MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF FUNGAL POPULATIONS POSSIBLY INVOLVED IN THE BIOLOGICAL ALTERATION OF STONES IN HISTORICAL BUILDINGS

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SUMMARY

The deterioration process of historical building is progressive and irreversible, and the timing and mode of impact are different depending on the characteristics of building materials used, local microclimate, air pollution, presence of specific flora and fauna. The chemical and microbiological characterisation of building materials is mandatory in preventing and eventually recovering degradation effects. Ideally, the analysis of structural stones should be complete, efficient, rapid, and non destructive when dealing with a precious or unique construction.

The investigation has been performed on a private historical building made using calcarenite stones and sited between the archaeological site of Lavello, a little town located in the Basilicata Region (South Italy), and the industrial area surrounding this town. To study in progress the degradation of stone materials, a new building sample (ca. 1 m^3) was constructed by using the same stones (33x15cm), collected from a local quarry.

The intact calcarenite stone was characterised by using different methods of surface analysis (XRD, XPS, SEM), and exposed to outdoor conditions. The analyses of the stone material were repeated after three and six months to early evaluate the progression of alterations and the forward modifications of calcarenite structure.

After only three months of the new building sample exposure, the adopted analytical methods were able to provide a series of data, which allowed the assessment of the incipient modification of the stone surfaces. The degradation appeared worsened performing the same observations on sixth month replicates, suggesting that environmental conditions modified the structure and the compactness of stones and favoured the biological colonization of surfaces especially in the South–East direction of prevailing winds. For this reason the presence of fungi on the stones' surface was investigated and a morphological and molecular characterization of sampled fungi was performed. Several genera and species of fungi, possibly, involved in degradation were found. The most frequent colonies belonged to *Alternaria* (*A. infectoria*, *A. citri* and *Alternaria* sp.), *Coprinopsis* sp., *Penicillium piceum*, *Fusatrium equiseti* and *Scytalidium termophilus*.

Key words: historical building, surface analysis, surface modification, biological alteration, *Alternaria*, *Coprinopsis sp.*, *Penicillium piceum*, *Fusatrium equiseti*, *Scytalidium thermophilum*

INTRODUCTION

Deterioration of cultural heritage is a severe problem in European cities and urban developments and its preservation constitute a worldwide worry.

Stone monuments represent an important part of cultural heritage, but, due to their prevalently outdoor location, they are generally subject to a complex series of weathering and decay processes [Van Grieken *et al.*, 1998; Albertano, 1995; Caneva *et al.*, 1992].

The stone degradation process concerning monuments is progressive and irreversible, and timing and mode of impact are different depending on the characteristics of the monument

(location, orientation, mineralogical and structural properties), microclimate (temperature, humidity, solar radiation, wind regime, precipitations), air pollution (particulate, concentrations of SO_2, NOx, CO_2), presence of specific flora and fauna that are settling the buildings [Warscheid *et al.*,2000; Dornieden *et al.*, 2000; Ortega-Calvo *et al.*, 1995](Figure 1).

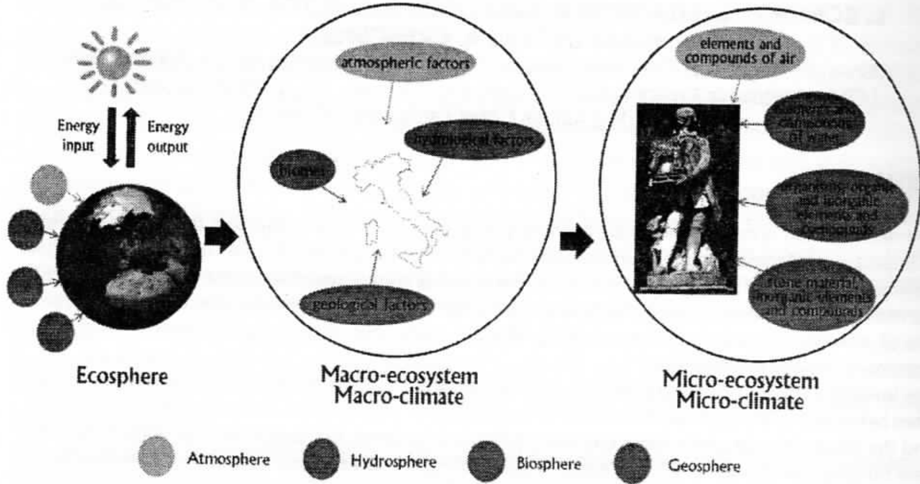

Figure 1. Ecological hierarchy of an artwork ecosystem as part of the ecosphere (http://cdn.intechopen.com/pdfs/20152/InTech-biodiversity_on_stone_artifacts.pdf)

The harmful effect exerted by microrganism colonising monument surfaces is scientifically known as bio deterioration. Lichens, algae, bacteria and fungi may affect or even cause physicochemical changes of the substrate, resulting in aesthetic, bio-geophysical and bio-geochemical damages. Indeed, biological colonizers can generate deterioration of stone surfaces through a variety of mechanisms, such as biofilm formation, biocorrosion caused by excretion of corrosive organic and inorganic acids, redox processes of mineral lattice cations, physical penetration and production of pigments [Papida *et al.*, 2000; Diakumaku *et al.*,1995; Saiz-Jimenez,1995]. It should be pointed out that biodeterioration usually results from a complex interaction between co-existing microorganisms and depends on environmental and climatic conditions, the nature of the art-stones and the bioreceptivity of whole materials used in the building [Dornieden *et al.*, 2000; Nugari *et al.*, 2009; Macedo *et al.*, 2009, Gaylarde *et al.*, 2005; Bellinzoni *et al.*, 2003; Caneva G *et al.*, 1995]. In general, high roughness and macroporosity have been demonstrated to give rise to high bioreceptivity [Guillitte and Dreesen, 1995; Tiano, 2002; Barberousse *et al.*, 2006].

Calcarenite is the lithotype widely used in the past as building material in the Mediterranean area [Warscheid and Braams, 2000)] and the artistic heritage of Southern Italy offers a remarkable example of this classic and typical architecture. Calcarenite is characterized by high percentage of calcium carbonate and high porosity; for this reason it is particularly sensitive to chemical, physical and biological agents. Indeed, the critical factor is the solubility of calcium carbonate, which is low in pure water but increases as the percentage of water dissolved carbon dioxide increases. In unpolluted areas, rainfall can gradually give rise to the dissolution

of calcium carbonate and, consequently, accelerate further decay up to stone surface crumbling [Schaffer, 1932]. This 'natural' deterioration is promoted and accelerated by acid pollutants in the atmosphere (especially sulphur dioxide and nitrogen oxides) produced by industries and other human activities [Welton *et al.* 2003].

The scope of the present paper was the morphological and molecular characteristisation of fungal populations colonizing stone surfaces and possibly involved in the biological alteration of calcarenite in historical buildings, aiming at the identification of remediation techniques based on natural methods.

MATERIALS AND METHODS

Experimental plan

The investigation has been performed on a private historical building (Figure 2a) constructed using calcarenite stones and sited between the archaeological site of Lavello, a little town located in the Basilicata Region (South of Italy), and the industrial area surrounding this town. To study in progress the degradation of stone materials, a new building sample (ca. 1 m3) was constructed by using the same material (33x15cm), collected from a local quarry in the territory of Gravina municipality, South of Italy (Figure 2b), and placed close to the historical building. Not destructive sampling was performed at the interval of three months. The samples were stored at 4°C.

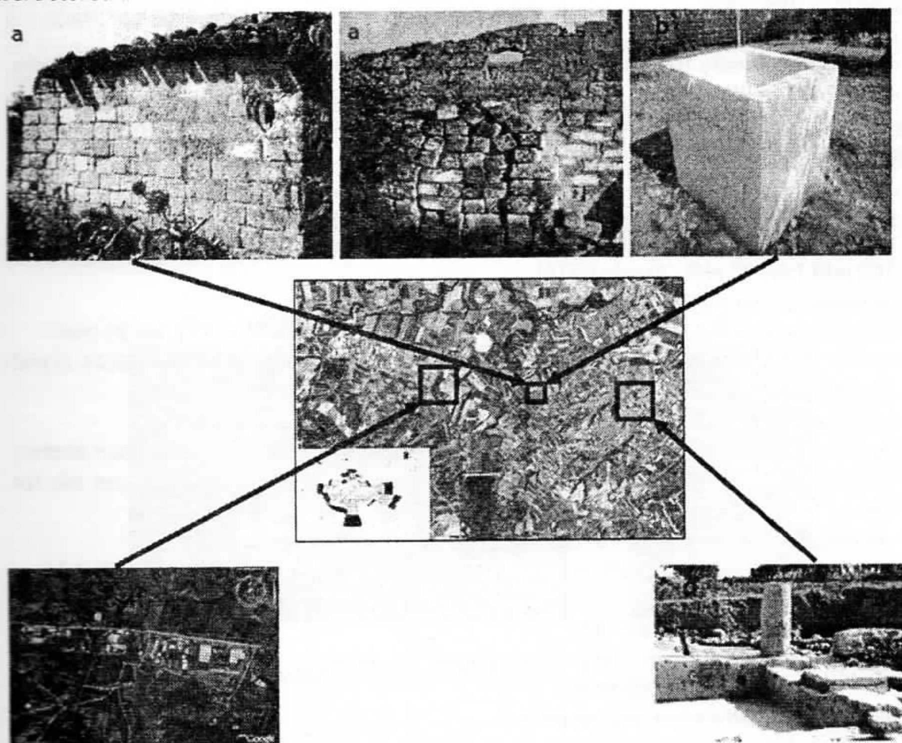

Figure 2. Old (a) and new (b) buildings concerned, and their location between the archaeological site (c) and the industrial site (d).

Microclimate parameters and air quality

Meteorological and air quality data were provided by ARPAB, the Agency of Environmental Pollution Monitoring of Basilicata Region. The meteorological and air quality stations were sited close to the new building sample and 11 Km far from the industrial area of S. Nicola di Melfi.

Surface analysis

Scraped powder and some little fragments were examined with different complementary techniques, namely: X-ray diffraction (XRD) by using Philips X'pert Pro MPD (The Netherlands), X-ray induced photoelectron spectroscopy (XPS) by using a Leybold LH X1 spectrometer (Cologne, Germany) equipped with a Mg/Al double anode, scanning electron microscopy (SEM) equipped with energy dispersive spectrometer (EDS) as detector.
SEM investigations were carried out on polished thin sections, after graphite sputter-coating of the samples. Two SEM instruments, a S360 (Cambridge Instruments) and an EVO-50XVP (LEO50XVP Karl Zeiss Group, Germany) were used. Microanalyses were conducted using an Oxford-Link EDS instrument equipped with a Ge detector and a 0.4-mm-thickned Super Atmosphere Thin Window (SATW).

Biological analysis

To molecularly identify the six recovered fungi, genomic DNA was extracted from mycelia grown at 24°C on PDA (Potato Dextrose Agar; Oxoyd, UK) 9 cm diameter plates, added with streptomycin 200 µg/ml (IBI Scientific, USA), following the procedure described by Raeder and Broda (1985), with the following modifications:

- grind mycelium fragments in a mortar containing liquid nitrogen;
- transfer powder in an Eppendorf tube containing 500 µl lysis buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl pH8, 20 mM EDTA);
- incubate 15 min at 55°C then centrifuge 5 min at 12.000 rpm;
- recover the liquid phase, add 10 µl RNAse 10 mg/ml and incubate at 37°C for 30 min;
- recover the surnatant, add 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and mix for 1 min;
- centrifuge 10 min at 13,000 rpm;
- recover the surnatant and repeat the extraction with phenol-chloroform-isoamyl alcohol;
- recover the surnatant, add 1 volume of chloroform-isoamyl alcohol (24:1) and mix for 1min;
- centrifuge 10 min at 13,000 rpm and recover the surnatant;
- add 2 volumes of absolute ethanol and 20 µl of NaCl 5 M, mix and incubate at least 1 h at -20°C;
- centrifuge 10 min at 13,000 rpm;
- discard the liquid and add 70% ethanol to wash the pellet;
- centrifuge 10 min at 13,000 rpm;
- discard the liquid and air-dry the pellet;
- re-suspend the pellet in 30-40 µl of sterile water.

PCR and sequence analysis

An aliquot (2 µl) of extracted DNA was used as template for PCR reaction with primers ITS1 and ITS4 (White *et al.*, 1990), specific for ITS regions in the cluster of ribosomal genes of fungi. Incubation mixtures for PCR amplifications contained DNA template, 1X reaction buffer, 2.0 mM $MgCl_2$, 200 µM each dNTP, 400 nM forward and reverse primers and 2.5 U of Taq DNA polymerase (Sigma-Aldrich, USA). All reagents were mixed and maintained at 95°C for 3 min. Thirty-eight cycles of PCR were performed by heating at 94°C 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final period at 72°C for 10 min. PCR products of the expected size (about 550 bp) were purified using Nucleospin Extract Kit (Macherey-Nagel, Germany) and sequenced (Stazione Zoologica "Anton Dohrn", Italy). The obtained sequences were analysed using BLASTn program with default parameters
(http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990).

RESULTS AND DISCUSSION

After only three months of the new building sample exposure, the adopted analytical methods were able to provide a series of data, which allowed the assessment of the incipient modification of the stone surfaces. The degradation appeared worsened performing the same observations on sixth month replicates, suggesting that environmental conditions modified the structure and the compactness of stones and favoured the biological colonization of surfaces especially in the South–East direction of prevailing winds. Fungi colonizing the stone surfaces of the new building sample as well as the historical building were identified as shown in the figure 3 and in table 1 and 2.
Figure 3 shows the electrophoresis analysis of PCR purified products. Table 1 reports percent of nucleotidic identity between ITS sequences of our isolates compared with those registered in GenBank. We identified the Alternaria (*A. infectoria*, *A. citri* and *Alternaria* sp.), *Coprinopsis* sp. *Penicillium piceum*, *Fusatrium equiseti* and *Scytalidium thermophilum*. Table 2 reports the main information about the isolates present in GenBank resulted the most similar to the isolates recovered from stones.

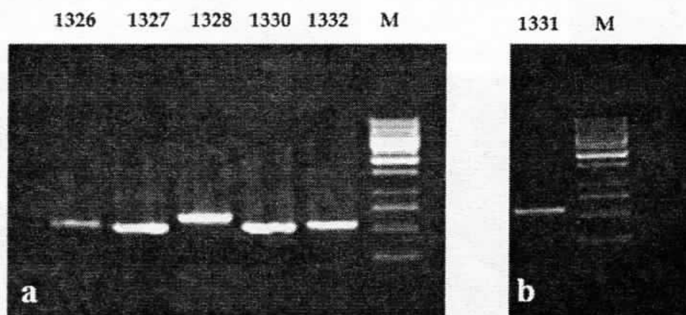

Figure 3 a-b. Electrophoresis analysis of PCR purified products. M = Molecular marker 1 kb (Fermentas, Canada)

Table 1. Results of ITS sequence comparisons between isolates in GenBank and those recovered from stones.

Sample	GenBank accession number (fungal species)	Sequence identity (%)
North	GU584952 (*Alternaria infectoria*)	99.8
East	JN541212 (*Alternaria alternata*)	100
East	HQ433353 (*Coprinopsis cinerea*)	99.8
South	JF776161 (*Fusarium equiseti*)	100
South	AY787846 (*Penicillium piceum*)	100
East	AY154705 (*Alternaria citri*)	100
North	*Scytalidium thermophilum*	100

Table 2. Main characteristics of isolates present in GenBank resulted the most similar to those recovered from stones.

Species and GenBank accession number	Source	Geographic area
Alternaria infectoria (GU584952)	grapevine	Austria
Alternaria alternata (JN541212)	*Bauhinia seminarioi*	Ecuador
Coprinopsis cinerea (HQ433353)	aspecific	France
Fusarium equiseti (JF776161)	*Paris polyphylla* var. chinensis	Chine
Penicillium piceum (AY787846)	aspecific	USA
Alternaria citri (AY154705)	*Citrus nobilis*	Iran
Scytalidium thermophilum	*Not specified*	China

Those colonisers were mainly found on the new building sample surface exposed to South–East direction, that of prevailing winds, but on old historical building were also detected inside the calcarenite stones. According to Cuezva (2008), Cuezva *et al.* (2009), and Jurado *et al.* (2009) scanning electron microscopy (SEM) confirmed fungal colonisation (Figure 4).

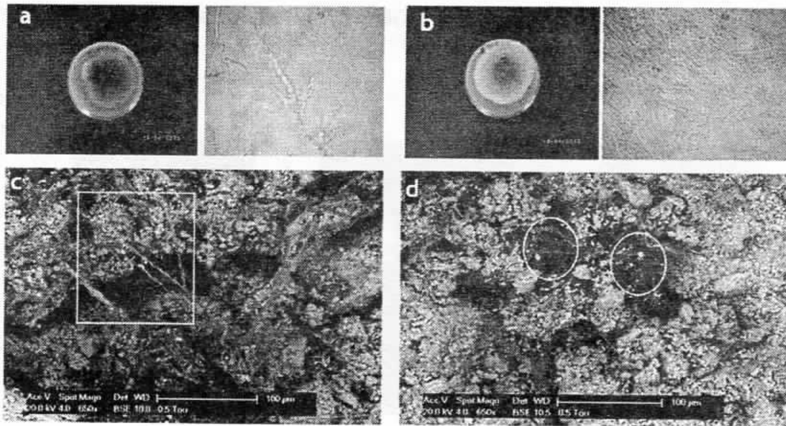

Figure 4 a-b-c-d. Fungal colonies identified in vitro (a and b) and confirmed by SEM analysis (C and D).

Although the mentioned fungal species are typically soil fungi, our findings are in accordance to the results of Šimonoviǻova *et al.* (2004) who found a considerable number of fungal colonies belonging to the same genus and species reported in the present paper. The identified microfungi can cause discoloration as well as mechanical exfoliation of building stones, which

phenomena were observed in our investigation through the mechanical hyphae penetration, and presence of different pigments (Alternaria) and biofilm, as those normally produced by some species of genus Alternaria and Penicillium. Alternaria are reported as dominant species among microfungi found on mineral substrates (Šimonoviãova *et al.*, 2004). Alternaria alternate and Penicillium piceum were also the most common species more recently isolated from mineral substrates (Milica V. Ljaljeviã Grbiã and Jelena B. Vukojeviã, 2009).
Unfortunately, between the identified fungi was found *Alternaria infectoria* (figure 5) to be able to induce both stone deterioration and some health problems to humans (e.g. dermatitis and allergies).

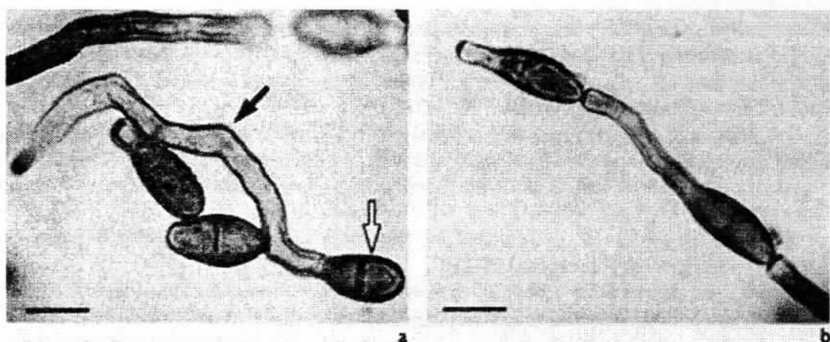

Figure 5. Lactophenol cotton blue mount of *A. infectoria.* (a) Microscopic appearance of conidia (open arrow) with apical outgrowth of long, geniculate secondary conidiophore (pseudorostrata) (closed arrow). (b) Chain formation of conidia, separated by pseudorostrata. Bars, 10 μm (Teysir Halaby *et al.*2001)

CONCLUSION

Biological infections and the intensity of biodeterioration processes are strongly influenced by water availability (Grant 1982, John 1988, Ortega-Calvo *et al.* 1995, Bellinzoni *et al.* 2003). This relationship is affected by material-specific parameters, like porosity and permeability, environmental conditions of the site and exposure (Warscheid and Braams, 2000). The establishment of fungi on rocks is possible even without the pioneering participation of phototrophic organisms. Fungi are able to penetrate into the rock material by hyphal growth combined with corrosive activity, due to excretion of organic acids or by oxidation of mineral-forming cations, preferably iron and manganese. Their deterioration activities also include discoloration of stone surface, due to the excretion of melanin by dematiaceous fungi (Warscheid and Braams, 2000). It has been reported that fungi comprise a significant component of microbiota in a wide range of rocks including sandstone, granite, limestone, marble and gypsum (Burford *et al.*, 2003). Šimonoviãova *et al.* (2004) ascertained the presence of 36 different microfungi on stones in hypogean cemetery in Bratislava. In the Earth's lithosphere, fungi are of fundamental importance as decomposer organisms, being ubiquitous in subaerial and subsoil environments. The ability of fungi to interact with minerals, metals, metalloids and organic compounds through biomechanical and biochemical processes, makes them ideally suited as biological weathering agents of rock and building stone. Biological and mycological investigations may be a very important part of good conservation practices, and cannot be ignored in modern conservation concept which includes close collaboration between art and science. The

progress of this work should be the development of new tools based on antagonist microorganisms and/or bioactive ingredients produced by them as biological cleaning agents for a natural remediation (bioremediation) of deteriorated monuments. Those biocides will be tested in laboratory and later on in situ, then compared with traditional cleaning methods.

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