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Monitoring the effects of different conservation treatments on paper-infecting fungi

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ABSTRACT

Fungi are among the most degradative organisms inducing biodeterioration of paper-based items of cultural heritage. Appropriate conservation measures and restoration treatments to deal with fungal infections include mechanical, chemical, and biological methods, which entail effects on the paper itself and health hazards for humans. Three different conservation treatments, namely freeze-drying, gamma rays, and ethylene oxide fumigation, were compared and monitored to assess their short- (one month, T1) and long-term (one year, T2) effectiveness to inhibit fungal growth. After the inoculation with fungi possessing cellulose hydrolysis ability — *Chaetomium globosum*, *Trichoderma viride*, and *Cladosporium cladosporioides* — as single strains or as a mixture, different quality paper samples were treated and screened for fungal viability by culture-dependent and -independent techniques.

Results derived from both strategies were contradictory. Both gamma irradiation and EtO fumigation showed full efficacy as disinfecting agents when evaluated with cultivation techniques. However, when using molecular analyses, the application of gamma rays showed a short-term reduction in DNA recovery and DNA fragmentation; the latter phenomenon was also observed in a minor degree in samples treated with freeze-drying. When RNA was used as an indicator of long-term fungal viability, differences in the RNA recovery from samples treated with freeze-drying or gamma rays could be observed in samples inoculated with the mixed culture. Only the treatment with ethylene oxide proved negative for both DNA and RNA recovery. Therefore, DNA fragmentation after an ethylene oxide treatment can hamper future paleogenetic and archaeological molecular studies on the objects.

1. Introduction

A major part of our cultural heritage has been recorded in various forms on paper for centuries and is therefore vulnerable to biodeterioration of its organic components through fungi (Nol et al., 2001; Corte et al., 2003; Cappitelli and Sorlini, 2005; Michaelsen et al., 2006, 2009; Rakotonirainy et al., 2007; Zotti and Ferroni, 2008; Mesquita et al., 2009) and, in a minor way, bacteria (De Paolis and Lippi, 2008; Jurado et al., 2010; Michaelsen et al., 2010), resulting in both structural and aesthetic damage.

Fungal contamination is considered a major concern for libraries or archives full of paper-based books and documents. For the storage and maintenance of this often valuable material, it is crucial not only to control active fungal growth, i.e., hyphae, mycelium, or mould, but also to remove or reduce the amount of fungal ascospores and conidia. The structural nature of ascospores as progenitors of future growth allows the fungi to survive severe conditions, and ascospores are consequently harder to inactivate than the vegetative hyphae. In unfavorable conditions resting spores have low water content and their metabolism is inactivated but reversible (Florian, 1993; Deacon, 2005). Hence, any treatment to conserve objects of cultural value should be directed toward the spores, as the vegetative hyphae are relatively easy to control by physical removal and through monitoring of the storage climate, in terms of temperature, relative humidity, and activity of water (Nitterus, 2000). Fungi are an increasing and dominant problem for archives and museums and thus the prevention of their growth and the development of appropriate treatment measures for contaminated objects are a challenge for restorers, curators, and scientists (Sterflinger, 2010).

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A broad spectrum of chemical and non-chemical components has been used to sanitize microfungi attacking paper-made objects in an attempt to inhibit degradation (Magaudda, 2004). In this study we chose three different conservation treatments for paper to compare their effectiveness over the short- and long-term, namely freeze-drying, gamma rays, and ethylene oxide fumigation.

A commonly used strategy in paper conservation is freeze-drying, a method in which the water is frozen and then removed by sublimation, i.e., it goes from the solid phase to water vapor, bypassing the liquid phase. Sublimation allows the water to be removed without the effects of water evaporative forces, which can cause dimensional changes. In addition, freeze-drying with dehydration can kill hydrated conidia and germinating conidia and it stops the growth of fungal mycelia and bacterial cells (Sussman, 1966; Mazur, 1968; Florian, 2002). Yet if the moisture content in the thawed materials remains high, resting dry conidia may be activated. Freezing can also increase the porosity and thickness of organic materials and make them more hygroscopic. Although it cannot be considered a disinfecting treatment, freeze-drying is still the most effective method known for the physical, chemical, and biological stabilization of water-damaged archival and library materials, especially when large quantities are involved and time is of the essence (Schmidt, 1985; McCleary, 1987; Walsh, 1988; Parker, 1989; Florian, 1990).

The application of gamma rays in conservation science dates back to the 1960s, when the radio-resistance of significant mould fungi from goods of cultural value was tested (Belyakova, 1960). High-energy electromagnetic radiation is deeply penetrating and biocidal through the denaturation and cleavage of nucleic acids, which leads to a simultaneous and indiscriminate devitalisation of all living organisms (Magaudda, 2004). Gamma irradiation is successfully used to sterilize laboratory and hospital utensils and food, but it can have unwanted side effects when applied in paper conservation where high irradiation doses, which are often required in repeated doses, can result in cumulative depolymerization of the underlying cellulose in the paper. Severe aging characteristics, such as lowered folding endurance and tear resistance, increased yellowing, and general embrittlement, have been reported in paper treated with gamma rays (Butterfield, 1987; Adamo et al., 1998), whereas more recent studies have suggested that the damage in terms of mechanical–physical properties is not significant (Adamo et al., 2001; Gonzalez et al., 2002). The observed effects of gamma rays on fungi from paper have confirmed that radiation treatment of books and documents is effective, as there was no fungal growth detectable in cultivation studies (Jörg et al., 1992; Da Silva et al., 2006).

Finally, ethylene oxide (EtO) has been widely used for sterilization of objects of cultural heritage since 1933 (Ballard and Baer, 1986). Ethylene oxide does not require activation energy, is used at room temperature, and expresses the high reactivity and diffusivity required for the inactivation of microorganisms (Mendes et al., 2007). By adding alkyl groups to DNA, RNA, or proteins, EtO prevents normal cellular metabolism and the ability to reproduce (Rutala and Weber, 1999). This ability to act as an alkylating agent was widely taken to indicate a carcinogenic potential, an assumption that has subsequently been proven for EtO and some of its residuals (Bolt, 1996; Angerer et al., 1998), leading to a ban on EtO for the practice of paper conservation in many countries. Additionally, a study found paper material fumigated with EtO to be more susceptible to microbial attack after fumigation (Valentin, 1986). This phenomenon is not fully understood but it is claimed that ethylene glycol, formed as a by-product during fumigation, activates spores that contaminate the object in further storage (Florian, 1993). In this study, the effects of these three conservation treatments on fungal spores and mycelium viability and activity were investigated by culture-dependent and -independent strategies. The main goals were: (a) to compare the efficacy of molecular versus cultivation techniques as models for the monitoring of conservation treatments, (b) to evaluate massive and aggressive disinfecting treatments on DNA integrity, and (c) to use rRNA analyses as a method of determining long-term viability of fungi.

With this end, two paper grades were used for the in-vitro inoculation of spores from different fungal species to obtain infected paper samples. The physiological state of the fungal strains was monitored over the short- and long-term after the treatments to determine time-dependent effects of the treatments on fungal viability. These effects were examined by culturing and on a molecular level by generating DNA-denaturing gradient gel electrophoresis (DNA-DGGE) profiles. As only metabolically active cells produce RNA, we used the ability to retrieve fungal-specific rRNA directly from the paper samples as an indicator of the viability of the respective fungi after the samples had been left for a year for eventual regrowth. This strategy is commonly used in bacterial ecology and is based on the fact that metabolically active species transcribe more rRNA for ribosome synthesis than do inactive species (Prosser, 2002). As RNA is also highly unstable in the environment, the detection of RNA in an environmental sample has been used as a strategy for detecting the most active microorganisms within a natural community (Gonzalez et al., 2006). To our knowledge this is the first study combining molecular methods including RNA detection of fungi with monitoring of conservation treatments for paper-made objects.

2. Materials and methods

2.1. Sample preparation and cultivation

Fungal growth was induced in vitro by inoculating two types of paper with three fungal strains previously isolated: *Chaetomium globosum* Kunze, *Cladosporium cladosporioides* (Fres.) de Vries, and *Trichoderma viride* Pers. (Michaelsen et al., 2006). Paper A was a Whatman paper type (Whatman 1 CHR category No. 3001917, not glued and not sized, 100% cotton linter, pH 6.5–7.0) consisting of pure cellulose with low ash content, and paper D was a “Mezzofino” type paper with a high lignin content, namely a naturally aged rag paper produced by the Istituto Poligrafico dello Stato in 1976 (Gallo et al., 1999), No. 200953, consisting of bleached cellulose (45%), wood pulp treated with sulphites (25%), wood pulp from softwood (20%), glue (3%), aluminum sulfate (5%), and kaolin (2%), pH 4.5. Each paper sample existed as a set of small discs (about 1 cm diameter) cut with a puncher (12.5 mm disc punch n. T5443, Agar Scientific Ltd., Stansted, Essex, England).

Fungal strains were grown on malt extract agar (2%) to obtain colonies with mature fruiting bodies or reproductive structures. Conidia and ascospores were harvested with a sterile cotton swab and diluted in water with Tween 80 (0.01%, Sigma, Italia) to obtain solutions with a standard concentration of about 5000 spores/ml. A further dilution was performed with a Czapek broth, to obtain 50 spores μl^{-1}. The water vapor-sterilized paper samples were inoculated with 50 μl of broth each to provide a physiological starter for germination. Each inoculum was applied to the sample disc in a single spot. About 100% relative humidity (RH) was maintained with distilled water during fungal growth in double-bottom glass containers. Samples were kept in a thermostatic cell at 27 °C for 7 days (*C. cladosporioides* and *T. viride*), and 14 days (*Ch. globosum* and mixed inocula).

Chaetomium is a genus of filamentous fungi (phylum *Ascomycota*, class *Sordariomycetes*) encompassing species that typically possess densely setose, ovoid to pyriform ostiolate ascomata, clavate asci, and pigmented, one-celled ascospores (Samson et al., 2000). Species