Journal of Cultural Heritage 67 (2024) 194–202

Contents lists available at ScienceDirect

Journal of Cultural Heritage

journal homepage: www.elsevier.com/locate/culher

Original article

Isolation and identification of fungal biodeteriogens from the wall of a cultural heritage church and potential applicability of antifungal proteins in protection

Kinga Dán[a,b], Sándor Kocsubé[c], Liliána Tóth[a], Attila Farkas[d], Gábor Rákhely[a,e], László Galgóczy[a,f,*]

[a] Department of Biotechnology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary
[b] Doctoral School of Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary
[c] Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary
[d] Institute of Plant Biology, HUN-REN Biological Research Centre, Hungarian Research Network, Szeged, Hungary
[e] Institute of Biophysics, HUN-REN Biological Research Centre, Hungarian Research Network, Szeged, Hungary
[f] Institute of Biochemistry, HUN-REN Biological Research Centre, Hungarian Research Network, Szeged, Hungary

ARTICLE INFO

Article history:
Received 16 October 2023
Accepted 4 March 2024

Keywords:
Antifungal protein
Biodeteriorated wall
Fungus
Neosartorya (Aspergillus) fischeri
Preventive protection

ABSTRACT

Fungi are able to permanently damage cultural heritage buildings and stone monuments. Protection against these biodeteriogen fungi is challenging because it may require the use of drastic techniques to remove fungal colonies and chemicals, which can cause further damage, especially on mural paintings. Natural biocides are less damaging, greener, and safer and mainly include essential oils and plant-derived substances. Studies on the applicability of purified antifungal proteins in the conservation of cultural heritage buildings are lacking. In this study, we isolated mold mycoflora from the biodeteriorated inner walls of the Calvinist church of Kézdialbis (Kézdialbis, Romania) and investigated the *in vitro* antifungal efficacy of *Neosartorya* (*Aspergillus*) *fischeri* antifungal proteins, NFAP and NFAP2 against them. Antifungal susceptibility testing revealed that these proteins inhibited the growth of the most commonly isolated indoor biodeteriogen fungi from the site (*Aspergillus creber, Aureobasidium pullulans, Cladosporium* sp., *Penicillium chrysogenum,* and *Parengyodontium album*) and not common ones (*Mucor hiemalis, Sarocladium kiliense*) with different spectra and efficacies. A phytopathogen and an entomopathogen species (*Pseudopithomyces chartarum* and *Beauveria pseudobassiana*, respectively) were also isolated and described first as fungal biodeteriogens of the inner walls. Among them, *P. chartarum* was susceptible to NFAP2. Next, we tested the applicability of NFAP and NFAP2 in protecting cultural heritage buildings in a wall model experiment against *A. creber*. Topical application of both antifungal proteins significantly decreased the area infected by fungi. Scanning electron microscopy of the wall models showed that *A. creber* had reduced growth and conidiation in the presence of NFAP and NFAP2. This inhibitory activity was maintained for a long time. Strong UV irradiation decreased the protective effect of both proteins in the wall model. Taking into account these results, the high stability of NFAP and NFAP2 in harsh environments, and their bulk recombinant producibility, we suggest that both can be used in the protection and posttreatment of painted walls of cultural heritage buildings to clean up mural painting or to inhibit mold recolonization after mechanical, physical, or chemical decontamination.

Abbreviations: AFP, antifungal protein; ITS, internal transcribed spacer; LCM, low-cationic medium; MEA, malt extract agar; MIC, minimum inhibitory concentration; NCBI, National Center for Biotechnology Information; NFAP, *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein; NFAP2, *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein 2; PDA, potato dextrose agar; PDB, potato dextrose broth; RPB2, DNA-directed RNA polymerase II subunit 2; SEM, scanning electron microscopy; SZMC, Szeged Microbiology Collection; UV, ultraviolet.

* Corresponding author at: Department of Biotechnology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, 6726 Szeged, Hungary.
E-mail address: galgoczi@bio.u-szeged.hu (L. Galgóczy).

https://doi.org/10.1016/j.culher.2024.03.002

1. Introduction

Cultural heritage is a unique and irreplaceable bridge between the past and the future; therefore, preserving it is unquestionably important. For this reason, it is essential to identify agents that cause deterioration and eliminate them in a targeted manner, which do not cause further damage to objects of cultural heritage [1]. The biodeterioration of cultural heritage, when living organisms cause undesirable degradation, is a significant problem globally [2–4]. Microorganisms (bacteria, archaea, fungi, algae, and lichens) are considered the main causative agents [5], which can damage objects of cultural heritage, such as artworks, buildings, murals, sculptures, and monuments. These microorganisms can grow on various substrates, such as textile [6], paper [7], wood [7–9], mural painting [10–13], and stone [3,5]. Among these microorganisms (especially microscopic fungi) are considered the main biodeteriogens of stones and wall paintings [5,10,13,14]. These materials represent extreme habitats as they are low in nutrients and have low water activity. Nevertheless, fungi colonize them due to their high adaptation capacity to harsh environmental conditions and utilize all organic and inorganic components of stone and mural artworks as nutrients [14,15]. The fungal biodeteriogens of stone monuments can be divided into two major morphological and ecological groups according to the environmental conditions under which they grow on the surface: 1) Hyphomycetes, including species belonging to the mold genera *Alternaria, Cladosporium, Epicoccum, Aureobasidium*, and *Phoma*, are dominant in moderate or humid climates, and 2) black yeasts (*Hortaea, Sarcinomyces, Coniosporium, Capnobotryella, Exophiala*, and *Trimmatostroma*) are dominant in arid and semiarid environments [15]. These fungi destroy stone and wall both mechanically and chemically, penetrating their materials and mortars, causing breakup and fragmentation [2,5]. Secretion of pigments and other coloring agents into the environment leads to chromatic variations of the monument surface and significantly decreases its esthetic value [3].

Over the years, several different indirect and direct methods have been developed for the conservation of cultural heritage and the removal of microbial colonies (including microscopic fungi) from infected sites. Indirect methods are based on the control of environmental conditions, such as humidity, temperature, and nutrients, to inhibit fungal colonization. These methods are always coupled with a direct method, which generally uses mechanical, physical, or chemical tools to remove microbial colonies from the infected object [16]. Mechanical methods including application of vacuum cleaners, scalpels, spatulas, or lasers often fail, because they cannot completely remove microbial colonies from the contaminated surface [17]. Physical methods, such as low-frequency electrical systems, heat, and ultraviolet (UV) or gamma irradiation, are also unable to completely remove microorganisms from monuments. Additionally, they have undesirable effects, for example, UV irradiation can be dangerous for the user [17]. The most effective and widely used method for the conservation and decontamination of cultural heritage involves the surface application of biocides, such as Algophase, anatase, and Glifene SL [17]. These biocides eliminate and inhibit growth of microorganisms [16,17]; however, they are neither economical nor convenient, because they lose efficacy over time and must be reapplied. Repetitive application of biocides can further damage heritage objects, be harmful to human health and the environment, and result in the emergence of resistant fungal species [18]. Therefore, several biocides have recently been withdrawn from the market [16], generating a substantial demand for new and safe cultural heritage conservation strategies. Natural substances represent promising alternatives; some have been proven to be effective, such as essential oils [19], juglone [20], capsaicin [21], secondary metabolites of lichens, and compounds produced by microorganisms (*e.g.*, biosurfactant lipopeptides, *Trichoderma harzianum* cell filtrates) [18].

Mold-secreted antifungal proteins (AFPs) represent promising compounds to fight against human and plant pathogenic fungi [22,23]. The mold, *Neosartorya* (*Aspergillus*) *fischeri* NRRL 181, secretes two small, cysteine-rich, cationic and highly stable AFPs, namely NFAP and NFAP2 (Supplementary Table 1). Both inhibit mold growth *in vitro* [24–26] and are considered as safe as topical drugs and biofungicides [25–28]. Their beneficial characteristics, such as water solubility, as well as high stability against protease degradation and in extreme environmental conditions [29,30], indicate that they can be safely used as topical fungicides in aqueous solution to protect buildings of cultural heritage.

2. Research aims

In this study, we investigated the potential of NFAP and NFAP2 in protecting cultural heritage buildings against mold contamination. We isolated and identified fungal biodeteriogens from the inner painted walls of the Calvinist church of Kézdialbis (Romania) and investigated their *in vitro* susceptibility to NFAP and NFAP2. In addition, we tested the protective effect of both proteins against mold contamination in wall model experiments. According to our results, both peptides inhibited the growth of some isolated biodeteriorating fungi in the investigated concentration range, and application of their aqueous solution on the surface of the wall model significantly reduced the infected area. These results indicate that aqueous solutions of both AFPs can be considered for protection or posttreatment of painted walls of cultural heritage buildings to inhibit mold (re)colonization after mechanical, physical, or chemical decontamination.

3. Materials and methods

3.1. Sampling site and sampling

The fungal samples were taken from the Calvinist church of Kézdialbis, Romania (45° 56′ 06″ N, 26° 00′ 21″ E), which was built in the 1330s, based on the excavated baptismal font. The church has undergone several reconstructions and expansions over the centuries, and the arch ribs from the 16th century and the ornamental wall paintings from the 17th century are still visible. This monument is one of the well-preserved examples of Calvinist church interiors painted from the Protestant Reformation period in Europe [31].

Fungal samples were isolated with sterile cotton swabs from the discolored inner walls (Fig. 1); inoculated into cryotubes containing malt extract agar slants (MEA; Sigma-Aldrich, St. Louis, MO, USA), supplemented with antibiotic cocktails ampicillin, streptomycin, kanamycin, tetracycline; 100 μg mL^{-1} of each; and incubated at 25 °C for 10 days. The fungi were streaked on MEA plates supplemented with the same antibiotic cocktail and incubated at 25 °C for 7 days. Fungal suspensions were prepared from individual fungal colonies in spore buffer [0.9% (m/v%) NaCl and 0.01% (v/v%) Tween 80]. Then 5 μL of the suspensions were spotted onto the center of MEA and potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO, USA) plates and incubated at 25 °C for 7 days for microscopic investigation. The isolates were maintained on MEA and PDA agar slants at 4 °C. All isolated and identified fungi were deposited in the Szeged Microbiology Collection (SZMC; Szeged, Hungary) under the strain numbers indicated in Table 1.

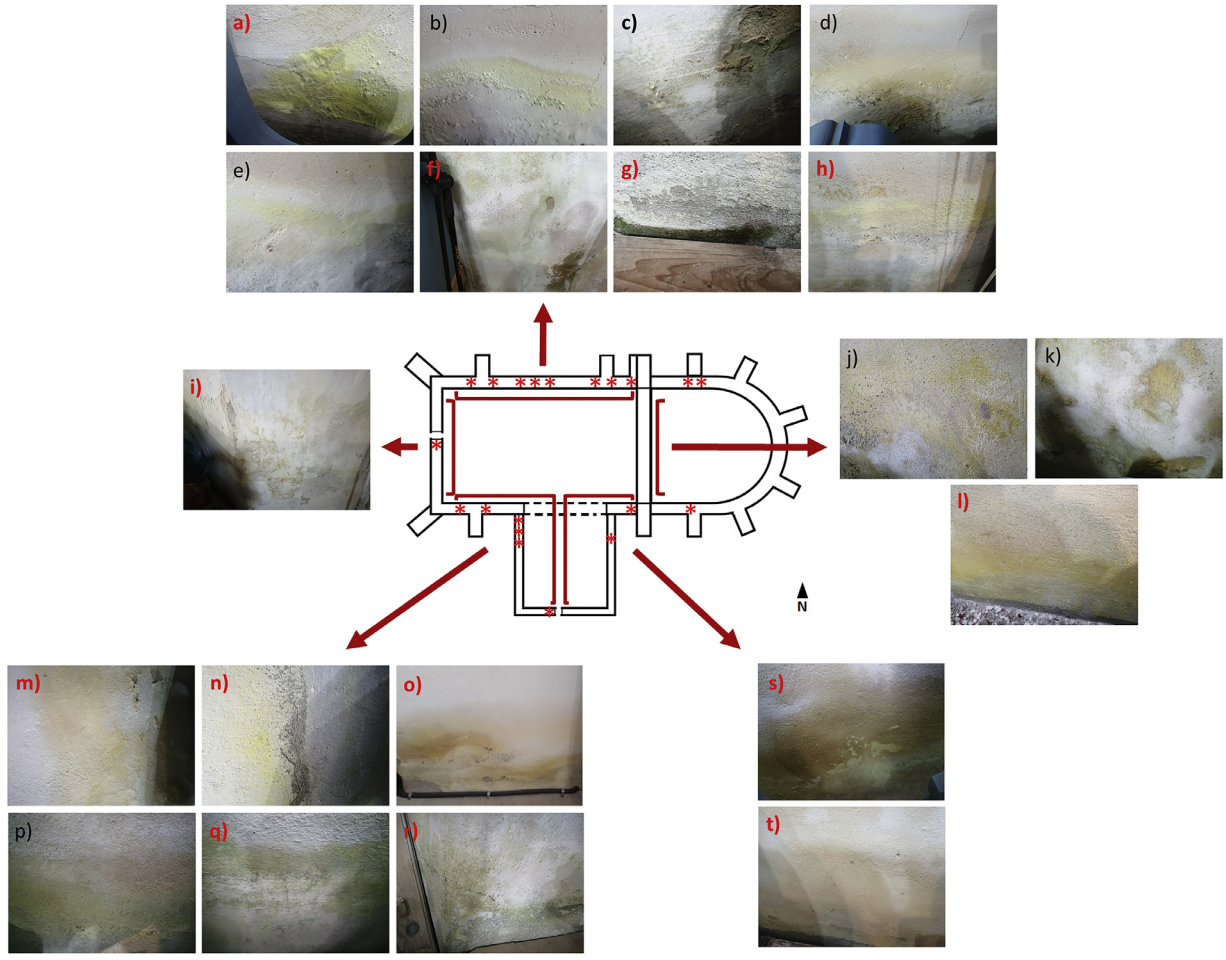

Fig. 1. Delineation and fungal contamination of the inner walls of the Calvinist church of Kézdialbis. Locations of sampling sites (a-t) are indicated with red asterisks. Bold red letters indicate the identified fungi from the inner walls: *Beauveria pseudobassiana* (a), *Sarocladium kiliense* (b), *Sarocladium kiliense* (f), *Mucor* sp. (g), *Aspergillus creber* (h), *Cladosporium* sp. (i), *Penicillium chrysogenum*, *Aspergillus* (*jensenii*) *creber*, and *Parengyodontium album* (l), *Aspergillus* (*jensenii*) *cereber* and *Parengyodontium album* (m), *Cladosporium* sp. (n), *Parengyodontium album* (o), *Cladosporium* sp. (q), *Pseudopithomyces chartarum*, *Aureobasidium pullulans* (r), *Parengyodontium album* (s), *Aspergillus* (jensenii) *creber*, *Cladosporium* sp. (t). Source of the delineation: Cova Design Studio, 2000.; Sepsiszentgyörgy, Romania.

Table 1
Identified fungi from the contaminated inner walls of the Calvinist church of Kézdialbis, based on the similarity of partial DNA sequence of the internal transcribed region (ITS1–5.8S rRNA-ITS2) and DNA-directed RNA polymerase II subunit 2 (RPB2) encoding genes with those available in the GenBank of the National Center for Biotechnology Information (NCBI).

Species	ITS1-5.8S rRNA-ITS2 sequences					RPB2 sequences			
	SZMC	Acc. No.[1]	Query cover	Per. Ident.	Acc. No.[2]	Acc. No.[1]	Query cover	Per. Ident.	Acc. No.[2]
Aspergillus creber	28230	OR564025	99%	99.62%	ON790425.1	OR608346	98%	99.72%	LN898915.1
*Aspergillus (jensenii) creber**	28229	OR564023	99%	99.81%	MH725588.1	OR608347	96%	100%	LN898928.1
Aureobasidium pullulans	28233	OR569671	99%	99.05%	KR912256.1	OR608345	100%	99.91%	DQ470906.1
Beauveria pseudobassiana	28226	OR544477	98%	99.63%	ON479319.1	OR555873	98%	99.84%	MN523620.1
Cladosporium (*allicinum*) sp.**	28228	OR564027	98%	100%	OM237215.1	OR612086	91%	98.90%	MF951411.1
Cladosporium (*cladosporioides*) sp.**	28227	OR544489	96%	99.81%	OK149113.1	OR612085	76%	96.20%	KX288432.1
Mucor (*hiemalis*) sp.**	28234	OR564030	99%	97%	OQ975663.1	OR612087	100%	92.74%	EF014398.1
Parengyodontium album	28231	OR564024	98%	100%	OL457280.1	OR612033	n.d.	n.d.	OL904994.1
Penicillium chrysogenum	28235	OR564029	98%	100%	KR011762.1	OR607646	100%	100%	JF909937.1
Pseudopithomyces chartarum	28232	OR569668	99%	98.56%	KF876827.1	OR607645	96%	100%	OU641536.1
Sarocladium kiliense	28362	OR564028	99%	99.63%	MZ818340.1	OR612032	99%	100%	OQ454241.1

Acc. No[1]: accession number of the deposited partial gene sequence of the isolated mold, Acc. No[2]: accession number of the gene showing the highest similarity in NCBI GeneBank, Per. Ident.: sequence identity in percentage, SZMC: Szeged Microbiology Collection (University of Szeged, Szeged, Hungary). n.d.: not determined due to unverified sequence is available in NCBI GenBank.

* : According to a recent taxonomic revision, *Aspergillus jensenii* is a synonym of *Aspergillus creber* [54].

** : On the basis of the relatively low query cover and/or sequence identity, we speculate that *Cladosporium* spp. and *Mucor* sp. represent undescribed species.

3.2. Light microscopy

The micromorphologies of isolated fungi grown on MEA and PDA agar plates were investigated and visualized using a light microscope (Olympus BX53, Olympus Life Science, Hamburg, Germany) and photographed with an Olympus XC50 CCD color camera (Olympus Life Science, Hamburg, Germany) using a non-invasive adhesive tape method. Lactophenol cotton blue solution [5 µL, 20% (v/v%) glycerol, 40% (v/v%) water, 40% (m/v%) phenol, 0.2% (m/v%) cotton blue, 40% (v/v%) lactic acid] was dropped onto microscope slides and an adhesive tape, which was touched to the surface of the fungal colonies with conidiophores, was placed on it and covered with cover glass.

3.3. Genomic DNA extraction

To extract genomic DNA, the fungi were grown in 20 mL of potato dextrose broth (PDB; Sigma-Aldrich, St. Louis, MO, USA) at 25 °C for 7 days with continuous shaking at 160 rpm. Mycelia were harvested by filtration and frozen at −80 °C. The frozen mycelia were crushed with sterile sand in a 1:1 ratio with a pestle in a microcentrifuge tube, mixed with 300 µL of 10% (m/v%) *N*-lauryl sarcosine and 1 µL of 10 mg mL^{-1} RNase A (Thermo Fisher Scientific, Waltham, MA, United States), vortexed for 2 min, incubated at 65 °C for 10 min and at 4 °C for 10 min, mixed with 300 µL of 5 M sodium acetate, vortexed for 1 min, and centrifuged at 13,400 × *g* for 15 min at 4 °C. The supernatant was transferred to a new microcentrifuge tube and mixed with 500 µL of ice-cold 70% (v/v%) isopropanol. The tube was inverted 10 times, incubated at room temperature for 10 min, and centrifuged at 13,400 × *g* for 20 min at 4 °C. The supernatant was removed and 500 µL of ice cold 70% ethanol (v/v%) was added to the pellet. The tube was centrifuged at 13,400 × *g* for 5 min at 4 °C. The supernatant was removed, and the DNA pellet was dried and dissolved in 30 µL of molecular grade water.

3.4. Molecular identification of fungal isolates

The isolated fungi were identified at the species or genus level based on the DNA sequence of the internal transcribed spacer region (ITS; ITS1–5.8S rRNA-ITS2) and partial DNA-directed RNA polymerase II subunit 2 (RPB2) amplified using primer pairs ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ (Tm: 61.0 °C) and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ (Tm: 55.3 °C) [32]; and RPB2–3bF: 5′-GGWGGWTAYTTYATATYAATGG-3′ (Tm: 55.3 °C) and fRPB2–7cR: 5′-CCCATRGCTTGYTTRCCCAT-3′ (Tm: 58.3 °C) [33], respectively. The compositions of the PCR mixtures are listed in Supplementary Table 2. The PCR conditions were denaturation at 98 °C for 3 min; 30 cycles of denaturation at 98 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min. The PCR products were purified with a PCR and DNA Cleanup Kit (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions, and subjected to Sanger sequencing (Eurofins Genomics; Ebersberg, Germany). The obtained sequences were compared with sequences available in the GenBank of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool. The identification criterion at the species level was at least 96% of the query coverage and identity of the molecular markers studied. All obtained sequences were deposited in the NCBI GenBank under the accession numbers indicated in Table 1.

3.5. Antifungal protein production

Recombinant NFAP and NFAP2 were produced in a *Penicillium chrysogenum*-based expression system and purified by cation exchange chromatography, as described [34,35]. Briefly, *P. chrysogenum* strains transformed with the NFAP or NFAP2 expression plasmid were cultured in *P. chrysogenum* minimal medium [(2% sucrose, 0.3% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4 \times 7\ H_2O$, 0.005% $FeSO_4 \times 7\ H_2O$ (w/v%), 0.25% 1 M potassium phosphate buffer pH 5.8, 0.01% trace element solution (v/v%); trace element solution: 0.1% $FeSO_4 \times 7\ H_2O$, 0.9% $ZnSO_4 \times 7\ H_2O$, 0.4% $CuSO_4 \times 5\ H_2O$, 0.01% $MnSO_4 \times H_2O$, 0.01% H_3BO_3, 0.01% $Na_2MoO_4 \times 2\ H_2O$ (w/v%)] for 96 h at 25 °C with continuous shaking at 160 rpm. After incubation, recombinant proteins were purified from the cell-free and ultrafiltrated supernatant (Ultracell30 kDa Ultrafiltartion Discs, regenerated cellulose; Millipore, Billerica, MA, USA) by cation exchange chromatography on Bio-Scale™ Mini Macro-Prep R High S column (Bio-Rad Laboratories, Hercules, CA, USA) using the NGC Chromatography System (Bio-Rad Laboratories, Hercules, CA, USA). To reach maximum purity (~100%), semipreparative reversed-phase high-performance liquid chromatography was used, as described [28].

3.6. Antifungal susceptibility testing

Broth microdilution susceptibility test was performed according to Tóth et al. (2016) [30]. The test was carried out in low-cationic medium [LCM: 0.5% glucose, 0.25% yeast extract, 0.0125% peptone (w/v%)] to determine the minimum inhibitory concentrations (MICs) of NFAP and NFAP2 against all isolated fungal species. Briefly, 100 µL of NFAP or NFAP2 (3.125–50 $\mu g\ mL^{-1}$, twofold dilutions in LCM) were mixed with 100 µL of 2×10^5 conidia mL^{-1} in LCM in the wells of a flat bottom 96-well microtiter plate (TC Plate 96 Well, Suspension, F; Sarstedt, Nümbrecht, Germany). The plates were incubated without shaking at 25 °C for 72 h. Absorbance (OD_{620}) was measured with a microtiter plate reader (Thermo Electron Multiskan Ascent Microplate Reader; Thermo Fisher Scientific, Waltham, MA, United States). MIC was defined as the lowest concentration of an AFP that reduces fungal growth to ≤5% compared to the untreated control (set to 100%). Susceptibility tests were repeated at least two times, including two technical replicates.

3.7. Wall model

To prepare the wall model, 10 g of Glatt skim coat powder (Ceresit, CT 127; Henkel, Düsseldorf, Germany) were mixed with 6.5 mL of distilled water. Then 1 g of this solution was plastered on a 2.5 × 2.5 cm square region of a microscope slide. Its surface was painted with 0.5 mL of white wall paint (Dulux Absolute White; AkzoNobel Coatings Zrt., Budapest, Hungary). The prepared model was sterilized with UV irradiation under a laminar flow germicide lamp (Hera Safe; Thermo Electron Corporation, Waltham, MA, USA) for 25 min. Subsequently, 3 × 200 µL PDB was streaked and dried on the surface. Then 10 µL of 2×10^6 conidia mL^{-1} of *Aspergillus creber* conidial suspension (diluted in PDB) was dropped in the middle of the wall model. To treat the wall model with AFPs, a 3 × 100 µL of 16 × MIC NFAP or NFAP2 solution (100 µg mL^{-1}) in water was sprayed onto the surface and dried before dropping the conidial suspension. To mimic the humid condition and cold bridge of the walls where fungi preferably grow, the wall models were laid on wet paper towels and incubated at room temperature (21 °C–25 °C) for 10 days in a closed plastic box above ice containing transparent polystyrene box (Supplementary Fig. 1). The wall models were subjected to image analysis to measure the size of the infected area and scanning electron microscopy (SEM). This experiment was repeated three times with three technical replications.

To demonstrate the long-term protective effect of NFAP and NFAP2, five-five representative wall models from the above exper-

iment was further incubated for three months in a transparent plastic box on the laboratory table at room temperature.

To investigate the aging effect of UV irradiation on the protective effect of NFAP and NFAP2, wall models were exposed to UV irradiation for 30 min with a distance of 25 cm under the germicide lamp of a laminar flow box (Hera Safe; Thermo Electron Corporation, Waltham, MA, USA) immediately after application of the proteins on the surface, then were infected and incubated for 10 days as described above. This experiment was repeated two times with three technical replicates.

3.8. Image analysis

The percentage of fungus-infected area in the images of the wall models was calculated using the Analyze Particles tool of ImageJ3 software [36] after conversion to an 8-bit image and applying the auto threshold (Max Entropy) to convert the fungal colonies to black spots.

3.9. Scanning electron microscopy

Representative parts of the mural wall models were cut and fixed with 2.5% (v/v%) glutaraldehyde and 0.05 M cacodylate buffer (pH = 7.2) in phosphate buffered saline (PBS; pH = 7.4) at 4 °C overnight; dehydrated with a graded series of ethanol [30%, 50%, 70%, 80% (v/v%)] each at 4 °C for 24 h; and stored in 100% (v/v%) ethanol at 4 °C overnight. Then they were dried with a Quorum K850 critical point dryer (Quorum Technologies, Laughton, UK), followed by a 12 nm gold coating, and observed under a JEOL JSM-7100F/LV (JEOL USA, Peabody, MA, USA) field emission scanning electron microscope (JEOL Ltd., Tokyo, Japan).

3.10. Statistical analysis

A paired *t*-test was used to calculate significant differences ($p < 0.05$) between fungus-infected areas of AFP-treated and untreated wall models (Statistics Kingdom online platform, 2023.; https://www.statskingdom.com/paired-t-test-calculator.html).

4. Results and discussion

4.1. Fungal biodeteriogens in the inner walls of the Calvinist church of Kézdialbis

The mold mycoflora of biodeteriorated inner walls of the Calvinist church of Kézdialbis was monitored (Fig. 1). In total, 25 mold samples were collected from contaminated and discolored corners. Of the collected samples, 18 isolates grew on MEA slants in cryotubes under the applied incubation conditions. The results of molecular identification based on sequencing of ITS1–5.8S rRNA-ITS2 and partial RPB2 indicated that the 18 isolates represent 11 species belonging to nine genera (Table 1). The macro- and micromorphology of the isolated fungi supported the results of the molecular identification method (Supplementary Figs. 2–5). Most isolated species are common indoor fungi and are known as biodeteriogens of cultural heritage, such as *A. creber* [37,38], *Aureobasidium pullulans* [39,40], *Cladosporium allicinum, Cladosporium cladosporoides* [41,42,43], and *P. chrysogenum* [44,45]. *Parengyodontium album* has frequently been reported to be a biodeteriogen of indoor environments of cultural heritage [46]. *Mucor hiemalis* [47,48] and *Sarocladium kiliense* [49] are not common indoor fungi, but are described as members of the microflora of mold-contaminated heritage objects. *Pseudopithomyces chartarum,* a plant pathogenic species [50] and not a frequent indoor mold [51], has not been isolated from fungus-infected inner walls of cultural heritage buildings until this study (Supplementary Figs. 2–5j). *Beauveria pseudobassiana* is an important entomopathogenic fungus [52] that has not been described as an indoor and biodeteriogen mold. To our knowledge, this is the first report on *B. pseudobassiana* in the literature (Supplementary Figs. 2–5d). This species probably entered the church through pine wood furniture and colonized the inner wall. Based on the relatively low percent of the query cover or sequence identity, we hypothesize that the isolated *Cladosporium* spp. are not *C. allicinum* or *C. cladosporoides* and the *Mucor* sp. is not *M. hiemalis* (Table 1), and they may represent undescribed species. However, further characterization and phylogenetic studies are needed to support this hypothesis. We refer to them as *Cladosporium* sp. and *Mucor* sp. in the paper.

4.2. Susceptibility of isolated biodeteriogen fungi to NFAP and NFAP2

The susceptibilities of the isolated and identified biodeteriogen fungi to 3.125–50 μg mL^{-1} NFAP and NFAP2 were tested using a broth microdilution assay. The MIC values of NFAP were in the range of 3.125 μg mL^{-1} to >50 μg mL^{-1}, and *P. album* SZMC 28231 was the most susceptible (MIC: 3.125 μg mL^{-1}). Total growth inhibition was not observed for *A. pullulans* SZMC 28233, *B. pseudobassiana* SZMC 28226, and *Cladosporium* sp. SZMC 28228 even at the highest concentration of NFAP (50 μg mL^{-1}); however, it caused 47 ± 3%, 78 ± 2%, and 87 ± 5% growth inhibition, respectively. The MIC values of NFAP2 ranged from 6.25 μg mL^{-1} to >50 μg mL^{-1}. *A. creber* SZMC 28230 (MIC: 6.25 μg mL^{-1}) was most and *B. pseudobassiana* SZMC 28226, *P. chartarum* SZMC 28232, and *P. chrysogenum* SZMC 28235 were least susceptible to NFAP2. For these isolates, no reduction in fungal growth was observed at 50 μg mL^{-1} NFAP2. This NFAP2 concentration did not cause full growth inhibition of *P. album* SZMC 28231 and *Mucor* sp. SZMC 28234; however, it reduced growth to 28 ± 6% and 32 ± 2%, respectively, compared with untreated controls. In summary, the antifungal spectrum of NFAP and NFAP2 is different with a small overlap (*A. creber* SZMC 28230, *Mucor* sp. SZMC 28234, *P. album* SZMC 28231, and *S. kiliense* SZMC 28362), and neither could inhibit the growth of some isolated fungi, such as *B. pseudobassiana* SZMC 28226, *Cladosporium* sp. SZMC 28228 and *Cladsoporium* sp. SZMC 28227, in the concentration range investigated. The observed MIC values are summarized in Table 2. These results correspond to our susceptibility testing results on phytopathogenic fungi [26], *i.e.*, the antifungal spectra of NFAP and NFAP2 show differences with small overlap. The results of our susceptibility test extend to the *in vitro* antifungal spectrum of NFAP and NFAP2, because neither has been investigated against isolated species.

4.3. Protective effect of NFAP and NFAP2 against A. creber in a wall model

The antifungal active tertiary structures of NFAP and NFAP2 are stabilized by three intramolecular disulfide bridges between cysteine residues rendering them high stability under harsh environmental conditions (such as high temperature and a wide pH range) and resistance against protease degradation [25,29,30,35,54,55]. The melting temperature of these proteins is above 70 °C, and after thermal unfolding they are able to properly refold in minutes at room temperature [30,54,55]. The remarkable antifungal efficacy of NFAP and NFAP2 against isolated indoor biodeteriogen fungi together with the high stability mentioned above suggests their applicability as topical agents on inner walls to prevent mold contamination (Table 2). This hypothesis was tested in a wall model where fungi can grow [13]. To this end, we made a wall model and infected it with *A. creber* SZMC 28230, one of the most widespread indoor mold that can produce the mycotoxin sterigmatocystin [37] and has a high biodeterioration ability [56]. *A. creber*

Table 2
Minimum inhibitory concentrations of NFAP and NFAP2 against isolated and identified fungi from the contaminated inner walls of the Calvinist church of Kézdialbis.

Mold	MIC (μg mL^{-1})	
	NFAP	NFAP2
Aspergillus creber SZMC 28230	6.25	6.25
Aspergillus (*jensenii*) *creber*[*] SZMC 28229	>50	50
Aureobasidium pullulans SZMC 28233	>50 (52 ± 7%)[**]	50
Beauveria pseudobassiana SZMC 28226	>50	>50
Cladosporium (*cladosporioides*) sp.[**] SZMC 28227	>50	>50
Cladosporium (*allicinum*) sp.[**] SZMC 28228	>50	50
Mucor (*hiemalis*) sp.[**] SZMC 28234	>50 (21 ± 8%)[**]	>50 (32 ± 2%)[**]
Parengyodontium album SZMC 28231	3.125	>50 (28 ± 2%)[**]
Penicillium chrysogenum SZMC 28235	12.5	>50
Pseudopithomyces chartarum SZMC 28232	6.25	>50
Sarocladium kiliense SZMC 28236	>50 (12 ± 5%)[**]	12.5

MIC: Minimum inhibitory concentration, NFAP, NFAP2: *Neosartorya* (*Aspergillus*) *fischeri* NRRL 181 antifungal protein and antifungal protein 2, respectively. Growth percentages (mean ± SD) are indicated in brackets, when complete growth inhibition was not observed at 50 μg mL^{-1} AFP concentration.

[*] According to a recent taxonomic revision, *Aspergillus jensenii* is a synonym of *Aspergillus creber* [53].

[**] Based on the relatively low query cover and/or sequence identity, we speculate that *Cladosporium* spp. and *Mucor* sp. represent undescribed species. [***]: To calculate the reduced growth, the absorbance (OD_{620}) of untreated control was considered 100 % growth.

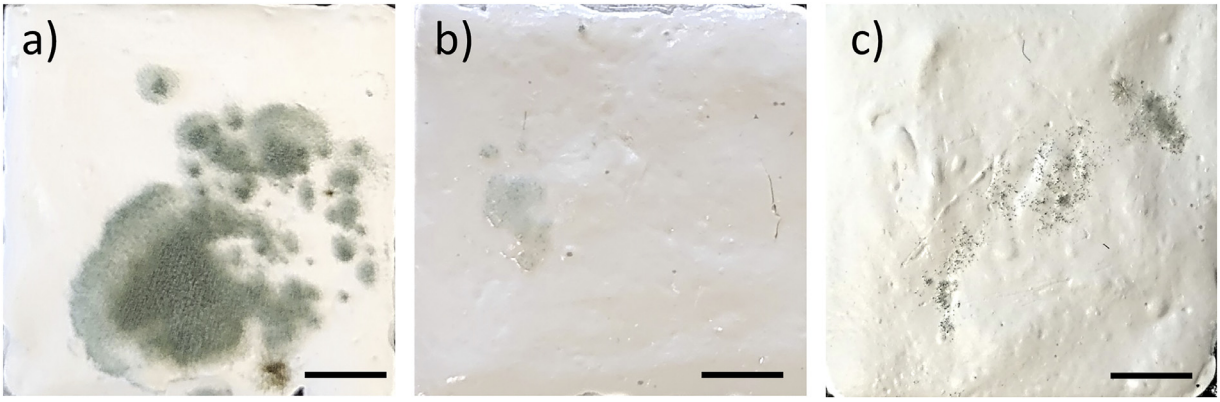

Fig. 2. Representative pictures of the wall model contaminated with *Aspergillus creber* SZMC 28230 without (a) and with NFAP (b), or NFAP2 (c) treatment. NFAP and NFAP2: *Neosartorya* (*Aspergillus*) *fischeri* antifungal proteins 1 and 2, respectively. Scale bar: 0.5 cm.

SZMC 28230 could colonize the surface of the model under the applied incubation conditions (Fig. 2a). When the surface was treated with an aqueous solution of NFAP or NFAP2 prior to infection, *A. creber* SZMC 28230 showed remarkably reduced growth (Fig. 2b and c, respectively). Image analysis was performed to determine the percentage of fungus-infected area. NFAP significantly reduced the size of the infected area from 21.6 ± 10.7% to 8.2 ± 10.5% ($p = 0.006762$, $t = -3.6221$, $n = 9$, $\bar{xd} = -13.4556$, Sd = 11.1446), whereas NFAP2 decreased the area from 9.5 ± 4.7% to 3.7 ± 3.1% ($p = 0.002516$, $t = -4.3289$, $n = 9$, $\bar{xd} = -5.8111$, Sd = 4.0272). The long-term protective ability of NFAP and NFAP2 at room temperature and exposure to dim sunlight was demonstrated by the experiment when five-five demonstrative wall models from the previous experiments were further incubated for three months on a laboratory table. After this long incubation period, both AFPs maintained protective ability as significant reduction in the fungus-infected area was still observed (Supplementary Fig. 6.). This reduction was 17.2%±7.1% to 3.3%±1.5% ($p = 0.01992$, $t = -3.7516$, $n = 5$, $\bar{x}d = -13.9$, Sd = 8.2849), and 7.1%±3.8% to 0.9%±0.7% ($p = 0.02844$, $t = -3.3548$, $n = 5$, $\bar{x}d = -6.14$, Sd = 4.0924) in the case of NFAP and NFAP2, respectively. To test the possible aging effect of high-dose UV irradiation on the antifungal efficacy of NFAP and NFAP2, wall models were irradiated with the germicide lamp of a laminar flow box. This treatment decreased the protective effect of NFAP and NFAP2, and slight or no significant reduction was observed in the fungus-infected area (Supplementary Fig. 7.). This reduction was 14.2 ±4.7% to 8.6%±4.4% ($p = 0.04827$, $t = -2.5997$, $n = 6$, $\bar{x}d = -5.5167$, Sd = 5.1979) and 14.8%±4.8% to 9 ±3.2% ($p = 0.113$, $t = -1.9195$, $n = 6$, $\bar{x}d = -5.7333$, Sd = 7.3162) in the case of NFAP and NFAP2, respectively. The percentages of infected areas calculated from independent experiments are shown in Supplementary Tables 3, 4, and 5. Several compounds of natural origin (*e.g.*, essential oils, plant extracts, secondary metabolites produced by microbes and lichen, and microbial enzymes) and biocontrol microorganisms (bacteriophages and bacteria) have a high potential to control microbial deterioration of cultural heritage sites. They represent alternatives that are safer for humans and more environmentally friendly than chemical biocides [57]. In a comprehensive review of the literature, Fidanza et al. (2019) reported that natural biocides (mainly essential oils and plant substances) can be used to conserve stone cultural heritage, but did not report the applicability of purified AFPs [16]. Based on this review and a literature search in the PubMed database in 2019–2023, we believe that our study is the first to report the potential use of AFPs in the conservation of heritage sites. However, an artificial aging experiment indicated that UV irradiation can decrease the protective effect of NFAP and NFAP2 possibly by disrupting disulfide bridges that stabilize the antifungal active tertiary structure [54,55]. This phenomenon was demonstrated with other disulfide bond-stabilized proteins [58,59]. It has to be noted that such a

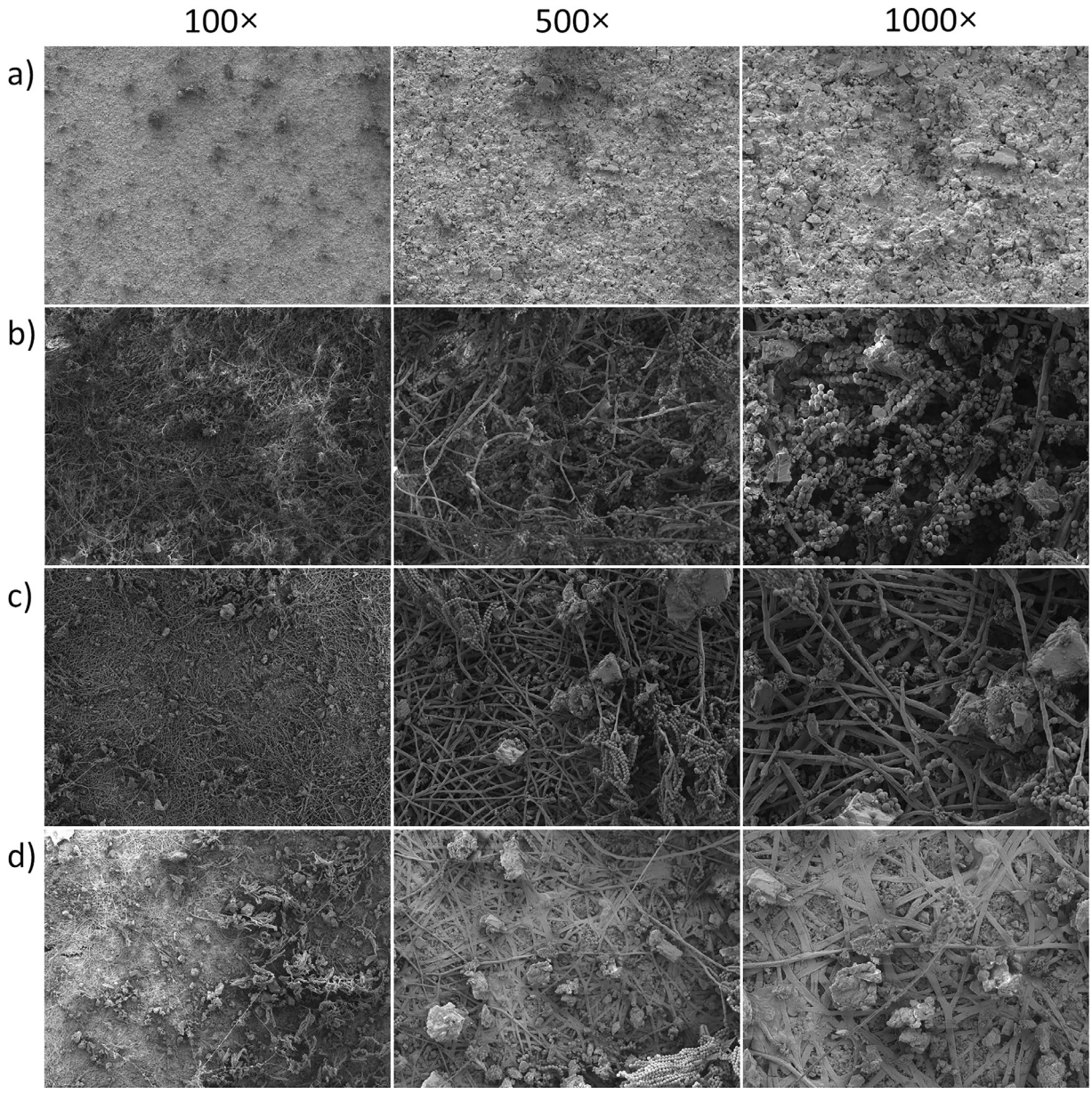

Fig. 3. Scanning electron microscopy of *Aspergillus creber* SZMC 28230 contamination on wall model. Untreated/uninfected (a), untreated/infected (b), NFAP- (c), and NFAP2-treated (d). NFAP and NFAP2 treatments were performed with 16 × MIC (100 μg mL^{-1}). Scale bars: 100 ×: 100 μm, 500 × and 1000 ×: 10 μm.

strong UV irradiation by dim sunlight in an indoor environment cannot be possible than that applied in the artificial aging experiment. In addition, relatively large amounts of NFAP and NFAP2 can be recombinantly produced using the fermentable, generally recognized as safe fungus *P. chrysogenum* [35,16]. This can make their industrial production economic in the near future.

4.4. Scanning electron microscopy analysis of wall models

SEM analysis of the wall model was performed to investigate the antifungal effect of NFAP and NFAP2. The uninfected/untreated wall model had a rough surface with small plaques (Fig. 3a). Without AFP treatment, the contaminated areas of the model were colonized by *A. creber*. The fungus was able to develop a dense mycelial network with several conidiophores (Fig. 3b). NFAP (Fig. 3c) and NFAP2 (Fig. 3d) treatment reduced the density of the mycelial network and the number of conidiophores in the contaminated area (Fig. 3c and d). In these samples, the rough surface of the wall model was visible, indicating the fungal growth inhibitory effects of NFAP and NFAP2 (Fig. 3c and d).

5. Conclusions

In this study, we isolated fungal biodeteriogens from the inner walls of a cultural heritage church. The molecular identification and macromorphological identification of these fungi indicated that most isolates are indoor fungi and destroy cultural heritage buildings. Among them, two undescribed *Cladosporium* species were detected. To our knowledge, this study is the first to report that *P. chartarum* and *B. pseudobassiana* (a phytopathogenic and entomopathogenic fungus, respectively) are biodeteriogen species that grow on the inner walls of buildings. Extracellular AFPs from *N.* (*A.*) *fischeri* (NFAP and NFAP2) inhibited the growth of the isolated fungi with varying efficacy. NFAP and NFAP2 significantly reduced *A. creber* contamination in a wall model experiment, however, strong UV irradiation decreased their protective effect. Based

on these results, we propose that NFAP and NFAP2, highly stable proteins in harsh environments, can have high potency as natural biocides for the protection and conservation of inner walls of cultural heritage buildings. In addition, their application can decrease the concentration of fungal spores in the indoor area.

However, in our study, we only examined the deteriogen effects of fungi and recommended an alternative procedure to eliminate them from indoor heritage sites by topical application of AFPs. This approach may have the risk that, after the elimination of contagious fungi, other species will appear on the wall, such as bacteria, cyanobacteria, algae, or fungal species resistant to AFPs. An excellent example of this problem is the Lascaux Cave in France, where after a species-specific biocide treatment, a new fungal species appeared and spread suddenly [60]. Other biodeteriogen species can appear not only as a consequence of taxon-specific treatment, but it can be a result of increased recolonization ability of the species. Recolonization always occurs sooner or later after decontamination [61,62]. Therefore, *in situ* experiments are necessary to detect the possibility of the risks mentioned above after the sole application of NFAP and NFAP2 as natural biocides. Previous studies have demonstrated that NFAP and NFAP2 have different broad antifungal spectra with small overlaps [26,27], and can interact additively with each other [26], and NFAP2 synergistically with antifungal compounds [35]. According to these observations, it is possible that altering the application of NFAP and NFAP2 or their combination with other chemical and mechanical methods may overcome the risk of the recolonization by fungi.

Our results presented in this study provided insight into new natural biocides (*viz.* AFPs) derived from fungi that can be applied for the protection of cultural heritage buildings, which are mainly restricted to essential oils and plant substances today.

Acknowledgements

K.D. holds a Székely Forerunner Fellowship from the Forerunner Federation USA, and fellowship from ELTE Márton Áron Special College. The present work of L.G. was financed by the Hungarian National Research, Development and Innovation Office - NKFIH, FK 134343 project. The open access publishing was supported by the University of Szeged Open Access Fund; Grant number: 6590. The authors would like to thank Željko Savković (Institute of Botany and Botanical Garden 'Jevremovac', Faculty of Biology, University of Belgrade) for the constructive discussions and criticisms during the investigations. The authors would like to thank to Györgyi Váradi (Department of Medical Chemistry, Albert Szent-Györgyi Medical School, University of Szeged) for the semipreparative reversed-phase high-performance liquid chromatography purification of the investigated antifungal proteins.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.culher.2024.03.002.

References

[1] T. Branysova, K. Demnerova, M. Durovic, H. Stiborova, Microbial biodeterioration of cultural heritage and identification of the active agents over the last two decades, J. Cult. Herit. 55 (2022) 245–260, doi:10.1016/j.culher.2022.03.013.

[2] M. Stupar, M.L. Grbić, G.S. Simić, A. Jelikić, J. Vukojević, M. Sabovljević, A subaerial biofilms investigation and new approach in biocide application in cultural heritage conservation: holy Virgin Church (Gradac Monastery, Serbia), Indoor Built Environ. 23 (2014) 584–593, doi:10.1177/1420326X12466753.

[3] M.L. Grbić, G. Šimić, M. Stupar, A. Jelikić, M. Sabovljević, Maja Dordevic, J. Vukojevic, Biodiversity's hidden treasure: biodeteriorated archaeological tombstones of Serbia, Curr. Sci. 112 (2017) 304–310, doi:10.18520/cs/v112/i02/304-310.

[4] X. Liu, Y. Qian, Y. Wang, F. Wu, W. Wang, J.D. Gu, Innovative approaches for the processes involved in microbial biodeterioration of cultural heritage materials, Curr. Opin. Biotechnol. 75 (2022) 102716, doi:10.1016/j.copbio.2022.102716.

[5] Ž. Savković, N. Unković, M. Stupar, M. Franković, M. Jovanović, S. Erić, K. Šarić, S. Stanković, I. Dimkić, J. Vukojevic, M.L. Grbić, Diversity and biodeteriorative potential of fungal dwellers on ancient stone stela, Int. Biodeterior. Biodegrad. 115 (2016) 212–223, doi:10.1016/j.ibiod.2016.08.027.

[6] K. Kavkler, A. Demšar, Č. Tavzes, C. Gostinčar, P. Zalar, Discolouration of fungal stains on cotton textiles, Int. Biodeterior. Biodegrad. 172 (2022) 105427, doi:10.1016/j.ibiod.2022.105427.

[7] M.L. Grbić, M. Stupar, J. Vukojević, I. Maričić, N. Bungur, Molds in museum environments: biodeterioration of art photographs and wooden sculptures, Arch. Biol. Sci. 65 (2013) 955–962, doi:10.2298/ABS1303955G.

[8] Z. Liu, T. Fu, C. Hu, D. Shen, N. Macchioni, L. Sozzi, Y. Chen, J. Liu, X. Tian, Q. Ge, Z. Feng, H. Liu, Z. Zhang, J. Pan, Microbial community analysis and biodeterioration of waterlogged archaeological wood from the Nanhai No. 1 shipwreck during storage, Sci. Rep. 8 (2018) 7170, doi:10.1038/s41598-018-25484-8.

[9] Y.S. Kim, A.P. Singh, Wood as cultural heritage material and its deterioration by biotic and abiotic agents, Second. Xylem Biol. Orig. Funct. Appl. (2016) 233–257, doi:10.1016/B978-0-12-802185-9.00012-7.

[10] N. Unković, M.L. Grbić, G. Subakov-Simić, M. Stupar, J. Vukojević, A. Jelikić, D. Stanojević, Biodeteriogenic and toxigenic agents on 17th century mural paintings and façade of the old church of the Holy Ascension (Veliki Krćimir, Serbia), Indoor Built Environ. 25 (2016) 826–837, doi:10.1177/1420326X15587178.

[11] A.A. Gorbushina, J. Heyrman, T. Dornieden, M. Gonzalez-Delvalle, W.E. Krumbein, L. Laiz, K. Petersen, C. Saiz-Jimenez, J. Swings, Bacterial and fungal diversity and biodeterioration problems in mural painting environments of St. Martins church (Greene-Kreiensen, Germany), Int. Biodeterior. Biodegrad. 53 (2004) 13–24, doi:10.1016/j.ibiod.2003.07.003.

[12] O. Pepe, L. Sannino, S. Palomba, M. Anastasio, G. Blaiotta, F. Villani, G. Moschetti, Heterotrophic microorganisms in deteriorated medieval wall paintings in southern Italian churches, Microbiol. Res. 165 (2010) 21–32, doi:10.1016/j.micres.2008.03.005.

[13] N. Unković, M.L. Grbić, M. Stupar, Ž. Savković, A. Jelikić, D. Stanojević, J. Vukojević, Fungal-induced deterioration of mural paintings: *in situ* and mock-model microscopy analyses, Microsc. Microanal. 22 (2016) 410–421, doi:10.1017/S1431927616000544.

[14] N. Unković, I. Dimkić, M. Stupar, S. Stanković, J. Vukojević, M.L. Grbić, Biodegradative potential of fungal isolates from sacral ambient: *in vitro* study as risk assessment implication for the conservation of wall paintings, PLoS ONE 13 (2018) e0190922, doi:10.1371/journal.pone.0190922.

[15] K. Sterflinger, Fungi: their role in deterioration of cultural heritage, Fungal Biol. Rev. 24 (2010) 47–55, doi:10.1016/j.fbr.2010.03.003.

[16] M.R. Fidanza, G. Caneva, Natural biocides for the conservation of stone cultural heritage: a review, J. Cult. Herit. 38 (2019) 271–286, doi:10.1016/j.culher.2019.01.005.

[17] M.A. Kakakhel, F. Wu, J.D. Gu, H. Feng, K. Shah, W. Wang, Controlling biodeterioration of cultural heritage objects with biocides: a review, Int. Biodeterior. Biodegrad. 143 (2019) 104721, doi:10.1016/j.ibiod.2019.104721.

[18] D. Pinna, Can we do without biocides to cope with biofilms and lichens on stone heritage? Int. Biodeterior. Biodegrad. 172 (2022) 105437, doi:10.1016/j.ibiod.2022.105437.

[19] M. Stupar, M.L. Grbić, A. Džamić, N. Unković, M. Ristić, J. Vukojević, Antifungal activity of *Helichrysum italicum* (Roth) G. Don (Asteraceae) essential oil against fungi isolated from cultural heritage objects, Arch. Biol. Sci. 66 (2014) 1539–1545, doi:10.2298/ABS1404539S.

[20] S. Pfendler, F. Borderie, F. Bousta, L. Alaoui-Sosse, B. Alaoui-Sosse, L. Aleya, Comparison of biocides, allelopathic substances and UV-C as treatments for biofilm proliferation on heritage monuments, J. Cult. Herit. 33 (2018) 117–124, doi:10.1016/j.culher.2018.03.016.

[21] D. Maxim, L. Buc, M.I. Moza, O. Chachula, T. Biotin, Preliminary antifungal investigation of ten biocides against moulds from two different church frescoes, Ann. RSCB (2012) 17.

[22] L. Galgóczy, A. Yap, F. Marx, Cysteine-rich antifungal proteins from filamentous fungi are promising bioactive natural compounds in anti-*Candida* therapy, Isr. J. Chem. 59 (2019) 360–370, doi:10.1002/ijch.201800168.

[23] M. Gandía, A. Kakar, M. Giner-Llorca, J. Holzknecht, P. Martínez-Culebras, L. Galgóczy, F. Marx, J.F. Marcos, P. Manzanares, Potential of antifungal proteins (Afps) to control penicillium postharvest fruit decay, J. Fungi 7 (2021) 449, doi:10.3390/jof7060449.

[24] M. Virágh, D. Vörös, Z. Kele, L. Kovács, Á. Fizil, G. Lakatos, G. Maróti, Gy. Batta, C. Vágvölgyi, L. Galgóczy, Production of a defensin-like antifungal protein NFAP from *Neosartorya fischeri* in *Pichia pastoris* and its antifungal activity against filamentous fungal isolates from human infections, Protein Expr. Purif. 94 (2014) 79–84, doi:10.1016/j.pep.2013.11.003.

[25] L. Tóth, G. Váradi, É. Boros, A. Borics, H. Ficze, I. Nagy, G.K. Tóth, G. Rákhely, F. Marx, L. Galgóczy, Biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein NFAP and novel synthetic γ -Core peptides, Front. Microbiol. 11 (2020) 820, doi:10.3389/fmicb.2020.00820.

[26] L. Tóth, P. Poór, A. Ördög, G. Váradi, A. Farkas, C. Papp, G. Bende, G.K. Tóth, G. Rákhely, F. Marx, L. Galgóczy, The combination of *Neosartorya* (*Aspergillus*) *fischeri* antifungal proteins with rationally designed γ-core peptide derivatives is effective for plant and crop protection, BioControl 67 (2022) 249–262, doi:10.1007/s10526-022-10132-y.

[27] R. Kovács, F. Nagy, Z. Tóth, L. Forgács, L. Tóth, G. Váradi, G.K. Tóth, K. Vadászi, A.M. Borman, L. Majoros, L. Galgóczy, The *Neosartorya fischeri* antifungal protein 2 (NFAP2): a new potential weapon against multidrug-resistant *Candida auris* biofilms, Int. J. Mol. Sci. 22 (2021) 771, doi:10.3390/ijms22020771.
[28] R. Kovács, J. Holzknecht, Z. Hargitai, C. Papp, A. Farkas, A. Borics, L. Tóth, G. Váradi, G.K. Tóth, I. Kovács, S. Dubrac, L. Majoros, F. Marx, L. Galgóczy, *In vivo* applicability of *Neosartorya fischeri* antifungal protein 2 (NFAP2) in treatment of vulvovaginal candidiasis, Antimicrob. Agents Chemother. 63 (2019) e01777-18, doi:10.1128/AAC.01777-18.
[29] L. Kovács, M. Virágh, M. Takó, T. Papp, C. Vágvölgyi, L. Galgóczy, Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP), Peptides 32 (2011) 1724–1731, doi:10.1016/j.peptides.2011.06.022.
[30] L. Tóth, Z. Kele, A. Borics, L.G. Nagy, G. Váradi, M. Virágh, M. Takó, C. Vágvölgyi, L. Galgóczy, NFAP2, a novel cysteine-rich anti-yeast protein from *Neosartorya fischeri* NRRL 181: isolation and characterization, AMB Express 6 (2016) 75, doi:10.1186/s13568-016-0250-8.
[31] D. Danielisz, Theses of the doctoral dissertation entitled Calvinist Church Architecture of Trei Scaune (Háromszék), Budapest University of Technology and Economics Department of History of Architecture and Monument Preservation Csonka Pál Doctoral School, 2021 https://repozitorium.omikk.bme.hu/bitstream/handle/10890/28334/tezis_eng.pdf?sequence=4&isAllowed=y.
[32] T.J. White, T.D. Bruns, S.B. Lee, J.W. Taylor, Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for phylogenetics, in: PCR Protocols: A guide to Methods and Applications, Academic Press, 1990, pp. 315–322.
[33] Y.J. Liu, S. Whelen, B.D. Hall, Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit, Mol. Biol. Evol. 16 (1999) 1799–1808, doi:10.1093/oxfordjournals.molbev.a026092.
[34] C. Sonderegger, L. Galgóczy, S. Garrigues, Á. Fizil, A. Borics, P. Manzanares, N. Hegedüs, A. Huber, J.F. Marcos, G. Batta, F. Marx, A *Penicillium chrysogenum*-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses, Microb. Cell Fact. 15 (2016) 192, doi:10.1186/s12934-016-0586-4.
[35] L. Tóth, G. Váradi, A. Borics, G. Batta, Z. Kele, Á. Vendrinszky, R. Tóth, H. Ficze, G.K. Tóth, C. Vágvölgyi, F. Marx, L. Galgóczy, Anti-candidal activity and functional mapping of recombinant and synthetic *Neosartorya fischeri* antifungal protein 2 (NFAP2), Front. Microbiol. 9 (2018) 393, doi:10.3389/fmicb.2018.00393.
[36] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat Methods 9 (2012) 671–675, doi:10.1038/nmeth.2089.
[37] A. Géry, V. Séguin, J. Bonhomme, D. Garon, Draft genome sequences of three airborne *Aspergilli* series *Versicolores*, Mycobiology 50 (2022) 66–68, doi:10.1080/12298093.2022.2028437.
[38] G. Caneva, D. Isola, H.J. Lee, Y.J. Chung, Biological risk for hypogea: shared data from Etruscan tombs in Italy and ancient tombs of the Baekje Dynasty in Republic of Korea, Appl. Sci. 10 (2020) 6104, doi:10.3390/app10176104.
[39] P. Segura-Medina, M.H. Vargas, J.M. Aguilar-Romero, J.L. Arreola-Ramírez, J.L. Miguel-Reyes, J. Salas-Hernández, Mold burden in house dust and its relationship with asthma control, Respir. Med. 150 (2019) 74–80, doi:10.1016/j.rmed.2019.02.014.
[40] D. Isola, F. Bartoli, P. Meloni, G. Caneva, L. Zucconi, Black fungi and stone heritage conservation: ecological and metabolic assays for evaluating colonization potential and responses to traditional biocides, Appl. Sci. 12 (2022) 2038, doi:10.3390/app12042038.
[41] K. Bensch, J.Z. Groenewald, M. Meijer, J. Dijksterhuis, Ž. Jurjević, B. Andersen, J. Houbraken, P.W. Crous, R.A. Samson, *Cladosporium* species in indoor environments, Stud. Mycol. 89 (2018) 177–301, doi:10.1016/j.simyco.2018.03.002.
[42] D.S. Paiva, L. Fernandes, E. Pereira, J. Trovão, N. Mesquita, I. Tiago, A. Portugal, Exploring differences in culturable fungal diversity using standard freezing incubation-a case study in the limestones of Lemos Pantheon (Portugal), J. Fungi 9 (2023) 501, doi:10.3390/jof9040501.
[43] H. Paiva de Carvalho, N. Mesquita, J. Trovão, S.F. Rodríguez, A.C. Pinheiro, V. Gomes, A. Alcoforado, F. Gil, A. Portugal, Fungal contamination of paintings and wooden sculptures inside the storage room of a museum: are current norms and reference values adequate? J. Cult. Herit. 34 (2018) 268–276, doi:10.1016/j.culher.2018.05.001.
[44] G. Reboux, A.P. Bellanger, S. Roussel, F. Grenouillet, S. Sornin, R. Piarroux, J.C. Dalphin, L. Millon, Indoor mold concentration in Eastern France, Indoor Air 19 (2009) 446–453, doi:10.1111/j.1600-0668.2009.00598.x.
[45] J. Trovão, A. Portugal, F. Soares, D.S. Paiva, N. Mesquita, C. Coelho, A.C. Pinheiro, L. Catarino, F. Gil, I. Tiago, Fungal diversity and distribution across distinct biodeterioration phenomena in limestone walls of the old cathedral of Coimbra, UNESCO World Heritage Site, Int. Biodeterior. Biodegrad. 142 (2019) 91–102, doi:10.1016/j.ibiod.2019.05.008.
[46] J. Leplat, A. François, F. Bousta, *Parengyodontium album*, a frequently reported fungal species in the cultural heritage environment, Fungal Biol. Rev. 34 (2020) 126135, doi:10.1016/j.fbr.2020.06.002.
[47] M. Kacprzak, M. Stolarska, P. Lis, Culturable airborne fungi communities in naturally ventilated indoor spaces of old residential buildings in Poland, Build. Serv. Eng. Res. Technol. 44 (2023) 659–675, doi:10.1177/01436244231205363.
[48] A. Arya, A.R. Shah, S. Sadasivan, Indoor aeromycoflora of Baroda museum and deterioration of Egyptian mummy, Curr. Sci. 81 (2001) 793–799 https://www.jstor.org/stable/24106399.
[49] S.E. Favero-Longo, F. Brigadeci, A. Segimiro, S. Voyron, M. Cardinali, M. Girlanda, R. Piervittori, Biocide efficacy and consolidant effect on the mycoflora of historical stuccos in indoor environment, J. Cult. Herit. 34 (2018) 33–42, doi:10.1016/j.culher.2018.03.017.
[50] Cecilia Roux, *Leptosphaerulina chartarum* sp.nov., the teleomorph of *Pithomyces chartarum*, Trans. Br. Mycol. 86 (1986) 319–323, doi:10.1016/S0007-1536(86)80163-2.
[51] B.V. Chauhan, D.H. Jones, G. Banerjee, S. Agrawal, I.M. Sulaiman, C. Jia, P. Banerjee, Indoor bacterial and fungal burden in "Moldy" *versus* "Non-Moldy" homes: a case study employing advanced sequencing techniques in a US Metropolitan Area, Pathogens 12 (2023) 1006, doi:10.3390/pathogens12081006.
[52] Y. Wang, D. Tang, D. Duan, Y. Wang, H. Yu, Morphology, molecular characterization, and virulence of *Beauveria pseudobassiana* isolated from different hosts, J. Invertebr. Pathol. 172 (2020) 107333, doi:10.1016/j.jip.2020.107333.
[53] F. Sklenář, K. Glässnerová, Ž. Jurjević, J. Houbraken, R.A. Samson, C.M. Visagie, N. Yilmaz, J. Gené, J. Cano, A.J. Chen, A. Nováková, T. Yaguchi, M. Kolařík, V. Hubka, Taxonomy of *Aspergillus* series *Versicolores*: species reduction and lessons learned about intraspecific variability, Stud. Mycol. 102 (2022) 53–93, doi:10.3114/sim.2022.102.02.
[54] L. Galgóczy, A. Borics, M. Virágh, H. Ficze, G. Váradi, Z. Kele, F. Marx, Structural determinants of *Neosartorya fischeri* antifungal protein (NFAP) for folding, stability and antifungal activity, Sci. Rep. 7 (2017) 1963, doi:10.1038/s41598-017-02234-w.
[55] G. Váradi, Z. Kele, A. Czajlik, A. Borics, G. Bende, C. Papp, G. Rákhely, G.K. Tóth, Gy. Batta, L. Galgóczy, Hard nut to crack: solving the disulfide linkage pattern of the *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein 2, Protein Sci. 32 (2023) e4692, doi:10.1002/pro.4692.
[56] S.M. Romero, S. Giudicessi, R. Vitale, Is the fungus *Aspergillus* a threat to cultural heritage, J. Cult. Herit. 51 (2021) 107–124, doi:10.1016/j.culher.2021.08.002.
[57] F. Cappitelli, C. Cattò, F. Villa, The control of cultural heritage microbial deterioration, Microorganisms 8 (2020) 1542, doi:10.3390/microorganisms8101542.
[58] M. Correia, M.T. Neves-Petersen, A. Parracino, A. Kold di Gennaro, S.B. Petersen, Photophysics, photochemistry and energetics of UV light induced disulphide bridge disruption in apo-α-lactalbumin, J. Fluoresc. 22 (2012) 323–337, doi:10.1007/s10895-011-0963-7.
[59] P. Wongkongkathep, H. Li, X. Zhang, R.R. Loo, R.R. Julian, J.A. Loo, Enhancing protein disulfide bond cleavage by UV excitation and electron capture dissociation for top-down mass spectrometry, Int. J. Mass Spectrom. 390 (2015) 137–145, doi:10.1016/j.ijms.2015.07.008.
[60] P.M. Martin-Sanchez, A. Nováková, F. Bastian, C. Alabouvette, C. Saiz-Jimenez, Use of biocides for the control of fungal outbreaks in subterranean environments: the case of the Lascaux Cave in France, Environ. Sci. Technol. 46 (2012) 3762–3770, doi:10.1021/es2040625.
[61] M. Villar-dePablo, C. Ascaso, E. Rodríguez-Pérez, M. Urizal, J. Wierzchos, S. Pérez-Ortega, A. de Los Ríos, Innovative approaches to accurately assess the effectiveness of biocide-based treatments to fight biodeterioration of cultural heritage monuments, Sci. Total Environ. 897 (2023) 165318, doi:10.1016/j.scitotenv.2023.165318.
[62] G. Toreno, L. Zucconi, G. Caneva, P. Meloni, D. Isola, Recolonization dynamics of marble monuments after cleaning treatments: a nine-year follow-up study, Sci. Total Environ. 912 (2024) 169350, doi:10.1016/j.scitotenv.2023.169350.