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Research article

# Smelling the difference: separation of healthy and infected button mushrooms via microbial volatile organic compounds

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ABSTRACT

In the literature there is a lack of consensus regarding mushroom volatiles; most of the studies identify only a few volatiles. This study deals with button mushrooms, their emitted volatiles, and the main changes during infections (green mould and cobweb disease) in a time series experiment. Emitted volatile profiles were determined using HS-SPME-GC-MS coupled analytical technique. The separation of healthy and infected mushroom samples was done using different multivariate statistical methods (PCA, PLS-DA, HeatMap). The main volatile compounds were also determined. As a result, several compounds were found to successfully distinguish healthy (bisabolene, cymene, myrtenol, D-limonene, *etc.*) and infected (thujopsene, cedr-8-ene, chamigrene, patchulane, longifolene, *etc.*), mushroom samples, and an early disease detection was achieved. Results can be used for further investigation of infected mushroom identification in an early stage in packaged mushroom products. Furthermore, these results could help to identify infections in commercially available mushrooms, thus increasing shelf-life in super/hypermarkets.

## 1. Introduction

Mushrooms are consumed as a delicacy for their texture and flavour throughout the world. Consuming mushrooms helps to maintain a healthy human diet due to their high protein, essential amino acid, fiber, and low-fat content [1]. Many studies have already demonstrated that edible mushrooms serve as good sources of bioactive substances *e.g.* functional polysaccharides, terpenes, peptides, glycoproteins, mineral elements, unsaturated fatty acids, and antioxidants (vitamin E and vitamin C) [2–4]. Numerous studies introduced the benefits of mushroom consumption; antagonistic effects against viral pathogens and carcinogens improved antioxidant defence, and immune responses among many others [3,5–7]. Additionally, the nutritional characteristics of edible mushrooms can also be improved by various technological processes. Vitamin D-enhanced mushrooms are produced using artificial lightning at a certain wavelength during mushroom growing, resulting in the only non-animal food products with substantial amounts of bioavailable vitamin D [8]. Thus, mushrooms can be used both as functional food and as an ingredient in functional food products. Many cultures and civilizations around the world use wild edible mushrooms in their cuisine or traditional medicine [9] however, the white button mushroom (*Agaricus bisporus*) is the most consumed mushroom species in Western countries. Today's consumers usually buy mushrooms in super- or hypermarkets, where button mushroom is the most widespread due to the well-defined and established production technologies. Therefore, the examination of healthy and infected button mushrooms is essential, especially the

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examination of post-harvest infections to protect human health.

Button mushroom production is highly susceptible to the infections of different mould species, such as wet bubble disease (*Mycogone perniciosa*), dry bubble disease (*Lecanicillium fungicola*), cobweb disease (*Dactylium dendroides*), and green mould disease (*Trichoderma aggressivum*) [10–13]. If these harmful microorganisms appear during production, mushroom mycelium growth will be obstructed, thus reducing the average yield. One of the best tools to examine mushrooms quality is capturing and analyzing their emitted volatiles. The mushroom species show characteristic aroma profiles [14]. The typical mushroom flavour is formed by a series of aliphatic eight-carbon (C8) components, such as 1-octen-3-ol, 2-octen-1-ol, 3-octanol, 1-octanol, 1-octen-3-one, and 3-octanone. Moreover, 1-octen-3-ol is described as a "mushroom-like flavour" [15]. Despite the high consumption of mushrooms, only a few studies deal with their aroma profiles [16] and even less with the white button mushroom's aroma.

To preserve the quality of the final product, the presence of these microorganisms should be followed by sensitive analytical techniques. Detection of microbial volatile organic compounds (MVOCs) of harmful microorganisms is a useful tool for monitoring mushroom production. A sampling of emitted volatile organic compounds (VOCs) in the air has been proven to be a viable low-cost and fast method [17]. Several studies discuss the detection of different VOCs and MVOCs [18–23], although gas chromatography coupled with mass spectrometry (GC–MS) is still the most used analytical method.

Solid-phase microextraction (SPME) is so far the best and easiest solution for extracting volatiles from the air. The SPME approach was created and introduced by Arthur et al. in 1989 [24], and since then, it has undergone several improvements [25]. The extraction of volatile and semi-volatile organic compounds from environmental, biological, and food samples using SPME sampling in combination with GC-MS has now become a successfully applied routine approach [26–28]. Several papers have been already published dealing with the analysis of MVOCs using a headspace SPME (HS-SPME) sampling system [20,29–33].

The main objective of this study was to describe the similarities and differences between the MVOC profiles of *Agaricus bisporus* and its two main diseases; green mould and cobweb disease. Furthermore, temporal analysis of the MVOC profile changes was aimed to describe the evolution of diseases.

## 2. Materials and methods

### 2.1. Sample preparation

Button mushroom (*Agaricus bisporus*) obtained from a local producer was infected with two mould-related diseases: *Trichoderma aggressivum* (green mould disease) and *Dactylium dendroides* (cobweb disease). Fungal strains were obtained from the culture collection of the Hungarian University of Agriculture and Life Sciences, Department of Vegetable and Mushroom Growing. Moulds were harvested from potato-dextrose agar (PDA, 39 g $L^{-1}$) under a sterile box. Sterile water was used to prepare spore suspension ($10^6$). *Agaricus bisporus* samples were placed into a sterile container having 0.5 L airspace, and then 500 μl spore suspension was pipetted to the sample surface. Control samples were prepared by pipetting 500 μl of sterile water on the surface of mushroom samples. All infections were done in triplicate. Afterwards, containers were immediately closed. Two holes were drilled into the sample containers. One was closed with a septum, the other was stuffed with sterile cotton. Septum enabled sampling without opening the container, while sterile cotton provided an aerobe environment in a "semi-closed" container. The day of infection was set as the zero point of the measurements. Sample collection was carried out for eight days, on the 1st, 2nd, 3rd, 4th, 5th 8th days after infection. On the 2nd, 3rd, 5th' and 8th days sampling were implemented in the mornings and afternoons also. In the following K denotes the control sample (*A. bisporus*), T denotes mushroom infected with *T. aggressivum*, and D denotes mushroom infected with *D. dendroides*. Numbers (1-5, and 8) mark the infection days. As an example, 5T denotes a mushroom infected with *T. aggressivum* measured on day 5. Alkane standard solution ($C_8$–$C_{20}$) (Merck, Germany) was used to calculate the Kováts retention index (RI). All measures were done in triplicate.

### 2.2. Analytical measurements

The headspace solid-phase microextraction (HS-SPME) technique was used to capture the volatiles from the air above the mushroom samples. 65 μm Stable Flex™ polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber was used to adsorb volatile compounds. Prior to its initial usage, a bi-polar PDMS/DVB coated silica fiber (Supelco, 65 μm partially crosslinked, 24 gauge) was conditioned at 250 °C for 30 min. Samplings were performed through a septum. All samples were kept at a constant temperature of 23 °C, and the extraction process was 20 min.

A non-polar HP-5MS ((5 %-phenyl)-methylpolysiloxane; 30 m, 0.25 mm i.d., 0.25 μm film, Agilent Technologies) column was utilized in conjunction with an Agilent 6890 Gas Chromatograph (GC) coupled with a 5975 C MSD Mass Spectrometer (MS). An optimized oven temperature program was used [37]. The oven temperature program started at 50 °C and increased from there to 120 °C at 20 °C $min^{-1}$, then to 170 °C at 40 °C $min^{-1}$, to 190 °C at 25 °C $min^{-1}$, to 280 °C at 40 °C $min^{-1}$, and ultimately to 300 °C (2 min hold) at 50 °C $min^{-1}$. The inlet temperature was held constant at 250 °C during the measurements. Hydrogen gas was used as carrier gas with a constant 1.2 mL $min^{-1}$ flow. In the MS instrument, the ionsource temperature was set to 230 °C while the quadrupole temperature was held at 150 °C. Positive electron ionization (EI+) was used, with an electron energy level of 70 eV. The detector was operated in scan mode for values between 33 and 500 *m/z*. Perfluorotributylamine (PFTB) was used each day before the measurements to tune the MS instrument. The GC and MS parameters were controlled with Agilent Enhanced MSD ChemStation software. Agilent MassHunter Workstation Qualitative Analysis B.08.00 software was used to evaluate and compare the chromatograms. For compound identification Agilent NIST 2017 Mass Spectral Library was used. The identification results were additionally verified using two other libraries (W9N08 and W10N11). Each measures were done in triplicate.

**Table 1**
List of emitted volatiles of *Agaricus bisporus* and its two main diseases. MW: molecular weight in Da, MF: average match factor in % according to NIST libraries, n.d.: no data found in NIST libraries, K: control mushroom, Ta: mushroom infected with *Trichoderma aggressivum*, Da: mushroom infected with *Dactylium dendroides*. K, Ta, Da columns show the summarized changes (tendency) during the examined time period (day 2, day 5 and day 8).

| RI lit | $RI_{measured}$ | Compound | Formula | CAS number | MW | MF | K | Ta | Da |
|---|---|---|---|---|---|---|---|---|---|
| **Volatile changes of *T. aggressivum*** | | | | | | | | | |
| **Increasing tendency** | | | | | | | | | |
| n.d. | 877 | E,E-2,4-Octadien-1-ol | $C_8H_{14}O$ | 69668-94-6 | 126 | 86.1 | | | |
| **Maximum at day 5** | | | | | | | | | |
| 800 | 797 | Octane | $C_8H_{18}$ | 111-65-9 | 114 | 86.1 | | | |
| 900 | 893 | Nonane | $C_9H_{20}$ | 111-84-2 | 128 | 72.0 | | | |
| 1000 | 999 | Decane | $C_{10}H_{22}$ | 124-18-5 | 142 | 70.0 | | | |
| 1030 | 1018 | D-Limonene | $C_{10}H_{16}$ | 5989-27-5 | 136 | 80.0 | | | |
| 1324 | 1311 | Megastigma-4,6(Z),8(Z)-triene | $C_{13}H_{20}$ | 71186-25-9 | 176 | 82.0 | | | |
| 1405 | 1395 | Longifolene | $C_{15}H_{24}$ | 475-20-7 | 204 | 78.6 | | | |
| 1411 | 1399 | Cedr-8-ene | $C_{15}H_{24}$ | 469-61-4 | 204 | 86.1 | | | |
| 1428 | 1419 | Cedr-8(15)-ene (β-Cedrene) | $C_{15}H_{24}$ | 546-28-1 | 204 | 86.0 | | | |
| 1429 | 1420 | cis-(-)-Thujopsene | $C_{15}H_{24}$ | 470-40-6 | 204 | 65.6 | | | |
| 1445 | 1440 | β-Elemene | $C_{15}H_{24}$ | 515-13-9 | 204 | 78.1 | | | |
| n.d. | 1448 | 2-Pentanone, 4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]hept-2-yl)- | $C_{14}H_{24}O_2$ | 97306-61-1 | 224 | 73.3 | | | |
| 1461 | 1457 | Isocaryophyllene | $C_{15}H_{24}$ | 999191-48-7 | 204 | 91.0 | | | |
| 1474 | 1462 | β-Cadinene | $C_{15}H_{24}$ | 523-47-7 | 204 | 77.6 | | | |
| n.d. | 1465 | Patchulane | $C_{15}H_{26}$ | 19078-35-4 | 206 | 80.0 | | | |
| 1479 | 1469 | β-Guaiene | $C_{15}H_{24}$ | 88-84-6 | 204 | 88.0 | | | |
| 1485 | 1470 | Aromadendrane | $C_{15}H_{26}$ | 6790-78-9 | 206 | 75.7 | | | |
| 1508 | 1489 | α-Chamigrene | $C_{15}H_{24}$ | 19912-83-5 | 204 | 78.4 | | | |
| 1524 | 1509 | (+)-δ-Cadinene | $C_{15}H_{24}$ | 483-76-1 | 204 | 83.7 | | | |
| 1552 | 1543 | Isopatchoulane | $C_{15}H_{26}$ | 3724-42-3 | 206 | 73.0 | | | |
| **Decreasing tendency** | | | | | | | | | |
| 1165 | 1157 | Isomenthone | $C_{10}H_{18}O$ | 491-07-6 | 154 | 66.8 | | | |
| **Volatile changes in diseases (both *T. aggressivum*, *D. dendroides*)** | | | | | | | | | |
| **Increasing tendency** | | | | | | | | | |
| 730 | 723 | Disulfide, dimethyl | $C_2H_6S_2$ | 624-92-0 | 94 | 97.4 | | | |
| 773 | 765 | Toluene | $C_7H_8$ | 108-88-3 | 92 | 83.0 | | | |
| 820 | 809 | 1,3-Octadiene | $C_8H_{14}$ | 1002-33-1 | 110 | 94.0 | | | |
| 840 | 827 | 4-Octyne | $C_8H_{14}$ | 1942-45-6 | 110 | 85.3 | | | |
| 889 | 879 | 1-Nonene | $C_9H_{18}$ | 124-11-8 | 126 | 76.7 | | | |
| 890 | 885 | Styrene | $C_8H_8$ | 100-42-5 | 104 | 95.0 | | | |
| 920 | 911 | Anisole (Benzene, methoxy-) | $C_7H_8O$ | 100-66-3 | 108 | 72.6 | | | |
| n.d. | 921 | Octa-2,4,6-triene | $C_8H_{16}O$ | 928-68-7 | 128 | 68.0 | | | |
| 957 | 945 | 2-Heptanone-6-methyl | $C_8H_{16}O$ | 22104-78-5 | 128 | 85.7 | | | |
| 1067 | 1048 | 2-Octen-1-ol | $C_9H_{14}S$ | 4861-58-9 | 154 | 88.0 | | | |
| **Decreasing tendency** | | | | | | | | | |
| 1023 | 1006 | m-Cymene | $C_{10}H_{14}$ | 535-77-3 | 134 | 80.3 | | | |
| n.d. | 1004 | 2-Decen-1-ol, (E)- | $C_{10}H_{20}O$ | 18409-18-2 | 156 | 82.0 | | | |
| 1065 | 1051 | Acetophenone | $C_8H_8O$ | 98-86-2 | 120 | 74.6 | | | |
| 1300 | 1289 | Tridecane | $C_{13}H_{28}$ | 629-50-5 | 148 | 71.0 | | | |
| n.d. | 1443 | 4-(2,2-Dimethyl-6-methylenecyclohexylidene)-3-methylbutan-2-one | $C_{14}H_{22}O$ | 93175-74-7 | 206 | 70.0 | | | |
| 1444 | 1451 | β-Caryophyllene | $C_{15}H_{24}$ | 87-44-5 | 204 | 71.0 | | | |
| 1508 | 1490 | cis-α-Bisabolene | $C_{15}H_{24}$ | 29837-07-8 | 204 | 70.0 | | | |
| 2108 | 2089 | Bisphenol A | $C_{15}H_{16}O_2$ | 80-05-7 | 228 | 86,2 | | | |
| - | 2075 | Unidentified compound1 | $C_{12}H_{26}O_2$ | | 202 | | | | |
| - | 2080 | Unidentified compound2 | $C_{12}H_{26}O_2$ | | 202 | | | | |
| **Volatile changes of *D. dendroides*** | | | | | | | | | |
| **Increasing tendency** | | | | | | | | | |
| 848 | 832 | 2-Hexanone, 4-methyl- | $C_7H_{14}O$ | 105-42-0 | 114 | 88.6 | | | |
| 911 | 902 | 2-Nonene | $C_9H_{18}$ | 6434-77-1 | 126 | 70.0 | | | |
| 986 | 973 | 3-Octanone | $C_8H_{16}O$ | 106-68-3 | 128 | 81.6 | | | |
| 1036 | 1031 | Cyclooctane | $C_8H_{16}$ | 292-64-8 | 136 | 75.0 | | | |
| n.d. | 1062 | 3-Heptanone, 5-ethyl-4-methyl- | $C_{10}H_{20}O$ | 27607-63-2 | 156 | 68.0 | | | |
| 1090 | 1079 | 2,4-Heptadienal, 2,4-dimethyl- | $C_9H_{14}O$ | 42452-48-2 | 138 | 76.2 | | | |
| 1091 | 1081 | 1-Undecene | $C_{11}H_{22}$ | 821-95-4 | 154 | 92.3 | | | |
| 1092 | 1085 | 5-Undecene | $C_{11}H_{22}$ | 4941-53-1 | 154 | 81.8 | | | |
| 1100 | 1095 | Undecane | $C_{11}H_{24}$ | 1120-21-4 | 156 | 92.3 | | | |
| 1113 | 1099 | 1,4-Undecadiene, (E)- | $C_{11}H_{20}$ | 55976-13-1 | 152 | 88.5 | | | |
| 1123 | 1114 | 3-Undecene | $C_{11}H_{22}$ | 60669-40-1 | 154 | 80.5 | | | |
| 1190 | 1172 | 1-Dodecene | $C_{12}H_{24}$ | 112-41-4 | 168 | 86.4 | | | |
| **Decreasing tendency** | | | | | | | | | |
| 850 | 843 | Ethylbenzene | $C_8H_{10}$ | 100-41-4 | 106 | 86.2 | | | |
| 889 | 875 | o-Xylene | $C_8H_{10}$ | 95-47-6 | 106 | 79.9 | | | |
| 1190 | 1175 | Myrtenol | $C_{10}H_{16}O$ | 515-00-4 | 152 | 67.0 | | | |
| **Minimum at day 5** | | | | | | | | | |
| 1190 | 1181 | 4-propylanisole (Benzene, 1-methoxy-4-propyl-) | $C_{10}H_{14}O$ | 104-45-0 | 150 | 83.9 | | | |
| 1200 | 1196 | Dodecane | $C_{12}H_{26}$ | 112-40-3 | 170 | 72.3 | | | |

### 2.3. Statistical analysis

Principal component analysis (PCA) was applied to reduce the dimensionality of the input data. PCA is primarily a dimension reduction technique that takes the original input variables (intensity values of the measured compounds) and compresses them into a smaller number of uncorrelated principal components (PCs). The created PCs then can be used to visualize the results on scores plots (representing the row vectors, or samples) and loading plots (representing the column vectors, or components). BiPlots are also widely used to present the information of scores and loading plots at the same time; hence the connections between the samples and components can be analyzed more easily. The created PCs can also be used as input variables to other statistical techniques, such as classification algorithms. Classification methods can create models describing the differences in predefined classes (infected *vs.* control). Partial least squares discriminant analysis (PLS-DA) was used to classify the samples and to prove if the intensity changes of the samples can be indicative of the elapsed time from the infection of the samples. Additionally, the connections among the samples and compounds were plotted by HeatMap which uses correlation analysis and agglomerative hierarchical clustering to group the components increasing or decreasing over time. All calculations were done using XL-STAT 2019.1.3 software (Addinsoft, New York, USA) [34].

## 3. Results

### 3.1. Volatile compositions

Volatile organic compounds of *Agaricus bisporus* and its two main diseases (*Trichoderma aggressivum* and *Dactylium dendroides*) were identified by comparing their mass spectra to standard mass spectra from the National Institute of Standards and Technology MS spectral library (NIST17). During the evaluation process, 104 compounds were found. Thereafter, false-positive hits were excluded, and finally, 82 compounds were picked up (SM_1.). From these, 70 compounds were successfully identified using the background correction process. Then the obtained background-free mass spectrum was identified using the NIST MS Search library. Compounds having at least 60% (ID % $\geq$ 60%) match factor value were accepted only when they were present in at least ten parallel measures. Exceptions were made for those compounds appearing only at the beginning or at the end of the examined time period. Table 1 shows the main temporal changes of compounds over the experiment and SM_2 indicates the significant differences between control (healthy) and infected samples. Significant differences between control samples and infected samples were tested by Wilcoxon matched pair statistical test ($\alpha = 0.05$) and marked with bold letters. In general, SPME sampling method can be characterized by a relatively high standard deviation value. Therefore there are only a few cases where significant differences can be found between healthy and infected samples, mostly on the 5th and 8th days. However, the tendency (increasing, decreasing) can be clearly seen from the results (Table 1 column K, Ta, Da).

*Trichoderma aggressivum* and *Dactylium dendroides* both caused brown spots on the mushroom surface. After a while, both infections caused small mouldy spots on the cap, then *T. aggressivum* produced green (Figure 1.), while *D. dendroides* produced white/grey spores at the end of the examined period. Two different mould-growing phases were determined; mycelium growing phase and spore production phase. Volatiles appearing and/or increasing until the 5th day was considered as compounds related to the mycelium growing phase, while volatiles showing a continuous increasing tendency until the end of the examined period (8th day) or appearing on the 8th day were considered as compounds related to spore production. According to these developmental phases, 15 compounds may refer to *T. aggressivum*, while 27 compounds may refer to *D. dendroides* sporulation. There were 14 common volatile compounds found that increased during the examined period. These compounds may predict the presence of infections. In the case of *T. aggressivum,* 18 compounds showed increasing intensity until day 5, then changed their tendency and started to decrease. The majority of these compounds are sesquiterpenes (longifolene, cedr-8-ene, cedr-8(15)ene, *cis*-(−)thujopsene, β-elemene, isocaryophyllene, β-cadinene,


**Figure 1.** *Trichoderma aggressivum* (green mould infection) on day 5 and day 10. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

β-guaiene, α-chamigrene, (+)-δ-cadiene), and were found to be produced by moulds. These 14 common volatile compounds were also linked to the mycelium growing phase. A limited number of compounds showed decreased intensity values during the examined period possibly due to transformation and/or decomposition caused by mould activity. On the other hand, infections might have affected the normal mushroom metabolism, therefore changing the normal volatile pattern of *Agaricus bisporus*, too.

### 3.2. Data analysis

Measurements were done for eight days, with three parallels on the first day, and six parallels on the other sampling days in the case of all 82 compounds, thus the obtained data enabled the use of multivariate statistical methods. Principal Component Analysis (PCA) was applied to determine relevant compounds, while Partial Least Squares- Differentiation Analysis (PLS-DA) distinguished the samples and HeatMap showed temporal relationships among samples and volatiles.

#### 3.2.1. Principal Component Analysis (PCA) – determination of relevant compounds

PCA was run to reduce dimensionality and scores plots were created to see whether there are any connections between samples and

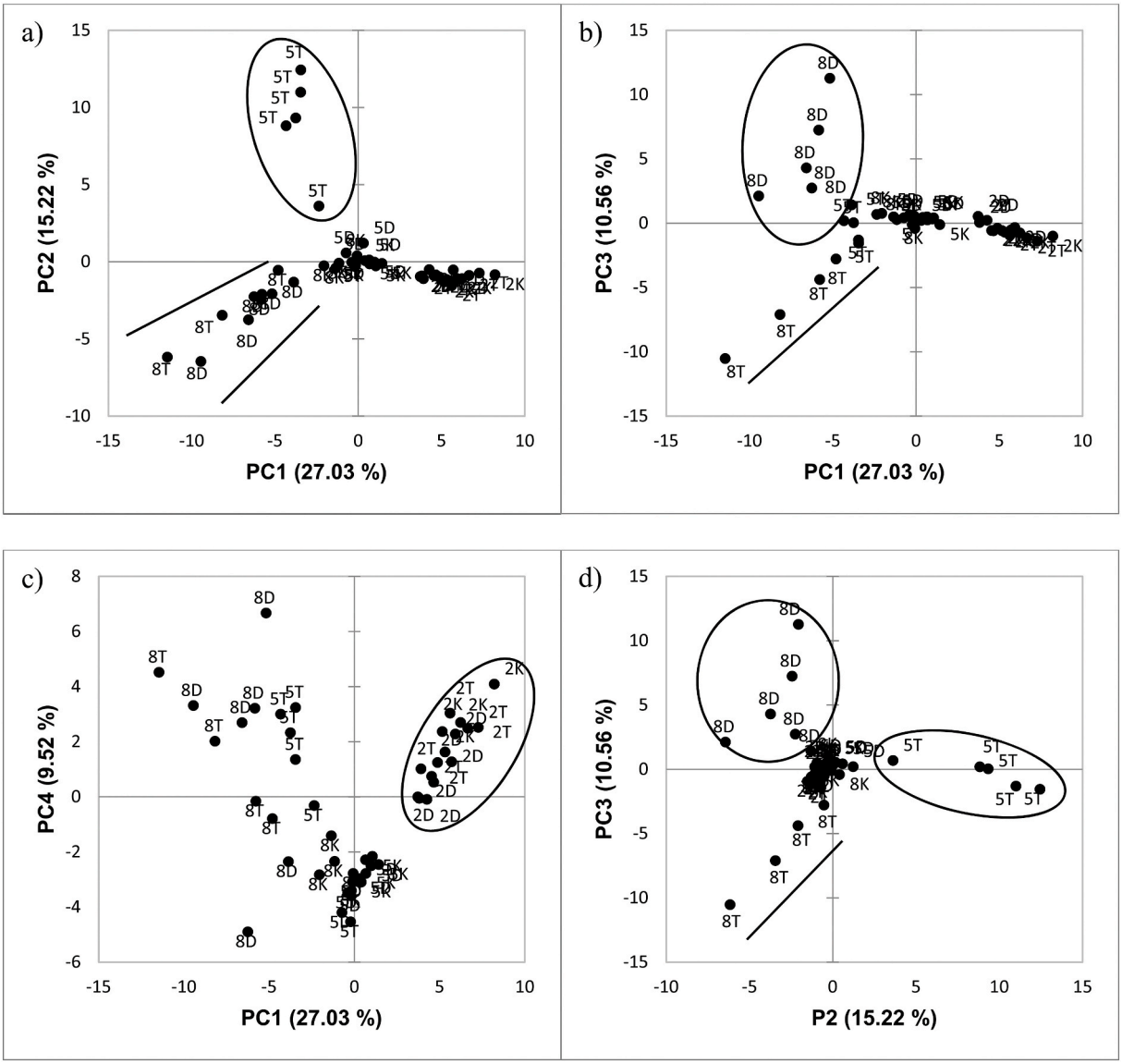


**Figure 2.** PCA scores plots of the first four principal components (PCs). a) PC1 and PC2 - explained variance 42.2%, b) PC1 and PC2 - explained variance 37.59%, c) PC1 and PC4 - explained variance 36.54%, and d) PC2 and PC3 - explained variance 25.78%. K: control (*A. bisporus*), T: mushroom infected with *T. aggressivum*, D: mushroom infected with *D. dendroides*. 2, 5, and 8 mark the infection days. (for example, 5T denotes a mushroom infected with *T. aggressivum*, measured on day 5).

sampling days. Detailed analysis of the created PCs shows the volatile components affecting the differences. Figure 2 shows the first four principal component score plots results.

PC1 has a main effect on distinguishing sampling days (Figure 2 a,b,c). Among others acetophenone, *p*-aminotoluene, β-caryophyllene, 4-hydroxy-β-ionone, *cis*-α-bisabolene, and 1,4-dibutoxybutane compounds affect the separation of day 2, while 1,3-octadiene, 1-nonene, (+)-δ-cadinene and 4-(2,2-dimethyl-6-methylenecyclohexylidene)-3-methylbutan-2-one are responsible for different positions of day 8 along PC1.

PC2 defines variables explaining differences between 5T and 8T samples (Figure 2 a,d). Megastigma-4,6(Z),8(Z)-triene, *cis*-(−)-thujopsene, β-cadinene, patchulane, isocaryophyllene, cedr-8-ene, β-guaiene, aromadendrane, α-chamigrene, and 2-pentanone, 4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]hept-2-yl)- play a key role to differentiate 5T from others. These compounds may refer to the mycelium growth phase of *T. aggressivum* as presented in Table 1.

PC3 is composed of variables explaining the differences between mushrooms infected by *T. aggressivum* and *D. dendroides* on day 8 (Figure 2 b,d). 2-nonene, decane, 3-undecene, undecane, and 5-undecene explained the differentiation of 8D samples, which results are in accordance with Table 1, namely these compounds' intensity values jumped at the end of the examined period. Similar results were found in the case of *T. aggressivum* infected samples; E,E−2,4-octadien-1-ol increased continuously on day 8, therefore it has a main effect to separate 8T along PC3. Surprisingly, two compounds (2-pentylthiophene and oxime-, methoxy-phenyl-) increased in the case of both diseases, although these differentiate only *T. aggressivum* infection and do not show any role in the case of *D. dendroides* infection.

### 3.2.2. *Partial Least Squares Discriminant Analysis (PLS-DA) – distinguishing different groups*

Partial Least Squares Discriminant Analysis was used to create groups and discriminate control samples and different diseases based on PCs defined by PCA. First of all, PLS-DA was run to discriminate sampling days (Figure 3a). 5K and 8K are located close to 2K, 2T, and 2D, meaning that control samples did not change significantly during the examined time period. According to these results,

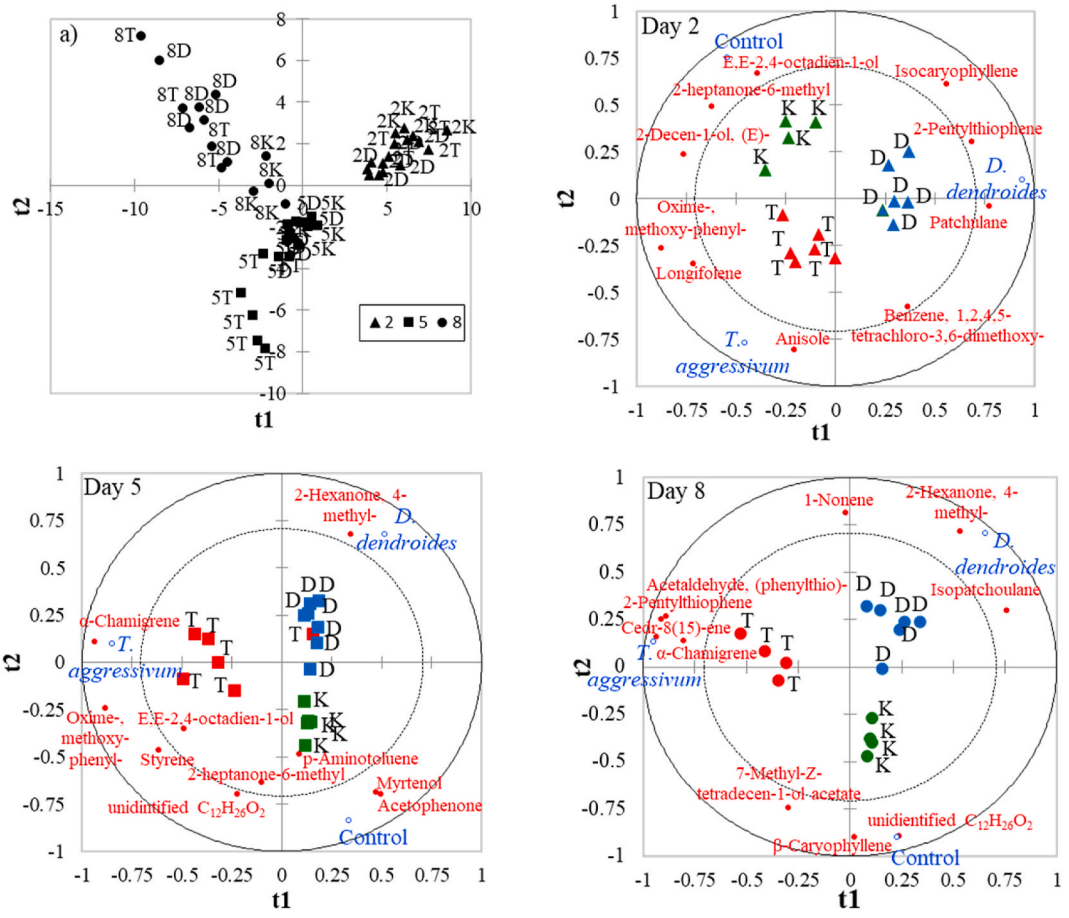


**Figure 3.** Partial Least Squares Discriminant Analysis results of different days and samples. a) shows discrimination of days: day 2, day 5, and day 8., while b), c), and d) shows discrimination of samples on day 2 (b), day 5 (c), and day 8 (d). 2: samples from day 2 (marked with triangles), 5: samples from day 5 (marked with squares), 8: samples from day 8 (marked with dots). K: control *Agaricus bisporus* samples, T: mushroom samples infected with *T. aggressivum*, D: mushroom samples infected with *D. dendroides*.

differences between days exist, therefore the next step is to examine whether samples (control and infected) can be discriminated against within a given day.

According to PLS-DA results, uninfected samples were placed in different groups on day 2. One 5T sample has been misclassified possibly due to the slower evolution of infection at day 5. Finally, samples on day 8 showed 100% discrimination (Figure. 3., Day 8), additionally, within-group distances tend to decrease as time passes. It means, that compounds such as anisole, isocaryophyllene, benzene, 1,2,4,5-tetrachloro-3,6-dimethoxy-, longifolene, patchulane, 2-decen-1-ol, (E)- distinguish control samples from infected (*T. aggressivum*, *D. dendroides*) samples at a very early stage of infections. Certain compounds play a key role in the discrimination of even two days. For example, oxime-, methoxy-phenyl-, E,E−2,4-octadien-1-ol, and 2-heptanone-6-methyl were found in the first ten most discriminating volatiles at day 2 and day 5. α-chamigrene and 2-hexanone, 4-methyl- were found on day 5 and day 8, while only 2-penthylthiophene was found on day 2 and day 8.

### 3.3. HeatMap – temporal relationships among samples and volatiles

The main hypothesis was that volatiles from control samples will not or just slightly change over time, while volatiles from infected samples will significantly change. To visualize the relationship among samples and volatile compounds over time, HeatMap was created based on the original 82 compounds (Figure 4).

HeatMap uses colours to indicate correlations between samples and volatiles. The green colour indicates strong positive, while the red colour indicates strong negative correlations between samples and volatile compounds. HeatMap runs cluster analysis to cluster samples and volatiles presented on the top and the left side in Figure 4, respectively. Based on the obtained pattern, three main sections were determined.

In the first section (S1), the correlation is changing among samples and volatiles during the examined time period. Samples on the first day form one cluster, as they show strong positive correlations with many volatiles (*e.g.* β-caryophyllene, *m*-cymene, D-limonene). However, as time passes these positive correlations change, and a strong negative correlation was found on the eighth day. Most of these compounds have a decreasing tendency according to Table 1. To sum up, the presence and high intensities of the compounds located in S1 indicate that the infection is in an early stage.

The right side of the second section (S2) presents a huge green spot (Figure 4.). This means that compounds located here show a strong positive correlation with infected samples at day 8 (8T, 8D) creating the group of compounds indicating an advanced stage of

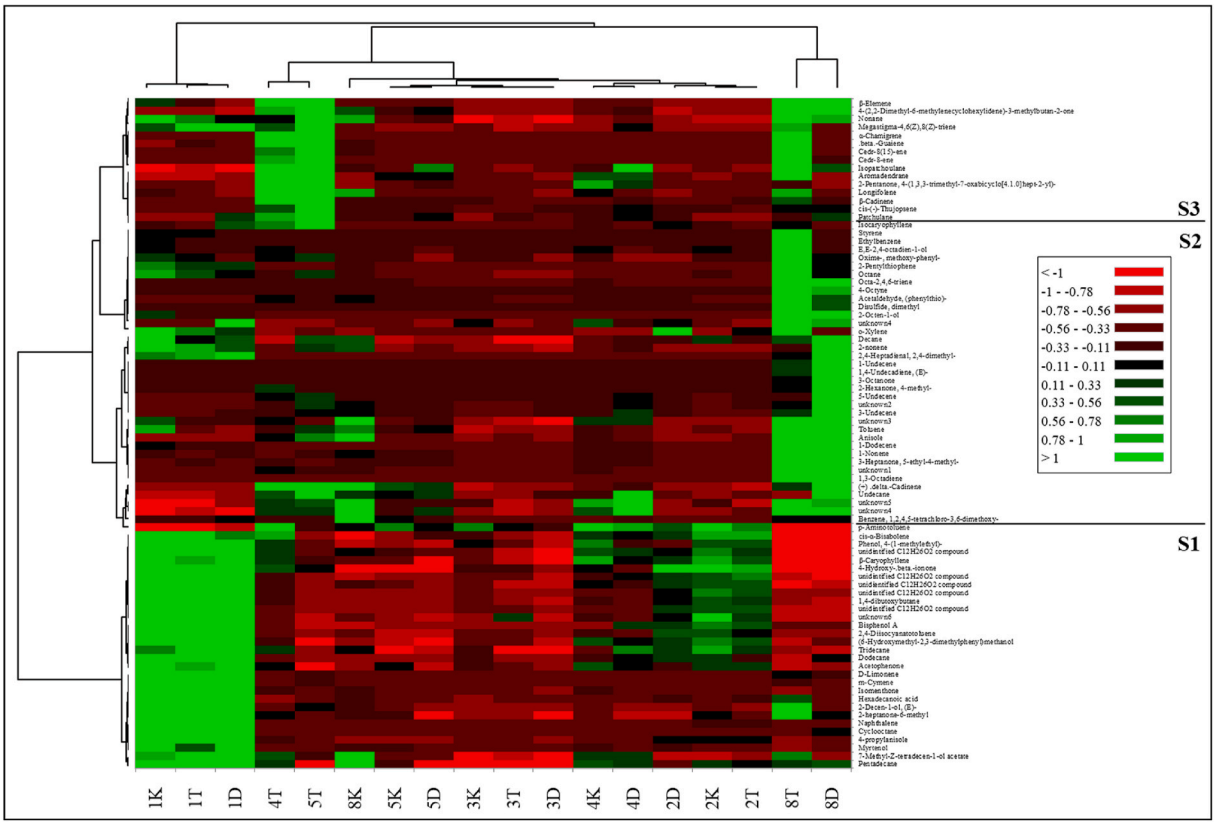


**Figure 4.** HeatMap visualizing temporal relationships among samples and volatiles on different days. K: control (*A. bisporus*), T: mushroom infected with *T. aggressivum*, D: mushroom infected with *D. dendroides*. 2, 5 and 8 mark the infection days. S1, S2 and S3 show different sections.

infection. Section 2 (S2) includes compounds such as styrene, octa-2,4,6-triene, 4-octyne, 2-octen-1-ol, decane, 2-nonene, 2,4-heptadienal, 2,4-dimethyl-, 1-undecene, 1,4-undecadiene, (E)-, 3-octanone, 2-hexanone, 4-methyl-, 5-undecene, 3-undecene, 1-dodecene, 1-nonene, 1,3-octadiene, undecane, octane, (+)-δ-cadinene, ethylbenzene, o-xylene, toluene, and others. Most of these compounds also have a continuously increasing tendency in time according to Table 1. To sum up, the presence and high intensities of the compounds located on the right side of S2 indicate the sporulation of green and cobweb moulds.

The third section (S3) includes volatiles having a strong positive correlation with green mould-infected samples (4T, 5T, 8T). There is a strong positive correlation among β-elemene, nonane, α-chamigrene, β-guaiene, cedr-8-ene, cedr-8(15)-ene, isopatchoulane, longifolene, β-cadinene, *cis*-(−)-thujopsene, patchulane, isocaryophyllene and samples infected with *T. aggressivum*. The above-mentioned compounds have been determined as green mould mycelium indicators. To sum up, the presence of the compounds located in S3 indicates the presence of green mould disease on mushrooms.

## 4. Discussion

104 compounds were found during the experiment out of which 70 compounds were successfully identified. Several of them have been already described in the literature as mushroom volatiles. D-limonene has a citrus, mint, and fruity-like odour and has been already found in 11 edible mushroom species using the HS-SPME method [35,36]. Additionally, our earlier study also identified D-limonene as a biomarker of green mould disease [37]. There are contradictory results about 3-octanone, which has a musty, mushroom, and herbal-like odour. Some authors described 3-octanone in *Cantharellus cibarius* and *Boletus edulis* species but were not able to find it in *A. bisporus* [35]. On the other hand, Cronin et al. described 3-octanone only in *Amanita rubescens* but not in *C. cibarius* and *B. edulis* [36]. Additionally, Radványi et al. found 3-octanone in only *A. bisporus* mycelium [37], while Costa et al. found 3-octanone as the main volatile compound of chopped mushroom [38]. Supposedly, the presence of 3-octanone could mean the degradation or damage of the mushroom surface. Interestingly, 3-octanone was also found as a marker compound of *Trichoderma atroviride* [39]. Based on these, 3-octanone seems to be an infection-indicator compound in the case of *Agaricus bisporus*.

2-octen-1-ol has already been described as a main eight-carbon compound in mushroom (*A. bisporus*) and mushroom food dressings but is also found as a main volatile in several edible mushroom species [10,38].

## 5. Conclusion

*Agaricus bisporus* production is a very mould-sensitive process. If the mushroom is infected during production, it could mean a serious problem during the sale. Therefore, infections must be monitored not only in the case of mushroom production but also during post-harvest processes. To achieve this goal, two diseases (green mould and cobweb disease) were analyzed on *A. bisporus*. Different statistical methods (PCA, PLS-DA, HeatMap) were used to distinguish infected samples from healthy mushrooms to achieve a very early disease detection.

Several compounds were identified and classified according to their main effect:

- 14 compounds indicate a healthy sample status: *cis*-α-bisabolene; β-caryophyllene; tridecane; acetophenone; cymene; isomenthone; 2-decen-1-ol; myrtenol; 2,4-diisocyanatotoluene; dodecane; 4-propylanisole; D-limonene; 2-heptanone-6-methyl; cyclooctane.
- 15 compounds indicate *T. aggressivum* infections, three of them -marked in italic-indicates the disease in an early stage: megastigma-4,6,8-triene; thujopsene; β-cadinene; *patchulane*; *isocaryophyllene*; cedr-8-ene; β-guaiene; aromadendrane; α-chamigrene; 2-pentanone,4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]hept-2-yl)-; β-elemene; nonane; cedr-8(15)-ene; isopatchoulane; *longifolene*.
- Only 5 compounds have the main effect to distinguish cobweb disease: 2-nonene; decane; 3-undecene, undecane, and 5-undecene.

These results can be used for further investigation of infected mushroom identification in an early stage in packaged mushroom products. Furthermore, these results could help to identify infections in commercially available mushrooms, thus increasing shelf-life in super/hypermarkets.

## Declarations

### *Author contribution statement*

Dalma Radványi, Ph.D: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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*Data availability statement*

Data included in article/supp. material/referenced in article.

*Declaration of interest's statement*

The authors declare no competing interests.

*Additional information*

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