Research article

Fungal deterioration of limestone false-door monument

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ABSTRACT

Unfortunately, monuments all over the world may become discolored and degraded as a result of the growth and activity of fungi. Biodeterioration is an irreversible damage that is caused by microbial colonization on the surface of buildings. Different fungi were isolated from limestone False-door in Kom Aushim museum- El-Fayoum governorate, Egypt. These include; *Aspergillus niger, A. fumigatus, A. sulphureus, A. flavus, Alternaria alternata, Alternaria spp.,* and *Cladosporium herbarium.* Fungal grow on modern limestone surfaces after 60 days of infection. Transmission electron microscope demonstrated the penetration and presence of fungal threads inside limestone. Environmental Scanning electron microscope attached with EDX Unit revealed an increase in carbon and magnesium ions from 9.16 to 12.17% and 1.41–1.51%, respectively after fungal infection of limestone, while other ions decreased after infection; aluminum from 1.96 to 1.39%, silicon from 7.40 to 3.57%, potassium from 0.44 to 0.41%, calcium from 41.41 to 35.04 % and iron from 1.08 to 0.90 %. *p*-Chloro-*m*-crysol is the most potent to inhibit the growth of isolated fungi at MIC 50 ppm for most fungal species.

1. Introduction

A false-door, built into the west wall of the tomb chapel, was the focal point of the cult of the deceased in ancient Egypt. Bard (1999) reported that the Ancient Egyptians believed that the False-door was a threshold between the worlds of the living and the dead, and through which a deity or the spirit of the deceased could enter and exit. In pharaonic period the limestone was extensively used in the Egyptian buildings (Tawfik, 2015). The nature of the limestone structure and the environmental conditions influence the extent of microbial colonization and the biodeterioration processes. Most the world's stone cultural heritage monuments have suffered severe and irreversible degradation and deterioration from microorganisms (Lan et al., 2010; Cuezva et al., 2012; Saiz-Jimenez et al., 2012; Morón-Ríos et al., 2017). Therefore numerous geological and microbiological studies were performed on limestone of buildings (Ammar and El-Deeb, 1992; Borghi et al., 2015; Tawfik, 2015).

Biodeterioration by microorganisms is predominantly observed not only on items of cultural heritage, stone artifacts such as historical moments but on modern materials, buildings, museums and private collections (Warscheid and Braams, 2000; Lan et al., 2010; Abd El-Ghany, 2013; Gu et al., 2013). Biodeterioration of archeological stones showed both prokaryotic and eukaryotic organisms contribute to stone deterioration. Mosses and liverworts have received comparatively less attention because their impact has been considered primarily aesthetic (Tomaselli, 2003). Early scientific papers suggested that the coexistence of living organisms on stone increased its sensitivity to damage through their water-binding capacity, the mineralogy, porosity, surface roughness and capacity to collect water and organic material will control its receptivity (Guillitte, 1995; Krumbein and Gobushina, 1995). Fungi have a major deteriogenic potential than bacteria, as they secrete more levels of organic acids (Palmer and Hirsch, 1991; Makky and Abdel Ghany, 2009; Abdel Ghany and Al Abboud, 2014; Abdelghany et al., 2017; Abdel Ghany et al., 2018), and have been detected on degraded stone buildings in temperate and tropical zones (Warscheid and Braams, 2000; Gaylarde and Gaylarde, 2005). In addition, these fungi may cause physical degradation of stone by the growth of hyphal networks through the pore space system (Urzi et al., 2000).

Inorganic compounds of limestone are considered good substrates for a major number of diverse microorganisms. So, the fungi are able to obtain several elements that they need for their metabolism from

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limestone by solubilization through production of various organic acids (Warscheid et al., 1991). Several fungal species produced calcium oxalates and may be capable of modulating or regulating biomineralization on limestone surfaces depending on the species with which they interact (Morón-Ríos et al., 2017).

According to previous study (Feng et al.,1998), culturable fungi from six ancient caves were isolated, and several genera comprise *Aspergillus* and *Cladosporium*, were associated with accelerated aging of cementing materials and the discoloration of mural paintings. Three xerophilic fungi (*Eurotium amstelodami, E. chevalieri, E. repens*), and six non-xerophilic strains (*Alternaria alternata, Aspergillus terrus, Aspergillus versicolor, Cladosporium herbarum, Fusarium moniliforme* and *Penicillium chrysogenium*) were isolated from Al-Shatby and El-Anfoushi archeological tombs in El-Anfoushi and Al-Shatby Alexandria- Egypt, respectively (Hala and Neveen, 2011).

The effect of tourists and environmental factors were considered the main factors contributing to biodeterioration of historic buildings and stone monuments (Wang et al., 2010). A previous reports (Tiano et al., 1995; Li et al., 2016) demonstrated that the ecological factors including light intensity, temperature, pH and air humidity affect the number, types and distribution of limestones colonizing species. In addition, the impact of NO_2 and SO_2 concentration on the bacterial and fungal communities' diversity was evaluated on Stone Monuments (Li et al., 2016).

Microorganisms causing biodeterioration on monuments have usually been controlled by biocides but the biocides have since been shown to be effective for only a short period of time (Gu et al., 2013). Study on the activity of biocide products in the elimination of the biological agents that cause a biodeterioration on stone monuments was reported (Nugari and Salvadori, 2003); Abdelhafez et al. (2012), also proved that the biocide products generally have a different effect on eukaryotic and prokaryotic microorganisms. The present work is aimed at recognizing and identify the fungi cause of limestone False-door in the Egyptian museum biodeterioration and suggest methods of treatment.

2. Materials and methods

2.1. The site of fungal isolation

False-door present in Kom Aushim museum- El-Fayoum governorate, Egypt composed of limestone from Ancient Egyptians. The interior conditions in the museum including relative humidity 55–60%, artificial light 50–150 lux and temperature 20 °C.

2.2. Fungal isolation from sample collection

False-door infected colonies (Fig. 1) are taken by sterilized swabs, then suspended into a sterile saline solution. Suspension (1ml) was spread on a surface of sterilized plates containing Czapek's agar media and by scratching the infected area by a sterilized surgical cutter (scalpel) directly on a surface of sterilized agar plates containing a suitable medium.

2.3. Airborne fungal flora inside the museum

Six sterilized Petri dishes containing Czapek's agar media are opened in different parts of the Egyptian museum containing False-door for 60 min to isolate the airborne fungal flora inside the museum.

2.4. Purification and characterization of causative fungal isolates

Different growth media including Czapek's agar, potato dextrose agar, and oatmeal agar were used for fungal isolation and identification. After an incubation period (6 days) all Petri dishes containing fungal isolates from the False-door and airborne fungal flora from inside the museum, the isolates of the causative organisms were purified and then investigated microscopically. Identification of the isolates were accomplished depending on the macroscopic characteristics such as colony diameter, texture, pigmentation, margin appearance, style, coloration, texture, density, colony elevation, and exudates secretion, to establish a morphotype classification, microscopic features were evaluated by observing slides prepared from secondary cultures attending to determine color, size, and morphology of the vegetative and reproductive structures by using the KS 300 measuring program for performing of all measurements and comparing it with that are present in the identification references (Gilman, 1998; Domsch et al., 1980a and b; Klich, 2001).

2.5. Infection of modern limestone with fungal isolates

Modern pieces of limestone from El-mokatam zone Egypt, the specimen was used to determine the effect of fungal isolates on the deterioration of these modern pieces. The pieces were inoculated with the spore suspension of each specific isolate by spraying 5 ml of a suspension of each fungal isolate, containing 0.5×10^6 spores/ml. The infected modern pieces were incubated for 60 days at ambient temperature and 60–70% humidity. Morphological and chemical properties of limestone pieces were determined before and after inoculation as described below.

2.6. Morphological of limestone before and after fungal infection

Morphological observations of limestone infected by fungal isolates after 60 days at room temperature, obtained by Binocular dissecting stereomicroscope. Chemical properties of modern limestone before and after infection were determined by Environmental Scanning electron microscope (ESEM) as follow:

Fig. 1. Fungal infection on limestone (Black spots) of False-door.

ESEM Model Quanta 250 FEG (Field Emission Gun) attached with EDX Unit (Energy Dispersive X-ray Analyses) with accelerating voltage 30 Kv, magnification 14x up to 10^6 used to analyze specimen elements at magnification power ranging from 500–10000X (Ministry of petroleum, the Egyptian mineral resources authority, Central laboratories sector). *Scanning Electron Microscope* (SEM) equipped with *Energy Dispersive Spectrum* (EDS) microanalysis detector is an excellent tool to analyze the archaeological objects (Goldstein et al., 2003 and Tite et al., 1982). Moreover, it is non-destructive and capable of providing characteristic information on the composition and percentage of minerals present in samples (Palanivel and Meyvel, 2010).

2.7. Determination of minimal inhibitory concentration (MIC) of antifungal agents against the isolated fungi

Three commercially available fungicides were purchased from Aldrich Company (Germany) and used for testing their effect against the isolated fungi and determining their minimal inhibitory concentrations. These fungicides were Dichloroxylenol, thymol, *p*-chloro-*m*-crysol. Stock solution of each fungicide was prepared by dissolving 1 g/L acetone 95%. Gradient concentrations of each fungicide ranged from 12ppm to 200 ppm were prepared by diluting the stock solution with acetone. One ml of fungal spore suspension was spread on Czapek's agar plate. A cork pourer was used to make three pores in each Czapek's agar plate. In one plate 100μl of each concentration (from 12 ppm to 200 ppm) of the tested fungicides were placed in each pore. Plates were incubated at 30 °C for 3 days comparing with control plates (acetone instead of fungicides). The minimal inhibitory concentration (MIC) was determined by measuring the inhibition zone (Brantner et al., 1993).

2.8. In vivio treatment of false-door

The brushing method was used for the treatment of the spots spread all over the False-door with using p-chloro-*m*-crysol. For the brushing method, 50 ppm of p-chloro-*m*-crysol was applied to the False-door surface by 5 brushing intervals over a period of 1 h. Each brushing interval lasted 1 min followed by a 5-min break.

3. Results and discussion

Airborne detection of fungi in museum revealed the presence of several fungal species including *Aspergillus niger, A. fumigatus, A. sulphureus* and *A. flavus* (Figs. 2 and 3). On the other hand, the fungal swabs cultures from False-door contained the same fungal species of aspergilli, with the addition to other genera including *Alternaria alternata, Alternaria sp.* and *Cladosporium herbarum* (Figs. 2 and 3). The diversity of airborne fungi in museum confirms that a large number of propagules may

Fig. 2. Fungal isolates colonies from airborn of Museum(A) and False-door (F).

potentially reach and colonize the False-door surface. Therefore, according to our findings, limestone surfaces could act as a reservoir of fungal species and function as a fungal source via the dispersion of species under optimum conditions. Recently Farooq et al. (2015) reported that several fungal species including *Aspergillus fumigatus, A. flavus, A. niger, Cladosporium herbarum, Curvularia lunata, Dematium spp., Fusarium oxysporum, Mucor hiemalis, Penicillium chrysogenum, P. frequentans, Alternaria alternata* and *Rhizopus oryzae* were widespread on stone monuments in Dharmarajika Stupa (One of the eight shrines constructed in the 3rd century BC during the reign of Emperor Ashoka of the Mauryan dynasty). Our results detected pigment producing fungi such as *Alternaria alternata, Alternaria spp.,Cladosporium herbarum*. Therefore, blackening of antiquities structures is often due to the colonization by darkly pigmented fungi containing melanin. Several studies indicated that the fungal community contained melanin at some or all of their reproductive stages (74% of the species), in which the dominant fungi isolated from monuments also contained pigmentation (Gorbushina et al., 2002; Lan et al., 2010). Some investigator considers lichens and fungi to be the primary agents leading to limestone decay, while others have suggested that biological attack might be followed by chemical and physical agents (Dakal and Arora, 2012; Li et al., 2016).

Morphological examination by Binocular dissecting stereomicroscope revealed fungal infiltration, a mat of filamentous fungal threads or hyphaeon limestone sample after infection, black spots and slight roughness was appeared (Fig. 4). The fungal colonization of buildings may initiate shortly after construction but the forming of biofilm usually takes numerous years (Barberousse et al., 2006; Gómez-Cornelio et al., 2012; Adamson et al., 2013). Scanning electron micrograph of limestone after fungal infection demonstrated the penetration and presence of fungal threads inside limestone (Fig. 5). Fungi may cause physical biodegradation of stone by the growth of hyphal networks through the pore space system (Urzi et al., 2000). Sterflinger (2000) suggested that *Aspergillus niger, Penicillium simplissimum* and *Scopulariopsis brevicaulis* were important deteriogenic fungi attacking siliceous stone. Various metabolic substances excreted by fungi are coloured, leading to significant aesthetic alterations, and physical stress (Sterflinger, 2000; Burford et al., 2003).

EDX microanalysis of limestone prior and after infection (Fig. 6 and Table 1) with mixture of isolated fungi (*Aspergillus niger, A. fumigatus, A. sulphureus, A. flavus, Alternaria alternata, Alternaria sp.* and *Cladosporium herbarum*) showed that the samples essentially consist of carbon (C) calcium (Ca), silicon (Si), magnesium (Mg), aluminum (Al), potassium (K), iron (Fe). Data recorded in Table 1 show that significant increase detected in carbon and magnesium from 9.16 to 12.17% and 1.41–1.51%, respectively, while other ions decreased after infection; Al from 1.96 to 1.39%, Si from 7.40 to 3.57%, K from 0.44 to 0.41%, Ca from 41.41 to 35.04 % and iron from 1.08 to 0.90 %. Caneva and Salvadori (1989) stated that the deterioration of limestone is due to the action of excreted oxalic and citric acids, the acids by numerous species of fungi, function as chelating agents that can leach iron, calcium, magnesium, from the stone surface. The release of calcium from the tested limestone samples after fungal degradation was detected by Hala and Neveen (2011).

Chemical formulates with biocidal action were used for biodeterioration processes control. Minimal inhibitory concentration (MIC) of tested antifungal compounds was differed depending on the type of antifungal compound and fungal species (Table 2). In general, MIC patterns of p-chloro-*m*-crysol did not show much variation between fungal isolates. Therefore p-chloro-*m*-crysol represent the most potent to inhibit the growth of all isolated fungi at MIC (50 ppm), however, MIC of dichloroxylenol was less than *p*-chloro-meta-crysol for particular isolates including *A. flavus* and *A. sulphureus*. MIC values of Thymol and dichloroxylenol against different fungal isolates in the current study were in the range of 12–100ppm. Biocides have a broad-spectrum activity against microbial growth on inanimate surfaces and living tissue. Their action mechanisms have been extensively studied, as has fungal
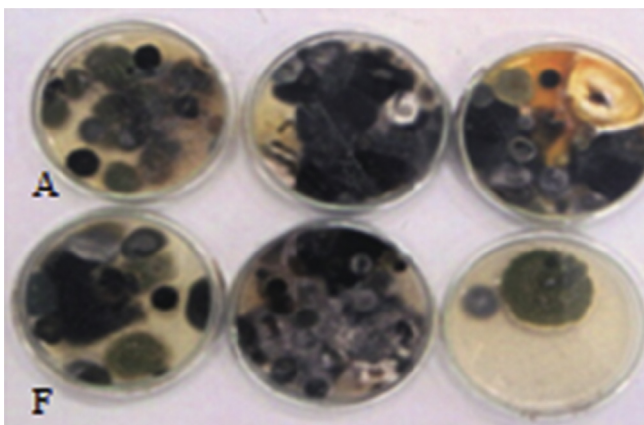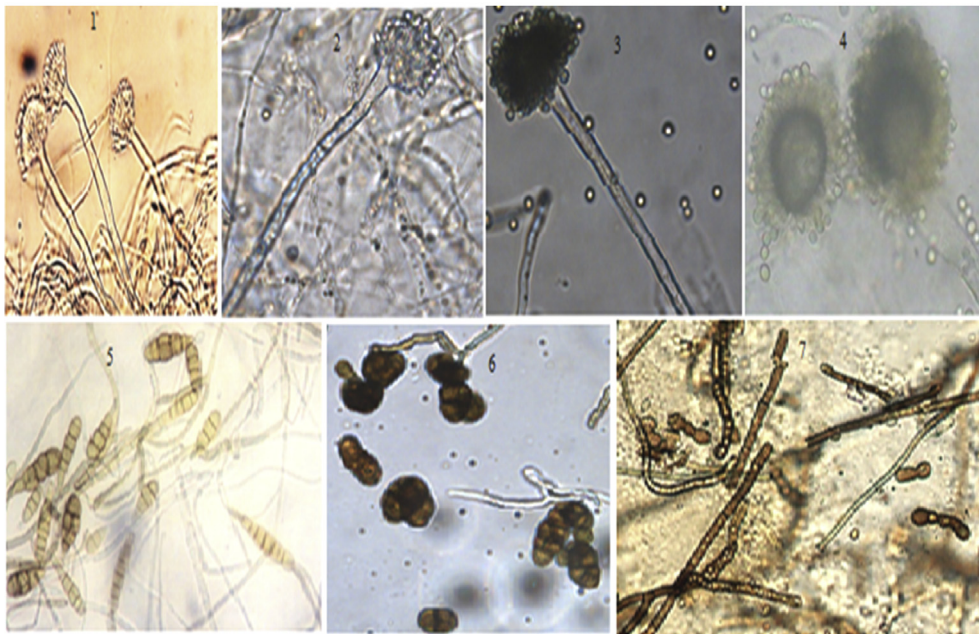
Fig. 3. Morphological character (X-400) of *Aspergillus sulphureus* 1, *A. fumigatus* 2, *A. flavus* 3, *A. niger* 4, *Alternaria sp.* 5, *Alternaria alternata* 6 and *Cladosporium herbarum* 7.
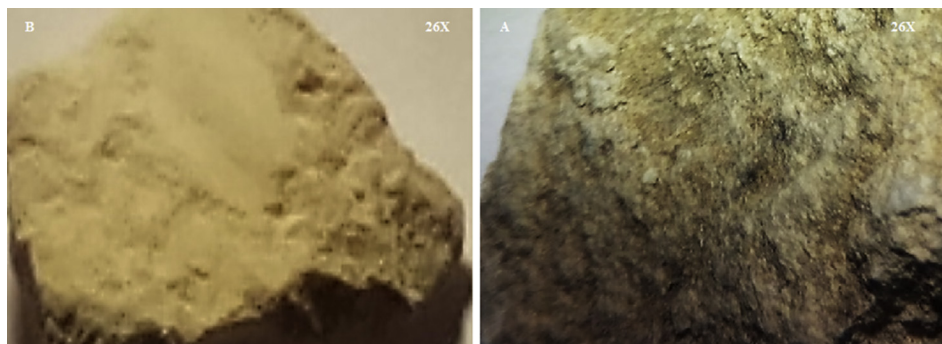

Fig. 4. Binocular Stereo microscopes of modernlime stone sample before infection(B). and after infection (A) for 60 days.
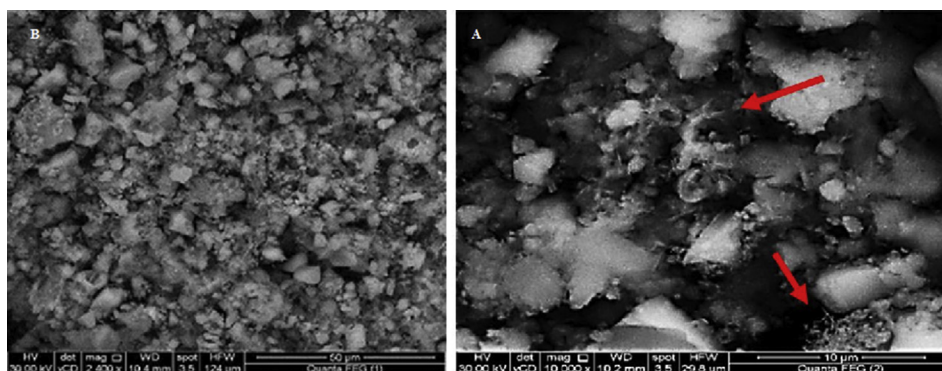

Fig. 5. Scanning electron micrograph of modern limestone sample prior infection(B). Magnification x2400, scale bar = 50microns and after infection(A) Magnification X10 000.

resistance to them (Sandle et al., 2014; Kalkanci et al., 2015).

Earlier, Ammar and El-Deeb (1992) used a mixture of povidone-iodine and dichloroxylenol for the treatment of microbial biodeterioration on limestone inside Tut Ankh Amon tomb, Luxer Egypt. Fungal isolates from limestone including *Aspergillus niger, A. flavus, A. sulphureus, Cladosporium herbarum, Alternaria alternata, Stachybotrys*

chartarum were inhibited by 600 ppm of dichloroxylenol and 400 ppm thymol as MIC (Abdelhafez et al., 2012) confirmed that 600 ppm of dichloroxylenol and 400 ppm thymol were the MIC that prevents *Aspergillus niger, A. flavus, A. sulphureus, Cladosporium herbarum, Alternaria alternata, Stachybotrys chartarum* which isolated from limestone. pH and conductivity of limestone were recorded to confirm the fungicidal or
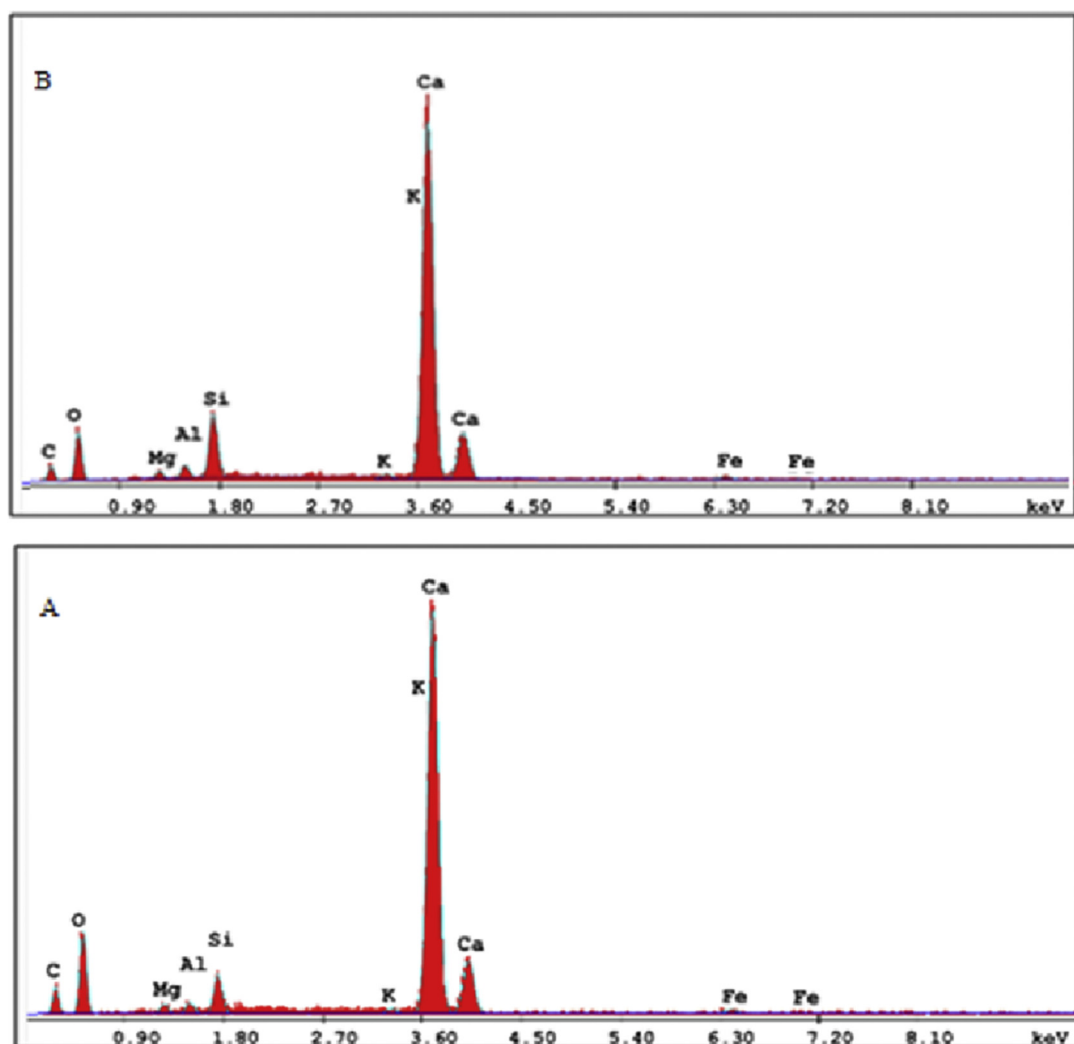
Fig. 6. EDX-spectrum of limestone before(B) and after (A) fungal infection.

Table 1
Chemical analysis of elements in modern limestone sample before and after fungal infection for 2 months.

Element	Element (Wt %)	
	Before fungal infection	After fungal infection
Carbon	9.61	12.17
Calcium	41.41	35.04
Silicon	7.40	3.57
Magnesium	1.41	1.51
Aluminum	1.96	1.39
Potassium	0.44	0.41
Oxygen	36.67	45.02
Iron	1.08	0.90

Table 2
Minimum inhibitory concentration (ppm) of fungicides against fungal isolates from limestone.

Fungus test	Dichloroxylenol		Thymol		p-chloro-m-crysol	
	*Mean diam. (mm)	MIC (ppm)	*Mean diam. (mm)	MIC (ppm)	*Mean diam. (mm)	MIC (ppm)
A. flavus	20	12	21	50	31	50
A. sulphureus	22	15	24	50	35	50
Alternaria alternata	24	100	42	100	24	50
Altrnaria sp	18	50	20	12	27	25
Cladosporium herbarum	20	100	20	100	22	50

* Inhibition zone diameter (mm).

fungistatic of fungicides used *invivid*, where pH and conductivity decreased by the addition of solvent (Aceton) and fungicide (*p*-chloro-*m*-crysol) to the limestone (Table 3), this decrease gave an unfavorable condition for the microbial growth. *p*-chloro-*m*-crysol at a concentrationof 50 ppm in acetone was applied tothe False-door by using the brush method (Fig. 7). The efficiency of the most potent inhibit material biocide (p-chloro-m-crysol at 50 ppm) can be determined according to the result of microbial swabs taken from the False-door after complete applying the microbial treatment, these results were recorded (Table 4). Data recorded in the current study indicate that there is no microbial growth appeared in the plates after finished the incubation period, which

gave excellent results for the antifungal used (*P*-chloro-m-crysol) and applying method.

4. Conclusion

The False-door Monument are a sign of human civilization in egypt. A specific distribution of fungi across the limestone False-door Monument was observed. Numerous fungi were evidenced, indicating the high heterogeneity of occurring mycoflora. The presence of some fungal species can cause many aesthetical damages of world fame archaeological

Table 3
pH and conductivity of limestone.

sample	pH	Conductivity
Limestone	7.8	96.8
Acetone	7.7	59.2
Limestone + Acetone	6.0	42.5
Limestone + Acteone + *p*-chloro-*m*-crysol	6.5	4.8

Fig. 7. *p*-chloro-*m*-crysol at concentration 50 ppm applying for False-door. Treatment by brush method.

Table 4
Swab culture from False-door after applying biocide *p*-chloro-*m*-crysol at 50 ppm by brush method.

Application Time (Day)	Different localities of False-door				
	Upper left	Upper right	Center	Down left	Down right
Direct treatment	-ve*	-ve*	-ve*	-ve*	-ve*
1	-ve*	-ve*	-ve*	-ve*	-ve*
2	-ve*	-ve*	-ve*	-ve*	-ve*
60	-ve*	-ve*	-ve*	-ve*	-ve*

*-ve, fungal colonies not grow.

because these fungi have been reported as biodeteriogens of cultural heritage especially stone based monuments. Therefore detection and characterization of biodeterioration are necessary before any restoration and conservation treatments. Our results confirmed the well-known efficacy of low concentrations of biocides, and suggested the potential usage of P-chloro-m-crysol as agents in limestone False-door Monument and other limestone buildings conservation.

Declarations

Author contribution statement

Tarek M. Abdel Ghany: Conceived and designed the experiments; Wrote the paper.

Omar A.M.: Conceived and designed the experiments; Analyzed and interpreted the data.

Fatma M Elwkeel: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Mohamed A. Al Abboud: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Alawlaqi M.M. Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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