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Fungal deterioration of medieval wall fresco determined by analysing small fragments containing copper

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Abstract

A medieval fresco at the Chapel of the Holy nail, Siena was analysed to determine microbiological causes of deterioration of the painted surface or fresco micro-layer. Fragments of fresco a few mm wide were sampled under sterile conditions and some were incubated in mineral medium to isolate microbial species growing on endogenous carbon sources. Quiescent spores of fungi were detected. After 18 h fungal hyphae grew from turgid spores and pH dropped from 5.5 to 5.0, suggesting organic acid production during growth. Light microscopy (LM) and transmission electron microscopy (TEM) were used to study the ultrastructure of the fungi. Molecular studies involving sequencing 18S rDNA led to identification of *Penicillium crysogenum*. Element concentrations in the painted layer were analysed by scanning electron microscopy (SEM) with energy dispersive X-ray microanalysis of the fresco micro-layer before and after incubation of fragments. The main finding was that total carbon decreased from 26% to 13% while copper increased from 5% to 28%. The high concentration of copper and the acid pH inhibiting bacterial proliferation, indicated *P. crysogenum* as the main agent of deterioration. Hypotheses are formulated with regard to the high concentrations of copper useful for conservation of fresco substrates.

Keywords: Biodeterioration; Copper; Hypha; Light and electron microscopy; Microanalysis; Molecular biology; *Penicillium crysogenum*; Spores

1. Introduction

Recent studies have shown that substrates of medieval paintings are very stable because they contain high concentrations of heavy metals such as lead, copper and mercury (Ciferri, 1999) and organic substances of animal origin, such as egg yolk rich in lipids (Huang et al., 2003), or fig latex containing fatty acids (Hosamani and Pattanashettar, 2003), fig leaves containing powerful triterpenoids which are persistent irritants, or unripe figs containing acrid and caustic proteolytic enzymes (Saeed and Sabir, 2002). These organic solvents are the main binders of inorganic pigments, which skilled medieval artists used to colour temperas for frescoes (Cennini, 1437).

Growth of microorganisms on paintings may cause aesthetic and structural damage. Frescoes may be colonized by communities of organisms that interact with the substrate at different stages (Ciferri, 1999). The process is related to the bioreceptivity of the painted surface (Urzì and Realini, 1998). Light and sufficiently warm temperatures combined with rich nutrients in the medieval substrate create suitable conditions for reproduction of quiescent fungal spores (Gorbushina et al., 2004). Those sensitive to humidity and heat emit hyphae that penetrate the substrate, with mechanically destructive effects (Berner et al., 1997). As colonization proceeds, the smooth surface of the painting is modified: pigments initially compact and resistant to attack by various agents become rough and bioreactive (Giullitte, 1995). These events give rise to exfoliation, cracking and loss of the coloured patina (Ciferri, 1999).

A complex problem is the interaction between biodeterioration and the effects of biodegradation (Eggins and

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Oxley, 2001). Certain deteriogenic fungi change cell surface molecules, causing genetic and epigenetic recombinations (Vestrepen et al., 2004). Their metabolic products cause further chemical damage (Angell and Chamberlain, 1991; Beech, 2004). The capacity of fungi to dissolve carbonates depends on available carbon sources, such as oxalic and citric acids which may mobilize cations with chelating activity (Hirsch et al., 1995).

The aim of this study was to select the main agent of deterioration, a pioneer organism able to growth only on organic carbon residues of fresco surface, in the medieval Chapel of the Holy nail, Santa Maria della Scala, Siena. The fungal and microbial populations attacking fresco substrates were identified and characterized by recent molecular and genomic biology techniques involving amplification of DNA region with high polymorphism (Rölleke et al., 1998).

2. Materials and methods

2.1. Chapel history

The chapel had been deconsecrated and was used as the hospital archives for a period beginning in the 1960s (Berenson, 1968). The frescoes then coexisted with long rows of shelves packed with books and clinical records where bacteria and microorganisms carried by the respiratory tracts of hospital staff (Flamming, 2001) circulated together with quiescent spores transported by insects that settled on the porous painted walls (Gorbushina and Petersen, 2000). Being hygroscopic, the wooden shelves of the archives absorbed humidity in the air and rising damp in the walls to some extent. In spring and summer when the temperature exceeded 20 °C a warm-humid microclimate was established. Under these poorly aired conditions, condensation often formed on the ceiling.

The environmental factors triggering deterioration included relative humidity and temperature. The high vaulted ceiling permitted air stratification with temperature gradients that could change in relation to external temperature (Camuffo, 1998). The rectangular room measures 15 × 10 m with thick walls about 6 m high. Small closed unequal windows with thick opaque glass created humidity gradients that affected microclimatic conditions in the room.

Prompted by the Florence flood of 1966 that damaged the artistic treasures of that city, the Istituto Centrale del Restauro in Rome recommended that acrylic resins such as Paraloid B 72 be used against fungal contamination (Bassi and Giacobini, 2001).

In the 1970s, only constantly damp frescoed ceilings of the chapel were treated with acrylic resin. The result was encouraging and those treated with Paraloid B 72 no longer harbour fungi, however close examination showed a shiny yellowish plastic patina. The walls did not have urgent problems of deterioration due to the presence of the books, and were not treated with acrylic resins.

In the 1990s, the medieval hospital of Santa Maria della Scala was vacated and the hospital archives were removed from the Crucifixion Chapel. In the next few years, the frescoes not treated with Paraloid B 72 deteriorated rapidly due to direct contact with humidity. The present study was concerned with a detail of the chapel wall bearing 600-year-old frescoes by Vecchietta. A preliminary survey was conducted into the causes of deterioration. Deterioration of Vecchietta's Crucifixion was very evident (Fig. 1): part of original blue of the sky had turned green and parts of the fresco were discoloured and/or had lost their coloured patina.

2.2. Sampling and culture

Sampling was performed according to recommendations Normal procedures (3/1980). Ten irregular fragments (approximately 1 × 3 mm and 0.5 mm thick) of the painted surface were obtained from zone A (Fig. 1 arrow), with sterile tweezers in collaboration with restorers, before restoration scaffolding was erected. All samples were placed in sterile plastic tubes and numbered. Seven fragments were inoculated in solid medium containing per liter 1 g $MgSO_4 \cdot 7H_2O$, 0.7 g KCl, 2 g KH_2PO_4, 3 g Na_2HPO_4, 1 g NH_4NO_3, 15% agar (Difco) and incubated for 18 h at 28 °C (Baldi et al., 2003). Final pH was 5.5. Petri dishes containing medium without painted fragments were used as controls.

2.3. Microscope observations and microanalysis

In order to observe the microorganisms responsible for the fresco deterioration in situ, light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) with energy dispersive X-ray microanalysis (EDAX) of superficial substrates were used.

Two incubated wall fragments were observed by light microscope (Zeiss Axiophot) to check for fungal growth and hyphal development. The same specimens were also observed by TEM (Philips Morgagni 268D). This involved fixing in 3% glutaraldehyde for 30 min and 1% osmium tetroxide for the same time, then dehydrating in increasing concentrations of ethanol (40%, 60%, 80%, 100%) at room temperature. The samples were gradually infiltrated in Spurr's low viscosity embedding resin (Spurr, 1969) and polymerized at 70 °C for 7 h. After polymerization, they were sectioned with an LKB III ultramicrotome using a diamond knife. The thin sections were collected on copper grids, stained for 3 min in 2% uranyl acetate and 2% lead citrate and observed by TEM.

Three original fragments and three from the culture medium were glued to standard vacuum-clean stubs and coated with graphite (Edwards,

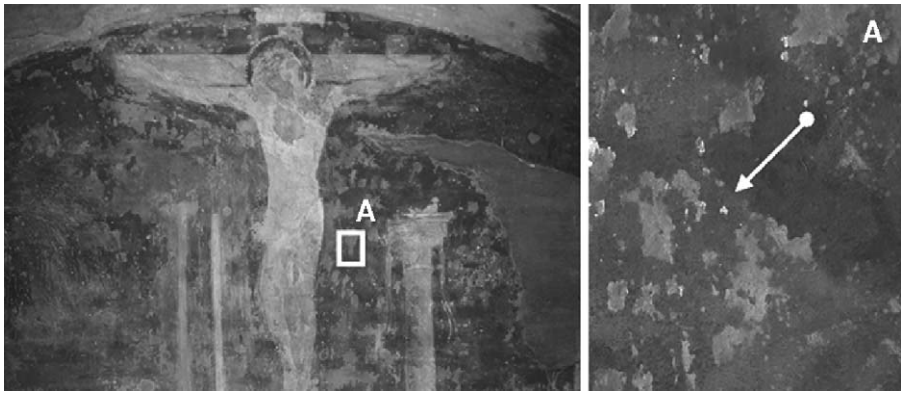

Fig. 1. Fresco in the medieval Chapel of the Holy Nail, Siena, showing crucifixion and site (A) of fragment sampling.

carbon scancoat, S150A) and observed by SEM (Philips XL20). The instrument was equipped with an EDAX DX4 probe for energy-dispersive X-ray microanalysis used at 20 kV acceleration voltage for determining elements of the pictorial surface. Concentrations had an approximated error of 1%. Mean concentrations and standards deviations of each element were calculated from five random determinations on different spots of analysed sample. The X-ray beam was 4 μm wide and penetrated to a depth of 2 μm.

2.4. DNA amplification and sequencing

To identify the pioneer microorganism involved in fresco deterioration, direct DNA extraction from incubated wall fragments was performed. Sterile mQ water (10 μl) was added to two wall fragments cultures, isolated in 0.2 ml PCR tubes. Samples were exposed to one-step heat shock cycles at 95 °C for 30 min. After heat-shock, samples were placed on ice until PCR mixes were complete. PCR conditions, run in a two-phase protocol (pre-amplification and amplification), were slightly modified from Jasalavich et al. (2000). An MJ DNA thermal cycler was used in all amplification experiments. Internal transcribed spacer (ITS) region of nuclear-encoded ribosomal RNA genes was amplified with ITS1-F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). ITS1-F primer is designed specifically to amplify the ITS region of basidiomycetes, and ITS4 is considered a universal primer for fungal rDNA. Primers 341f and 907 (Gurtner et al., 2000) used in the secondary amplification were designed to amplify fragments corresponding to the *Escherichia coli* 16S rDNA sequence from positions 341 and 926. PCR amplification mixes consisted of Taq buffer 1X (Amersham Biosciences), 200 M dNTP, 50 pmol forward primer and 50 pmol reverse primer, 5U Taq DNA polymerase (Amersham Biosciences) and sterile water to a final volume of 100 l.

For pre-amplifications, after an initial denaturing step at 95 °C for 5 min, 40 PCR cycles, consisting of 35-s exposure at 95 °C, 35-s exposure at 53 °C, and 60-s exposure at 72 °C, were performed. A final extension step was added, exposing samples for 10 min at 72 °C. Secondary amplification was performed using 0.5 l of the first PCR mix as template under the same amplification conditions. Secondary amplification products were loaded into 1% agarose gel and run at 60 V in TAE 1X. The PCR products were incorporated with Et-Br and visualized on a transilluminator to check the specificity of the PCR amplification reaction. The PCR products were cloned in *E. coli* by TOPO-TA cloning kit (Invitrogen). DNA sequencing of recombinant TOPO vectors was done according to the user manual of the Autoread sequencing kit (Amersham Biosciences) on an ALFexpressII automated sequencer (Amersham Biosciences). The sequences obtained were analysed by BLAST search in Genbank and CLUSTAL W multiple alignment software was used to produce the phylogram within the genus *Penicillium*. The consensus sequences were obtained by analysis of five different recombinant TOPO vectors harbouring the same PCR amplification fragment.

3. Results and discussion

3.1. LM, TEM and SEM analysis

The fungus that grew in the mineral medium containing inorganic nutrients and only organic carbon residues found in the original fresco fragments was the pioneer organism and possibly the agent responsible for biodeterioration of the frescoes in the Crucifixion Chapel of Siena Medieval Hospital. This approach excludes the growth of other opportunistic microorganisms for short incubations (18 h). LM analysis showed mycelium production when culture was prolonged for a few days (Fig. 2).

TEM analysis of longitudinal sections of hyphae (Fig. 3a) showed well-defined electrondense cytoplasma

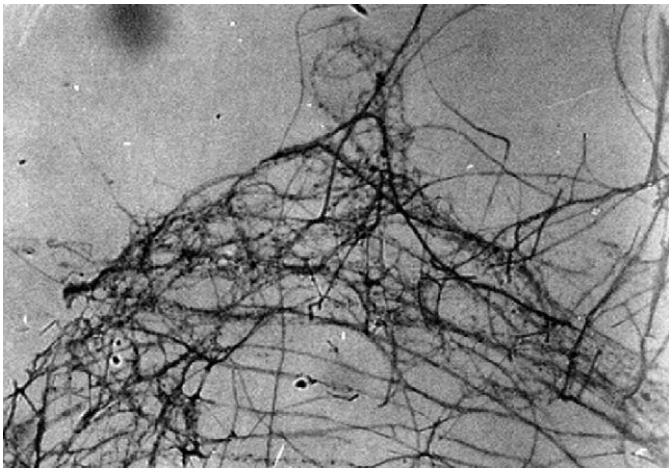

Fig. 2. Light microscope image showing hyphal growth after a few days of culture (hypha sections measure about 1.5 μm).

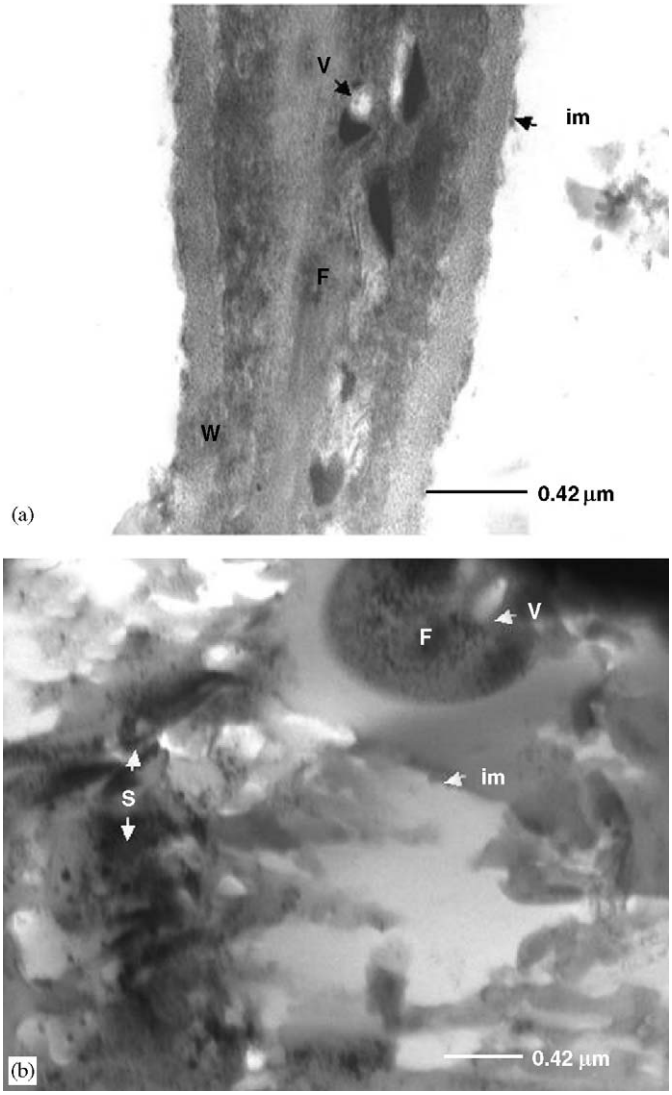

Fig. 3. (a) Longitudinal section of hyphae F, showing cell wall w, cytoplasm with vacuole v and matrix im; (b) cross-section of hyphae-substrate showing pictorial substrate s, hyphae cytoplasm and cytoplasmic vesicle v (bar = 0.42 μm).

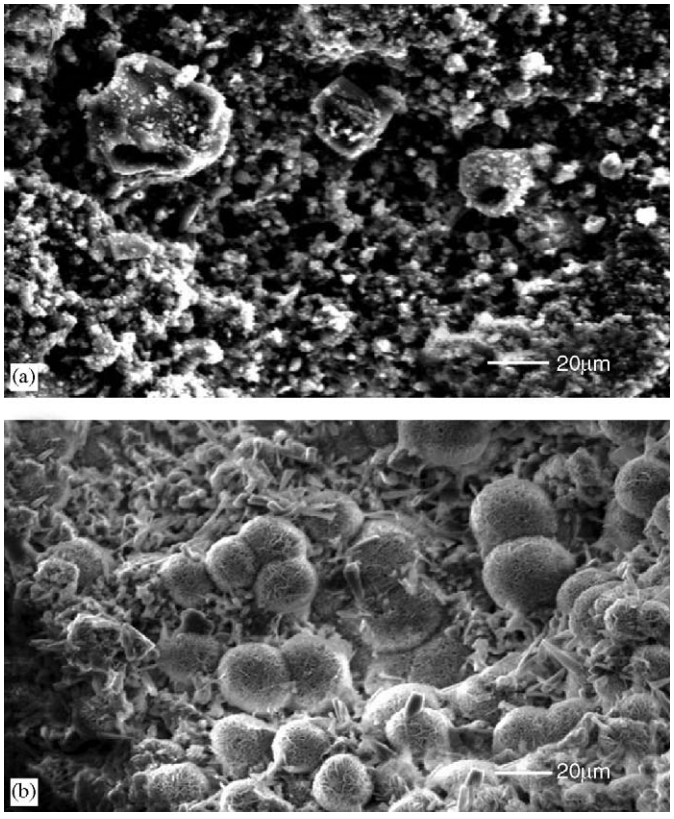

Fig. 4. (a) External surface of microfragment showing quiescent fungal spores; (b) fungal spores on microfragment surface proliferating in culture medium (bar = 20 µm).

rich in vacuoles and lipids as well as interfacial matrix. Cross-sections showed interaction between fungus wall and substrate with visible electrondense layers of metabolic precipitate around hyphae (Fig. 3b). This confirmed the hypothesis that the hyphae isolated penetrated the substrate, breaking it up by direct mechanical action and causing disintegration of the coloured patina, as commonly found (Ciferri, 1999).

SEM observation of original fragments showed cracks and rough surface containing fungal spores (Fig. 4), quiescent due to long desiccation. When the fragment was incubated in mineral medium, the spores became turgid, showing walls with species-specific ornamentation, and emitting juvenile hyphae.

3.2. Molecular study of the organism isolated

The ITS1-F and ITS4 primers used in the pre-amplification step selectively amplify basidiomycete ITS sequence. PCR showed a unique 512 bp product from the cultured fragments. The PCR product was cloned and sequenced. BLAST confirmed the specificity of amplification of fungal rDNA, and the most significant score was obtained in the genus *Penicillium*. Different *Penicillium* species were significantly recognized, although *Penicillium chrysogenum* (accession number AY373903) was assigned the highest

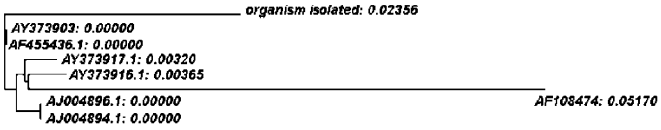

Fig. 5. Phylogram of *Penicillium crysogenum* and other related *Penicillium* and *Eupenicillium* spp. produced by CLUSTALW. The following Genbank accessions were compared: organism isolated from fresco micro-layer, AY373903 *P. crysogenum*, AF455436.1 *P. commune*, AY373917.1 *P. griseofulvum*, AY373916.1 *P. glandicola*, AJ004896.1 *P. dipodomys*, AJ004894.1 *P. nalgiovense*, 4AF108474 *Aspergillus niger*.

blast score (410). The *P. chrysogenum* intergenic rDNA region was aligned with 12 different accessions of *Penicillium* spp. by Clustal W. The phylogram revealed genetic distances from *P. crysogenum* and other major *Penicillium* spp. (Fig. 5). rDNA exists as a multiple-copy gene family consisting of very similar DNA sequences arranged in tandem. Each repeate unit has coding regions for one major transcript, punctuated by one or more intergenic regions. The ITS region is perhaps the most widely sequenced DNA region in fungi. It has typically been used for molecular systematics at species level, and even within species to distinguish races in relation to their geographical origin or distribution.

No results were obtained by using the 341f and 907r primers designed to amplify the *E. coli* 16S rDNA sequence. The reason for using fungus- and bacteria-specific primers was to give the DNA amplification test the greatest chance of selectively amplifying and identifying different organisms in the fresco substrate. Microorganism populations may consist of complex mixtures, including many bacterial and fungal species (Gurtner et al., 2000; Bassi and Giacobini, 2001). The fact that ribosomal genes are present in several copies per genome provides good detection sensitivity even when there is shortage of starting material as in our case. What DNA testing can tell us depends largely on the extensive sequence information available in databases. The present results demonstrate that the PCR-based ITS region of ribosomal DNA can be successfully amplified from the fungi colonizing the fresco. Molecular data processing by Blast search was sensitive for distinguishing ITS regions of different organisms and indicated *P. chrysogenum*, a common isolate from mural paintings (Ciferri, 1999), as the prevalent organism. This indication is in line with the results of SEM/TEM analysis. The origin of this fungus in the chapel may be paper particles from the archive shelves (Montemartini et al., 2003). The room hosted the archives for about 30 years. Since paper absorbs humidity, it may provide a good incubation medium for the spores of this fungus.

3.3. Element composition of fresco fragments

Concentrations of elements on the surface, before and after incubation in mineral medium, were significantly different (Fig. 6). The percentage of total carbon dropped from 26% to 13% after 18 h incubation. The carbon was

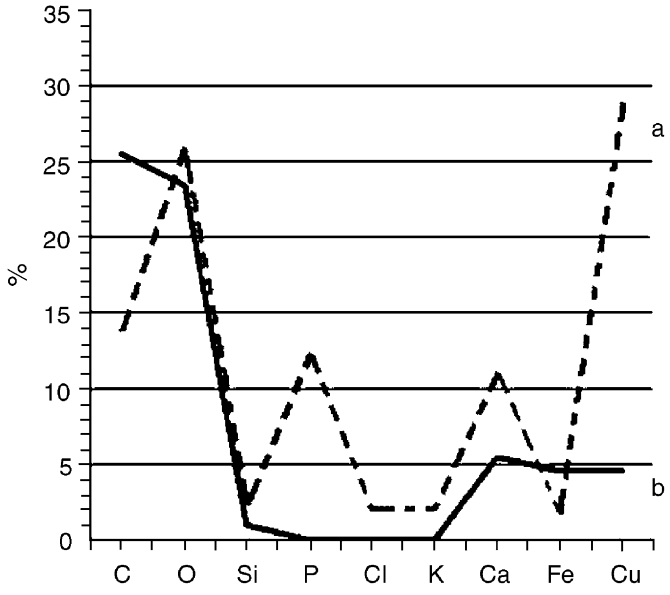

Fig. 6. X-ray microanalysis of the substrate: (a) with and (b) without hyphae.

consumed by *P. crysogenum*, which colonizes organic glue and binders mixed with mineral pigments commonly used in the 14th century (Cennini, 1437), washed over the inorganic carbon of lime used in the supporting plaster. The initial relatively acid solution (pH 5.5), favourable for fungal growth, suggests the presence of plant glue, such as the acid latex of figs and/or fig leaf extracts (Hosamani and Pattanashettar, 2003; Umerie et al., 2004), commonly used by medieval painters. Exogenous carbon sources, such as people's breath and sweat, lamp-black residues and dust particles, cannot be excluded. All these organic substances could induce the proliferation of *P. crysogenum* which was responsible for decreasing total carbon content of the patina. The initial pH dropped from 5.5 to 5.0 after 18 h incubation, suggesting fermentation of organic carbon. *P. crysogenum* is known to produce citric acid and/or oxalic acid from sugars (Vinze and Ambekar, 1986); these acids react with metals causing them to precipitate (Stokes and Lindsay, 1979; Clausen et al., 2000). The acidity inhibits bacterial proliferation (Bruce et al., 2003) and triggers fungal growth. For example, fungal cellulase activity is optimal at pH 5.5 (Castellanos et al., 1995). *P. crysogenum* is the famous fungus from which penicillin and other antibiotics are derived (Elander, 2003).

The other element which changed drastically on the surface layer was copper. This metal was not added to the medium and the original fragment contained up to 5%. This concentration could arise from the inorganic pigment azurite, a blue pigment consisting of copper carbonate hydrate $[Cu_3(CO_3)_2(OH)_2]$ (Matteini and Moles, 1990). Azurite is a cheap substitute for the more stable lapis lazuli (a complex alumino-silicate mineral) used for example by Giotto in the Scrovegni Chapel frescoes (Padua, Italy; 14th century). With time and humidity, azurite spontaneously converts to malachite $[Cu_2(CO_3)(OH)_2]$ and CO_2, conferring a light green colour to the frescoes. Today the painted sky of the crucifixion in the 'Santo Chiodo' chapel is visibly green.

In incubated fragments, the concentration of copper on the surface increased up to 28%. This concentration is five times higher than in the original fragment. Humar et al. (2004) reported that some painted surfaces colonized by fungi show an increase in copper content. Our explanation for this unexpectedly high concentration is that copper was leached from the fragment and concentrated in fungal hyphae coating the surface. Organic acids produced *P. crysogenum* can dissolve carbonates (Ciferri, 1999) and leach Cu which may precipitate on the fungal walls due to oxalic acid production (Stokes and Lindsay, 1979; Clausen et al., 2000). The electrondense layer matrix around fungal hyphae in Fig. 2b could be an area with higher copper concentrations responsible for conferring high Cu-tolerance to our strain of *P. chrysogenum*. This species is known to grow at high Cu concentrations (Bagy et al., 1991; el-Meleigy, 1992; Tan and Cheng, 2003). High levels of Cu together with acid pH and biosynthesis of antibiotic leads to further microbial selection on the mural.

Other elements such as phosphorus, chlorine, potassium and calcium increased after incubation because they were present in the mineral medium; others like oxygen, silicon and iron were similar in the original and incubated fragments.

The growth of *P. chrysogenum* therefore not only causes mechanical destruction of the pictorial patina, but also fading of colour due to leaching of Cu from the mineral pigment. Since the chapel was restored, the murals have been protected from fungal growth by controlling ambient temperature and humidity.

4. Conclusion

These results show that high concentration of copper reduced the biodiversity of microorganisms in the pictorial surface as already reported by Mitchell and Gu (2000). The biodeterioration was particularly effective in the area of the crucifixion where the wall was not treated with resin and organic carbon residues had been enriched with different types of deposits in the course of time. The addition of nutrient salts diluted in water favoured rapid proliferation of fungal spores and later the formation of mycelium on endogenous carbon sources. The fungal hyphae chelated cations and fungal metabolism leached copper from the pictorial surface with organic acids (decrease in pH from 5.5 to 5). While the acidic environment inhibited the growth of bacteria, *P. crysogenum* thrived, producing hyphae and physically breaking up the fresco surface. The fungus also concentrated copper fivefold in its hyphae with respect to the original fragments. This strain of *P. crysogenum* is particularly tolerant to copper, the source of which was copper carbonates such as azurite. Vecchietta used this common inorganic pigment instead of the

precious lapis lazuli. Azurite is unstable and eventually transformed into malachite (green colour) and under the action of organic acids, dissolved to copper ions and CO_2.

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