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Biological characteristics of some terrestrial fungi on different substrates

Abstract

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Key words: phytopathogenic fungi, hedera *Helix extract*, ITS sequences, archaeological bones, FTIR, microscopy, onychomycosis, naftifine, carotenoids.

I. Literature review

1. The importance of fungi in human activity

Fungi are ubiquitous organisms with a significant economic impact on human life, influencing a wide range of anthropic activities. They are especially important for the agricultural, the food, medical and pharmaceutical industries (Kendrick, 2001) and biotechnology (May and Adams, 1997). Fungi also have an important ecological role, being currently studied to clarify some fundamental issues related to their biodiversity, classification and evolution (Lange, 2014).

The effect of the fungi in the above-mentioned areas is positive as they can be used for brewing and bakery products, in industrial fermentations, for the production of enzymes and pharmaceutical compounds or as a source of food. On the other hand, they can have a negative effect as they can cause significant financial loss destroying food, construction materials or diseases for plants, animals and humans (Kendrick, 2001; Mueller *et al.*, 2011).

2. Terrestrial mycobiome

Soil is considered a terrestrial ecosystem with great taxonomic and functional diversity with regard to the associated fungi (Orgiazzi *et al.*, 2013). The totality of the fungi was defined by the term mycobiome. The diversity of the terrestrial mycobiome is closely correlated with the soil stratification and the type of vegetation present, the fungi ensuring the physiological and ecological links of the ecosystem (Dighton, 2016). The dimensions of the terrestrial fungi range from unicellular yeast and microscopic mold to large mushrooms (Tonge *et al.*, 2014).

Knowing the exact structure of the fungal communities present in the soil leads to a better understanding of specific ecological interactions with plants, pesticides or other microorganisms (Baldrian *et al.*, 2012). There are numerous studies presenting identification methods, from traditional variants to classical and modern molecular methods (Guarro et al., 1999; Atkins and Clark, 2004)

2.1. Characterizing the terrestrial mycobiome

Classical identification methods for fungi are based on phenotypic and observable characteristics. The defining features of fungi are related to their nutrition, vegetative state, cell

wall type, nucleus type, breeding strategy, distribution in nature and their ecological importance. Fungi are heterotrophic organisms, which uptake the nutrients through absorption from the environment, generally immobile, with the vegetative body called mycelium. The cell wall consists of glucans, chitin and cellulose, the cells being single or multiple nucleated, haploid or diploid. They can multiply sexually, asexually or parasexually (Webster and Weber, 2007).

Most commonly, classical analysis involves the microscopic analysis of samples for a faithful observation of the characters of interest. The classic optical microscope was updated to modern techniques involving contrast microscopy, fluorescence, cytochemistry and electron microscopy (Guarro *et al.*, 1999). The drawbacks of the classical methods of identification are: they are time consuming, are not quantitative, and they can lead to misidentification of the species of interest (Atkins and Clark, 2004).

The exact identification of a species based on morphological characters involves in the first place its isolation on a culture medium (Gupta *et al.*, 1993). Optimization of growth conditions has begun a few decades ago (Harley, 1934) and developed continuously so that nowadays there are general nutrient environments used for a wide range of species or selective media specially designed for a particular species, taking into account all its nutritional needs (Atlas, 2010). The limitations of classical identification methods can be solved using molecular techniques. Nucleic acids became the most commonly used target molecules in the identification of soil fungi communities with the emergence of Polymerase Chain Reaction (PCR) (White *et al.*, 1990). The development of sequencing technology allows the high-throughput analysis of the terrestrial mycobiome, providing a better way of accessing information related to its diversity, functional and ecological relationships between communities and their evolution (Lindahl *et al.*, 2013).

One of the challenges of the molecular analysis of terrestrial fungal communities is the choice of the most appropriate barcode to obtain the best taxonomic identification. The development of universal primers is focused on finding conserved regions in the DNA molecule, characteristic to fungi, but at the same time having some genetic variability, allowing the differentiation to species level (Stielow *et al.*, 2015).

The new sequencing techniques, pyrosequencing and Next-Generation Sequencing (NGS) led to the appearance of a large number of soil fungal sequences stored in specialized sequences databases: FungiBD (Stajich *et al.*, 2012), PHYMYCO-BD (Mahé *et al.*, 2012). For the analysis of the obtained sequences *via* metabarcoding analysis new pipelines were developed: CLOTU

(Kumar *et al.*, 2011), PLUTOF (Abarenkov *et al.*, 2010), PIPITS (Gweon *et al.*, 2015), (Bálint *et al.*, 2014).

The characterization of terrestrial mycobiome involves not only reviewing the diversity of the fungal species, but also determining the functions that fungi perform in its structure. Identifying a DNA sequence characteristic to a species does not clarify rather the DNA belongs to a living cell actively involved in the use of a particular substrate as a source of food or a dead or dormant cell; with no involvement in the ecosystem's functioning (Blagodatskaya and Kuzyakov, 2013).

2.2. The role of fungi in the terrestrial ecosystem

Fungi have some basic functions in the terrestrial ecosystem. The saprophytic fungi are mainly involved in the decomposition of organic substrates, providing carbon, nitrogen, sulfur and phosphorus in the nutrient circuit. Besides the saprophytes, fungi are often found in symbiotic associations especially with the roots of the mycorrhizal plants or are parasites on a wide range of nutritional substrates (Paul, 2014).

2.3. Environmental factors that influence the terrestrial mycobiome

The environmental factors determine temporal and spatial variations in the structure of fungal communities, being decisive for the abundance and diversity of the constituent species (Buee *et al.*, 2009). The main factors influencing the fungal communities directly are the soil typology and horizons, humidity, temperature (which varies seasonally in most climate zones), pH, and the amount of organic substances available (Tedersoo *et al.*, 2007).

3. Terrestrial fungi potentially harmful for humans

The effect of the terrestrial fungi on human society can be negative when fungi cause material damage (De Lucca, 2007). A few contexts in which humans can face the harmful consequences of soil fungal activities are the destruction of agricultural crops (Termorshuizen and Jeger, 2008; Alabouvette *et al.*, 2009), the destruction of cultural heritage (Marchiafava *et al.*, 1974; Kozlov and Kisternaya, 2014; Wahab *et al.*, 2015) or potential pathogens for human health (De Lucca, 2007).

4. Control/preservation/treatment

As a natural resource, the soil is mainly exploited through agriculture (White, 2013). Soil health is a concept used in literature to characterize its quality of natural resource (Kibblewhite *et*

al., 2008). The soil health is defined taking into account its physical, chemical and biological properties, considering heavily anthropic ecosystems such as lands used for agriculture and horticulture as well as natural ecosystems represented by grasslands and forests (Frąc *et al.*, 2018). Regarding the biological component of soil health, fungi play a decisive role. Their increased diversity implies improved soil quality and increased agricultural productivity (Ramachandra *et al.*, 2018). The use of fungicides (Baćmaga *et al.*, 2016) and fertilizers (Weber *et al.*, 2013) is one of the most common agricultural practices in reducing soil fungal diversity.

At the same time, the soil represents the environment for the buried objects of cultural heritage. Its conservation potential is appreciated based on its capacity to develop and maintain an *in situ* balance between archaeological artifacts and soil particles after burial for as long as possible (Kibblewhite *et al.*, 2015). Factors that alter the balance achieved (change of drainage level, fertilizer and fungicide use in superficial soil layers) mainly aim at changing the soil acidity and the content of organic and mineral substances (Davidson and Wilson, 2006), affecting the community structure of present microbes (Rousk *et al.*, 2009). Many of the species of microorganisms that come into contact with the archaeological objects during their deposition into the soil are transferred, post-excavation, to the storage spaces where the environmental factors (humidity, temperature, oxygenation level) change radically, leading to changing the conservative balance reached *in situ* (Le Cabec and Toussaint, 2017).

For the medical field, the irrational use of fungicides is a serious problem, causing the emergence of resistant fungal communities transferable from the agricultural field to the clinical one (Ribas e Ribas *et al.*, 2016).

II. Materials and methods

1. Isolation sources

1.1. Plants

Phytopathogenic fungi were identified on various substrates such as ornamental plants (*Rosa* spp., *Tulipa* spp. and *Galdiolus* spp.), crop plants (*Daucus carota* and *Allium cepa*) and medicinal plants (*Ranunculus ficaria*).

1.2. Archaeological samples

In order to highlight the potential degrading effect of fungi on archaeological bones, samples, obviously affected by a microbial attack, were selected from the archaeological sites of Feleacu (Cluj County), Tărian (Bihor County) and Turdaş (Hunedoara County). The effect of fungi was also investigated on charred vegetal debris, represented by cereal caryopsis, recovered from the archaeological site of Cetatea Capidava (Constanţa County).

1.3. Soil

The soil samples analyzed come from the archaeological sites of Tărian, Feleacu, Turdaş (associated with the corresponding osteological remains), and Capidava. The pedological conditions differ between the mentioned sites as well as the environmental conditions, the high humidity that characterizes the sites of Feleacu, Turdaş and Tărian is absent from the site of Capidava.

1.4. Human toenail

Toenail-affected fragments were harvested from an 85-year-old hepatitis B virus (HBV) with a sterile nail kit.

2. Samples preparation

2.1. Plants

Plants visibly affected by the fungal attack were used as a source for inoculations and DNA direct extractions without any treatment prior to the actual analysis.

2.2. Archaeological samples

2.2.1. Osteological samples

Bones surfaces visibly attacked by fungi were cleaned with ultrapure water (PCR grade water, Jena Bioscience, Jena, Germany). For each sample, 1g of powder was obtained from the areas with obvious coloring using a dental micromotor (Marathon-3 Champion, Saeyang Microtech, Daegu, South Korea) and autoclaved dental cutters.

2.2.2. Charred vegetal debris

Unlike the bone samples affected by microbial attack, the cereal caryopsis could not be cleaned due to their increased perishability. They were exposed to ultraviolet light for 10 minutes on each side (using UVIlink, UVITEC, Cambridge, UK).

2.2.3. Soil

The soil samples analyzed did not undergo treatments prior to the actual analysis

2.3. Human toenail

The nail fragments collected were sterilized in 20% ethanol for one minute.

3. Methods of analysis

3.1. Molecular methods

3.1.1. Isolation of fungal species associated with archaeological bones

3.1.1.1. Culture media

The Czapek-agar selective medium containing: 30 g/L sucrose, 15 g/L agar, 3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 1 g/L KCl, 0.5 g/L $MgSO_4•7H_2O$, 0.01 g/L $FeSO_4•7H_2O$, 2% soluble starch, and 50 µg/mL chloramphenicol at pH 7.3 was used to isolate and obtain pure cultures for phytopathogenic fungi. All media were sterilized by autoclaving at 121 °C for 30 minutes.

To obtain pure cultures of *Dumontinia tuberosa,* potato dextrose agar medium (PDA) containing 500 g/L potato, 20 g/L glucose, 15 g/L agar, and 50 µg/mL chloramphenicol and the Czapek starch medium containing 30 g/L sucrose, 15 g/L agar, 3 g/L $NaNO_3$, 1 g /L K_2HPO_4, 1 g/L KCl, 0.5 g/L $MgSO_4•7H_2O$, 0.01 g/L $FeSO_4•7H_2O$, 2% soluble starch, and 50 µg/mL

chloramphenicol at pH 7.3 were used. All media were sterilized by autoclaving at 121 °C for 30 minutes.

For the isolation of the species present on the archaeological samples and the associated soil from Tărian, Feleacu and Turdaş, the Sabouraud-agar culture medium was used, containing: 40 g/L glucose, 10 g/L peptone, 15 g/L agar, and 50 µg/mL chloramphenicol at pH 5.6 (Atlas, 2010). After inoculation, the plates were incubated for 5 days at 22 °C

To identify the fungal species causing onychomycosis in humans, Sabouraud-agar medium was used together with Yeast Extract-Peptone-Dextrose medium (YPD) containing 0.5% peptone, 0.3% yeast extract, 0.5% glucose, and 30 mg/L chloramphenicol. After inoculation, the plates were incubated for 3 days at 25 °C. The fungal cultures inoculated in YPD liquid medium were prepared in 50 mL Falcon tubes containing 10 mL medium and were grown at 22 °C for three days with shaking on a rotor at 100 rpm (Orbital GFL Shaker 3017, Gesellschaf für Labortechnik mbH, Burgwedel, Germany).

3.1.1.1. Inoculation

The pure phytopathogenic fungi colonies were obtained by inoculating the Petri dishes with Czapek-agar medium in the central point with spores/mycelium from the affected plant materials. To obtain pure fungal colonies from the surface of the archaeological bones and soil samples, 300 mg of the powder obtained from the outer bone tissue and 100 mg of soil respectively were hydrated with 500 µl of ultrapure water for 2 hours at room temperature. 300 µl of the mixture was distributed on the Sabouraud-agar plates (Arora, 2003). The nail fragments were used to inoculate in three-point the Petri dishes with Sabouraud-agar medium (Hankin and Anagnostakis, 1975).

3.1.1.2. DNA extraction

The DNA was extracted from each isolated colony on culture medium using the Animal and Fungi DNA Preparation Kit (Jena Bioscience, Jena, Germany) according to the manufacturer's instructions.

3.1.2. Direct assessment of fungal diversity

3.1.2.1. DNA extraction

From the osteological samples and related soil from the archaeological sites, DNA was directly extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad,

USA) form 200 mg of bone powder and 200 mg of soil respectively for each of the samples according to manufacturer's indications. For the extraction of DNA from vegetal debris, the proposed protocol was used (Hofreiter *et al.*, 2000).

3.1.3. Molecular markers

The identification of fungal species was carried out based on the region of the internal transcribed spacer (ITS), as a 520 base pair fragment was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3 ') and ITS4 (5'-TCCTCCGCTTATTCGATATGC-3') (White *et al.*, 1990)

3.1.4. Amplificarea fragmentelor de interes

The Polymerase Chain Reaction (PCR) provided the amplification of the desired fragments. The reaction mixture for a final volume of 25 µl contained: 1X MangoTaq Colored Reaction Buffer (Bioline), 2.5 mM $MgCl_2$ (Bioline), 0.2 mM dNTP (Bioline), 0.5 mM of each primer (Macrogen Sequencing Service, Korea), 1.25 U MangoTaq (Bioline), and 2 µl DNA template in each PCR run. Amplification was carried out under following conditions: initial denaturation 95 °C for 5 min; 35 cycles of 95 °C for 30 sec (denaturation), 56 °C for 30 sec (annealing) 72 °C for 30 sec (elongation) and final elongation at 72 °C for 5 min. The DNA fragments generated by PCR were extracted from the agarose gel (Favorgen, Ping-Tung, Taiwan) and sequenced using the standard sequencing commercial service at Macrogen Inc. (Seoul, South Korea). The DNA sequences were submitted to GenBank under accession numbers MF373466-MF373487.

3.1.5. Evaluation of the amplification efficiency

The amplification products were migrated into agarose gel (1.5% agarose, 0.5 µg/mL ethidium bromide). The desired size fragments were purified with the FavorPrep ™ Gel / PCR Purification Kit (FAVORGEN, Ping-Tung, Taiwan) according to the manufacturer's instructions.

3.1.6. Cloning the fragments of interest

The fragments amplified from the DNA extracted directly from the osteological and vegetal archaeological samples and the additional soil were cloned using the CloneJET ™ PCR Cloning Kit (ThermoFisher Scientific, Waltham, USA) following the manufacturer's recommendations. For the transformation, competent cells of *Escherichia coli* were used, the

ampicillin-resistant strain, DH5α. For each sample, 16 colonies were randomly selected and subsequently inoculated into 5 mL of liquid LB medium for the plasmids extraction. The plasmids were purified using the GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, Waltham, USA), according to the manufacturer's specifications. Verification of the presence of the fragments of interest was accomplished by digestion with the restriction enzyme Bgl II (ThermoFisher Scientific, Waltham, USA).

3.1.7. Sequencing

Fragments generated by the amplification of DNA isolated from the colonies developed on the culture media were sequenced using ITS1 primer (White *et al.*, 1990). For the cloned fragments the sequencing primer was pJET1.2F from the cloning vector. Sequencing was performed using the service provided by Macrogen Inc. (Seoul, South Korea).

3.1.8. Sequence analysis

The obtained sequences were manually verified using the BioEdit version 7.0.9 (Tom Hall, Ibis Biosciences). The sequences were compared to those present in the National Center for Biotechnology Information databases (NCBI) (https://www.ncbi.nlm.nih.gov/pubmed) using the BLASTN (Basic Local Alignment Search Tool) to identify similar sequences (Altschul *et al.*, 1997). The sequences were deposited in the GenBank database under the access numbers MF373466- MF373487, and MF399498-MF399539.

3.1.9. qPCR (quantitative Polymerase Chain Reaction)

The primers used for quantification of fungal DNA molecules were FR1 (5'-AICCATTCAATCGGTAIT-3') and FF390 (5'-CGATAACGAACGAGACCT-3') (Chemidlin Prevost-Boure *et al.*, 2011), targeting a 390 bp fragment in the 18S rRNA gene. In order to obtain the standard curve, we used previously sequenced pJET1.2 plasmids containing the SSU fragment. Amplifications were carried out in the Rotor-Gene® Q (QIAGEN, Hilden, Germany) using SensiFASTTM SYBR No-ROX Kit (Bioline, London, UK) in a total volume of 20 µL: 1x of SensiFAST SYBR No-ROX Mix, 0.5 mM of each primer, 1.5 µL of DNA and 6.5 µL of water. A three-step PCR program consisting of 40 cycles was used as follows: 95 °C for 3 min, 95 °C for 10 sec, 56 °C for 15 sec, 72 °C for 15 sec. Two replicates were used for each sample analysis, as well as for NTC (No Template Control).

3.2. Physical methods

3.2.1. FTIR (Fourier Transform Infrared Spectroscopy)

In order to characterize the properties of the bone's surface, an area of approximately 5-6-mm^2 from the non-discolored outer layer of each selected osteological sample, adjoining the visible stains, was abraded with a dental micro-motor (Marathon-3 Champion, Saeyang Microtech, Daegu, South Korea). For the FTIR analysis, 2 mg of bone powder was homogenized with 200 mg of KBr using a mortar and a pestle (Surovell and Stiner, 2001). The mixture was pelleted using a hydraulic press. The FTIR absorption spectra were recorded with a JASCO 4100 spectrometer (Jasco, Tokyo, Japan), at room temperature, in the 400–6000 cm^{-1} spectral range with a spectral resolution of 4 cm^{-1}.

3.2.2 Electronic microscopy

Small fragments were removed from the discolored surface of each bone, using dental tweezers, and were examined using scanning electron microscopy. The samples were covered with a thin layer (5 nm) of gold (Au). The coating was applied in a Q150R ES automatic Sputter Coater, in an argon atmosphere. SEM images were recorded using an FEI Quanta 3D FEG dual beam electron microscope (FEI, Eindhoven, Netherlands) operating at an accelerating voltage of 30 kV. (Flegler *et al.*, 1993).

Electronic scanning and transmission microscopy images for the pathogenic fungal samples were obtained with the JEOL JEM 1010 electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan) from the Electronic Microscopy Center ”Professor Constantin Crăciun” of Babeş-Bolyai University. For scanning electron microscopy (SEM), the samples were critical-point dried in liquid CO_2 mounted on sticky carbon tabs and sputter-coated with gold (10 nm). For transmission electron microscopy (TEM), the fixed and dried samples were infiltrated with resin, deposited onto colloidal-carbon-coated copper grids and negatively stained with lead citrate and uranyl acetate. The grids were examined by SEM with a JEOL JSM 5510 LV electron microscope (Vánky, 1994) and by TEM with a JEOL JEM 1010 electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan) (Hayat, 2000). For electron microscopy samples, the chemicals used were glutaraldehyde, resin (Epon 812), lead citrate, uranyl acetate, bismuth subnitrate (Electron Microscopy Sciences, Fort Washington, USA); sticky carbon tabs, colloidal carbon coated grids (Agar Scientific, Cambridge, England).

4. Methods of treatment

4.1. Testing plant extracts with antifungal activity

Hedera helix was collected from the Alexandru Borza Botanical Garden of Cluj-Napoca, and a voucher specimen (CL 664210) is deposited at the Herbarium of Babeş-Bolyai University, Cluj-Napoca, Romania. The plant extract was prepared by a cold repercolation method (Sundaram and Gurumoorthi, 2012) at room temperature for 3 days small fragments (0.5–1 cm) of ivy leaves, which were extracted with 50% ethanol (Merck, Bucureşti, Romania) in the Mycology Laboratory of Babeş-Bolyai University, Cluj-Napoca, Romania. The Drug Extract Ratio (DER) was 1:1.

The antifungal activity of the *Hedera helix* leaf extract, expressed as MIC, was assessed by the agar dilution assay and was compared to the antimycotic drug fluconazole (2 mg × mL–1, Krka, Novo Mesto, Slovenia) and a control (nutritive medium and ethanol). The results were interpreted by calculating the percentage of mycelial growth inhibition (P) at each concentration, by using the formula $P = (C–T) \times 100/C$, where C is the diameter of the control colony and T is the diameter of the treated colony (Nidiry and Babu, 2005).

4.2. Testing commercial antifungal products naftifine and bifonazole

Two pharmaceutical antifungal products, Exoderil (containing 10 mg/mL of naftifine as active compound, Sandoz GmbH Kundl Austria) and Canespor (containing 10 mg/mL of bifonazole as active compound, KVP Pharma + Veterinär Produkte GmbH, Kiel, Germany), were tested against the pathogenic fungi causing onychomycosis. Minimum inhibitory concentrations (MIC50), the minimum concentrations of compound required to inhibit 50% of the population in terms of OD600nm values, for both antifungal agents was determined in liquid media and expressed in terms of the active compound concentration, followed by comparison with available data. The tested concentrations were 0, 0.005, 0.01, 0.025, 0.05, 0.1, 0.2, 0.6, 1.2, 3.6, 10.8, 50, 100, 500, and 1000 mg/L for naftifine and 0, 0.015, 0.03, 0.06, 0.12, 1, 10, 50, 100, 500, and 1000 mg/L for bifonazole, respectively (Carrillo-Munoz *et al.*, 1999).

4.3. Tests for depigmentation/repigmentation

For the species, *Rhodotorula mucilaginosa*, isolated from both archaeological bone and human toenail, the effect of naftifine as an inhibitor of carotenoid synthesis was highlighted. Depigmented colonies were obtained by resuspension of a *R. mucilaginosa* control colony in 1

mg/mL solution of naftifine diluted with 20% ethanol prior to inoculation on the culture medium. The method was inspired by practice recommendations of naftifine hydrochloride dosage and administration.

An alternative depigmentation strategy of *R. mucilaginosa* colonies involving naftifine consisted in the introduction of the substance at concentrations of 0.1 mg/mL, 0.2 mg/mL in the SDA growth medium prior to inoculation. The depigmented colonies were obtained after incubation at 22 °C for 3 days. Reinoculation of depigmented colonies on growth medium without naftifine resulted in the repigmentation of the inoculated colonies. The experiments were conducted in triplicate at the Department of Chemistry of the Faculty of Chemistry and Chemical Engineering of Babeş-Bolyai University.

4.4. Highlighting the role of carotenoids

The concentration of 1.2 mg/L naftifine in YPD medium was chosen for subsequent investigations. An inoculum of 20 µL was used for 10 mL culture media. Two sets of experiments were carried out for *R. mucilaginosa* inoculated in YPD medium without naftifine and with naftifine. *R. mucilaginosa* previously treated with 1.2 mg/L naftifne was also used for inoculation in YPD medium without naftifne. For the frst experiment, afer inoculation the Erlenmeyer flasks were exposed to UV radiation for 40 minutes in a biological safety cabinet. For the second (hypoxic) experiment, flasks inoculated with *R. mucilaginosa* were sealed afer argon was bubbled for 60 s in each flask.

4.5. Measuring the amount of carotenoids

Cells from 2 mL of homogenous suspension were harvested and washed three times with 1 mL 0.9% NaCl solution. 900 µL were centrifuged and the saline solution was discarded. The pellet was resuspended in 1.8 mL of DMSO, immediately followed by vigorous shaking for complete cell disruption (transformation of the suspension into a clear solution) and carotenoid extraction. Te clear solution was further centrifuged at 20000 g speed for cell debris deposition. The UV-vis spectra of the clear supernatant were measured between 200 and 800 nm using a Varian Cary 5000 UV-Vis-NIR Spectrophotometer (Agilent Technologies). Absorbance at 512 nm (maximum wavelength for these samples) was taken as a marker of the total carotenoid content.

III. Results and discussion

1. Phytopathogenic fungi

1.1. Testing *Hedera helix* extract

The antifungal activity of the *Hedera helix* extract was determined by testing its various concentrations by incorporating the extract into Czapek-agar growth medium. The extract was compared with the commercial antifungic fluconazole, which was also introduced into the growth medium and with a control in which ethanol was added to Czapek-agar medium (Table 1).

Taking into consideration some of the most frequent diseases of vegetables and ornamentals, 6 phytopathogenic fungi (*Aspergillus niger, Botrytis cinerea, B. tulipae, Fusarium oxysporum* f. sp. *tulipae, Penicillium gladioli,* and *Sclerotinia sclerotiorum*) were selected for testing the antifungal properties of the *Hedera helix* leaf extract. The extract inhibited the germination and growth of the plant pathogenic fungi in a concentration-dependent manner. The minimum inhibitory concentration (MIC) varied between 10% and 14% ($P \leq 0.05$) for the leaf extract compared to 8% and 30% ($P \leq 0.05$) for fluconazole (Table 1). These results are comparable with those obtained in our previous study for the ivy flower extract (MIC = 8–12%) and fruit extract (MIC = 10–14%).

Ivy leaf extract inhibited the fungi causing grey mould (*Botrytis cinerea*, *B. tulipae*), blue mould (*Penicillium gladioli*) and white rot (*Sclerotinia sclerotiorum*) of plants. The MIC for all of these fungi was 10%. Less of an inhibitory effect was recorded in the case of *Fusarium oxysporum* f. sp. *tulipae*, with a MIC of 12%. The most resistant of all of the selected fungi was *Aspergillus niger*, with a MIC of 14%. At any rate, the obtained results are better than those for fluconazole (Table 1), which makes the ivy leaf extract a promising biocontrol agent for plant pathogenic fungi.

Table 1 *In vitro* effect of the *Hedera helix* leaf extract. P – mycelial growth inhibition, [a] – the effect of the *Hedera helix* extract, [b] – the effect of fluconazole, C – control; results are the mean±SD of 4 replicates.

Species	*Hedera helix* extract (1:1) concentration (%)	Colony's diameter (mm)	P[a] (%)	Fluconazole concentration		Colony's diameter (mm)	P[b] (%)
				(%)	(mg/mL)		
Aspergillus niger	C	22	0	C		22	0
	6	18	18.18±0.81	10	0.2	11	54.13±0.50
	8	15	31.81±0.50	20	0.4	7	68.18±0.50
	10	8	63.63±0.57	25	0.5	3	86.36±0.57
	12	2	90.90±0.57	30	0.6	0	100
	14		100				
Botrytis cinerea	C	65	0	C		65	0
	3	44	32.30±0.50	2	0.04	40	38.46±0.50
	6	11	83.07±0.81	6	0.12	20	69.23±0.95
	8	4	93.84±0.95	10	0.2	3	95.38±0.50
	10	0	100	12	0.24	0	100
Botrytis tulipae	C	62	0	C		62	0
	3	41	33.87±0.50	2	0.04	50	19.35±0.50
	6	10	83.87±0.50	6	0.12	24	61.29±0.81
	8	3	95.16±0.57	10	0.20	3	95.16±0.50
	10	0	100	12	0.24	0	100
Fusarium oxysporum	C	32	0	C		32	0
	3	29	9.37±0.57	2	0.04	20	37.5±0.50
	6	17	46.87±0.50	6	0.12	8	75± 0.50
	8	12	62.5± 0.50	8	0.16	2	93.75±0.57
	10	4	87.5± 0.81	10	0.20	0	100
	12	0	100				
Penicillium gladioli	C	15	0	C		15	0
	3	11	26.66± 0.50	10	0.2	11	26.66±0.50
	6	7	53.33±0.81	16	0.32	9	40±0.50
	8	3	80±0.57	20	0.4	6	60± 0.50
	10	0	100	25	0.5	3	80±0.50
				30	0.6	0	100
Sclerotinia sclerotiorum	C	64	0	C		64	0
	3	31	51.56±0.50	2	0.04	30	53.12±0.50
	6	15	76.56±0.57	4	0.08	15	76.56±0.50
	8	3	95.31±0.81	6	0.12	4	93.75±0.57
	10	0	100	8	0.16	0	100

2. Fungi associated with archaeological samples

2.1. Fungi associated with archaeological bones

2.1.1. Assessing the degradation status of archaeological bones

Visual assessment of analyzed bones led to the identification of obvious surface changes in the form of discoloration foci and/or biofilm build-up. All samples showed clear white to black discoloration foci, and samples from Feleacu displayed also significant biofilm build-up. Physical anthropological investigation carried out following standard guidelines records mild surface degradation of bone and assigns a taphonomic score of 0 to samples from Tărian and Turdaș, and score 3 for the Feleacu sample, where score 0 signifies no weathering associated modifications and score 5, advanced surface degradation of bone with loss of its structural integrity.

The discoloration centers were examined with the aid of a scanning electron microscope and significant differences were observed between samples. The images for the Tărian and Turdaș samples did not show any evident microbiota being present on the surface of the bone, while the Feleacu sample exhibits an abundant fungal (*Stachybotrys chartarum*) biofilm. No consistent bioerosion markers (pitting, boring or tunnelling) (Marchiafava *et al.*, 1974; Hackett, 1981) were observed during SEM examination of the samples from Tărian and Feleacu. In the Turdaș sample, some pitting of 6 microns in diameter could be observed.

For all three bone samples, IR-SF (Infrared Splitting Factor) and C/P (carbonate to phosphate ratio) were calculated to assess mineral phase changes associated with degradation, and Am/P (amide to phosphate ratio) was established in order to estimate comparative loss of organic phase (proteins, predominantly collagen). The IR-SF index was calculated by adding the heights of the peaks at 565 and 605 cm^{-1}, after 2 points linear baseline substraction from under each peak of interest, representing the absorption signals specific for phosphate mineral groups from crystalline hydroxyapatite (the mineral phase of a bone structure) (Berna *et al.*, 2004) and dividing the sum by the height of the minimum recorded between the split peaks (Surovell and Stiner, 2001). The C/P was calculated by dividing the absorbance value at 1415 cm^{-1} by the absorbance value at 1035 cm^{-1} (Wright and Schwarcz, 1996) and the Am/P (linearly related to mineral content) was calculated by reporting the absorbance at 1640 cm^{-1} by the absorbance at 1035 cm^{-1} (Trueman et al., 2004). Raised IR-SF values indicate an increase in crystallinity, a common consequence of post-mortem alteration of the bone mineral phase. Archaeological bones have usually lower or

higher C/P values than fresh bone, low C/P values designating loss of carbonate from bone, and high C/P values being associated to the presence of carbonates in the burial environment. Low Am/P values are indicative of bone organic phase loss.

We compared the values obtained for the three mentioned indices, between the analyzed bone samples of archaeological origin. The sample from Turdaş presents the highest IR-SF (3.02) value correlated to lowest C/P (0.32) and Am/P (0.237) values. In contrast, the Tărian sample shows lowest IR-SF (2.14) value correlated to highest C/P (0.473) and Am/P (0.713) indices. The obtained values are apparently correlated with the age of the samples as the archaeological site from Turdaş dates to the 5th millennium BC and the one from Tărian is more recent, dating to the 19th century AD. Values obtained for the Feleacu sample (IR-SF=2.55; C/P=0.527; Am/P=0.477) are intermediate, and the archaeological site is radiocarbon dated between 13th-16th centuries AD. Nevertheless, the low number of samples, the absence of replicates/site and the multitude of factors, other than historical age, (skeletal element, sample preparation (Hollund, 2013)), presence of bacteria affecting the bone structure (Müller et al., 2011)) affect these values, and therefore limit the power of this correlation.

When compared to other data reported in the literature for archaeological and modern bone samples, the indices have similar values to archaeological samples, with no evident biodegradation patterns retrieved from floodable soils (Hollund, 2013). Frequently, the values for IR-SF range between 1 and 3.4 for modern bones (Hollund, 2013, Lebon et al., 2010, Müller et al., 2011) and between 3.4 and 4.6 in case of archaeological samples (Trueman et al., 2004, Berna et al., 2004). The C/P ratio varies from 0.15 to 0.35 in archaeological samples and is usually 0.28 for modern samples (Lebon et al., 2010). The Am/P values are usually 0.98 for modern bones and lower for archaeological samples (Hollund, 2013).

The C/P index value from Feleacu is highest among the studied samples. The IR-SF and C/P ratio were previously described to be linearly (Trueman et al., 2008) inversely (Bartelink et al., 2014) correlated, suggesting that C/P index for Feleacu should have been lower than the one from Tărian. The values for C/P, frequently reported for archaeological bones samples range between 0.15 and 0.35 (Lebon et al., 2010), indicating a possible calcite deposition on the surface of the bone from Feleacu.

The values of the FT-IR indices, IR-SF, C/P and Am/P, are apparently correlated with the chronology of the sites, but given the limited number of samples analyzed in this study and the

multitude of factors affecting mineral and organic bone structure, the cause of the physico-chemical structural rearrangements cannot be absolutely attributed to the historical age of the processed samples. On the other hand, the values reported here are similar to those obtained for other previously described archaeological samples (Trueman *et al.*, 2004; Lebon *et al.*, 2010; Hollund, 2013) as no significant decreases in the carbonate content or in the amounts of collagen, an usual result of microbial driven alterations (Müller *et al.*, 2011), were observed.

2.1.2 Species isolated on culture medium

The species of fungi isolated from the archeological samples from Feleacu, Turdaş, and Tărian are summarized in Table 2. Based on the ITS sequence, 14 species and 2 genera of fungi belonging to the classes Sordariomycetes, Eurotiomycetes, Leotiomycetes, Dothideomycetes (Ascomycota), Microbotryomycetes, Tremellomycetes (Basidiomycota) and Mortierellacetes (Mucoromycota). Representatives of the Ascomycota phylum dominate relative to those in the Mocoromycota and Basidiomycota, a situation that overlaps with that described in the literature, on the frequency of the three phyla in the structure of soil mycobioms (Dai *et al.*, 2013; Benucci *et al.*, 2018).

On the archaeological bones, the identified fungi were of *Stachybotrys chartarum* (Feleacu), *Aspergillus versicolor*, *Aspergillus ustus* and *Rhodotorula mucilaginosa* (Turdaş), *Penicillium chrysogenum*, *Cryptococcus saitoi* and *Rhodosporidiobolus ruineniae* (Tărian). In the soil samples, a greater number of species were found. In the soil from Feleacu were identified the species of fungi *Stachybotrys chartarum, Penicillium chrysogenum, Aspergillus versicolor*, and a species of the genus *Geomyces*. In the sample from Turdaş were identified the species *Pseudogymnoascus pannorum, Mortierella alpina, Dactylonectria macrodidyma* and *Entoleuca mammata*, and in the sample from Tărian the species *Stachybotrys chartarum, Penicillium chrysogenum, Pseudogymnoascus pannorum, Alternaria alternata, Ilyonectria radicicola, Solicoccozyma aeria*, and a species of the genus *Thielavia* (Table 2).

Table 2 The fungal species identified in bone and soil samples from Feleacu, Turdaş, and Tărian based on the ITS sequence after isolation on Sabouraud-agar medium.

Source	Site	Species [NCBI accession number]	Class	Phylum
Bone	Feleacu	*Stachybotrys chartarum* [MF373466]	Sordariomycetes	Ascomycota
Soil	Feleacu	*Stachybotrys chartarum* [MF373467]	Sordariomycetes	Ascomycota
		Penicillium chrysogenum [MF373468]	Eurotiomycetes	Ascomycota
		Aspergillus versicolor [MF373469]	Eurotiomycetes	Ascomycota
		Geomyces sp. [MF373470]	Leotiomycetes	Ascomycota
Bone	Turdaş	*Aspergillus versicolor* [MF373471]	Eurotiomycetes	Ascomycota
		Aspergillus ustus [MF373472]	Eurotiomycetes	Ascomycota
		Rhodotorula mucilaginosa [MF373473]	Microbotryomycetes	Basidiomycota
Soil	Turdaş	*Pseudogymnoascus pannorum* [MF373474]	Leotiomycetes	Ascomycota
		Mortierella alpina [MF373475]	Mortierellacetes	Mucoromycota
		Dactylonectria macrodidyma [MF373476]	Sordariomycetes	Ascomycota
		Entoleuca mammata [MF373477]	Sordariomycetes	Ascomycota
Bone	Tărian	*Penicillium chrysogenum* [MF373478]	Eurotiomycetes	Ascomycota
		Cryptococcus saitoi [MF373479]	Tremellomycetes	Basidiomycota
		Rhodosporidiobolus ruineniae [MF373480]	Microbotryomycetes	Basidiomycota
Soil	Tărian	*Stachybotrys chartarum* [MF373481]	Sordariomycetes	Ascomycota
		Penicillium chrysogenum [MF373482]	Eurotiomycetes	Ascomycota
		Pseudogymnoascus pannorum [MF373483]	Leotiomycetes	Ascomycota
		Alternaria alternata [MF373484]	Dothideomycetes	Ascomycota
		Ilyonectria radicicola [MF373485]	Sordariomycetes	Ascomycota
		Solicoccozyma aeria [MF373486]	Tremellomycetes	Basidiomycota
		Thielavia sp. [MF373487]	Sordariomycetes	Ascomycota

The fungal species *S. chartarum*, *P. chrysogenum*, *A. versicolor*, and *P. pannorum* were identified in several isolates from cultivated bone/soil samples. The species are actively involved in the colonization of the archaeological bones in the storage rooms and their presence was frequently reported in indoor air (Verdier *et al.*, 2014; Ruga *et al.*, 2015), and on objects with historical importance (Foladi *et al.*, 2013; Pitre *et al.*, 2013; Kavkler *et al.*, 2015). The original source for these species cannot be absolutely determined, but the presence of all of them in one or more soil samples seems to indicate that they entered storage environment with the samples, contaminated the storage boxes and became ubiquitous in the storage rooms air.

The only species with direct *ex situ* biological degradation potential is *P. chrysogenum,* as it can produce collagenases (de Albuquerque Wanderley *et al.*, 2016). These enzymes cannot nevertheless degrade the collagen present in the bone structure as it is embedded in the mineral

matrix (Trueman and Martill, 2002). This species does not produce important quantities of acids (Van Den Berg *et al.*, 2008) that can potentially dissolve the inorganic content of the bone (Kendall *et al.*, 2017). Thus, the most probable scenario is that *P. chrysogenum* cannot use the bone as a nutrient source, and due to its absence in the sample displaying microscopical pitting (Turdaş) it is highly unlikely to have the ability to mechanically alter the bone surface.

The collected bones were deposited in cardboard boxes and stored for periods ranging from 2 (Tărian) to 4 years (Feleacu). The cellulosic substrate of the storage boxes can be easily metabolized as many of the cultured species (*S. chartarum*, *P. chrysogenum*, *A. versicolor*, *Alternaria alternata, Thielavia sp.*) have cellulolytic activity (Zyani *et al.*, 2009) thus becoming nutritive substrates for these species.

2.1.3. Direct assessment of fungal communities colonizing the archaeological samples

The species of fungi and green algae identified on the archaeological bones of Feleacu, Turdaş, and Tărian respectively in the adjacent soils are summarized in Table 3. Based on the ITS sequence, 33 species and 2 genera of fungi belonging to the classes Peronosporea (Oomycota), Saccharomycetes Sordariomycetes, Eurotiomycetes, Leotiomycetes, Dothideomycetes (Ascomycota), Microbotryomycetes, Tremellomycetes, Agaricomycetes (Basidiomycota) and green algae belonging to the class Chlorophyceae (Chlorophyta of the kingdom Plantae) were identified.

On the archaeological bones, the identified fungi were of *Stachybotrys chartarum, Stachybotrys echinata, Chaetomium murorum,* and *Thielavia hyalocarpa* (Feleacu), *Stachybotrys chartarum, Aspergillus versicolor, and Alternaria alternata* (Turdaş), *Stachybotrys chartarum, Cryptococcus albidus, Daedaleopsis confragosa, Omphalina mutila, Cladosporium herbarum, Rhodosporidiobolus runinae, Epicoccum nigrum,* and *Volutella ciliata* (Tărian) (Table 3). In the soil from Feleacu were identified the species of fungi *Stachybotrys chartarum, Acremonium persicinum, Stachybotrys eucylindrospora, Stachybotrys chlorohalonata, Stachybotrys kampalensis, Penicillium solitum*, and *Aspergillus nomius.* In the sample from Turdaş were identified the species *Stachybotrys chartarum, Phialophora hyalina, Ramaria decurrens* şi *Chaetomidium leptoderma*, and in the sample from *Kazachstania barnetti, Bremia lactucae, Neocudoniella radicella, Entoloma asterosporum*, *Epicoccum nigrum* and a species of the genus *Thelebolus* (Table 3).

Table 3 The fungal and green algae species identified in bone and soil samples from Feleacu, Turdaş, and Tărian based on the ITS sequence.

Source	Site	Species [NCBI accession number]	Number of clones	Class	Phylum
Bone	Feleacu	*Stachybotrys chartarum* [MF399498]	13	Sordariomycetes	Ascomycota
		Stachybotrys echinata [MF399499]	5	Sordariomycetes	Ascomycota
		Chaetomium murorum [MF399500]	2	Sordariomycetes	Ascomycota
		Thielavia hyalocarpa [MF399501]	3	Sordariomycetes	Ascomycota
		Uncultured sp. [MF399502]	1		
Soil	Feleacu	*Stachybotrys chartarum* [MF399503]	7	Sordariomycetes	Ascomycota
		Acremonium persicinum [MF399504]	2	Sordariomycetes	Ascomycota
		Stachybotrys eucylindrospora [MF399505]	7	Sordariomycetes	Ascomycota
		Stachybotrys chlorohalonata [MF399506]	1	Sordariomycetes	Ascomycota
		Stachybotrys kampalensis [MF399507]	5	Sordariomycetes	Ascomycota
		Penicillium solitum [MF399508]	1	Eurotiomycetes	Ascomycota
		Aspergillus nomius [MF399509]	1	Eurotiomycetes	Ascomycota
Bone	Turdaş	*Stachybotrys chartarum* [MF399510]	5	Sordariomycetes	Ascomycota
		Aspergillus versicolor [MF399511]	4	Eurotiomycetes	Ascomycota
		Tetracystis sp. [MF399512]	8	Chlorophyceae	Chlorophyta
		Spongiochloris spongiosa [MF399513]	2	Chlorophyceae	Chlorophyta
		Chlorosarcinopsis eremi [MF399514]	2	Chlorophyceae	Chlorophyta
		Alternaria alternata [MF399515]	2	Dothideomycetes	Ascomycota
		Uncultured sp. [MF399516]	1		
Soil	Turdaş	*Stachybotrys chartarum* [MF399517]	5	Sordariomycetes	Ascomycota
		Phialophora hyalina [MF399518]	10	Eurotiomycetes	Ascomycota
		Ramaria decurrens [MF399519]	2	Agaricomycetes	Basidiomycota
		Chaetomidium leptoderma [MF399520]	6	Sordariomycetes	Ascomycota
		Uncultured sp. [MF399521]	1		
Bone	Tărian	*Stachybotrys chartarum* [MF399522]	2	Sordariomycetes	Ascomycota
		Cryptococcus albidus [MF399523]	3	Tremellomycetes	Basidiomycota
		Daedaleopsis confragosa [MF399524]	2	Agaricomycetes	Basidiomycota
		Omphalina mutila [MF399525]	2	Agaricomycetes	Basidiomycota
		Cladosporium herbarum [MF399526]	2	Dothideomycetes	Ascomycota
		Rhodosporidiobolus runinae [MF399527]	3	Microbotryomycetes	Basidiomycota
		Epicoccum nigrum [MF399528]	7	Dothideomycetes	Ascomycota
		Volutella ciliata [MF399529]	2	Sordariomycetes	Ascomycota
		Uncultured sp. [MF399530]	1		
Soil	Tărian	*Kazachstania barnetti* [MF399531]	3	Saccharomycetes	Ascomycota
		Bremia lactucae [MF399532]	2	Peronosporea	Oomycota
		Thelebolus sp. [MF399533]	2	Leotiomycetes	Ascomycota
		Neocudoniella radicella [MF399534]	2	Leotiomycetes	Ascomycota
		Entoloma asterosporum [MF399535]	2	Agaricomycetes	Basidiomycota
		Epicoccum nigrum [MF399536]	3	Dothideomycetes	Ascomycota
		Tetracystis sp. [MF399537]	6	Chlorophyceae	Chlorophyta
		Spongiochloris spongiosa [MF399538]	3	Chlorophyceae	Chlorophyta
		Uncultured sp. [MF399539]	1		

Most of the species identified for bone and soil samples from the three archaeological sites are commonly found in the soil (Kuhn and Ghannoum, 2003) and storage facilities for historical artifacts (Koul and Upadhyay, 2018). For the samples from Feleacu it is noticeable the presence of species of the genus *Stachybotrys*. In addition to the species *S. chartarum*, present in the samples from Turdaş and Tărian, four other species of the genus *Stachybotrys* as *S. echinata*, *S. eucylindrospora*, *S. chlorohalonata*, and *S. kampalensis*, are characteristic of the site of Feleacu. The high number of species of the *Stachybotrys* species, only in the Feleacu samples, brings into question the possibility that they were the point of penetration of the fungi into the storage facility. The cellulosic material of the storage boxes used for samples storage favors their spread. At the same time, the genus is ubiquitous, the conclusion above being influenced by the small number of clones analyzed (24 clones/sample (bone/soil)/archaeological site).

2.1.4. Quantification of fungal DNA

The qPCR results show that the fungal community (as inferred from the number of 18S rDNA fragment copies/mg bone powder/soil) present in the Tărian soil sample has the largest size (3.34E+09), while lowest value was obtained for the Turdaş soil sample (9.28E+02). There is a difference of seven orders of magnitude between the two samples. For the Feleacu and Turdaş archaeological sites, similar values of fungal communities' sizes were obtained when comparing soil samples (Feleacu: 2.63E+06; Turdaş: 9.28E+02) to bone samples (Feleacu: 8.07E+06; Turdaş: 5.14E+03), with slightly higher values in bone samples. Interestingly, for Tărian, the size of the fungal community present in soil (3.34E+09) is six orders of magnitude higher than the one analyzed from bone (5.14E+03).

The obtained values could be explained by the fact that some fungal cells might have adhered or could be incorporated into superficial bone tissue (Garland, 1989), in contrast to soil particles that could be constantly washed by ambient water molecules. For the sample from Tărian the size of the community from soil (3.34E+09 molecules/mg) is larger than the one from the archaeological bone (5.14E + 03 molecules/mg), the Tarian soil providing favorable conditions to the development of the fungal community (Jumpponen *et al.*, 2010). The dimensions of the communities cannot be explained simply by the interpretation of the values as being inherent to an *in situ* context of the samples, given the fact that the presence of possible contaminants induced during excavation and sample analysis, respectively during storage, has been signaled.

2.1.5. FTIR

Fourier Transform Infrared Spectroscopy allows the recording of particular vibration modalities of the main types of chemical bonds (C-C, C-H, C-O, H-O) from the cellular structure of the fungi. Several types of bonds can be used as discriminants for the identification of fungal species, with spectra being recorded between 600-4000 cm^{-1} (Salman *et al.*, 2010).

FTIR spectra were recorded for all of the colonies isolated on culture medium, but only 5 of the 20 spectra were usable in the spectrophotometric confirmation assay. Two of the samples originate from the surface of the archeological bone from Tărian, being the molecularly identified as TARIAN_1 - *Cryptococcus saitoi*, TARIAN_3 - *Rhodosporidiobolus ruineniae*, one sample is from the soil from Tărian identified as TARIAN_4 - *Stachybotrys chartarum*, and two samples are from the soil from Turdaş being identified as TURDAS_5 - *Pseudogymnoascus pannorum* and TURDAS_7 - *Entoleuca mammata* (Table 2).

For none of the mentioned species, FTIR spectra have been recorded in the literature until now, therefore there are no possibilities of comparison for the obtained spectra. At the same time, the mentioned spectra are not identical to other spectra of known species, which may be authentic, but their autenticity has not been confirmed by multiple spectral measurements obtained from multiple independent colonies of the same species.

2.2. Fungi associated with charred vegetal remains

For the carbonized caryopsis from Capidava, the amplification of the DNA fragment for the ITS sequence was not achieved. Frequently, cereals discovered in archaeological sites are charred (Bilgic et al., 2016). The phenomenon is often due to secondary fires occurring in the archaeological sites (Matuzeviciute et al., 2018) or to their deposition in carbonized form (Gustafsson, 2000). Most of the time, the burning process is associated with a good preservation of the archaeological plants (Antolín and Buxó, 2011). The good preservation refers not only to morphological features, but also to the success of the amplification of ancient DNA molecules, endogenous to plant material (Bilgic et al., 2016).

In the associated soil, 4 species of the Class Dothideomycetes (Ascomycota) were identified. The species identified predominantly belong to the genus *Alternaria* (*A. tenuissima, A. alternata, A. brasssicae*) and *Aureobasidium* (*A. pullulans*) and are common in the soil, being

characterized as phytopathogens for a large number of plants (Pethybridge et al. 2006; Huang et al., 2009).

The number of fungal DNA molecules was also quantified for the soil sample from Capidava. The values were expressed as the number of copies of 18S RNA gene/mg and subsequently compared with the values obtained for similar samples from the sites of Feleacu, Turdaş, and Tărian. The size of the Capidava fungal community (7.67E + 03) is higher than on present in the sample from Turdaş (9.28E + 02) but lower than those recorded in Tărian (3.34E + 09) and Feleacu (8.75E + 05) samples. Differences in population size can be explained by different environmental conditions, marked by the lack of humidity for the Capidava sample, which nevertheless favors the development of a more numerous fungal community than in Turdaş sample.

3. Pathogenic fungi for humans

3.1. *Rhodotorula mucilaginosa* as onychomycosis agent

Onychomycosis is a multifactorial nail fungal infection. It is an important public health problem because of its high prevalence, high rates of recurrence and progression to chronic lesions. Onychomycosis accounts for 50% of all nail diseases. It afects 2–13% of the general population, and this percentage increases with age, reaching up to 40% in the elderly. Tickened, discoloured, deformed nails without pain are the most common symptoms of a fungal nail infection (Cengiz *et al.*, 2018). Although the clinical picture can be very suggestive, the diagnosis should be confrmed by a routine method before starting treatment. The most commonly used methods are direct potassium hydroxide (KOH) examination, culture, and, to a lesser extent, nail biopsy. The diagnosis of onychomycosis is made when one or more of the three diagnostic tests are positive. Other tests are expensive and require the use of specialized equipment and materials (Velasquez-Agudelo and Cardona-Arias, 2017).

Common pathogens in onychomycosis are dermatophytes, nondermatophyte moulds (NDMs) and yeasts (Westerberg and Voyack, 2013).

Rhodotorula mucilaginosa is one of the human pathogens causing onychomycosis (da Cunha *et al.*, 2009). The presence of *R. mucilaginosa* was confirmed on the basis of the ITS sequence in the sample obtained from the infected nail. *R. mucilaginosa* is a basidiomycetous yeast. It presents only spheroidal to oval budding cells (2.5–6.5× 6.5–14.0 μm) with carotenoid pigments and without the rudimentary formation of hyphae (Frengova and Beshkova, 2009).

Carotenoids can act as vitamin A precursors (Goodwin, 1952), having colouring and antioxidant properties (Mata-Gómez *et al.*, 2014). Carotenoids are widely produced pigments found in algae, yeasts and plants. Their main functions are reactive oxygen species scavenging and protection against photo-oxidative damage (El-Banna *et al.*, 2012). Four main pigments, torularhodin, torulene, beta-carotene and gamma-carotene, are synthesized by *R. mucilaginosa* species, and carotenoid production is usually enhanced by stress factors such as UV exposure, oxidative stress or osmotic stress (Mata-Gómez *et al.*, 2014). Torularhodin was shown to be correlated with the survival of the cells under UV-B light (Moliné *et al.*, 2010). Beta-carotene and torularhodin also showed antioxidant activity, preventing hyperoxia-induced cytotoxicity (Moore and Breedveld, 1989).

3.2. Testing commercial antifungal products naftifine and bifonazole against *Rhodotorula mucilaginosa*

On the species *R. mucilaginosa* was tested the antifungal effect of naftifine (the active substance in the pharmaceutical product Exoderil in which its concentration is 10 mg/mL). The MIC50 was determined based on the optical densities (OD) at 600 nm of the yeast cultures in YPD liquid medium at concentrations of 0.005, 0.01, 0.025, 0.05, 0.1, 0.2, 0.6, 1.2, 3.6, 10.8, 50, 100, 500, 1000 mg / L naftifine. The MIC50 value of 55 ± 14 mg/L is similar to the value of 0.5 mg/L reported in literature for the naftifine tested on the species *R. rubra* (formerly known as *R. mucilaginosa*) (Carrillo-Munoz *et al.*, 1999).

For low concentrations of naftifine, depigmentation of the analyzed colonies was observed without affecting cell growth. The effect of the compound on depigmentation and the minimal concentration required for depigmentation (MDC) were determined by tracking the absorbance at 512 nm, concurrently with the optical density at 600 nm. The decrease in carotenoid content (total content being estimated by the absorbance value at 512 nm) correlated directly with the increase of naftifine. The MDC value, 0.088 ± 0.02 mg/L, is comparable to the value of 0.008 mg/L reported in the literature as the naftifine inhibitory concentration in carotenoid synthesis for the strain *Staphylococcus aureus* (Chen et al., 2016).

At the cellular level, naftifine affects the last enzyme (farnesyl diphosphate synthase) involved in the synthesis of isoprenoid isopentenyl diphosphate (IPP). IPP is a common product in the ergosterol and lycopene synthesis pathways (Verwaal *et al.*, 2007). Low levels of naftifine leads to the decrease of the cellular lycopene (Chen *et al.*, 2016) at the expense of ergosterol (essential role in cell membrane structure) affected only by higher concentrations of naftifine (Carrillo-Munoz *et al.*, 1999).

UV-Vis spectra were measured for the carotenoids extracted from naftifine treated *R. mucilaginosa* colonies. The absorption peaks of the three major cellular pigments were recorded as follows: β - carotene (475 nm), torulen (512 nm) and torularhodin (544 nm) (Perrier et al., 1995). The peak conformation changes with the increase in the naftifine concentration tested, indicating an increase in the relative β-carotene content with the decrease in torularhodin and torulene content. For concentrations greater than 1.2 mg/L naftifine, carotenoid synthesis is totally inhibited.

Another commercial antifungal product, bifonazole (the active substance in the Canespor pharmaceutical product with a concentration of 10 mg/mL), was tested against *R. mucilaginosa*. MIC50 was determined based on OD at 600 nm of cultures in liquid YPD medium at concentrations of 0, 0.005, 0.01, 0.025, 0.05, 0.1, 0.2, 0.4, 1.2, 2.4, 10, 50, 100, 500, 1000 mg/L bifonazole. The MIC50 value of 4.4 ± 0.8 mg/L is similar to that reported in the literature for bifonazole tested *for R. rubra* (formerly *R. mucilaginosa*) (Carrillo-Munoz *et al.*, 1999). Compared with the MIC50 obtained for naftifine, the MIC50 value of bifonazole is smaller by an order of magnitude.

Unlike naftifine, bifonazole does not interfere with the synthetic pathway of carotenoids. The antifungal effect is due to the inhibition of the ergosterol synthesis by affecting the activity of HMG-CoA reductase responsible for demethylation of 4,4 ', 14-trimethylsterols (Berg *et al.*, 1984).

3.3. Highlighting the role of carotenoids

Carotenoids have a role in protecting cells under stress conditions (oxidative stress, UV exposure). Blocking their synthesis may be correlated with the existence of cells less resistant to stress conditions. The carotenoid content (by absorbance at 512 nm) and cell growth (by optical density at 600 nm) were evaluated concomitantly for *R. mucilaginosa* control colonies, naftifine treated colonies and repigmented colonies under normal growth conditions, respectively stress represented by hypoxia and exposure to UV.

Thus, control colonies were compared with naftifine-treated control colonies (1.2 mg/L), with repigmented colonies, control colonies exposed to UV light for 40 minutes, with naftifine treated colonies (1.2 mg/L) exposed to UV light for 40 minutes, with control colonies argon-bubbled for 60 seconds and with naftifine treated colonies (1.2 mg/L) argon-bubbled for 60 seconds.

The applied treatments slightly influence the cell growth rate, thus the OD600 values *R. mucilaginosa* for control colonies, respectively for naftifine-treated and repigmented, remain constant, around 3 (with a slight increase in OD600 for the repigmented colonies). For the samples exposed to UV light, the values of the OD600 decrease to about 2.5 and for those developed in the hypoxic environment, the values of the OD decrease even more to 2. In both cases of UV exposure and in the hypoxic environment, the OD600 values recorded for naftifine-treated colonies are higher than for pigmented homologous colonies.

The total carotenoid content is the highest for the *R. mucilaginosa* control colony and has similar values for the repigmented colony. Exposure to UV and hypoxic conditions resulted in a decrease in carotenoid content (from 0.18 in control to 0.03 in the sample exposed to UV and 0.04 in the argon-bubbled sample), independent to the presence of naftifine. Naftifine-treated colonies have comparable values of Abs512, the obtained values were 0.016 for control, respectively 0.006 for the samples exposed to UV light and 0.007 for the samples argon-bubbled.

The better cell survival rate of naftifine-treated colonies (1.2 mg/L) under stress conditions compared to the pigmented stressed colonies may be due to the stimulation of the ergosterol synthesis in the context of blocking the carotenoid synthesis pathway by naftifine (Carrillo-Munoz *et al.*, 1999). At cellular level, ergosterol plays a fundamental role in the membrane system, including the cell membrane itself, its microdomains (represented by lipid membranes with membrane glycoproteins, important in cell signaling, membrane transport and membrane fluidity), cytoplasmic fat droplets, vacuoles and mitochondria (Lv *et al.*, 2016). The fungicidal effect of UV light on yeasts is primarily due to the destruction of the cell membranes (Ozcelik, 2007). Naftifine influences the ultrastructure of *R. mucilaginosa* cells, with more voluminous lipid droplets in the cytoplasm of cells at the concentration of 1 mg/mL, possibly correlated with increasing ergosterol (Lv *et al.*, 2016).

IV. Conclusions

The ivy leaf ethanolic extract exhibited antifungal activity against the tested phytopathogenic fungi (*Aspergillus niger, Botrytis cinerea, B. tulipae, Fusarium oxysporum* f. sp. *tulipae, Penicillium gladioli* and *Sclerotinia sclerotiorum*) in a dose-dependent manner with MIC of varying between 10% to 14%. The results were comparable to those obtained for a synthetic drug (fluconazole), which makes ivy leaf extract a cost-effective and a potent herbal control agent for the treatment of plant diseases, such as grey mold, plant wilt and white rot.

The study of the bone/soil associated communities in the archaeological sites of Feleacu, Turdaş and Tărian led to the identification of fungal species frequently reported in the literature in various soil samples or in the ambient environment of enclosed spaces represented by storage areas or rooms of living. The evaluation of the fungal community is discussed in relation to the conservation status of excavated archaeological bones before and during their storage/processing. It was attempted to establish the structure of the fungal community at the time of the archaeological excavation and to follow the evolution of endogenous species and exogenous contaminants, in order to assess their potential as degradative agents and as risk factors for the health of the researchers. An interdisciplinary approach involving macroscopic visual evaluation, scanning electron microscopy, Fourier transform infrared spectroscopy and fungal barcodes (ITS, ARN18S gene) was used on samples previously isolated on culture medium or analyzed directly without isolation.

Rhodotorula mucilaginosa was identified and confirmed molecularly as an agent of onychomycosis in an 85-year-old patient with chronic hepatitis B virus. At the same time, the species was also isolated from the archaeological site of Turdaş. For the antifungal agents naftifine (10 mg/mL, active substance in Exoderil) and bifonazole (10 mg/mL, active substance in Canespor) was showed the fungicidal effect against *R. mucilaginosa*, determining the MIC50 values. The mechanisms of the antifungal action of naftifine and bifonazole in *R. mucilaginosa* cells were similar, affecting the biosynthetic pathway of ergosterol. Only naftifine affects the biosynthetic pathway of carotenoids, causing depigmentation of *R. mucilaginosa* colonies. Depigmentation is a reversible process, the yeast cells resuming carotenoid production after transfer to culture medium without naftifine. The UV-vis spectrometry allowed detection of the changes in the carotenoid content in depigmented yeast cells, revealing variations in the composition of the main carotenoids (torularhodin, torulen, β-carotene).

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