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Detection and Identification of Microbial Volatile Organic Compounds of the Green Mold Disease:
MVOC Profile on Different Media

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ABSTRACT

Button mushrooms are one of the most commonly cultivated mushroom species facing different risks e.g.: viral, bacterial and fungal diseases. One of the most common problems is caused by Trichoderma aggressivum, or 'green mould' disease. The presence or absence of mushroom disease-related moulds can sufficiently be detected from the air by headspace solid-phase microextraction coupled gas chromatography-mass spectrometry (HS SPME GC-MS) via their emitted microbial volatile organic compounds (MVOCs). In the present study, HS SPME GC-MS was used to explore the volatile secondary metabolites released by T. aggressivum f. europaeum on different nutrient-rich and -poor media. The MVOC pattern of green mould was determined, then media-dependent and independent biomarkers were also identified during metabolomic experiments. The presented results provide the basics of a green mould identification system which helps producers reducing yield loss, new directions for researchers in mapping the metabolomic pathways of T. aggressivum and new tools for policy makers in mushroom quality control.

KEYWORDS

Biomarkers, Different Media, Environmental Chemistry, MVOC (Microbial Volatile Organic Compound) Pattern, SPME (Solid-Phase Microextraction) Sampling, Trichoderma Aggressivum

LITERATURE REVIEW

Mushroom production showed an increasing tendency in the past few years. Button mushroom (*Agaricus bisporus*) gives one-third of the total amount of mushroom production (Chang & Miles, 2004; Largeteau & Savoie, 2010). Unfortunately, it is excessively sensitive to different diseases, such

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as viral, fungal (*Lecanicillium fungicola* – dry bubble disease, *Mycogene perniciosa* – wet bubble disease, *Trichoderma aggressivum* – green mould disease), and bacterial diseases (*Pseudomonas* spp.) (Fletcher, 1990).

Trichoderma aggressivum is known as the most harmful mould in mushroom production. Green mould is able to proliferate on mushroom compost, while the mushroom mycelium growth is obstructed, therefore it causes retarded, low-quality mushroom fruiting body instead of healthy, high-quality products (O'Brien, Grogan, & Kavanagh, 2014). Moreover, *T. aggressivum* produces extracellular enzymes, toxic secondary metabolites, and volatile organic compounds. As a consequence, not only the quality and the amount of yield decreases but the use of crop models is also obstructed (Papajorgji, Clark, & Jallas, 2009). Green mould disease is hard to recognize in the initial days during its long vegetative growth phase, the mould lawn is white (button mushroom mycelium is white as well); the colour of mould changes only when the green spores occur (after 2-4 days) (Largeteau & Savoie, 2010). *Trichoderma* species educed metabolite control system, which enables them to survive extreme environmental conditions, like low oxygen (Silva, Steindorff, & Monteiro, 2014). Green mould is also known as the most aggressive mould species in mushroom cultivation. At the initial phase, mould mycelium grows simultaneously with mushroom mycelium; mould can use compost substrates as carbon source via extracellular enzymes (Krupke, Castle, & Rinker, 2003). As soon as *Trichoderma* produce green spores, the mushroom growth is decreased, while the growth of green mould rapidly increases. After green mould sporulation, the button mushroom mycelium is obstructed by the mould (Górski, Sobieralski, Siwulski, Frąszczak, & Sas-Golak, 2014; Mamoun, Lapicco, Savoie, & Olivier, 2000; Mamoun, Savoie, & Olivier, 2000; Williams, Clarkson, Mills, & Cooper, 2003). One mushroom growth inhibitor compound produced by *T. aggressivum* is 3,4-dihydro-8-hydroxy-3-methylisocoumarin. This compound has not been noticed in not aggressive *Trichoderma* isolates (Krupke et al., 2003). In order to preserve the high quality of mushroom compost, this harmful microorganism must be detected as soon as possible and the quality of the compost should be continuously monitored and controlled.

A novel approach to fight against infections is volatile organic compound (VOC) examination. VOCs can be used as specific biomarkers or ecological indicators to describe or identify different species or groups of fungi (Muller et al., 2013). Microbial volatile organic compounds (MVOCs) are emitted during microorganism's metabolite pathways. Several microorganisms emit volatile compounds to evolve interactions (Tirranen & Gitelson, 2006). In 2013, Lemfack and his co-workers built an MVOC database (Lemfack, Nickel, Dunkel, Preissner, & Piechulla, 2014), which contains more than 10 000 species and their VOCs, and it is online available.

Several paper deals with examination of *Trichoderm*a fungi's volatile compounds, however, only *T. atroviride* (2-heptanone; 1-octen-3-ol; 3-octanone; 2-pentyl furan 3-octanol; 6-α-phellandrene; α-terpinene; β-phellandrene; 2-nonanone; phenylethyl alcohol; β-farnesene; α-curcumene (Stoppacher, Kluger, Zeilinger, Krska, & Schuhmacher, 2010)) and *T. harzianum* (butyric acid, ethyl ester; 2-methyl butyric acid, ethyl ester; phenylethanol; 2,6,-dimethyl-2,4,6-octatriene (Fiedler, Schütz, & Geh, 2001)) have been examined in most of the cases. Volatile metabolites of *T. aggressivum* were only researched by Krupke and his co-workers in 2003 (Krupke et al., 2003).

Trichoderma species emitted mostly different terpenes (Berg, Kemami Wangun, Nkengfack, & Schlegel, 2004; Cardoza et al., 2011; Reino, Guerrero, Hernández-Galán, & Collado, 2008). The diversity of *Trichoderma* species can be demonstrated according to its metabolite profile (Gupta et al., 2014). Alternative identification of different mould species can be done using their emitted volatile metabolite compounds (Naznin et al., 2014; Zhang, Askim, Zhong, Orlean, & Suslick, 2014).

Several fungi species can be distinguished using different coupled analytical techniques. MVOCs emitted by fungi can be captured, analysed and monitored with headspace solid-phase microextraction combination with gas chromatography-mass spectrometry (HS-SPME-GC-MS) from the air directly above the sample (Claeson, Levin, Blomquist, & Sunesson, 2002; Dong et al., 2015; Kluger, Zeilinger, Wiesenberger, Schöfbeck, & Schuhmacher, 2013; Matysik, Herbarth, & Mueller,

2008; Muller et al., 2013; Polizzi et al., 2012; Radványi, Gere, Jókai, & Fodor, 2014; Van Lancker et al., 2008; Wady, Bunte, Pehrson, & Larsson, 2003). Moulds can be separated based on their MVOC compounds, however, the evaluation process could be difficult and time-consuming. An alternative solution to reduce analysis time is detrended fluctuation analysis (DFA), which enables a fast and reliable separation of different samples based solely on raw chromatograms (Radványi et al., 2016).

In the present study, HS-SPME-GC-MS was used to explore the volatile secondary metabolites released by *Trichoderma aggressivum* on four different media.

OBJECTIVES

The main objective of the presented work is to identify media-independent biomarkers of *Trichoderma aggressivum* on four media and three modified media in order to create the basics of a prediction system. Secondary aim is to identify the media-dependent biomarkers which help us to understand the changes of microbial volatile organic compound (MVOC) profiles of *Trichoderma aggressivum*.

MATERIALS AND METHODS

Microorganism

Trichoderma aggressivum f. *europaeum* (10^7-10^8 spores/mL) species was inoculated on different media. The number of spores was counted in Burker chamber. The fungal strain was obtained from the culture collection of Szent István University, Department of Vegetable and Mushroom Growing.

Media

Two nutrient-rich media (malt extract agar - MEA and potato-dextrose agar - PDA) and two nutrient-decreased media (compost agar - CA) and water agar - WA) were used to inoculate *T. aggressivum*. According to mushroom growers, green mould is able to grow on mushroom compost, therefore compost agar was made from dried and grinded Phase II mushroom compost. Modified compost agars (with different sugars) were also used to grow *T. aggressivum*. Table 4 in the Appendix summarizes the ingredients of different media. All ingredients and mushroom compost were provided by Biofungi Ltd. Slant agar was made under sterile box with 5 mL sterilised media, which was transferred into 20 mL HS vials. After agar solidification, 500 µL spore suspension was pipetted to the surface of slant agar in the HS vial, then the vial was closed immediately.

Solid-Phase Microextraction Sampling

Extraction of volatile compounds from headspace was performed using solid-phase microextraction (SPME) consisting of a fused silica fiber coated with polydimethylsiloxane/divinylbenzene (65 µm Stable Flex™ PDMS/DVB fiber, Supelco, Bellefonte, PA, USA). Fiber was chosen based on literature data (Crespo, Pedrini, Juárez, & Dal Bello, 2008; Malheiro, Guedes de Pinho, Soares, César da Silva Ferreira, & Baptista, 2013; Sági-Kiss & Fodor, 2011; Stoppacher et al., 2010). The microorganism was kept at 24 °C and monitored from the inoculation to the seventh day. The fiber was passed through the septum of the vial and exposed to the volatiles derived from *T. aggressivum*. After 15 min absorption, the fiber was retracted into the needle and removed from the HS vial to perform GC-MS analysis. All measures were done in triplicate.

Gas Chromatography-Mass Spectrometry Analysis

After injection of SPME needle into the GC injector, MVOCs were desorbed from the fiber at 250 °C (4 min). An Agilent 6890 Gas Chromatograph coupled with a 5975 C MSD Mass Spectrometer was used to analyse the volatiles. A non-polar HP-5MS ((5%-phenyl)-methylpolysiloxane; 30 m, 0.25 mm i.d., 0.25 µm film, Agilent Technologies) capillary column was used, which is excellent for

volatile compounds. The split valve was closed during desorption period and the inlet temperature was held at 250 °C. Optimised column program was used; it began at 50 °C and increased to 150 °C at 20 °C/min, then to 170 °C at 40 °C/min, to 190 °C at 25 °C/min, to 280 °C at 40 °C/min, and finally to 300 °C (2 min hold) at 50 °C/min. Hydrogen 6.0 was used as a carrier gas with a constant 1.2 mL/min flow to accelerate the separation on the column.

The mass fragments were detected by quadrupole MS, where the ion source temperature was set at 230 °C and the quadrupole temperature was held at 150 °C. Positive electron ionization (EI+) was used, with an electron energy level of 70 eV. The MS was tuned using perfluorotributylamine (PFTB) before the measurements. Agilent Enhanced MSD ChemStation software handled the GC and MS parameters and Agilent MassHunter Qualitative Analysis B.06.00 software was used for evaluation and comparison of the chromatograms. The collected spectra were analysed using NIST Mass Spectral Search Program (NIST/EPA/NIH/Mass Library, version 2.0, Wiley 10th edition). The analysis was conducted following and earlier work of the authors (Radványi, Gere, Jókai, & Fodor, 2014).

RESULTS AND DISCUSSION

Analysis of Green Mould Growth on Different Media

A spore suspension of *T. aggressivum* f. *europaeum* obtained from the same strain was analysed during the experiment. After inoculation on different media, MVOCs emitted by green mould were monitored and the microorganism growth (mycelium growth, spore production) were also examined until seven days. The green mould grew faster on nutrient-rich media (PDA and MEA) at 24 °C, as expected. The appropriate temperature and media composition enhance the mould growth (Samuels, Dodd, Gams, Castlebury, & Petrini, 2002; Sobieralski, Siwulski, & Frużyńska-Jóźwiak, 2009; Stoppacher et al., 2010); the mycelium growth commenced on the second day on nutrient-rich media. After initial mycelium growth, green spores appeared at the edge of the mycelium lawn and finally the spores suffused with the white mycelium lawn (S 1.).

The mycelium growth on nutrient-decreased media (CA, WA) was negligible, only white spots appeared (S 1.), however; production of green spores was faster within this mycelium spots. Green mould grew better on MEA and PDA media, therefore it emitted more volatile metabolites on these media. The main mould specific volatiles, terpenes (Cardoza et al., 2011; Hermosa, Cardoza, Rubio, Gutiérrez, & Monte, 2014; Reino et al., 2008), appeared around the sixth minute on the total ion chromatogram (TIC) (Figure 1).

Identification of Volatile Metabolites

The intensity of MVOC's is usually much lower than the compound's intensity of ordinary SPME measures, where the compounds have approximately 10^7-10^8 intensity values. Owing to these low-intensity values, automatic evaluation software – such as Mass Hunter Unknown Analysis – cannot be used properly for compound deconvolution, identification or integration (data not shown). In order to get reliable results, peak intensities were categorised according to the abundance values (Figure 2).

Compounds having $4.0*10^5$ intensity values were classified into the first group (High Intensity Compounds, HIC). The mass spectra of compounds having high intensity values have a low background interference; therefore background correction (background subtraction) was not needed. High intensity compounds can be easily identified using mass spectrum libraries (*e.g.*: NIST library).

Most of the compounds had $1.2\text{-}3.0*10^5$ intensity values (Medium Intensity Compounds, MIC) while compounds having lower intensity values were passed into the third group (Low Intensity Compounds, LIC) (Figure 2). Medium intensity compounds (MIC) could have background interference, therefore background correction is needed in these cases. The examined compound mass spectrum can be disturbed with other (not relevant) compounds fragment (background).

Figure 1. MVOC pattern of T. aggressivum on different media. The ellipse indicates the main mould specific volatiles (terpenes).

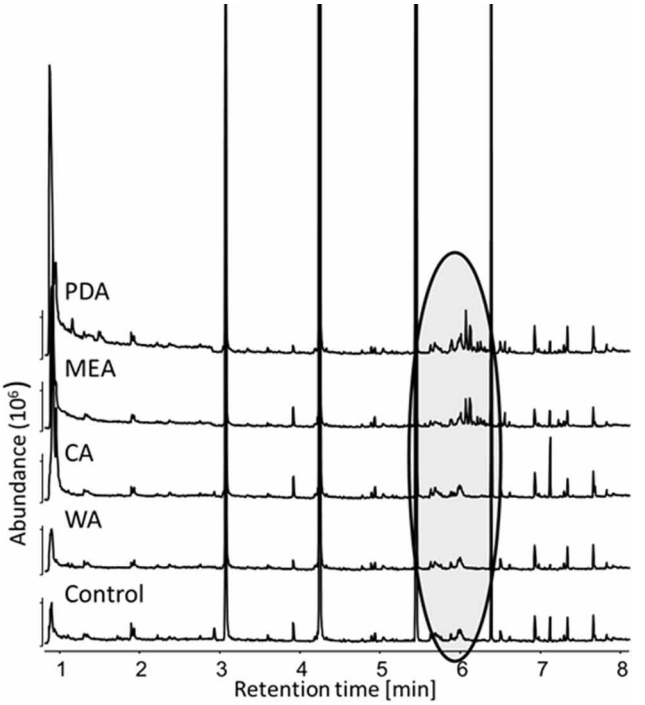
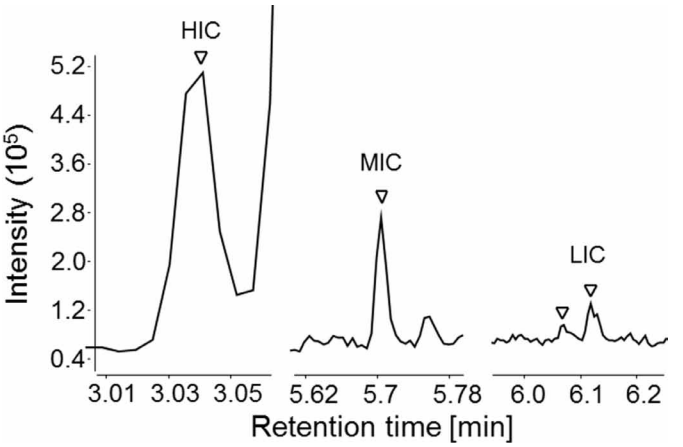


Figure 2. High (≥ 4.0*10^5), Medium (1.2-3.0*10^5) and Low (≤ 10^5) Intensity compounds. HIG: High Intensity Compounds, MIG: Medium Intensity Compounds, LIG: Low Intensity Compounds



After background correction, the examined compound mass spectrum is not disturbed by any other compounds' fragments, therefore a clean compound mass spectrum is available. The obtained background-free mass spectrum was properly identified using NIST mass spectrum library. Compounds identity value having at least 60% (ID % ≥ 60%) was accepted only when they were present in all three parallel measures.

In most of the cases, low intensity compounds (LIC), mainly those compounds which had nearly as low intensity values as the limit of detection (LOD), caused problems. During LIC compounds' peak

area determination, automatic integration algorithm integrated more peaks into one compound or could not find some compounds; hence manual deconvolution was used. Mass spectrum of compounds was corrected with the background. However, it could be a problem that the fragment ions of the examined compounds do not have much higher intensity values than the interfering background fragment ions. Thus, the examined compound fragment ions could be lost from the mass spectra during background subtraction. In these cases, incorrect identification could be achieved. The presence of molecular ion ($M^{\cdot+}$), and its accurate m/z value could mean a stable point during unknown screening and unknown compound identification. Besides the molecular ion, retention index (RI) could serve some additional information about the unknown compounds. NIST library also contains data from retention parameters, such as different compound-related, column- and temperature program-dependent retention indices. Retention index from the library was compared to the measured and identified compounds to achieve proper and reliable identification.

Examination of Trichoderma Aggressivum's Biomarkers

Determination of marker compounds. In the first step, MVOC pattern of *Trichoderma aggressivum* was defined on all different media, where 150 compounds were found. Thereafter, false positive hits were excluded, and 56 compounds were picked up which may refer to mould presence. Moreover, 12 compounds were eliminated which couldn't be linked directly to the presence of *T. aggressivum*. As a result, 44 compounds were chosen as biomarkers of *T. aggressivum* in case of four different media (MEA, PDA, CA, WA). These 44 biomarkers were grouped regarding different media (Table 1).

3 compounds (1,2-dimethyl benzene; 3-octanone; 2-pinen-4-ol) were found from the chosen 44 biomarkers, which appeared in all different media. More common biomarkers (18 compounds) were found on nutrient-rich media (PDA, MEA); which is shown by the "MEA-PDA markers" column of Table 1. In summary, 21 (18+3) compounds were determined as common biomarkers of *T. aggressivum.* Moreover, 10 marker compounds were found during *T. aggressivum* growth only on PDA media and 13 marker compounds only on MEA media (Table 1. "Media-dependent markers"). Furthermore, 12 "hypothetical" marker compounds (ethylbenzene; 2-nitroterephthalamide; ethyl (1-adamantylamino)carbothioylcarbamate; benzenesulfonamide, N-(bicyclo[2.2.1]hept-5-en-2-ylmethyl)-4-(5-oxo-3-pyrrolidinyl)-; 3-formyl-N-methyl-9-[phenylethynyl]dibenzo[2,3-a: 5,6-a'] (1,4)-thiazine; 2-nonynoic acid; beta-resorcylic acid; unknown (t_R= 2.93, 3.60, 4.88, 6.01 and 7.40 min)) were found which may also indicate the presence of green mould, but more experiment is needed to verify this result.

Table 1. Differentiation of biomarkers according to media (MEA-malt extract agar, PDA-potato-dextrose agar, CA-dried compost agar, WA-water agar)

<table>
<tr><th></th><th>Media-Dependent Markers (10+13)</th><th>MEA-PDA Markers (18)</th><th>Media-Independent Markers (3)</th></tr>
<tr><td>PDA</td><td>2-propanone; isobutyl chloride; 1-propanol, 2-methyl; longifolene; unknowns (t_R=5.38, 5.48, 5.58, 5.76, 5.81 and 5.84 min)</td><td rowspan="2">octane; adamantan-2-ol, 4-bromo-, cis; alpha-humulene; cedr-8-ene; beta-cubebene; beta-copaene; beta-ylange; patchoulane; (5Z)-5-pentadecen-7-yne; ledane; gamma-elemene; (2Z,6E)-farnesol; unidentified C15H26 derivatives (t_R=6.30 and 6.33 min); unknown (t_R=3.10, 3.36, 6.26 and 7.67 min)</td><td rowspan="2">1,2-dimethyl benzene; 3-octanone; 2-pinen-4-ol</td></tr>
<tr><td>MEA</td><td>benzeneethanamine; tyrene; tetracyclo[5.3.1.1(2,6).0(4,9)]dodecane; beta-caryophyllen; 8,11-octadecadiynoic acid, methyl ester; 4-(2,2-dimethyl-6-methylenecyclohexyl)butanal; unidentified 2H-pyran derivatives (t_R=4.68 min); unknowns (t_R= 4.33, 4.61, 5.15, 6.16, 6.48, 7.32 min)</td></tr>
<tr><td>CA</td><td colspan="2">-</td><td rowspan="2"></td></tr>
<tr><td>WA</td><td colspan="2">-</td></tr>
</table>

The identified marker compounds were compared to literature data; those compounds were mostly searched which indicate the presence of *Trichoderma* spp. Seven compounds were found (2-methyl-1-propanol; octane; ethylbenzene; 1,2-diethyl-benzene; 3-octanone; beta-elemene; beta-caryophyllene) which obviously refer to the presence of *Trichoderma* spp. More *Trichoderma* spp.-related compounds (479 specific compounds) can be found in the literature (Siddiquee, 2014).

Monitoring of Biomarkers

After successful identification, compound intensity changes were tracked during the examined period. An example of intensity changes is shown in Figure 3.

On the first day, cedr-8-ene appeared on the TIC chromatogram, then the intensity is increased as the mould grew on the media. After an intensity maximum, decreased tendency was observed and finally it was not detectable on the seventh day. Marker compounds were grouped according to their intensity change tendencies (monotone increasing, showing intensity maximum, monotone decreasing or having fluctuating intensity change tendency).

Figure 3. Intensity changes of cedr-8-ene according to the examined days on MEA media

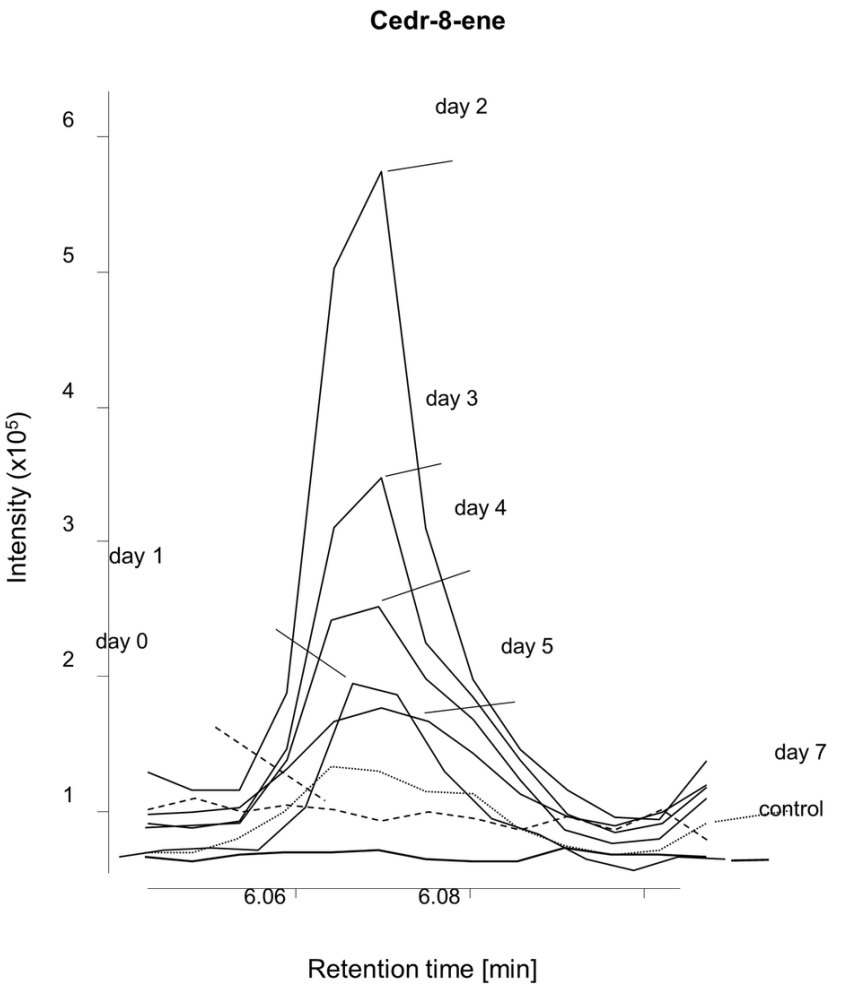


Table 2. Volatile biomarkers emitted by T. aggressivum: (a) On PDA media; (b) On MEA media. ID%: identity value during identification, RI: retention index

(a)	Biomarkers on PDA						
t_R (min)	Compound Name	Chemical Formula	RI Database	RI Counted	CAS Number	Molecular Weight	ID%
1.01	2-propanone	C_3H_6O	n.a.	-	67-64-1	58.0	67.38
1.19	isobutyl chloride	C_4H_9Cl	n.a.	-	513-36-0	92.0	79.16
1.22	1-propanol, 2-methyl	$C_4H_{10}O$	n.a.	-	78-83-1	74.1	71.25
5.38	unknown	-	-	1276	-	-	-
5.48	unknown	-	-	1289	-	-	-
5.58	unknown	-	-	1303	-	-	-
5.70	longifolene	$C_{15}H_{24}$	n.a.	1324	475-20-7	204.2	82.15
5.76	unknown	-	-	1335	-	-	-
5.81	unknown	-	-	1344	-	-	-
5.84	unknown-	-	-	1349	-	-	-
(b)	**Biomarkers on MEA**						
t_R (min)	Compound Name	Chemical Formula	RI Database	RI Counted	CAS Number	Molecular Weight	ID%
2.90	benzeneethanamine	$C_9H_{13}N$	n.a.	950	60-15-1	135.1	64.62
4.33	unknown	-	-	1133	-	-	-
4.61	unknown	-	-	1168	-	-	-
4.68	unidentified 2H-pyran derivatives	-	-	1176	-	-	-
5.15	unknown	-	-	1241	-	-	-
5.56	tyrene	$C_7H_5Cl_3O$	1468	1300	87-40-1	194.2	62.47
5.98	tetracyclo[5.3.1.1(2,6).0(4,9)] dodecane	$C_{12}H_{17}D$	n.a.	1374	2000099-51-5	163.1	68.27
6.16	unknown	-	-	1407	-	-	-
6.23	beta-caryophyllen	$C_{15}H_{24}$	n.a.	1422	87-44-5	204.2	72.71
6.48	unknown	-	-	1474	-	-	-
7.00	8,11-octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	2112	1599	18202-23-8	290.2	69.61
7.25	4-(2,2-dimethyl-6-methylenecyclohexyl)butanal	$C_{15}H_{26}O$	1468	1662	95452-13-4	194.2	66.62
7.32	unknown	-	-	1680	-	-	-

Intensity Changes of Common and Media-Dependent Compounds

Trichoderma aggressivum inoculated on PDA emitted 10 specific biomarkers (Table 2a), which occurred only on this media. Four out of ten compounds were identified successfully: 2-propanone; isobutyl chloride; 1-propanol, 2-methyl and longifolene. *T. aggressivum* inoculated on MEA emitted 13 specific biomarkers (Table 2b) during the examined period. Very low intensity values ($< 10^5$) were registered in the case of these marker compounds, therefore, the identification was not totally complete; only six compounds were identified succsessfully.

Table 3. Intensity changes of common and media-dependent compounds

(a) Common Compounds			
Monotone Increasing	**Intensity Maximum on the Second Day**	**Intensity Maximum on the Second or Third Day**	**Fluctuating**
octane, **3-octanone**	adamantan-2-ol, 4-bromo-, cis; **2-pinen-4-ol**; two unknown compounds (t_R=3.36, 6.26 min); alpha-humulene; cedr-8-ene; beta-cubebene, patchulene, ledane	**1,2-dimethyl-benzene**; beta-copaene; beta-ylangene; unidentified $C_{15}H_{26}$ derivatives (t_R=6.30 min); (5Z)-5-pentadecen-7-yne; (2Z,6E)-farnesol, gamma-elemene	unknown compounds (t_R=3.10, 7.67 min), unidentified $C_{15}H_{26}$ derivative (t_R=6.33 min)
(b) PDA Media-Dependent Biomarkers			
Monotone Increasing	**Intensity Maximum on the Second Day**	**Intensity Maximum on the Second or Third Day**	**Fluctuating**
2-propanone; unknown (t_R=5.48 min); longifolene	isobutyl chloride and 1-porpanol, 2-methyl	-	unknown compounds (t_R=5.38; 5.58; 5.76; 5.81, 5.84 min)
(c) MEA Media-Dependent Biomarkers			
Monotone Decreasing	**Intensity Maximum on the Second Day**	**Intensity Maximum on the Second or Third Day**	**Fluctuating**
unknown compound (t_R=4.61 min)	benzeneethanamine; unknown compounds (t_R=4.33; 5.15, 6.16 min); unidentified 2H-pyran derivatives (t_R=4.68 min) *tyrene and beta-caryophyllen * Tetracyclo[5.3.1.1(2,6).0(4,9)] dodecane ***	unknown compounds (t_R=6.48, 7.32 min), 4-(2,2-dimethyl-6-methylenecyclohexyl) butanal	8,11-octadecadiynoic acid, methyl ester *tyrene and beta-caryophyllen**

* Tyrene and beta-caryophyllene had also intensity maximum at the second day, but later their intensity fluctuated, too. ** Tetracyclo[5.3.1.1(2,6).0(4,9)] dodecane had intensity maximum on the first day.

Common compounds and their parameters were collected into S 6. Average value of compound peak area of the parallel measures was used to analyse intensity changes and to visualise the results. Table 3 shows the different groups according to intensity change; media-independent markers are highlighted.

The microorganism's growth was also examined during the monitoring experiments (Figure 4).

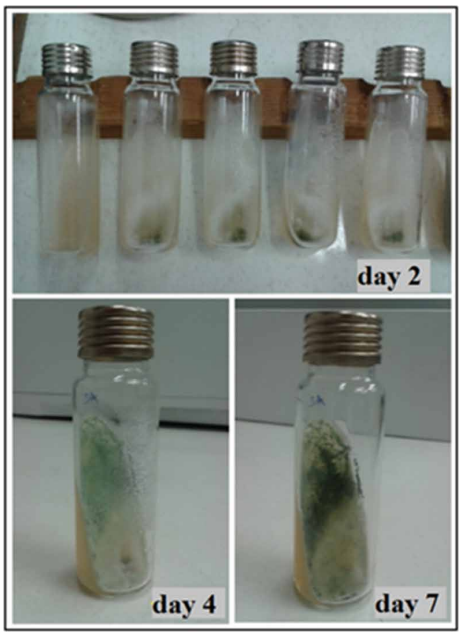
The results from microorganism growth and monitoring experiments were compared. According to our results, marker compounds appeared at different growing phases of the microorganism. Biomarkers emitted at the first few days are linked to the mycelium growth phase because active white mycelium growth was experienced at these days (Figure 4). Compounds having intensity maximum on the second or third day showed different intensity maximums on different media (MEA or PDA). If an examined compound appears earlier on MEA media it could mean that the microorganism could have faster metabolism on MEA, than PDA. It means, that the microorganism is able to utilize and consume the nutrients from MEA more easily, thus the accretion, the colony development was also faster on this media.

Compounds having a monotone increasing tendency may refer to green spore production, especially those whose intensity value jumped after the third day.

Examination of Marker Compounds on Modified Compost Agar

According to the results, *T. aggressivum* grew faster on nutrient-rich media (MEA and PDA), as expected. The green mould was not able to grow on water agar, only scattered green spore production was observed. Moreover, *T. aggressivum* was not able to grow on dried compost agar however, it occurs mainly on mushroom compost causing green mould-disease. Remarkable green mould growth was expected in the case of compost agar. Some biomarkers of green mould inoculated on MEA

Figure 4. Green mould growth on MEA substrata. This figure shows the mycelium growth phase at the second day and the green spore production phase from the fourth day. The seventh day shows the final state of the microorganism (picture taken by the author DR).



and PDA media showed time shift in their occurrence. Differences in carbohydrate resources could cause this time shift.

Taking into consideration previous conclusions, different sugars (mannitol, maltose, and dextrose) were added to the dried mushroom compost agar. These sugar types were chosen according to the literature (Komon-Zelazowska et al., 2007; Kubicek, Bissett, Druzhinina, Kullnig-Gradinger, & Szakacs, 2003). Dextrose (PDA-like agar) and maltose (MEA-like agar) added to CA was chosen to compare the results and mannitol was also added to CA to analyse sugar alcohol beside mono- and disaccharides. *T. aggressivum* was inoculated on these modified compost agars and the emitted MVOCs were analysed.

Green mould growth was monitored for seven days in this experiment, too. Instead of fast growing on carbohydrate-rich media, green spore production was also noticeable on the edge of these media. More compounds were detected in this part of the experiment compared to the original measures. 3-octanone, 3-octanol, and tyrene appeared on all three modified media moreover, 1,2-dimethyl benzene and 2-pinen-4-ol were also observed in the case of media added extra maltose. An unknown compound having 3.10 minute retention time could not be identified in the original measures however, on modified media, it was identified as 3-octanol. According to the results, two additional emitted compounds (tyrene and 3-octanol) act as *Trichoderma aggressivum* marker compound.

CONCLUSION

According to the literature, bisporic button mushroom is one of the most commonly cultivated mushroom species all over the world. One of the most common fungal problems in mushoroom production is caused *Trichoderma aggressivum,* called green mould disease. Presence of green mould can be determined by Headspace-solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS).

The microbial volatile organic compound (MVOC) pattern of green mould (*T. aggressivum f. europaeum*) was determined on different media. After MVOC determination, the biomarkers were also identified. Different evaluation processes were established, since these biomarker compounds had highly different intensity values, which requires intensity-dependent evaluation processes, in order to get proper results.

Media-independent (3 markers), and media-dependent (10 markers on PDA, 13 markers on MEA) biomarkers were determined from the selected markers, moreover from the nutrient-rich media derived marker compounds (18 markers) were also determined. After successful identification and classification of biomarkers, the changes in their intensity values were examined. Furthermore, the changes of marker compounds were linked to the inoculated microorganisms' growth phase (mycelium growth or green spore production phase) on four different media. Twelve hypothetical marker compounds were also found in addition to the defined and identified biomarkers, which may indicate the presence of mould in some cases.

Some biomarkers of green mould inoculated on MEA and PDA media showed time shift in their occurrence, which could cause by the differences in carbohydrate resources. Taking into consideration previous conclusions, different sugars (mannitol, maltose, and dextrose) were added to the dried mushroom compost agar. According to results, two more emitted compounds act as *Trichoderma aggressivum* marker compound. These marker compounds can later be used in quality control systems which are able to identify the presence of green mould disease in an early phase, therefore the producers will have more time to prevent yield loss.

As a result, HS-SPME-GC-MS coupled analytical technique was suitable to detect the presence of green mould disease. The *Trichoderma* MVOC pattern on different media was successfully determined. Using this method, fast mould disease detection could be achieved in the field of mushroom production. A further step for researchers would be to develop smaller, cheaper and low-energy cost devices which are able to identify the presence of the selected biomarkers. It is also important to mention that the stability of the identified biomarkers should also be checked on other media.

The above displayed sampling and evaluation processes may be able to detect different indoor moulds by remote sensing (Yatsenko, Cifarelli, Boyko, & Pardalos, 2009). The indoor moulds causing so-called sick building syndrome (SBS) could also be examined. Furthermore, the method can also be extended from food analysis to environmental monitoring; thus, several harmful diseases could be prevented such as asthma, allergic reactions or headaches.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

Berg, A., Kemami Wangun, H. V., Nkengfack, A. E., & Schlegel, B. (2004). Lignoren, a new sesquiterpenoid metabolite from Trichoderma lignorum HKI 0257. *Journal of Basic Microbiology*, *44*(4), 317–319. doi:10.1002/jobm.200410383 PMID:15266604

Cardoza, R. E., Malmierca, M. G., Hermosa, M. R., Alexander, N. J., McCormick, S. P., Proctor, R. H., & Gutierrez, S. et al. (2011). Identification of loci and functional characterization of trichothecene biosynthesis genes in filamentous fungi of the genus Trichoderma. *Applied and Environmental Microbiologynvironmental Microbiology*, *77*(14), 4867–4877. doi:10.1128/AEM.00595-11 PMID:21642405

Chang, S.-T., & Miles, P. G. (2004). *Mushrooms.Cultivation, Nutritional Value, Medicinal Effect, and Environmental Impact (2nd ed.)*. Boca Raton: CRC Press LLC. doi:10.1201/9780203492086

Claeson, A.-S., Levin, J.-O., Blomquist, G., & Sunesson, A.-L. (2002). Volatile metabolites from microorganisms grown on humid building materials and synthetic media. *Journal of Environmental Monitoring*, *4*(5), 667–672. doi:10.1039/B202571J PMID:12400912

Crespo, R., Pedrini, N., Juárez, M. P., & Dal Bello, G. M. (2008). Volatile organic compounds released by the entomopathogenic fungus Beauveria bassiana. *Microbiological Research*, *163*(2), 148–151. doi:10.1016/j.micres.2006.03.013 PMID:16733086

Dong, L., Liu, R., Dong, H., Piao, Y., Hu, X., Li, C., & Zhao, C. et al. (2015). Volatile metabolite profiling of malt contaminated by Fusarium poae during malting. *Journal of Cereal Science*, *66*, 37–45. doi:10.1016/j.jcs.2015.09.006

Fiedler, K., Schütz, E., & Geh, S. (2001). Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. *International Journal of Hygiene and Environmental Health*, *204*(2), 111–121. doi:10.1078/1438-4639-00094 PMID:11759153

Fletcher, J. T. (1990). *Trichoderma and Penicillium diseases of Agaricus bisporus*. London, UK.

Górski, R., Sobieralski, K., Siwulski, M., Frąszczak, B., & Sas-Golak, I. (2014). The effect of Trichoderma isolates, from family mushroom growing farms, on the yield of four Agaricus bisporus (Lange) Imbach strains. *Journal of Plant Protection Research*, *54*(1), 24–27. doi:10.2478/jppr-2014-0016

Gupta, V., Schmoll, M., Herrera-Estrella, A., Upadhyay, R., Druzhinina, I., & Tuohy, M. (2014). *Biotechnology and Biology of Trichoderma* (1st ed.). Amsterdam: Elsevier Science BV.

Hermosa, R., Cardoza, R. E., Rubio, M. B., Gutiérrez, S., & Monte, E. (2014). Secondary Metabolism and Antimicrobial Metabolites of Trichoderma. In V. Gupta, M. Schmoll, A. Herrera-Estrella, R. Upadhyay, I. Druzhinina, & M. Tuohy (Eds.), *Biotechnology and Biology of Trichoderma* (1st ed., pp. 125–137). Elsevier. doi:10.1016/B978-0-444-59576-8.00010-2

Kluger, B., Zeilinger, S., Wiesenberger, G., Schöfbeck, D., & Schuhmacher, R. (2013). Detection and Identification of Fungal Microbial Volatile Organic Compounds by HS-SPME-GC–MS. In V. K. Gupta, M. G. Tuohy, M. Ayyachamy, K. M. Turner, & A. O'Donovan (Eds.), *Laboratory Protocols in Fungal Biology* (pp. 455–465). New York: Springer. doi:10.1007/978-1-4614-2356-0_42

Komon-Zelazowska, M., Bissett, J., Zafari, D., Hatvani, L., Manczinger, L., Woo, S., & Druzhinina, I. S. et al. (2007). Genetically closely related but phenotypically divergent Trichoderma species cause green mold disease in oyster mushroom farms worldwide. *Applied and Environmental Microbiology*, *73*(22), 7415–7426. doi:10.1128/AEM.01059-07 PMID:17827333

Krupke, O. A., Castle, A. J., & Rinker, D. L. (2003). The North American mushroom competitor, Trichoderma aggressivum f. aggressivum, produces antifungal compounds in mushroom compost that inhibit mycelial growth of the commercial mushroom Agaricus bisporus. *Mycological Research*, *107*(12), 1467–1475. doi:10.1017/S0953756203008621 PMID:15000247

Kubicek, C. P., Bissett, J., Druzhinina, I., Kullnig-Gradinger, C., & Szakacs, G. (2003). Genetic and metabolic diversity of Trichoderma: A case study on South-East Asian isolates. *Fungal Genetics and Biology*, *38*(3), 310–319. doi:10.1016/S1087-1845(02)00583-2 PMID:12684020

Largeteau, M. L., & Savoie, J.-M. (2010). Microbially induced diseases of Agaricus bisporus: Biochemical mechanisms and impact on commercial mushroom production. *Applied Microbiology and Biotechnology*, *86*(1), 63–73. doi:10.1007/s00253-010-2445-2 PMID:20127233

Lemfack, M. C., Nickel, J., Dunkel, M., Preissner, R., & Piechulla, B. (2014). mVOC: A database of microbial volatiles. *Nucleic Acids Research*, *42*, D744–D748. doi:10.1093/nar/gkt1250 PMID:24311565

Malheiro, R., Guedes de Pinho, P., Soares, S., César da Silva Ferreira, A., & Baptista, P. (2013). Volatile biomarkers for wild mushrooms species discrimination. *Food Research International*, *54*(1), 186–194. doi:10.1016/j.foodres.2013.06.010

Mamoun, M. L., Lapicco, R., Savoie, J. M., & Olivier, J. M. (2000). Green mould disease in France : Trichoderma harzianum Th2 and other species causing damages on mushroom farms. In Science and Cultivation of Edible Fungi (pp. 625–632). Maastricht.

Mamoun, M. L., Savoie, J.-M., & Olivier, J.-M. (2000). Interactions between the Pathogen Trichoderma harzianum Th2 and Agaricus bisporus in Mushroom Compost. *Mycologia*, *92*(2), 233–240. doi:10.1080/00275514.2000.12061150

Matysik, S., Herbarth, O., & Mueller, A. (2008). Determination of volatile metabolites originating from mould growth on wall paper and synthetic media. *Journal of Microbiological Methods*, *75*(2), 182–187. doi:10.1016/j.mimet.2008.05.027 PMID:18577403

Muller, A., Faubert, P., Hagen, M., Zu Castell, W., Polle, A., Schnitzler, J.-P., & Rosenkranz, M. (2013). Volatile profiles of fungi-chemotyping of species and ecological functions. *Fungal Genetics and Biology*. *FG & B*, *54*, 25–33. doi:10.1016/j.fgb.2013.02.005 PMID:23474123

Naznin, H. A., Kiyohara, D., Kimura, M., Miyazawa, M., Shimizu, M., & Hyakumachi, M. (2014). Systemic resistance induced by volatile organic compounds emitted by plant growth-promoting fungi in Arabidopsis thaliana. *PLoS One*, *9*(1), e86882. doi:10.1371/journal.pone.0086882 PMID:24475190

O'Brien, M., Grogan, H., & Kavanagh, K. (2014). Proteomic response of Trichoderma aggressivum f. europaeum to Agaricus bisporus tissue and mushroom compost. *Fungal Biology*, *118*(9–10), 785–791. doi:10.1016/j.funbio.2014.06.004 PMID:25209637

Papajorgji, P., Clark, R., & Jallas, E. (2009). The Model Driven Architecture Model Driven Architecture MDA Approach: A Framework for Developing Complex Agricultural Systems. In Advances in Modeling Agricultural Systems (pp. 1–18). Academic Press. doi:10.1007/978-0-387-75181-8_1

Polizzi, V., Adams, A., Malysheva, S. V., De Saeger, S., Van Peteghem, C., Moretti, A., & De Kimpe, N. et al. (2012). Identification of volatile markers for indoor fungal growth and chemotaxonomic classification of Aspergillus species. *Fungal Biology*, *116*(9), 941–953. doi:10.1016/j.funbio.2012.06.001 PMID:22954337

Radványi, D., Gere, A., Jókai, Z., & Fodor, P. (2014). Rapid evaluation technique to differentiate mushroom disease-related moulds by detecting microbial volatile organic compounds using HS-SPME-GC-MS. *Analytical and Bioanalytical Chemistry,* (2), 537-545. doi:10.1007/s00216-014-8302-x

Radványi, D., Gere, A., Sipos, L., Kovács, S., Jókai, Z., & Fodor, P. (2016). Discrimination of mushroom disease-related mould species based solely on unprocessed chromatograms. *Journal of Chemometrics*, *30*(4), 197–202. doi:10.1002/cem.2777

Reino, J. L., Guerrero, R. F., Hernández-Galán, R., & Collado, I. G. (2008). Secondary metabolites from species of the biocontrol agent Trichoderma. *Phytochemistry Reviews*, *7*(1), 89–123. doi:10.1007/s11101-006-9032-2

Sági-Kiss, V., & Fodor, P. (2011). Development of a SPME-GC-MS method for spoilage detection in case of plums inoculated with Penicillium expansum. *Acta Alimentaria*, *40*(Suppl.), 188–197. doi:10.1556/AAlim.40.2011.Suppl.18

Samuels, G. J., Dodd, S. L., Gams, W., Castlebury, L. A., & Petrini, O. (2002). Trichoderma species associated with the green mold epidemic of commercially grown Agaricus bisporus. *Mycologia*, *94*(1), 146–170. doi:10.1080/15572536.2003.11833257 PMID:21156486

Siddiquee, S. (2014). Biotechnology and Biology of Trichoderma. In Biotechnology and Biology of Trichoderma. Academic Press. doi:10.1016/B978-0-444-59576-8.00011-4

Silva, R. N., Steindorff, A. S., & Monteiro, V. N. (2014). Metabolic Diversity of Trichoderma. In V. G. Gupta, M. Schmoll, A. Herrera-Estrella, R. S. Upadhyay, I. Druzhinina, & M. Tuohy (Eds.), *Biotechnology and Biology of Trichoderma* (1st ed., pp. 363–376). Elsevier. doi:10.1016/B978-0-444-59576-8.00027-8

Sobieralski, K., Siwulski, M., & Frużyńska-Jóźwiak, D. (2009). Growth of Aggressive Isolates of Trichoderma aggressivum f. europaeum in Dependence on Temperature and Medium. *Phytopathologia*, *53*(1), 11–18.

Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R., & Schuhmacher, R. (2010). Identification and profiling of volatile metabolites of the biocontrol fungus Trichoderma atroviride by HS-SPME-GC-MS. *Journal of Microbiological Methods*, *81*(2), 187–193. doi:10.1016/j.mimet.2010.03.011 PMID:20302890

Tirranen, L. S., & Gitelson, I. I. (2006). The role of volatile metabolites in microbial communities of the LSS higher plant link. *Advances in Space Research*, *38*(6), 1227–1232. doi:10.1016/j.asr.2006.02.038

Van Lancker, F., Adams, A., Delmulle, B., De Saeger, S., Moretti, A., Van Peteghem, C., & De Kimpe, N. (2008). Use of headspace SPME-GC-MS for the analysis of the volatiles produced by indoor molds grown on different substrates. *Journal of Environmental Monitoring*, *10*(10), 1127–1133. doi:10.1039/b808608g PMID:18843388

Wady, L., Bunte, A., Pehrson, C., & Larsson, L. (2003). Use of gas chromatography-mass spectrometry/solid phase microextraction for the identification of MVOCs from moldy building materials. *Journal of Microbiological Methods*, *52*(3), 325–332. doi:10.1016/S0167-7012(02)00190-2 PMID:12531501

Williams, J., Clarkson, J. M., Mills, P. R., & Cooper, R. M. (2003). Saprotrophic and Mycoparasitic Components of Aggressiveness of Trichoderma harzianum Groups toward Commercial Mushroom Agaricus bisporus. *Applied and Environmental Microbiology*, *69*(7), 4192–4199. doi:10.1128/AEM.69.7.4192-4199.2003 PMID:12839799

Yatsenko, V., Cifarelli, C., Boyko, N., & Pardalos, P. M. (2009). Modeling and Device Development for Chlorophyll Estimation in Vegetation. In Advances in Modeling Agricultural Systems (pp. 421–431). Academic Press. doi:10.1007/978-0-387-75181-8_20

Zhang, Y., Askim, J. R., Zhong, W., Orlean, P., & Suslick, K. S. (2014). Identification of pathogenic fungi with an optoelectronic nose. *Analyst (London)*, *139*(8), 1922–1928. doi:10.1039/C3AN02112B PMID:24570999

APPENDIX

Table 4. List of ingredients for 1000 mL media

Media		Ingredients	Bacteriological Agar [g]	Sugar [g]	Peptone Water [mL]
MEA	malt extract agar	15 g malt extract	16	-	1000
PDA	potato dextrose agar	39 g potato dextrose agar	-	-	1000
WA	water agar	-	16	-	1000
CA	mushroom compost agar	10 g dried mushroom compost	16	-	1000
Modified Media					
CAD	compost agar with extra dextrose	10 g dried mushroom compost	16	2.0 g dextrose	1000
CAMa	compost agar with extra maltose	10 g dried mushroom compost	16	2.0 g maltose	1000
CAMt	compost agar with extra mannitol	10 g dried mushroom compost	16	2.0 g mannitol	1000

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