Biocide efficacy and consolidant effect on the mycoflora of historical stuccos in indoor environment

BIOCIDE EFFICACY AND CONSOLIDANT EFFECT ON THE MYCOFLORA OF HISTORICAL STUCCOS IN INDOOR ENVIRONMENT

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Abstract
Investigations are needed to address and optimize the use of biocides and restoration materials with reference to the fungal diversity which often characterizes cultural heritage surfaces.
This work aimed to examine the diversity of fungi responsible of aesthetic decay on the stuccos of the vault of a religious building in Torino (NW-Italy), and to evaluate the sensitivity of the detected set of species to widely used biocidal products (benzalkonium chloride, isothiazolinones, sulphamide derivatives) and their application solvents. The effect of four commercial consolidants on their potential (re-)colonization following restoration interventions was also assessed.
Four different deterioration phenomena were related to the occurrence of *Chaetomium murale*, *Stachybotrys chartarum*, *Penicillium chrysogenum* and *Sarocladium kiliense*, respectively. Surface receptivity to the different species -identified on morphological and molecular bases- was related to slightly different thermo-hygrometric conditions, the distribution of painted surfaces, salts, and local remnants of cellulose poultice used in past restoration interventions.
Specific sensitivity to two solvents and ten different biocide treatments was evaluated in terms of inhibition of mycelial growth from transplanted inocula at 9 (T1) and 27 (T2) days after the incubation. The different solvents and biocide products differently affected growth and/or pigmentation of the four species. Only 40% of the ten performed biocide treatments determined the growth inhibition of all the examined species at both T1 and T2. In other cases, inhibition observed at T1 for *C. murorum*, *S. chartharum* and *S. kiliense*, was followed at T2 by the colonization of inhibition zones.
The mycelial growth on an oligotrophic culture medium poured with four commercial consolidants was evaluated one year after the incubation. All the species displayed some growth from the inocula, with a scarse biomass being only observed in negative (water) controls and upon one consolidant treatment. The three other products strongly supported a higher growth of at least two of the examined fungal species with respect to negative controls.

In conclusion, different sensitivity of each fungal species for most biocidal treatments and stimulation by consolidants indicate that species-specific assays of products are necessary to calibrate and optimize restoration works. In particular, effective inhibition of fungi by biocides, and potential stimulation by consolidants, should be evaluated after several weeks and months, respectively, since short-term monitoring may be misleading.

Highlights

Four fungal species cause deterioration on the stuccoed vault of a heritage building.
Morphological identifications are improved by molecular analyses on fungal isolates.
Species-specific sensitivity is recognizable for most of assayed biocide treatments.
Most of assayed consolidants stimulate the growth of the detected species.
Species-specific assays are necessary to address the use of restoration products.

Keywords
cultural heritage, deterioration, fungi, mycelial growth, restoration products, species-specific sensitivity

1. Research aims

A scientific research involving art scientists and professional restorers was carried out to characterize the fungal colonization on stuccos of the vault of a religious building and to define suitable materials for restoration. The study aimed to analyze and compare the effects of several commercial biocides and consolidants on different microfungi of interest for cultural heritage conservation. The importance of the identification of biodeterioration agents and of ad-hoc tests on the efficacy/influence of commercial restoration products to define restoration strategies on mineral materials was examined.

2. Introduction

Fungi are agents of physical and chemical deterioration processes on both organic and inorganic cultural heritage materials [1-3]. The porosity of artificial stone materials (sensu [4]), including mortars, plasters, stuccos and wall-paintings, favours the growth of mycelia: hyphae often determine micro-disaggregation/decohesion and detachment of surface layers of the substrate, and release metabolites with acidic and chelating functions, driving mineral leaching, or pigments, determining aesthetic decay [3-5]. High relative humidity and/or capillarity or condensation phenomena, together with the occurrence of organic materials (glues, binders) in the inorganic matrix, particularly support conspicuous fungal colonization [3, 6, 7]. Fungal colonization is thus a common phenomenon which restorers have to face on outdoor surfaces [8], but also in several indoor environments, as churches and historical buildings, where microclimate conditions can be hardly controlled [4, 9, 10].

Although the use of physical methods has been recently encouraged [11], biocides are still the weapon most widely used by restorers to control biodeteriogens on stonework [12-14]. Some researchers already showed that different fungal species may display different sensitivity to different biocide products [e.g. 15]. However, as in the case of lichen-forming fungi [16], specific patterns of sensitivity have been still poorly characterized, and with reference to a limited set of case studies in terms of fungal species and biocides, suggesting the need for further investigations. Accordingly, restorers mostly use biocides without taking into consideration the potential diversity of fungal communities on different mineral materials; moreover, many commercial products are distributed as generally effective against fungi at a unique recommended concentration, but species on which they were assayed are not clarified [e.g. 17]. With regard to fungal communities on stuccos, knowledge on the efficacy of commonly used biocidal products against different fungal species has been scanty produced, although experimental trials on alternative control strategies, using plant extracts and/or nanoparticles, have been run [18, 19].

Restorers also use many other products to control the physical and chemical decay of mineral materials, including consolidants and water-repellents. Some studies highlighted the potential interference of such restoration materials and biocides, reciprocally affecting their effectiveness [20, 21]. Moreover, re-colonization processes were correlated with the usage of restoration materials as organic nutrients [22]. Also in this case, however, the effects determined on different fungal species have been rarely evaluated.

This work aimed to examine the diversity of fungi responsible of aesthetic decay on the stuccos of the vault of a religious building in Torino (NW-Italy), and to evaluate the sensitivity of the detected set of species to widely used biocidal products (benzalkonium chloride, isothiazolinones, sulphamide derivatives) and their application solvents. The effect of four commercial consolidants on their potential (re-)colonization following restoration interventions was also assessed. Fungal colonization was characterized by combining *in situ* microscopical observations, culturing and molecular analyses. The effects of biocides and consolidants were assayed *in vitro*. The research did not aim to rank the performance of the different products, as each product was not tested in all

possible concentrations, application methods and treatment cycles. Conversely, the objective was to verify if different species showed similar or different responses to each kind of restoration product.

3. Materials and methods

3.1. Investigation site and microclimatic analysis

The sacristy of the Church of the Fraternity of Holy Trinity in Torino (Italy), dating back to the XVIII century, displays a NW-SE oriented rectangular shape (5.9 x 9.2 m) and a composite vault (vault impost 5.0 m high; vault height: 2.40 m) with polycentric arches (Fig. 1A). These are decorated with stuccos which contour a central wall painting (Melchizedek's sacrifice). The East side of the vault was long subjected to a water seepage condition (definitely controlled/eliminated in 2008), which determined efflorescence and sub-efflorescence phenomena, yielding decohesion processes and aesthetic damage. Materials used in past restoration, including cellulose pulp, also possibly supported the recent decay.
The thermo-hygrometric conditions of the vault were monitored from March to June 2012 following the Italian Standard UNI10829, i.e. two times per day (in the morning and in the evening), four days per week. Measures of the surface temperature of the vault were acquired along a grid of 15 points using a IR-thermometer Testo 845. Air temperature and relative humidity were measured in correspondence of each point of the grid at 5.5 m of height from the sacristy floor using Testo 175-H2 data-loggers.

3.2 Sampling, isolation and identification of microfungi

Fungal colonization was surveyed on the whole surface of the vault with the naked eye and using a SCALAR Digital Microscope DG-3, and mapped using the CAD software.

Samples were taken from surface points displaying macroscopically different deterioration phenomena possibly related to fungal growth (3 samples per each of four different deterioration types), using sterile lancets or cotton swabs. The samples were observed using a light microscope Olympus CX40 and a preliminary identification of fungi associated to the different deterioration types was run on the basis of macro- and microscopic criteria with the help of pertinent keys and literature [23, 34].

The samples were also inoculated on MEA and on the nutrient-poor BG11 [25] to simulate the oligotrophic conditions of mineral substrates as stuccos and wall paintings [see 26, 27]. The plates were incubated at 16°C in the dark. After 2-6 weeks, the obtained cultures were examined under light microscopy and/or analyzed with regard to the ITS region (ITS1-5.8S-ITS2) of rDNA to confirm/improve their identification at the species level. DNA extraction was performed using the DNeasy Plant Minikit (Qiagen). PCRs were performed in 50 µl volume containing 2 µl of DNA template, 33 µl of distilled sterile water, 5 µl buffer, 4 µl of 2.5 mM dNTPs, 2 µl each of ITS1F-ITS4 primers [28] at a concentration of 10 pmol/µl and 2 µl of DNA Polymerase (Red Taq High Fidelity, 1U/µl). The PCR products and the low mass ladder marker were separated by electrophoresis in 1.5% agarose gels containing ethidium bromide for 30' at 99 V in the tris-acetate EDTA (TAE) buffer and photographed using VersaDocTM Model 4000 Imaging System (Bio-Rad Laboratories, UK). The PCR products were purified using QiaQuick PCR purification Kit (Q-BIOgene, Heidelberg) and directly sequenced using the same primers by Beckman Coulter Genomics (UK). Sequences of the strains were deposited in the National Center for Biotechnology Information database (accession numbers: MG280779-MG280781). The reverse and forward sequences were aligned and assembled using the Staden Package software. The consensus sequences obtained were compared with those of the database of the National Centre for Biotechnology Information (NCBI), Bethesda, Md. (BLAST-search, http://www.ncbi.nlm.nih.gov/BLAST/ [29]).

Phylogenetic analyses were conducted in MEGA6 [30] by means of the Maximum Likelihood (ML) method based on the General Time Reversible model [31] to improve the species-level identification of two fungal isolates assigned to the genera *Chaetomium* and *Acremonium* (*Sarocladium*). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. The sequences used to infer phylogenetic analysis were chosen on the basis of BLASTn results [29] and according to the outcomes of recent phylogenetic studies [32, 33].

3.3 Biocide and consolidant treatments

One mm diameter discs of mycelium were cut from the edge of the colonies of four different fungi, isolated on MEA from the sacristy vault, and inoculated on new plastic Petri plates containing the same culture medium or BG11.

Specific sensitivity to biocides was evaluated in terms of inhibition of mycelial growth from transplanted inocula [34]. Fifty microlitres of six commercial biocides (Table 1) were poured with a pipette Transferpette® digital 20-200 µl (Brand, Germany) directly on each inoculum on MEA, the products variously spreading on the culture medium depending on their nature (Fig. S1). MEA provides fungi with enough nutrients to stimulate their metabolic activity [35] and rapidly clarify the biocide effects on their growth. All the biocides were prepared according to the producer's instructions in terms of final concentration and usable solvents (more than one solvent for the same product was tested when suggested). Products including benzalkonium chloride (BZC) were diluted to assay the same concentration of active principle (0.25%). Products including isothiazolinones (IT) and sulphamide derivatives (SD) were prepared at the recommended concentration. The different solvents used in the biocide preparation (deionized water, isopropanol, white spirit) were used as negative controls. Six replicates were analyzed for each case study. Growth measurements on the mycelia were carried out 9 (T1) and 27 (T2) days after the incubation (T0) at 15°C. Colony areas were measured by image analysis using AutoCAD software (Autodesk, San Rafael, CA) (Fig. S1). The results were statistically analyzed by means of ANOVA with post hoc Tukey test using Systat 10.2.

Fifty microlitres of four commercial consolidants (Table 2), prepared according to the producer's instruction, were poured directly on each inoculum on the oligotrophic culture medium BG11 [25], representative of the oligotrophic conditions of the stucco surface. The colony morphology upon the different treatments was described one year after the incubation at 15°C. The effect of consolidant applications was semiquantitatively evaluated on the basis of the mycelial growth/biomass observed on the surface of culture medium.

Table 1 - Biocides and solvents applied in the experiments.

Compound categories	Product	Producer	Active principle	Solvent	Code	Recommended concentration	Used concentration
Solvents	Deionized water	-	-	-	H_2O	-	-
	Isopropanol	Sinopia S.a.S. (Torino, Italy)	-	-	i-PrOH	-	100%
	White Spirit	Sinopia S.a.S. (Torino, Italy)	-	-	WS	-	100%
Benzalkonium chloride	Benzalkonium chloride >95% (T)	Fluka (Buchs, Switzerland)	Benzalkonium chloride (pure salt) [dodecyl dimethyl benzyl ammonium chloride]	Water	BZC1/H_2O	-	0.25%
				Water:isopropanol (50:50)	BZC1/ i-PrOH	-	0.25%
	Benzalconio cloruro	Sinopia S.a.S. (Torino, Italy)	Benzalkonium chloride (50% in water) [dodecyl dimethyl benzyl ammonium chloride]	Water	BZC2/H_2O	0.3-2%	0.5%
	DesNovo	Bresciani S.r.l. (Milano, Italy)	Benzalkonium chloride (10% in water) [dodecyl dimethyl benzyl ammonium chloride]	Water	BZC3/H_2O	0.5-10%	2.50%
Isothiazolinones	Biotin T	C.T.S. S.r.l. (Altavilla Vicentina, Italy)	N-octyl-isothiazolinone + didecyl-dimethyl ammonium chloride	Water	BT/H_2O	1-3%	2%
	Biotin R	C.T.S. S.r.l. (Altavilla Vicentina, Italy)	N-octyl-isothiazolinone + 3-iodoprop-2-ynyl N-butylcarbamate	Isopropanol	BR/ i-PrOH	3-5%	4%
				White Spirit	BR/WS	3-5%	4%
	Sinoctan PS	Sinopia S.a.S. (Torino, Italy)	4,5 Dichloro-2-octyl-isothiazolinone + Iodopropynyl-butyl -carbamate	White Spirit	SN/WS	3-5%	4%
Sulphamide derivatives	Lichenicida 264	Bresciani S.r.l. (Milano, Italy)	n,n-dimethyl-n'-phenyl-n'-fluoro-dichloromethylthio-sulphamide	Isopropanol	LI/i-PrOH	1-2%	1%
				White Spirit	LI/WS		1%

Table 2 - Consolidants applied in the experiments.

Product	Producer	Substance	Dispersion mediumr	Code
Ludox ® P-X30	Grace (MD, USA)	Colloidal Silica (av. particle size 15 nm)	Water (alkaline aqueous colloidal dispersion)	Ludox
Estel ® 1000	C.T.S. S.r.l. (Altavilla Vicentina, Italy)	Tetraethyl orthosilicate (particle size 30 nm)	White Spirit	Estel
MICROACRIL CV40	Bresciani S.r.l. (Milano, Italy)	Butyl acrylate, methyl methacrylate copolymer (particle size 40 nm)	Aqueous microemulsion of acrylic polymer	Microacril
Dispersion K52	Kremer Pigments (NY, USA)	Acrylic acid ester copolymer (particle size 20-40 nm))	Aqueous dispersion of acrylic polymer	K52

4 Results

4.1 Fungal colonization and vault microclimate

The survey of the stuccoed vault revealed four different deterioration phenomena related to the occurrence of filamentous fungi: (a) (sub-)millimetric black dots, (b) millimetric to centimetric black stains; (c) submillimetric greenish dots, also observed on the wall painting, and (d) whitish decimetric patinas (Figs. 1B and 2).
Black dots corresponded to globose, hairy perithecia of a *Chaetomium* species (Figs. 2A-B). Mycelia isolated from the perithecia grew diffusely on MEA, yielding colonies which were whitish and dark pigmented on the front and reverse sides of culture medium, respectively. The best blast hits were GeneBank sequences of *Thielavia* spp. and *Chaetomium* spp. (99% of sequence identity for both genera). The phylogenetic analysis indicated that the obtained ITS sequence cluster into the *C. murorum* clade (Fig. S2).
Dematiaceous compact mycelia with characteristic conidiophores, assigned to *Stachybotrys chartarum* (Ehrenb.) S. Hughes, were responsible for the black stains (Figs. 2C-D). On MEA, similar compact colonies were isolated, which were dematiaceous on both the front and reverse side of the culture medium.
Greenish dots corresponded to fertile colonies of *Penicillium chrysogenum* Thom. (Figs. 2E-F), locally associated with *Aspergillus* sp. On MEA, isolated colonies were similarly greenish on the front side, whitish to cream-coloured on the reverse. The ITS sequence of this isolate was compared with those present in the database of the Westerdijk Fungal Biodiversity Institute (http://www.westerdijkinstitute.nl) and best blast hits were with *Penicillium chrysogenum* sequences (99.52% similarity with the ITS sequence from CBS 132205).
Whitish patinas were related to periodic outbreaks of white tuft mycelium of an *Acremonium* species (Fig. 2G-H). On MEA, compact whitish colonies, with a cream-coloured reverse, bearing conidiophores, grew on the surface of the culture medium. Best blast hits included GeneBank sequences of *Sarocladium kiliense* (Grütz) Summerb. (formerly *Acremonium kiliense* Grütz; e.g. LT615283:100% similarity) and *Sarocladium strictum* (W. Gams) Summerb. (formerly *Acremonium strictum* W. Gams; e.g. KP082947: 100% similarity). The ITS sequence clustered into the *S. kiliense* clade in the ML tree (Fig. S3).
Mycelia and reproductive structures of *Chaetomium murorum*, *Stachybotrys chartarum*, and *Sarocladium kiliense* were mostly localized in the north-eastern corner of the vault (Fig. 1B), which had been affected in the past by water seepage, exhibited diffuse salt efflorescence and featured slightly lower surface temperatures than the opposite south-eastern corner (Fig. 3), where the colonies of *Penicillium chrysogenum* were instead observed. Air temperature and relative humidity measured in representative mornings from April to June ranged between 12 and 25°C and between 50 and 70%, respectively. Remnants of cellulose poultice used in past restoration interventions were observed where *C. murorum* and *S. chartarum* occurred.

Fig. 1 – Vault of the Church of the Fraternity of Holy Trinity in Torino (Italy). A. Stucco decorations contour the central wall painting of the Melchizedek's sacrifice. B. Distribution of deterioration phenomena related to fungal colonization: black dots by *Chaetomium murorum* (black dots), black stains by *Stachybotrys chartarum* (black spots), green stains by *Penicillium chrysogenum* (green/grey spots), whitish patinas by *Sarocladium kiliense* (blue/grey striped area).

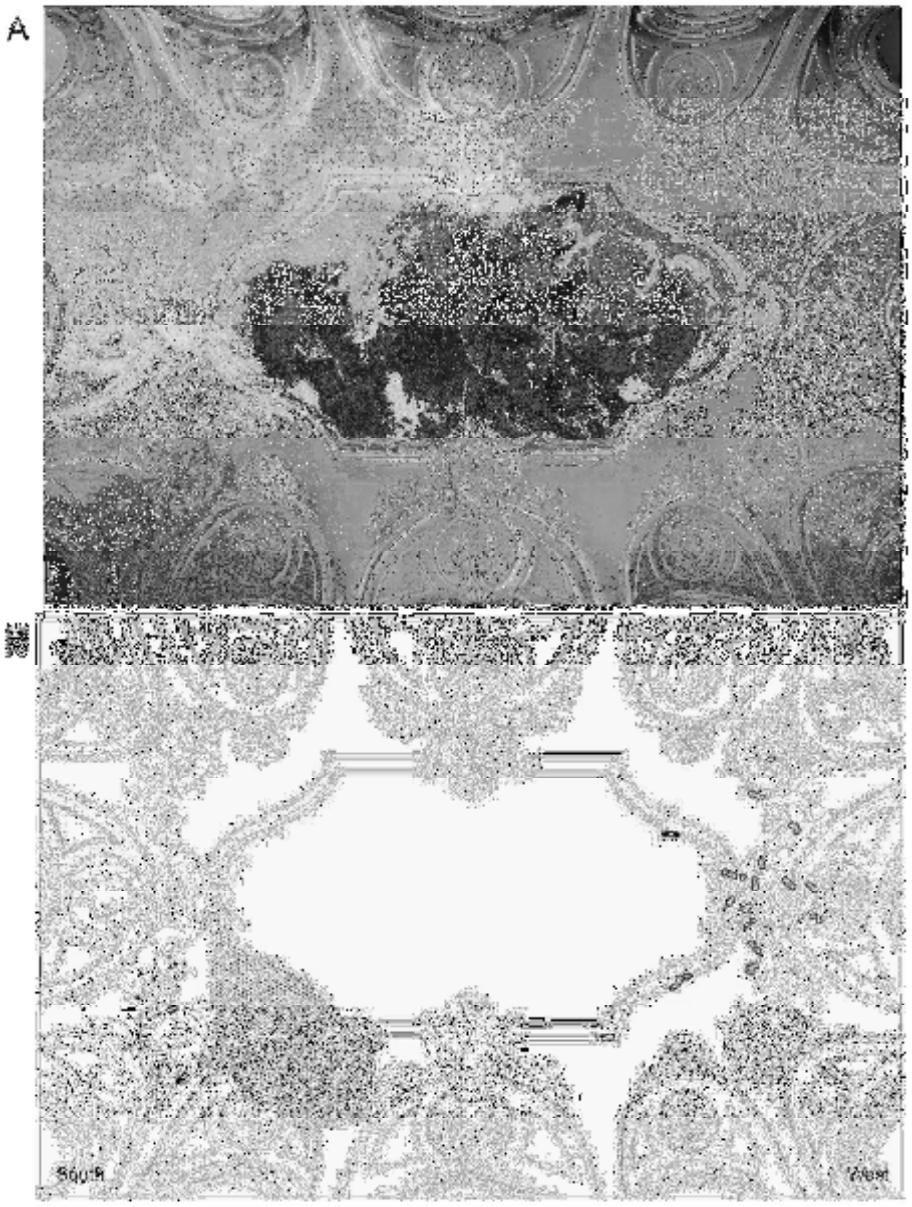


Fig. 2 – Deterioration phenomena of the stuccoed vault surface related to fungal colonization (A, C, E, G) and microscopic details of causing species (B, D, F, H): A-B, black dots by *Chaetomium murorum*; C-D, black stains by *Stachybotrys chartarum*, E-F, green stains by *Penicillium chrysogenum*.; G-H, whitish patinas by *Sarocladium kiliense*. Scale bars: 20 µm (B, D, F), 500 µm (H). Yellow square in A, C, E, G: 1.2 × 1.2 cm.

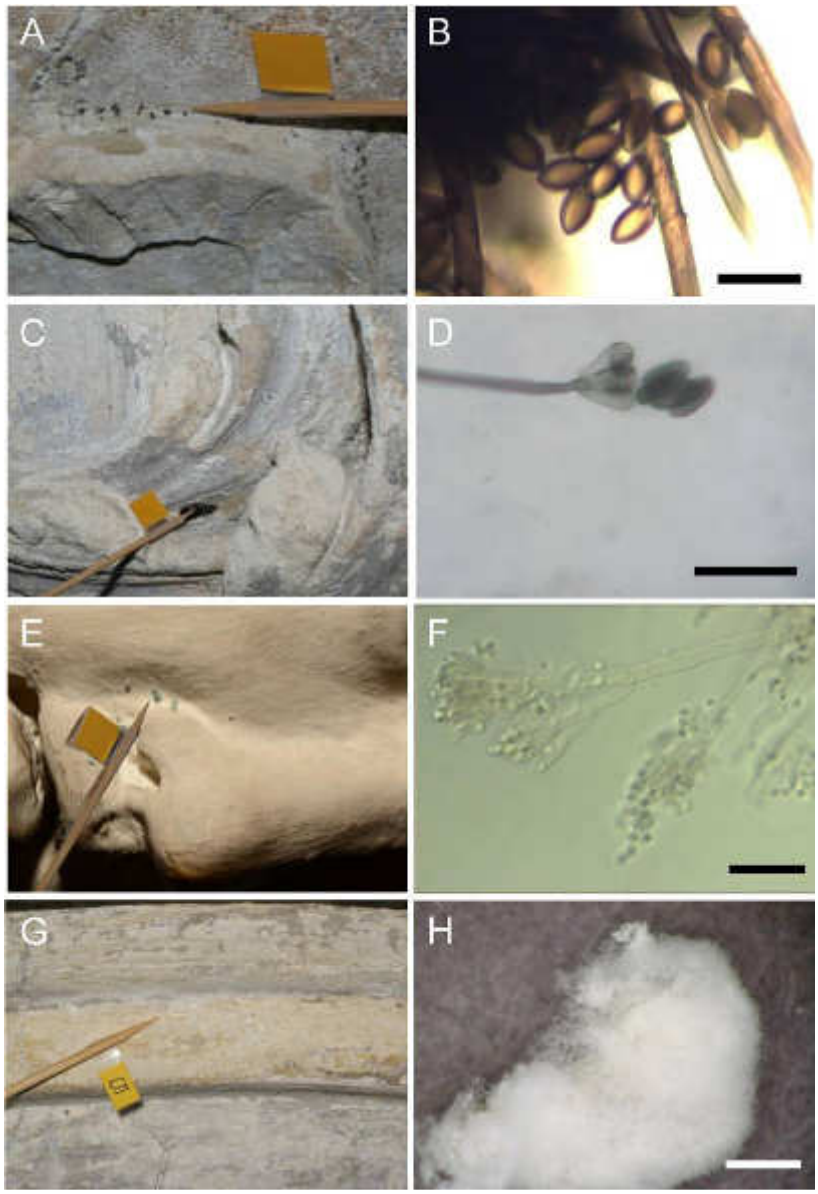
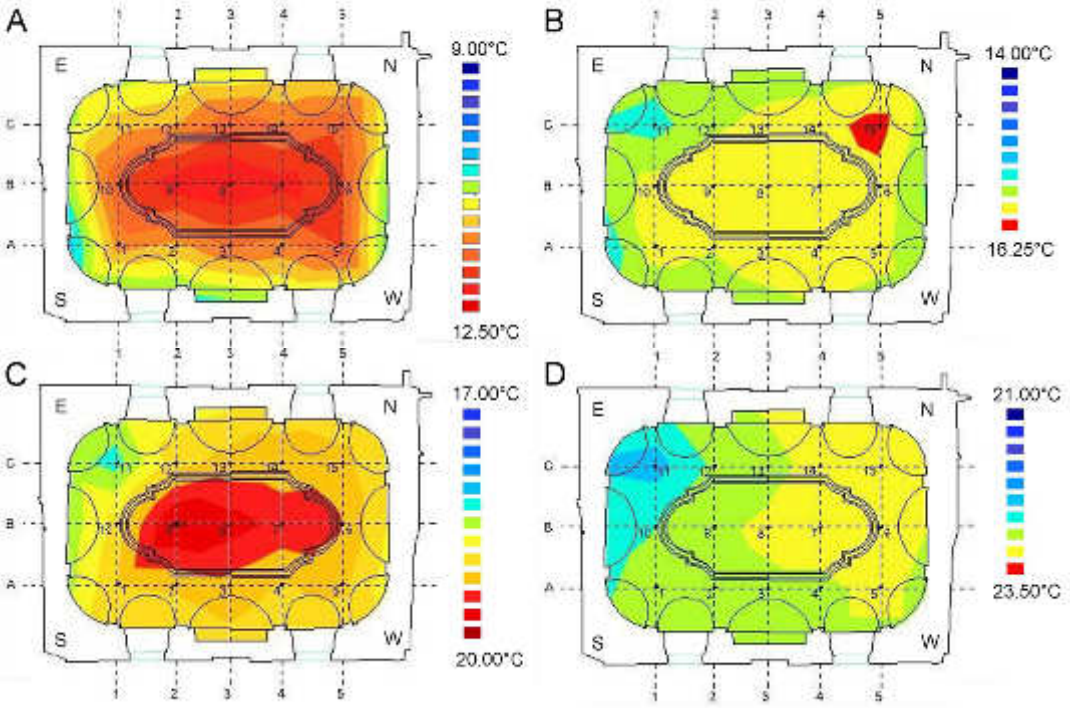


Fig. 3 – Temperature gradients at the vault surface measured in mornings representative of the microclimate conditions monitored in March (A), April (B), May (C) and June (D).



4.2 Biocide efficacy

The different solvents and biocide products differently affected the growth and/or the pigmentation of the four fungal species isolated from the sacristy vault (Fig. 4; Table 3).
When treated with deionized water only, *C. murorum* and *S. chartarum* showed similar areal growth at both T1 and T2, while the initial growth (T1) of *S. kiliense* was slower, as much as the final growth (T2) of *P. chrysogenum* (Fig. 4). Organic solvents alone did not determine a significant modification of the mycelial growth with respect to water controls (with the exception of a growth reduction of 55% observed for *S. kiliense* upon the treatment with isopropanol). However, the reverse side of *C. murorum* colonies appeared pinkish rather than dematiaceous, while the reverse side of *P. chrysogenum* colonies appeared darker.
Only 40% of the ten performed biocide treatments determined the same effect, i.e. growth inhibition, on all the examined species (Table 3). BZC preparations in water, including the pure compound and two commercial products, only inhibited the growth of *S. kiliense*. The growth of *S. chartarum* and *C. murorum* was only observed at the T2 timepoint, and in the case of the former species was significantly reduced (from -65 to -85%), while the mycelia of the latter species generally lost the dematiaceous appearance of their reverse side, appearing whitish-pinkish. The areal growth of *P. chrysogenum* at T1 was even higher than in water controls, but did not show further increases at T2. BZC prepared with isopropanol did not inhibit any of the tested species, while the other biocides prepared in isopropanol, including the isothiazolinone-containing BR and the sulfamide-derivative LI, inhibited the growth of all species. BR prepared in WS and the isothiazolinone-containing BT prepared in deionized water also inhibited all species. By contrast, the SN prepared in WS, after an initial inhibition observed at T1, significantly, but only partially (from -45% to -75%) reduced the growth observed at T2 for *C. murorum* (-75%), *S. chartarum* (-60%) and *S. kiliense* (-45%). It was instead effective against *P. chrysogenum*. Differently, LI prepared in WS inhibited *C. murorum* and *S. chartarum*, while some growth was observed at T2 for *P. chrysogenum* and *S. kiliense*. Further observations performed after three further weeks for these two species confirmed the viability of colonies for which some growth was detected between T1 and T2 (data not shown).

4.3 Consolidant effect

All the species displayed some mycelial growth from inocula on the BG11 medium, poured with deionized water only (negative controls) or covered with consolidants. However, a scarse fungal biomass was observed in all the water control assays for *C. murorum*, *S. chartarum* and *S. kiliense*, while *P. chrysogenum* only better developed on the oligotrophic medium. A similar scarse growth, and even a lower growth in the case of *P. chrysogenum*, was observed upon the treatment with K52. Ludox P X-30 did not significantly affect the biomass development of *S. chartarum* and *S. kiliense* , but it strongly supported a higher growth of *C. murorum* and *P. chrysogenum*. The other two commercial consolidants determined a higher biomass development for all the four species with respect to the negative controls (Table 4).

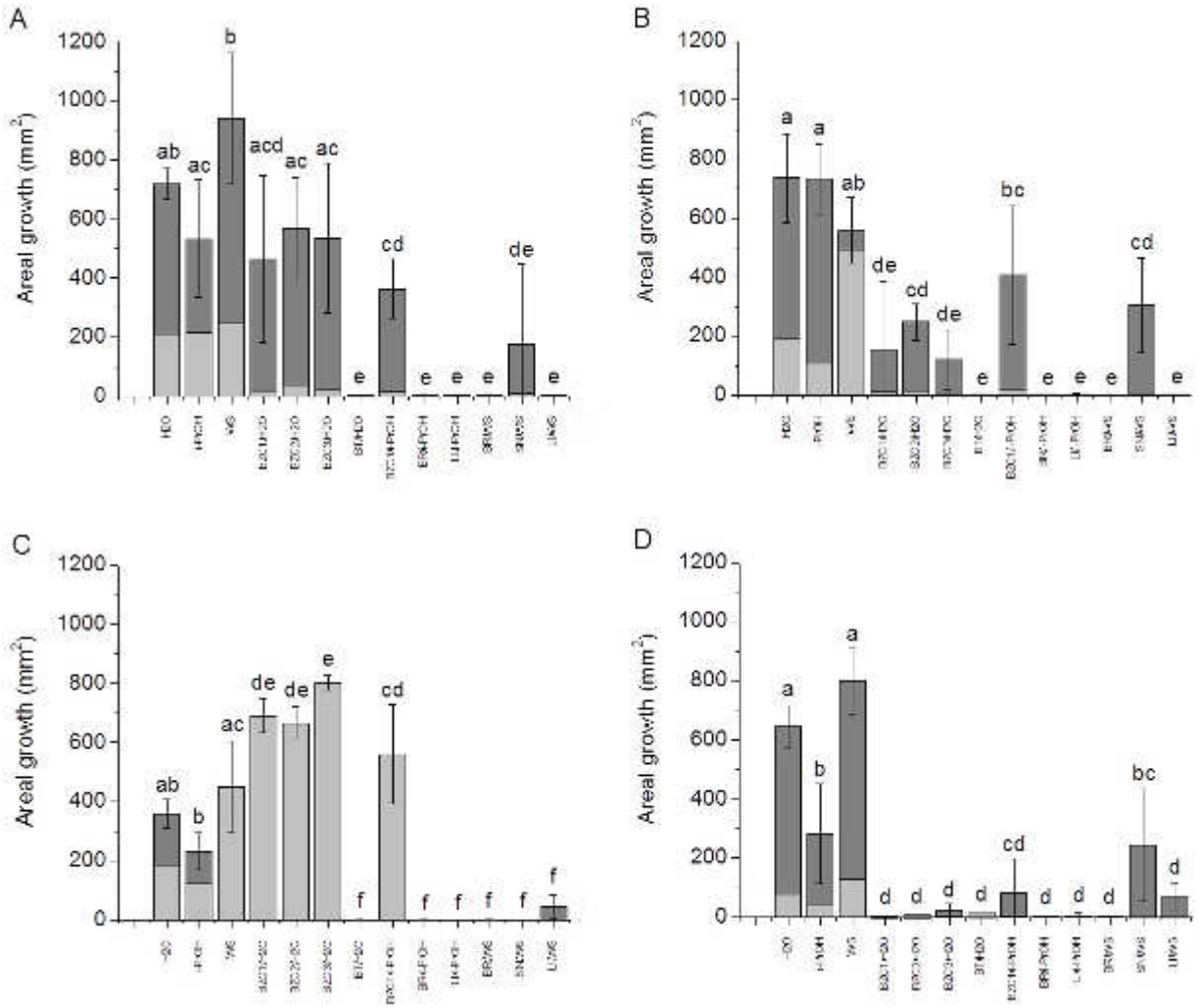
Table 3 - Synoptic comparison of biocide (and organic solvent) efficacy against the fungi isolated from the stuccoed vault with respect to negative controls (H_2O): =, no significant difference in areal growth at T2; +, significant higher growth; -, significant lower growth, but no inhibition; ┼, growth inhibition. Products which determine the same effect on all the fungi are marked in bold.

Code	*Chaetomium s.l.*	*S. chartarum*	*Penicillium sp.*	*Acremonium s.l.*
i-PrOH	=	=	=	-
WS	+	=	=	=
BZC1/H_2O	=	-	+	┼
BZC1/ i-PrOH	-	-	+	-
BZC2/H_2O	=	-	+	┼
BZC3/H_2O	=	-	+	┼
BT/H_2O	┼	┼	┼	┼
BR/ i-PrOH	┼	┼	┼	┼
BR/WS	┼	┼	┼	┼
SN/WS	-	-	┼	-
LI/i-PrOH	┼	┼	┼	┼
LI/WS	┼	┼	-	-

Table 4. Semiquantitative evaluation of the mycelial growth observed for *Chaetomium murorum*, *Stachybotrys chartarum*, *Penicillium chrysogenum* and *Sarocladium kiliense* isolated from the vault and inoculated for one year on the oligotrophic BG11 culture medium on which deionized water (control) and four commercial consolidants were poured. Mycelial biomass: scarse (+), well developed (++), abundant (+++), very abundant (++++) (representative images are reported in Fig. S4). Details on consolidant products are reported in Table 2.

Compound	*C. murorum*	*S. chartarum*	*P. chrysogenum*	*S. kiliense*
H_2O	+	+	++	+
Ludox	+++	+	+++	+
Estel	+++	+++	++++	+++
Microacril	++	++	++	++
K 52	+	+	+	+

Fig. 4 - Effect of biocides and solvents on the areal growth of (A) *Chaetomium murorum*, (B) *Stachybotrys chartarum*, (C) *Penicillium chrysogenum*, and (D) *Sarocladium kiliense*, isolated from the stuccoed vault of the Church of the Fraternity of Holy Trinity in Torino (Italy). Columns indicate areal growth at T1 (av.; light grey sector) and at T2 (av. ± SD; dark grey sector. For each species, columns which do not share at least one letter are statistically different at T2 (ANOVA, Tukey's test, $p<0.05$). Biocide and solvent abbreviations are listed in Table 1.



5 Discussion

Mild temperature and high relative humidity (>50%) measured at the surface, and in proximity, of the stuccoed vault provided optimal conditions for the growth of mesophilic fungi, as frequently reported for indoor environments of religious buildings in temperate areas, where the microclimate conditions are often uncontrolled [4, 36]. However, conducive microclimate conditions shall go along sufficient nutrient availability to support the growth of fungi or other heterotrophic microorganisms on mineral materials, as wall paintings or stuccos [6, 37, with refs. therein]. Remarkably, two out of the four observed bio-deterioration phenomena were related to the vault colonization by cellulolytic fungi, *Chaetomium murorum* and *Stachybotrys chartarum* [38]. The growth of such species is most likely unrelated to the oligotrophic stucco surface or to external organic depositions, but rather dependent on a modified receptivity of the substrate due to past restoration interventions (tertiary receptivity, sensu [39]), which left remnants of cellulose poultice available for fungal nutrition. *Acremonium* species are known for rapid growth and biomass production, with consequent triggering for human infections by pathogenic strains [e.g. 40], but also troubles for the conservation of the indoor stone cultural heritage, as rock paintings in caves and religious buildings [41 with refs. therein, 42]. Species of the cosmopolitan genus *Sarocladium* (formerly, *Acremonium*), such as *S. kiliense,* were shown to be able to degrade a wide range of complex organic substances, including dyes [43]. *Penicillium* species are among the most frequent species reported on artificial stone materials, such as frescoes, because of their high resistance of high salt concentrations [15, 38]. Some authors indicate *Penicilium* spp. as the major deteriogens of painted surfaces in temperate climates [44]. Accordingly, we observed *P. chrysogenum* on stuccos, but also on painted surfaces, which may particularly support its occurrence. Notably, *P. chrysogenum* was detected on the slightly drier side of the surveyed vault, less affected by salt efflorescence than the opposite side, where the other three species were found. In the examined case, however, the past water damage did not generally increase fungal colonization, in contrast with other reports [e.g. 45 with refs. therein].

Different nutritional requirements of the four species could account for their different sensitivity to most of the performed biocidal treatments. Fast growth of *S. kiliense*, may account for rapid biocide consumption. This may explain the inhibition by the relatively low BZC concentration we tested (0.25%), which was instead ineffective against the other fungal species. Faster growth observed at T1 for *P. chrysogenum* upon BZC treatments, with respect to controls, and successive inhibition, suggest that BZC was initially used as nutrient, as reported for other microorganisms (e.g. the bacterium *Aeromonas hydrophila*; [46]), or that the compound initially promoted growth stimulation as a response to stress, and only in a second phase exhibited a toxic effect. Accordingly, sub-inhibitory concentrations of BZC were shown to enhance the growth of some fungal strains [47]. In other works, the application of BZC at the maximum recommended concentrations determined the inhibition of all the tested strains [15, 48 with refs. therein]. However, the opportunity to adopt high BZC concentrations should take into account the risk of favouring BZC-resistant microorganisms, including gram-negative bacteria, *Pseudomonas* species [49] and microorganisms potentially using these nitrogen-containing biocides as nutrients [50]. Different mechanisms of action of the different products may also significantly regulate the observed variability in biocidal effects: BZC interferes with biological membranes by changing their structure and permeability; IT oxidates thiol-containing cytoplasmic and membrane-bound compounds, yielding metabolic inhibition; sulphamide derivatives (such as LI) inhibit respiration [51-53]. Higher susceptibility of fungi to IT than to BZC was already reported [e.g. 54], but this may also depend on the applied concentrations. Indeed, the IT-containing SN/WS, with a ten times lower final concentration of the active principle (approx. 0.02% in SN/WS with respect to 0.5-1.0% in BR/WS and BR/i-PrOH, was only effective in inhibiting one out of the four tested fungal strains. Nevertheless, this finding confirms the different sensitivity of each fungal species/strain to a given biocide application, and the opportunity of performing ad-hoc/species-specific assays to calibrate suitable products and minimum effective concentrations suitable to control fungal deterioration. A

routine use of high concentrations may indeed imply negative effects for restorer safety and possibly stimulate the development of resistant strains [55]. In this sense, the development of alternative, less-impacting chemical solutions [18, 48], or affordable physical methods [11], to control microorganisms has to be strongly encouraged. Meanwhile, the traditional application of biocides should be optimized by better calibrating application strategies.
Inhibition by all the biocide treatments observed at T1, after few days, for *C. murorum*, *S. chartharum* and *S. kiliense*, followed by a significant mycelial growth in the successive weeks, between T1 and T2, was in agreement with recent results on biocide effects of BZC against cave fungi [54]. The latter fungi were indeed inhibited by BZC and IT for a few days after the biocide application, but afterwards resumed growth and colonized previous inhibition zones. Accordingly, the monitoring of fungal susceptibility to biocides should be planned for periods longer than one-two weeks to collect reliable data on effective inhibition.
Even longer periods should possibly be monitored to reliably assay the suitability of consolidant products which do not stimulate fungal growth. Fungal growth stimulation observed on an oligotrophic culture medium one year after consolidant application, for all but one of the tested products, confirmed that restoration products may significantly support recolonization processes which are frequently observed on a time term of months or few years after restoration interventions [22]. Due to the strong ability of fungi to degrade organics, including recalcitrant polymers, compounds and/or additives in restoration products may serve as nutrient for some strains [22]. Accordingly, especially in outdoor environments, the application of consolidants and other restoration products combined with biocidal products, including nanocomposites, have already been recommended [56, 57], but such a combination is so far not routinely used by restorers, and should be tested on a wide spectrum of biodeteriogens.

6 Conclusion

The nutrient-poor stuccoed vault supported a low diversity of fungal species, which were nevertheless responsible of remarkable and heterogeneous deterioration phenomena, causing aesthetic decay. Different sensitivity of each species for most biocidal treatments and stimulation by consolidants indicate that species-specific assays of products are necessary to calibrate and optimize restoration works. In particular, BZC in the low range of recommended concentrations (0.25%) was demonstrated ineffective against most of the tested fungal strains. The IT-containing BR and BT displayed the best effectiveness against all the strains, and BT, prepared in deionized water, was finally selected for the restoration work to avoid the use of solvents. It is worth to remark that effective inhibition of fungi by biocides, and potential stimulation by consolidants, should be evaluated after several weeks and months, respectively, since short-term monitoring may be misleading. Moreover, it is necessary to be aware that any treatment may in some way affect the microbiological environment, as exemplified here by the presence of cellulolytic fungi on the stuccos, due to the fact that cellulose poultice was left during past restoration interventions.

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