

Identification of fungal strains isolated from buildings of cultural importance in Romania and antagonistic relationships amongst them

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Abstract

Specialists involved in the restoration and preservation of cultural monument buildings are confronted with the issue of biodegradation - the irreversible altering of the material structure by living organisms, such as bacteria, algae, fungi, lichens or insects. The current study is targeted on the microscopic fungi attack of different construction materials from buildings of cultural importance, aiming to identify the involved species, as well as to investigate their antagonistic relationships. The phenotypic identification, based on the evaluation of macroscopic and microscopic features was followed by MALDI-TOF analysis and molecular characterization based on the sequencing of the ITS (*Internal Transcribed Spacer*) taxonomic marker. The results showed a predominance of the genera *Penicillium*, *Aspergillus*, *Rhizopus* for the affected building materials. The antagonism tests have shown a significant inhibitory effect of the *Trichoderma* sp. and *Mucor* sp. strains upon the other isolates.

Keywords: architecture, heritage, biodegradation, MALDI-TOF, antagonism

1. Introduction

From the early years of monuments' restoration and preservation, scientists have confronted the issue of biodegradation, defined as the irreversible altering of the material structure by living organisms, such as bacteria, algae, fungi, lichens, insects, etc. (WONG et al. [1]). Being by far the most active and efficient biodegrading living organisms, fungi have always played a key-role in shaping the landscape, whether natural or anthropic. Therefore, it is nowadays worth studying their impact on the built environment, old or new, considering that these heterotrophic organisms were among the first of large dimensions to colonize the earth, and their degrading and biomass recycling activity resulted in the creation of soil and landscape, as we know them today (by mineral dissolution, production of external

metabolites, decomposing of organic matter, water and nutrient absorption and retention, symbiotic relationships etc.).

Beside their beneficial role, fungi are also involved in biodegradation of different materials by different mechanisms, such as: the alteration of the material integrity through their physical presence and the exerted forces, the production of organic (acetic, oxalic, gluconic) or inorganic (nitric, sulfuric, carbonic) acids, pollutants metabolism, production of exoenzymes, biofilms development, etc. (SCHEERER, [2]; STERFLINGER et al. [3]). The result of the degradation process is the alteration of the physical and chemical structure, followed by changes of aspect, corrosion, bad smell, staining, permeation, rotting, loss of resistance, some of them with a direct impact on the health state of the inhabitants.(HUCKFELDT, [4])

In the case of stone, for example, the degradation can induce the staining of material in black-dark brown [3], caused by the *Dematiaceae* (CIFERRI et al. [5]); green-caused by algae or associations; yellow, marking the presence of the carotenoids. A physical altering of the stone is the *pitting* process, which starts with punctual drills caused by microorganisms that in time excavate the material creating large pores (CANEVA [6]).

Wood, too, can suffer staining, especially from mold-producing *Ascomycetes*; rotting is by far the most severe biodegradation of wood, known to be of three types: brown, white, and wet. (HUCKFELDT, [7]).

Studying the antagonism among different strains of biodegrading microorganisms is a starting point for identifying and extracting naturally produced antifungal compounds, specifically active against biodegrading species.

2. Materials and Methods

2.1. Isolation and phenotypic identification

The samples were collected during the winter of 2017 from 3 buildings dating from the 19th century, two of them rated class B (local importance) located in the Neamt County and the third situated in Bucharest, in a protected area, severely affected by a fire, followed by intensive exposure to natural elements. The samples were collected from a wide range of building materials, both structural (pillars, beams, walls) and non-structural (cladding, woodwork, carpentry).

The fungal attack of the wood consists of cellulose and hemicellulose decomposing, by means of hydrolytic enzymes: exoglucanase, endoglucanase, cellobiase, in the case of brown and wet rot and lignin degradation (MOORE et al. [8]), by lignin-peroxidase, laccase and manganese peroxidase, in the case of dry rot.

The stone or cement-made materials provide support for fungal growth by nourishing them with chemoorganotrophic organisms, by water retention determined by porosity, or a by offering a nourishing mineral composition. In turn, the fungi degrade the material by producing organic acids (citric, oxalic, lactic, fumaric acid) which dissolve the mineral (CWALINA [9]).

The collected samples consisted of pieces of building materials, with sizes of approx. 1 cm³, from different construction elements: pillars, walls, base, beams, cladding, and woodwork. The samples were placed on Sabouraud Dextrose Agar growth medium (SDA), and incubated at 28°C ± 2° C for 14 days. After this step, spores from each individual colony growing on the plate were transferred to a new plate with SDA.

2.1. Identification

Each purified colony recovered on SDA was characterized macroscopically, on aspects regarding color, growth distribution, texture. The phenotypic identification at the genus level was confirmed microscopically, using the Lactophenol Cotton Blue (LCB) technique. The solution has three components: phenol, which kills the living organisms, lactic acid, which preserves the structure of the fungi, and cotton blue, which stains the chitin in the cellular wall. The reproductive structures were visualized at the AxioCam MRc microscopy professional digital camera and image acquisition and analysis software ZEN Imaging 2012.

2.2.MALDI-TOF Analysis

From a total number of ~100 isolated purified colonies, The Matrix Assisted Laser Desorption Ionization (MALDI-TOF) was performed on 30 representative plates, from which 11 have been successfully identified. The MALDI-TOF analysis implies a laser beam which strikes a matrix of small organic molecules, transforming them into gas ions without fragmentation. The Time of Flight refers to the time required by each ion to reach a detector, correlated with their molecular mass/ electric charge ratio. This type of analysis is only relevant when comparing to a database, therefore, a large amount of the tested strains could not be determined.

2.3.ITS Sequencing

For determination through sequencing of the ITS region, strains belonging to *Penicillium* sp., as the most frequently isolated genus, and to *Trichoderma* sp., for the known potential of this genus to produce metabolites with antifungal properties. The DNA extraction from the two mycelia was performed using the commercial kit from REDEExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich). The extracted DNA integrity was further reconfirmed by electrophoresis in 1% agarose gel. The ITS1 and ITS4 regions were amplified through PCR with the primers 5'-TCCGTAGGTGAACCTGCGG-3'- for ITS1 and 5'- TCCTCCGCTTATTGATATGC-3' for ITS4. The amplification reaction components and conditions are shown in Table 1, 2 (MARTIN [10]).

The PCR products were stained for visualization with ethidium bromide (EB) (10 µg / ml) and migrated through electrophoresis in a 1% agarose gel and the resulted amplicon was assessed in comparison to a specific molecular weight marker (100bp, Thermo Scientific) (WHITE, [11]).

Table 1. Reaction components used in the PCR reactions.

Concentration						Final volume
primer	MgCl ₂	dNTP	DNA Taq-pol	Reaction buffer	DNA	
0,5µM	1,2mM	2µM	0,2 U	1x	10x	20µl

Table 2. Amplification programs of PCR reactions.

The gene and the size of the amplicon	Amplification program				
	Denaturation	No. of cycles	Denaturation	Anealing	Extension
ITS=600bp	95°C for 5 min	35	95°C,1 min	55°C,60 sec	72°C,8 min

The amplicons were then sequenced using an ABI 3730xl DNA Analyser with a 10 s annealing time at 55°C (Applied Biosystems) and the sequences were analyzed using ChromasLite 2.1.1 (<http://www.technelysium.com.au>). The NCBI BLAST (Basic Local Alignment Search Tool) database was used in order to assess the similarity with published sequences, belonging to identified fungal species. Sequencing was carried out at the Beijing Genomics Institute (Beijing, China).

Further, the fungal strains were tested for antagonism relationships amongst each other (SESAN, [12]), for the purpose of identifying potential antifungal compounds.

3. Results and Conclusions

A total number of 100 isolates was recovered and identified by examining the macroscopic and microscopic features as belonging to 9 fungal genera, as shown in the Table 3, with a predominance of *Rhizopus*, *Aspergillus*, *Fusarium* and *Penicillium* genera (Fig. 2) and a wide diversity at species level, revealed by MALDI-TOF (Table 4).

Table 3. The distribution of the fungal strains isolated from different monument buildings.

No.	Building	Caradja, NT	Catargi, NT	Delavrancea, B
	Genus			

	Material	Carp.	W. str	Cladd.	Wood.str	Cladd.	Cladd.1	Cladd.2	W. str.
1.	<i>Rhizopus</i>	x	x	x	x	x	x	x	x
2.	<i>Aspergillus</i>	x	x	x	x	x		x	
3.	<i>Fusarium</i>		x		x	x	x	x	x
4.	<i>Mucor</i>	x	x		x	x	x	x	x
5.	<i>Trichoderma</i>		x			x			
6.	<i>Penicillium</i>		x			x		x	x
7.	<i>Alternaria</i>		x			x		x	x
8.	<i>Epicoccum</i>							x	
9.	<i>Botrytis</i>	x	x						

Abbrev.: **Caradja, NT** (Aristide Caradja Mansion, Grumăzești, Neamț County Code: NT-IV-m-B-10762) **Catargi, NT** (Catargi fam. Mansion, Tupilați, Neamț County Code: NT-II-a-B-10723), **Delavrancea, B** (Str. Barbu Ștefănescu Delavrancea nr. 24, Bucharest), **carp**-carpentry; **W. str.**- wooden structure; **cladd.**-cladding.

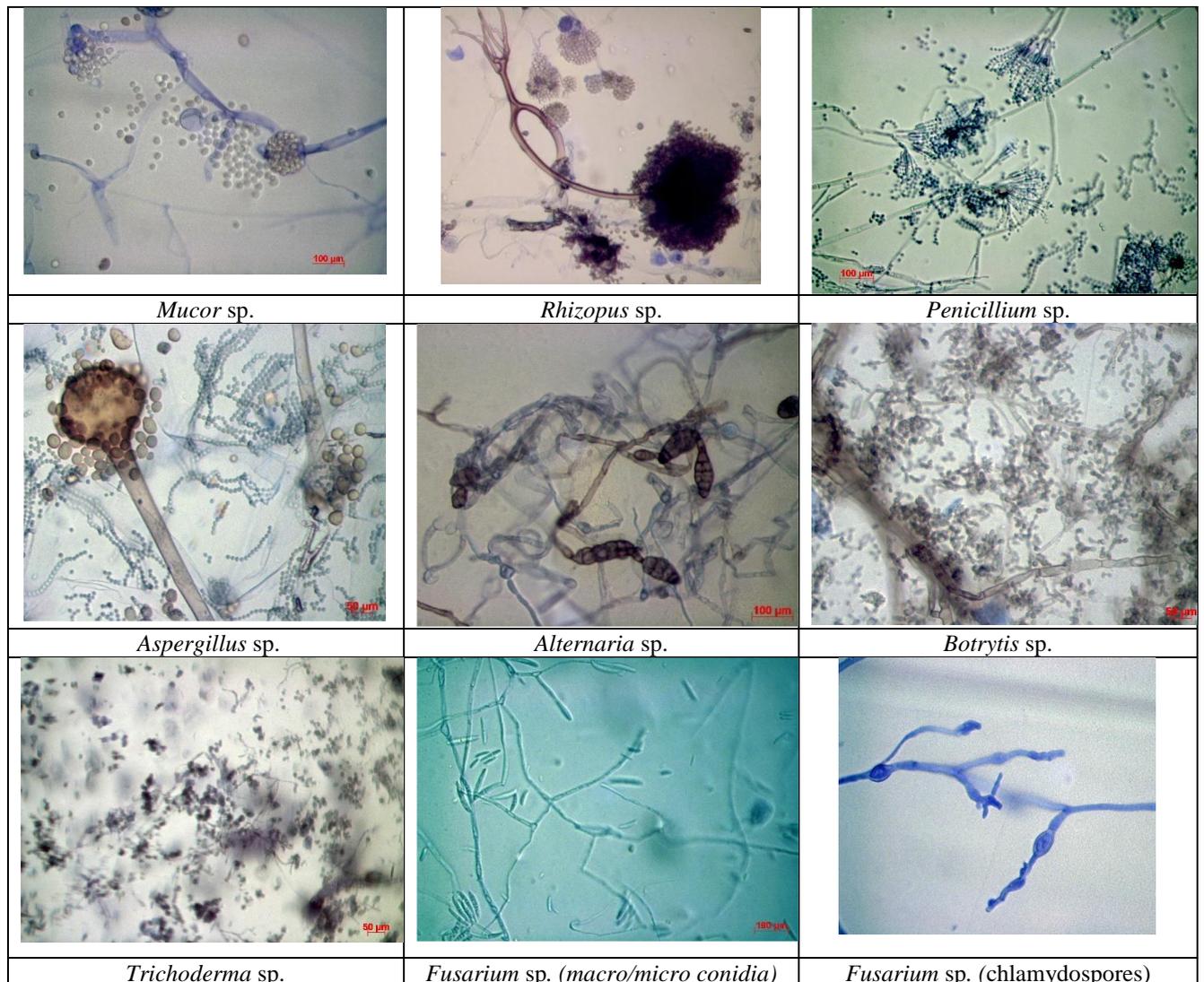


Figure 2. Microscopic aspect of the isolated fungi, belonging to different genera (cotton blue staining, x40)

Table 4. Fungal species identified by MALDI-TOF

Plate no.	SPECIES	SOURCE	MATERIAL
19	<i>Rhizopus stolonifer</i>	Caradja, NT	Wooden beam
14	<i>Rhizopus stolonifer</i>	Catargi, NT	Cladding
65	<i>Penicillium brevicompactum</i>	Caradja, NT	Wooden beam
20	<i>Penicillium crysogenum</i>	Caradja, NT	Wooden beam
67	<i>Penicillium crysogenum</i>	Catargi, NT	Masonry walls
71	<i>Penicillium crysogenum</i>	Catargi, NT	Masonry walls
110	<i>Penicillium digitatum</i>	Caradja, NT	Cladding
84	<i>Mucor circinelloides</i>	Catargi, NT	Wooden beam
46	<i>Fusarium proliferatum</i>	Delavrancea, B	Interior cladding
41	<i>Penicillium crysogenum</i>	Catargi, NT	Masonry walls

The molecular identification of the two *Penicillium* and *Trichoderma* isolates allowed the identification of *Penicillium chrysogenum* (accession no. GenBank database JN851002.1) and *Trichoderma longibrachyatum* (accession no. GenBank database - MH731276.1) species, with 99% identity.

Antagonism studies

Living in associations with different species, in their competition for resources and territory, fungi develop survival natural strategies, such as: colonization and spore germination speed, substrate usage ability, or ways of eliminating competitors (production of enzymes (STEYAERT, [13]) by the production of toxins, volatile compounds - acting at distance, these compounds can affect the competitor's germination capacity, the shape of the mycelium and the enzyme production ability) (HUMPHRIS, [14]). The *Trichoderma* sp. isolates, for instance, are known for their antifungal activity, due to the production of cellulase, chitinase, xylanase, pectinase, glucanase, lipase, amylase, protease or volatile compounds. For revealing the antagonism among the recovered isolates, the plates were divided into two equal parts, and the strains were streaked in the middle of each one, in spots with approximately the same diameter. Figures 3 and 4 reveal naturally occurring (after the first isolation) or induced antagonism experiments and moments of the attack, revealed microscopically: coiling (REMADI et al.) [15] (*Trichoderma* wraps its hyphae around the host); hyphae anastomosis as a reaction of the host; chlamydospores production inside the host as a final stage before the lysis of the cellular wall (BLASZCZYK, [16]).



Naturally developed antagonism: *Trichoderma*, *Penicillium*, *Fusarium*.



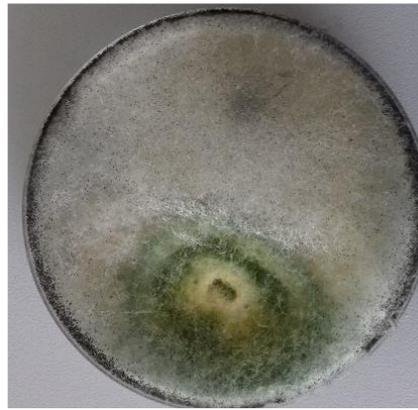
.Naturally developed antagonism: *Fusarium*-*Trichoderma*.



Antagonism *Fusarium*-*Mucor*.



Trichoderma-*Fusarium*.

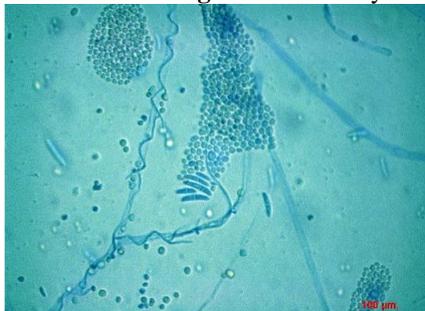


Trichoderma-*Mucor*.



Fusarium-*Mucor*.

Figure 3. Naturally occurring or experimental antagonisms among different fungal isolates.



Coiling - *Trichoderma* coiling around *Fusarium*.



Hyphal anastomosis due to degradation.



Formation of chlamydospores in the final stage of the attack.

Figure 4. Microscopic aspects of fungal mycelia developed in antagonism experiments.

It can be concluded that the identified *Ascomycetes* have a certain affinity for the studied materials, as show by other similar studies, such as: URZI et al., 1992, noted that the Acropolis marble is a host for species of *Torula*, *Alternaria*, *Pythomyces*, *Urocladium* (URZI et al [17]); Gomez- Alarcon et al. (1994) isolated *Fusarium* and *Penicillium* isolates from the walls of the church Carrascosa del Campo, Spain; the same authors studied the monuments of Alcalá de Henares, Spain, invaded by different species of *Alternaria*, *Penicillium*, *Phoma*, *Trichoderma* and *Ulocladium* genera; for concrete or cement-made cladding, CWALINA, 2008 identified isolates belonging to *Aspergillus*, *Ceratostomella*,

Cladosporium, *Fusarium*, *Hormoconis*, *Hormodendrum*, *Penicillium* and *Spicardia* genera, as main sources of biodeterioration.

In Romania, Berinde, 1986 described the representative species that cause the mold of the wood, as belonging to the genera *Penicillium*, *Aspergillus*, *Trichoderma*, *Alternaria*, *Paecilomyces*, *Cladosporium*, *Aureobasidium*, *Bispora*, *Neurospora*, *Mucor*, *Chaetomium*. (BERINDE, [18]). Barbu et al, 1983, marked the presence of fungi (*Alternaria alternata*, *Trichoderma viridae*, *Fusarium oxisporum*, *Cladosporium herbarum*, *Verticillium* sp.) on the stone ruins of Dacian citadels (BARBU et al [19]). In 2007 Radulescu and colab. have isolated species belonging to *Penicillium*, *Alternaria* and *Cladosporium* from the surface of different types of heritage textile provided by the National Museum of the Romanian Peasant in Bucharest (Radulescu et al [20]).

Similar molecular identification studies based on the sequencing of the ITS1/ITS2 region was carried out by Sterflinger who identified *Penicillium crysogenum* as having an affinity for chitinous substrates, and species of the genus *Trichoderma* (*T. viridae*, *T. harzianum*) for paper and cellulose textiles (STERFLINGER, [21]). The ITS1/ITS2 sequencing of the same spacer region on fungi isolated from 17th century textiles showed the predominance of *Aspergillus penicilloides* (LECH, [22]). Through the same method, the predominance of species belonging to *Hypochnicium* genus in the tomb belonging to the Han dynasty, China, was revealed (LIU et al. [23]).

Conclusion

In the present study, the phenotypic and molecular characterization of the filamentous fungi isolated from different buildings dating from the 19th century, rated class B of heritage in Romania revealed the presence of nine genera mostly belonging to the *Rhizopus*, *Aspergillus*, *Fusarium*, *Penicillium* genera. Out of these, many strains showed inhibitory activity in antagonism experiments and will be further studied for the isolation and characterization of antifungal compounds.

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