Supporting Information

The Use of Biocides for the Control of Fungal Outbreaks in Subterranean Environments: The Case of the Lascaux Cave in France

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Materials and Methods

Nucleic acid extraction and amplification

Total nucleic acids from environmental samples (Table 1) were extracted using the method described by Griffiths et al.[1], which allowed the parallel analysis of DNA and RNA. The samples were homogenized in hexadecyl trimethyl ammonium bromide extraction buffer. Extracted nucleic acids were resuspended in 80 µl of sterile ultrapure water. For RNA isolation, a 50 µl aliquot was subjected to a DNase digestion step using an RNase-Free DNase (Quiagen GmbH, Hilden, Germany), and the RNA was purified using the RNeasy MinElute Cleanup kit (Quiagen GmbH, Hilden, Germany), according to the manufacturer's instructions.

Genomic DNA from isolated melanized fungi was extracted by scraping the mycelium from the plates and transferring it to a 1.5 ml Eppendorf tube containing 500 µl lysing buffer and glass beads. The mixture was shaken in a cell disrupter Fast Prep-24 (MP Biomedicals, Solon, OH, USA) at full speed for 3 min. The DNA was purified by phenol/chloroform extraction and ethanol precipitation.

Fungal ITS regions (ITS1, 5.8S rDNA and ITS2) from the environmental samples and the isolated strains were PCR-amplified using the primers ITS1 and ITS4.[2] PCR reactions were performed in a BioRad iCycler thermal cycler (BioRad, Hercules, CA, USA). The PCR reaction mixture (50 µl) consisted of 5 µl 10X PCR buffer, 2 µl $MgCl_2$ (50 mM), 0.5 µl of each primer (50 µM), 5 µl dNTP (2 mM each), 10-20 ng DNA template, 0.25 µl Taq polymerase (5 U/µl) (Bioline, GC Biotech, The Netherlands) and 34.75 µl sterile ultrapure water. Cycling parameters were 94ºC for 2 min, followed by 35 cycles of 94ºC for 1 min, 50ºC for 1 min, and 72ºC for 1 min with a final extension at 72ºC for 5 min. Reactions were performed in duplicate, and negative controls (containing no DNA) were included in each PCR reaction. All amplification products were electrophoresed on 1.5% (w/v) agarose gels, stained with SYBR Green I (Roche Diagnostics, Mannheim, Germany) and visualized under UV light.

For RNA analysis, cDNA of ITS regions was synthesized from 4 µl of purified RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and the ITS4 primer according to Anderson and Parkin.[3] Control reactions containing no Superscript II were performed for each sample along with one reaction containing no RNA. RT-PCR was performed with the ITS1 and ITS4 primers, using 1-2 µl of cDNA diluted 1:10 as a template. PCR conditions were the same as for DNA. An additional negative control containing no cDNA was also included in each RT-PCR. All amplification products were purified with a JETquick PCR Purification Spin kit (Genomed, Löhne, Germany) and stored at -20ºC for later analysis.

One µl of purified PCR products from each environmental sample was used as a template in a nested PCR to produce products for DGGE analysis using the primers ITS1-GC (containing a GC clamp on its 5′ end) and ITS2.[2] The cycling conditions were previously described by Michaelsen et al.[4] Before DGGE, PCR products were analyzed by agarose gel electrophoresis as described above.

Clone libraries

ITS clone libraries were constructed to analyze the community structure of five selected environmental samples (Table 1). The fungal species present (DNA) and the metabolically active species (RNA) were independently analyzed in three samples. The purified ITS-PCR products were ligated into the pGEM-T Easy Vector (Promega, Milan, Italy) and then transformed into One Shot Max Efficiency DH5α-T1 Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA). Transformants were randomly selected by plating onto Luria Bertani (LB) ampicillin (100 µg/ml) agar plates with isopropyl-ß-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-d-galactopyranoside, and incubated at 37ºC overnight. Selected library clones (white colonies) were PCR-screened directly for the presence of inserts using the T7-promoter and pUC/M13-reverse primers; 1 µl of colonies suspended in sterile ultrapure water was used as template. Clones with inserts were grown in LB ampicillin broth at 37ºC overnight and preserved in 40% w/v sterile

glycerol. Fifty µl aliquots were stored at -80ºC and similar aliquots were sent to Macrogen Inc. (Seoul, Korea) for plasmid purification and sequencing using the same primer set.

The ITS sequences from the pool of inserts were manually checked, and low quality and very short sequences were removed. The presence of chimeras was tested using the Chimera Test application provided by the Fungal Metagenomics Project at the University of Alaska, Fairbanks (http://www.borealfungi.uaf.edu), which compares separate BLAST searches using the ITS1 versus ITS2 regions of each clone sequence. Putative chimeric sequences were excluded from further analysis. The sequences were aligned using MUSCLE,[5] and the respective distance matrices were generated by PHYLIP.[6] The resulting matrices were used as input for DOTUR[7] in order to assign sequences to Operational Taxonomic Units (OTUs) using the furthest neighbor algorithm at different evolutionary distances (0-10 %) and to generate the respective rarefaction curves. For each OTU, one clone was chosen as a representative sequence. A similarity search was performed using the NCBI BLASTn algorithm for identification (National Centre for Biotechnology Information, (http://www.ncbi.nlm.nih.gov/). The results were compared with those provided by the BLAST search on the Fungal Metagenomics Project's website as detailed above.

Denaturing gradient gel electrophoresis (DGGE) analysis

For the community fingerprint of all environmental samples detailed in Table 1, 20 µl of PCR products amplified using the primers ITS1-GC and ITS2 (containing the ITS1 region) were analyzed by DGGE. Gel electrophoresis was performed as previously described by Muyzer et al.[8] in 0.5X TAE (20mM Tris, 10mM acetate, 0.5mM Na_2EDTA; pH 7.8) with 8% (w/v) acrylamide gels containing a gradient of denaturants of 25-45%. The gels were run in a D-Code System (BioRad, Hercules, CA, USA) for 16h in TAE 0.5X buffer at 60ºC and constant voltage of 100 V. After electrophoresis, gels were stained in an ethidium bromide solution and visualized under UV light.

Markers containing the PCR products (primers ITS1-GC/ITS2) from seven different fungal strains frequently isolated from the Lascaux Cave were designed to allow comparative analysis of DGGE patterns from different gels, and as a quality control for the gel run. The reference species used were: *Exophila* sp., *Geomyces pannorum*, *Cladosporium* sp., *Cosmospora* sp., *Penicillium* sp., *Acremonium nepalense* and *Ochroconis lascauxensis*. For identification of bands in the community fingerprint from the five cloned environmental samples (Table 1), representative clones of the major OTUs were included in the DGGE analysis. Some additional interesting bands were excised from the DGGE gels for identification. DNA from these bands was extracted using the QIAEX II Kit (Quiagen GmbH, Hilden, Germany), and re-amplified using the primers ITS1/ITS2 with the same conditions as ITS1/ITS4. The sequences of the excised bands were obtained and analyzed as described above for identification of fungal strains. To elucidate identification of short sequences from DGGE bands, we compared these sequences with those from isolated strains and representative clones of OTUs.

Isolation and identification of melanized fungi

Dematiaceous fungi were isolated from the four samples collected in February 2011 (Table 1). They were homogenized in a sterile solution of Tween 80 (0.01%), and four tenfold serial dilutions (10^{-1}-10^{-4}) were performed. Aliquots of 100 µl from each dilution were plated on selective culture media, dichloran rose bengal chloramphenicol agar (DRBCA) and erythritol chloramphenicol agar (ECA). The dishes were incubated at 15ºC for two months to allow the development of slow-growing melanized colonies. The pure cultures were stored at 5ºC for further study. For estimation of morphological characters, the selected strains were cultivated at 25ºC in the dark for 40 days on malt extract agar (MEA) and potato dextrose agar (PDA), and optical microscopy was performed. Molecular identification of strains was based on the analysis of internal transcribed spacer regions (ITS) sequences from rDNA. DNA extraction and amplification protocols are described above. Purified PCR products were sequenced in both directions by Secugen (CSIC,

Madrid, Spain) using an ABI 3700 sequencer (Applied Biosystems). The sequences were aligned and edited using BIOEDIT 7.0.5.3 and a similarity search was performed using the NCBI BLASTn algorithm for identification (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

Phylogenetic relationships

Phylogenetic relationships between isolated strains and representative OTUs belonging to Herpotrichiellaceae family, which includes the main black yeast genera, were estimated. The closest related sequences from GenBank were included in this study. The analyses were conducted using MEGA 5.0[9] with the Neighbor-Joining method.[10] Gaps were treated as missing data, the Kimura 2 parameter substitution model was used, and bootstrap values were generated using 1000 replicates. Additionally, the resulting topology was compared with results from other treeing algorithms, including the Maximum-Likelihood and Maximum-Parsimony methods.

Use of aromatic compounds as carbon sources

Three aromatic compounds were used: vanillic acid, sodium *p*-toluene sulfonate and toluene for evaluating the use of possible carbon sources present in the cave. Different fungal strains were cultured in dishes containing Czapek-Dox agar without a carbon source, with sucrose or with one of the aromatic compounds (vanillic acid or sodium *p*-toluene sulfonate). Three different concentrations were used: 0.01, 0.05 and 0.25 % carbon source. For toluene, the method described by Zhao et al.[11] was adopted. Petri dishes were incubated at 22ºC for 40 days. Relative growth was estimated in relation to the negative control (without a carbon source).

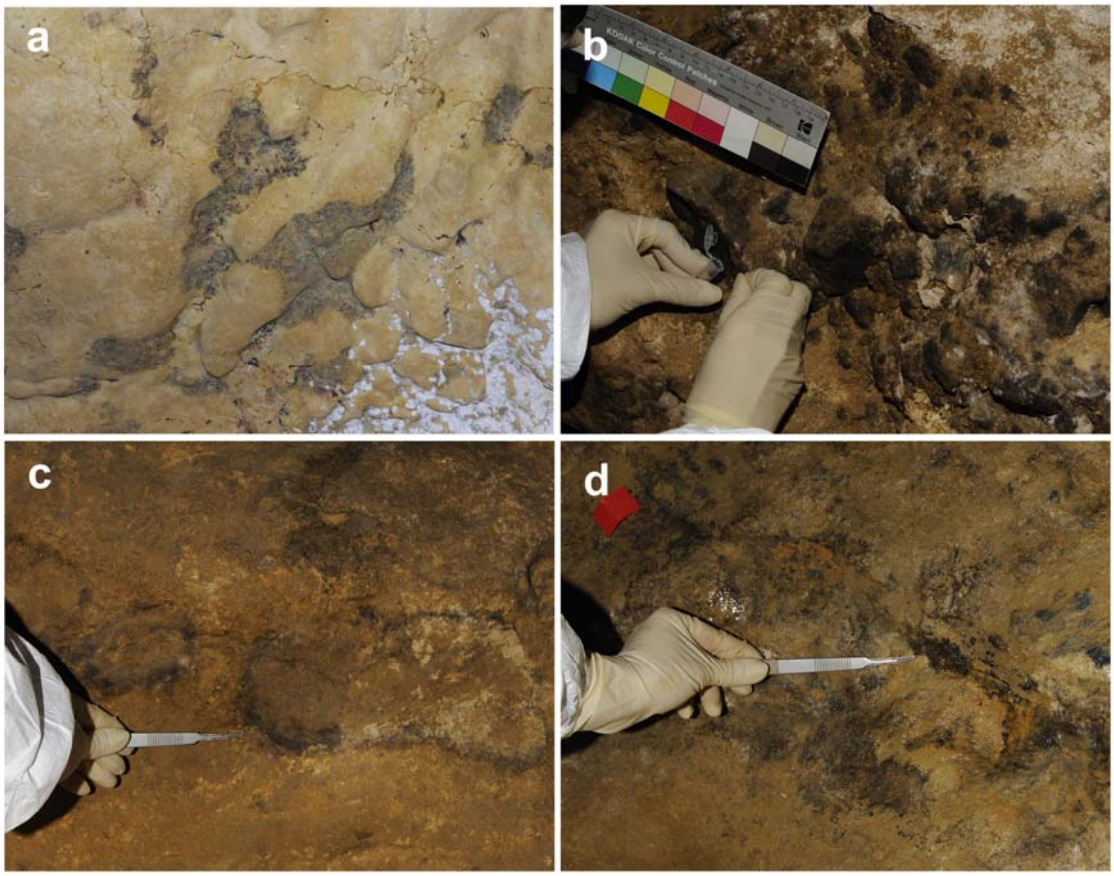

Figure S1. Black stains samples used in the study of fungal communities by the construction of clone libraries. **a.** Sample C08 in the Passageway-left side, before cleaning and biocide applications. **b**. Sample M1 in the Passageway-right side. **c**. Sample M6 in the Apse, forming a circular ring. **D**. Sample M8 in the Great Hall of Bulls, an area with constant humidity.

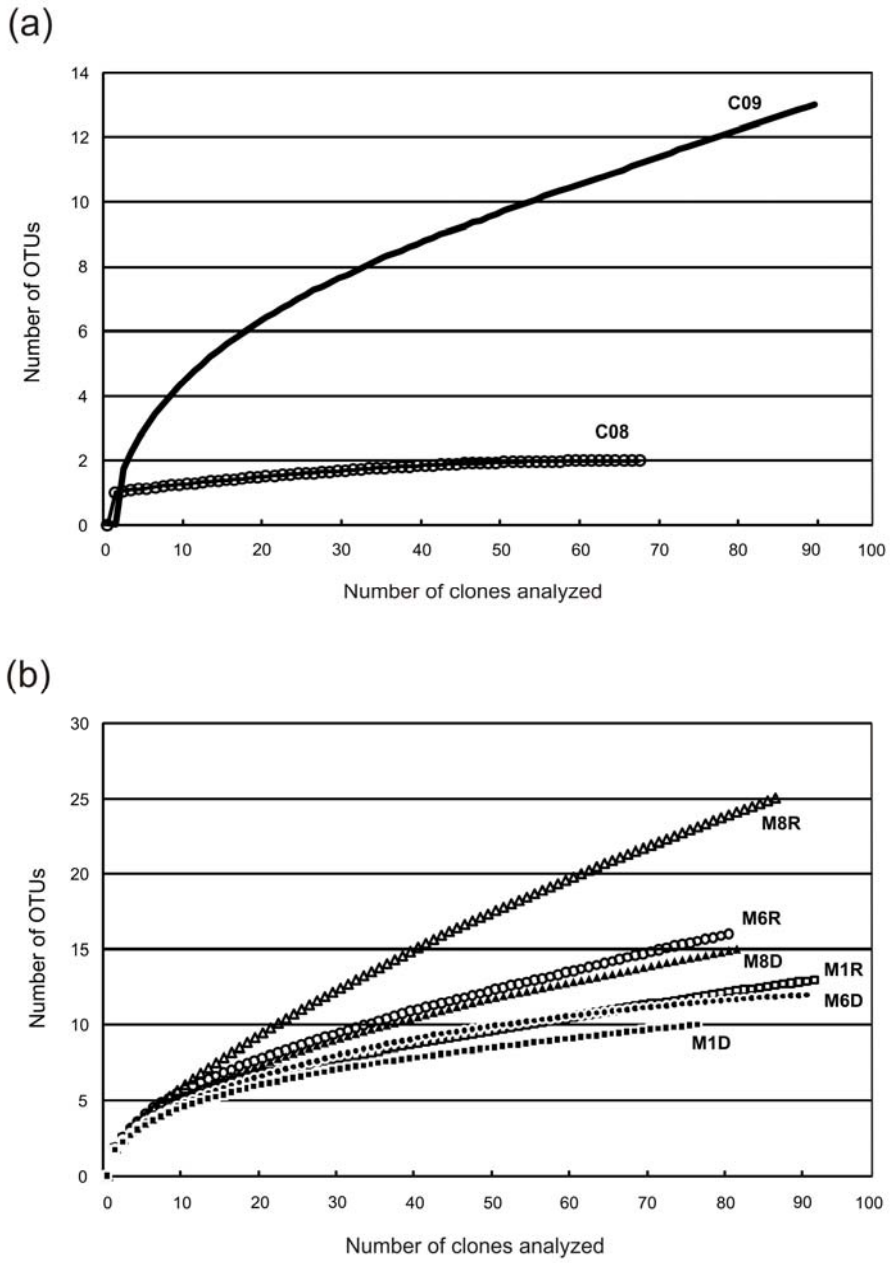

Figure S2. Rarefaction curves using ITS sequences from cloned environmental samples collected in 2008-09 (**a**) and 2010 (**b**). Curves have been calculated at evolutionary distance of 5%.

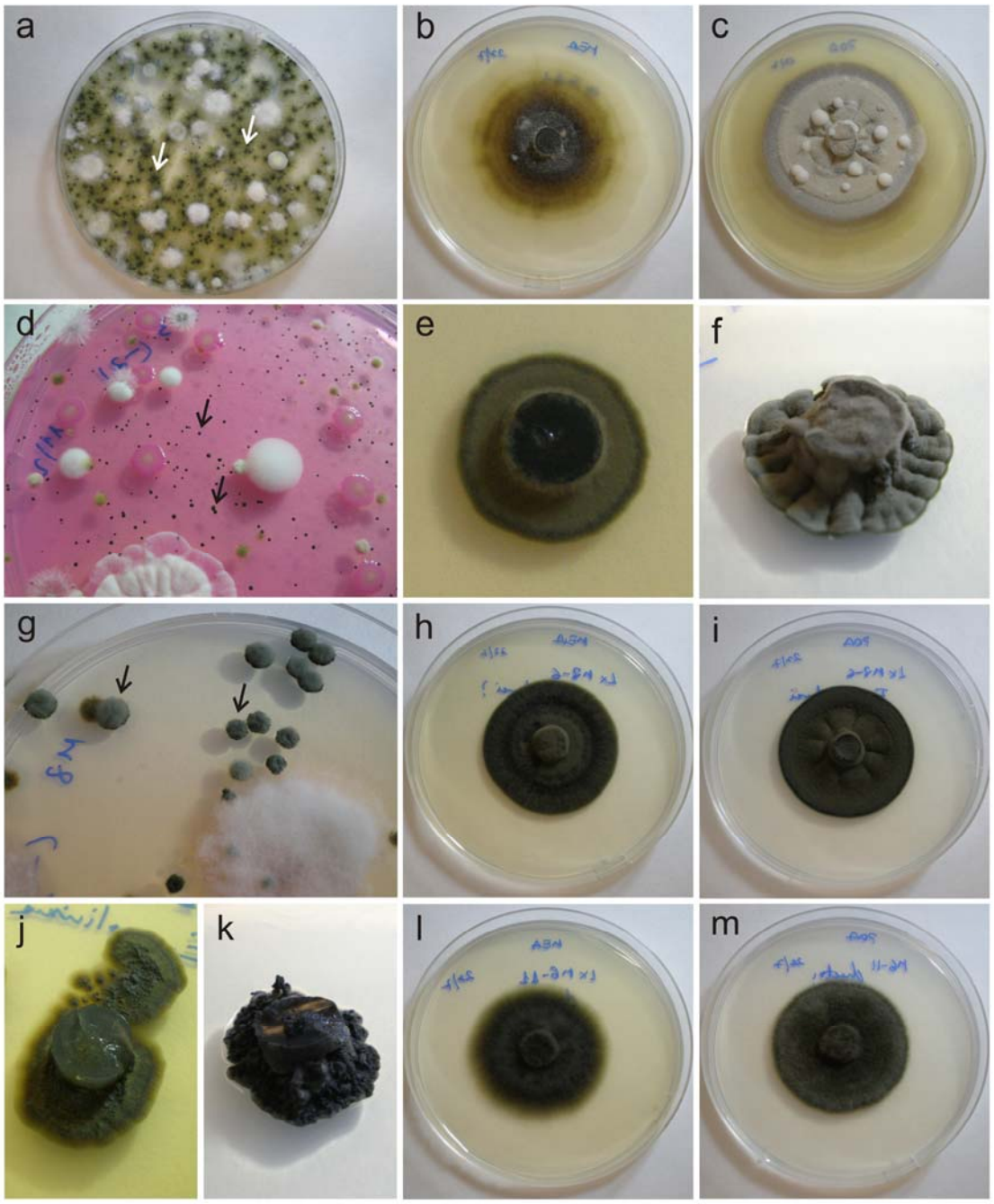

Figure S3. Isolation of melanized fungi and their colony morphology. **a**. *Acremonium nepalense* colonies grown on ECA at 15ºC for 25 days, isolated from the M6 black stain. Colonies of *A. nepalense*, grown on MEA (**b**) and PDA (**c**) at 25ºC for 40 days. **d**. *Exophiala castellanii* colonies grown on DCRBA at 15ºC for 10 days, isolated from the M3 pink-violet stain. Colonies of *E. castellanii*, grown on MEA (**e**) and PDA (**f**) at 25ºC for 40 days. **g**. *Exophiala* sp. colonies grown on ECA at 15ºC for 45 days, isolated from the M8 black stain. Colonies of *Exophiala* sp., grown on MEA (**h**) and PDA (**i**) at 25ºC for 40 days. Colonies of *Exophiala moniliae* (**j** and **k**) and Herpotrichiellaceae sp. (**l** and **m**) grown on MEA and PDA, respectively, at 25ºC for 40 days.

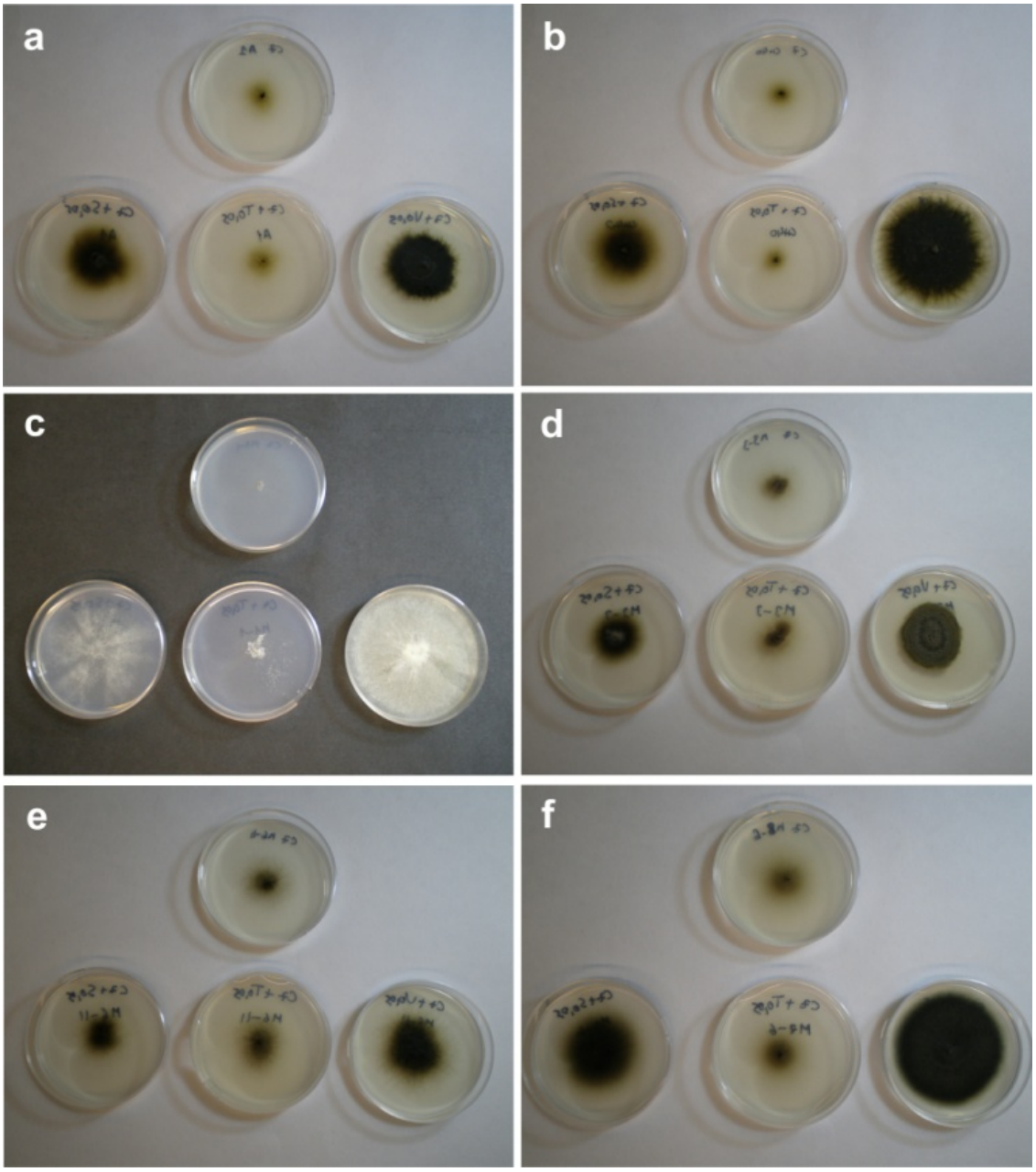

Figure S4. Use of carbon sources by selected strains. **a**. *Ochroconis lascauxensis.* **b**. *Ochroconis anomala*. **c**. *Acremonium nepalense*. **d**. *Exophiala castellanii*. **e**. Herpotrichillaceae sp. **f**. *Exophiala* sp. Position of the Petri dishes: negative control, no carbon source (top), positive control with sucrose (bottom-left), sodium *p*-toluene sulfonate (bottom-center), vanillic acid (bottom-right). All tested compounds provided identical concentration of carbon to the culture medium (equivalent to sucrose 0.05%).

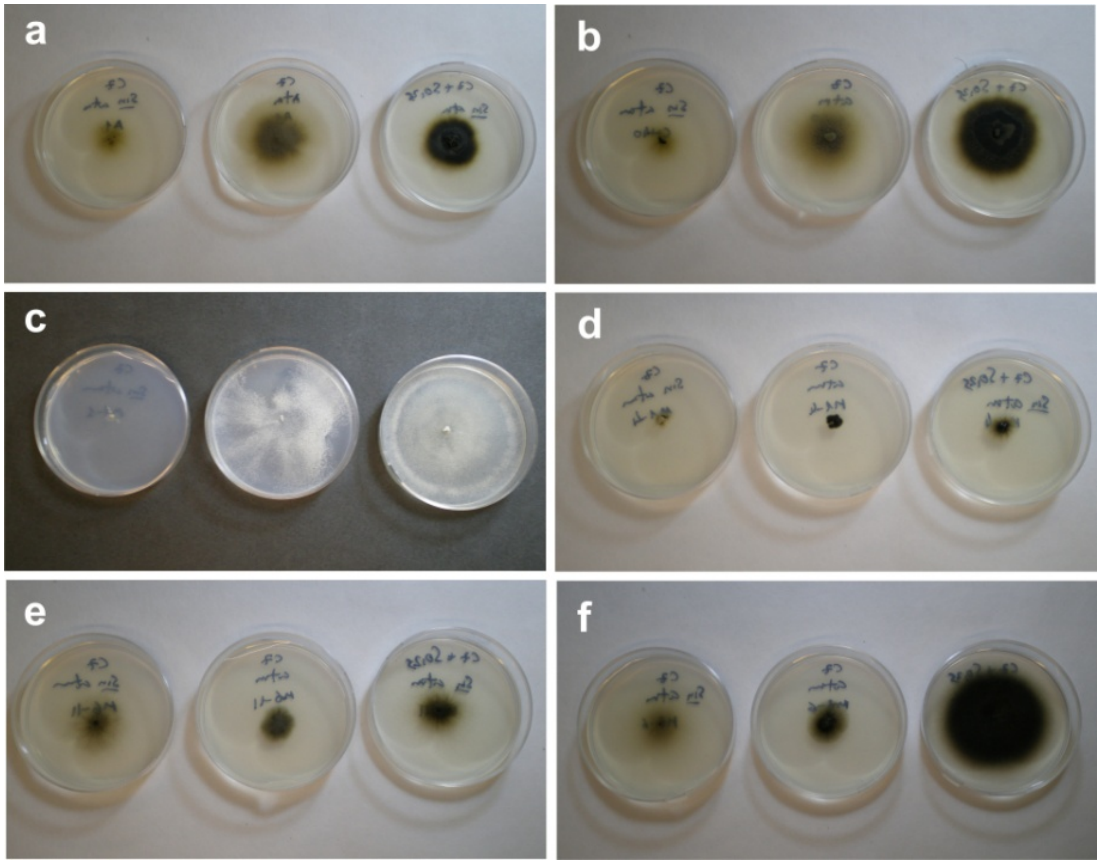

Figure S5. Use of toluene as carbon source by selected strains: **a**. *Ochoroconis lascauxensis*. **b**. *Ochroconis anomala*. **c**. *Acremonium nepalense*. **d**. *Exophiala moniliae*. **e**. Herpotrichillaceae sp. **f**. *Exophiala* sp. Position of Petri dishes: negative control, no carbon source (left), toluene saturated atmosphere (center), positive control with sucrose (right).

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