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SAPROXYLIC INSECTS AND FUNGI IN FORESTS OF THE REPUBLIC OF MOLDOVA

Oana-Alina BOIU-SICUIA[1, 2], Inna RASTIMEŞINA[3], Olga POSTOLACHI[3], Svetlana BACAL[4, 5], Galina BUŞMACHIU[4, 5]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, 011464, Bucharest, Romania
[2]Research-Development Institute for Plant Protection, 8 Ion Ionescu de la Brad Blvd, 013813, Bucharest, Romania
[3]Institute of Microbiology and Biotechnology of Technical University of Moldova, 1 Academiei Street, MD-2028, Chisinau, Republic of Moldova
[4]Moldova State University, Institute of Zoology, 1 Academiei Street, MD-2028, Chisinau, Republic of Moldova
[5]Plaiul Fagului Nature Reserve, Radenii Vechi, Ungheni District, MD 3642, Republic of Moldova

Corresponding author email: svetabacal@yahoo.com

Abstract

Climatic chances, and most precisely extended drought, have a negative impact on forests ecosystems. Such conditions can increase the incidence of pests and diseases in the forests, and change the behavior of saproxylic insects and fungi, which migrate from the dead wood to the stressed trees. The aim of this study is to reveal the fungal species associated with saproxylic insects collected from debilitated trees found in the strictly protected area of the Plaiul Fagului Nature Reserve. A total of 21 fungal strains were isolated from the insects' body of coleopteran species Platypus cylindrus, Scolytus carpini, Stereocorynes truncorum and Xyleborus monographus collected from Quercus petraea trees - edifier of the European-type natural forests in the Republic of Moldova. This study is the first one describing the fungal diversity associated with saproxylic insects in the Republic of Moldova.

Key words*: saproxylic insects, fungal diversity, natural forests, pest, diseases.*

INTRODUCTION

In recent years, in the Republic of Moldova, forests are affected by long-term droughts. This abiotic stress triggered the attack of saproxylic pests with xylophagous appetite. Infested trees dry out within a few years and show fine sawdust on their stem, holes of various sizes and shapes, and oozing sap. Moreover, sections cut into the wood show also fungal infections.

To our knowledge, there are no studies regarding the relationships between saproxylic coleopterans and wood associated fungi, in the Republic of Moldova. However, many variables can be considered. For instance, there could be no direct interactions between such pests and fungi. The xylophagous insects could only create holes within the living trees that simplify the entering of the fungi inside the wood (Termorshuizen, 2016). Therefore, the saprophytic fungi can switch either to a pathogenic behavior after interring into debilitated trees, or they can establish as endophytic colonizers inside the wood.

There could also be other suppositions, such as a commensal relationship, where the fungi are randomly transported by the insect (Tiberi et al., 2002). However, there is not excluded that some xylophagous insects could associate themselves with various types of fungi able to degrade the wood, this way providing for the insects those nutrients and growth factors they need. While in return, the fungus benefits from the transport to the food source (Tiberi et al., 2016). Moreover, some fungi could be used by the insects, also as an alternative feeding resource. Such saproxylic insects are known to be mycetophagous (Olenici and Fodor, 2021).

To contribute with knowledge to this field, the current study was focused on analyzing the

fungal microbiota found to be transported by saproxylic insects collected from Moldavian forests. This study is the first to our knowledge identifying wood-inhabiting fungi associated with saproxylic insects.

MATERIALS AND METHODS

Sampling

In between 2022 and 2023 xylophagous beetles were collected from Plaiul Fagului Scientific Reserve, Republic of Moldova. Trunk traps were used to collect these beetles. To reveal if these saproxylic coleopteran species are potential vectors for fungal transmission certain laboratory procedures of analysis were performed.

Fungal isolation

The collected saproxylic beetles were brought to laboratory and cleaned of impurities by washing with sterile saline solution. The insects were then placed in sterile 1.5 ml microtubes, macerated for 1 hour in 1 ml of sterile distilled water, ground and vortexed. After infusion, decimal dilutions were prepared and inoculated on malt extract agar medium (MEA) (Millipore). The plates were incubated for approximately 10 days at 25 to 26°C, and periodically examined. Isolates were than purified on MEA, and stored in the Laboratory of Soil Microbiology (LSM), of the Institute of Microbiology and Biotechnology, of Technical University of Moldova.

Molecular identification of fungi

Fresh fungal cultures were used for genomic DNA extraction. The fungal biomass, ~ 100 mg, was aseptically transferred in sterile screw cap tubes and mechanically lysed by bead beating for 90 seconds, using a Mini Bead Beater-8 homogenizer (BioSpec, Bartlesville, OK, USA) in order to improve DNA extraction. The fungal DNA was extracted and purified using the ZR Fungal/Bacterial MiniPrep™ commercial kit (ZymoResearch, SUA) according to the manufacturer instructions. The DNA quantity and purity were determined using the SpectraMax® QuickDrop™ Micro-Volume Spectrophotometer (Molecular Devices, San Jose, CA, USA).

The resulting DNA was used as template in PCR reaction for the ITS1-5.8S-ITS4 region amplification. In the PCR reaction ITS1: 5'-TCC GTA GGT GAA CCT GCG G -3' (Invitrogen) and ITS4: 5'- TCC TCC GCT TAT TGA TAT GC -3' (Invitrogen) primers were used. The PCR was performed in a 50 µl reaction volume, with ~ 10 ng of template DNA. The PCR mix contained 1X Green Buffer with $MgCl_2$ included, 0.2 mM dNTPs (Thermo-Scientific, Baltics, UAB, Vilnius, Lithuania), 0.5 µM of each primer, and 0.2 U of DreamTaq DNA Polymerase (Thermo-Scientific, Baltics, UAB, Vilnius, Lithuania), all mixed in sterile MilliQ water. The amplification program was set up in 3 steps, an initial denaturation step of 4 min at 94°C, followed by a 35 cycles step of 1 min at 94°C for denaturation, 1 min at 45°C for primers' annealing, 2 min at 72°C for elongation, followed by a last step of 10 min at 72°C for the final elongation. The PCR products were revealed through agarose gel electrophoresis.

The PCR products were migrated in 1% gel electrophoresis with ethidium bromide in 0.5X TBE buffer, in comparison to a 100 bp DNA ladder (ThermoScientific, Baltics, UAB, Vilnius, Lithuania) used to estimate the molecular weight of the PCR products. The electrophoretic profiles were visualized under UV light using the BioDoc-It Imaging System (Ultra-Violet Products Ltd., Upland, CA, USA). The PCR products were then sent for purification and paired-end sequencing, to CeMIA (Cellular and Molecular Immunological Applications, Greece). The Sanger dideoxy sequencing method was used for analysis. The partial sequences obtained with the forward and reverse primers were aligned using the BioEdit program. The assembled sequences were subjected to the online NBLAST software for taxonomic identification. Based on the sequences similarities with other microorganisms found in the National Center for Biotechnology Information (NCBI) database, the analyzed samples were identified at specie or genus level. The accurate partial sequences of the ITS1-5.8S-ITS4 region were submitted to the GenBank online database of the National Institutes of Health (NIH), which is available to the public access.

RESULTS AND DISCUSSIONS

A total of 21 fungal strains were isolated from the insects' body of *Platypus cylindrus* (Fabricius, 1972), *Scolytus carpini* (Ratzeburg, 1837), *Stereocorynes truncorum* (E.F.Germar, 1823) and *Xyleborus monographus* (Fabricius, 1972) collected from *Quercus petraea* trees.
The purified strains were included in the National Collection of Nonpathogenic Microorganisms (CNMN) of the Institute of Microbiology and Biotechnology, of Technical University of Moldova, were they received a collection number (Table 1).

Table 1. Saproxylic beetle transmitted fungi

	Saproxylic coleopteran species			
Fungal strain	***P. c.***	***S. c.***	***S. t.***	***X. m.***
LP-CNMN-01			+	+
LP-CNMN-02		+	+	
LP-CNMN-03	+	+		+
LP-CNMN-04		+		+
LP-CNMN-05		+	+	
LP-CX-09			+	
LP-CNMN-10		+		
LP-CNMN-11		+		
LP-CNMN-12		+		
LP-CNMN-13			+	
LP-CNMN-14			+	
LP-CNMN-15	+			
LP-CNMN-16	+			
LP-CNMN-17	+			
LP-CNMN-18				+
LP-CNMN-19				+
LP-CX-20				+
LP-CX-21	+			
LP-CNMN-22			+	
LP-CX-23				+
LP-CNMN-24			+	
Total transmitted fungi	**5**	**7**	**8**	**7**
Legend: *P. c.* is *Platypus cylindrus*, *S. c.* is *Scolytus carpini*, *S. t.* is *Stereocorynes truncorum*, while *X. m.* is *Xyleborus monographus.*				

Some fungal strains were present in more than one coleopteran species (Table 1). This is the case where similar morphological fungi were isolated from insects collected from the same tree.
From each of the 21 purified fungal strains subjected to molecular identification was amplified the ITS1-5.8S-ITS2 region. The resulting amplification product was subjected to Sanger dideoxy sequencing. After assembling the paired-end sequencing results, most of the fungi were identified at species level (18 strains). However, 3 of the strains, *Lophiostoma* sp. LP-CX-09, *Myrmecridium* sp. LP-CX-20 and *Cladosporium* sp. LP-CX-23, were identified only to Genus level.
The sequences of the ITS1-5.8S-ITS2 region, for each strain, are currently available on the open access GenBank on-line database, based on their accession number (Table 2).

Table 2. Fungal strains identification

Fungal strain	**Molecular identification**	**Sequence length**	**GenBank accession number**
LP-CNMN-01	*Alternaria infectoria*	576 bp	PP351369.1
LP-CNMN-02	*Cladosporium cladosporioides*	543 bp	PP351370.1
LP-CNMN-03	*Cladosporium herbarum*	540 bp	PP351371.1
LP-CNMN-04	*C. herbarum*	541 bp	PP351372.1
LP-CNMN-05	*Alternaria alternata*	561 bp	PP351373.1
LP-CX-09	*Lophiostoma* sp.	526 bp	PP351377.1
LP-CNMN-10	*Acrodontium salmoneum*	333 bp	PP351378.1
LP-CNMN-11	*Metschnikowia pulcherrima*	371 bp	PP351379.1
LP-CNMN-12	*A. alternata*	560 bp	PP351380.1
LP-CNMN-13	*Alternaria tenuissima*	550 bp	PP351381.1
LP-CNMN-14	*Penicillium citreonigrum*	564 bp	PP351382.1
LP-CNMN-15	*Peniophora cinerea*	584 bp	PP351383.1
LP-CNMN-16	*Sarocladium bacillisporum*	535 bp	PP351384.1
LP-CNMN-17	*Aureobasidium pullulans*	599 bp	PP351385.1
LP-CNMN-18	*A. pullulans*	563 bp	PP351386.1
LP-CNMN-19	*Filobasidium magnum*	609 bp	PP351387.1
LP-CX-20	*Myrmecridium* sp.	548 bp	PP351388.1
LP-CX-21	*Botrytis cinerea*	521 bp	PP351389.1
LP-CNMN-22	*Fomes fomentarius*	625 bp	PP351390.1
LP-CX-23	*Cladosporium* sp.	543 bp	PP351391.1
LP-CNMN-24	*A. alternata*	562 bp	PP351392.1

According to the sequencing results, LP-CNMN-01 strain was attributed to *Alternaria infectoria*. This mold species is ubiquitous in various ecosystems, and also has human pathogenicity potential (Silva et al., 2014). Various metabolites can be produced by *A. infectoria*, but the pyranones compounds, novae-zelandins A and B, 4Z-infectopyrone and infectopyrone, are considered chemotaxonomic

markers for this species and related *Alternaria* spp. molds (Lou et al., 2013).

The LP-CNMN-05, LP-CNMN-12 and LP-CNMN-24 strains were identified as *Alternaria alternata*, while LP-CNMN-13 was attributed to *Alternaria tenuissima*. DiGirolomo et al. (2020) have also isolated such fungal species from Nitidulidae beetles. They isolated *A. alternata* from *Glischrochilus sanguinolentus* and *Carpophilus corticinus*, while *A. tenuissima* was isolated from *Glischrochilus fasciatus* (DiGirolomo et al., 2020). Both of these fungi are able to grow endophytically. However, they could switch to a pathogenic behavior, creating phytosanitary problems to numerous herbaceous and woody plant species. *A. alternata* can also develop as saprophyte on dead wood, or as animals' pathogen (DiGirolomo et al., 2020).

Cladosporium spp. fungi, were also identified among the isolated fungi. LP-CNMN-02 strain was identified as *Cladosporium cladosporioides*, while LP-CNMN-03 and LP-CNMN-04 as *Cladosporium herbarum*. These mold species are known as endophytic or saprotrophic on woody substrate, but some can cause infections in plants, animals or they can parasitize lichens (DiGirolomo et al., 2020). Potential transmission of *Cladosporium* spp. fungi by insects of the Nitidulidae family is also sustained by DiGirolomo et al. (2020), which isolated *C. cladosporioides*, *C. pseudocladosporioides*, *C. ramotenellum* and other unidentified *Cladosporium* spp. from the insects' body (DiGirolomo et al., 2020). Moreover, different species of *Cladosporium* spp., including *C. cladosporioides* and *C. herbarum*, are normal to be found on decaying oak wood (Behnke-Borowczyk et al., 2018; Weatherhead, 2021). Robert Jankowiak (2008) mentions the presence of *C. cladosporioides* mold in association with oak wood of *Quercus robur*, infested with larvae of *Curculio glandium* and *Kenneliola* spp. But *C. cladosporioides* is also mentioned among the molds found on *Quercus petraea* oak wood, in decaying stage 1 (logs lying on the ground for 1-3 years) and stage 2 (logs lying on the ground for 5-20 years) (Behnke-Borowczy et al., 2018).

As the *C. herbarum* LP-CNMN-03 and LP-CNMN-04 strains revealed some sequence similarities of high query cover and identity percent, within the ITS1-5,8S-ITS2 region, with some strains of *C. allicinum* and *C. sinuosum*, supplementary information were searched about these molds. Thus, in the United States of America, more specifically in Utah, such species of endophytic fungi were isolated and identified in *Quercus gambelii* oak leaves. *C. herbarum, C. sinuosum*, and one isolate of unidentified *Cladosporium* sp. were found to be saprotrophic on oak wood (Weatherhead, 2021). Moreover, molecular genetics studies reported that *C. allicinum*, *C. herbarum* and *C. sinuosum* are conspecific within the *C. herbarum* complex (Bensch et al., 2012).

Due to the low number of DNA sequences related to the ITS1-5,8S-ITS2 region for *Lophiostoma* genus, within the GeneBank database, the LP-CX-09 strain could not be identified to species level. *Lophiostoma corticola* and other *Lophiostoma* sp. were found among the *Quercus robur* colonizers, in the 10 to 50-year-old oak trunks wounds (Marčiulynas et al., 2023).

LP-CNMN-10 strain was identified as *Acrodontium salmoneum*. This species is mentioned in seeds, on mites, in caves air and soil (Kubátová et al., 2001), or in rooms with structural dampness (www.moldguy.ca).

Yeasts were also isolated within this study. The LP-CNMN-11 strain was identified as *Metschnikowia pulcherrima*. The presence of *Metschnikowia* spp. yeasts on decaying wood is mentioned by numerous research groups (Wang et al., 2009, 2010; Guo et al., 2012, Hui et al., 2013).

LP-CNMN-16 strain was affiliated to *Sarocladium bacillisporum* species. High similarity within the ITS1-5.8S-ITS2 region sequence was obtained between this strains and various references. A 99% query cover and 100% identity were seen with the CBS 388.67 reference strain isolated from the Netherlands soil; a 99.81% identity was obtained with the IMI 113161 (syn. CBS 425.67) reference strain isolated from the soil of deciduous forests in Ontario, Canada; and a 99.63% identity was found with the CBS 212.79 reference strain isolated in Romania from insects. Numerous species of *Sarocladium* are reported to be

present in plant tissues or decaying plant material (Giraldo et al., 2015).

Strains no. LP-CNMN-17 and LP-CNMN-18 were both identified as *Aureobasidium pullulans.* However, they show different growth morphology. This is not surprising, and can be explained by the dimorphism found in this species (Reeslev et al., 1993). The *A. pullulans* LP-CNMN-17 strain show a yeast-like growth, whereas LP-CNMN-18 strain reveals a brown, filamentous growth.

Behnke-Borowczy et al. (2018) also reported species of yeasts and molds isolated from decaying oak wood in various stages of decay. Among the mutual identified species there are *Aureobasidium pullulans* (LP-CNMN-17 and LP-CNMN-18 strains from our study), *Botrytis cinerea* (LP-CX-21), *Filobasidium* sp. (*F. magnum* LP-CNMN-19), *Penicillium citreonigrum* (LP-CNMN-14), and *Peniophora* sp. (*P. cienerea* LP-CNMN-15), respectively.

CONCLUSIONS

This study is the first one describing the fungal diversity associated with saproxylic insects in the Republic of Moldova. Identifying the spectrum of fungi isolated from the saproxylic beetles collected from Moldavian forests, it can be concluded that the obtained results are sustained by scientific data revealed by other research groups in the field.

Platypus cylindrus, commonly known as the oak pinhole borer, was found to carry *Botrytis cinerea*, *Cladosporium herbarum*, *Peniophora cinerea*, *Sarocladium bacillisporum* moulds and *Aureobasidium pullulans* yeast.

Scolytus carpini bark beetle transported *Acrodontium salmoneum, Alternaria alternata, Cladosporium cladosporioides, C. herbarum* moulds and *Metschnikowia pulcherrima* yeast.

From the *Stereocorynes truncorum* pest beetle were isolated *Alternaria alternata, A. infectoria, A. tenuissima, Cladosporium cladosporioides, Fomes fomentarius, Lophiostoma* sp. and *Penicillium citreonigrum* moulds.

On the *Xyleborus monographus* oak borer were found *Alternaria infectoria, Cladosporium* spp., *Aureobasidium pullulans, Filobasidium magnum* and *Myrmecridium* sp. fungi.

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