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MITIGATION APPROACH TO AVOID FUNGAL COLONISATION OF POROUS LIMESTONE

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Abstract

This work highlights the importance and eficacy of BEVOTECH solutions to inhibit fungal development on rock surfaces. These natural, ecofriendly and safe biocompounds can overcome the biodecay processes, constituting an alternative and innovative solution for rock materials preservation. The three BEVOTECH biocompounds promoted efficient inhibition levels for Aspergillus, Penicillium, Cladosporium and Acremonium, being completely safe for rock materials, do not inducing any alteration on colour, texture and structure of the stones. Mitigation strategies using combinatory application of these new solutions seems to be an efficient strategy to control and eliminate a complex population that usually colonise Cultural Heritage assets. According to the efficiency and safety of these novel biocompounds, their implementation on the plan of conservation and intervention process needs to be outlined and include on mitigation strategies to prevent, control and minimise biodeterioration, to contribute for the preservation and safeguard of these cultural assets.

Keywords*: Biocolonisation; Alteration stone; Bioactive compounds; Mitigation strategies*

Introduction

The Convent of Christ in Tomar (Portugal) have being affected over the years by structural and aesthetic damages like biofilms formation, coloured stains appearing/emergence, delamination and detachment of some stone fragments. During the last two years our research group has developed several works around this imponent monument, studying the materials used and the alteration phenomena that induce stones decay [1], and the results suggest the active role of the microorganisms on the deterioration of these materials and whose preservation may be threatened. To face this problem, several efforts have been accomplished in our laboratory on the development of mitigation strategies that eliminate microbial proliferation.

However, there is still a long way to go in this field, since the nature of the stone materials and their dynamic relations with their physical environments is altered to some degree, whether directly or indirectly [2]. On the other hand, the microbial growth on stone building surfaces can also lead alteration processes, which are supported/favoured by appropriate combination of humidity, warmth, light and bioreceptivity [3]. The presence of biocontamination is a huge problem that affects a significant percentage of the world's cultural

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heritage made of stone materials like archeological antiquities, including architectural monuments, statues, tombstones, stelae and among others, once they induce biodecay. Thus, to control the biodegradation/biodeterioration process, different approaches can be used, as such as: indirect control by altering environmental conditions, mechanical removal of biodeteriogens, physical eradication methods and chemical/biocidal treatment. However, the strategy to control these alteration processes must be include a polyphasic and interdisciplinary approaches that considers the history and condition of the artefact as well as physical and chemical damaging factors [4].

The mechanical methods to clean the surfaces include the use of scalpels, spatulas, scrapers and vacuum cleaners. This approach does not add new material on the substrates, there is a danger of cells or microbial spores remaining on the material, which are able to reactivate in favourable conditions and induce alteration processes. The physical methods are also being applied with this purpose and are based on the application of ultraviolet light, gamma rays, high-frequency current and low-frequency electrical current. However, low temperatures, pressure reduction and ultrasound can also be used. However, the most efficient method is irradiation with UV between 200-300nm, however it is necessary to take into account that UV light has a poor penetration power and can lead photodegradation of pigments and organic materials. The use of UV-C irradiation is an alternative to chemical products because this process does not generate pollution phenomena and the physical support remains unaltered. UV-C irradiation is harmful to living organisms due to its short wavelength, which confers highly energetic photons and germicidal properties upon these organisms, compromising the viability and metabolic activity of the microorganisms [5].

Gamma-irradiation can have several advantages for the conservation of objects of cultural heritage. It is highly penetrating and therefore very efficient in killing microbial communities colonising these objects. Furthermore, this technique is of use to conservators as it is not producing hazardous traces for paintings, it does not cause the formation of secondary radioactivity nor the formation of toxic residues and it is cost attractive. The required dose of gamma irradiation depends on the contamination level, the microbial diversity and its capacity for irradiation resistance. Nevertheless, gamma irradiation is not suitable for large paintings and it does not have a long-lasting effect. Beyond this limitation, a major problem in using gamma irradiation to eliminate colonising microorganisms is the possible deterioration of the object to preserve. The colour stability might be affected as chemical and physical properties of pigments may be changed due to gamma irradiation [6-8].

Laser cleaning, in comparison with conventional mechanical and chemical cleaning is a precise and versatile non-contact method. It has lower environmental and health-related side effects and prevents damage to underlying substrates by through self-controlling mechanisms. The cleaning of artworks by laser irradiation is a contemporary technique with many practical advantages (precision, rapidity, localised action, etc.). The main problem in the case of the biological degradation of artworks is a complete elimination of biodeteriogens and the treatment efficiency (removal of all active organisms).

The chemical treatments frequently applied have high efficiency in the microbial elimination, however, the main problem is the toxicity of the compounds used. The commercial biocides available are mainly alcohols, aldehydes, organic acids, carbon acid esters, phenols and their derivatives, halogenated compounds, metals and metal-organic substances, among others. Compounds like quaternary ammonium salts, metals and metal organic substances and heterocyclic organic products, have been widely applied for the control of microbial growth on artworks. Among the products currently used, quaternary ammonium salts are a group of substances widely applied in artworks treatment due to its broad-spectrum action and low toxicity. The antimicrobial effect of quaternary ammonium compounds is probably based on the

inactivation of proteins and enzymes and the detrimental impact on the microbial cell membrane. Their effectiveness is dependent on their chemical structure, such as the presence of an aromatic ring structure and the respective length of the four radicals. These compounds affect a broad microbial spectrum ranging from bacteria, fungi to algae and lichens, however possess high toxicity levels [9, 10].

Thus, in general, one biocide must to efficiently kill all microorganisms, have no/low toxicity for the operator and a low risk for the environment, does not interfere with materials, and offer protection against microorganisms for a long period of time. In fact, the efficiency of a chemical treatment depends on the concentration, microbial diversity and persistence of its action through time. There are different ways of application as such as spraying, brushing, applying poultices, injection or fumigation. Biocidal treatments have to be undertaken during dry weather. In windy conditions, excessive unwanted removal of biocidal spray may pose health and environmental risks. These compounds are commonly applied in repairing, cleaning and maintenance of artworks. Their application aims to prevent and/or control microbial growth. In this way, biocides can be applied before conservation-intervention process to eliminate microorganisms already present, and, after the intervention as preventive effect to slow down the re-colonisation of restored surfaces [11-24].

Althout the inhibition capacity, these biocides possess high toxicity which can represent an environmental risk. In this way, besides the ability to control biological growth against biological agents, the requirements for a good biocide are: high effectiveness against biodeteriogens, absence of interference with the constituent materials, low toxicity to human health and low risk of environmental pollution.

Thus, a greater effort has been undertaken to develop safer, environmental friendly, less costly and highly effective management methods that pose less risk to these monuments and, consequently, to humans (usually visitors) and the environment.

Emerging alternatives, such as nanotechnology, more dedicated to overcoming deficiencies observed in conventional management, are being suggested. It is now recognized that nanoparticles (NP) with dimensions less than 100nm possess distinctive physico-chemical properties, which may be useful in solving several world issues related to medicine, agriculture and environment. Silver nanoparticles (AgNP) have different physicochemical characteristics than the bulk matter, which make them more reactive and more highly effective against a broad spectrum of microorganisms [25, 26]. In our laboratory, an investigation group has been dedicated on the synthesis and characterisation of calcium and magnesium hydroxides nanoparticles for consolidation of mural paintings. This research is the initial part of an ongoing project which aims to develop new synthetic strategies towards novel and innovative materials for preservation and restoration of old renders, whose preliminary results suggest huge potential [26].

Due to the limitations related with the use of chemical compounds, natural products represent a huge potential source of compounds with antimicrobial properties, which can be an useful and advantageous alternative for the chemical products. Natural substances with antimicrobial action have been identified from a very wide range of sources, including plants, microorganisms and animals. In this way, several strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been referred to produce lipopeptides. In response to nutritional stress, a variety of processes are activated by *Bacillus* strains, including sporulation, synthesis of extracellular degradative enzymes and antibiotic production [27-31]. Several studies are being performed in HERCULES laboratory in the development of green solutions, whose added value is to be eco-friendly and without negative effects on the environment or human beings, and for the artworks. These biotechnological based compounds are being tested in several artwork

materials like monument stone building monuments, mural paintings whose antagonistic action against many fungal strains was proved [32, 33].

In this paper an outlook on the design and application of green solutions for mitigation uses, based on natural biocompounds, is reported, whose main concern is the safeguard of stone building monuments and rock materials.

Materials and Methods

Sampling site and microbial population

Seventeen filamentous fungi previously isolated from stone materials of Convent of Christ in Tomar (Ançã limestone) with evident chromatic and structural alterations, belonging to HERCULES Laboratory – Évora University microorganisms' collection, were selected to perform antifungal assays and simulation tests. Each fungal strain was cultured in MEA slants, during 7 days at 28ºC.

Antifungal assays

Fungal inhibition was tested in the presence of different commercial and bionatural compounds. Three natural biocompounds (3 mg/mL) - BEVOTECH-11, BEVOTECH-14 and BEVOTECH-16 - obtained by biotechnological methodologies, using different strains of *Bacillus* sp. CCMI 1051, CCMI 1052 and CCMI 1053, referred to produce bioactive compounds which has a great potential to suppress biodeteriogenic fungi growth [32], were tested on stone materials. A positive control Micostatin® (Nystatin 4000UI/mL) and commercial chemical compounds Preventol PN® 0.1% (sodium 2, 3, 4, 5, 6-pentachlorophenplate) was also evaluated against the predominant fungi isolated, under sterile conditions. The commercial and natural lipopeptide compounds [32, 34, 35] were tested at different concentrations against the several fungal isolates from Convent of Christ by agar incorporation method [36].

Cultures of each microorganism were prepared in Malt Extract Agar (MEA) slants and incubated at 28ºC for 7 days. Fungal spore suspensions were prepared by adding a loopful of hyphae and spores in 15mL of NaCl 0.85% solution. The suspension was filtered by sterilised triple gauze and incorporated (10^5CFU/mL) in MEA at 45ºC. Sterile filter paper discs (Macherey-Nagel 827 ATD) were placed on agar and impregnated with 20µL of biocide. The Petri dishes were incubated at 28ºC for 4-5 days. Antimicrobial activity was evaluated accordingly to the inhibition halo developed around the disc. The assays were performed in triplicate, and the results presented as average ± standard deviation (SD).

Data were evaluated statistically using the SPSS® 24.0 software for Windows Copyright©, Microsoft Corporation, by descriptive parameters and by One-way ANOVA in order to determine statistically significant differences at the 95% confidence level ($p < 0.05$). The population variances homogeneity was confirmed by Levene test and multiple average comparisons were evaluated by Tukey test, being considered significant values those whose probability of occurrence is greater than 95% ($p < 0.05$).

Simulation assays

The lithotype used to perform the simulation assays was Ançã limestone, a light colour limestone, between yellowish and bluish-white, oolithic texture, without veins and very soft surfaces. This rock material is originating from Ançã region, located in the centre of Portugal, and is widely used on the Portuguese architecture and sculpture.

Stone fragments (2×2×1.5cm) were washed, dried and sterilised. Four fungal fresh cultures selected strains were prepared in slants with MEA and growth at 28ºC during five days. After that, the inoculum of each microorganism was prepared, harvesting the cells with physiologic serum and applied on the sterile stone fragments. The slabs were inoculated with each inoculum (10^5CFU/mL) and after the natural biocompounds (BEVOTECH-11,

BEVOTECH-14 and BEVOTECH-16) were applied. The development of each assay was monitored and recorded weekly, for a month, using a Nikon D3100 camera. During this period, the cell viability of the fungal strains was assessed by MTT based method [37, 38] on four different strains (MTT represent total dehydrogenase activity reported as Abs570nm in each assay). At the end of the simulation assay, each limestone slab were analysed by scanning electron microscopy, in a HITACHI 3700N variable pressure scanning electron microscope (VP-SEM) coupled with a Bruker XFlash 5010 energy dispersive X-ray (EDX) spectrometer with an accelerating voltage of 20kV and backscattered electrons mode, to evaluate the microstructural integrity of the rock material and to understand the mechanisms action of the fungal population.

Results and Discussion

A previous study performed on the stone materials of Convent of Christ affected by biofilms, stains and structural degradation revealed that this altered areas are strongly linked with biocontamination [1]. Biofilms formation, coloured stains and structural degradation can be observed on this historical monument, whose main colonisers are bacteria, fungi, cyanobacteria and algae. Attending the high level of biocontamination detected, mitigation strategies would be implemented and effective biocides need to be tested to try to eliminate the microbiota present and control their propagation.

Firstly, antimicrobial trials were performed against the fungal isolates from Convent of Christ, in order to evaluate the inhibition capacity of natural (BEVOTECH-11, BEVOTECH-14 and BEVOTECH-16) and commercial biocides (Mycostatin® and Preventol PN® 0.1%). The results revealed that the seventeen filamentous fungi tested were inhibited by the commercial and natural biocides tested (Table 1). Mycoscatin® was used as positive control, once this compound is currently known for their antifungal capacity [39, 40]**.** This capacity was also proved for these filamentous fungi, however for the majority of the isolates, the commercial and natural compounds revealed high inhibition capacity. Although the inhibition capacity of Preventol® ≥1%, their application in real context is not advised due to the high toxicity of this compound [11-24], being necessary use low concentration (Preventol® 0.1%) or find new solutions. According to this, our work focuses on the implementation of new, green and safe alternatives to mitigate microbial proliferation on stone materials. We present three natural biocompounds that promoted efficient inhibition levels, being equal or higher than Preventol® 0.1%.

The antifungal assays allowed good inhibition results, revealing that the natural compounds can present an ecological, safe and green solution face to the commercial compounds available on the market. The natural compounds possess equal or higher inhibition capacity than chemical products getting also total inhibition (Table 1).

The antifungal tests allowed to verify that *Acremonium* (CC11 and CC13) fungi were the most inhibited by BEVOTECH compounds, while, the isolate CC2 was the less inhibited.

The promising capability of these biocompounds to inhibit fungal strains was also detected for *Penicillium* sp. and *Cladosporium* sp..

These results highlighted the effectiveness of these BEVOTECH compounds and their use rather than the toxic commercial compounds. Beyond the effectiveness, it was necessary to evaluate the efficiency and safety for stone materials. In this way, simulation assays with the same stone materials used on Convent of Christ (Ançã limestones) were inoculated with fungal isolates from this monument and treated with BEVOTECH solutions.

Table 1. Antifungal activity of commercial and natural compounds against filamentous fungi isolated from Convent of Christ.

Fungal isolates		Inhibition halo (mm)				
		Nystatin	**Prev. 0.1%**	**BEV-11**	**BEV-14**	**BEV-16**
CC_1	*Cladosporium* sp. 1	12.7 ± 0.5 [a]	N.I. [b]	18.4 ± 1.3 [c]	15.2 ± 0.4 [d]	14.3 ± 0.7 [d]
CC_2	Mycellium 1	17.7 ± 2.8 [a]	16.9 ± 2.1 [a]	22.6 ± 1.0 [b]	20.2 ± 1.6[b,c]	19.2 ± 2.1 [a,c]
CC_3	*Aspergillus* sp.1	17.1 ± 1.5 [a]	16.0 ± 1.0 [b]	15.8 ± 0.7 [a]	15.1 ± 0.9[a,c]	13.6 ± 0.7 [d]
CC_4	*Penicillium* sp.1	16.1 ± 1.7 [a]	T.I. [b]	30.1 ± 1.9 [c]	28.0 ± 3.7 [c]	22.9 ± 3.1 [d]
CC_5	Mycellium 2	23.4 ± 2.8 [a]	21.3 ± 2.8 [a]	24.8 ± 2.4 [a]	26.6 ± 3.4 [a]	24.7 ± 3.9 [a]
CC_6	*Penicillium* sp.2	18.3 ± 2.5 [a]	27.1 ± 2.1 [b]	23.9 ± 3.6 [c]	27.8 ± 2.5[b,d]	18.8 ± 1.5 [a]
CC_7	*Cladosporium* sp. 2	16.1 ± 4.2 [a]	15.0 ± 0.9 [a]	27.0 ± 1.5 [b]	27.8 ± 1.2 [b]	21.8 ± 1.4 [c]
CC_8	Mycellium 3	19.9 ± 2.5 [a]	27.7 ± 5.2 [b]	35.4 ± 4.2 [c]	41.4 ± 8.8 [d]	31.4 ± 5.5[b,c,e]
CC_9	*Penicillium* sp.3	17.3 ± 3.9 [a]	14.0 ± 0.9 [b]	33.2 ± 1.7 [c]	29.7 ± 1.7 [d]	22.2 ± 1.4 [e]
CC_10	Mycellium 4	16.1 ± 1.8 [a]	28.1 ± 8.4 [b]	35 ± 3.6 [c]	30.3 ± 1.9[b,c]	22.4 ± 1.9 [d]
CC_11	*Acremonium* sp.1	14.2 ± 0.9 [a]	23.2 ± 3.3 [b]	38.4 ± 1.9 [c]	T.I. [d]	41.9 ± 2.4 [e]
CC_12	*Penicillium* sp.4	21.4 ± 4.0 [a]	T.I. [b]	34.8 ± 3.3 [c]	32.3 ± 3.3 [c]	22.0 ± 1.7 [a]
CC_13	*Acremonium* sp.2	15.1 ± 1.3 [a]	20.7 ± 1.2 [b]	T.I. [c]	T.I. [c]	40.2 ± 0.8 [d]
CC_14	Mycellium 5	14 ± 2.1 [a]	19.4 ± 1.2 [b]	38.8 ± 1.1 [c]	35 ± 2.7 [d]	29.7 ± 2.2 [e]
CC_15	*Mycellium 6*	12 ± 0.0 [a]	15.8 ± 0.7 [b]	27.3 ± 1.7 [c]	24.1 ± 1.2 [d]	21.7 ± 1.0 [e]
CC_16	*Penicillium sp.5*	22.1 ± 1.9 [a]	15.7 ± 1.5 [b]	32.2 ± 2.8 [c]	34.7 ± 2.6 [c]	22.6 ± 2.2 [a]
CC_17	*Penicillium* sp.6	25.6 ± 2.4 [a]	13.0 ± 0.0 [b]	22.9 ± 1.4 [c]	22.3 ± 1.2 [c]	17.0 ± 0.7 [d]

T.I. – total inhibion; N.I. – no inhibition

* Different letters (a - f) following the values indicate significant differences ($p<0.05$) for each isolate in the presence of the several biocides tested. Values of each determination represents means ± SD (n=9).

The rock slabs (without and with fungal isolates) were monitored by a month and the results showed the capacity of these fungi to colonise and proliferate on rock materials (Fig. 1), evidencing the bioreceptivity of these materials and the consequently biodegradation/biodeterioration. SEM micrographies evidenced the capacity of the fungal population to produce biofilms on the surfaces and ability to proliferate in depth on the rock microstructure. This behaviour can explain the damages that affect the rock materials as such as biological patinas, cracks and detachments., which alter the structure and stability, affecting mechanical properties, superficial absorbency/hydrophobicity, diffusivity, and thermohygric behaviour, effects also reported by Warscheid and other authors [20, 41-43].

These rock slabs were monitored in the presence of the natural biocompounds - BEVOTECH-11, BEVOTECH-14 and BEVOTECH-16 – to evaluate, *in situ*, their inhibition capacity. Through SEM-BSE we were able to evaluate treatment with the BEVOTECH compounds which revealed that these three compounds are completely safe for rock materials, do not induce any alteration on the colour, texture and structure of the stones. Furthermore, these biocompounds allowed great capacity to inhibit fungal proliferation and high efficiency to stop their growth (Fig. 1). BEVOTECH-11 and BEVOTECH-14 allowed highest inhibition levels than BEVOTECH-16 (Table1), however, these BEVOTECH solutions showed different antifungal activity according to the microorganism present. This behaviour point off a combined application of a consortium of biocides to maximize the efficiency of the antimicrobial treatment.

Alongside this, biochemical tests to cells viability assessment (MTT assays) revealed a decrease of viable cells, corroborating the efficiency of these new solutions to inhibit microbial proliferation (Fig. 2).

In fact, the natural biocompounds revealed effective inhibition capacity, both for *in vitro* and *in situ* assays. Figure 3 shows the SEM and SEM-EDX micrographs of Ançã limestone, control and treated samples, which revealed that these BEVOTECH solutions do not induce alterations on the rock materials, the material composition do not suffer alteration, the surface do not change neither damages were detected.

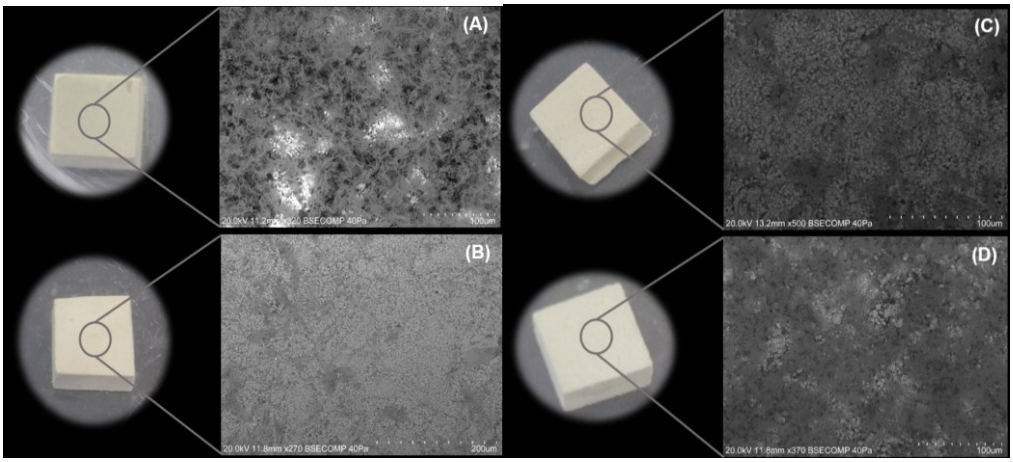

Fig. 1. SEM analysis of Ançã limestone non-treated (A) and treated with BEVOTECH-11 (B), BEVOTECH-14 (C) and BEVOTECH-16 (D), to evaluate the antimicrobiological capacity to inhibit fungal proliferation.

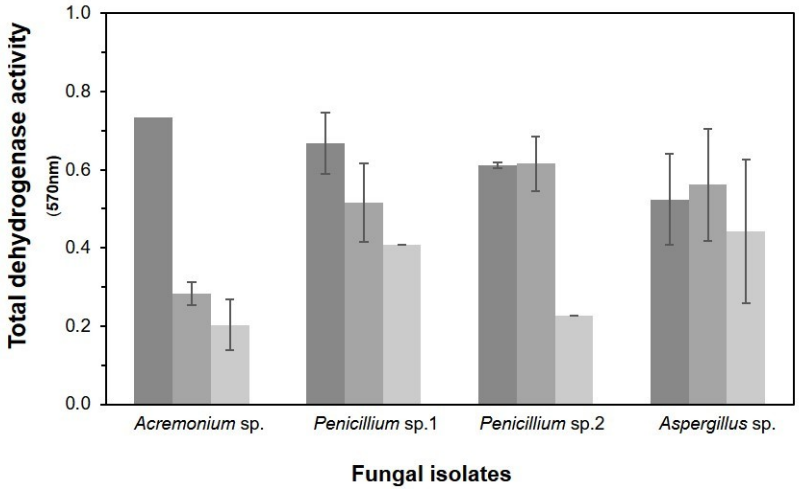

Fig. 2. Metabolic activity assessment of fungal proliferation/inhibition on rock materials (■ - t_0; ■ - t_{7d}; ■ - t_{30d})

Bevotech compounds seem to be a great alternative to the chemical compounds and a green/ecofriendly solution for Cultural Heritage safeguard plan. Its combined application allows potentiating its action spectrum and prolonging its inhibitory effect.

According to these results, this work suggest that these compounds can be used on the biocontrol and be implemented on safeguard of cultural heritage assets which will be include long-term monitorisation, dynamic population assessment, to early detection and prevention of possible recolonisation.

The findings emphasize the need of proper and look forward diagnosis, treatment and intervention processes, using complementary approaches and straightforward strategies based on Heritage Sciences guidelines to prevent and safeguard our monuments (Fig. 4).

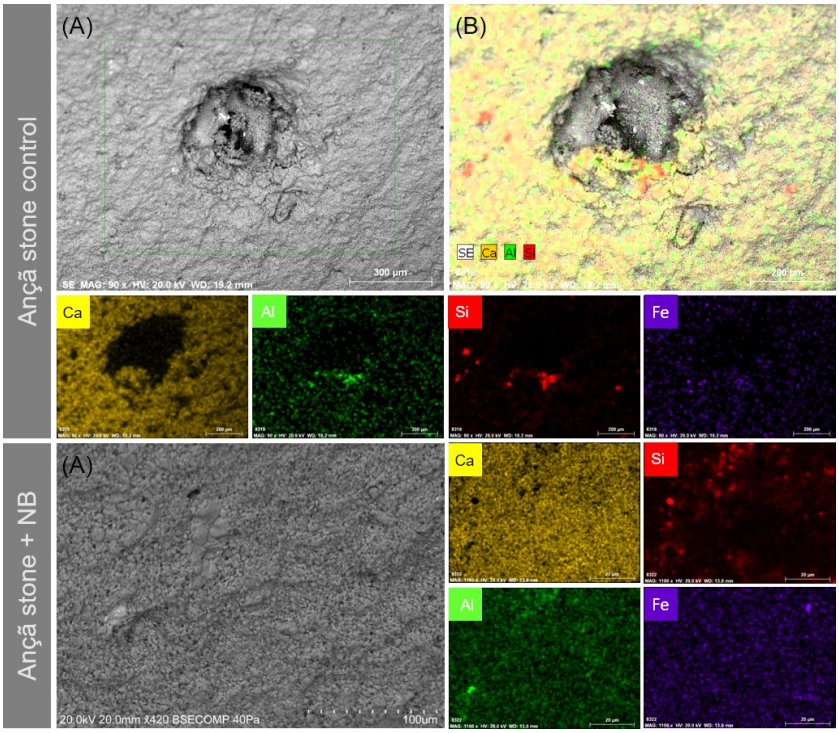

Fig. 3. SEM (A) and SEM-EDX (B) analysis with 2D mapping and individual map of several elements of Ançã limestone control and with natural biocompounds (NB) treatment.

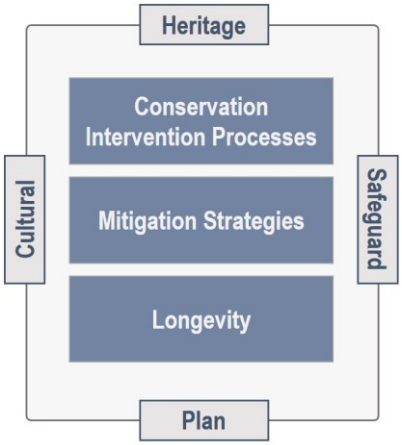

Fig. 4. Plan for successful measures to biodeterioration control and for cultural heritage assets safeguard

Conclusions

This work constitute an integrated approach to elucidate the ability of biological activity to deteriorate building stone materials compromising their aesthetic quality and structural properties and a strategy to eliminate these undesirable colonisers.

The novel biocompounds developed -BEVOTECH- represent a groundbreaking alternative to the commercial and toxic chemical products to be applied in fungal mitigation

strategies of limestone monuments. Their application on biocontrol can contribute for the longevity of the cultural assets, and, to control and prevent possible recolonisation which will be implemented as preventive conservation measures.

The efficacy of these novel biocompounds encourages us to continue and explore their inhibitory capacity on other biological agents and support materials, on complex population, on individual photosynthetic cells and photosynthetic cell clusters. Moreover, the implementation of new formulations and the combined application of these biocompounds are compulsory to increase their spectrum of action.

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