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Morphology of Fungal Stains on Paper Characterized with Multi-Scale and Multi-Sensory Surface Metrology

Hanna Szczepanowska,[1], Thomas G. Mathia[2], and Patrice Belin[3],

[1]MCI, Smithsonian Institution, Suitland, Maryland
[2]C.N.R.S. Ecole Centrale de Lyon, Laboratoire de Tribologie et Dynamique des Systèmes, Guy de Collongue Ecully, France
[3]Altimet, Thonon les Bains, France

Summary: Pigmentation of paper induced by fungi is one of the most complex phenomenons because it involves living organisms growing on a heterogeneous paper substrate. A novel approach to the study of interfaces of fungi and paper in black stains produced by pigmented Dematiaceous fungi with meristematic growth was undertaken applying surface metrology techniques: confocal laser scanning microscope (CLSM) white-light, confocal chromatic aberration profilometer (LCA) and scanning electron microscope in variable pressure (SEM-VP); fungal morphology was examined with transmitted light microscopy (TLM). The role of paper topography and surface morphology in fungi-induced biodeterioration was investigated elucidating some of the dynamic interactions of fungi with paper, spatial distribution of biological deposits, inclusions in paper matrix, and patterns of fungal growth on paper thus contributing to a better understanding of biodeterioration of paper-based cultural heritage. So far, to the authors' knowledge, there are no published reports on the investigation of interfaces of bio-stains and paper utilizing surface metrology techniques. SCANNING 36: 76–85, 2014. © 2013 Wiley Periodicals, Inc.

Key words: fungal stains, confocal optical microscopy, surface topography of fungal deposit on paper

Conflicts of interest: None.

Address for reprints: Hanna Szczepanowska, MCI, Smithsonian Institution, 4210 Silver Hill Rd, Suitland, MD 20572.
E-mail: szczepanowskah@si.edu www.si.edu/mci

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Introduction

Biodeterioration of art works and documents on paper is a particularly complex phenomenon because it involves living microorganisms interacting with highly heterogeneous materials, such as paper and art media. Bio-deterioration of cultural heritage refers to decay of culturally, historically or artistically significant objects induced by microorganisms, such as bacteria, fungi or lichens.

Pigmentation induced by fungi disfigures cultural heritage objects diminishing their artistic and monetary value, yet has received little attention. Many fungal species produce pigmentation which serves different functions in the microbes' life. Phylogenetic diversity of pigmented microbial species and the chemical diversity of the pigments themselves preclude a single unifying hypothesis for their evolution. In general, pigmentation produced by microorganisms results from their complex bio-chemical processes occurring in their cell structures and are defined as secondary metabolites (Medentsev and Akimenko, '98; Gomez et al., 2001; Liu and Victor, 2009). Black pigmentation of Dematiateous fungi is attributed to melanin which is shielding fungal cells from damaging UV radiation. Pigments are deposited in the cell walls of mycelium and/or fruiting structures. Pigmentation is one form of fungal response to light, fungal patterns of growth in a substrate is another, referred to phototropism. Fungi also respond to surface topography, known as thigmotropism, which has been extensively studied in plant pathogens (Tucker and Talbot, 2010) and human infectious pathogens (Perera *et al.*, '97), but not in biodetrioration of cultural material. The aim of this investigation was to better understand some aspects of the relationship between surface morphology of paper and black pigmented fungal growth and stains' formations. Morphology of fungal species and spectroscopic characteristics of their pigments dictated the choice of optical instruments for this investigation. The authors are proposing a novel

approach of a systematic, multi-scale regime adapted from the fields of surface metrology and traceology to the investigation of biodeteriorated cultural material (Mathia *et al.*, 2011).

Materials and Methods

Fungal Deposits

Sample material, paper with fungal deposits, was a single sheet of paper manufactured ca 17th century obtained from the collection of the Archives in Mdina, Malta (Fig. 1). Two types of fungal deposits were investigated, and referred to as stain type A and B. Stain type A, of average diameter 1.5–2 mm, was formed by congregation of dark pigmented fungal cells, in chains or dispersed as individual cells (Fig. 2). Stain type B consisted of macroscopic fruiting structures discerned visually as black pin-hole size inclusions (Fig. 3). Twenty-two stains produced by cell clusters (stains A) and ten fruiting structures (stains B) were analyzed.

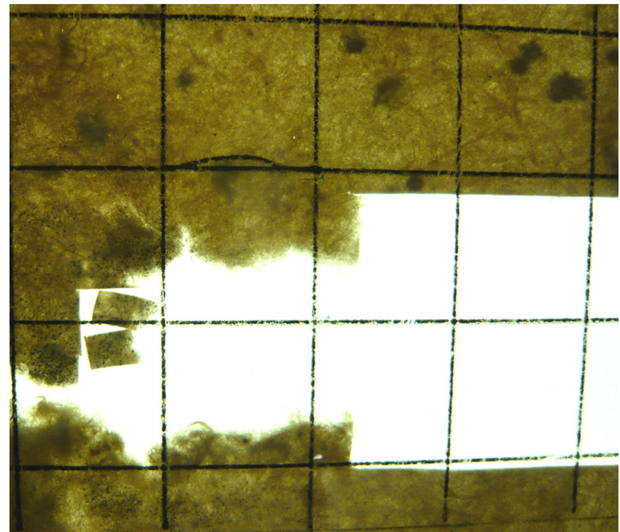

Fig 1. Fragment of a hand-made paper, 17th century, Maltese Collection, infested by fungi viewed in transmitted light. Type A black stain are predominant on this paper, average size of each stain 1.5mm x 2mm, visible in the upper part of the paper. Type B, on two small cut samples (left side, center) are fruiting bodies, visible as black dots. Scale bar: 10 mm corresponding to the side of each square.

Paper Substrate

The morphology of paper fibers examined in transmitted light microscopy (TLM) and scanning electron microscopy in variable pressure (SEM–VP) indicated that cotton was predominantly used as paper fiber. No other type of fibers was detected qualifying the paper as 100% rag paper (meaning that cotton rags were used as raw material). The Biuret test and Bradford assay confirmed presence of protenacious material indicating gelatin sizing. Gelatin has been used

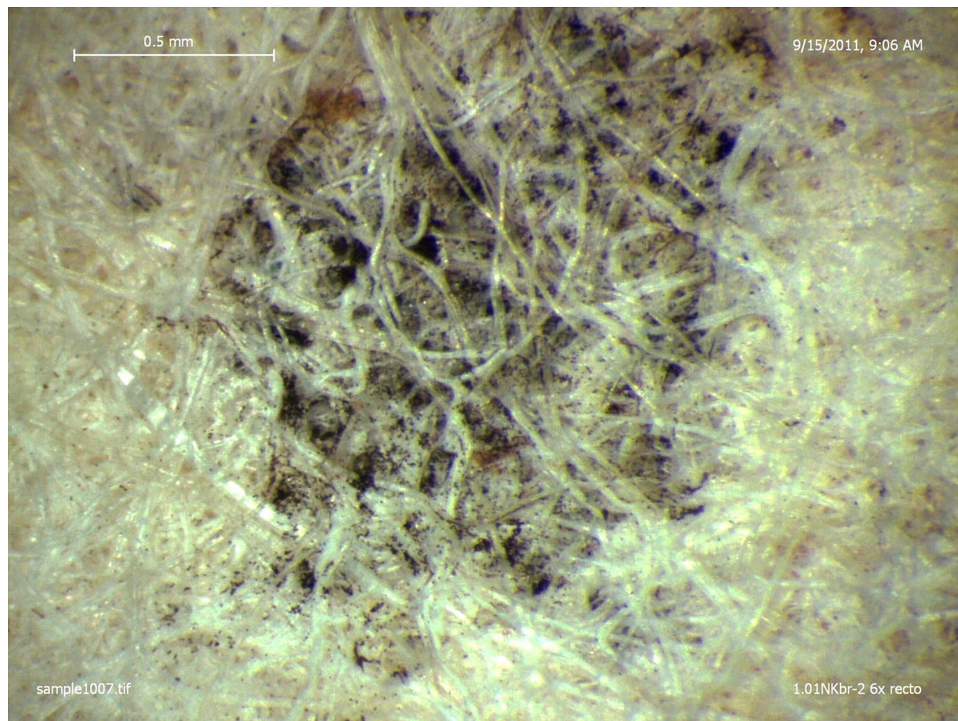

Fig 2. Stain A was formed by pigmented cells clustered in the recessed areas of the paper matrix. Stereo-micrograph, 6x, Zoom Stereomicroscope, Wild M8 Heerbrugg. Scale bar: 0.5mm.

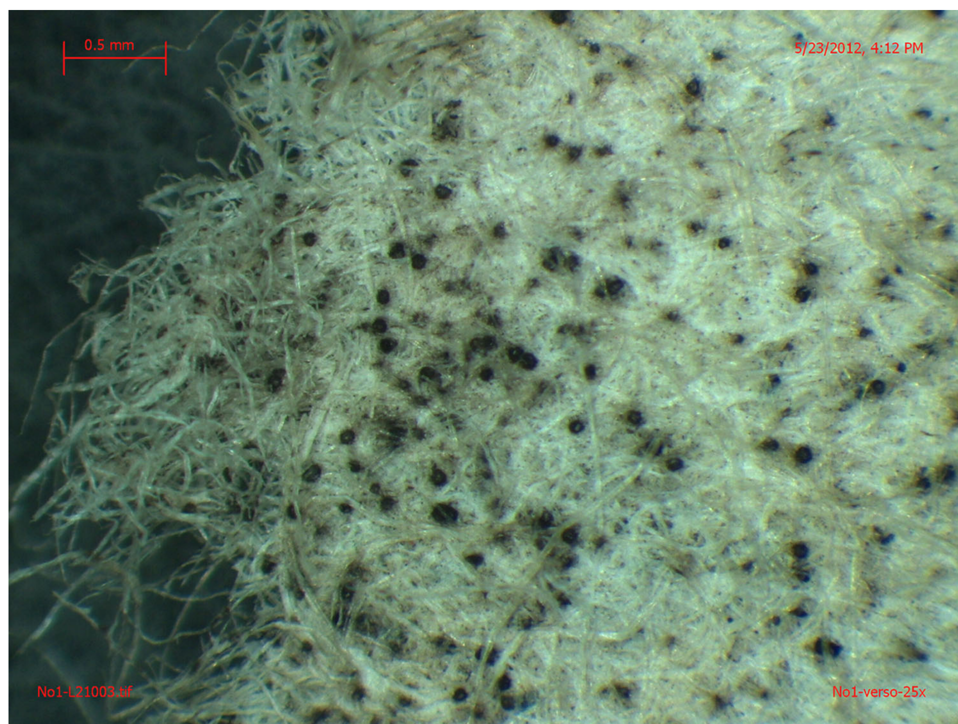

Fig 3. Stain B was formed by dark pigmented individual fruiting bodies attached to paper fibers. Stereo-micrograph, 50x, Wild M8 Heerbrugg Stereomicroscope. Scale bar: 0.5mm.

historically as paper sizing through six centuries (Hunter '78; Banik and Bruckle, 2011).

Instruments

Morphological characterization of fungal deposits and spatial distribution of fungal pigmented cells and fruiting bodies were analyzed with the stereomicroscope, Wild M8 Heerbrugg, Zoom Stereomicroscope with LED light ring, Volpi, Swiss. Magnification ranged 6x-50x. The digital camera, Leica EC 3, Heerbrugg, captured the digital images. Paper remained in stable environment for the duration for the experiment; room temperature 22°C and relative humidity 45–52%. Conditions of the environment were monitored with Elsec 764 Environmental Monitor; Littlemore Scientific Engineering, UK.

Morphology of individual fungal deposits was characterized using transmitted light microscope, Leica DM LM, in phase contrast illumination, dark field and bright light illuminations. Magnification ranged: 50×, 100×, 200× and 400×, power source: Leica AC volts 0–15.

Interfaces of fungal deposits and paper matrix were investigated with the scanning electron microscopy in variable pressure. SEM–VP Hitachi S 3700N was used, magnification range: 500–3,000×, and 40 Pa pressure.

Interfaces of fungal deposits and paper as well as paper topography were imaged with 3D confocal laser scanning microscope (CLSM) Keyence VK-9700 series at magnification 1,000×. Violet light, 408 nm wavelength was used as source of light. Photoelectron multiplier allowed measuring samples with low surface reflectance and steep slope gradient.

Surface topography and fungal deposits was measured with white-light, confocal chromatic aberration profilometer (LAC) ALTIMET Altisurf500. Working distance of the optical pen was 3.3 mm with measuring range 110 μm.

Examination Methods

The methods of investigation replicated the real-life situation of examining cultural heritage material which often does not permit sampling of the original object. The selected areas of paper with fungal deposits were investigated *in situ*, applying non-contact optical sensors using white light, chromatic aberration profilometer (Fig. 4). Examination in SEM–VP was the only exception because it required minute samples of paper with bio-mass.

Results and Discussion

Pigmented Fungal Deposits and Paper Substrate

Transmitted light and incidental light microscopy of bio-mass is traditionally carried out on biological specimens, providing information about their general

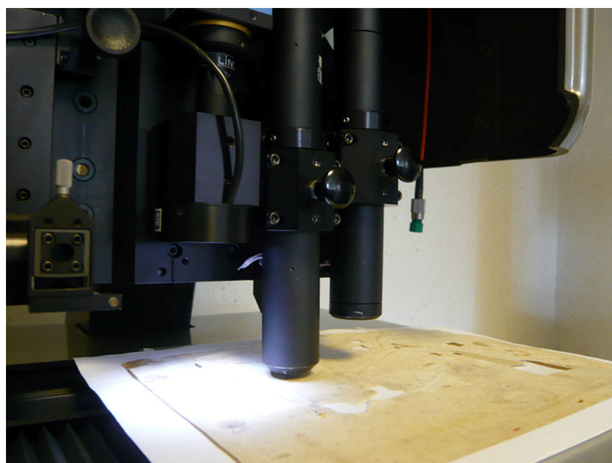

Fig 4. Non-contact, in situ measurement of fungal deposits on paper with white light, chromatic aberration profilometer, ALTIMET Altisurf500. The advantage of this instrument is its ability to measure surfaces without destructive sampling of the original material. Details of the measuring probe are specified in Fig. 11.

shapes, forms and dimensions. Morphology of dark brown and nearly black fungal cells in stain type A indicated pattern of individual cells cluster and chain-like formations. Dimensions of cells were consistent, 6–9 μm, larger than the average size of fungal conidia. The size indicated that the cells are swollen mycelium, rather then propagulates, such as spores or conidia. Cells showed evidence of isodiametric enlargements, thick and pigmented cell walls (Fig. 5). These morphological features and their size characterize meristematic fungi, sometimes referred to as microcolonial fungi (Sterflinger et al., ’99).

Stains type B were formed by single perithecial heads, of average diameter 50–95 μm (Fig. 6).

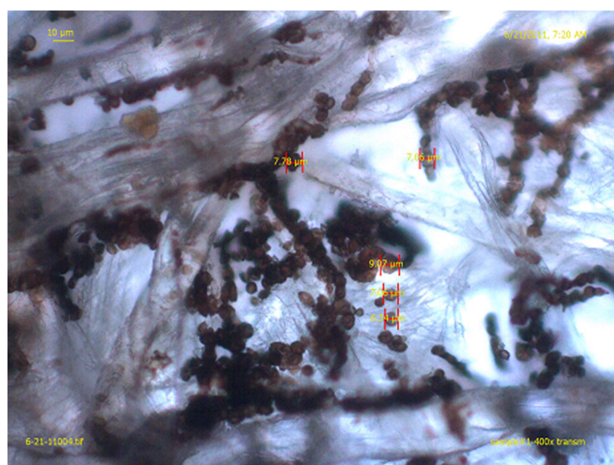

Fig 5. Stain A. Pigmented fungal cells are trailing on paper fibers, partially touching the surface of the paper fibers. Size of individual cells in chains is 6-9μm. Transmitted light microscope, 400x, Leica DMLM. Scale bar: 10 μm

Pigmentation was restricted to the fruiting structures and short mycelia wrapped around the paper fibers or trailing on the surface of fibers. Characteristics of the perithecial heads indicated fungus that belongs to the genus *Chaetomium;* fungi of this genus are typically found on paper, utilizing cellulose as the source of nutrient (Von Arx et al., ’86; Szczepanowska and Cavaliere, 2000).

Investigation of interfaces between fungal cells and paper surface in stains A, using SEM–VP revealed attachment of individual cells to the surface of fibers without involvement of mycelium or any forms of structural filaments (Fig. 7). Based on the current, fragmentary knowledge of how the cells attach to surfaces, stability of that attachment may be attributed to either extracellular adhesins (adhesive –like, protenecious material, produced by cells upon contact with surfaces) (Epstein and Nicholson, 2006; Schumacher et al., 2008; Gamarra et al., 2010) or physical, energetic forces involving electron-charged surfaces (Van Loosdrecht et al., ’89; Wang et al., 2010). Fruiting structures in stain B utilized paper fibers as a form of anchorage (Fig. 8).

Instruments

Transmitted light microscopy and stereomicroscope were used to characterize fungal morphology. That initial examination guided the choice of optical instrumentation, types of optical sensors and light sources.

The choice of non-contact, optical instruments was dictated by their ability to measure the surface features on a micro-scale and to produce repeatable results *in situ*, without the need of removing samples from the original material. Two optical instruments were used: confocal laser scanning microscope (CLSM) Keyence VK9700 and white-light, confocal chromatic aberration profilometer (LCA) ALTIMET Altisurf500. Each instrument offered different output providing complementary information.

Imaging Confocal Laser Scanning Microscope (CLSM)

CLSM combines lateral scanning (x,y direction) with confocal probe using laser as the light source. The surface height (z direction) is “sensed” based on depth of focus of the objective. The highest signal on the image correlates with the highest position on the surface. The advantage of confocal microscopes is in its high numerical aperture and a high measurability of local slope (Artigas, 2011). CLSM, VK9700, used violet light (408 nm) as a light source. It produced high-resolution

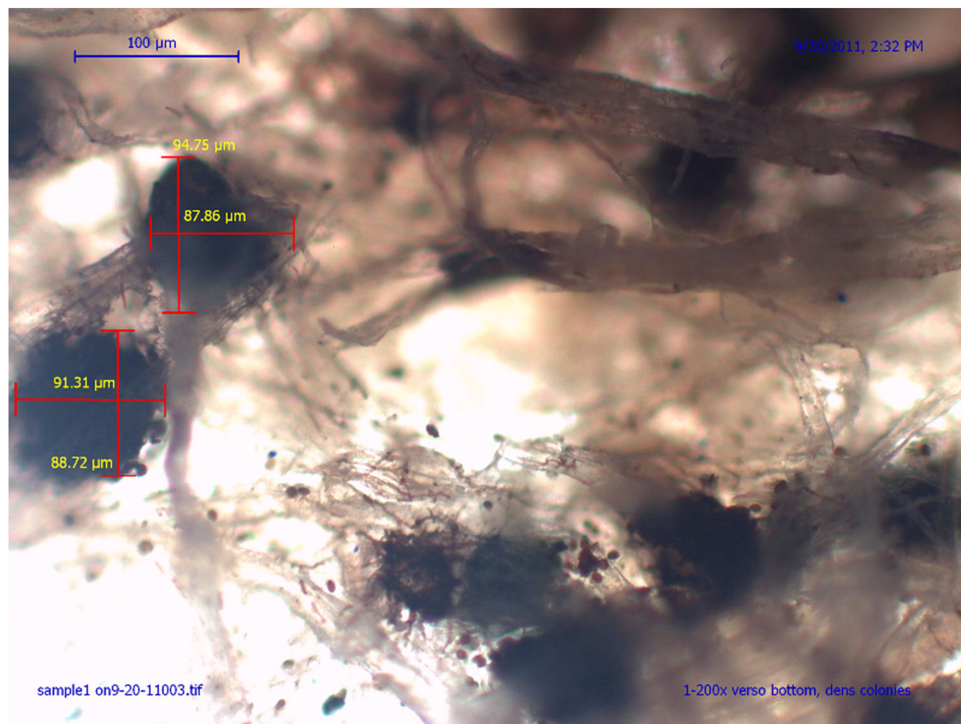

Fig 6. Stain B. Dark pigmented fruiting structures (perithecial heads), are attached to paper fibers with short mycelia. Their size varied; here the diameter is 87-94 µm. Transmitted light microscope, 200x, Leica DMLM. Scale bar: 100 µm

images of fungi on the surface revealing spatial distribution of individual cells and chains formations in the paper matrix (Fig. 9). The highest points on the surface are lighter indicating their higher altitude. The final image was created by overlay of laser scans and white light imaging combined in this model of CLSM (Keyence, personal communication, 2012).

Based on the imaging generated by CLSM we hypothesized that the surface topography, specifically paper roughness, provided either physical anchorage to fungal formations and/or protection against removal of cells by external forces, such as air movements. It is also possible that water retention is greater in those areas, making them attractive for spores' germination. What we can state based on our investigation is that fungal cells were closely positioned next to the paper fibers in stains A and nested in crevices in stains B. Optical sectioning of the sample was undertaken (results not shown). However, in order to unequivocally differentiate between peaks formed by fungal deposits from peaks

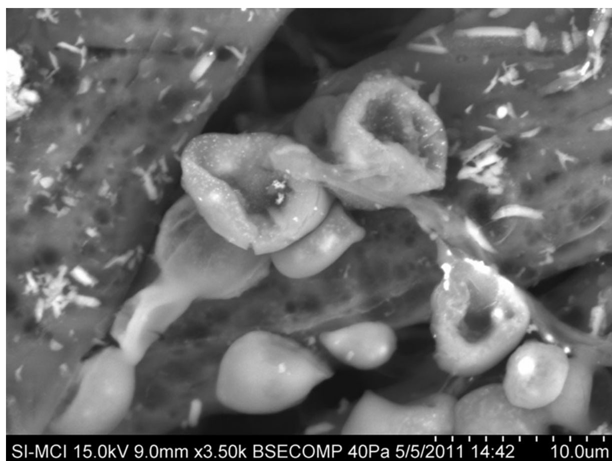

Fig 7. Stain A. SEM-VP micrograph of interfaces of pigmented cells and paper fibers reveals attachment of cells either via extracellular material (protenatious adhesines) or by electron-charge of their surface. SEM-VP Hitachi S 3700N, magnification 2000x, BSE, pressure 40Pa, scale bar, 20 µm.

Fig 8. Stain B. SEM-VP micrograph of the pigmented fruiting body anchored to paper fibers. Paper fibers are used as a physical support for the fruiting structure. SEM-VP Hitachi S 3700N, magnification 800x, BSE, pressure 40.Pa, power of electron beam 10.0kV, scale bar, 50 µm.

Fig 9. Stain A. Surface morphology of paper with fungal cells captured with 3D confocal laser scanning microscope (CLSM). Laser scanning over fungal cells in xyz direction produced 3D image with accurate height of the cells and paper fibers. Distribution of semi-spherical cells is visualized with orange color on the background of brown fibers. Lighter coloration of cells is due to their elevated position o the surface of fibers. Keyence VX9700 series at magnification 1000x, scale bar: 10 μm.

formed by paper fibers close comparative evaluation of imaging and measurements obtained from other sources would be necessary.

Investigation of the fungal stains, type A, using confocal laser scanning microscope, revealed co-dependence of fungal deposits and paper topography. Analysis of both sides of paper sheet indicated distinct differences in roughness on each side. Surface topography impacted fungal growth and distribution of pigmented deposits. The rough side (Fig. 9) of the entire paper sheet showed evidence of much greater amount of pigmented fungal cells than the smoother side

Fig 10. Reverse of stain A shows smooth surface of paper fibers with minimal fungal growth. Lesser amount of fungal deposits was consistent on all stains, throughout the entire reverse side of the paper sheet. Keyence VK9700 at magnification 1000x, scale bar: 10 μm.

(Fig. 10). Consequently, that resulted in more prominent staining of the rough side.

Confocal, While Light Chromatic Aberration Profilometer (LCA)

Another optical instrument selected for this investigation was LCA, white light, confocal chromatic aberration profilometer, ALTIMET Altisurf500. It is based on chromatic dispersion of white light, generating the height information by focus of a specific wavelength on the surface. The white light is imaged through a chromatic objective along *z*-axis providing color-coding along that optical axis (Fig. 11). When an object is present in the color field, a unique wavelength is perfectly focused at its surface and reflected into the optical system. The backscattered beam passes through a filtering pinhole into a spectrometer which analysis which wavelength has been perfectly focused on the object determining its position (Pruss et al., 2005; STIL, 2011).

In addition to investigating the features of fungal inclusions, heterogeneity of the paper matrix was considered in the interpretation of the generated measurements. Paper fibers are tubular, or ribbon like filaments, thus light was reflected in different directions from all sides of the fibers encountered during the measurement. Precise surface measuring optical sensor provided information about the paper fibers, for example detecting and measuring the characteristic longitudinal twist of cotton fibers in addition to measuring the surface distribution and depth of fungal inclusions (Fig. 12).

Spherical shape and low-reflection of light from fungal inclusions in both type of stains, (A and B) required a magnifier of high photometric efficiency. Furthermore, due to spherical shape of inclusions in stain B the intensity of the collected signal decreased with increasing slop angle. That was another factor considered in selecting a suitable optical instrument, which could handle measurements of semi-spherical shapes.

The spatial distribution of dark pigmented fungal cells in stain A were detected, measured and imaged with ALTIMET Altisurf500. Placement of cells near and on the surface of paper fibers was consisted with the TLM and SEM–VP examinations. Additional information was obtained by measuring the altitude of fungal deposits, which reached the height of 75–80 μm (Fig. 13).

The results of measuring inclusions in stain B, using LCA were puzzling, indicating loss of data. Non-measured points can result from many causes, such as the surface height being above or below the measuring range, the focal point falling into a "hole," the slop being too steep, or reflective/absorptive characteristics of the material (Blateyron, 2011). In case of fungal inclusions the areas of data "voids" corresponded to the shape and

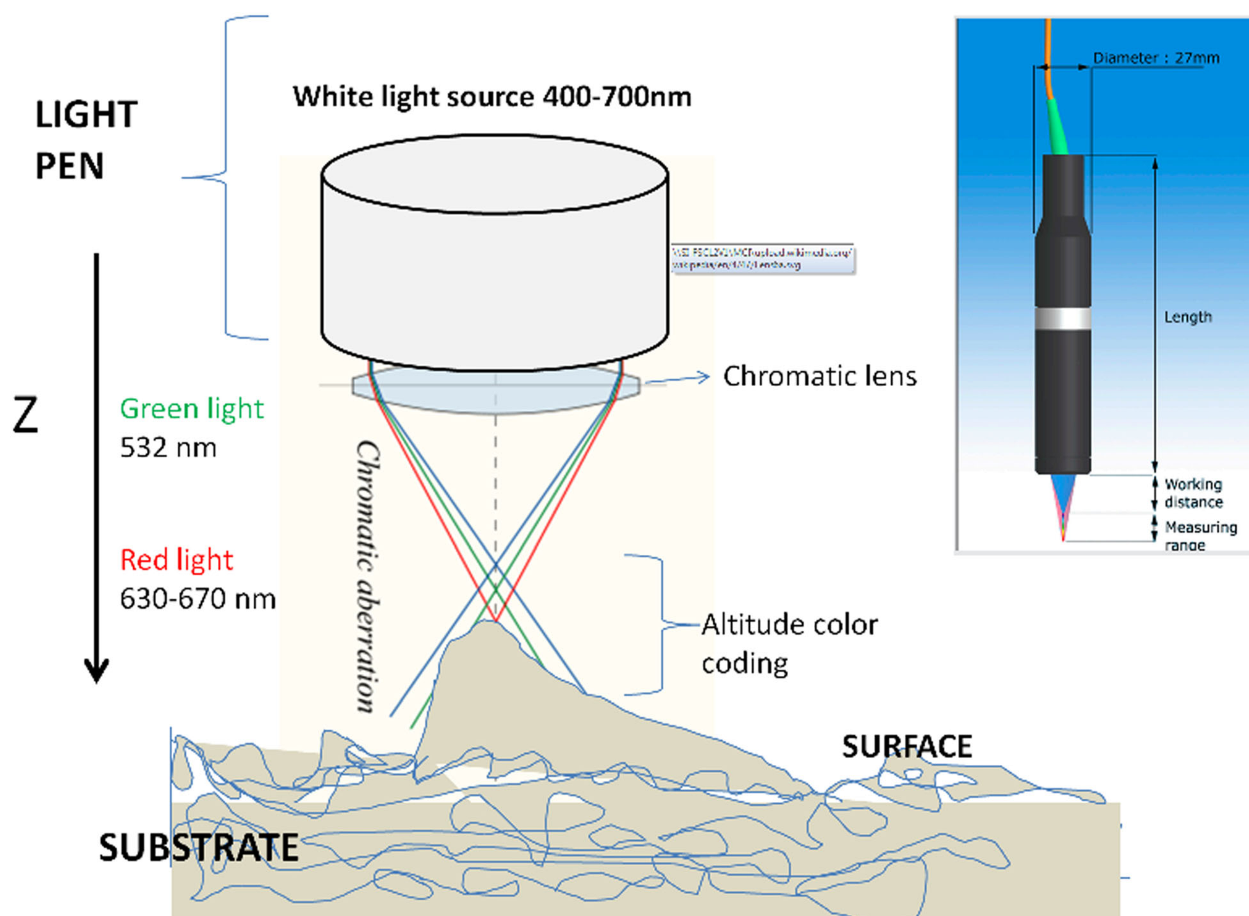

Fig 11. Basic setup of the white light, confocal chromatic aberration profilometer, ALTIMET Altisurf500. The working distance of the optical sensor was 3mm and measuring range 110µm.

location of fungi. The 3D map shows the areas of fungal inclusions nesting in the paper matrix (Fig. 14). Furthermore, the "voids" were not uniform, showing that some signals were received and analyzed (Fig. 15). Most likely that was light reflected from the paper fibers below the inclusions which provided information on the depth of their placement in the paper matrix. That has been confirmed by the partial profile obtained from the measured points (Fig. 16A,B). Filling in the missing measure-points which is a common practice of post-processing, would erase the information about location and shape of fungal inclusions.

Knowing that the black or dark brown pigmented fungi are almost always associated with melanin which is known to absorb portion of visible and UV light, the lack of data may be attributed to the characteristics of the pigment. Attempts to analyze them by mass-spectroscopy by other researchers yield poor fingerprint spectra (Buskirk et al., 2011; Bochenek and Gudowska-Nowak, 2003). Advancements of phytophotobiology will benefit other fields concerned with dark pigmented fungi.

Preliminary Conclusions of Work in Progress

Application of optical sensors to measure heterogenous substrate (paper) with biological deposits (fungal mycelium and/or fruiting structures) is a novel approach to the study of interfaces of paper and fungi. The sequence of observation regime, from macro through micro and next to the surface metrology is essential for the meaningful interpretation of the results. Each mode of investigation, evaluation of surface topography, morphology of bio-mass, and surface analysis of bio-mass deposit complemented each other revealing new aspects of bio-stains and their interactions with the substrate.

Detailed studies of the bio-mass distribution in the substrate revealed a broad range of fungal interactions with the substrate (paper fibers). That diversity of interactions is directly related to the morphological characteristics of different fungal species and unique topography of each substrate on which bio-mass was formed. Only partial understanding of the interfacial phenomena of the bio-pigments and substrates was

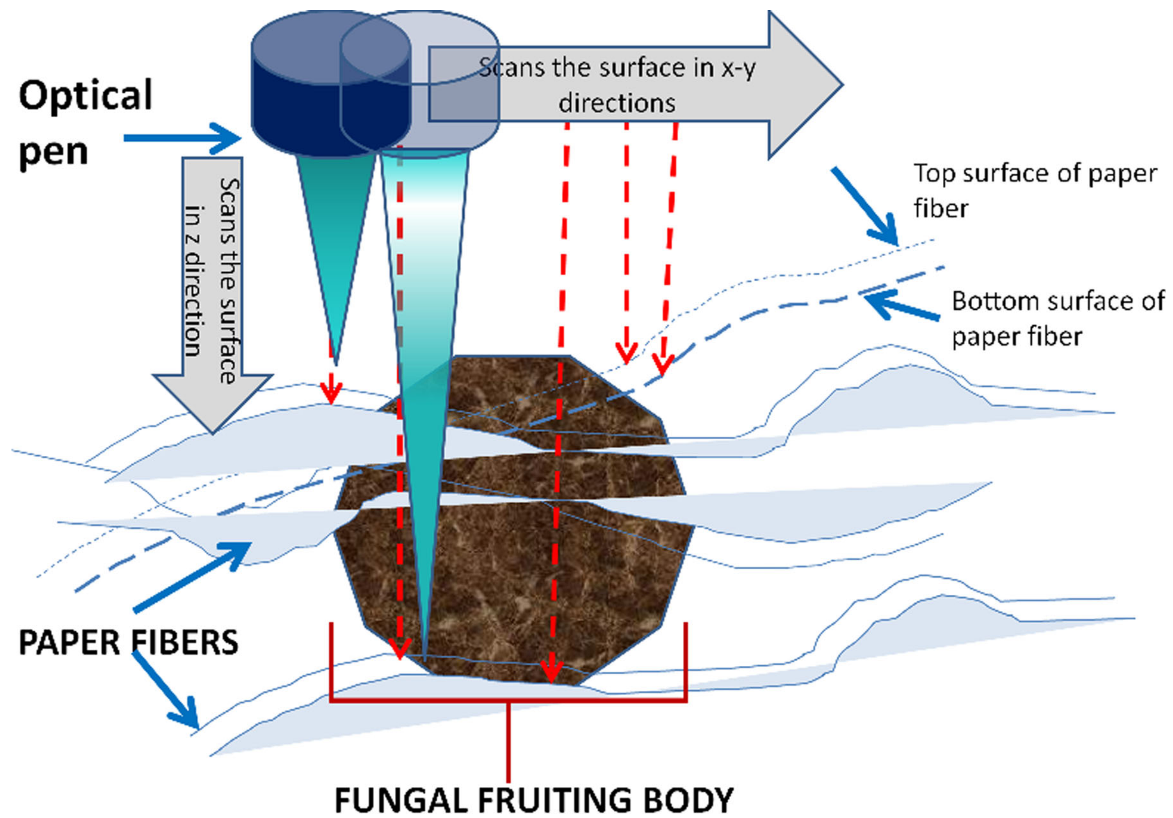

Fig 12. Optical sensor measures fungal inclusion (perithecial head) in paper matrix. Light is reflected from different planes, surface of paper fibers and surface of fungal inclusions. Pigment in fungal cells is unevenly distributed resulting in partial reflection or absorption of light from optical instrument. Absorption of light produced image indicating loss of data corresponding in shape to fungal inclusions. Partial light signals detected by the precise optical sensor of ALTIMET Altisurf500 permitted to locate the base of the inclusions thus indicating the depth of inclusions in the matrix (see details Fig. 16).

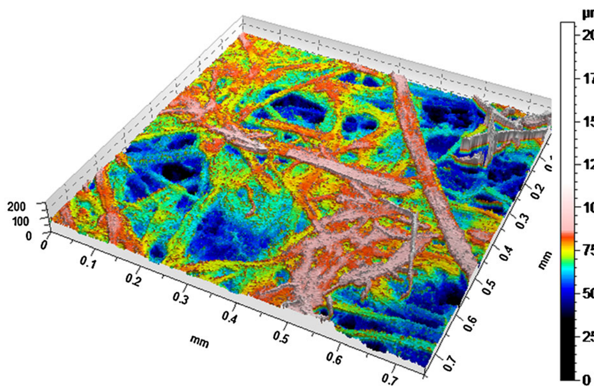

Fig 13. Stain A. Surface topography of fungal cells on paper surface, acquired with white-light, confocal chromatic aberration profilometer ALTIMET AltiSurf500. Small, spherical deposits of fungal cells of uniform height are located on and along the fibers, their altitude reaching at z 75-80μm. Measurement duration: 6h4min, spacing 1.00 μm. Area measured: x: 750μm, y: 750μm, z: 235μm.

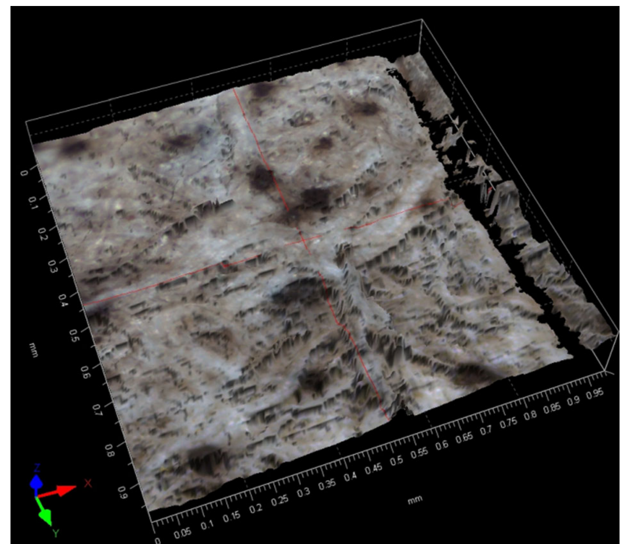

Fig 14. Stain B. 3D visualization of dark pigmented fruiting structures nested in the paper matrix. Measured are 0.95mm x 0.95mm, spacing of the acquired measurements 1μm,optical sensor size 2 μm. White light, confocal chromatic aberration profilometer ALTIMET AltiSurf500.

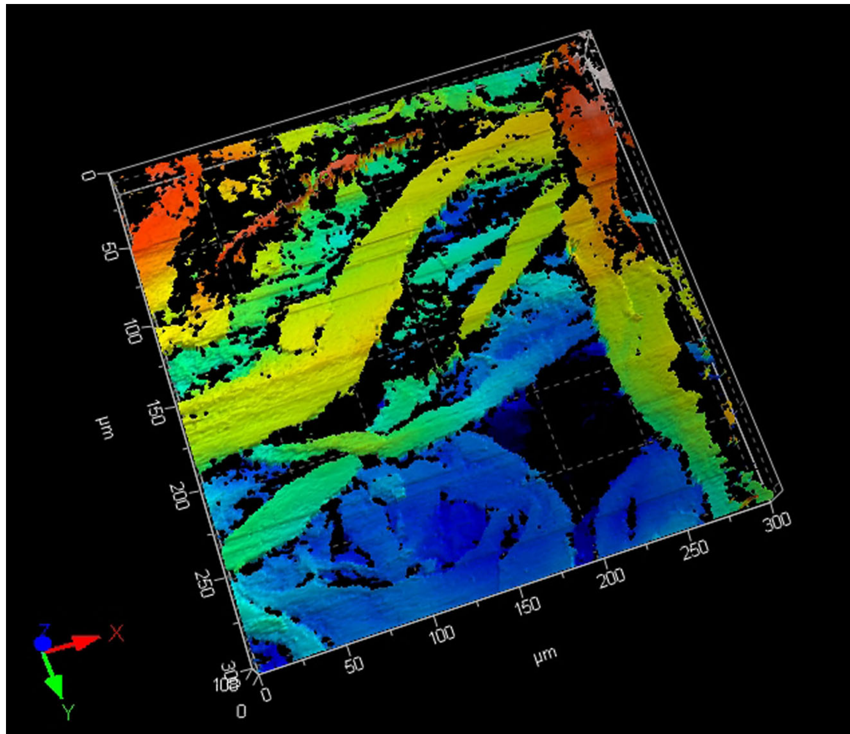

Fig 15. Stain B. Surface topography of paper with fruiting structures. Missing data, visible as black 'voids' corresponded in shape and size with fungal inclusions is attributed to the partial light absorption by melanin, black pigment in fungal fruiting structures. The shape of the 'voids' and partially missing data provided information most likely about pigment distribution, lesser amount of which permits light penetration and acquisition of data.

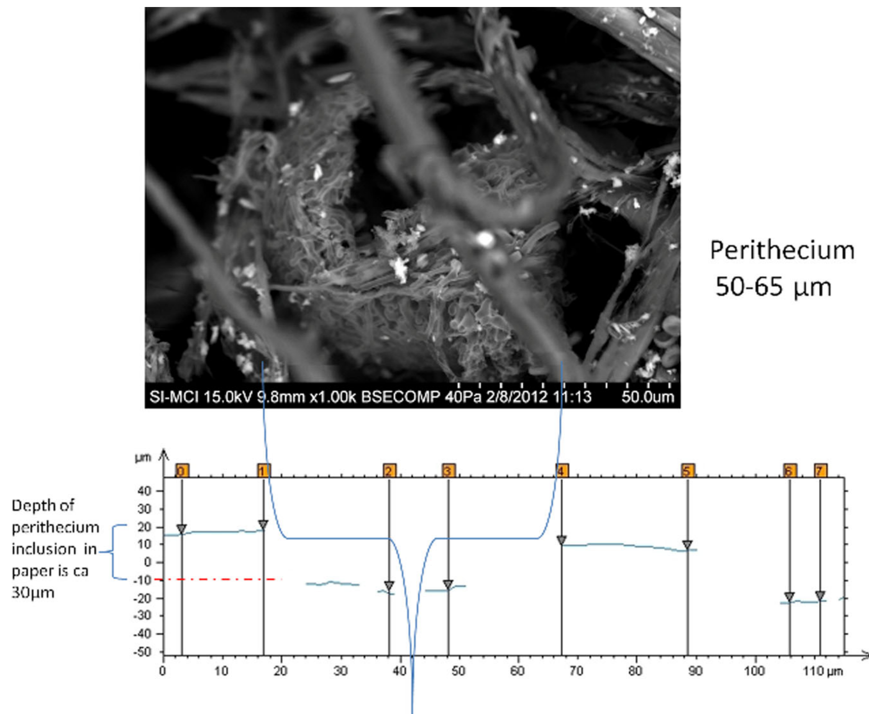

Fig 16. Stain B, close-up of the fruiting structure shown as SEM micrograph, upper part of the figure, and its profile, below the SEM micrograph. Missing points in the profile correspond with the shape and diameter of the inclusion. The obtained data indicated the depth of the inclusion in paper matrix, ca. 32 µm. The diameter of that particular fruiting structure was ca 50 µm. X-axis shows diameter, ca 50 microns of the fruiting structure, indicated by 'lack of data' in a range between 17 and 67 microns. Z-axis shows the depth the fruiting structure in the paper matrix, 32 µm. Considering that the top surface measurement is recorded at 16 µm the lowest signal where the fruiting structure is located reaches (minus) −18 µm. Note that the scale is centered, showing '0' in the middle of Z axis. It was determined based on a mean square base line displaying altitude from that line.

gained using a broad range of analytical instruments. The most puzzling observation of "voids" in 3D topography maps cannot be fully explained due to limited knowledge of fungal melanin light absorption and distribution in fungal cells (Walker *et al.*, 2010). Although this phenomena may be caused by not yet identified responses of fungal structures to light. Further research particularly in phytophotobiology may elucidate this observation.

Combining the traditional, optical light microscopy with the surface metrological techniques and instruments such as confocal laser scanning microscopy provided information about spatial inter-relations between the bio-mass and substrate, such as depth of fungal inclusions. However, many unknown behavioral patterns of microorganisms, varied environmental conditions in which they grow and diverse composition of substrates require to look at each case as a unique, multi-faceted problem. As the research continues more information will be available, elucidating this complex phenomenon of bio-mass interaction with substrates.

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