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# IN VITRO COLONIZATION EXPERIMENTS FOR THE ASSESSMENT OF MYCELIAL GROWTH ON A TUFF SUBSTRATUM BY A *FUSARIUM SOLANI* STRAIN ISOLATED FROM THE OPLONTIS (NAPLES, ITALY) ARCHAEOLOGICAL SITE

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***Abstract***

In order to investigate the mycelial structure of rock-inhabiting fungi, an *in vitro* colonization test has been set with a low carbon source supply. A surface overlay documentation of the spreading colonies and their hyphal branching was observed both by metallurgical microscopy and fluorescence microscopy with the use of a fluorescent chitin and cellulose binding dye, calcofluor, during the whole experiment. The thickness of the fungal mat was also measured in central, medial and distal areas of the colony for each tuff tile, using a metallurgical microscope. Finally, after 20 days the tiles were also observed with CLS-microscope and all the photographic documentation was used for a segmentation image analysis on Fiji software to calculate the overlay and the volume of the mycelium. Our findings confirm that *in vitro* experiments coupled with microscopic observations are useful tools to evaluate and quantify fungal biomass on a stone substratum, especially in the early steps of fungal colonization.

***Keywords***: *Fusarium solani, colonization of hyphae, primary bioreceptivity, image analysis, CLSM confocal microscopy*

## Introduction

Fungi can be a serious threat for cultural heritage and artworks and are among the major agents of microbial deterioration of building stones [1] since they can establish on monuments made of different lithic materials, including granite, limestone, marble, sandstone [2-4]. Climatic conditions may be harsh and may also not allow rapid growth of mycelia; nevertheless, fungi can develop a low profile growth, forming small colonies or unicellular aggregates with a high surface to volume ratio that allows them to thrive also under the limiting and fluctuating conditions of open environments [5-6]. Dispersal of fungi is achieved through the formation and propagation of spores which can rest on bare substrates, also in the presence of very reduced organic sources, thanks to loose structure colonies at the microbe - mineral surface that helps the cells to adhere to the substratum and to assimilate nutrients [7]. In this respect, under the reduced availability of nutrients typical of lithic materials the first organism that colonize a virgin substratum will gain a primary advantage [8].

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Fungal biofilms accelerate the weathering processes of lithic materials by enhancing the irregularities of the surface (ridges and groove), penetrating inside the pores of the stone [9], and also constructing new ducts and cavities into intact mineral material [10].

In a survey conducted on the Roman monuments of Oplontis, Italy, we have observed a frequent occurrence of patinas caused by fungal growth on tuff walls: the following efforts to isolate these organisms led to the identification, among other less frequent genera, of fungal strains belonging to *Fusarium solani*. *Fusarium* is a genus of filamentous fungi (Hypocreales, Ascomycota,) commonly retrieved as soil contaminant and plant pathogen [11]; but also frequently found as a component of biofilms deteriorating stone monuments [12-14].

The understanding of the early steps of microbial colonization is a challenging issue for the biology of biofilms. Complicate interactions occur in biofilms, which involve any possible microbial individual in the formation of communities that are able to persist and flourish in extremely variable and harsh environments such as rocks and building materials. Though fungi appear to behave as a late colonizer of biofilm, in the opportune environmental conditions they can also act as pioneers, starting wide patinas that lead to the discoloration and the alteration of the material [15, 16].

Here we present an *in vitro* laboratory test coupled with optical, fluorescence and confocal microscopy, to study the early steps of the colonization of yellow tuff tiles by a *Fusarium solani* strain (ACUF 016f) isolated from the archaeological site of Oplontis. Literature is poor as regards the assessment of fungal growth on stone substrate [17, 18]; in most cases, the inoculum of the fungus is not reproducible or the initial medium supply is composed of more than one carbon compound, giving misleading results in the following observations. One of the issues linked to the use of small inoculum and limiting carbon source, is the subsequent way of determining the biomass and the colonization degree of the colony. Hyphae can be thin and translucent for non-melanized fungi, and difficult to detect on a matt substratum [19].

Few studies have been performed on thickness variability of biofilms growing on hard substrates [20-22], with the aim of providing a quantitative basis for analysis of microscale architecture of biofilms [23, 24], especially for the investigation of bacterial populations [25]. In order to investigate the mycelial structure of rock-inhabiting fungi, the growth of F. solani on yellow Neapolitan tuff tiles has been followed in sealed glass chambers under low sucrose concentration and high Relative Humidity (RH). This *in vitro* model allowed us to shed light on bi and three-dimensional evolution of the fungal mycelium by using a polyphasic approach based on the use of epifluorescence and metallurgical microscopy in bright field combined with CLS-microscopy.

## Material and Methods

### *Sampling site description and identification of a* Fusarium solani *strain*

The strain used for this experiment has been collected in a survey at the Oplontis archaeological site (Fig. 1, top and left bottom). Sample was collected from the external tuff walls of the residential villa, known as the "Villa of Poppea" with sterile scalpels, and grown in the lab on PDM agarized medium. Fungal hyphae were isolated with the aid of a stereomicroscope and then separately cultivated, using the same culture medium. The identification of a *F. solani* strain (Fig. 1, right bottom) was initially assessed on the basis of morphological observations, and confirmed by molecular analysis. DNA was extracted with a modified Doyle and Doyle DNA extraction protocol [25] and used for a Polymerase Chain Reaction with primers targeting ITS spacers (primer forward 5'-TCCGTAGGTGAACCTGCGG-3'; primer reverse 5'-TTCAAAGATTCGATGATTCAC-3'). PCR was carried out in a 25 µl aliquot containing approximately 100ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10x

buffer), supplemented to give a final concentration of 2.5 mM MgCl2, 1.25U of Taq polymerase (EconoTaq, Lucigen), and 0.5 pmol of each primer. Amplification was run in an Applied Biosystem 2720 thermal cycler. The profile used was 5 min at 95°C, 33 cycles of 95°C for 30s, 60°C for 45s, and 72°C 45s, and a final elongation step of 7 min at 72°C.


**Fig. 1.** Detail of the archaeological site of Oplonti (top), detail of the biofilm on the surface of a tuff wall (bottom left) from which it was isolated *F. solani* (bottom right).

PCR product was evaluated on 1.4% (w/v) agarose gel in an electrophoretic run and purified using QIAquick® PCR Purification kit (Qiagen Inc, Valencia, CA, USA). Sequence reaction was obtained with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Foster City, CA), purified in automation using the Agencourt CleanSEQ Dye terminator removal Kit (Agencourt Bioscience Corporation, 500 Cummins Center, Suite 2450, Beverly MA 01915 - USA) and a robotic station Biomek FX (Beckman Coulter, Fullerton, CA). The product was analyzed by an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems). The amplification primers were used as the sequencing primers.

Nucleotide sequence similarity was determined by using BLAST version 2.0 (National Center for Biotechnology Information databases). The isolated strain of *F. solani* was maintained following the protocol by *McGinnis et al.* [27].

***Roughness and Porosity analysis, Petrographic data***

In many archeological sites the use of local stone for architectural purposes very frequent; in Campania region (Italy) the large availability of volcanic products as Neapolitan Yellow Tuff (NYT) and their easy workability, determined their great utilization since the earliest times [28]**.** The lithic samples used in in vitro tests were taken from the caves Neapolitan Yellow Tuff (NYT) of Quarto (Napoli). NYT samples were cut especially with measures suitable for the needs of this experiment (average size 3x3x1 cm).

According to standard ISO 4287:1997 [29] the roughness parameters were evaluated on each sample with an ALPA© RT-20 palmar rugosimeter. All measurements were performed in triplicate, and data acquisition was conducted using the Measurement Studio Lite 1.0.3.96 software. The porosity of tuff lithic samples was assessed through mercury porosimetry

(Autopore 4, Micromeritics®). The water absorption coefficient (WAC) of the tiles (mean ± sd) was calculated according to Barberousse et al. [30]. Eight lithic samples were held at a constant temperature (21°C) and relative humidity (51-55%) for two weeks. The density (mean ± sd) of the tuff was obtained by evaluating the ratio weight/volume.

***Laboratory strains and Culturing conditions***

All the tuff tiles used in the experiments were washed with sterile water, dried and displaced in triplicate in glass chambers, which were tyndallized. The tiles were then watered at their maximum absorbance capacity with sterile Bold's Basal Medium (BBM) [31] added with sucrose 12g/L, according to *Jeger et al.* [32].

*F. solani* conidia were obtained from a 5 days old colony treated for 1 minute with PBS-Tween20 solution at the final concentration of 0,5% and mechanically scraped with a sterile handle. The suspension was then recovered and filtered through a sterile gauze, conveniently diluted and the number of conidia per milliliter was determined through a direct microscopic count in a Bürker blood-counting chamber. In all the experiments the inoculum consisted of 5000 conidia suspended in 5µl of sterile distilled water, injected with a pipette tip in the middle of each tile. During the whole time of observation, no bacterial contamination was found. One more glass chamber was prepared with tuff tiles watered with distilled water instead of nutritive medium and kept until the end of the experiment as a control.

To evaluate the fungal growth, every 4 days three tuff tiles from each of the three glass chambers were analyzed, and once the measures were taken they were discarded.

**F. solani** ***growth under different sucrose concentrations or relative air humidities***

Preliminary experiments were carried out to evaluate the kinetic of *F. solani* fungal growth according to Table (1). Six different concentrations of sucrose in the medium were used, ranging from 1.5 g/L to 48 g/L, at a constant RH of 100%. The fungal overlay was then assessed by digital image analysis. The relationship between relative humidity and *F. solani* growth on tuff tiles was assessed according to *Häubner et al.* [33]. In the bottom of four glass chambers were poured distilled water or saturated salt solutions of NaCl, $KNO_3$, $Ca(NO_3)_2$, to provide respectively 100, 93, 85, 76%, relative air humidity; the tiles inoculated with the fungal spores were positioned on perforated ceramic grilles placed at about two cm from the solutions, and each glass chamber was covered with a glass lid and sealed with silicone foam.

Both RH and sucrose experiments were monitored for 12 days: every 4 days, 3 samples were photographed with a digital camera. Digital image analysis was applied to quantify the growth of the mycelium on the stone samples inoculated at different sucrose concentration or at different values of Relative Humidity. The photographic recording of each tile was performed at 3 incubation times (4, 8 and 12 days) with a digital camera (Nikon D5100 with Nikkor 50mm objective). The conventional RGB color images obtained with the digital camera were used to quantify the colonization area on each lithic sample with the program *Trainable Weka Segmentation* [34, 35] a plugin of open source image processing package *Fiji*, an open source image processing package [36] and also at http://www.fiji.sc.

**F. solani** ***growth in constant nutrients and moisture***

The methods previously described were used to select the best experimental conditions to be adopted in the following test, planned to describe the mycelial structure of *F. solani* biofilm on tuff tiles.

The tests were performed at 12 g/l sucrose and at 100% RH and lasted three weeks. In this set of experiments, ten points of observation were chosen for each tile, as previously described, and the observations were carried out at an interval of four days.

For the assessment of the fungal mat architecture on the lithic substrate, five glass chambers were prepared for the test, under the same experimental conditions. Fifteen tuff tiles

were inoculated with *F. solani* conidia and also distributed in the 5 glass chambers at 100% RH as previously described. The experiment lasted three weeks and was repeated two times.

Each tile was virtually divided into three zones ranging from the middle to the external borders of the tile and 10 points of observation, 2 central (near the inoculum), 4 median (average distance of 9 mm from the center of the sample) and 4 distal (average distance of 18 mm from the center of the sample) were selected (Fig. 2, left). To evaluate the fungal growth, the experiment was monitored for 20 days: every 4 days, three tuff tiles from each of the five glass chambers were analyzed, and once the measures were taken they were discarded**.**

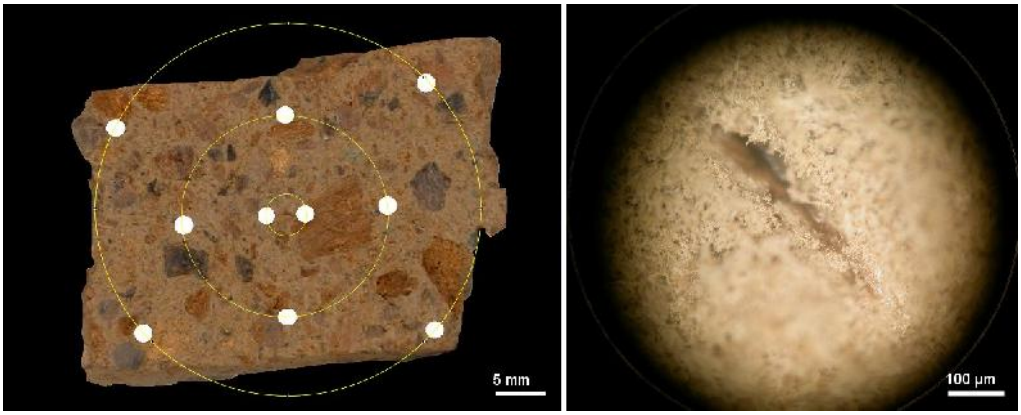


**Fig. 2.** Representation of the 10 measuring points of the biofilms of *F. solani* on the tuff tile (left); an example of biofilm cutting for thickness calculation (right), metallurgical microscope photos.

Each set of measurements was performed for three weeks at an interval of 4 days in the following way:

1. Quantification of the colonization area. The samples of tuff tiles were photographed with a digital camera. From the digitized images, the coverage area was calculated by means of WEKA segmentation showing the colonized areas of the tile, and by using Analyze Particle.
2. Measurement of the fungal thickness. In the 10 selected points, the hyphal network was cut with a pen cutter (Fig. 2, right) equipped with blade oblique (WLXY®, model number: WL9309). According to Bakke and Olsson [37] the thickness values were determined with a metallurgical microscope (Leitz Wetzlar Ortholux Microscope) with an objective 4x.
3. Measurement of the mat surface texture. The same tiles used for the measurement of thickness were then sprayed with Calcofluor White 1% [38], a fluorescent dye that binds cellulose and chitin in the fungal wall The observations were carried out on the 10 selected points with an epifluorescence microscope (Nikon Eclipse E800) at 20x magnification, using the DAPI filter (excitation 395 to 415 nm, emission from 455 nm). A photographic documentation was collected and later used for computer image analysis, with the threshold tool of *Fiji* software, to evaluate the coverage area of the hyphae network.

The results obtained by the triplicates for any given set of measurements for both the thickness and surface data were used as means for each observation point and then plotted with their respective standard errors.

At the end of the experiments (20$^{th}$ day), the growth of *F. solani* on three tuff tiles was also analyzed with a Confocal Microscope Zeiss LSM700 (software Zen 2011) by capturing images at 10x. The fluorescence of hyphae was recorded in one channel using the Calcofluor white to label the hyphae with excitation beams at 405-458 nm and emission at 415-505 nm

(blue-green channel). The images from stacks were captured at 13.46 µm intervals. For each biofilm, 3 replicates were used for taking Z-stacks images. The substratum area of the image of the epilithic stack was 1024 x 1024 pixel (640.174 x 640.174 µm). The number of images in each stack varied according to the thickness of the biofilm. Fiji was also used to evaluate the area of the fluorescence photographs and all stacked CLSM images. The images have been previously converted to 8-bit and then resampled by using the tool Threshold [39-41]. *Comstat2* [42], *3D Manager* [43] and *DiameterJ* [44] tools were used to determine the volume, thickness, roughness and features of the hyphae of each Z-stacks [45].

**Table 1.** Number of tiles (in triplicates) and sucrose and RH conditions for each set of experiments.

| | **Tuff tiles** | **RH** (%) | **Sucrose concentration** (g/L) | **Observation time** (1t = 4 days) | **Duration** (days) |
|---|---|---|---|---|---|
| Different RH | 9 | 76<br>85<br>93<br>100 | 12 | 3 | 12 |
| Different sucrose | 9 | 100 | 1.5<br>3<br>6<br>12<br>24<br>48 | 3 | 12 |
| Constant RH and sucrose | 15 | 100 | 12 | 5 | 20 |

## Results and Discussion

### *Roughness and Porosity analysis, Petrographic data*

The NYT shows its typical assemblage of prevailing epigenetic phases (phillipsite, chabazite and analcime), feldspar, and a minor amount of mica, hydrated iron oxides and volcanic glass [46]. The roughness profile was calculated considering 1600 sample points for each lithic sample to determine the average roughness (Ra = 19.32), the root mean square surface roughness (Rq = 23.21), mean roughness depth (Rz = 85.68) and maximum or total roughness (Rt = 101.83). The porosity parameters of tuff lithic samples were calculated, along with density and water adsorption coefficient (Table 2).

**Table 2.** Water adsorption coefficient (WAC), porosity and roughness of tuff tile.

| WAC ($g\ dm^{-2}\ min^{-1/2}$) | Porosity | | | | Roughness | | | |
|---|---|---|---|---|---|---|---|---|
| | Density ($g\ cm^{-3}$) | Porosity (%) | Average pore diameter (nm) | Total pore area ($m^2/g$) | Ra | Rq | Rz | Rt |
| $49.28^{-5}$ | 1.461 | 56.63 | 247.8 | 9.47 | 19.32 | 23.21 | 85.68 | 101.83 |

### *Preliminary assessment of* **F. solani** *growth under different sucrose concentrations or relative air humidities*

A development of fungal hyphae on tuff was observed since the first week of incubation, as reported for other accelerated test of stone colonization by fungi [47].

At all the selected sucrose concentrations a *F. solani* fungal mat developed on the surface of the tiles, and a linear increase of surface coverage was observed between 1.5 and 24 g/L of sucrose concentration (Fig. 3, left). At 24 and 48 g/L sucrose, the surface of the tiles was completely covered by *F. solani* hyphae at the end of the experiments. According to experimental data of *Chertov et al.* [48] and the mathematical model developed by *Picioreanu* et al. [49], the spread of *F. solani* depended on nutrient concentration, and sucrose

concentration lower than 24 g/L that did not allow the complete coverage of the tiles during the course of the tests were considered as limiting.

The effect of the atmospheric relative humidity on *F. solani* growth was assessed in the range 76-100% RH. In these experiments, a sucrose concentration of 12 g/L was used to supplement the inorganic culture medium (Fig. 3, right). The results of digital image analysis show that HR levels from 82 to 100% equally supported the growth of *F. solani* during the whole experiment. It is known that microorganisms can survive also under very reduced water availability, but in that case, they are not able to grow [49]. *F. solani* growth is dependent on relative humidity (Fig. 4) but, contrary to what reported by *Dubey and Jain* [50] fungal growth on the tuff stone was observed also at 76% RH, probably due to the high water adsorption coefficient of tuff stone and its ability to retain moisture.

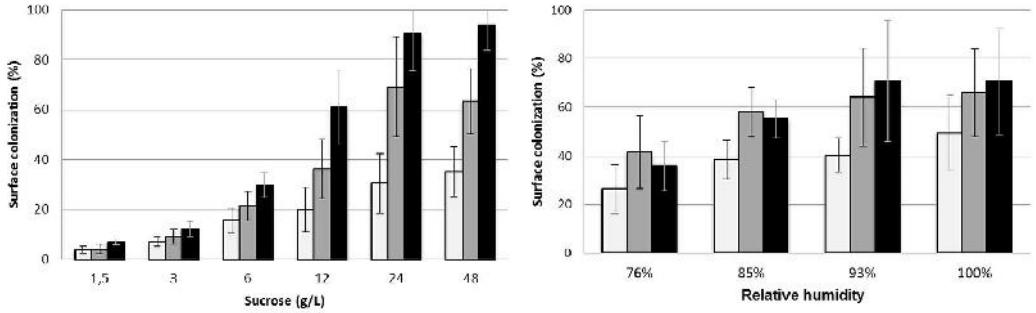


**Fig. 3.** Growth (percentage coverage) of *F. solani* on tufa tiles: different concentrations of nutrients and at 100% RH (left), different 100% RH and to sucrose concentration of 12 g/L (right) at 4 (light grey ), 8 (dark grey ) and 12 (black ) days incubation.

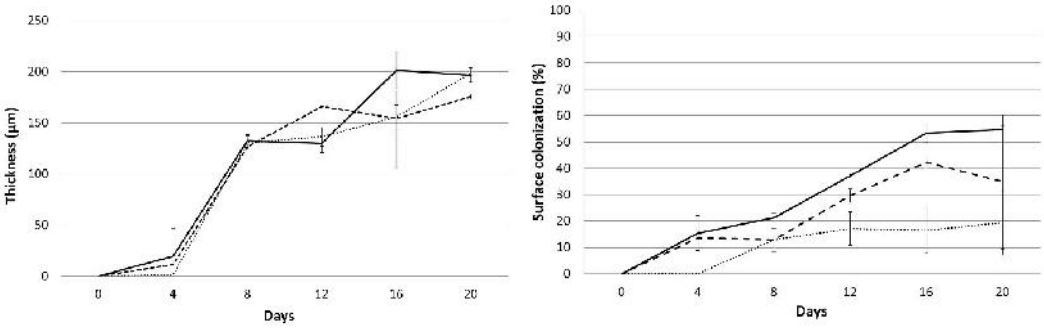


**Fig. 4.** Percentage of thickness of biofilm growth on tufa tiles (left), surface colonization on tufa tiles (right); points: central (——), median (- - -), distal (· · ·).

### F. solani *mat structure under constant nutrients and moisture*

The growth of hyphal filaments was observed in all the observation points corresponding to central, median and distal regions of the tiles (Fig. 4, left). In the central region, the aerial mycelium increases its thickness during the first 15 days of incubation, reaching a plateau at the 16th day. The maximum average thickness appears to be fixed to about 200 µm for all the considered regions on the tile. A similar pattern, albeit shifted in the time, was also observed in the median region, where the plateau was reached at the 20$^{th}$ day, whereas in the distal region *F. solani* growth was significantly lower, and the hyphae were unable to colonize all available space in the course of the experiments, due to limiting available nutrients (Fig. 4, right). In this region a noteworthy development of conidiophores was observed (*not shown*).

Fungal growth on tuff tiles results in a mycelium with a reticulate texture, with open spaces that separate the fungal mat, leading to a patchy structure of the fungal growth. The

development of a reticulate texture could be due to change in the growth direction of filaments, as reported for cyanobacterial growth on lithic substrates [51]. The surface texture of the hyphae (sprayed with fluorescent brightener Calcofluor White) during the colonization of the substrate was observed with an epifluorescence microscope. Data show that in each region of the tile there is a constant increase of the hyphal branching over the time, that are leading to a progressive reduction of the void dimension in the texture.

Surface texture and thickness are not related ($R^2$ = 0.45), suggesting that in our experimental conditions the hyphal organization is oriented toward a higher superficial branching degree more than increasing the thickness of the mycelium.

In order to assess if the spatial organization of hyphal branching is subject to major changes along the layers forming the mat, and/or in the three regions of the tile, we performed a three-dimensional evaluation of the hyphal branching by CLS-microscopy (Fig. 5). At the end of the 20th day of incubation, the tuff tiles were sprayed with Calcofluor white and then analyzed with a confocal microscope in order to evaluate some structural parameters in the central, medial and distal region of the biofilm (Table 3, top). Z-Stacks analysis has shown a clear resemblance of structural patterns of hyphae in the central and median regions. To evaluate the characteristics of the hyphae network, we analyzed z-stack MIP images with Fiji's *DiameterJ* plugin (Table 3, bottom).

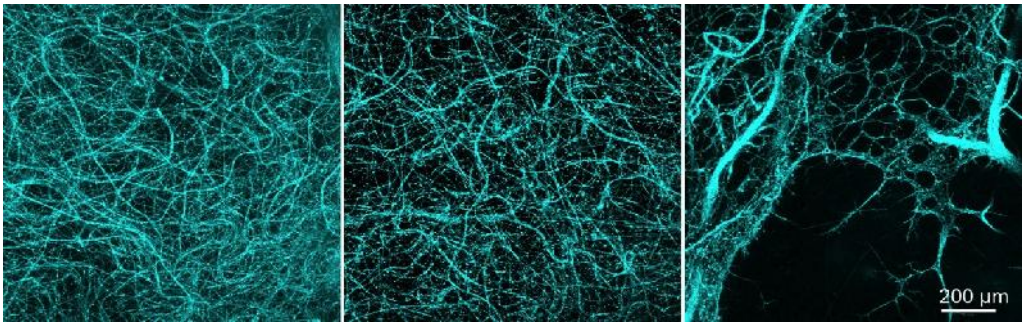


**Fig. 5.** MIPs of *F. solani* biofilm on lithic tuff tile, central (left), median (central) and distal (right) z-stack to the biofilm growth surface.

**Table 3.** Analysis of various architectural parameters of *F. solani* biofilm

| | Central | Median | Distal |
|---|---|---|---|
| **Area MIP (%)** | 44.112±1.822 | 35.941±3.862 | 11.878±0.665 |
| **Biomass (µm$^3$)** | 32780434.778±1051337.997 | 33470831.368±2373961.800 | 20381191.392±19922166.572 |
| **Thikness average** | 215.7985±113.608 | 220.940±3.080 | 125.140±64.875 |
| **Thikness max** | 420.000±141.421 | 334.500±11.313 | 240.000±133.643 |
| **Ra** | 0.7355±0.057 | 0.645±0.041 | 1.417±0.286 |
| **Mean pore area (µm$^2$)** | 28.514±5.499 | 30.497±3.156 | 34.803±8.2654 |
| **Max. pore area (µm$^2$)** | 1241.723±614.373 | 2654.363±258.238 | 3734.674±1821.298 |
| **Number of pores** | 719±226.981 | 964±151.228 | 270±98.558 |
| **n° of intersections** | 31213±620.386 | 50680±1380.868 | 14984±1569.448 |
| **Diameter mean (µm)** | 1.176±0.215 | 1.87±0.561 | 4.867±1.256 |
| **Diameter max (µm)** | 4.575±2.121 | 10.457±4.85 | 68.071±10.684 |

According to the consideration of Matsuura and Miyazima [52], that larger leading hyphae continue to extend, whereas secondary hyphae seem to be more sensitive to nutrient depleting conditions, we observed a prevalent presence of very thick hyphae in the distal region of the tuff tiles.

The three-dimensional architecture of fungal biofilm on different substrates is largely influenced by the environment: mat formation by *Saccharomyces cerevisiae* was grown on medium containing a low agar concentration showed a radial pattern of growth, with a central

hub made of a complex network of cells and radial spokes, originated from the hub. This structure was influenced by the viscosity of the medium, and nutrient availability [53]. Similarly, CSLM analyses performed on *Fusarium* populations grown on soft contact lens revealed that biofilm thickness was limited by the water content of lenses and that the architecture of fungal mat was dependent on surface characteristic of lenses, albeit remaining uniform at the center and periphery [53-55].

In our experiments, the structural features of yellow Neapolitan tuff seem to dictate a different three-dimensional structure of *Fusarium solani* mats. Table 3 shows that the roughness of the mat increases from central to distal regions of the tiles, these latter showing an almost double roughness compared to the central and median regions. Moreover, there is an increase in the average and maximum size of the hyphae network voids from the central to the distal region of the tile, and also the values of the mean and maximum diameters of the hyphae show an increase from central towards distal regions of the tile. Finally, the number of intersections of hyphae has a drastic decline from the center to the borders of the tile (Table 3). All these results concur to indicate that the mycelium in the central and median regions is compact and consists of a very intricate network of prevalently thin filaments, and that is homogeneous along the stacks. Conversely, in the distal areas, the mycelial network is loose and double-stranded filaments prevail. Also, in this case, no vertical zonation of hyphal architecture was evidenced.

## Conclusions

In vitro colonization experiments can be useful to understand the role of environmental parameters such as relative humidity, temperature, light intensity and nutrients on the fungal three-dimensional organization. Our tests have indicated that *F. solani* is able to colonize the tiles also in very limiting conditions of carbon supply and relative humidity and that the yellow Neapolitan tuff is a very bioreceptive material. Using the in vitro model, we showed that the colonization of the tuff tiles exhibited a well-defined pattern:

- The surface roughness of the biofilm and mean and maximum diameters of the hyphae showed an increase from central towards distal regions;
- the number of intersections of fungal filaments declined from the center to the borders of the mat, causing the increase of the average and maximum size of the voids in the distal region of the mycelium.

A combination of microscopical techniques (epifluorescence, metallurgical microscopy in bright field and CLS-microscopy) can be successfully applied to assess the first steps of fungal colonization on stone substrate, especially to evaluate thickness and structural texture of the mycelium, less investigated parameters in biodeterioration studies although they play a key role in the establishment of mature biofilms pioneered by fungi.

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