Detection of *Trichoderma aggressivum* Green Mould During Spawn-run

Johan Baars[1], Jo Rutjens[1] and Roland Mumm[1,2]

[1]Plant Research International, P. O. Box 16, Wageningen 6700AA, The Netherlands; [2]Centre for BioSystems Genomics, P. O. Box 98, Wageningen 6700AB, The Netherlands

Email: Johan. Baars@ wur. nl; Jo. Rutjens@ wur. nl; Roland. Mumm@ wur. nl

Since 2006, 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 method that allows early detection of *Trichoderma* green mould.

Agaricus bisporus and *T. aggressivum* use volatiles to affect each other's growth rate. We tested the possibility of detecting *Trichoderma* green mould using the volatiles that are produced during spawn run.

We used an untargeted metabolomics approach to compare the volatiles emitted by non-infected compost with those of infected compost. A proof of principle for this approach was obtained in an experimental model containing 300 g of freshly spawned, well aerated phase 2 compost. This model compared a development into fully infected compost with a normal spawn-run.

In commercial practice of phase 3 composting, tunnels are likely partially infected. We therefore tested whether the method would also work in 50 kg batches of partially infected compost. The potential of using headspace volatiles asa detection method to detect *T. aggressivum*in the process air of the tunnels, avoiding the need to sample inside the tunnel is discussed.

Agaricus bisporus, *Trichoderma aggressivum*, Green Mould, Volatiles, Compost, Headspace Analysis, Gas-chromatography Mass Spectrometry, Metabolomics

Introduction

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. This centralised system of compost production allows a high level of hygiene. However, if an infection of compost occurs, the problems affect a large number of mushroom farms.

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 an early detection of *Trichoderma* green mould.

Although there are PCR methods available for diagnosis of *Trichoderma* green mould (Chen et al., 1999;

Pudelko and Pyzalski, 2010; Staniaszek et al., 2010), during spawn run the compost is inaccessible for sampling. 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., (1998) 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. Using small quantities of compost, Baars et al. (2011) showed that volatiles produced during spawn run in *Trichoderma aggressivum* infected compost differ from those in non-infected compost. However, these results were obtained in a system that compares fully infected compost with completely uninfected compost. Such a situation will probably never occur in a real life situation. In this paper we describe results of experiments that compare partially infected composts with uninfected compost. Main goal is to eventually develop 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

For all experiments commercial spawn of *A. bisporus* strain A15 (Sylvan Inc.) was used. *Trichoderma aggressivum* (MES 13067) was isolated from a diseased commercial crop in the Netherlands in 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).

For infection experiments, large containers were constructed that could hold up to 50 kg of compost (Fig. 1). The containers had 2 different compartments. A shallow tray was used asa bottom. This shallow tray

Fig. 1 Containers used for spawn run.

was fitted with a ventilator that blew air into the container. On top of the bottom container, a large container was fitted with air tight sealing. From this container the bottom was removed and replaced with a metal rack on top. Portions of 50 kg of spawned compost were placed in the large container on top of a nylon mat (such as normally used in composting facilities to empty the room) that rested on the metal rack. The compost was stacked in 6 layers of about 5 cm thick (about 8.5 kg/layer), separated by a nylon gauze. The container was closed by a shallow tray placed upside down on top of the middle container. A flexible hose was fitted to the top tray and to the bathroom ventilator fitted to the bottom tray. This allowed us to recirculate air through the compost. Fresh air was supplied to the containers using a membrane pump.

Infection was established by inserting 2 sorghum grains that were overgrown with *T. aggressivum* mycelium and 2 spawn grains (strain A15) into the top layer of compost. Controls were left uninfected. Subsequently, the containers were closed and incubated for 17 days under continuous aeration by recirculating air through the compost layer. For this the containers (3 × infected and 3 controls) were placed in a growing room at the Mushroom Farm of Wageningen UR. Four containers were fitted with temperature sensors to record temperature during spawn run. When the temperature of the compost increased, temperature in the growing room was lowered in order to maintain compost temperature below 30℃. At the outletsof the containers, volatiles from process air were collected at time points T = 1 days, T = 10 days and T = 14 days.

At the end of the experiment, the different layers of compost were taken out of the containers and overlaid with a metal gauze, orifices sized 12 cm × 9 cm. At each intersection of the wires small samples of compost were taken to determine the extent to which the compost was infected with *T. aggressivum*. Compost samples were placed on malt extract agar and incubated at 28℃ to see whether *Trichoderma* mycelium would grow from them. Based on the locations at which *Trichoderma* was found in the compost, an estimate was made of the amount of infected compost.

Volatiles produced by the compost were collected on stainless steel cartridges filled with 200mg of Tenax TA (20/35 mesh, Grace Alltech) at the start of spawn run (T = 1), after 10d (T = 10), and 14d (T = 14) for 1 hour at an air flow rate of 150mL/min using a handheld vacuum pump. In some experiments additional samples were taken at T = 12 days and T = 18 days. After sampling the cartridges were capped on both sides and stored until analysis.

Headspace samples were analysed by coupled gas chromatography-mass spectrometry (GC-MS) with a Thermo TraceGC Ultra connected to a Thermo Trace DSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA). Before thermodesorption, the cartridges filled with Tenax TA were flushed with helium at 30mL/min for 15 min to remove moisture and oxygen. After flushing the collected volatiles were desorbed from the Tenax traps at 250℃ (Ultra; Markes, Llantrisant, UK) for 4min with a helium flow of 30mL/min. The released compounds were focused on an electrically cooled sorbent trap (Unity; Markes, Llantrisant, UK) at a temperature of 4℃. Volatiles were injected on the analytical column (ZB-5Msi, 30 m × 0.25 mm ID, 1.0 μm-film thickness, Phenomenex, USA) in splitless mode by ballistic heating of the cold trap to 260℃ for 3 min. A constant column flow was set to 1mL/min. The temperature program started at 40℃ (3.5 min hold) and rose 10℃/min to 280℃ (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.

We used an untargeted metabolomics approach to analyse the GC-MS raw data (Hall, 2011). GC-MS raw data were processed by using MetAlign software (Lommen, 2009) to extract and align the mass signals ($s/n \geqslant 3$). Mass signals that were below a 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 (Tikunov et al., 2005, 2011). The eluted compounds were identified using Xcalibur software (Thermo, 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 and Kratz(1963) and were compared with those authentic reference standards and 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, Sweden) (Eriksson, 2006). Data were log-transformed and then variables were mean-centred, and pareto scaled.

Results and Discussion

After spawn run, the containers were opened to assess the level of infection in the compost. The different layers of compost were taken out of the container and laid side by side (Fig. 2). The top three layers of compost were visibly infected at the end of spawn run. Infected areas were clearly recognised as dark spots with less mycelium growth in the compost. To assess the extent to which *T. aggressivum* had expanded into the compost, samples were taken at regular distances and were incubated on maltextract agar (50 mg/kg Penicillium G and Streptomycin added). The results show that the *T. aggressivum* mycelium did not extend far beyond the visible infection marks.

Fig. 2 Infection marks visible in the compost at the end of spawn run. From front to back, the top layer and the layers below are shown.

A partial infection was achieved since about 5% – 14% of the compost were infected by *T. aggressivum* mycelium. The amount of infected compost, however, differed between the different experiments (Table 1).

Table 1 Amounts of infected compost after point inoculation with *Trichoderma aggressivum*

Exp.	Container 1	Container 2	Container 3	Average
27020	1.9 – 3.9 kg	n. d.	2.9 – 4.4 kg	5% – 8% of the compost
27030	3.9 – 4.9 kg	2.9 – 3.6 kg	2.6 – 4.2 kg	6% – 8% of the compost
27060	3.3 – 7.3 kg	4.3 – 5.5 kg	4.3 – 5.2 kg	8% – 12% of the compost
27130	2.4 – 4.9 kg	3.3 – 4.9 kg	7.7 – 10.6 kg	9% – 14% of the compost

Data of volatiles profiles from three out of the four replicate experiments were pooled and subjected to principal component analysis (PCA). A total of 356 reconstructed volatiles were retained and used in the statistical analysis. PCA showed that the volatile profiles T = 1clearly differed from the volatile blends of later time points(T = 10, T = 12, T = 14 and T = 18, Fig. 3, left panel). The T = 1 samples are characterised by relative high abundances of styrene and xylene, two benzene derivatives that likely originate from the mini-tunnels (Fig. 3, right panel). Interestingly, some samples being located in the upper left quadrant of the score plot (Fig. 3, left) are characterised by a high content of chloroform and some other small organo-halogen compounds. Yet all containers were filled with subsamples of the same batch of compost. It remains to be investigated what the factors are determining the considerable production of organo-halogens in some containers but not in others irregarding of ainfection with *T. aggressivum*.

Grove (1981) has shown that out of sodium chloride, *A. bisporus* is able to produce tetrachloro-1,4-dimethoxybenzene. This compound is a secondary metabolite that is produced by many basidiomycetes. However, this compounds is not among those produced by the compost. Chloroform can also be produced by other basidiomycete fungi (de Jong and Field, 1997). Depending on the substrate, also *Trichoderma* speciesare able to produce chloroform (Bruce et al., 2000).

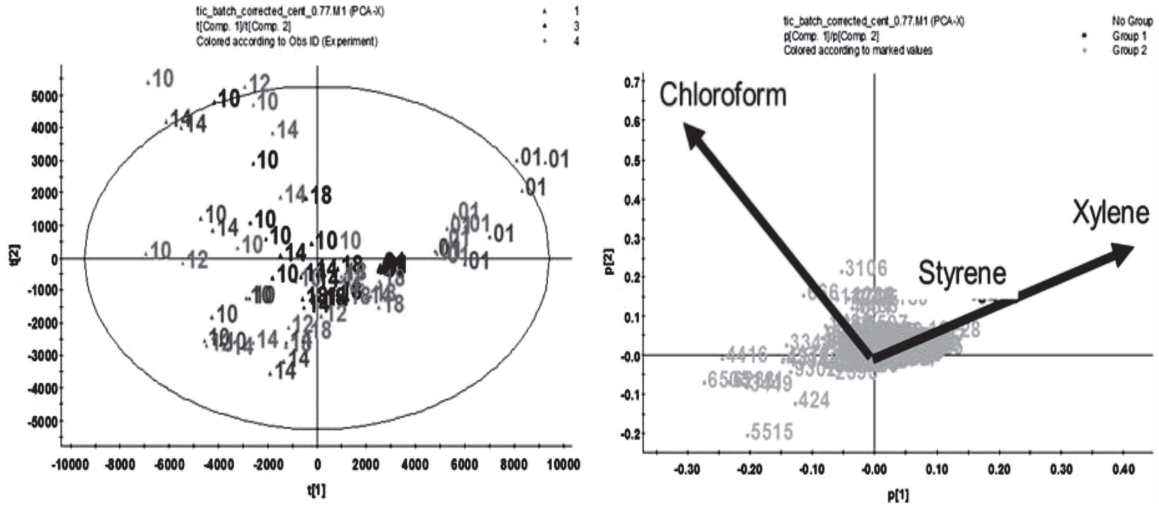

Fig. 3 PCA analysis of volatile profiles of *T. aggressivum* and non-infected compost. Left panel: Score plot of the first 2 PCs explaining 68% of the variation. Different colours indicate different experiments that were pooled. Right panel: Loading plot of the first 2 PCs.

The remaining data were used to find volatiles that are able to discriminate infected and uninfected compost as early as possible during spawn run. After 10 days of spawn run the differences between the volatile patterns were not distinct enough to allow discrimination of infected and uninfected compost. After 12, 14 and 18 days of spawn run the differences between the volatile patterns became more pronounced.

Using OPLS-DA, an attempt was made to identify which volatiles are characteristic for *T. aggressivum* infected compost. Out of the twelve most reliably discriminating volatiles, eight were sesquiterpenes, two were C_8-compounds, one was a benzene and one a ketone. Sesquiterpenesare a chemical class with many members that show antifungal activity (Stadler and Sterner, 1998; Abraham, 2001; Cheng et al., 2005). This antifungal activity is for instance demonstrated by changes in the linear growth rate of the target organism or changes in its enzymatic activity (Strobel et al., 2001). These effects can be both stimulating or inhibiting

(Dick and Hutchinson, 1966; Schoeman et al., 1996; Mackie and Wheatley, 1999).

The results described here, indicate that it may be possible to use volatiles to discriminate compost that is partially infected with *Trichoderma aggressivum* from non-infected compost no earlier than after 12 days of spawn run.

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