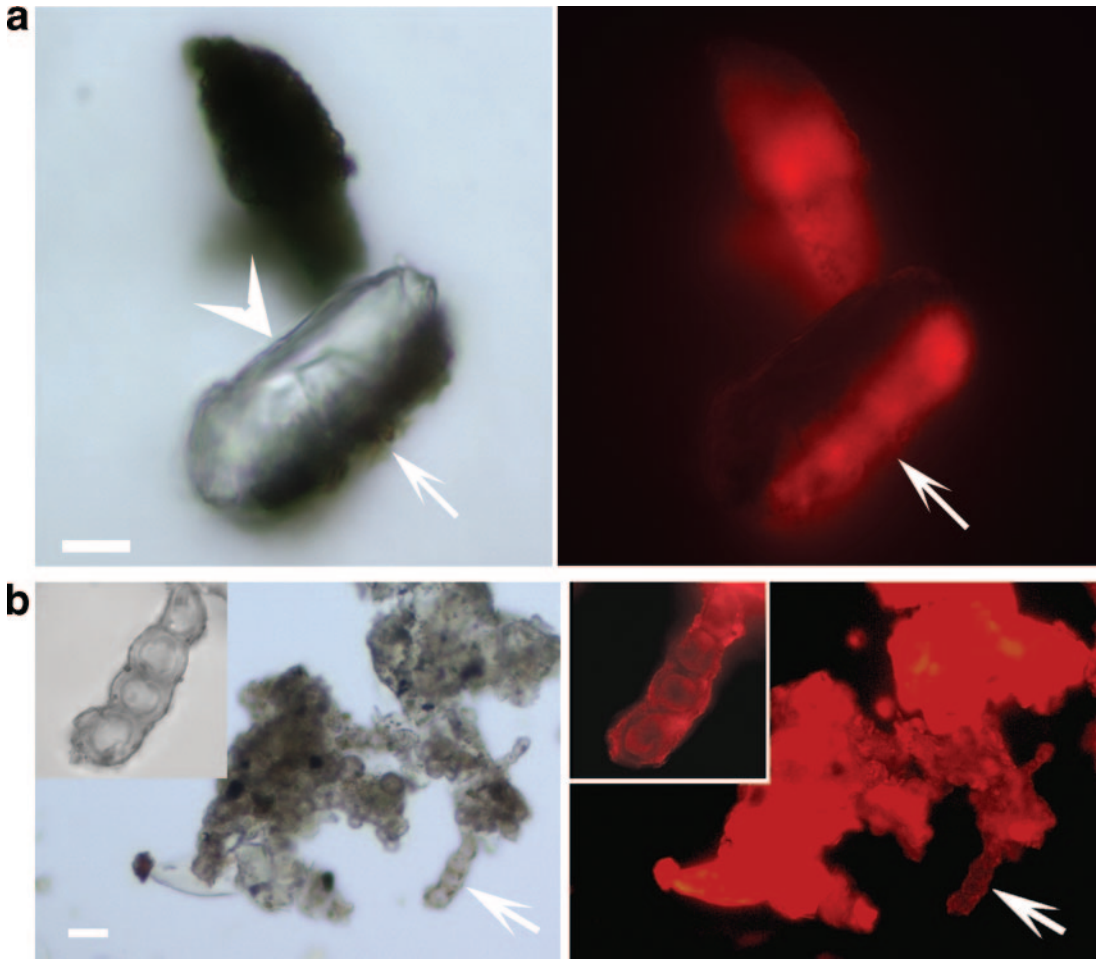

FIG. 3. (a) A chip of marble sample 19O029, with fungus coating part of the surface. The marble (arrowhead) did not fluoresce, whereas the fungal coat (arrow) fluoresced intensely. Scale bar, 20 μm. (b) A view (×20 magnification) of 13E036, with a magnified inset (×100). Scale bar, 10 μm.

tures labeled by melanin-binding antibody as shown in Fig. 3. As expected, the marble did not fluoresce whereas the fungal coat (indicated by an arrow) fluoresced intensely (Fig. 3a). In the magnified inset in Fig. 3b, the typical meristematic growth is clearly visible.

DISCUSSION

Many tests of acrylics, polymers commonly used in conservation treatments, have been carried out to evaluate their chemical and physical stability. These tests proved that these materials are generally a good choice for the consolidation and protection of stone. In contrast, few studies have been carried out on the evaluation of the susceptibility to biological attack of synthetic materials used in cultural heritage conservation (5–7). In recent years, black fungi have been recognized as the most conspicuous and probably the most damaging organisms attacking the surfaces of stone monuments (11). We report for the first time on the systematic occurrence of dematiaceous meristematic fungi on stone samples consolidated and protected with naturally aged acrylic resins. Numerous studies have dealt with the analysis of meristematic fungi on rocks and historical structures made of natural stone, but none has taken into account the importance of the presence of aged synthetic resins for this kind of microorganism. It is worth mentioning that fungal growth on synthetic polymers has been proven previously, even though freshly dried acrylic resins have seemed to be among the compounds least susceptible to fungal attack in laboratory conditions (5, 6). Synthetic resins on monuments show advanced chemical and physical degradation, such as yellowing and cracking, after about 30 years of environmental aging; this chemical and physical degradation is in turn likely to facilitate biological degradation. Under UV irradiation the main degradation pathway of acrylics is chain scission (18). The oligomers produced by UV irradiation are surely more easily attacked by fungi than the high-molecular-weight polymers from which they originated. In particular, photooxidation of poly-isobutylmethacrylate is quite efficient due to the branched isobutyl group of the polymer side chain (8). In this paper, a decay process of the coating, enhanced by biological growth, was proved from the recovery from the Milan cathedral marble surface of a material with proteinaceous features which forms a noncontinuous patina, replacing the polymeric film. Actually, FTIR spectra of samples collected

from adjacent areas indicate the presence of the partially decayed acrylic resin where microbial colonization is not noticeable and the presence of a proteinaceous material where the fungal growth was assessed.

A pioneer study on this topic was carried out by Pinna and Salvadori (22), who made optical and electron microscopic observations of meristematic fungal growth in cracks and fissures treated with acrylic resins on the facade of Tempio Malatestiano in Rimini (Italy). However, no further investigation beyond documenting the presence of meristematic fungi has been reported by the above-mentioned authors.

In our research, the combined used of light microscopy and cultural methods revealed in all samples the presence of meristematic fungi, including those belonging to *Alternaria*, *Cladosporium*, *Epicoccum*, and other genera showing the features of black fungi. In the case of meristematic fungi, the main drawbacks of culture are the length of time (at least 1 month) that is necessary for growth and their morphological plasticity, which greatly prevents direct microscopic identification. As a consequence, DNA-based methods have been successfully applied to studies of dematiaceous fungus colonization on different kinds of sample environments; these methods include restriction fragment length polymorphism analysis (3, 10, 30, 31), random amplified polymorphic DNA analysis (34), and partial or complete 18S rRNA gene sequencing (2, 16, 25). For this reason we employed sequencing of DGGE bands to detect fungi independently from cultivation. We could identify sequences which showed 100% 18S rRNA gene similarity with sequences of both *Glyphium elatum* and *Coniosporium* spp. (Table 2). *Coniosporium* spp. have been isolated from ancient marbles in Turkey (accession no. AJ972863; H. Sert and K. Sterflinger, unpublished data) and Greece (27). The biodeteriorative potential of this genus has been investigated in detail for building stones of historical monuments (28, 30, 36). The dematiaceous fungi of the genera *Phoma* and *Alternaria* identified in two biodeteriorated samples are among the most conspicuous and probably the most damaging organisms identified as attacking and even penetrating the surfaces of stone monuments (33, 38). A number of experimental studies have shown the ability of some ectomycorrhizal fungi such as *Cenococcum geophilum* to dissolve calcium-bearing minerals (4, 13) present in the marble as calcium carbonate and calcium sulfate or gypsum. The alga *Trebouxia jamesii* has been detected by DGGE among the predominant species in some samples, since the primer annealing temperature was decreased for fungal 18S rRNA gene amplification. However, the finding of *T. jamesii* is interesting, because it is known that *T. jamesii* forms a lichen in association with *Evernia mesomorpha*, another meristematic fungus (21).

DGGE results are important for providing information on the presence and genera of black fungi. From a conservation practice viewpoint, evaluation of the spatial distribution of these fungi is also of great value to prevent further damage. As a consequence, we applied immunofluorescence techniques that confirmed the presence of melanin-producing fungi and in addition showed their locations and the presence of meristematic growth.

The results presented in this paper clearly indicate that while acrylics are stable and play the role of protectives and consolidants resisting damage from physical and chemical agents, this does not necessary mean that in the long term they are the best choice for conservation. In conclusion, we have demonstrated that stones protected by aged synthetic acrylics can be heavily colonized by black fungi; thus, acrylics, instead of preventing damage, could accelerate the decay process.

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