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Non-Toxic Fumigation and Alternative Control Techniques Against Fungal Colonization for Preserving Archaeological Oil Painting

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Abstract: In this study, samples were collected from the deteriorated parts of archeological oil painting of Ismael pasha exhibited in Al-Gizyra museum, Egypt. The tested oil painting grounds belonged to the period from beginning of the 19th century to the middle of the 20th in Egypt were analysed and fungal deterioration aspects were examined by different techniques such as Scanning Eectron Microscopy (SEM), X-ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR). The data show that calcium sulphate was the major component of the tested oil painting sample. Brittleness and deep cracks were observed as result of fungal damage. Seventeen different fungal species were isolated from the tested Ismael pasha oil painting, belonging to the genera of *Acremonium*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Curvularia*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium*, *Phoma*, *Rhizopus*, *Scopulariopsis*, *Stemphylium* and *Trichoderima*. The data reveal that *Cladosporium cladosporioides* contributed the broadest spectra in the tested oil painting. Screening for proteolytic and celluloytic enzyme activities indicate that the genera of *Cadosporium*, *Alternaria* and *Aspergilli* showed the highest significant enzyme activities. Comparative sensitivity to radiation against all isolated fungal species indicate that the treatment of the tested fungal species with diode laser lead to complete inhibition of all tested species after 15 min exposure time. Calcium sulfate at 0.10% concentration lead to the highest dry weight of *C. cladosporioides* (1.63 g/100 mL), while the change in pH was nearly non-significantly affected with sulphur concentration. Gel electrophoresis patterns of the most radioresistant species (*C. cladosporioides*) reveal the dramatic loss of essentially all major protein bands after laser irradiation.

Key words: Fungi, archaeological, radiation, laser, UV, gamma

INTRODUCTION

Paintings are essential components of cultural heritage all over the world and science can offer the necessary tools for the physical, chemical and structural characterization of the material to highlight the various stages of the biological degradation processes (Matteini and Moles, 2002). Paintings are composed of cellulosic support, a preparation layer and a paint layer, may be easily degraded by microorganisms, as may the materials (glues) used to prepare a ground layer thus, besides the organic nature of the support, it contain organic molecules that many microorganisms may utilize for growth, such as sugars, gums, polysaccharides, proteins and oils (Strzelczyk, 1981).

The role of microorganisms in the degradation of our cultural heritage was studied by Koestler *et al.* (1997). The microbial colonization of oil paintings was tested by Seves *et al.* (1996a, b). In painted works of art, the fungal deterioration processes can involve either a portion of the painting or all of its components (Strzelczyk *et al.*, 1987). Wide variations in the species isolated from different periods were reported in the analyses of the fungal flora present on paintings (Agrawal *et al.*, 1988).

The fungal flora attacking paintings include virtually all species of fungi because the variety of organic components of these works of art can represent a carbon source for practically all species, in addition, they show a great tolerance for environmental conditions and can use condensation moisture (Dhawan and Agrawal, 1986). The development of fungi on the surface of paintings induces aesthetical, mechanical and biochemical decay, where the growing mycelium spread over the paints masking design and colour, while the growth of hyphae and fruiting bodies inside the support can cause friability and loss of the paint layer (Tiano, 2001). Ionita (1973) isolated 26 different species of fungi from stains appearing on the frescoes from areas of efflorescence and from zones in which the painted layer was fissured and portions were breaking away from the support. The species that are just saprophytes living on the painted surface may be growing at the expense of other microorganisms

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colonizing the frescoes, however, the idea that fungi may be the primary microbiological agents responsible for degradation of art works is so entrenched (Guglielminetti *et al*., 1994). Fungi such as *Phanerochaete chrysosporium* or *Trameies versicolor* are effective at degrading and discoloring dye due to the production of enzymes (Jarosz-Wilkolazka *et al*., 2002). Effect of cultural conditions on cellulases from *Chaetomium indicum* and *Stachybotrys atra* isolated from monumental objects was recorded by Darwish *et al*. (2005).

The development of new technologies and materials to prevent fungal painting damage permits planning of restoration and conservation (Baldini, 1996). In order to control biodeterioration processes the control methods can be classified as mechanicals, physicals and chemicals. Traditional mechanical methods involve the physical removal of biodeteriogens either by hand or with tools, while mechanical methods can damage the substrate (Ionita, 1971). Chemical solvents have negative effects on the objects to be treated and not always effective against survival resistant or quiescent phase structures (Tiano, 2001). Murals restored were cleaned and treated with nystatin but showed the appearance of greenish brown to black spots on the painted surface (Sampo and Mosca, 1989). Physical methods include different types of radiation (gamma, UV and laser radiation). Gamma rays are a form of electromagnetic radiation used for sterilizing micro flora especially on organic materials such as paint (Van Der Molen *et al*., 1980). UV Solar radiation has a lethal effect on natural populations of culturable outdoor atmospheric microorganisms (Tong and Lighthart, 1996). UV irradiation used to disinfect indoor environments public shelters (Miller and Macher, 2000). There have been some reports about the effect of laser irradiation on microorganisms (Iwase *et al*., 1989). No significant harmful effect has occurred on the mechanical and physical properties of pure printing inks when applying ionizing radiation (Rocchetti *et al*., 2002). Several research groups confirmed the efficiency of ionizing radiation on biodeteriorating organisms (Magaudda, 2004).

The final goal of this research is to characterize a series of markers to be used for the analysis of deterioration aspects and diagnosis of incipient pathologies to the knowledge of fungal mycoflora that occurs on oil painting in order to propose the most appropriate preventative maintenance non-destructive techniques employed on the archaeological oil painting.

MATERIALS AND METHODS

Methods of measurement of fungal deterioration: Mineralogical properties have been primarily checked by (SEM and XRD) for inorganic materials and FTIR spectroscopy for organic products.

I-Scanning Electron Microscope (SEM) studies was carried out by using SEM Model Phillips XL30 with accelerating Voltage 25 K.V, X 420 and resolution for 50 μm was used to study the changes of surface morphology and particle size of the fungal deteriorated ground samples. II-X-ray Diffraction Analyses (XRD) were performed on Phillips X-ray diffraction equipment model pw/1840 with Ni filter, Cu radiation 1.54056 A°at 40 KV, 25 mA, 0.05 sec^{-1}. III-Fourier Transform Infrared Analysis (FTIR) allows the oil painting ground sample to identify the adhesive material used in oil painting ground (Rao, 1963).

Isolation and identification of fungal colonization: Czapek-Dox's agar media was used as isolation medium. The medium was brought up to 1000 mL with distilled water. Fifteen ml of this medium were cooled to just above the solidification and added to each Petri-dish. Swabbing with sterile cotton swabs and scalpel from markedly damaged surfaces of the tested oil painting with visible colonies of microscopic fungi was carried out. In the laboratory, swab samples were shaken mechanically for 10 min in 10 mL sterile distilled water and 1 mL aliquots of the resulting suspensions used to prepare spread plates on Czapeck's Dox agar. Plates were incubated in the dark at 27°C for 7 days and the microscopic fungi were identified using the diagnostic keys of Gilman (l957), Barnett and Hunter (1972) and Moubasher (l993).

Screening for proteolytic and celluloytic enzyme activities: Proteolytic activity in the culture medium was determined by the lysis clear zone method according to Badr, 1995, modified as follows: 50 μL clear cell free culture medium was inoculated (6 mm well) into proteinase indicator medium (0.5% gelatin, 2% agar and 0.05 M Tris-HCl at pH 8.0). Plates were incubated at 35°C for 24 h and then flooded with protienase indicator solution (15 g $HgCl_2$, 20 mL HCl and 60 mL distilled water). The clear zones were measured in mm. Cellulolytic clear zone (mm) method was carried out according to Toyama and Toyama (2000).

Comparative sensitivity to radiation against isolated fungi

Gamma irradiation: The source of irradiation used for the tested fungal species was Cobalt-60 gamma cell 3500. This source is located at amiddle Eastern Regional Radioisotopes Center for the Arab countries (Dokki, Cairo). The dose rate was 2.4 Gy min^{-1}. Fungal species were irradiated with dose of 25 kGy at exposure times (6, 12 and 18 h). III-UV radiation: Spore suspension of the isolated fungal species were prepared in saline solution (0.85%, w/v, NaCl containing a drop of Tween 80) from

7 days old slant and irradiated with Philips TUV -30-W-245 nm Lamp, type No. 57413-P/40 at a distance of 20 cm at exposure times (5, 10 and 15 min). The treated spores were kept in dark for 2 h to avoid photo activation repair. II-Laser irradiation: Laser source was located at the National Institute of Laser Enhanced Science (NILES), Cairo University. The laser used was solid state diod laser of wave length 650 nm type DC Brushless PAT. Pending Crop. Tokyo. Japan. It was applied at output level of 250 mW. The total energy delivered was 30 J at exposure times (5, 10 and 15 min).

All assays were conducted in triplicates. The irradiated spores were spread into plates containing Czapek-Dox's agar media for five days incubation period. The growing colonies were counted against the control plates. The changes in survival caused by exposure to irradiation were evaluated by determining the relative recovery of microorganisms. The relative recovery was estimated as a ratio of number of colonies obtained with the impinge to the total number of microorganisms.

Effect of different concentrations of sulfate salts on the growth of the most radioresistant species: A medium of Czapek-Dox's media with $CaSO_4$ salt as sulphate source was added to the flask containing the liquid medium at five concentrations (0.025, 0.050, 0.100, 0.200 and 0.400%) and non salt medium was used as control. Triplicate flasks which inoculated with the fungal discs were incubated at 27°C for 10 days. Final pH and dry weight were determined.

Gel electrophoresis: Gel electrophoresis was carried out to show the effect of the most efficient radiation source on the most resistant species. Extract proteins were analyzed with Sodium Dodecyl-sulphate-polyacrylamide Gel Electrophoesis (SDS-PAGE). The Laemmli SDS-PAGE discontinuous system with homogenous gel was used under reducing or non-reducing conditions (Laemmli, 1970). Gels were stained with Silver staining (Morrissey, 1981).

RESULTS AND DISCUSSION

Source of isolation: In the present work the selected oil painting is Ismael pasha painting and was obtained from Al-Gizyra museum belonged to rulers of the family of Muhammad Ali Pasha on canvas before given the title of khedive (1863-1867 AD), 73.5×59 cm, No.7/32 (Fig. 1). A scientific study of the grounds of these oil painting to know the kinds of these grounds and the main materials that used in this period from beginning of the 19th century to the middle of the 20th in Egypt and also to study the types of fungal deterioration. The ground layer of oil painting is the layer between the support and the paint layer (Fig. 2).

Fig. 1: Oil painting of Ismael Pasha

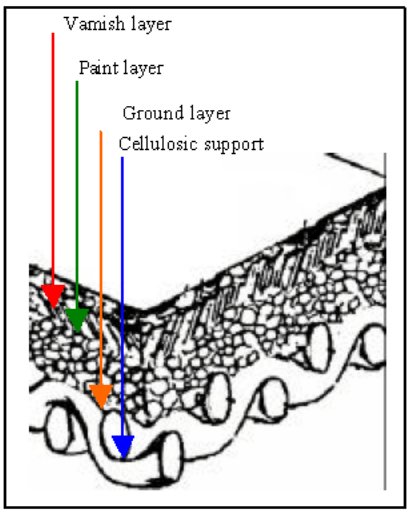

Fig. 2: Selection of the oil painting

Methods of measurement of fungal deterioration: Figure 3A and B declared fine and deep cracks at the ground of the tested oil painting of Ismael Pasha, Moreover, definite holes through the grains of the filling materials of the oil painting. Brittleness and deep cracks were observed due to losing the adhesive material as result of fungal damage. Tiano and Gargani, (1981) determined that direct microscopic examination of the microbial structures adhering to transparent cellulose tape pressed on the painted surface revealed the presence of fungal elements, such as hyphae, typical of most filamentous fungi.

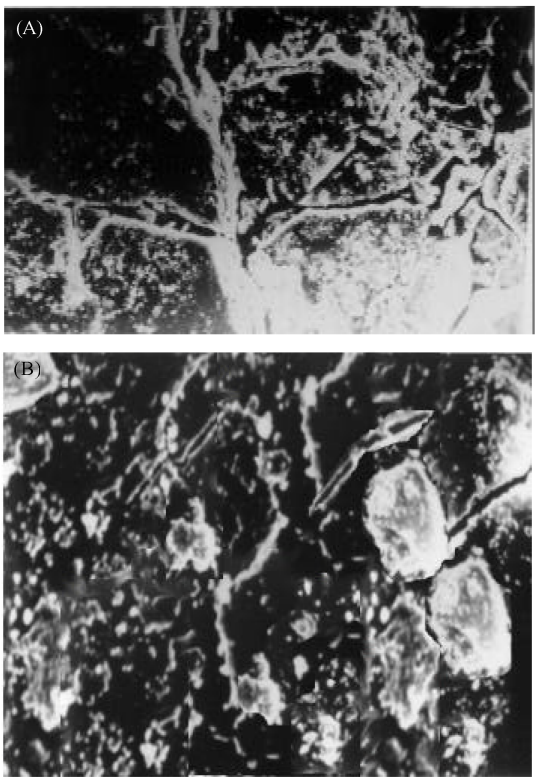

Fig. 3A, B: SEM photographs for different parts of deteriorated and stained deteriorated parts of the tested oil painting showing brittleness and deep cracks as result of fungal damage

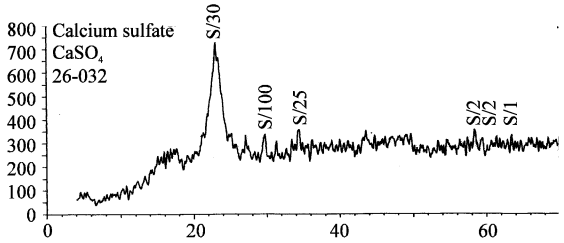

Fig. 4: X-ray diffraction patterns of the selected ground of tested Ismael pasha oil painting

The relative intensities of the diffraction peaks in the XRD spectra can be related to the degree of crystallinity. The ground of Ismael pasha selected oil painting analyses by XRD spectra is consists of anhydrite calcium sulfate $CaSO_4$ which may indicate that gypsum has been employed and transferred to anhydrite Smith and (Schwartzbam, 1961) due to deterioration factors (Byrne, 1995) (Fig. 4). The term gypsum corresponds to a dihydrate calcium sulphate but it is usually refers to a variety of different materials such as native calcium sulphate (Budavari, 1989). The morphology and mineral

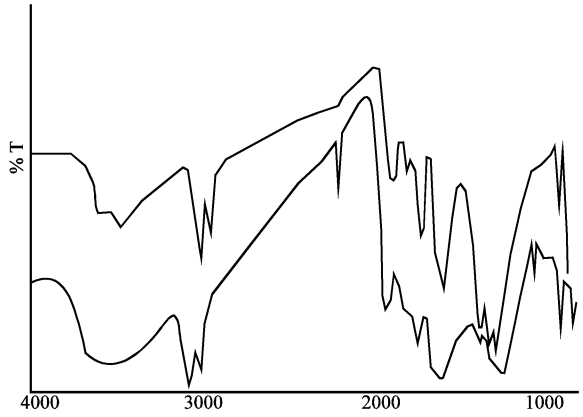

Fig. 5: FT IR. spectra patterns of the standard new animal glue (1) and adhesive material of the grounds of the selected oil paintings samples (2)

composition of the gesso used for the panel painting was studied by (Gómez *et al*., 1998).

Fourier Transform Infrared Analysis (FTIR) data showed that the adhesive material which indicate the white material of the ground of tested oil paintings is animal glue where patterns of the standard new animal glue is typically as the tested oil painting (Fig. 5). FTIR allows to classify the samples as sulphate base ground according to the identifying bands of sulphate at 1157 cm^{-1} which can be attributed to S-O stretching of the sulphate group. The occurrence of sulphation processes by FTIR is in agreement with XRD data. In this way, the principal component of the tested oil sample was found to be the calcium sulphate as that recorded by (Arbizzani *et al*., 2004). The strong band located at 3200 (NH2 amides and NH amine) could be due to the contribution of the hydrolyses of peptide linkage in protein molecule by protease enzyme of deteriorated fungal species. Extracellular enzymes may modify the colors as well as the stability of the painted layer and of the substrate (Agrawal *et al*., 1988). Saiz-Jimenez and Samson (1981) have shown that, at the beginning growth of fungi on a mural's surface caused hyphal penetration to the painted layer degrading some of its components (especially glues) which resulted in a decrease in the cohesion of the painted layers, thus giving rise to exfoliations, cracking and loss of the paint.

It can be shown that SEM observations together with FTIR and XRD results contribute significantly to interpret the way of provenance of the sample.

Frequency of occurrence of the fungal species isolated from tested oil painting: Fungi were isolated from the four different damaged areas of the tested oil painting in the

Table 1: Surveys of species and frequency of occurrence of fungi isolated from different sites on tested oil painting (colony/g dry material)

Fungal species	Count of species	Frequency of occurrence	Painting sites
Acremonium strictum	11	M	1 and 3
Alternaria alternata	13	M	2 and 3
Aspergillus niger	15	M	1, 2, 3 and 4
A. terreus	5	L	3
A. versicolor	13	M	2 and 3
Aureobasidium pullulans	7	H	1, 2, 3 and 4
Cladosporium cladosporioides	30	H	1, 2, 3 and 4
Curvularia geniculata	17	M	1 and 3
Fusarium chlamydosporum	15	M	1 and 3
Geotrichum candidum	10	L	3
Mucor hiemalis	13	M	3 and 4
Penicillium glabrum	12	M	1, 2, 3 and 4
Phoma herbarum	9	L	1
Rhizopus oryzae	11	M	1, 2, 3 and 4
Scopulariopsis brevicaulis	14	M	2 and 4
Stemphylium vesicaium	21	L	2
Trichoderima hamatum	25	M	1 and 2
Total count		268	
No. of species		17	

High (H) 20-30, Moderate (M) 10-20, Low (L) 0-10

museum. The extent of fungal growth was assessed visually. Seventeen different fungal species were isolated from the tested Ismael pasha oil painting belonging to the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Curvularia*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium*, *Phoma*, *Rhizopus*, *Scopulariopsis*, *Stemphylium* and *Trichoderima*. The result in Table 1 show that the genus *Cladosporium cladosporioides* contributed the broadest spectra. The obtained data is in accordance with that obtained by Nugari *et al*. (1993) who reported that *Cladosporium* one of the major biological agents and the most significant agent responsible for fresco degradation and De la Torre *et al*. (1991) stated that the genera which have been demonstrated being more abundant on the monuments investigated are: *Cladosporium*, *Penicillium*, *Trichoderma*, *Fusarium* and *Phoma* and also dark spots are attributed to the presence of fungi of the family of Dematiaceae which contains, water, organic solvents insoluble and melanin pigments inside the mycelium. Bacterial colonization of the painted surface had chemically modified some of the components of the paint, rendering them utilizable by the fungus (O'Neill, 1986). In the opinion of the investigators, *Cladosporium* is one of the most commonly isolated flora from frescoes because it is resistant to variations in external factors (temperature, humidity) (Agrawal *et al*., 1989). Berner *et al*. (1998) found that *Acremonium*, *Verticillium* and *Aspergillus* were mainly found to grow on the remaining fibers of the cellulose pulp used during restoration of painting. Fungal deterioration of paintings was discussed together with methods for their control by Sarbhoy *et al*. (1990). *Aspergillus* and *Penicillium* sp. were the most efficient in biodeterioration of restored frescoes (Sampo *et al*., 1990). Contamination of oil paintings by *Alternaria*, *Cladosporium*, *Fusarium*, *Aspergillus*, *Trichoderma* and *Penicillium* was tested by Inoue and Koyano (1991). On the other hand, *Aureobasidium pullulans* which being among the most frequent isolates from the tested oil painting, it did not grow well on any of these materials. Indeed, a previous report showed that *Aureobasidium pullulans* was unable to utilize hydroxyethylcellulose, a component of the paint, for growth but that it utilized this compound pretreated with cells of *Pseudomonas* or even with a cellulase produced by the bacterium (Schmitt, 1974). Among the species of fungi most frequently involved in deterioration of the paint layer species of *Penicillium*, *Aspergillus* and *Phomapigmentovora* disintegrate oil binders, *Aureobasidium* decompose oil binders, *Geotrichum* develop on casein binders, *Mucor* and *Rhizopus* attack glue (Menier, 1988). Most of the common dominant tested fungal species on the selected oil painting are asexual this finding is in agreement with Karen (1994) who stated that *Aspergillus*, *cladosporium*, *pullularia* and *penicillium* were asexual fungi in all museum materials around the world.

Screening for proteolytic and cellulolytic enzyme activities: The genera of *Cladosporium*, *Alternaria* and *Aspergilli* showed the highest significant cellulolytic and proteolytic activities which have the ability to hydrolyze the cellulolytic substances in the cellulolytic support and the proteinic substances in the animal glue of painting material. Darwish, 2001 investigated the fungal hydrolyses of cellulolytic support of oil painting (Fig. 6). Dhawan and Agrawal (1986) recorded that the cellulolytic support is the first part hydrolyzed by fungal species and also using of organic substances as animal glue increase the chance of biodeterioration by fungal species. The obtained results agree with that obtained by Seves *et al*. (1996) who stated that *Aspergillus niger* was found to possess cellulolytic and proteolytic activities on the paintings and also Kassim (1982) found that the highest yield of cellulase enzyme by a strain of *Aspergillus niger*. The superiority of cellulolytic enzymes depends on the mechanism of induction and catabolite depression of each species (Montenecourt *et al*., 1979). Cellulolytic fungi including *Alternaria*, *Ascochyta*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Humicola*, *Memnoniella*, *Penicillium*, *Scopulariopsis*, *Trichoderma* and *Trichothecium* spp. are the most common organisms responsible for the deterioration of various cultural paintings Lakshmikant *et al*. (1991). Fungal flora (*Cladosporium*, *Aspergillus*, *Alternaria*, *Penicillium* and

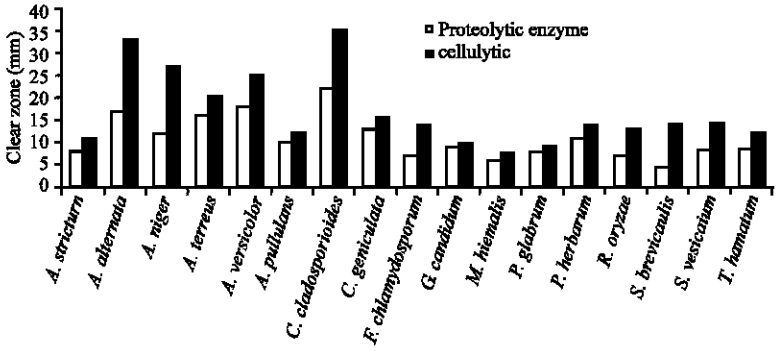

Fig. 6: Assay of cellulolytic enzyme activities (LSD at 0.05= 3.21) and proteolytic activities (LSD at 0.05 =4.73) of isolated fungal species

Fusarium) present on samples taken from deteriorated paintings and their direct involvement in enzymatic degradation of paints was tested by Guglielminetti *et al.* (1994).

Comparative sensitivity to radiation of isolated fungi: The survival percentages of all tested species decreased gradually by increasing exposure time of UV-radiation till reached to the minimum value at 15 min. The most resistant pigmented species was *C. cladosporioides* with significant survival percentage of 14.4% (Fig. 7a). These results agree with that obtained by (Boyd-Wilson *et al.*, 1998) who stated that the effects of short wavelength UV radiation on spores diminished as pigmentation increased. UV radiation is a major factor in *Alternaria solani* mortality (Rotem *et al.*, 1985). Caesar and Pearson (1983) found that UV radiation diminished the survival of ascospores of *Sclerotinia sclerotiorum*. The effects of germicidal effect of UV light on fungal flora was found by Menzies *et al.* (1999). Distributions of fungi were significantly lower when UV lamps were used (Levetin *et al.*, 2001). Evaluation of the UV irradiation induced changes in survivability of the tested fungal species suggests that some fungal species had a protection mechanism and have possibility to repair against UV radiation. Ulevihus' *et al.* (1999) stated that the high recovery potential of *A. niger* propagules and their ability to repair against UV radiation with the time.

The data in (Fig. 7b) reveal that the growth parameter of the tested fungal species decreased with increasing the exposure time of gamma radiation. The radioresistant fungal strains were *C. cladosporioides*, *A. pullulans* and *A. alternata* exhibited survival percentages 12.6, 10 and 7.6%, respectively after 18 h exposure time. The basic properties of paper are not significantly modified at gamma doses up to 10 kGy (Horakova and Martinek 1984). Physical control (ultraviolet rays, gamma rays) have been used especially against fungi in the treatment of archaeological objects (De Cleene, 1994).

Table 2: Effect of different concentrations of calcium sulfate on the dry weight gain and final pH of *C. cladosporioides* after 10 days growth

	C. cladosporioides	
Sulfate concentration (%)	Dry weight (g/100 mL)	Final pH
Control	1.12	7.00
0.025	0.50	7.50
0.050	0.91	7.16
0.100	1.63	7.33
0.200	0.72	7.00
0.400	0.46	6.80
LSD at 0.05	0.25	0.83

The treatment of the tested fungal species with diode laser lead to complete inhibition of all tested fungal species after 15 min exposure time (Fig. 7c). Difference in sensitivity to laser radiation at different exposure times may be due to the difference in cellular components. El-adly (1997) stated that the melanin pigment of some fungal species increases the absorbance of laser radiation and contributes in laser capture. Some microorganisms sensitized to killing by low power laser light (Wilson *et al.* 1993). Exposing the yeast cells to low power laser light rendering them susceptible to death (Dougherty *et al.* 1978). The concept of using a laser to treat painted surfaces is to avoid the use and the disadvantages of solvents (Swicklik, 1993). Laser irradiation was the most efficient radiation type for all tested species and *C. cladosporioides* was the most radioresistant species and will be used in the following experiment.

Effect of different concentrations of sulfate salts on the growth of *C. cladosporioides*: Calcium sulphate is the main component of the tested oil painting sample. Significant increases in mycelial dry weights with the increase in calcium sulfate concentration were evident until it reached its maximum value (1.63 g/100 mL) at 0.10% (Table 2). The change in pH was nearly non-significantly affected with sulphur concentration. The mechanism of fungal resistance against some salts may be explained on

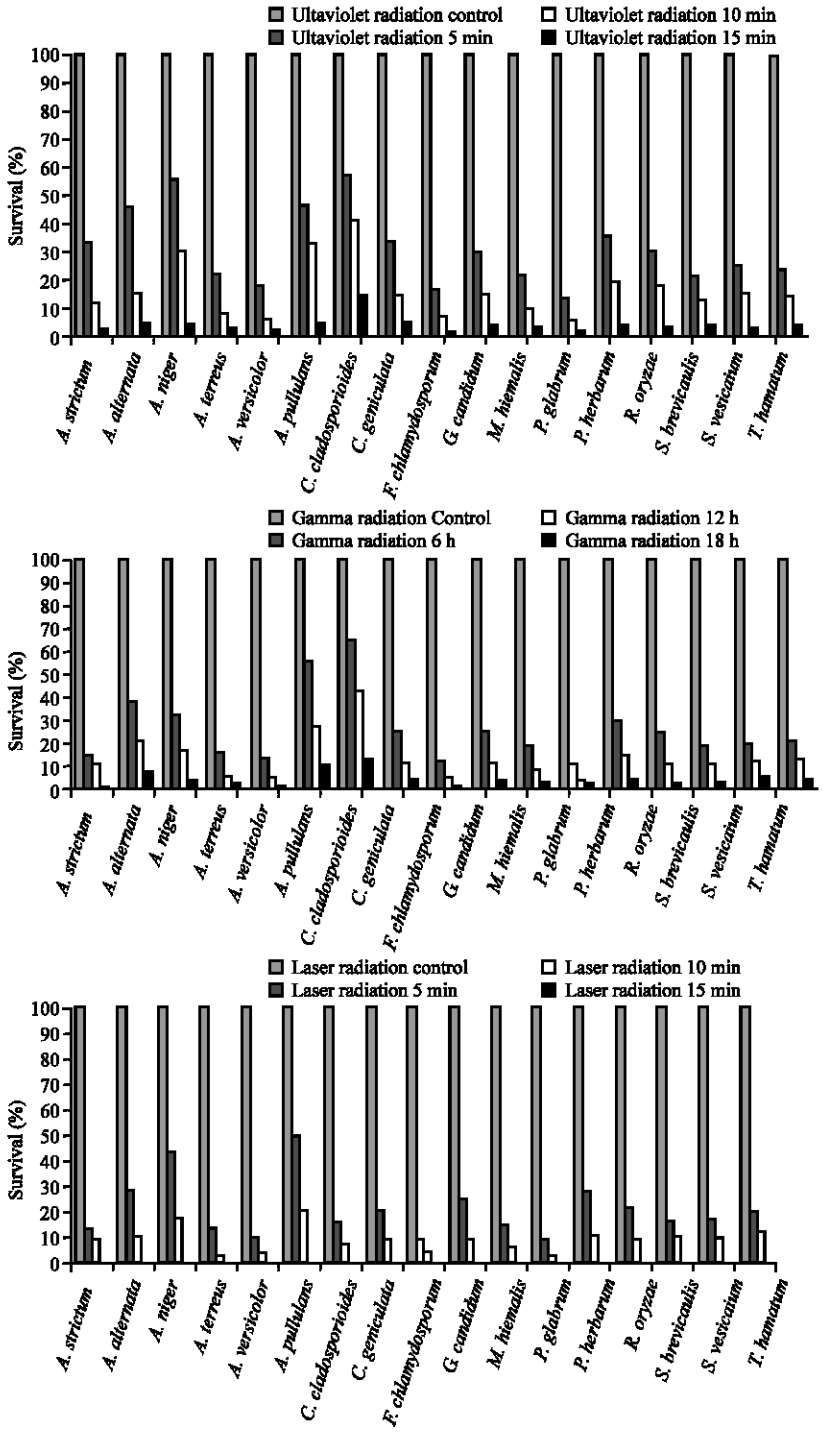

Fig. 7: a) UV-irradiation exposure times (LSD at 0.05 = 4.27), b) Gamma irradiation exposure times (LSD at 0.05 = 2.95) and c) Effect of laser-irradiation exposure times (LSD at 0.05 = 6.11) on the survival of isolated fungal species

the ability of some fungal species to accumulate metals on its spores (Somers, 1963) or by production of chlamydospores as *Aureobasidium pullulans* (Ross, 1982) or by secretion of melanin which stimulate tyrosine oxidase enzyme which can precipitate the toxic metal in protoplast (Gadd and Griffiths, 1980. LeDuy (1986) used the culture medium for enzyme production consists of $(NH_4)_2SO_4$, $MgSO_4$.

(a) (b)

Fig. 8: a) Non-irradiated protein of *C. cladosporioides*, b) Laser irradiated protein of *C. cladosporioides* loss of all bands except one. To confirm results non-irradiated *C. cladosporioides* and irradiated *C. cladosporioides* were run 2 times, all with consistent findings

Gel electrophoresis: Gel electrophoresis patterns of the most resistant species (*C. cladosporioides*) reveal the dramatic loss of essentially all major protein bands after laser irradiation (Fig. 8). The sterilizing quality of laser radiation is due to high-energy transfer that disrupts the DNA chains and breaks protein bonds (Hansen and Shaffer, 2001). Loss of all major protein bands of *Alternaria* spp. after ionizing irradiation was recorded by Dequen *et al*. (2005).

CONCLUSIONS

An alternative is the use of laser radiation, a promising treatment in the preservation field. The obtained results confirmed that laser radiation treatment of oil painting is extremely efficient. The laser preservation technology has brought a powerful way to save ancient painting from being damaged by moulds, guaranteeing a good quality of life for the painting employees and users.

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