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MORPHOLOGICAL CHARACTERISTICS AND VOC CONTENT OF AGRICULTURAL SUBSTRATES

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The physical-chemical analysis of agricultural substrates is essential for optimizing fungal growth in the production of mycelium-based biomaterials. Such analyses study the metabolism efficiency of inoculated agricultural residues, enhancing fungal biomass production and the development of biomaterials used in industries like packaging, construction and even textile applications. The research paper investigated the physical morphology and Volatile Organic Compounds (VOCs) content of five agricultural substrates that will be used in future studies for manufacturing of microbial biomaterials, consisted of 2 sources of barley straw, 2 sources of wheat straw and one source of maize cobs. Microscopical investigations highlighted the specific substrates morphology and can also provide detailed insights into fungal hyphae interactions, substrate colonization, and degradation of key components like lignin and cellulose. VOC analysis highlighted the existence of hexanal compound in all samples. Hexanal in agricultural substrates acts as a volatile organic compound with antifungal properties, helping control fungal growth and post-harvest diseases. These studies help optimize conditions for fungal growth and ensure the structural properties of the final biomaterials. VOCs emitted during fungal cultivation play a significant role in substrate quality monitoring. This combined approach is especially useful in large-scale industrial applications, where efficiency, sustainability, and safety are paramount.

Keywords: biomaterials, fungi, agricultural waste

INTRODUCTION

The physical-chemical analysis of agricultural substrates for obtaining fungal biomaterials is crucial for ensuring optimal growth conditions and substrate performance in the cultivation of fungi. These analyses help to understand substrate properties and adjust them for efficient fungal metabolism, biomass production, and, ultimately, the formation of biomaterials (Yang *et al*., 2021). By optimizing the physical and chemical properties of the substrate through the above analyses, fungi can efficiently metabolize the agricultural residues to produce mycelium-based biomaterials. These biomaterials have applications in: packaging materials, construction (e.g., mycelium bricks), textiles and leather alternatives, bio-composites etc. (Picco *et al*., 2023). Microscopical investigation of agricultural substrates is an important step in the production of fungal biomaterials. It provides insights into the interaction between fungi and the substrate, helping optimize conditions for fungal growth and biomaterial formation. There are some key reasons why this investigation method is useful in the field of biomaterial: 1) Microscopic analysis allows for detailed observation of fungal hyphae (filamentous structures of the mycelium) as they colonize and degrade the substrate. It helps assess the extent of colonization, the direction of growth, and how well the fungi penetrate the substrate (Li, 2013). 2) Agricultural substrates, such as straw, wood chips, or husks, are typically composed of lignin, cellulose, and hemicellulose. Microscopic investigation can reveal how effectively the fungal enzymes are breaking down these components (Andlar *et al*., 2018). 3) Fungi can modify the microstructure of the substrate as they grow. Microscopical techniques can reveal changes in porosity, pore size, and overall

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structural arrangement as fungal colonization progresses. 4) The fungal cell wall is composed of chitin, glucans, and other polysaccharides that contribute to the mechanical properties of the fungal mycelium. Microscopic techniques like fluorescence microscopy can help visualize the accumulation of fungal biomass and the composition of cell walls (Sydor *et al*., 2022). The analysis of volatile organic compounds (VOCs) in agricultural substrates plays a significant role in the production of fungal biomaterials. VOCs are small, volatile molecules that are emitted by both the substrate and the fungal cultures because of biological and chemical processes (Kaddes *et al*., 2019). Monitoring and understanding these compounds provide insights into the substrate's quality, fungal growth, and overall biomaterial production process. Agricultural substrates used for fungal growth undergo microbial decomposition, leading to the release of various VOCs such as alcohols, aldehydes, esters, and acids. These VOCs are by-products of the breakdown of lignin, cellulose, hemicellulose, and other organic materials. Agricultural substrates can be prone to contamination by bacteria, molds, or other microorganisms that can compete with the fungi for resources. Different substrates emit varying VOCs as they decompose. By analyzing the VOCs from different agricultural residues (e.g., straw, corn stover, sawdust, or rice husk), it is possible to understand how suitable a substrate is for fungal growth (Ventura-Aguilar *et al*., 2024). The VOC profile of fungal cultures can serve as a biomarker for the quality of the fungal biomaterial. Differences in VOC emissions can reflect variations in fungal growth rates, substrate degradation efficiency, or the presence of contaminants.

MATERIALS AND METHODS

Five types of agricultural substrates were subjected to both microscopy analysis and Volatile Organic Compounds (VOC) analysis: two types of barley straws (Laverda and Smarald varieties from Ilfov county), two types of wheat straws (Glosa C1 variety from Ilfov county and Anapurna C1 variety from Giurgiu County) and one type of maize cobs (Golden West GW 2122 (FAO 330) hybrid corn from Giurgiu County) (Fig. 1).

Barley straw #1 | Barley straw #2 | Wheat straw #1 | Wheat straw #2 | Maize cobs

Figure 1. Photos of agricultural substrate samples

The agricultural samples were mechanically cleaned of impurities (by shaking), then manually cut in small pieces of 5-10cm, for better handling. The samples were analyzed as they were, without any other preparations, at room temperature (25^{o}C +/- 2^{o}C; RH between 40%-50%), without sterilization (to avoid influencing the VOC concentration in the samples).

Optical Microscopy

Microscopic analysis refers to optical, opto-electronic or electronic methods of structural analysis and surface microtopography. Morphological modifications of the morphological components of agricultural substrates as well as the interaction between biological and cellulosic and polymeric materials can be characterized by optical and electron microscopic methods, which can provide important structural information. An Olympus SZ61-TR stereomicroscope (Fig. 2.a), with an additional trinocular module (for mounting the

DSLR camera for image acquisition) and “Off the Bench” illuminator (Fig. 2.b), was used for the characterization of the substrates by optical microscopy, at 0.67x and 3x magnification, with a zoom ratio of 6.7:1 (range 0.67-4.5x).

a b

Figure 2. SZ61-TR stereomicroscope

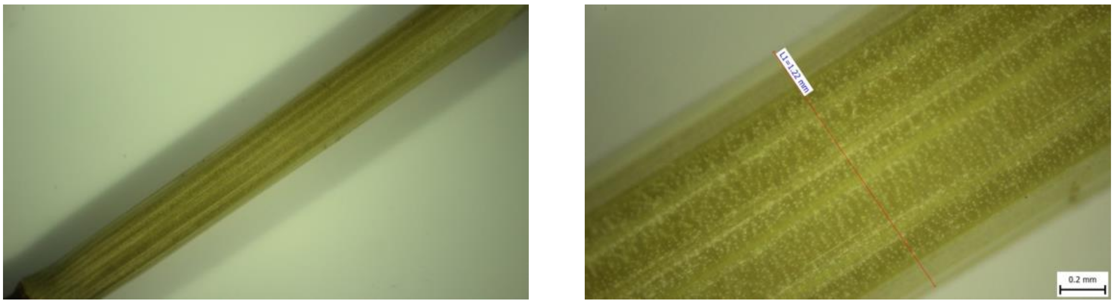
The surface morphology of the substrates was analyzed without prior preparation of the samples, and the visualization of the samples was done at 0.67x and 3x magnification using WHSZ10X-H/22 eyepieces. Image acquisition was done through a Canon EOS 1200D DSLR (18 megapixels, CMOS sensor, DIGIC image processor) and QuickPhoto Camera 3.1 image acquisition software.

Volatile Organic Compounds (VOC) Analysis

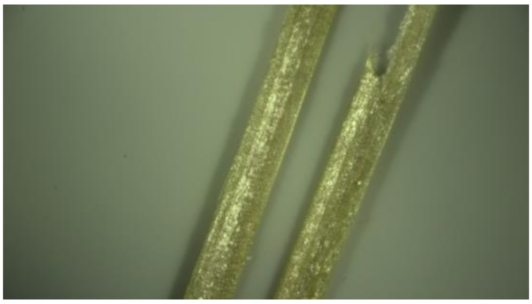
The analysis for the evaluation of volatile organic compounds was performed on the GC-MSD Agilent 6890N/ 5973 N Gas Chromatograph, headspace (HS) mode. The analysis was performed on Phenomenex Zebron™ ZB-5Msi capillary column, suitable for the analysis of pesticides, drugs, EPA methods, nitrosamines, phenols, with high selectivity, like that of 5% phenyl columns. The process parameters of the apparatus were as follows: HS parameters: vial temperature: 100°C; loop temperature: 120°C; transfer line temperature: 125°C. GC-MS parameters: capillary column: Phenomenex Zebron™ ZB-5MSi; length: 30 m; inner diameter 0.25 mm; layer thickness: 0.25 µm; injection system: splitless; injector temperature: 200°C; constant flow rate: 1 mL/min; carrier gas: helium; temperature program: 60°C to 280°C (5°C /min); injection volume: 1.0 µL; detector: MS, scan mode: 20-350 amu; auxiliary: 280°C.

RESULTS AND DISCUSSION

Microscopic analysis of the selected substrates allowed visualization of the surface morphology of each agricultural sample (Fig. 3-7).



0.67x (exterior) *3x (exterior detail)*

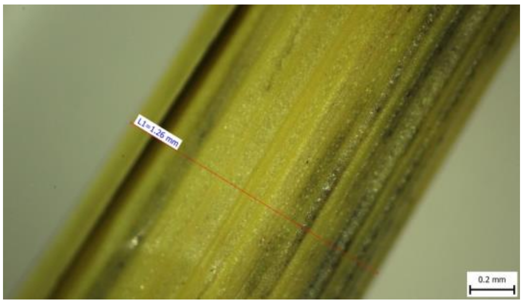


0.67x (interior)

0.67x

Figure 3. Optical microscopy barley straw (source 1)

0.67x (exterior)

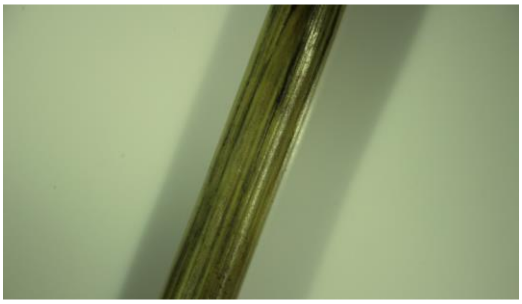


3x (exterior detail)

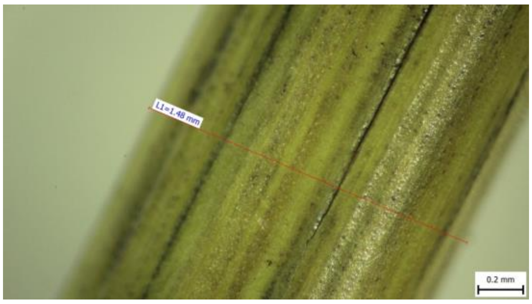
0.67x (interior)

0.67x

Figure 4. Optical microscopy barley straw (source 2)



0.67x (exterior)



3x (exterior detail)

0.67x (interior)

0.67x

Figure 5. Optical microscopy wheat straw (source 1)

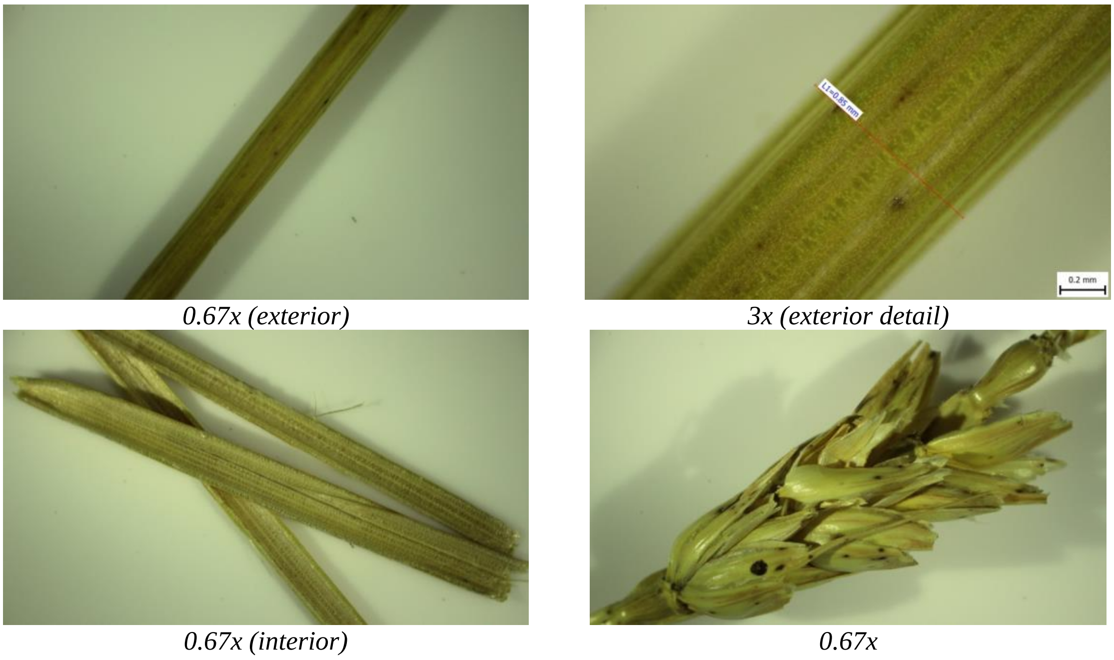


Figure 6. Optical microscopy wheat straw (source 2)

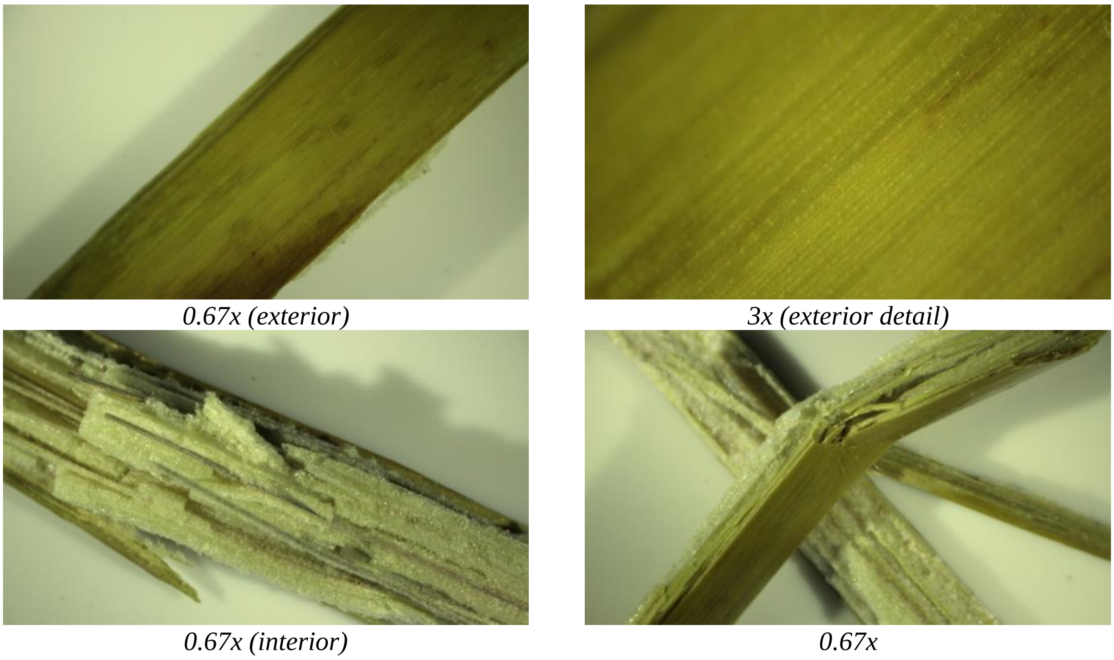


Figure 7. Optical microscopy maize cobs

The optical microscopy analyses performed on the five variants of agricultural substrates highlighted their specific surface morphology and can be used as a control tool for future experiments of inoculation of the substrates with macroscopical fungal strains. Optical microscopy shows a smooth exterior surface of the samples, which may indicate the need of a proper enzymatic digestion by the colonization strains. The substrates can also be pre-treated via physical chemical methods (treatment with $Ca(OH)_2$, cold fermentation, pre-treatment with hot water etc.), in order to increase their bio-availability, thus shorting the period needed for optimal colonization of the substrate. Also, optical microscopy is a fast and accurate method to check the development stage of the strain on the substrate, allowing to fine-tune the process of obtaining the final biomaterial demonstrator.

For future research, that will aim at using the tested substrates in obtaining microbial biomaterials, several key parameters must be considered. The substrate must be tailored to the specific type of biomaterial being produced, but the following general parameters can help ensure high productivity and efficiency: organic matter (ideal range: 40-60% dry matter); C/N ratio (ideal range: 20:1 to 30:1); acidity/alkalinity (ideal range: pH 6.0 – 7.5); moisture content (ideal range: 50-70%); water holding capacity (ideal range: high water retention with rapid drainage); nutrient availability (good traces of nitrogen, phosphorus, potassium, calcium, magnesium etc.); contaminant free (preferably, absence of heavy metals, pesticides, herbicides, pathogens – which can be removed by thermal sterilization of the substrate); thermal stability (ideal range: resistance to extreme temperature fluctuations). Another key factor is the texture and particle size, which has the purpose to support fungal growth by providing adequate surface area for nutrient exchange while maintaining good aeration and water retention.

Volatile organic compounds are a broad category of organic chemicals that have a high volatility, which means that they evaporate easily at room temperature. These compounds contain carbon and hydrogen atoms and may also include other atoms such as oxygen, nitrogen, sulphur and halogens (such as chlorine, fluorine or bromine). Among the most common examples of VOCs are well-known substances such as benzene, formaldehyde, toluene, acetone, ethanol, hexane etc. The overlapped chromatograms of the five agricultural biomass samples are showcased in Figure 8.

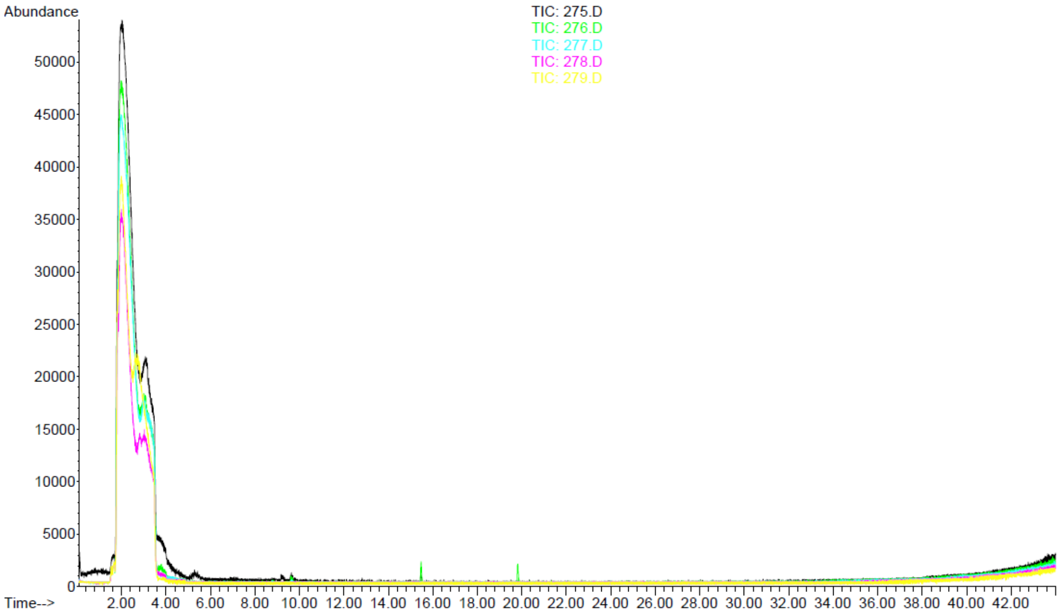


Figure 8. 275.D-barley straw #1; 276.D-barley straw #2; 277.D-wheat straw #1; 278.D-wheat straw #2; 279.D-maize cobs

The hexanal compound (Figure 9) was found in all samples, at retention time 3.80 min. It is an alkyl aldehyde, also called hexanaldehyde or caproaldehyde and is a volatile organic compound that imparts an odor similar to freshly cut grass as cis-3-hexenal (Gardini *et al.*, 1997).

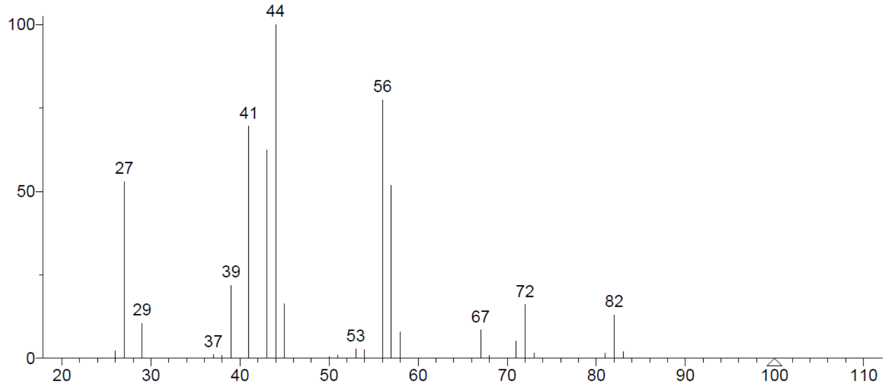


Figure 9. Hexanal mass spectra

In the literature, hexanal is described as a volatile organic compound with antifungal properties, being responsible for the reduction of post-harvest diseases (Song *et al*., 1996). The effect of hexanal on the germination of spores of *Botrytis cinerea*, *Monilinia fructicola*, *Penicillium expansum*, and on the mycelial growth of *Sclerotinia sclerotiorum*, *Alternaria alternata* and *Colletotrichum gloeosporioides* has been studied (Song *et al*., 2007). The effectiveness of hexanal in control methods is dependent on its concentration, the duration of treatment and the sensitivity of the fungal strain to hexanal vapors. Hexanal, an organic volatile agent naturally produced by plants, is commonly used as a food flavoring, and is generally known to be a safe compound. However, hexanal content does not pose an inhibitory threat to the colonization process, since the compound may be efficient against normal concentration airborne pathogens, when compared against the higher microbial concentration used in the manufacturing of biomaterials, in microbial inoculation phases. Also, one of the main steps in producing biomaterials is heat sterilization of the substrate, which helps with volatilization of parts of the compound. In previous research, the authors of the papers also obtained several prototypes based on barley, wheat and cobs substrates, using various fungal strains, and hexanal content did not limit the colonization process.

CONCLUSIONS

Microscopical investigation is a vital tool in understanding and optimizing the production of fungal biomaterials from agricultural substrates. It allows researchers and producers to monitor fungal growth, substrate degradation, hyphal interactions, and microbial contamination. By providing insights into the micro-level processes occurring during fungal cultivation, microscopical analysis enables the refinement of substrates and growth conditions, leading to high-quality, consistent biomaterials for industrial applications. VOCs play a critical role in the monitoring, optimization, and quality control of agricultural substrates used for fungal biomaterial production. Through VOC analysis, researchers and producers can ensure that the substrate is properly degraded, the fungal growth is healthy, contamination is controlled, and the final biomaterial meets the desired standards. This analysis provides a valuable tool for enhancing the efficiency and sustainability of the fungal biomaterial production process, particularly in large-scale or industrial applications (Attias *et al*., 2020). Volatile organic compounds can have adverse effects on human health, such as irritation of the eyes, nose and throat, headaches, dizziness and in severe cases, can cause damage to the liver, kidneys or central nervous system. Some VOCs are also known carcinogens. This is why it is important to determine these compounds in the selected agricultural substrates, as they are destined for composite biomaterials that must have the highest degree of biocompatibility.

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