Article

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Plant essential oils in controlling fungal colonization on wooden substrate

Abstract

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In order to control fungal colonization and the related deterioration process of cultural objects, several chemical compounds are actually utilised. These products generally are toxic, not biodegradable and persisting for long time in the environment, also acting on not-targeted biological systems. In this study, specifically to wooden cultural object, *Origanum vulgare* L. and *Thymus vulgaris* L. essential oils are proposed as green biocides to contrast the development of *Penicillium chrysogenum*, *Aspergillus flavus* and *Aspergillus niger* fungal species. The aim is replacing toxic products with natural molecules, proposing alternative methodologies supporting the using of non-toxic novel compounds safe for humans and environment.

Key words: wooden substrate biodeterioration, fungal colonization, aromatic plant, bioactive molecules, green biocides.

Introduction

The agricultural, cosmetic, food, pharmaceutical and sanitary industries have been interested for a long time to aromatic plants as source of essential oils (Abu-Shanab & al. 2004; Bakkali & al. 2008; Reichling & al. 2009; Karakaya & al. 2011; Petrovska 2012; Fernandez-Lopes & al. 2018). Particularly for Lamiaceae family, several plants represent a source of peculiar bioactive molecules applicable for different purposes, due to their antimicrobial and antioxidant properties or repellence in controlling insect infestation (Ebadollahi & al. 2020; Minh Chau & al. 2020). In this study the role of *Origanum vulgare* L. and *Thymus vulgaris* L. essential oils (EOs), as natural biocides to contrast the fungal colonization of wooden substrates is proposed.

The fungal taxa assayed, have been isolated and characterized, by a polyphasic approach, combining microscopy, *in vitro* culture and molecular techniques (Pasquarella & al. 2015; Palla & Barresi 2017; Riaz & al. 2017). Furthermore, the main chemical components of *O. vulgare* and *T. vulgaris* EOs were defined by Gas-Chromatography Mass-Spectrometry highlighting carvacrol and thymol and the related *p*-Cymene, as main compounds (Palla & al. 2020).

The antimicrobial activity of *O. vulgare* and *T. vulgaris* EOs has been *in vitro* evaluated by agar disc diffusion (ADD) method, showing different size in growth inhibition halos, related to the concentration of EOs solutions and to the different sensitivity of fungal colonies to chemical components. Instead, the biocide/biostatic activity was defined by micro-dilution tests, distinguishing the Minimum Inhibitory Concentration and Minimum Fungicidal Concentration (Barry 2007). The values for each EO were comprised between: MIC =1.2 – 2.8 microL/mL and MFC = 1.6 – 3.2 microL/mL.

These results, in addition to our other studies, lead us to suggest that *O. vulgare* and *T. vulgaris* essential oils can be used in controlling fungal colonization of wooden substrate, performing *green* strategies safe for humans and the environment (Palla 2019).

Materials and methods

Sampling

Specific sampling onto three selected areas of wooden surface (Fig. 1) was performed by sterile swabs, later employed to inoculate Sabouraud agar plates (incubated for 36/48 h at 30°C).

Fungal colonies

Isolated colonies and related reproductive structures were assessed by Wild (14X) or Leica (40X, after Lugol's staining) microscopes.

Genomic DNA extraction, *in vitro* amplification by Polymerase Chain Reactions (PCR) of target ribosomal DNA sequences (ITS=Internal Transcribed Sequence) and the electrophoresis of PCR products were performed in according to Browne & al. (2013), Biyik & al. (2016) and Di Carlo & al. (2017). The sequence of PCR products (about 600 bp in length) was determined by Operon sequencing service (Eurofins,

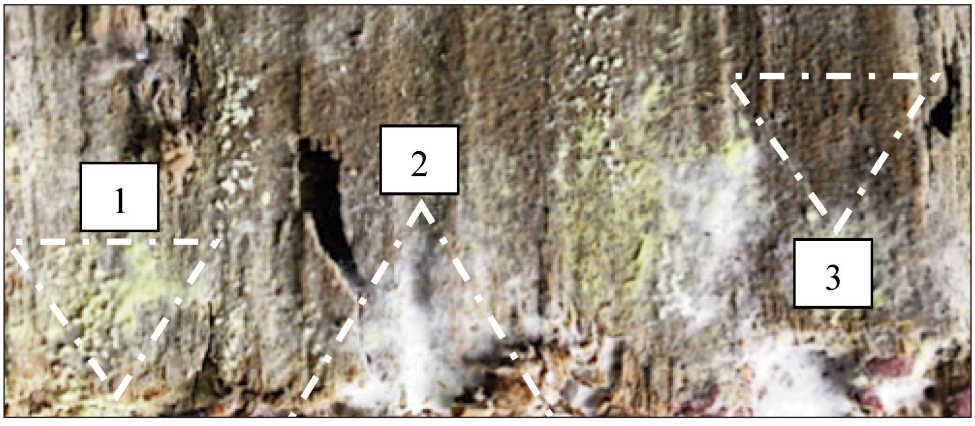

Fig. 1. Wooden substrate. Three areas (dashed by white lines) were selected basing on different morphology and pigmentation of patina: 1) green; 2) white; 3) brown.

Germany) and the sequences comparison (BLAST platform) allows the identification of fungal species (Altshul & al. 1990).

Essential Oil solutions

The *Origanum vulgare* L. and *Thymus vulgaris* L. solutions (12.5, 25, 50%) were obtained diluting 100% pure essences of EOs (do Terra) in 70% Ethanol.

Antimicrobial essays

The *Agar disc* diffusion method (ADD), one of routine antimicrobial *in vitro* test, has been performed placing paper disks (6 mm in diameter, Dutscher papier, France) moistened with (12.5, 25, 50%) EOs solutions, onto a fungal lawn growth on Sabouraud agar plates (seeded by conidia suspension = $1x10^4$ conidia/ml). The antimicrobial solution diffuses from the discs into the agar medium inhibiting fungal growth and generating an inhibition halo whose diameter is strictly related to antimicrobial activity (Balouiri & al. 2016; Barresi & al. 2017; Rotolo & al. 2018).

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were assessed in according to Barry (2007) by using the serial broth dilution method, performed in 96-wells microtiter, distinguishing between biocide or biostatic action. In each well, containing 30 µl of EO solution (at different concentration = 12.5%, 25%, 50%), an equal volume of microbial suspension ($1´10^6$ CFU/ml) was added. In order to facilitate the dispersion in the liquid medium, 0.1% of Tween 80 (not toxic for microbial cells) was added in the EO solution; as control, aqueous solutions of Benzalkonium chloride (0.2%, vol/vol) were utilized. Microbial growth, after 36/48 h of incubation at 30±1°C was evaluated by spectrophotometer (optical density at 500-600 nm). MIC value has been determined as the lowest concentration of the solution at which any visible growth corresponds (incubation 30±1°C, up to 7 days). The MFC was assayed as the lowest concentration of essential oil that completely inhibited the growth of fungi, specifically when the number of colonies, on antimicrobial-free sub-culture, indicated the 99.5% killing of the original inoculum. Each test was performed twice.

Exposure of colonized area to EO

The fungal colonized area was exposed to the volatile compounds of *O. vulgare* or *T. vulgaris* EOs, in a dedicated clean chamber (thermo-sealed barrier film), containing EOs solutions in 5 ml glass container. The thermo-hygrometric (Temp. and R.U.) values in/out of exposure chamber were constantly monitored by Oregon Scientific datalogger, as showed in Fig. 5.

Results

A diffuse colonization, mainly attributable to three fungal species *Aspergillus flavus*, *Aspergillus niger* and *Penicillium chrysogenum,* has been revealed by a polyphasic approach based on molecular investigation, *in vitro* culture and microscopy observations. In Figure 2, are showed the fungal colonies grew on Sabouraud agar and their reproductive structures, stained by Lugol's iodine reactive.

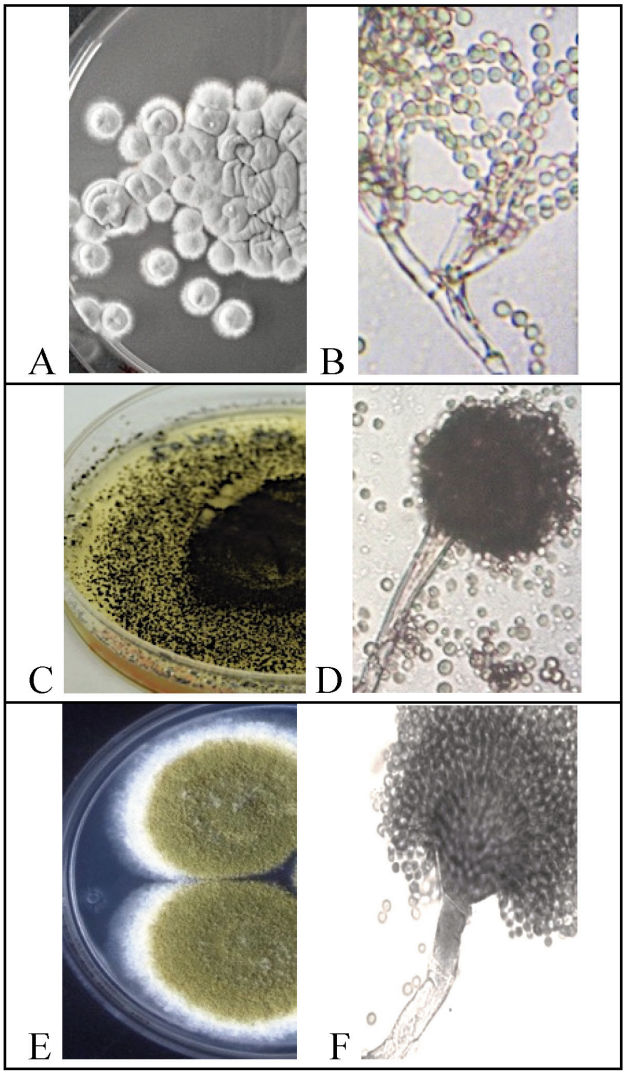

Fig. 2. Sub-cultures on Sabouraud agar (6 cm plates) of: A) *Penicillium chrysogenum*, C) *Aspergillus niger* and E) *Aspergillus* flavus fungal colonies; B, D, F) corresponding reproductive structures stained by Lugol's iodine reactive.

ADD *in vitro* assays, showed good activity of *O. vulgare* and *T. vulgaris* EOs *vs* three of isolated fungal species, highlighting different inhibition halos diameter in relationship to the fungal taxa and EO concentration (12.5, 25, 50%). Particularly, in Figure 3 are showed the growth inhibition halo, related to the 12.5% *O. vulgare* solution (halo diameter > 6mm), to 2% Benzalkonium-chloride commercial biocide (halo diameter < 6mm) and to 70% ET-OH solution (negligible).

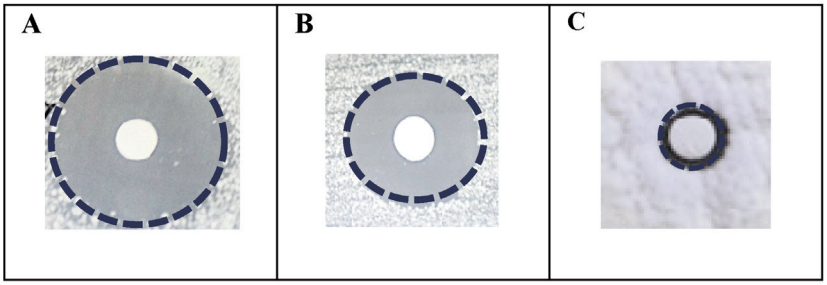

Fig. 3. Agar disc diffusion assays performed on Sabouraud agar seeded by *P. chrysogenum.* The growth inhibition halos (ih) are related to wetting the paper discs with: A) 12.5% *O. vulgare* (ih > 6 mm); B) 2% Benzalkonium chloride (ih <6 mm); C) 70% Et-OH solutions (ih close to zero).

The average diameter of inhibition halos in the agar diffusion disc assays (in triplicate samples), using 12.5% *O. vulgare* and *T. vulgaris* EOs, 2% Benzalkonium chloride and 70% Et-OH, is summarized in Fig. 4.

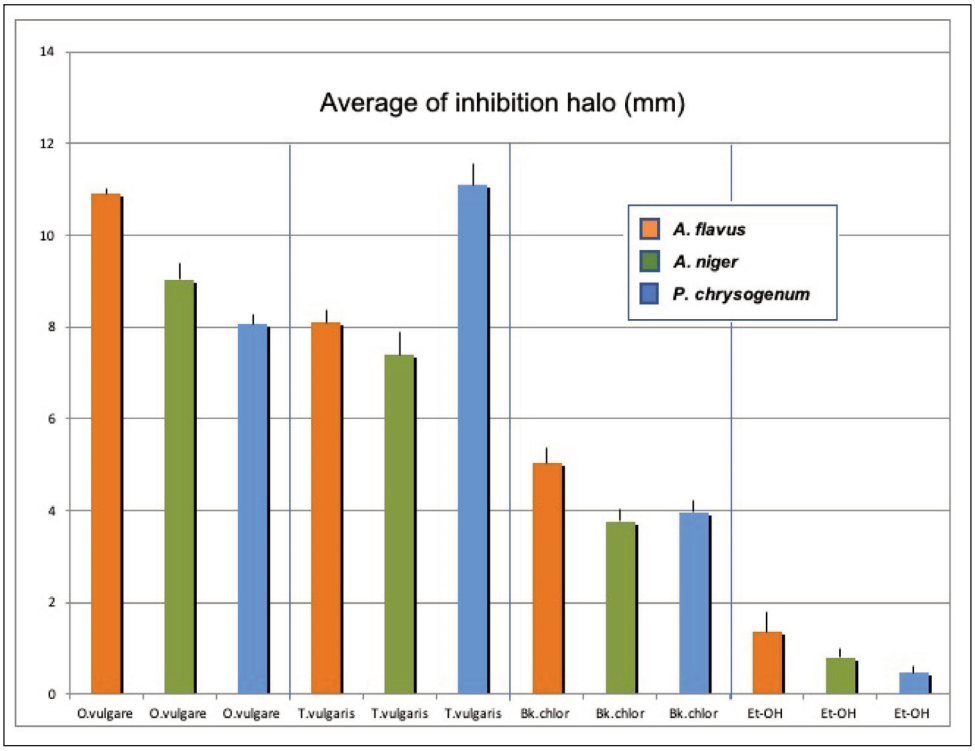

Fig. 4. Inhibition halo diameters (average of three replicates): significant values = ih > 6 mm.

The ADD assays performed (Sabouraud agar plate, 6 cm in diameter) by 25% EOs solutions showed i.h. greatest in diameter, reaching the complete growth inhibition of fungal colonies with the 50% EO solutions.

Based on these results, the 50% of *O. vulgare* or *T. vulgaris* solutions have been utilized for the exposure of wooden substrate to the volatile compounds, in a dedicated "clean chamber".

Particularly, the wooden substrate was exposed to *O. vulgare* or *T. vulgaris* volatile compounds for 30 days, continuously monitoring the thermo-hygrometric parameters inside/outside the clean chamber, Figure 5.

The action of EOs volatile compounds, mainly attributable to the carvacrol, *p*-cymene and thymol chemical compounds, could be probably improved by the low oxygen inside the micro-environment of exposure chamber (Elamin & al. 2019; Palla & al. 2020).

Discussion

Aromatic plants EOs contain complex mixtures of aliphatic, aromatic and terpenic compounds with a forceful role in plant-plant interaction and pollinators attraction (Theis & Lerdau 2003; Tholl 2006; Bakkali & al. 2008). During the century, essential oils, derived of over 2000 species from plant families such as Apiaceae, Lamiaceae, Myrtaceae, Rutaceae, Verbenaceae, Zingiberaceae, were applied for their antimicrobial-pesticidal properties (Isman & al. 2011; Petrovska 2012). A huge number of studies, highlighting the peculiar action of these bioactive substances, useful for sustainable approaches in agriculture, food storage, wood industries, have been recently developed, also in "green conservation strategies" of cultural heritage (Nerio Quintana & al. 2009; Tripath & al. 2009; Borrego & al. 2016; Salem & al.2016; Campos & al. 2019; Palla & al. 2020).In this work

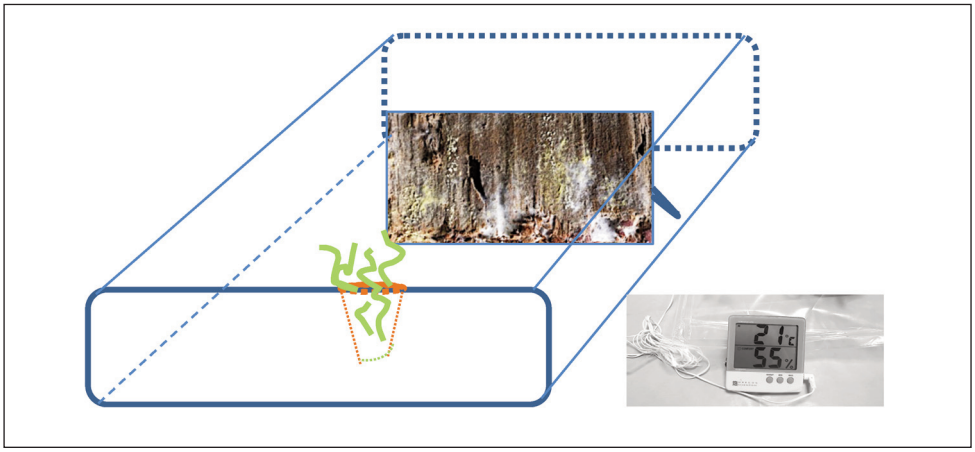

Fig. 5. Exposure of fungal colonized wooden substrate to volatile compounds of *O. vulgare* or *T. vulgaris* essential oil (dispensed in glass container). The "clean chamber" was assembled by gas-barrier thermo-sealed film. Temperature (21±2°C) and Relative Humidity (55±4%) values were constantly monitored by Oregon scientific datalogger equipped with microprobe, allowing the measurements of thermo-hygrometric values inside/outside the exposure chamber.

the *O. vulgare* and *T. vulgaris* essential oils, as well as in our previous studies performed by commercial and laboratory extracted EOs (*Allium sativum* L., *Calamintha nepeta* L., *Crithmum maritimum* L., *Melaleuca alternifolia* L., *Thuia plicata* L.), are proposed as valid alternative to chemical biocides (Di Carlo & al. 2017, Palla & al. 2019).

Conclusions

Fungi are widespread in nature and are dispersed in both indoor and outdoor environment by chlamidospores, conidia, sexual spores (De Leo & Urzì 2014). Metabolically more versatile than other biological systems, fungi are able to induce several bio-mechanical and enzymatic activities accelerating the deterioration of cultural objects (both of organic or inorganic matter). Influenced by thermo-hygrometric environmental parameters, fungi can utilize these substrates as a support in growing and nutritional source (Sterflinger 2010). Besides, in confined environments (archives, caves, hypogea, museums, libraries) the risk for human health (operators, visitors) must be also considered. Particularly, evaluating not only from harmful microbial species or their metabolic products (allergens, cellular debrides, spores, toxins), but also from the hazardous residues of biocide treatments for cultural object preservation.

This study is aimed to improve the knowledges, balancing preservation of cultural heritage for future generations and application of technical procedures safe for human health and environment.

Specifically, *Aspergillus flavus*, *Aspergillus niger* and *Penicillium chrysogenum* have been identified as main colonizers on wooden substrate. In order to eradicate the fungal colonization, the exposure to the volatile compounds of *O. vulgare* and *T. vulgaris* essential oils have been performed. Before exposition of cultural object, the antimicrobial activity of both essential oils has been *in vitro* evaluated against the isolated *Aspergillus and Penicillium* spp.

These results, in agreement with the related literature (Zambonelli & al. 2004; Soković & al. 2009; Tripathi & al. 2009; Lukas & al. 2015), confirm our previous studies on use of Lamiaceae plant essential oils as valid alternative to chemical commercial biopesticides (Rotolo & al. 2018; Edabollahi & al. 2020; Palla & al. 2020).

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