BIOTECHNOLOGY, A SOURCE OF KNOWLEDGE IN AGREEMENT WITH *GREEN STRATEGIES* FOR THE CONSERVATION OF CULTURAL ASSETS

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1. Introduction

The words, *"Prevention is better than cure"* represent a milestone for human health, at the same time however, the same words can unquestionably be shifted to the "*cure and preservation"* of cultural assets. Indeed, in order to perform adequate strategies, it is indispensable to limit any irreversible damage, characterize and quantify any microbial colonization that may induce the deterioration of constitutive heritage materials, and perform suitable antimicrobial interventions through the use of biotechnology.

As regards historic-artistic artifacts, biological, chemical and physical factors are able to act concomitantly to induce the degradation of both organic and inorganic artworks. The huge number of papers available in scientific literature attest that biotechnology plays a key role in medicine, agriculture and industry, with its importance becoming increasingly evident in the preservation and restoration of cultural assets.

Particularly in the last two decades relevant biotechnological applications in the field of artwork preservation have emerged opening the way for advanced innovation. This paper presents a summary of the basic and applied biotechnology research developed in the Laboratory of Biology and Biotechnology for Cultural Heritage (LaBBCH, University of Palermo, Italy). In addition, the further development of biotechnological techniques can be hypothesized, creating new opportunities for biologists, biotechnologists and artwork conservators, providing innovative strategies that are totally safe for artworks, operators and their environment.

Finally, biotechnological tools have a great potential of application thanks to the increasing interaction between the worlds of art and science.

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2. Biodeterioration

Several biological systems are able to induce “undesirable change in the properties of a material caused by the vital activities” (Hueck, 1968). Bacteria and fungi in particular, that are able to colonize many different nature artworks or present as environmental aerosol pollutants in relation to their metabolic versatility, represent complex problems for conservation. Moreover, specific thermo-hygrometric parameters can lead to higher concentrations of microbial units, enhancing the deterioration process (mainly due to the release of organic-inorganic acids, enzymes, pigments), altering the chemical, physical and material properties [1].

Deterioration is also the result of aerosol pollution (dust, grease, soot), resulting in the deposition of significantly reactive particles on the artwork surface [2, 3]. Frequently, biological, anthropogenic and pollution factors act simultaneously, thus inducing the formation of complex stratified layers as shown on the marble sculptured surface exposed to outdoor environments in Figure 1.

Figure 1. A complex multilayer on stonework surface, abiotic deposits and biological colonization, showing different aspects of: A) chromatic and morphological features; B) remarkable depth.

In order to characterize the taxa forming the microbial consortium on the surface of works of art, an integrated approach (microscopy, *in vitro* culture, molecular investigation) is needed [4].

Microscopy observation (using Lugol’s iodine staining), as shown in Figure 2, revealed fungal reproductive structures, attributable to A*spergillus* sp. and *Penicillium* sp.

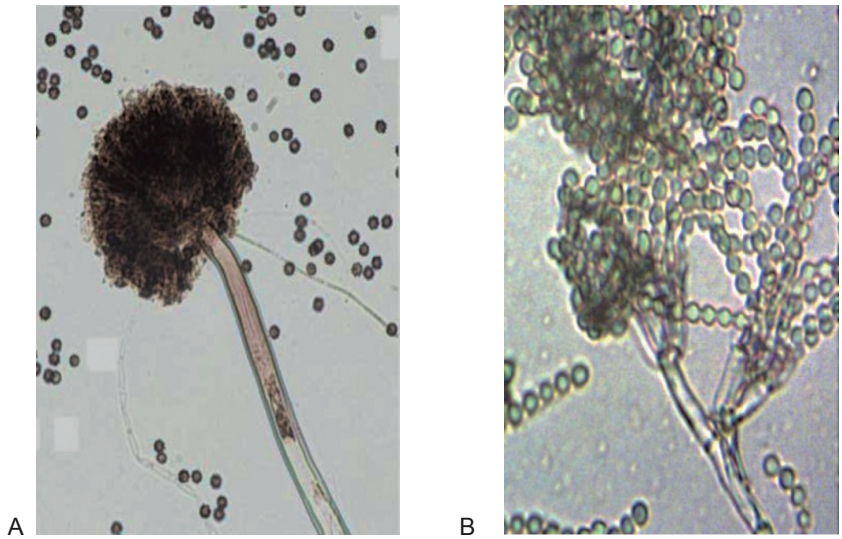

Figure 2. Microscopy analysis. Fungal structures, hyphae and conidia related to Aspergillus sp. (A) and Penicillium sp. (B), observed after staining by Lugol's iodine reagent.

A preliminary identification of fungal species was performed by *in vitro* culture on Sabouraud media, allowing the identification of the morphological profile of *Aspergillus flavus*, *Aspergillus niger*, *Alternaria alternata*, *Penicillium chrisogenum* colonies (Figure 3).

The third step in the integrated approach is molecular investigation, which contributes to confirming and completing the overview of the microbial colonizers. Specifically, the sequencing and sequence analysis of the DNA fragments, as products of the polymerase chain reactions (Figure 4), allow us to confirm the fungal species of *Aspergillus flavus*, *Aspergillus niger*, *Alternaria alternata*, *Penicillum chrisogenum,* as well as *Alternaria brassicicola.*

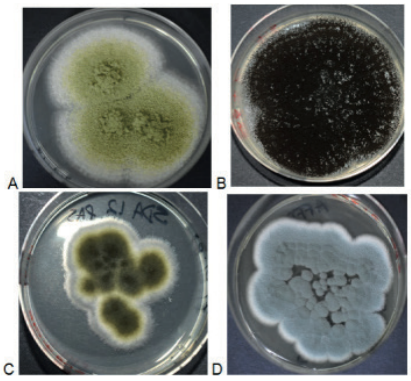

Figure 3. In vitro culture. Fungal colonies grown on Sabouroud-agar plate: (A) Aspergillus flavus; (B) Aspergillus niger; (C) Alternaria alternate; (D) Penicillium chrisogenum.

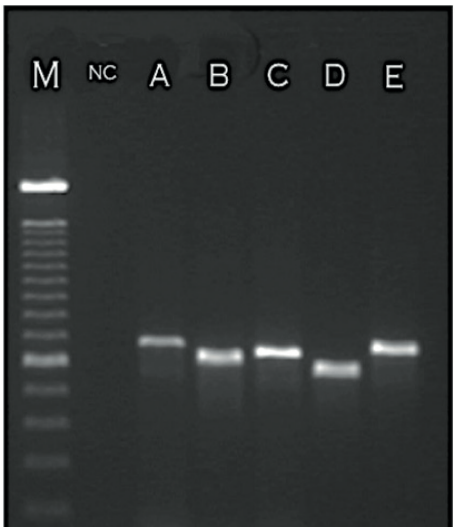

Figure 4. In vitro amplification of ITS rDNA region, using as a template the genomic microbial DNA directly extracted from biofilm. M = 100 bp DNA ladder; NC= negative-control; PCR reaction products corresponding to: A= Alternaria alternata; B=Aspergillus niger; C=Aspergillus flavus; D=Penicillium chrisogenum; E= Alternaria brassicola

3. Bioaerosol

The identification of the largest number of bacteria and fungi has a marked importance, also from the point of view of risk management in cultural heritage environments (CHE), specifically due to the presence of biological particles (allergens, cellular particles, spores, toxins), potentially dangerous for operator and visitor health [5 – 10]. As shown in Figure 5, an active sampling procedure of the CHE aerosol is outlined. Biological particles are collected using a Sartorius MD8 portable air sampler, equipped with sterile gelatine membrane filter.

The peculiarity of *gelatine membrane filters* is their solubility in aqueous solutions so, in addition to being used to inoculate agar media, it is possible to dissolve them in solution as 1xTE = 10mM Tris-HCl pH 7.5 /1 mM EDTA, performing the direct extraction of the microbial genomic DNA. The DNA will later be the template molecules in PCR reactions. Figure 5 schematically describes the integrated procedure utilized to reveal and identify microbial taxa colonizing the aerosol of CHE, such as archives.

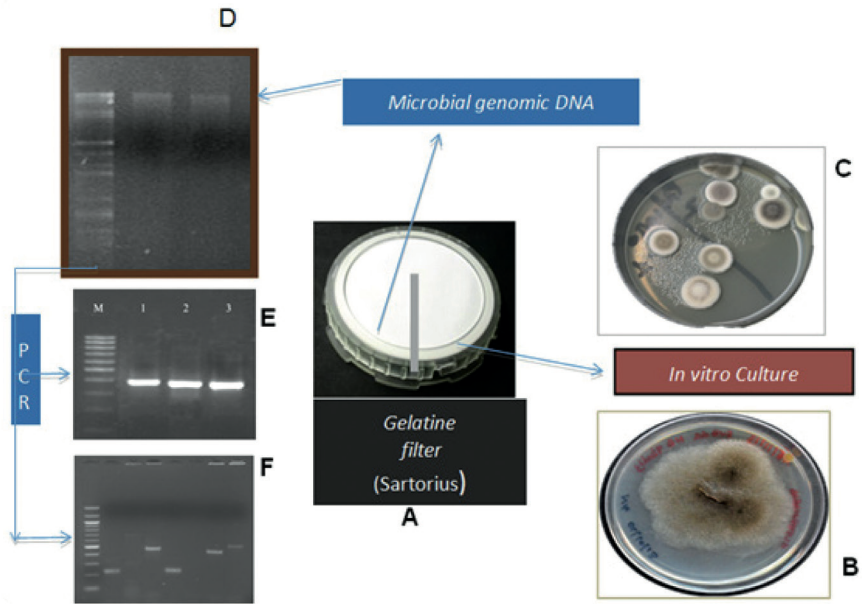

Figure 5. Scheme for analysis of CHE aerosol. Sampling by MD8 portable sampler equipped with gelatine membrane filter (A), the microbial taxa are identified combining an in vitro culture on Nutrient (B) or Sabouraud (C) agar media, and molecular investigation using the genomic microbial DNA (D) as a template in PCR reactions amplifying ITS rDNA regions (E, F).

4. Molecular biology investigation

As concerns genus/species identification, the molecular biology approach, based on the analysis of specific DNA genomic sequences was methodically applied to identify microbial colonizers on both inorganic and organic materials, exposed/stored in museums, archive libraries, deposits, etc. [11, 12]. Particularly by using the polymerase chain reaction (PCR) technique, an important tool for rapid identification of the biological systems, specific genomic regions can be *in vitro* amplified.

The corresponding amplification products are analysed through gel electrophoresis, followed by the resolving of the nucleotide composition (sequencing) and related analyses of sequence homology by consulting international nucleotide databases (e.g., NIH, USA; EMBL, Germany), performing phylogenetic analysis and also identifying novel microbial species [13, 14].

From our point of view, molecular investigation must be considered as part of an interdisciplinary approach, where the contribution of microscopy (Optical, SEM, CLSM) and *in vitro* culture (Nutrient / Sabouraud agar) are of comparable importance [9, 15-17].

5. Biological colonization control

The control of microbial colonization is generally performed by powerful biocides, chemical compounds with a broad spectrum of action against Gram+/- bacteria, brown and green algae, lichens, molds, micro-fungi [18-20]. The toxicity of many synthetic products is well understood [21] and some of them are utilized in preventive conservation or restoration procedures.

Recently, natural compounds (such as essential oils) have been tested to replace chemical compounds in order to develop alternative methods to control microbial colonization and to prevent repeated occurrences [5, 23-29].

The *in vitro* evaluation of antimicrobial activity can be performed by means of at least three methods defined as: *agar disk diffusion* (Fig. 6), *well diffusion* or *microdilution* [5, 22, 29-32].

Performing the *agar disk diffusion* assay, a paper disk (6 mm in diameter, wetted with 10 µl of essential oils (at different concentrations: from 6.25 to 100%) is placed on the surface of nutrient or Sabouraud agar plates (90 mm Petri dish) inoculated by a bacterial or fungal single colony culture at a concentration of 1×10^6 CFU/ml or $1x10^4$ conidia/ml [5]. After incubation for 24/48 hours at 30 ± 1ºC, confluent microbial growth was observed and the diameter (mm) of growth inhibition areas measured, as shown in Figure 6. The measurement of the growth inhibition halo (diameter in mm) allows the definition of antimicrobial activity (sensible > 9 mm, resistant < 6 mm).

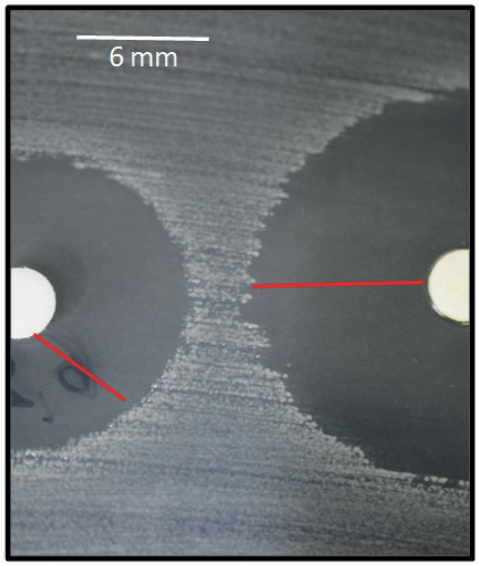

Figure 6. Agar disk diffusion method to test the antimicrobial activity of Thuja plicata Donn. essential oil (EO) vs Bacillus subtilis colonies. The inhibition halos are related to 6.25% (left) and 25% (right) EO. The red lines highlight that T. plicata EO already has antimicrobial activity at a lower percentage (6.25%).

A high susceptibility of microbial strains to *Thuja plicata* Donn. essential oil was also revealed for *Stafilococcus aureus* (Figure 7A) and *Penicillium chrysogenum* (Figure 7B).

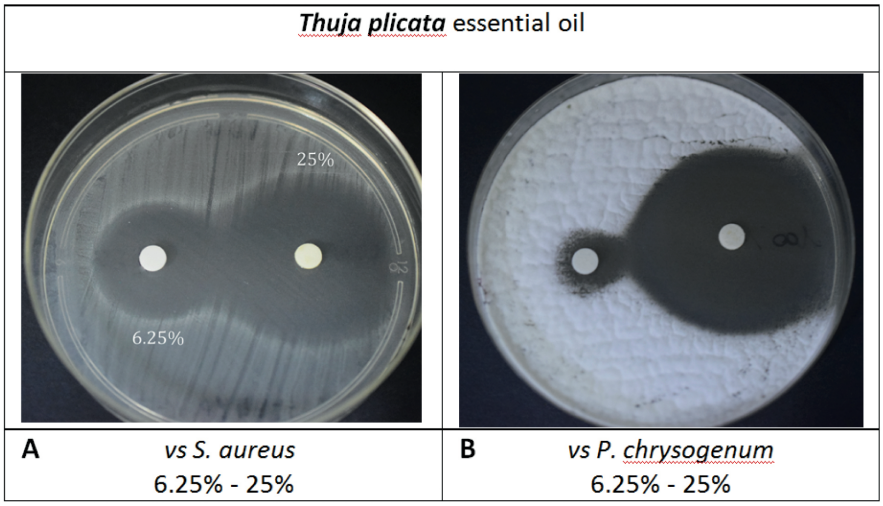

Figure 7. Agar disk diffusion method. The antimicrobial activity of Thuja plicata EO vs Stafilococcus aureus (A) and Penicillium chrysogenum colonies (B) is evident for both EO concentrations (6.25 and 25%).

Furthermore, *Thuja plicata* EO (12.5, 25% and 50%) showed peculiar growth inhibition activity also against *Micrococcus luteus* colonies; the antimicrobial activity was so high that the inhibition halos were confluent (Figure 8).

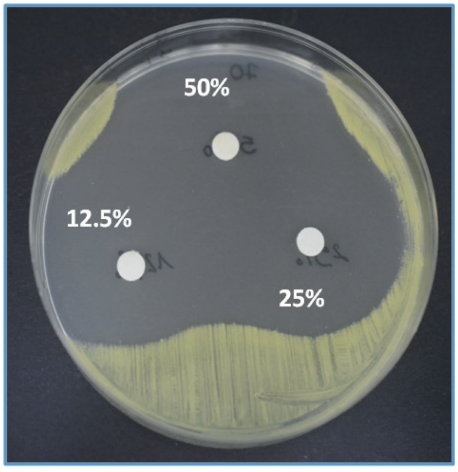

Figure 8. Agar disk diffusion method. Evident antimicrobial activity of Thuja plicata EO is shown vs Micrococcus luteus colonies.

In order to achieve information useful in comparing the sensibility of microbial species tested in this work to a commercial biocide, Benzalkonium Chloride (BC) was utilized in control assays. Specifically, a paper disk was wetted with 6.25% BC and after incubation at 30°C for 24/48 h, confluent microbial growth was observed except in the growth inhibition area (halo), Figure 9 [33, 34].

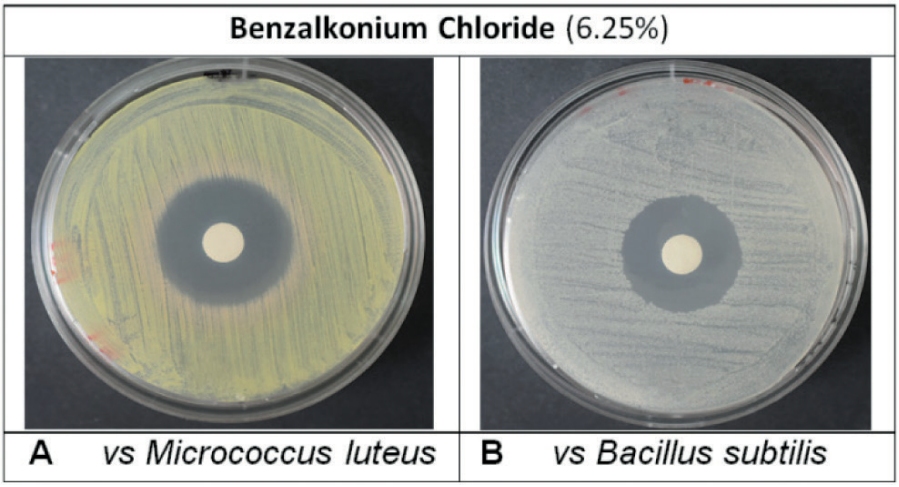

Figure 9. Agar disk diffusion method, control assays. Antimicrobial activity of Benzalkoniun chloride vs Micrococcus luteus (A) or Bacillus subtilis (B) colonies.

6. Conclusions

Microorganisms play a significant role in the biodeterioration of cultural assets and, depending on growth processes, development and metabolic activities can cause physical-chemical alterations and aesthetic damage to works of art with strong negative consequences for conservation strategies [35].

In this study we suggest an integrated approach to detect and identify the microorganisms colonizing artwork surfaces and the aerosol of cultural heritage environments, highlighting that molecular techniques definitely improve the sensitivity and specificity of diagnostic investigation.

The potential use of commercial (*Melaleuca alternifolia Origanum vulgare*, *Thymus vulgaris*, *Thuja plicata*) or laboratory distilled (*Allium sativum, Calamita nepeta, Crithmum maritmum,*) plant essential oils, together with the achievements reached during the *in vitro* and *in situ* applications to control the growth of bacterial (*Bacillus subtilis*, *Micrococcus luteus, Stafilococcus aureus*) or fungal (*Alternaria alternata*, *Alternaria brassicola, Aspergillus flavus, Aspergillum niger*, *Penicillium chrysogenum*) taxa, have led us to hypothesize their use as natural biocides, also focusing on their importance in safeguarding the environment and human health [27, 29]. Consistent with the hypothesis, encouraging results have recently been obtained using *Origanum vulgare* essential oil in the control of complex biofilm growth beneath mosaic tesserae [33].

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Biographical notes

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Summary

In this study, conservative strategies are applied in order to limit any irreversible damage and to characterize and quantify microbial colonization that may induce the deterioration of constitutive heritage materials; subsequently, suitable antimicrobial interventions are performed based on the use of biotechnology.

This paper presents a summary of the basic and applied biotechnology research developed in the Laboratory of Biology and Biotechnology for Cultural Heritage (LaBBCH, University of Palermo, Italy). It shows that biotechnology has an evidently important role in the preservation and restoration of cultural assets, also taking into consideration that *“Prevention is better than cure”,* a milestone in the field of human health that can be shifted and applied to the “*cure and preservation”* of cultural assets.

Moreover, biotechnological tools offer great potential for application, thanks to the increasing interaction between the worlds of art and science, thus opening the way for advanced innovation in the conservation and restoration field of art works.

Riassunto

In questo studio sono presentate alcune strategie conservative per caratterizzare e quantificare la colonizzazione microbica che può indurre il deterioramento dei materiali costitutivi di manufatti d’interesse storico-artistico, basate su protocolli biotecnologici.

Qui è presentata una sintesi della ricerca biotecnologica di base e applicata, condotta nel Laboratorio di Biologia e Biotecnologie per i Beni Culturali (LaBBCH). Ciò dimostra l’evidente ruolo della biotecnologia nella conservazione e nel restauro di beni culturali, sottolineando che “Prevenire è meglio che curare”, pietra miliare per la salute umana che può essere rivolta alla “cura e conservazione” dei beni culturali.

Inoltre, gli strumenti biotecnologici hanno un grande potenziale di applicazione grazie alla crescente interazione tra il mondo dell’arte e della scienza, aprendo la strada all’innovazione avanzata nel campo della conservazione / restauro di opere d’arte.