Mycogenic minerals formation by airborne *Aspergilli* and *Penicillia*

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Received: 02-06-2021 Accepted: 31-08-2021
Published: 15-09-2021

ABSTRACT
Formation of secondary mycogenic minerals, a phenomenon often reported on artworks, is heavily associated with both structural and aesthetic alterations of affected masterpieces. The aim of this study was to investigate capabilities of airborne *Aspergillus* and *Penicillium* species, common contaminants of works of art, to induce mineral formation *in vitro*. For this purpose, tested fungal isolates were cultivated on B4 medium, and optical microscopy and SEM-EDS techniques were applied to observe the morphology of crystals and to discern their chemical composition. Out of 34 isolates in total, mineral formation was documented in 14 *Aspergilli* and 15 *Penicillia* species.The predominant crystal in the investigated samples was calcium oxalate, while calcium carbonate crystals were only seldom reported. Biogenesis of secondary mycogenic minerals is probably due to the ability of investigated isolates to excrete different acidic metabolites into the substrata.Investigations of biomineralization of autochthonous airborne fungal isolates must not be neglected for adequate protection of stone-made cultural heritage objects.However, little focus is given to this phenomenon, especially concerning formation of oxalates via fungal metabolites.

Keywords: *Aspergillus*, biomineralization, calcium carbonate, calcium oxalate, *Penicillium*.

Formación de minerales micógenos por *Aspergilli* y *Penicillia* aerotransportados

RESUMEN
La formación de minerales micógenos secundarios, fenómeno que a menudo se documenta en las obras de arte, está fuertemente asociado con alteraciones tanto estructurales como estéticas de las obras maestras afectadas. El objetivo de este estudio fue investigar *in vitro* la capacidad de las especies de *Aspergillus* y *Penicillium* transmitidas por el aire, como algunos de los contaminantes más comunes de las obras de arte, para inducir la formación de minerales. Para ello, se cultivaron cepas de hongos en medio B4, y se aplicaron técnicas de Microscopía Óptica y SEM-EDS para observar la morfología de los cristales y aclarar la composición química. De un total de 34 cepas, se documentó la formación de minerales en 14 *Aspergilli* y 15 *Penicillia*. El oxalato de calcio fue predominante en las muestras investigadas, mientras que raras veces se reportan cristales de carbonato de calcio. La biogénesis de minerales micógenos secundarios probablemente se deba a la capacidad de los aislados investigados para excretar diferentes metabolitos ácidos en los sustratos. Los estudios de precipitación mineral de hongos autóctonos transmitidos por el aire no deben ser descuidados para una protección adecuada de los objetos del patrimonio cultural hechos en piedra.

Palabras claves: *Aspergillus*, biomineralización, carbonato cálcico, oxalato cálcico, *Penicillium*

INTRODUCTION

Fungi are omnipresent organisms capable of colonizing all substrata found in the natural environment. Many species have the ability to produce various primary or secondary metabolites that, individually or combined, are responsible for the decay of the colonized material.

Deterioration induced by fungi is nowadays a hot topic, investigated in numerous works with special regard on deterioration caused by pronounced enzymatic activities of fungi [1,2,3,4]. However, biomineralization is still a unsufficiently studied phenomenon, especially concerning the formation of oxalates via fungal metabolites [5]. Calcium oxalates, whewellite and weddellite, are frequently reported on cultural heritage objects and artifacts, and are associated with both structural and aesthetic alterations [6,7,8]. This most frequently occurs on mineral substrata, i.e. on rocks, building materials, and cultural heritage artifacts made from stone [9].

Although studies in this field usually deal with autochthonous isolates from cultural heritage objects, only

a few studies (Pangallo *et al.*, [10] and Unković *et al.*, [5]) have investigated the ability of airborne isolates to precipitate minerals on cultural heritage items. Fungal spores of *Aspergillus* and *Penicillium* species are light, small sized and easily dispersible by air flow [11]. Therefore, they can be easily transmitted to different substrata, with colonization occurring if favorable conditions are present.

Thereupon, the principal aim of this study was to contribute to the present knowledge of fungal induced formation of minerals by investigating on a collection of *Aspergillus* and *Penicillium* species, common airborne fungi, which frequently colonize various works of art.

MATERIALS AND METHODS

Tested fungi.

Airborne fungal isolates (34 in total) of *Aspergillus* and *Penicillium*/*Talaromyces* genera were selected from the Mycotheca of the University of Belgrade - Faculty of Biology (BEOFB) (table 1).

Tested fungi were previously isolated from indoor air of cultural heritage conservation premises in Belgrade and identified based on macromorphological characteristics of 7 days old colonies observed via stereomicroscope (Stemi DV4, Carl Zeiss, Oberkochen, Germany), and micromorphology of reproductive structures observed with microscope Axio Imager M.1 (Carl Zeiss, Oberkochen, Germany) with AxioVision Release 4.6 software.

Additionally, identification was confirmed by *ITS* and *β-tubulin*gene sequencing [3]. All isolates were maintained in cryovials filled with 1.5 ml of 30% glycerol and maintained at -75°C.

Biomineralization plate assay.

To assess *in vitro* biogenesis of secondary mycogenic minerals all selected fungal isolates were inoculated on B4 medium of the following composition (per liter): calcium acetate, 2.5 g; dextrose, 5 g; yeast extract, 4 g; agar, 14 g; 1000 mL dH2O [12,13]. Medium pH was adjusted to 8.0 with 4 M NaOH. Inoculated plates were incubated for 7 days on 25±1°C in thermostat (UE 500, Memmert, Germany).

The assay was performed in triplicate. As a control, the majority of fungal species were inoculated on MEA (malt extract, 40 g; agar, 15 g; 1000 mL dH2O; pH 6.8), with the exception of xerophilles and xerotolerants, which were inoculated on M40Y (sucrose, 400 g; malt extract, 20 g; yeast extract, 5 g; agar, 20 g; 1000 mL dH2O; pH 6.8) media. Incubation of control plates was in the same conditions as described above.

Optical Microscopy.

Mineral precipitation for positive fungal isolates was initially documented using Optical Microscopy. Fragments of medium, with aerial and submerged mycelium, were transferred onto glass slides and immersed in glycerin or stained with Lactophenol Cotton Blue. Formed minerals, and reproductive structures of interest samples, were observed via optical microscope Zeiss Axio Imager M.1 microscope equipped with the AxioVision Release 4.6 software (Carl Zeiss AG, Oberkochen, Germany).

SEM and EDS analyses.

To further assess morphology and determine chemical composition of obtained mineral phases, a Scanning Electron Microscope (JSM-6610LV, JEOL, Tokyo, Japan) coupled with X-max energy dispersive spectrometer (JEOL, Tokyo, Japan) was used. A total of 15 samples were randomly selected and analyzed (Nos. 1, 2, 5, 6, 8, 9, 12, 15, 19, 21, 20, 23, 29, 30, 31). Samples were covered with gold via BALTEC-SCD-005 (Wallruf, Germany) sputter coating instrument. Adequate internal standards were used for the chemical analyses. Obtained chemical analyses of unpolished samples, with detection limit of 0.1% for most elements, can be considered as semi-quantitative.

RESULTS AND DISCUSSION

Formation of secondary mycogenic minerals was observed for majority of tested fungal isolates, i.e. for 14 *Aspergilli* and 15 *Penicillia* (table 1). Different crystal forms, varying in morphology and dimensions were documented using optical microscopy (figures 1 and 2), and confirmed via SEM (figure 3). Two types of chemically and morphologically different components were discerned, calcium carbonate and calcium oxalate. Calcium carbonate forms, represented by irregular spherical and ellipsoidal forms (figure 1i, figure 2k, figure 3c and 3g) and confirmed by chemical composition obtained using EDS, were documented in only three isolates: *Aspergillus flavus*, *A. protuberus* and *Penicillium solitum* (tables 1 and 2).

Table 1. Investigated fungi and their ability to form secondary mycogenic minerals, with mineral chemical composition of selected isolates obtained via SEM-EDS.

No	Fungal isolate	Mineral production
1	*Aspergillus amstelodami* BEOFB3220m	+
2	*Aspergillus calidoustus* BEOFB3220m	+
3	*Aspergillus creber* BEOFB3250m	+
4	*Aspergillus domesticus* BEOFB3270m	-
5	*Aspergillus europaeus* BEOFB382m	O
6	*Aspergillus flavus* BEOFB315m	O, CC
7	*Aspergillus jensenii* BEOFB3200m	+
8	*Aspergillus melleus*BEOFB3180m	O
9	*Aspergillus niger* BEOFB345m	O
10	*Aspergillus penicillioides* BEOFB3190m	-
11	*Aspergillus proliferans* BEOFB3280m	+
12	*Aspergillus protuberus* BEOFB3240m	CC
13	*Aspergillus pseudoglaucus* BEOFB3170m	+
14	*Aspergillus ruber* BEOFB3150m	-
15	*Aspergillus sydowii* BEOFB3142m	O
16	*Aspergillus tabacinus* BEOFB3260m	+
17	*Aspergillus versicolor* BEOFB3133m	+
18	*Penicillium brevicompactum* BEOFB1102m	+
19	*Penicillium canescens* BEOFB11180m	+
20	*Penicillium carneum* BEOFB11150m	O
21	*Penicillium chrysogenum* BEOFB11120m	O
22	*Penicillium citreonigrum* BEOFB11190m	+
23	*Penicillium citrinum* BEOFB11110m	O
24	*Penicillium decumbens* BEOFB11160m	+
25	*Penicillium digitatum* BEOFB1112m	+
26	*Penicillium expansum* BEOFB11130m	+
27	*Penicillium glabrum* BEOFB11100m	+
28	*Penicillium sanguifluum* BEOFB11170m	-
29	*Penicillium solitum* BEOFB1190m	CC
30	*Penicillium ulaiense* BEOFB11140m	O
31	*Penicillium viridicatum* BEOFB11200m	O
32	*Talaromyces amestolkiae* BEOFB2610m	-
33	*Talaromyces sayulitensis* BEOFB2600m	+
34	*Talaromyces verruculosus* BEOFB2620m	+

(+): mineralization present but not analyzed; (-): mineralization absent; (O): oxalate minerals; (CC): calcium carbonate minerals.

Table 2. Mineral composition obtained via EDS of selected fungal isolates (isolate numbers correspond to the ones given in in table 1).

Mineral phase	Calcium oxalate					Calcium carbonate		
Isolate No.	**6**	**8**	**9**	**23**	**30**	**6**	**12**	**29**
C (wt%)	20.2	15.0	18.2	20.3	17.0	12.2	17.7	16.3
O (wt%)	52.1	57.5	55.5	53.9	60.2	46.3	47.7	31.2
Ca (wt%)	27.7	27.5	26.3	25.7	22.8	40.9	34.6	52.5
Total (wt%)	100	100	100	100	100	100	100	100

wt% - total weight percentage

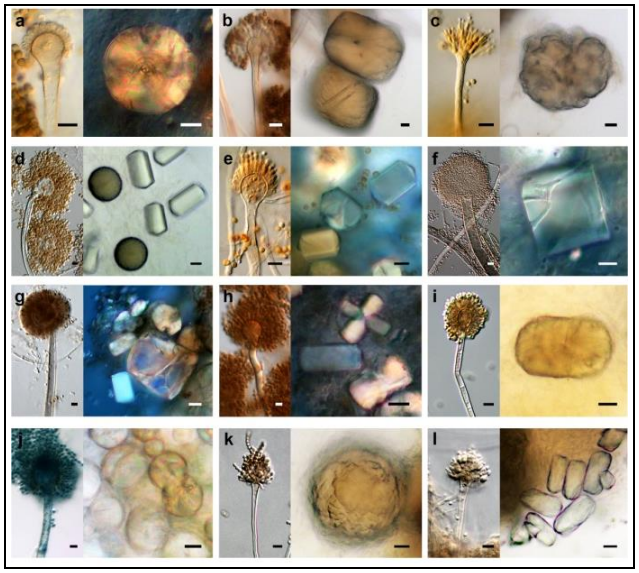

Fig. 1. Optical Microscopy of conidial apparatus (left) and mycogenic minerals (right) formed by investigated *Aspergillus* species: a) *Aspergillus amstelodami*; b) *A. calidoustus*; c) *A. creber*; d) *A. europaeus*; e) *A. flavus*; f) *A. melleus*; g) *A. niger*; h) *A. proliferans*; i) *A. protuberus*; j) *A. pseudoglaucus*; k) *A. sydowii*; l) *A. versicolor*. (scale bar 10 µm).

On the other hand, predominant oxalate minerals were represented with tetragonal prism combined with bipyramide, being observed in *A. europaeus* (figure 1d; figure 3a), *A. flavus* (figure 1e; figure 3b), *P. canescens* (figure 2b), *P. expansum* (figure 2i), *P. glabrum* (figure 2j), *P. ulaiense* (figure 2l, figure 3n) and *Talaromyces verruculosus* (figure 2o). In some cases, oxalate minerals were documented in the form of bipyramide, as in *P. chrysogenum* (figure 2d) and *P. viridicatum* (figure 2m; figure 3o) or as penetration twins (figure 3e). Described calcium oxalate forms correspond to weddellite. Numerous "hyphal prints" were frequently observed on the surface of crystal forms (figure 1f, figure 3b, 3d, 3f, 3m, 3n).

Fig. 2. Optical Microscopy of conidial aparatus (left) and mycogenic minerals (right) formed by investigated *Penicillium/Talaromyces* species: a) *Penicillium brevicompactum*; b) *P. canescens*; c) *P. carneum*; d) *P. chrysogenum*; e) *P. citreonigrum*; f) *P. citrinum*; g) *P. decumbens*; h) *P. digitatum*; i) *P. expansum*; j) *P. glabrum*; k) *P. solitum*; l) *P. ulaiense*; m) *P. viridicatum*; n) *Talaromyces sayulitensis*; o) *T. verruculosus*. (scale bar 10 µm).

Most of the tested fungi exhibited precipitation of secondary mycogenic minerals, which is in concurrence with the recent data reported by our research group (Savković *et al.* [8] and Unković *et al.* [5]) obtained using the same experimental procedures on fungi isolated from stone and wall paintings. The latter paper reported on mineral production for numerous *Aspergillus*/*Penicillium* isolates, including *A. europaeus*, *A. flavus* and *A. niger*,but not for *A. creber* and *A. versicolor* which were found positive in this research.

Pinzari *et al.*[7] also reported formation of oxalates on

paper substrate by *A. terreus*. On the other hand, biomineralization on B4 medium was not documented by any of the tested airborne fungi from Slovac National Gallery by Pangallo *et al.*[10] although this ability was confirmed for fungal isolates obtained from wooden sculptures. Mentioned discrepancies could be attributed to different metabolic profiles of fungal isolates, which could vary on both intra- and interspecies level. It should also be noted that oxalates of fungal origin were detected in foxing spots on deteriorated paper contaminated with airborne fungal material [14]. Furthermore, oxalate and calcite deposition associated with lichenized fungi was observed on stone monuments as orange-brownish pigmentations, so-called “scialbatura” [6].

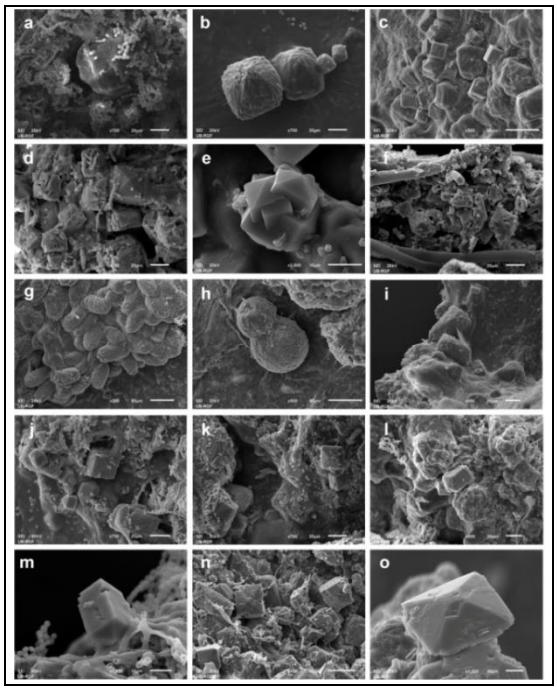

Fig. 3. SEM micrographs of mycogenic minerals formed in cultures of tested *Aspergilli* and *Penicillia*: a) *A. europaeus*; b) and c) *A. flavus*; d) and e) *A. mellus*; f) *A. niger*; g) *A. protuberus*; h) *A. sydowii*; i) *Penicillium canescens*; j) *P. carneum*; k) *P. chrysogenum*; l) *P. citirnum*; m) *P. solitum*; n) *P. ulaiense*; o) *P. viridicatum*. (scale bar 10 µm (e,m,o); scale bar 20 µm (a,b,d,f,i,j,k,l); scale bar 50 µm (c,g,h,n).

Biogenesis of secondary mycogenic minerals is probably due to the ability of investigated species to synthesize and excrete different inorganic and organic acids into the substrata.Various fungal species are known as potent acid producers. These organic acids can dissolve cations and/or chelate metals to form stable salt complexes [15, 16]. Oxalate production is most probably due to production of oxalic acid and its interaction with calcium acetate in B4 medium. On limestone substrata in natural environment, oxalates are formed via calcium carbonate solubilization by oxalic acid [17].Since excreted acids can solubilize calcium acetate, calcium ions are released and their interaction with CO_2 (as fungal respiration product) and H_2O (present in the B4 medium) can lead to the formation of calcium carbonate minerals [8] which explains why this mineral phase is occasionally being detected in our samples.Biogenesis of mycogenic oxalates is considered to be a fungal defensive mechanism exhibited in microenvironments with high concentrations of Ca^{2+} removed from substrata via the formation of stable salt complexes [18].

Morphology of mycogenic oxalates has even been considered in terms of taxonomical significance although this is highly debatable [8]. Also, in research presented here the ability of *A. europaeus*,*A. melleus, A. sydowi, P. carneum, P. citrinum, P.ulaiense*, and *P. viridicatum* to form oxalates, as well *A. protuberus* and *P.solitum* to precipitate calcium carbonates are reported for the first time [19].

In our study,the ability ofnumerous airborne *Aspergilli* and *Penicillia*isolates to precipitate minerals was demonstrated *in vitro* which indicates that biodeterioration potential of autochthonous fungi mustn't be neglected for adequate protection of cultural heritage objects, especially stone made buildings and monuments.

CONCLUSIONS

The majority of investigated *Aspergillus* and *Penicillium* species have demonstrated *in vitro* secondary mineral production. Calcium oxalate and calcium carbonate crystals were observed in tested samples with the predominance of oxalates.Therefore, we conclude that

investigating the ability of airborne fungal isolates to precipitate minerals is mandatory to properly assess structural and aesthetic alterations of cultural heritage objects.

ACKNOWLEDGEMENT

The study was supported by Ministry of Education, Science and Technological Development of Serbia (451-03-9/2021-14/ 200178).

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