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Biocidal Effect of Gamma Radiation on the Ecology of Filamentous Fungal Populations Associated with Stone Deterioration

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Abstract: This study focused on the identification of the filamentous fungi associated with soapstone samples exposed to outdoor conditions and the biocidal effect of gamma radiations on isolated fungal populations in Minas Gerais State in Brazil. Two soapstone blocks were placed outdoors under tropical environmental conditions for 12 months. A total of 9 filamentous fungal populations were identified on their surfaces, namely *Acremomium* (cf.) *alternatum*, *Alternaria alternata*, *Aspergillus fumigatus*, *Calcarisporium* (cf.) *arbuscula*, *Cladosporium cladosporioides*, *Curvularia lunata*, *Epicoccum nigrum*, *Fusarium equiseti* and *Penicillium citrinum.* The gamma radiation assay was then carried out as a test of biocidal action by exposing all fungal populations to the ionizing radiation. The results showed that only the *C. cladosporioides* species was resistant to this biocidal agent, since it was able to increase its population post exposure. Scanning electron microscopy images identified the microbial colonization on the soapstone blocks and the stone elementar composition was analyzed by energy dispersive X-ray spectrometry. After treatment, there was no structural and aesthetic alteration in the soapstone samples, and evidencing that gamma radiation can be used as a biocidal agent. However, the resistance of the black fungal population indicates caution in the choice of gamma irradiation as biocidal treatment.

Key words: Gamma radiation, biocidal, stone, ecology, filamentous fungi.

1. Introduction

During the colonial period in the State of Minas Gerais, Brazil, geological specimens such as soapstone (steatites) were used as important architectural elements, in church and palace coverings, fountains, ornamental and mainly sculptural architectures [1], especially in Baroque and Rococo art forms [2]. The soapstone was used mainly in pieces with finer and delicate carvings, such as medallions, images, sculptures and came in different shades owing to the variations in its mineralogical composition [3].

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The major component in soapstone is talc, with varying proportions of magnesite, carbonate, amphibole and opaque chlorite, with its gray coloring varying according to the distribution of the constituent minerals [4]. When weathered, they are brownish owing to oxides and iron hydroxides, and have macroscopic pores and cavities resulting from carbonate leaching [5].

Ionizing radiation is an alternative to commonly used biocidal treatment. Gamma irradiation of materials is used for sterilization and is often applied to materials for hospital use and in the food and pharmaceutical industries. The assay of irradiation in soapstone proposed in this work is unpublished, and therefore, there are no descriptions in the literature. A high-energy radiation can cause ionization where it is absorbed and can remove electrons from its orbitals in atoms or molecules, hence termed ionizing radiation [6]. When an ionizing radiation is absorbed by a biological material, it can act directly on critical targets in the cell. Nucleic acid molecules that are ionized and excited can lead to biological changes and even cell death. This explains the direct effect of radiation, which is the dominant process when dry spores of microorganisms are irradiated [7].

The use of stone in the construction of monuments and sculptures has had some consequences over time, like the appearance of patinas [8] such as black films or biofilms [9]. These patinas have been identified in granite substrates [10-12], marble [13], all over the world, like Italian stone monuments [14] and historical monuments in Brazil [15-17].

In addition to factors related to the microbial action on the stone surface, environmental conditions, such as temperature, relative humidity, osmotic variation and nutritional conditions, influence the prevalence of microorganisms in the colonized area [18]. For this reason, alternative research on biocidal action, such as the use of gamma radiation instead of chemical agents, is extremely relevant in order to avoid the impact of chemical agents on the environment and on stony historic monuments.

Herein, the results of the identification of filamentous fungi isolated from soapstone blocks before and after exposure to gamma irradiation were reported. Chemical and microscopy analyses were performed to investigate the interactions between microorganisms and the soapstone surface of dimensional soapstone, as seen in blocks from Minas Gerais, Brazil.

2. Material and Methods

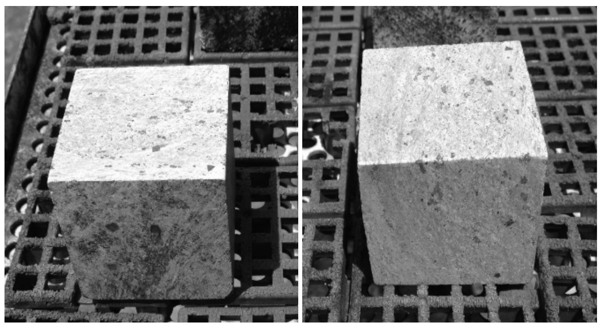
2.1 Sample Collection and Fungal Isolation

Two soapstone blocks of 5×5 cm^2 were exposed to outdoor tropical conditions for 12 months. Sample collections were made twice: the first was before the exposure to gamma radiation, and the second after gamma irradiation. A sterile spatula was used to obtain pulverized material from the surface of the stone blocks. The material was then placed in sterile Petri dishes and subsequently closed and transported to the Mycology Laboratory at Federal University of Minas Gerais for processing (Fig. 1).

A mixture of 1 mg of the pulverized material collected in 9 mL of saline solution was subjected to serial dilutions between 10^{-1} and 10^{-3} with 0.85% saline solution supplemented with 0.001% Tween 80. The prepared samples were plated on Czapek Dox Agar (Difco Laboratories, Detroit, MI, USA) by the spread plate method and incubated for 14 days at 27 °C [15]. Fungal colonies were isolated and identified by slide culture and visualization with light microscopy followed by DNA sequencing. CFUs (Colony Forming Units) were counted, and subcultures were made of any morphologically distinct colony observed in each sample. Long-term preservation of fungi was carried out at -80 °C in cryotubes with sterile 15% glycerol.

2.2 Fungal Identification

DNA extraction from filamentous fungi followed the protocol from Godinho, V. M., et al. [19]. The ITS (Internal Transcribed Spacer) region was amplified with the universal primers ITS1 and ITS4 [20]. The



(A) (B)

Fig. 1 Soapstone blocks (A and B) of dimensions 50 × 50 mm exposed outdoor in tropical conditions for 12 months before the gamma radiation assay.

amplification of ITS region was performed as described by Ferreira, M. C., et al. [21].

The amplicon was purified using the ExoSAP-IT PCR Clean-up kit (GE, Healthcare, Sunnyvale, CA, USA) according to the manufacturer's instructions. The bands purified were sequenced in a Sequenciator DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). The obtained sequences were analyzed with SeqManP with Lasergene software (DNASTAR Inc., Madison, WI, USA), and a consensus sequence was obtained using Bioedit v. 7.0.5.3 software (Carlsbad, ON, Canada). To achieve species-rank identification based on ITS, the consensus sequence was aligned with all sequences from related species retrieved from the NCBI (National Centre for Biotechnology Information) GenBank database using BLAST (Nucleotide Basic Local Alignment Search Tool) [22].

2.3 Gamma Irradiation Assay

The sensitivity of macromolecules to radiation is proportional to their molecular weight [23], and for the disinfection of filamentous fungi, a dose of 3 to 8 kGy is required [7]. The soapstone samples were exposed to gamma radiation for 97 minutes at rate 6,218.8 Gy/h at 10 cm. Stone samples were collected again after exposure.

2.4 SEM (Scanning Electron Microscopy) and EDS (Energy Dispersive X-Ray Spectroscopy)

SE (Secondary Electron) images obtained by SEM were used to evaluate the structural composition of the fungal community before gamma irradiation exposition. EDS is a relevant tool in the study of microscopic characterization of materials. The use of this tool in conjunction with BSE (Backscattered Electron) images allows better petrographic characterization. EDS detector installed in the SEM vacuum chamber measures the energy associated with the characteristic electrons transitions to determine which chemical elements are present in the sample. The samples were prepared with carbon coating and analyzed (Jeol 6360LV-Thermoloran).

3. Results

3.1 Chemical Characterization of Soapstone

The EDS analysis showed a similar pattern for the distribution of the chemical elements that made up both soapstone blocks (blocks A and B) (Fig. 2). In relation to block A (Fig. 3), the following elements were detected: ${}_{6}C$, ${}_{8}O$, ${}_{12}Mg$, ${}_{13}Al$, ${}_{14}Si$, ${}_{20}Ca$, ${}_{24}Cr$, ${}_{25}Mn$ and ${}_{26}Fe$. The analysis of block B showed the same chemical components, except for the absence of

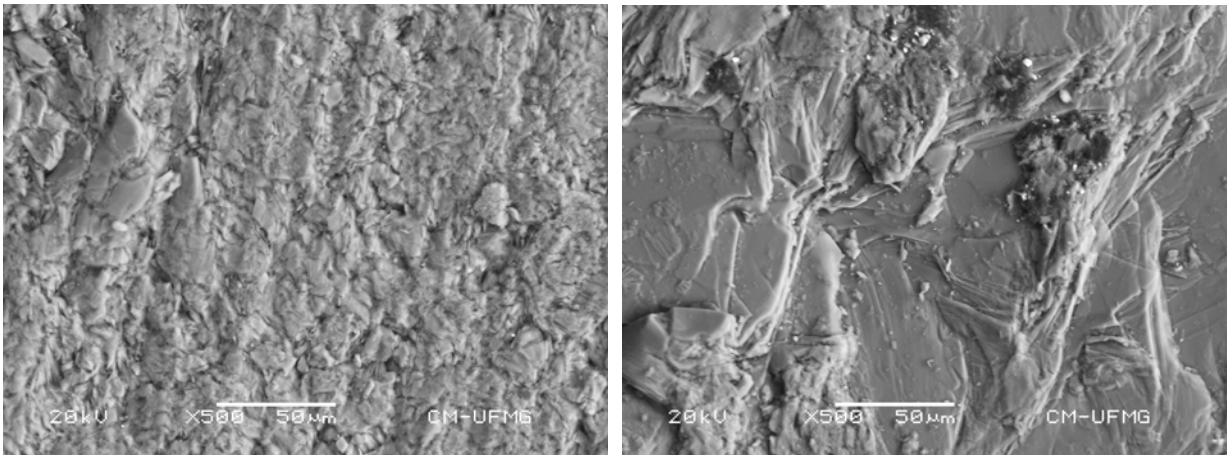


Fig. 2 BSE images from the surface of soapstone blocks analyzed by EDS: block A (left) and block B (right).

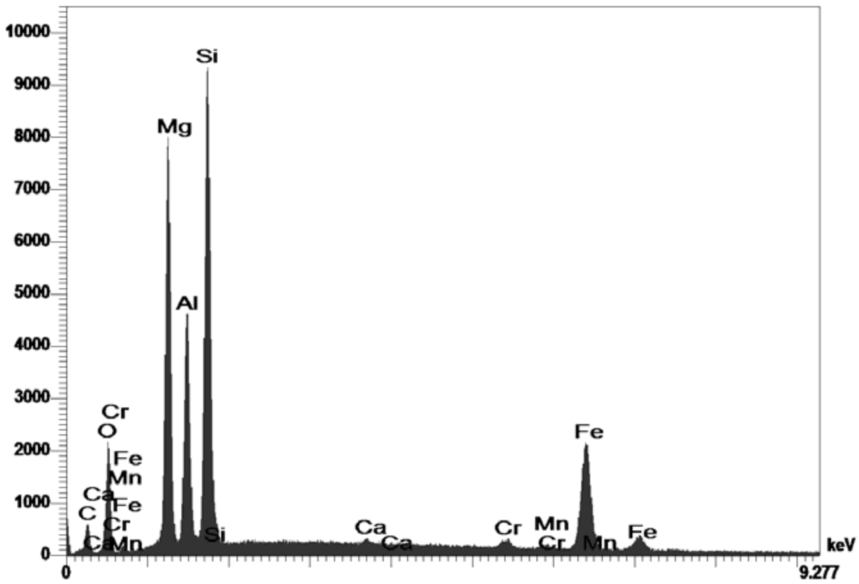


Fig. 3 X-ray fluorescence spectrum showing the chemical elements in soapstone block A.

Table 1 Fungal species colonizing the soapstone and the CFU/mL obtained before exposure to gamma radiation. Blocks A and B were outdoor exposed for 12 months under tropical conditions in Brazil.

Species of filamentous fungi	Top BLAST search results	Similarity	CFU/mL Block A	CFU/mL Block B
Acremomium (cf.) *alternatum*	AB693789.1	95%	50	40
Alternaria alternata	KT280009.1	99%	114	-
Aspergillus fumigatus	DS499594.1	99%	267	50
Calcarisporium (cf.) *arbuscula*	KC800713.1	95%	21	-
Cladosporium cladosporioides	KF619558.1	100%	253	57
Curvularia lunata	DQ836800.1	100%	-	46
Epicoccum nigrum	JN578611.1	99%	-	85
Fusarium equiseti	KJ412506.1	99%	-	27
Penicillium citrinum	NR121224.1	100%	102	89

CFU/mL = Colony Forming Unit.

manganese: $_{6}C$, $_{8}O$, $_{12}Mg$, $_{13}Al$, $_{14}Si$, $_{20}Ca$, $_{24}Cr$ and $_{26}Fe$.

3.2 Effect of Gamma Radiation on the Fungal Species Colonizing Soapstone Blocks

Table 1 shows the species of filamentous fungi colonizing primary soapstone before the gamma irradiation. After exposure to gamma radiation, a new collection was performed following the same parameters described, including the techniques of culture, isolation and fungal identification. Table 2 shows the effect of gamma irradiation on the fungal species that had colonized the soapstone block.

Exposure of the two blocks to gamma radiation caused a biocidal effect on 88.89% of the pioneer microbial communities. The only fungal species surviving the irradiation was *C. cladosporioides*.

An inherent characteristic of this species is the production of dark and black dyes, which may have functioned as a protection factor against the gamma radiation, thereby allowing its survival. The CFU/mL after exposure to gamma radiation revealed a high population of *C. cladosporioides*; however, there was an observed decrease in the diameter of the colonies in Petri dishes (data not shown).

3.3 Characterization of Fungal Populations by SE

SE images showed a region of irregular surface on

Table 2 Fungal species colonizing the soapstone and the CFU/mL obtained after exposure to gamma radiation. Both blocks were outdoor exposed for 12 months under tropical conditions in Brazil.

Species of filamentous fungi	Top BLAST search results	Similarity	CFU/mL Block A	CFU/mL Block B
Acremomium (cf.) *alternatum*	-	-	-	-
Alternaria alternata	-	-	-	-
Aspergillus fumigatus	-	-	-	-
Calcarisporium (cf.) *arbuscula*	-	-	-	-
Cladosporium cladosporioides	KF619558.1	100%	844	190
Curvularia lunata	-	-	-	-
Epicoccum nigrum	-	-	-	-
Fusarium equiseti	-	-	-	-
Penicillium citrinum	-	-	-	-

CFU/mL = Colony Forming Unit.

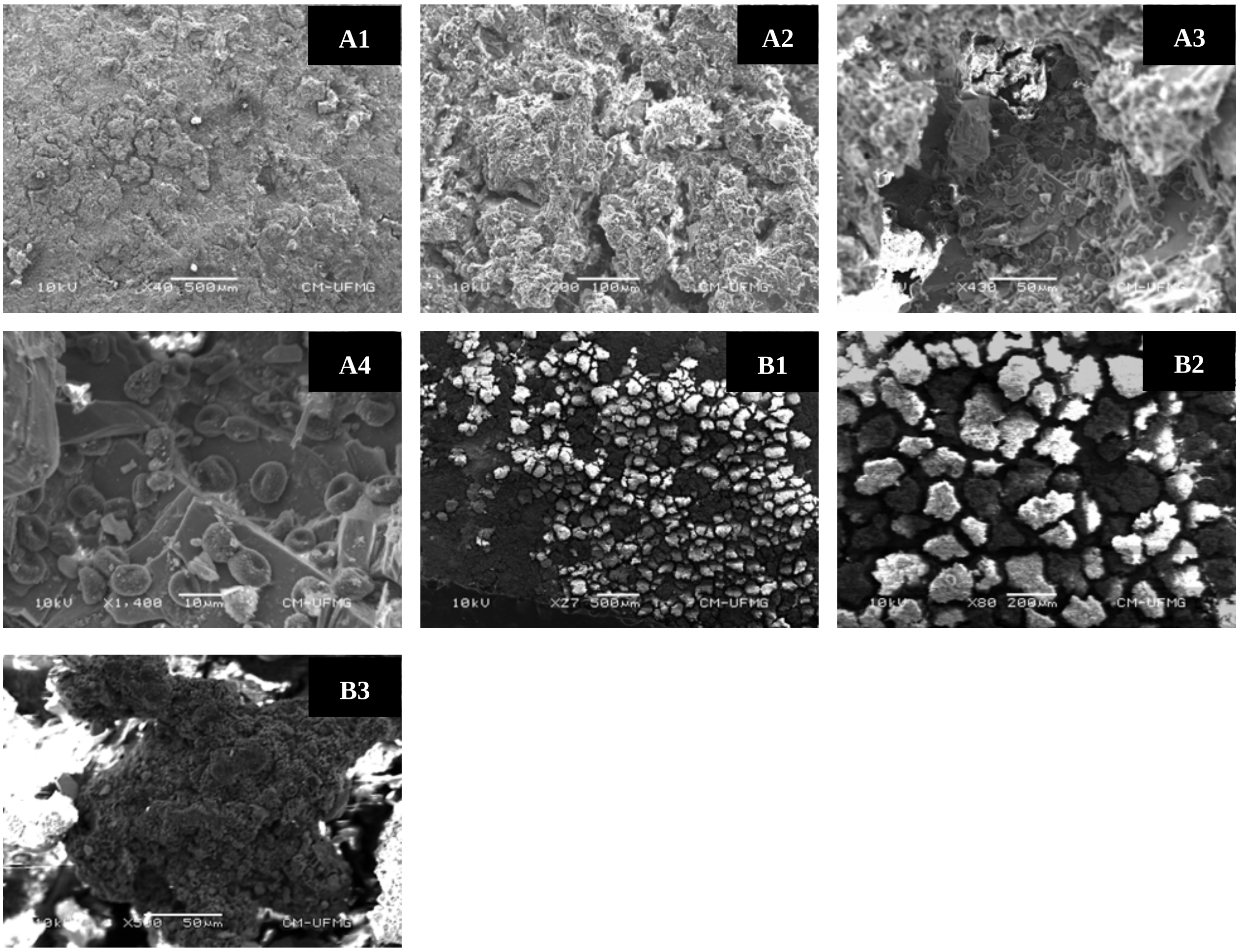


Fig. 4 SE images of soapstone blocks with fungal colonization: block A (A1, A2, A3, A4) and block B (B1, B2, B3).

both blocks that was colonized by filamentous fungi (Fig. 4). Fungal structures such as spores, hyphae and mycelia were observed on the soapstone blocks before exposure to gamma radiation.

4. Discussion

The susceptibility of fungi and spores to gamma radiation has been established in the literature. There are some studies showing the effects of ionizing radiation on filamentous fungi [6, 24, 25]. However, few studies are associated with the preservation of cultural and geological heritage, making this work an innovative tool in this area. This type of ionizing radiation produces changes that directly affect microbial DNA, in addition to the interaction of energy with the water molecules present in the organic substrate, leading to the formation of free radicals [26].

Saleh, Y. G., et al. [27] studied the resistance to ionizing radiation of some filamentous fungi such as, *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium* and *Penicillium* species. *Alternaria alternata*, *Curvularia lunata*, *Curvularia geniculata* and *C. cladosporioides* showed relatively more resistance to the effects of radioactivity.

Studies indicate that fungal spores from soil are eliminated (> 99%) when irradiated at an intensity of 10 kGy [28], which justifies the definition of this value for these soapstone tests. However, this value was not enough to eliminate the population of *C. cladosporioides*. This was the only species resistant to the exposure of gamma radiation. In addition, some strains have the metabolic capacity to produce black dye, such as melanin. This biopolymer is a type of black pigment that accumulates inside the mycelium and protects against UV rays and ionizing radiation [29]. Some authors have associated the radioresistance of some microorganisms with the melanization process of their cells [30].

Some species of *Alternaria* exhibit high resistance when exposed to higher doses than 4 kGy [24]. This fact led to the use of strains of *A. alternata* isolated from the radioisotope-contaminated environment around a Chernobyl nuclear reactor in Ukraine as a model for the genetic study of resistance to gamma radiation [31]. Analysis of the fungal microbiota in samples collected around the Chernobyl reactor revealed a predominance of black fungi [32].

Another study showed the effect of exposure to gamma radiation associated with melanin, in which melanized microorganisms were dominant in extreme environments contaminated with radiation, such as at Chernobyl, suggesting that the presence of melanin is beneficial to their life cycle. Thus, ionizing radiation could change the electronic properties of melanin to favor the growth of melanized microorganisms [29].

Other study published [33] showed that radiotrophic fungal species use melanin to convert gamma and beta radiation to chemical energy for growth.

Thus, these data support the resistance and prevalence of *C. cladosporioides* population after radioactive exposure in this study.

The ionizing radiation assay could be an alternative biocidal tool for the treatment of stone monuments. However, it is necessary to verify if the fungal species present is melanized or not, since radioactive exposure can aggravate the colonization and the permanence of melanized microorganisms on the stone surface.

In the present study, the species *C. cladosporioides* showed resistance to gamma radiation, despite a decrease in the diameter of the colonies. In addition, the count of CFU/mL after the radioactive exposure was higher, evidencing an increase in population.

5. Conclusions

The deleterious action of microorganisms on the geological heritage and the scarce data on the application of an innovative technology of biocidal action was the motivating factor for this research.

An alternative biocidal method of treatment with gamma radiation was proposed and was proven to be efficient in the elimination of the majority of filamentous fungal colonizers of stones. However, gamma radiation should be used with caution, since a deleterious effect was not observed for some species of black fungi; instead, it had an opposite effect of increasing the fungal population.

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