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Antifungal activity of selected essential oils and biocide benzalkonium chloride against the fungi isolated from cultural heritage objects

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ABSTRACT

The antifungal activity of *Origanum vulgare*, *Rosmarinus officinalis* and *Lavandula angustifolia* (Lamiaceae) essential oils and biocide benzalkonium chloride was investigated against fungi isolated from stone (*Bipolaris spicifera* and *Epicoccum nigrum*) and wooden substrata (*Aspergillus niger*, *Aspergillus ochraceus*, *Penicillium* sp. and *Trichoderma viride*) of cultural heritage objects. Carvacrol (64.06%) was the main component of *O. vulgare* essential oil, while linalool (37.61%) and linalool acetate (34.86%) dominated in *L. angustifolia* essential oil. The main component of *R. officinalis* essential oil was 1.8-cineole (44.28%). To determine fungistatic and fungicidal concentrations (MIC and MFC) micro-, macrodilution and microatmosphere methods were used. Mycelial growth and spore germination of fungal isolates were inhibited with different concentrations of antifungal agents. The oil of *O. vulgare* and biocide benzalkonium chloride displayed the strongest antifungal activities followed by *R. officinalis* and *L. angustifolia* essential oils. MIC and MFC values obtained in microatmosphere and microdilution method for *O. vulgare* essential oil ranged from 0.1 to 2.0 μL mL^{-1}, while for *R. officinalis* and *L. angustifolia* ranged from 10.0 to 100.0 μL mL^{-1}. The most susceptible fungus to essential oil treatments was *E. nigrum*. MIC and MFC values for benzalkonium chloride ranged from 0.1 to 4.0 μL mL^{-1}. Tested isolates, *A. niger* and *A. ochraceus*, were the most susceptible for biocide treatment.

1. Introduction

Fungi are widely recognized as major biodeteriogens of cultural heritage. They are capable of colonizing, degrading and altering a variety of materials, including the materials which have been used through the centuries for making cultural heritage monuments and artifacts (Sterflinger, 2010). Stone monuments in moderate and humid climates are usually colonized by fungal communities dominated by dematiaceous Hyphomycetes (Sterflinger and Krumbein, 1997; Urzì et al., 2001). Fungi on stone substrata, along with cyanobacteria and algae as phototrophic partners and heterotrophic bacteria, form specific microbial communities called subaerial biofilms (SABs) that develop on solid surfaces exposed to the atmosphere (Gorbushina and Broughton, 2009). The surfaces of stone monuments can be altered by fungal activity via hyphal penetration through the porous stone matrix (Kumar and Kumar, 1999; Sterflinger, 2000) and by production of organic acids and pigments (Gómez-Alarcón et al., 1994; Sharma et al., 2011). Pieces of art stored in museum depots or displayed in exhibition rooms can suffer the symptoms of fungal spoilage. Different materials used for artistic expression (such as wood, paper, textiles, leather, plastic, metal and clay) can be susceptible for fungal colonization and biodeteriorated through fungal growth and metabolic activity. Biodeterioration mechanisms of fungi are related to enzymatic hydrolysis of organic materials and the production and excretion of organic acids (Görs et al., 2007). Wooden artifacts are especially affected by fungal colonization due to cellulase production by certain filamentous fungi (Fazio et al., 2010). Prevention of mold growth on cultural heritage objects and artifacts is nowadays a significant challenge for restorers, conservators and architects (Sterflinger, 2010). Chemical treatments applied in cultural heritage conservation must be non-toxic and non-destructive (Stupar et al., 2012).

The objectives of this research were to evaluate in vitro effectiveness of the biocide benzalkonium chloride (BAC) and selected essential oils (EOs) as antifungal agents against fungi isolated from cultural heritage. BAC is widely used for the control of microbial growth in clinical and industrial environments (McBain et al., 2004). The biological activity of BAC is ascribed to its quaternary ammonium group (Mehta et al., 2007). Biocidal products containing quaternary ammonium compounds (QACs) are approved for conservation of cultural heritage monuments by the European Biocide Directive as relatively environmentally friendly

Abbreviations: SAB, subaerial biofilm; BAC, benzalkonium chloride; EO, essential oil; QAC, quaternary ammonium compound; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration.

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(Cooke, 2002). On the other hand, EOs and their derivatives are considered to be a possible substitute for controlling different types of biological settlement (Axinte et al., 2011). Antimicrobial activity of many EOs has been reviewed by Kalemba and Kunicka (2003), and many oils have been successfully applied in different fields of microbiological control. However, reports regarding the implementation of EOs in cultural heritage conservation are very scarce (e.g. Gatenby and Townley, 2003; Chung et al., 2003; Rakotonirainy and Lavédrine, 2005).

2. Materials and methods

2.1. Essential oils

The essential oils from selected aromatic plants from the Lamiaceae family *Origanum vulgare* L. (Frey + Lau, Ulzburg, Germany), *Rosmarinus officinalis* L. (Herba d.o.o. Belgrade, Serbia), and *Lavandula angustifolia* Miller (Frey + Lau, Ulzburg, Germany), were commercial samples obtained from the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade as a part of their collection. According to the manufacturer the quality of tested EOs corresponds to European Pharmacopeia 6 (Ph. Eur. 6.0, 2004).

2.2. Biocide

An aqueous solution of the biocide benzalkonium chloride (BAC) (50% (vol/vol)) was obtained from the Institute for Protection of Cultural Monuments in Serbia. Prior to experiment biocide was diluted in sterile distilled water to make a stock solution of final concentration 10% (vol/vol).

2.3. Tested fungal isolates

All fungi used in this study were isolated from different wooden and stone substrata of cultural heritage objects in Serbia (Table 1), identified to the species or genus level using appropriate identification keys. Isolated fungi were deposited to the Mycotheca of the Department for Algology, Mycology and Lichenology, Institute of Botany, Faculty of Biology, University of Belgrade. Isolates were maintained on malt extract agar (MEA), potato dextrose agar (PDA), stored at 4 °C and subcultured once a month.

2.4. Gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS)

Qualitative and quantitative analyses of the EOs were performed using GC and GC–MS. GC analysis of the oil was carried out on a GC HP-5890 II apparatus, equipped with a split–splitless injector, attached to a HP-5 column (25 m × 0.32 mm, 0.52 μm film thickness) and fitted with a FID. Carrier gas flow rate (H_2) was 1 mL/min, split ratio 1:30, injector temperature was 250 °C, detector temperature 300 °C, while the column temperature was linearly programmed from 40 to 240 °C (at 4 °C/min). The same analytical conditions were employed for GC–MS analysis, where a HP G 1800C Series II GCD system, equipped with a HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness) was used. The transfer line was heated to 260 °C. The mass spectra were acquired in EI mode (70 eV), in *m/z* range 40–400. Identification of individual EO components was accomplished by comparison of retention times with standard substances and by matching mass spectral data with those held in the Wiley 275 library of mass spectra. Confirmation was performed using AMDIS software and literature (Adams, 2007). Area percentages obtained by FID were used as the basis for quantitative analysis. The percentage composition of the oils was computed by the normalization method from the GC peak areas.

Table 1
Fungal isolates chosen for the study of antifungal activity of selected essential oils and biocide benzalkonium chloride.

Isolates	Substrata	Reference
Aspergillus niger Tiegh *Aspergillus ochraceus* G. Wilh *Penicillium* Link sp. *Trichoderma viride* Pers.	Wooden sculptures, Museum of Temporary Art, Serbia	Ljaljević Grbić et al. (2013)
Bipolaris spicifera (Bainier) Subram	Sandstone monument, Eifell Lock, Serbia	Ljaljević Grbić et al. (2009)
Epicoccum nigrum Link	Granite monument, Monument of the Unknown Hero, Serbia	Ljaljević Grbić et al. (2010)

2.5. Antifungal activity assays

The antifungal activity of selected EOs, and the biocide BAC, was investigated using three methods: micro-, macrodilution and microatmosphere methods. Microdilution and microatmosphere methods were used for testing the antifungal activity of EOs, while biocide BAC was tested using micro- and macrodilution methods.

2.5.1. Microatmosphere method

The following method allows the effect of volatile fractions of the EOs to be studied. The test was performed in sterile Petri plates of (85 mm diameter) containing 20 mL of MEA (Maruzzella and Sicurella, 1960). After inoculation of the fungi, using sterile needle under the stereomicroscope (Stemi DV4, Zeiss), at the center of the MEA, the Petri plates were overturned. A sterilized filter paper disc was placed in the center of the Petri plate lid soaked with various concentrations of EOs. For *O. vulgare*, concentrations of EO ranged from 0.1 to 2 μL mL^{-1}, while for *R. officinalis* and *L. angustifolia* EO concentrations ranged from 10 to 100 μL mL^{-1}. Plates were incubated for 3 weeks at room temperature, during which the growth of fungal colonies was monitored weekly. After incubation period, minimal inhibitory concentrations (MICs), defined as the lowest concentration of added EO with no visible fungal growth on MEA, were determined. Minimal fungicidal concentrations (MFCs) were determined by re-inoculation of treated inoculums onto sterile MEA. The lowest concentrations of EO giving no visible growth after re-inoculation were regarded as MFCs.

2.5.2. Macrodilution method

To investigate the antifungal activity of the biocide BAC, the modified mycelia growth assay with MEA was used (Ishii, 1995). The stock solution of BAC (10%, vol/vol), was further diluted in melted MEA in Petri plates to make final concentrations of the biocide ranging from 0.1 to 5 μL mL^{-1}. The fungi were inoculated at the center of the MEA. Plates were incubated for 3 weeks at room temperature. MIC and MFC values were determined in the same manner as in the microatmosphere method.

2.5.3. Microdilution method

The modified microdilution technique was used to determine the antifungal activity of EOs and BAC (Hanel and Raether, 1998; Daouk et al., 1995). Conidia were washed from the surface of the agar slants with sterile 0.85% saline containing 0.1% Tween 20 (vol/vol). The conidia suspension was adjusted with sterile 0.85% saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. The inocula were stored at −20 °C for further use. Dilutions of the inocula were cultured on solid MEA to verify the absence of contamination and to check the validity of the inocula.

Determination of the MICs was performed by a serial dilution technique using 96-well microtitre plates. Different volumes of investigated EOs and biocide BAC (10% (vol/vol)) were dissolved in malt extract broth (MEB) with fungal inoculums (10 μL) to make the same final concentrations, as those used in microatmosphere and macrodilution methods. The microplates were incubated for 72 h at 28 °C. The lowest