CAN VOLATILES EMITTED BY COMPOST DURING SPAWN RUN BE USED TO DETECT GREEN MOULD INFECTION EARLY?

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ABSTRACT

In recent years green mould (*Trichoderma aggressivum*) has presented big problems to the Dutch mushroom industry. *T. aggressivum* infects compost at a very early stage and in the Dutch situation infection most likely takes place at the compost yard. Even though compost producers in the Netherlands are very keen to prevent green mould problems, occasionally still a number of crops get infected. Therefore there is a need for a reliable method that allows early detection of *Trichoderma* green mould.

Although qPCR methods have been developed for quantitation of *T. aggressivum*, these cannot be used for detection in compost. In the Netherlands spawn run is performed in bulk at the compost yards and is referred to as phase 3 composting. During this process, spawned compost is incubated in tunnels and ventilated with large volumes of air to control compost temperature. During this process the compost is inaccessible for sampling.

Literature data showed that *Agaricus bisporus* and *T. aggressivum* use volatiles to affect each other's growth rate. We tested the possibility to detect *Trichoderma* green mould using the volatiles that are emitted during spawn run. This eventually could lead to a sophisticated non-invasive detection method of *T. aggressivum* in the process air of the tunnels, without the need to sample inside the tunnel during spawn run.

For this we compared volatiles that are produced in non-infected compost with volatiles that are produced in infected compost. In our experimental model, 300 g of phase 2 compost, is spawned and inserted in aerated glass vessels. Compost is colonised at an air temperature of 24°C. After 7, 10 and 14 days of spawn run, process air is sampled both in infected and non-infected cultures and analysed by coupled gas chromatography mass spectrometry (GC-MS). During this 14-day period white mushroom mycelium develops in the non-infected compost. In the infected compost the compost turns black with occasional tufts of white mycelium and green spores.

Volatile blends that are produced during normal compost colonisation (when *Agaricus bisporus* interacts with *Scytalidium thermophilum* and other micro flora present in compost) differ from those produced during colonisation of *T. aggressivum* infected compost. Some of the volatiles appear to be specific for *T. aggressivum* infected compost. Next to this also consistent differences in the overall pattern of volatile production are seen. Infections with *T. harzianum*, *T. atroviride*, an *Aspergillus* species, or Smokey mould (*Penicillium citreonigrum*) produce different volatile patterns. Significant differences between the volatile blends of infected and non-infected compost are visible after 7 days of compost colonisation. In commercial practice of phase 3 composting, tunnels are likely to be partially infected. On-going research is directed at studying larger amounts of compost that is only partially infected.

Keywords: *Trichoderma aggressivum*; button mushroom; *Agaricus bisporus*; volatiles; phase 3 compost; metabolomics.

INTRODUCTION

Many "green moulds" that occur in mushroom cultivation, are considered to be "weed-moulds". *Trichoderma aggressivum*, however, causes problems that are much worse, as it can totally wipe out crop production. The first reports of devastating green mould infection in the U.K. and Ireland date from around 1986 [1, 2, 3]. A few years later, severe green mould infections were experienced in Canada [4], the Eastern part of the U.S. [5], California [6], Spain [7] and France [8]. Despite being one of the main button mushroom producers, the Dutch industry remained spared of this disease for a long time. Nevertheless, in 2006 the first incidents were reported [9]. In about the same period, also the Hungarian industry experienced green mould problems [10].

Identifying the aggressive pathogen among the non-aggressive *Trichoderma* strains and species proved to be challenging. As morphological identification alone was not able to easily discriminate aggressive and non-aggressive strains [11], research focused on molecular techniques [12, 13, 14]. Eventually, Samuels et al. combined morphological characteristics and molecular data to reach a species description [15]. The aggressive *Trichoderma* strains, formerly known as *Trichoderma harzianum* biotypes 2 (European origin) and 4 (American origin) were renamed as *Trichoderma aggressivum* f. *europeanum* and *Trichoderma aggressivum* f. *aggressivum*, respectively. For diagnostic purposes several researchers published PCR based methods [16, 17].

T. aggressivum infects compost at a very early stage and in the Dutch situation infection most likely takes place at the compost yard. Unfortunately, diseased compost cannot be recognized at the compost yards. Even though compost producers in the Netherlands are very keen to prevent green mould problems, occasionally still a number of crops get infected. Therefore, there is a need for a reliable method that allows early detection of *Trichoderma* green mould.

In the Netherlands spawn run is performed in bulk at the compost yards and is referred to as phase 3 composting. During this process, spawned compost is incubated in tunnels and ventilated with large volumes of air to control compost temperature. During this process the compost is inaccessible for sampling of the compost. In addition, it is anticipated that small samples such as normally taken for PCR, may not be representative for the large volumes of compost that are produced. Also the short span of time between emptying of phase 3 tunnels and delivery of the compost at the growers renders PCR based methods less useful.

Mumpuni et al. [18] showed that *A. bisporus* and *T. aggressivum* use volatiles to affect each other's growth rate *in vitro*. This suggests that it may be possible to detect infected compost by analysing the volatiles present in the process air during phase 3 composting. Here we show results of experiments where we used chemical analysis of volatiles produced during phase 3 compost combined with an untargeted metabolomics approach to detect an infection with *T. aggressivum* at early stages. We demonstrate that this eventually could lead to a sophisticated non-invasive detection method of *T. aggressivum* in the process air of the tunnels, without the need to sample inside the tunnel during spawn run.

MATERIALS AND METHODS

Organisms and strains used. For all experiments commercial spawn of *A. bisporus* strain A15 (Sylvan Inc.) was used. Strains of *Trichoderma aggressivum* (MES 13067), *Trichoderma*

harzianum (MES 12998) and *Trichoderma atroviride* (MES 13083) were all isolated from diseased commercial crops in the Netherlands within the period 2007-2009. Species identity was determined by sequence analysis of the internal transcribed spacer of the ribosomal genes of part of the translation elongation factor 1α gene using the database of the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (http://www.isth.info/index.php). The strains of *Aspergillus fumigatus* (MES 11163) and smokey mould (*Penicillium citreonigrum*) were isolated from diseased crops in the Netherlands in 1994 and 2009, respectively, and species name was determined by microscopy.

Infection experiments. Portions of 300 g of spawned compost were placed in glass vessels on top of a nylon gauze sheet which in turn rests on a piece of oasis foam (Fig. 1). Infection was established by inserting 15 grains of sorghum that were overgrown with *T. aggressivum* mycelium into the compost. To establish infections of *T. harzianum*, *T. atroviride* and *P. citreonigrum*, 15 g of spawn grains overgrown with the pathogens was mixed with 5 g of mushroom spawn and used to inoculate 900 g of phase 2 compost. The spawned compost was divided in three equal portions and inserted into the experimental vessels. Controls were left uninfected. Subsequently, the vessels were closed and incubated for 3 weeks at 24 °C under continuous aeration by blowing purified air underneath the compost layer using a copper tube. At the outlet volatiles from process air were collected at different time points. Three vessels infected with *T. aggressivum* and three non-infected controls were incubated simultaneously.

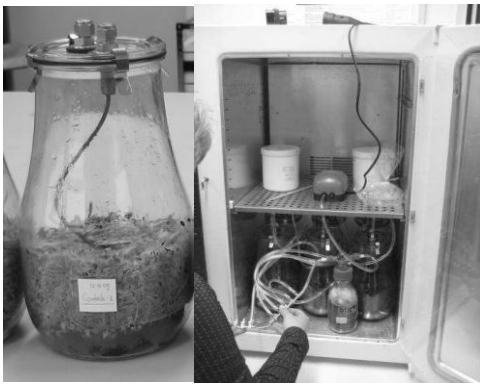

Figure 1: Experimental setup of compost experiments.
Portions of 300 g of compost were placed on top of a nylon gauze which in turn rests on a piece of oasis foam. Air is blown underneath the compost layer using a copper tube. At the outlet process air can be sampled.

Headspace sampling of volatiles. Volatiles produced by the compost were collected on stainless steel cartridges filled with 200 mg of Tenax TA (20/35 mesh, Grace Alltech) at the start of spawn run (T=0), after 7d (T=7), 10d (T=10), and 14d (T=14) for 1 hour at an air flow rate of 200 $ml.min^{-1}$. The cartridges were directly connected to the outlet if the vessel. During this period a volume of 12 litres of air was blown through the vessel. A vessel holds a volume of 2.5 litres, so during a sampling time of 1 hour, the volume of the vessels was refreshed about 5 times. After sampling the cartridges were capped on both sides and stored until analysis.

Chemical analysis of headspace volatiles. Headspace samples were analysed by coupled gas chromatography - mass spectrometry (GC-MS) with a Thermo TraceGC Ultra connected to a Thermo TraceDSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA). Before thermodesorption, the cartridges filled with Tenax TA were flushed with helium at 30 ml min^{-1} for 15 min to remove moisture and oxygen. After flushing the collected volatiles were

desorbed from the Tenax traps at 250 °C (Ultra; Markes, Llantrisant, UK) for 4 min with a helium flow of 30 ml min^{-1}. The released compounds were focused on an electrically cooled sorbent trap (Unity; Markes, Llantrisant, UK) at a temperature of 4 C. Volatiles were injected on the analytical column (ZB-5Msi, 30 m x 0.25 mm ID, 1.0 µm–film thickness, Phenomenex, USA) in splitless mode by ballistic heating of the cold trap to 260 C for 3 min. A constant column flow was set to 1ml/min^{-1}. The temperature program started at 40 C (3.5-min hold) and rose 10 C min^{-1} to 280 C (7-min hold). The column effluent was ionised by electron impact (EI) at 70 eV. Mass scanning was done from 45 to 450 *m*/*z* with a scan time of 5.13 scans s^{-1}.

Data processing. We used an untargeted metabolomics approach to analyse the GC-MS raw data [19]. GC-MS raw data were processed by using MetAlign software [20] to extract and align the mass signals (s/n≥ 3). Mass signals that were below an s/n of 3 were awarded randomized values between 2.4 and 3 times the calculated noise value. Only mass signals that were present in at least 2 samples were retained for analysis, all others were discarded. Signal redundancy per metabolite was removed by means of clustering and mass spectra were reconstructed [21]. The eluted compounds were identified using Xcalibur software (Thermo, Waltham, USA) by comparing the mass spectra with those of authentic reference standards or with NIST 08 and Wageningen natural compounds library spectra. Linear retention indices were calculated for each compound according to van den Dool & Kratz [22] and were compared with those published in the literature. The quantitative composition of the volatile blends was evaluated by principal components analysis (PCA) and orthogonal projection of latent structure discriminant analysis (OPLS-DA) using the software program SIMCA-P 12.0.1. (Umetrics AB, Umea, Sweden) [23]. Data were log-transformed and then variables were mean-centred, and pareto scaled.

RESULTS

Result of infections. An infection of compost with *T. aggressivum* was easy to establish when 15 spawn grains covered with *T. aggressivum* mycelium were inserted into 300 g of freshly spawned compost. Uninfected compost has a brownish colour with clearly visible white mycelium of *A. bisporus*. *T. aggressivum* infected compost is black with occasionally green tufts of spores. There is no visible sign of the white *A. bisporus* mycelium.

Lowering the number of infected spawn grains to 5 /300 g of freshly spawned compost still resulted in well infected compost after 10 days of spawn run. When using only one infected spawn grain, results were more variable, showing lots of *A. bisporus* mycelium by day 10 of spawn run. However, even from compost that did not show visible green mould symptoms by day 18 of spawn run, *T. aggressivum* could still be isolated. Infection with *T. harzianum*, *T. atroviride*, and *P. citreonigrum* was much more difficult to establish. Replacing 75% of the *A. bisporus* spawn used to inoculate phase 2 compost with *T. harzianum* colonized spawn resulted in some spots with green mould in the vicinity of spawn grains. However, most of the compost did not show visible signs of infection after 18 days of spawn run. In contrast, infection with *T. atroviride*, *Aspergillus* spp. and *P. citreonigrum* showed clearly visible signs of infection at the end of the experiment. Especially *T. atroviride* infected compost showed massive signs of infection with lots of pustules with green spores. After infection with an *Aspergillus* species the compost showed a less abundant presence of *A. bisporus* mycelium. After infection with *P. citreonigrum* signs of infection (lack of the white mycelium of *A. bisporus*) were somewhat slow to develop. Infection with *P. citreonigrum* was apparent after 18 days of spawn run. However, after a prolonged incubation (24 days) brownish spots of infection were seen throughout the whole compost layer.

Volatile production after infection with *T. aggressivum*. After data processing 578 potential volatile compounds were retained and relative intensities were subjected to PCA. PCA analysis resulted in a model with 5 significant PCs according to cross validation explaining 78% (R^2X) of the total variation (Q^2X=54%). There are no major differences in volatile patterns of infected and non-infected compost immediately after spawning (Fig. 2).

However, after 7, 10, and 14 days of spawn run the volatiles blends of *T. aggressivum* infected compost differ from those of non-infected compost. In uninfected compost, the largest differences in volatile pattern occur during the first 10 days of spawn run (Fig. 3).

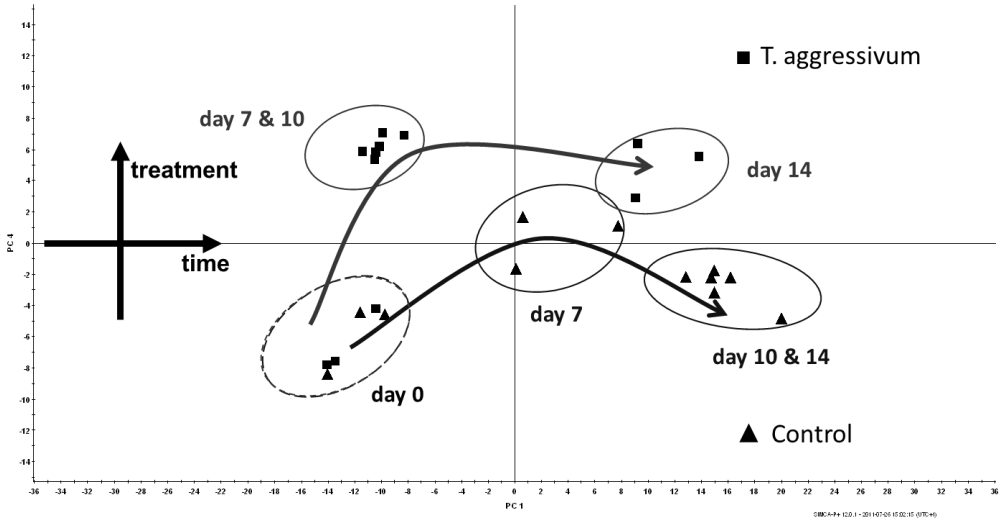

Figure 2: Principal Component Analysis the volatiles blends sampled during spawn run. PC 1 and PC 4 explaining 52% of the total variation are given. There are no major differences in volatile patterns in infected and non-infected compost immediately after spawning. However, after 7, 10 and 14 days of spawn run the volatiles in *T. aggressivum* infected compost differ from the volatiles in uninfected compost.

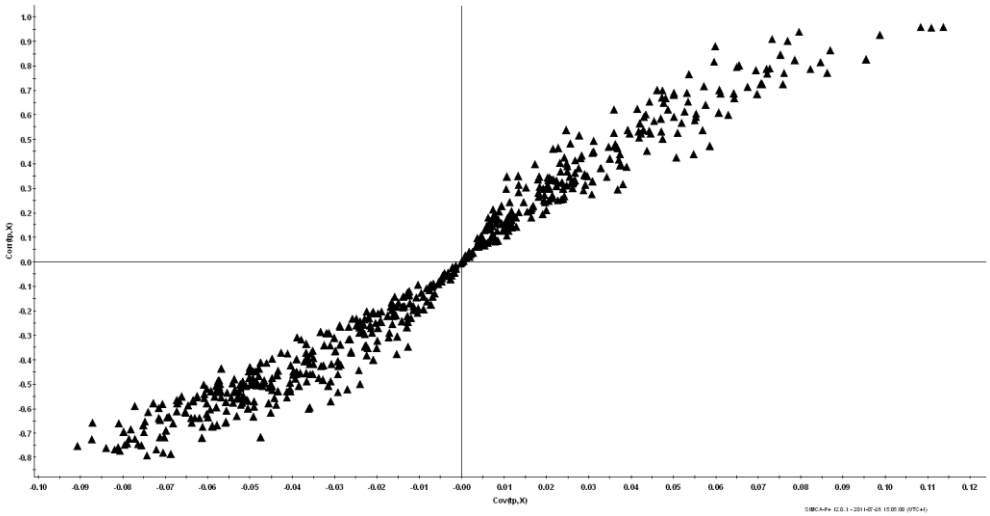

Figure 3: S-plot of an OPLS-DA of volatiles produced by *T. aggressivum* infected compost and non-infected compost.

The S-plot visualizes both the covariance and correlation loading profiles of the volatiles. Each triangle represents a volatile compound. Volatiles located at the upper right end of the plot are significantly more abundant in *T. aggressivum* infected compost on a 95% confidence level.

Volatile patterns in non-infected compost show no major differences after 10 and 14 days of spawn run. In *T. aggressivum* infected compost, the time course of volatile patterns is different. There are clear differences in volatile pattern between spawning and after 7 days of spawn run. Volatile patterns obtained after 7 and 10 days of spawn run do not differ much from each other. However, during the last 4 days of incubation, there are again differences in volatile pattern.
To determine which volatile compounds differ between *T. aggressivum* infected and non-infected compost, the samples of day 7, 10, and day 14 were pooled and the pooled samples were subjected to OPLS-DA. OPLS-DA is a supervised classification technique that aims to find a set of latent variables that discriminate the data according to the predefined treatments (infected *vs* non-infected) in the best possible way. Volatiles that were significantly different between infected and non-infected compost were selected from the so-called S-plot and jack-knifed confidence intervals (Figure 3). The S-plot visualizes the influence of volatiles in an OPLS-DA model. It is a scatter plot that combines the covariance and correlation loading profiles resulting from the predictive discriminant component [24]. This corresponds to combining the contribution or magnitude (covariance) with the effect and reliability (correlation) for the volatile compounds [24].The S-plot visualizes both the covariance and correlation between the volatiles and the modelled class designation [24]. Volatile compounds that are located at the upper right end of the S-shaped plot are significantly more abundant in *T. aggressivum* infected compost compared to non-infected compost (Figure 3). One of the volatiles that most reliably differs between *T. aggressivum* infected compost and non-infected compost is an unknown compound that from its mass spectral signature appears to be a sesquiterpene ($C_{15}H_{24}$). Next to this two monoterpenes (monoterpene 1 and monoterpene 2) are important to discriminate *T. aggressivum* infected compost and non-infected compost.

Specificity of potential marker volatiles of infected compost. Reliable volatile indicators for *T. aggressivum* infected compost should be specific for *T. aggressivum*. Infections of compost with *T. harzianum, T. atroviride, Aspergillus* species, or *P. citreonigrum* are considered to pose a lower risk than infection with *T. aggressivum*. Figures 5a-c show presence of the indicator sesquiterpene, monoterpene 1 and monoterpene 2 in the GC-chromatograms produced by the different pathogens. The sesquiterpene that is produced in *T. aggressivum* infected compost also appears to be produced in compost that is infected by *T. harzianum*, but appears absent in compost that is infected by *T. atroviride*, an *Aspergillus* species, and *P. citreonigrum*.

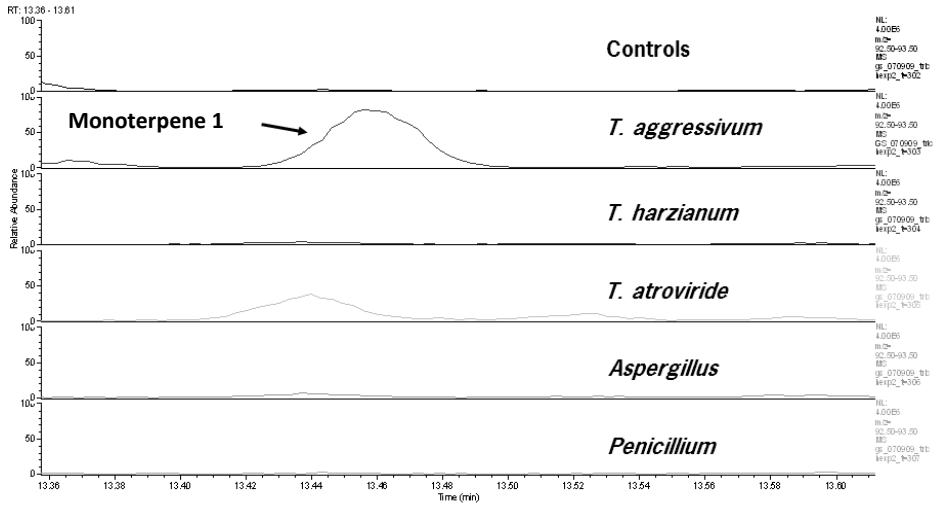

Figure 5a: Specificity of sesquiterpene as indicator of infection with *Trichoderma aggressivum*.

Figure 5b: Specificity of monoterpene 1 as indicator of infection with *Trichoderma aggressivum*.

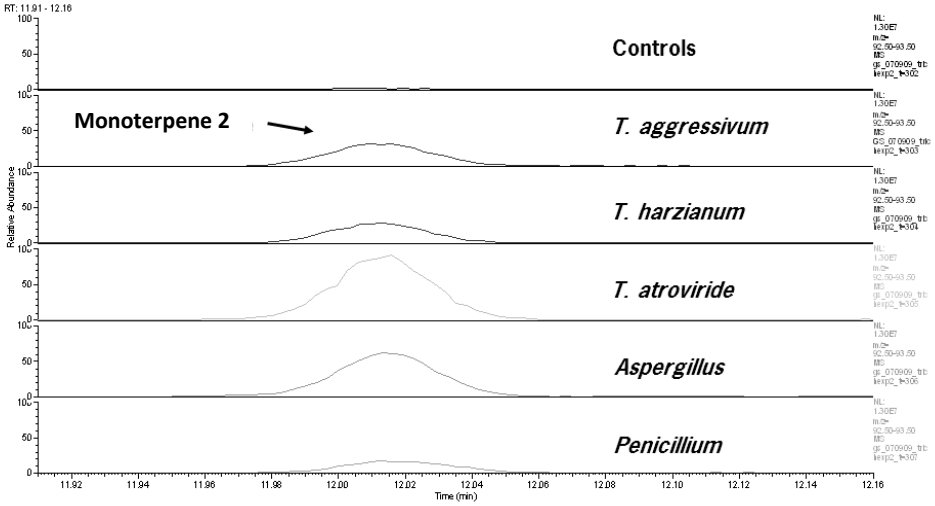

Figure 5c: Specificity of monoterpene 2 as indicator of infection with *Trichoderma aggressivum*.

The emission of monoterpene 1 appears to be truly specific for infection of compost by *T. aggressivum*. The emission of monoterpene 2 occurs in all infected composts and is therefore not useful to discriminate the pathogens.

DISCUSSION

The results of the experiments described here, were performed to study the possibility to identify *T. aggressivum* infected compost already during spawn run by the emitted volatiles. Mumpuni et al. [18] showed that volatiles (other than CO_2) produced by *T. aggressivum* were able to significantly reduce radial growth of *A. bisporus* colonies. The chemical nature of these volatiles was, however, not determined. For *T. harzianum* [25] and *T. atroviride* [26] some information on the chemical nature of the volatiles they produce is available. Also *A. bisporus* is known to produce a large number of volatiles. Some of these volatiles, for instance the C_8-compounds, are

involved in repelling pests and pathogens and controlling sporulation [27-31]. For instance, 1-octanol shows fungitoxic activity [32]. Especially *Trichoderma* species are known for producing antifungal volatiles [33]. The antifungal volatile lactone, 6-pentyl-pyrone (6-PAP), is well described as an antifungal compound produced by *T. harzianum* as secondary metabolite [34, 35, 36]. The production of volatiles is influenced by the substrate on which the mycelium is growing [25] and by the age of the mycelium [26].

In our experiments there is a change in the volatile pattern as spawn run is proceeding (both in *T. aggressivum* infected and non-infected compost). Differences become apparent already after 7 days of spawn run. Specific volatiles that are present in the process air of *T. aggressivum* infected compost but not in the uninfected compost are an as yet unidentified sesquiterpene, and the two monoterpenes. The unidentified sesquiterpene is present in process air of both *T. aggressivum* - and *T. harzianum* infected compost. The monoterpene 2 is present in the process air of all composts that were infected with pathogens. It is likely that this monoterpene is produced by *A. bisporus*. The monoterpene 1 appears to be specific for *T. aggressivum* infected compost.

Krupke et al. [37] studied growth inhibiting substances produced by *T. harzianum* and *T. aggressivum*. For this they extracted substances from infected compost and malt extract broth. When grown in malt extract broth, 3,4-dihydro-8-hydroxy-3-methylisocoumarin (mellein) was the major antifungal compounds produced by *T. aggressivum*. However, we could not detect this compound in the volatile blends of *T. aggressivum* infected compost. Nor were we able to identify 6-PAP in the volatile blends of *T. aggressivum* infected compost.

CONCLUSIONS

Compost infected with *T. aggressivum* can be distinguished from uninfected compost during spawn run, based on its volatile blend. Volatile blends are distinguishable already a few days (7-10d) after infection with *T. aggressivum.* Some terpenoids within the volatile blend might serve as indicators due to their specificity for *T. aggressivum*.

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