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"KultBaMikroo" Project: Preliminary Research on the Presence, Diversity, and Dynamics of Cellulolytic Fungi in the Air in the State Archive Building in Zadar

Projekt „KultBaMikroo": Preliminarno istraživanje prisutnosti, raznolikosti i dinamike celulolitičkih gljivica u zraku u prostorijama zgrade Državnog arhiva u Zadru

ABSTRACT

The presented methodology and preliminary results are part of the research in the interdisciplinary modular pilot project "Protection of Zadar's Cultural Heritage from the Negative Impact of Microorganisms" (KultBaMikroo), financed by the University of Zadar (IP.01.2021.12). The modular project is designed so that it can be carried out through several modules, whereby the types of cultural heritage artefacts on which the research is based can be changed with each module. The goal is to prevent book damage related to storage conditions and to provide guidelines for conservation and restoration. Moreover, as it is known that exposure to fungi is dangerous for human health, attention will be paid to the protection of employees and visitors on the premises where the research is carried out. The following modules are to addressed the issue of protecting paintings and immovable cultural heritage. In this first module, research was conducted at the premises of the State Archive in Zadar, which is a project partner, and at the Chapter Library of the Zadar Archdiocese. This overview includes only the preliminary results of the research conducted at the State Archive in Zadar, one of the most important archives in Croatia.

Ključne riječi: cellulolytic fungi, spores, cultural heritage, prevention, biological degradation, written matter

SAŽETAK

Prikazana metodologija rada i preliminarni rezultati dio su istraživanja provedenih kroz interdisciplinarni istraživački modularni pilot projekt „Zaštita zadarske kulturne baštine od negativnog utjecaja mikroorganizama" (KultBaMikroo) koji je financiran od strane Sveučilišta u Zadru (IP.01.2021.12.). Modularni projekt zamišljen je na način da se može provoditi kroz nekoliko modula, s tim da se kroz module mijenja vrsta građe kojom se projekt bavi. U ovom prvom istraživačkom modulu provode se istraživanja pisane građe u svrhu što bolje zaštite građe i ljudi koji borave u prostorima gdje se građa čuva. Planirano je da se u sljedećim modulima projekt baviti istraživanjima zaštite slikarske baštine, odnosno zaštitom nepokretne kulturne baštine. U ovom prvom modulu istraživanja su provođena u prostorima Državnog arhiva u Zadru, koji je partner na projektu, te u prostorima Kaptolske knjižnice Zadarske nadbiskupije u Zadru. Ovdje će biti prezentirani samo preliminarni rezultati istraživanja provedenih u Državnom arhivu u Zadru, jednom od najvažnijih arhiva u Hrvatskoj.

Keywords: celulolitičke gljivice, spore, kulturna baština, prevencija, biološka razgradnja, pisana građa

INTRODUCTION

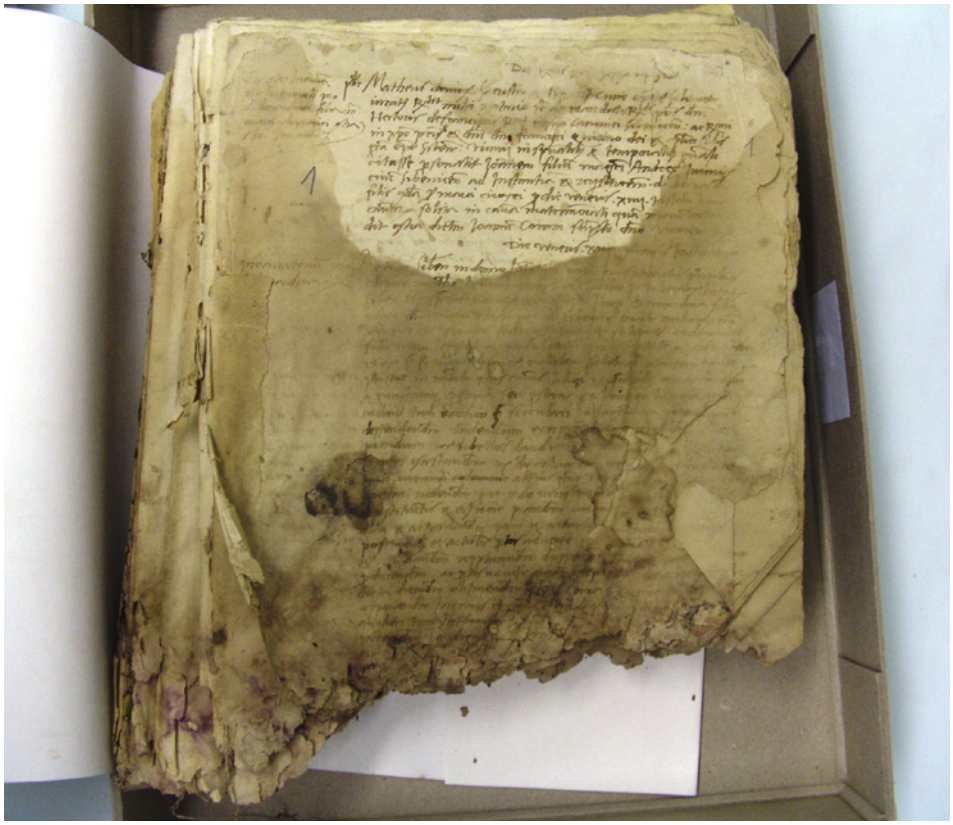
Aerobiology is a multidisciplinary science whose goal is to study the presence and significance of bioaerosol components in the air.[1] Bioaerosol is a collection of particles originating from the atmosphere that is present in the air long enough to allow monitoring of its quality. Bioaerosol also includes plant pollen and spores, microscopic invertebrates, bacterial and fungal spores, protists, viruses, bacteria, fragments of microorganisms, toxins, glucans, nucleic acid molecules, highly volatile metabolites, etc. Aeromycology is a sub-discipline of aerobiology whose task is the quantitative and qualitative study of the presence of fungal spores in the air. All types of material cultural heritage that contain organic molecules can be subject to the process of biological decomposition if the conditions are favourable, i.e. if there is a possibility of colonization by microorganisms. In that case, cultural heritage suffers physical and chemical damage.

Since fungi (moulds) are cosmopolitan, ubiquitous organisms, capable of colonizing various types of microenvironments, they are among the most important causes of biological degradation of wooden and book heritage (Fig. 1). Moulds are multicellular microscopic fungi consisting of a dense system of tubular cells without chlorophyll, and grow in the form of hyphae that reproduce asexually with conidia and spores. Conidia and mould spores are present in the air and dust, and under favourable conditions of humidity and temperature, they can begin to grow on a substrate containing organic molecules.

Fungi that colonize book matter can secrete cellulolytic, proteolytic, and ligninolytic exoenzymes that can break down the compounds of the paper. In order to analyse the mycoflora present in the air of the State Archive building in Zadar and propose possible measures to reduce the risk of colonization of the stored books by fungi, air sampling was carried out in six different rooms in the archive building over December 2021 and March 2022.

Sampling of microorganisms in the air was carried out using the "Air Sampler" device, which actively collects air in the form of an airstream onto a previously prepared nutrient medium in Petri dishes. Multiple nutrient media were used to cover a wide spectrum of fungi that favour different environmental conditions, therefore DG18[2] was used to isolate potential xerophilic, xerotolerant, osmophilic, and osmotolerant species, and OA[3] and MEA[4] were used for mesophilic species. At the same time, measurements of temperature and relative humidity were carried out on the premises because of their impact on the growth and development of microorganisms. The air was also sampled at the entrance of the institution to determine the fungi in the outdoor bioaerosol. After the incubation of the samples in laboratory conditions, fungi were isolated in pure cultures, morphologically identified, and tested for cellulose degradation.

The presence and concentration of fungal spores in closed rooms are associated with various types of automated and human activities, such as air conditioning, ventilation, hygiene systems, and others. Moreover, previously contaminated cultural heritage can be an additional source of fungal contamination of buildings where it is stored, especially if it is not handled adequately. In the said areas, aerobiological tests are crucial for detecting the source of contamination and for assessing the flow and major accumulation of airborne fungal spores. Aerobiological research is also important for determining daily and seasonal variations of fungal species. The monitoring of fungal bioaerosols provides information on the risks of contamination of stored cultural heritage and, more importantly, allows the assessment of the negative impact on human health. Therefore, it is important to regularly monitor the qualitative and quantitative structure of fungal bioaerosols, especially if potentially



1.
Handmade paper affected by moisture and mould

Papir ručne izrade napadnut vlagom i plijesni

new sources of contamination appear. Conservation can only be effectively carried out with a multidisciplinary approach that includes expert understanding of fungal biology and the implementation of new, modern, and practical methods. The aim and purpose of this scientific research is to determine the presence of fungi that produce cellulolytic enzymes in the State Archive building in Zadar.

Closed rooms where valuable cultural heritage is stored, such as museums, libraries, archives, etc. are mostly located in old, monumental buildings. However, very few of them can be considered as properly built and maintained for the adequate storage of archival and library matter, especially in terms of protection against microbiological threats. In such environments, many different factors can cause significant variations in temperature and humidity. This can lead to an increase in the concentration of bioaerosol, which in turn increases the possibility of a negative impact on stored cultural heritage as well as on human health. Over the past decade, the number of aerobiological studies investigating the negative impact of bio-deterioration by fungi on archival cultural heritage in buildings where it is stored has been increasing. Also, multidisciplinary studies on the environment of cultural heritage were carried out, including biological monitoring and microclimate analysis in order to define standardized protocols for book protection.

RESEARCH METHODOLOGY

Nutrient media

In order to isolate a wider spectrum of fungi that prefer different humidity, three different types of nutrient media were used: Malt Extract Agar (MEA), Oatmeal Agar (OA), and Dichloran-Glycerol Agar (DG18). The DG18 medium was used for the isolation of xerophilic and osmophilic species, while the OA and MEA media

were used for the isolation of mesophilic fungal species. A specific nutrient medium that contains cellulose, Carboxymethyl Cellulose (CMC), was used for the detection of cellulolytic activity of the fungus.

Monitoring of microclimatic parameters

Air temperature T (°C) and relative air humidity RH (%) were monitored in each room from December 2021 to April 2022 using the Data Logger LOG100/110/CRYO device. The air sampling was carried out in the following rooms: 1) Reading Room on the 3rd floor; 2) Room II-2 on the 2nd floor; 3) Room I-3 on the 1st floor; 4) Safe Vault on the 1st floor; 5) X-Party on the 1st floor; 6) Basement on the ground floor; 7) Entrance to the building.[5]

Air sampling and determining the concentration of spores (CFU)

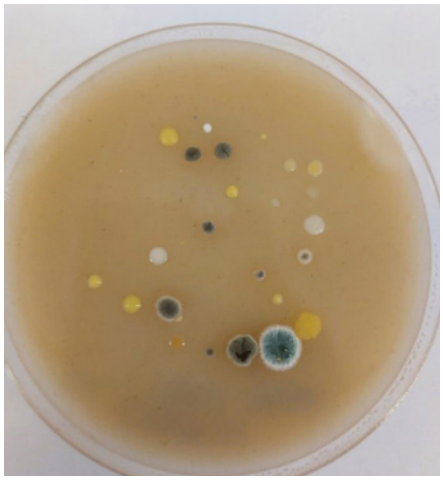
Mould spores were collected by the volumetric method of air sampling using the Air Sampler device (Fig. 2), which actively collects air onto a Petri dish with an airstream according to the method of Savković et al. (2019). In each of the six rooms, sampling was carried out at three different locations (door, centre, and window) at a height of 1.50 m. At each location, the air was collected on three different nutrient media. At the Entrance to the building, three samples were collected, also on three different nutrient media. Petri dishes with inoculated nutrient media were incubated in laboratory conditions at a temperature of 25°C for 7 days. Then the Colony Forming Unit (CFU) value was calculated from the number of mixed cultures grown on Petri dishes. The obtained values were corrected to the expected statistical abundance according to the method of Feller et al. (1950).

Isolation of pure cultures

To isolate pure cultures, each primary isolate from the mixed media (Fig. 3) was inoculated on the same type of media (MEA, OA, or DG18). Pure cultures were incubated at 25°C for 7 days (Fig. 4). After incubation, the morphological determination of fungi was carried out by examining their microscopic and macroscopic morphological characteristics (texture, colour) according to the Food and Indoor Fungi key (Samson et al., 2019).

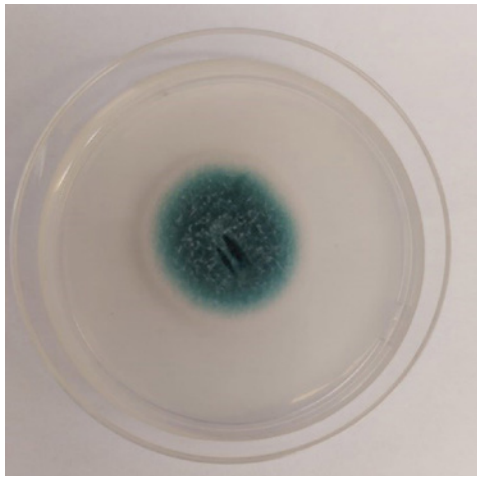
2.
Air sampling using the "Air Sampler" device

Uzorkovanje zraka uređajem „Air Sampler"



3.
Mixed culture of fungi on a nutrient medium

Mješovita kultura gljivica na hranjivoj podlozi



4.
Pure culture of fungal isolate on a nutrient medium

Čista kultura gljivica na hranjivoj podlozi

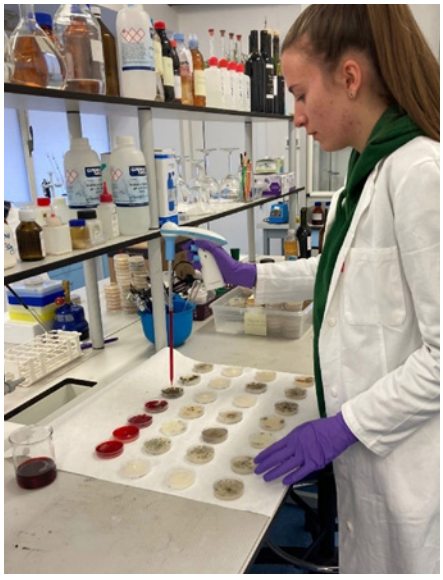
Cellulose degradation assay

The purpose of the cellulose degradation assay is to determine the cellulolytic activity of isolated fungi that present a threat to book matter in terms of bio-degradation. Previously obtained pure cultures were inoculated on CMC agar and incubated for 3 days at 25°C and 95% RH. Grown fungal colonies were stained with 0.1% Congo red solution (Fig. 5). After 15 minutes, the colonies were destained by adding 1M NaCl solution. An indicator of cellulase enzyme production is the appearance of a yellow circle around the colony (Fig. 6). The assay was performed according to the protocol of Savković et al. (2019).

RESULTS

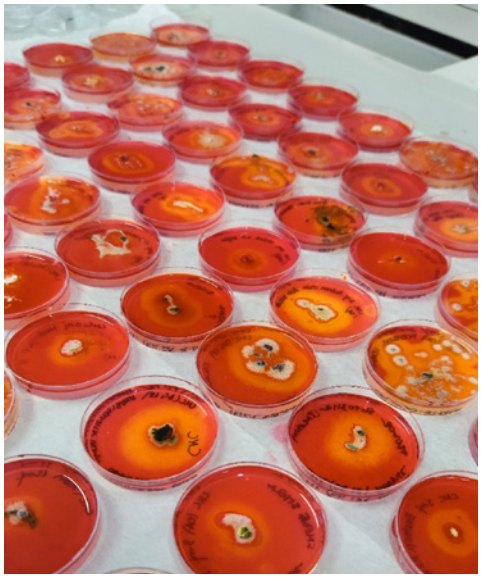
Correlation between microclimatic parameters and the concentration of spores in the air (CFU)

The obtained microclimate values on the "Data Logger" device showed that there were no extremes during the winter and spring seasons, i.e. the temperature deviated by 3°C from the average. The average air temperature was 19.9°C, and the average relative humidity was 43.9%.



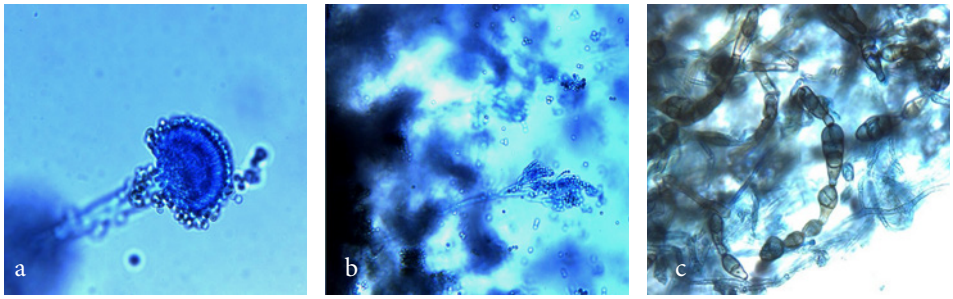
5.
Staining of cultures with Congo red solution

Bojanje kultura Congo red otopinom



6.
Confirmed cellulolytic activity of the tested fungal isolates

Potvrđena celulolitička aktivnost testiranih izolata



7.
Microscopic photography of isolates (P 10X40): a) *Aspergillus ssp.*, b) *Penicillium ssp.*, c) *Alternaria ssp*.

Mikroskopska determinacija izolata: a) *Aspergillus ssp.*, b) *Penicillium ssp.*, c) *Alternaria ssp.*

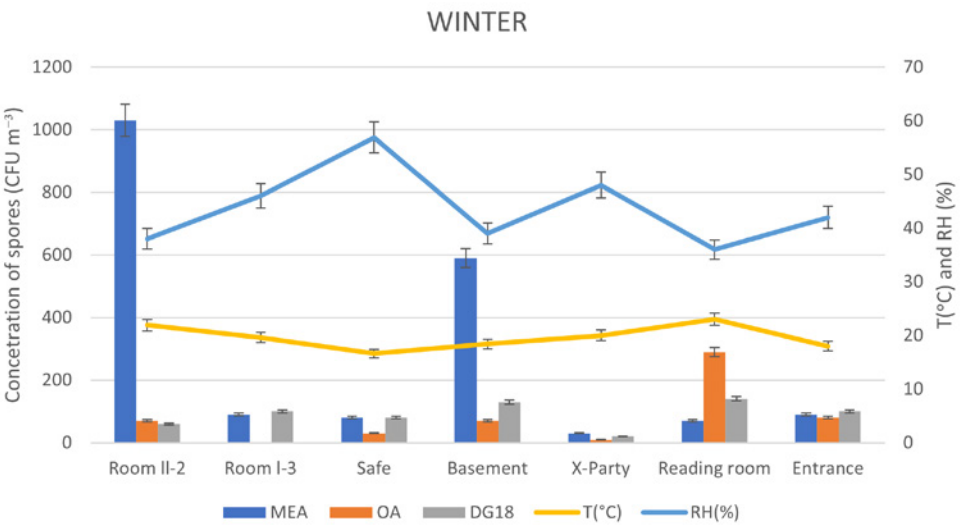
Analysis of the samples showed a relatively high concentration of fungal spores in some of the examined rooms in the Archive building during both seasons. It exceeded the value above 150 CFU m^3 prescribed in the standards for Italian libraries (MIBAC, 2001). Nevertheless, other standards recommend a value up to 1000 CFU m^3 for archival rooms that are used for storing cultural heritage (World Health Organization, 1990). According to these standards, the concentration in most rooms does not exceed the given limits, while for our research we used the Italian standard as a criterion.

According to the analysis of air samples taken at the State Archive, the concentration of fungal spores in the winter period was above the (Italian) prescribed limit in all rooms except in the X-Party on the 1st floor. Moreover, an elevated concentration of spores was recorded near the building (Entrance) during the winter period. According to the Italian standard, the tested rooms with a concentration of fungal spores above 150 CFU m^3 would be classified as contaminated, but it should be emphasized that the environmental conditions are not the same in Croatia and Italy.

Diversity of airborne fungi

Winter: During the winter period, a total of 51 Petri dishes were collected. Mixed cultures of fungi had grown on 41 dishes, while no colonies grew on the other 10 dishes. A total of 97 pure cultures were isolated from the nutrient media containing mixed cultures. Spore concentrations (CFU m^3) on different media (MEA, OA, DG18) in relation to the values of temperature and relative air humidity in the examined rooms and the Entrance to the building during winter sampling are shown in Graph 1.

Morphological determination (Fig. 7) established the following genera of cellulolytic fungi: *Aspergillus*, *Penicillium*, *Mucor*, *Alternaria*, *Cladosporium*, and *Rhizopus* (5).

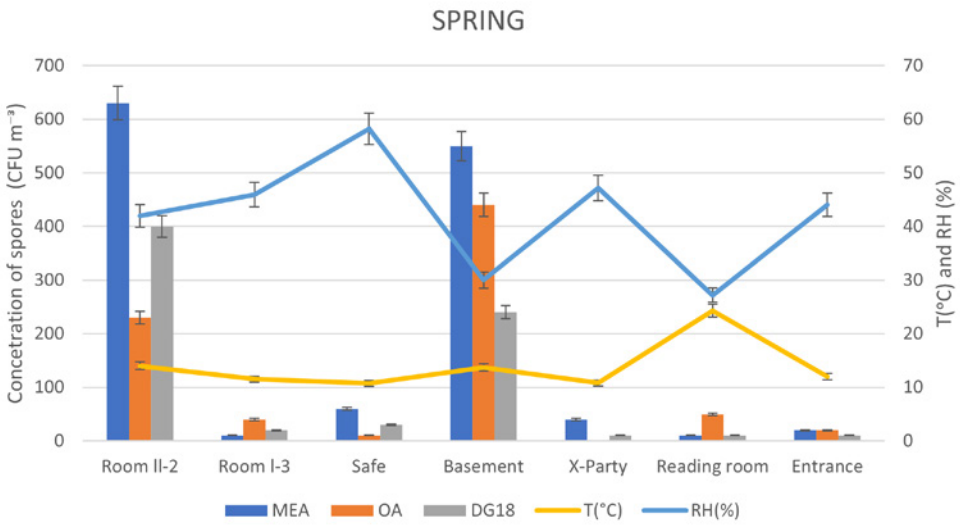


Graph 1.
Concentrations of spores (CFU m^3) on different media (MEA, OA, DG18) in relation to the values of temperature and relative air humidity in the examined rooms and the Entrance to the building during winter sampling

Koncentracije spora (CFU m^3) na različitim podlogama (MEA, OA, DG18) u odnosu na vrijednosti temperature i relativne vlažnosti zraka ispitivanih prostorija i ulaza u zgradu tijekom zimskog uzorkovanja

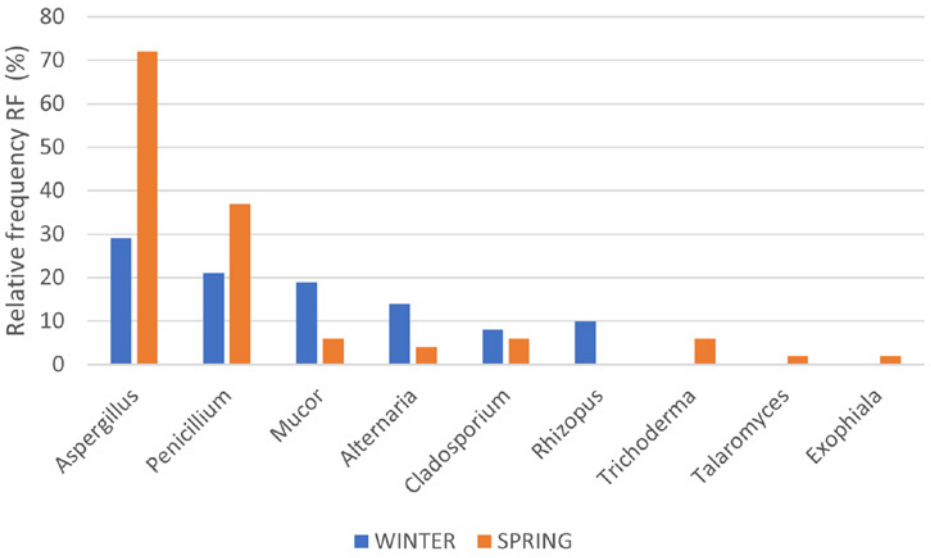
Graph 2.
Concentrations of spores (CFU m^3) on different media (MEA, OA, DG18) in relation to the values of temperature and relative air humidity in the examined rooms and the Entrance to the building during spring sampling

Koncentracije spora (CFU m^3) na različitim podlogama (MEA, OA, DG18) u odnosu na vrijednosti temperature i relativne vlažnosti zraka ispitivanih prostorija i ulaza u zgradu tijekom proljetnog uzorkovanja



Graph 3.
Relative frequency RF (%) of the presence of genera of cellulolytic fungi during the winter and spring periods

Relativna učestalost (RU) zastupljenosti rodova gljivica pozitivnih na razgradnju celuloze u zimskom i proljetnom razdoblju



Spring: After the incubation of samples collected during spring sampling, 38 Petri dishes showed the growth of mixed fungal cultures, while the other 13 showed no fungal colonies. A total of 81 pure fungal cultures were isolated from all mixed cultures. Spore concentrations (CFU m^3) on different media (MEA, OA, DG18) in relation to the values of temperature and relative air humidity in the examined rooms and the Entrance to the building during winter sampling are shown in Graph 2.

Relative frequency (RF) of cellulolytic fungal isolates

Relative frequencies (RF) of cellulolytic fungal isolates showed that the most abundant species in both seasons belonged to the genera *Aspergillus* and *Penicillium* (Graph 3). It was established that the genera *Trichoderma*, *Talaromyces*, and *Exophiala* were not present during the winter measurement, and that the genus *Rhizopus* was not present in the spring measurement. The highest frequency of cellulolytic fungi during spring and summer sampling was determined in the Basement. During winter sampling, the lowest value of cellulolytic fungi was shown in the X-Party, while during spring sampling, the lowest frequency was found in the Reading Room, X-Party, and Entrance.[6]

CONCLUSION

The highest concentration of fungal spores and conidia during both seasons was recorded in Room ll-2 and the Basement. Presumably, this depends on the purpose of a room, the intensity of its use, inadequate ventilation, or its location on the ground floor or in the basement. During winter and spring samplings, pure cultures were isolated in the highest percentage from the MEA nutrient medium. During both samplings, the largest number of pure cultures was isolated from the Basement and the least from X-Party. Given that there were no major microclimate differences between seasonal measurements, no significant change in the number of isolated cultures was recorded. By carrying out an assay to test cellulolytic activity using the CMC nutrient medium, it was determined that the majority of spores and/or conidia of cellulolytic fungi, which are responsible for the degradation of book matter were present in the State Archive building. Moreover, a higher prevalence of cellulolytic fungi was recorded in the spring period. During both seasons, the highest presence of cellulolytic fungi was recorded in the Basement, and the lowest in X-Party. In the Reading Room, a higher prevalence of cellulolytic fungi was recorded during the winter period, presumably because there were more users and the room was poorly ventilated.

Morphological determination methods showed that the most abundant species in both seasons belonged to the genera *Aspergillus* and *Penicillium*, followed by *Mucor*, *Alternaria*, and *Cladosporium*. Other species were not present in both seasons: the genera *Trichoderma*, *Talaromyces*, and *Exophiala* were absent during the winter season, and the genus *Rhizopus* during the spring measurement. According to previous research, fungal spores belonging to the species *Aspergillus* and *Penicillium* are most often present in closed spaces due to the formation of small-mass conidia that easily separate from the conidiophores, which makes their dispersal through the air easier. The obtained results of this research are in accordance with those of numerous earlier studies.

Exposure to high concentrations of fungal spores is known to adversely affect human health. A number of studies discuss the connection between the occurrence of respiratory problems and the presence of spores of micromycetes belonging to the genera *Penicillium* and *Alternaria*, while exposure to spores of species belonging to the *Aspergillus* genus have been linked to the occurrence of atopic dermatitis. According to our research results, spores and conidia of the mentioned genera of fungi are present in the State Archive building. Therefore, it would be desirable to undertake preventive measures to protect the health of employees and users, as well as valuable archival and library matter.

During this two-year project, air sampling and seasonal monitoring of microclimatic parameters will be continuously conducted in order to determine the causes of possible increased concentration of spores and the sources of contamination in the State Archives building. Based on the research results, it will be possible to work out guidelines for the storage of books in order to prevent further contamination by fungi. Monitoring of biological air contamination is essential for assessing the level of pollution and the potential risk for the preservation of cultural assets, and is the first step to a successful preventive strategy.

NOTES

[1] https://pum.unizd.hr/projekti-odjela/kultbamikroo

[2] Dichloran Glycerol Agar

[3] Oatmeal Agar

[4] Malt Extract Agar

[5] Inofficial names of rooms at the State Archive in Zadar, used for research purposes.

[6] Relative frequency (RF) positive fungal species were determined according to the method of Esquivel et al. (2003).