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ORIGINAL ARTICLE

# Formation of 6-n-pentyl-2H-pyran-2-one (6-PAP) and other volatiles by different *Trichoderma* species

**Henryk Jeleń · Lidia Błaszczyk · Jerzy Chełkowski · Katarzyna Rogowicz · Judyta Strakowska**

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**Abstract** Seventy-seven headspace*Trichoderma* isolates belonging to eight species—*T. atroviride*, *T. citrinoviride*, *T. hamatum*, *T. harzianum*, *T. koningii*, *T. viride*, *T. viridescens*, and *T. virens*—were screened for their ability to produce 6-n-pentyl-2H-pyran-2-one (6-PAP) and other volatiles using solid phase microextraction (SPME) coupled to gas chromatography–mass spectrometry (GC-MS). Intra- and interspecies variability was demonstrated in the 6-PAP synthesis. The most efficient producer of 6-PAP on potato dextrose agar (PDA) was *T. atroviride*. However, the variation in 6-PAP synthesis differed significantly among strains of this species, the lowest value being 33.4 μg (strain AN212), whereas the highest reached 1,426 μg per culture for strain AN35. Also, *T. viridescens* produced significant amounts of 6-PAP (200.1–526.3 μg for 11 out of 12 strains). Moderate producers were isolates belonging to *T. hamatum* (up to 155 μg) and *T. citrinoviride* (up to 200 μg). *Trichoderma viride* isolates showed very little production of 6-PP on PDA. No 6-PAP formation was detected in cultures of 25 isolates belonging to three species, *T. koningii*, *T. harzianum*, and *T. virens*. *Trichoderma atroviride* AN35, as the most efficient producer of 6-PAP, was selected to observe the dynamics of 6-PAP formation during growth on PDA at 20 °C for 6 days. A radical increase in 6-PAP production started after the fourth day of incubation, and the maximum expression was achieved in the final phase of the experiments, 6 days after inoculation. Apart from 6-PAP, over 40 other volatile compounds were detected in the survey of *Trichoderma* species. Among them, the most commonly produced substrates were 1-octene-3-ol, isoamyl alcohol, 3-octanone, cyclohept-3-en-1-one, 2-pentylfuran, linalol isobutyrate, toluene, D-limonene, and α-bergamotene. Seventeen of the detected compounds have never previously been reported as a secondary metabolite of *Trichoderma*.

**Keywords** *Trichoderma* · Metabolic diversity · Solid phase microextraction · Volatiles · 6-n-pentyl-2H-pyran-2-one · Antifungal compounds

H. Jeleń · K. Rogowicz
Faculty of Food Science and Nutrition, Poznań University of Life Sciences, Wojska Polskiego 31, 60-624 Poznań, Poland

L. Błaszczyk (✉) · J. Chełkowski · J. Strakowska
Institute of Plant Genetics, Polish Academy of Sciences, Strzeszyńska 34, 60-479 Poznań, Poland
e-mail: lgol@igr.poznan.pl

## Introduction

Species of the fungal genus *Trichoderma* (teleomorph in *Hypocrea*) are frequently encountered fungi, especially in soil and decaying wood (Klein and Eveleigh 1998; Kubicek et al. 2008; Jaklitsch 2009; Druzhinina et al. 2011; Friedl and Druzhinina 2012). Some of these species are known for their antagonistic activities towards plant pathogens, e.g., *Botritis cinerea*, *Fusarium* spp., *Pythium* spp., *Rhizoctonia solani*, *Verticillium dahilae*, and *Sclerotinia* spp. (Harman et al. 2004; Verma et al. 2007; Druzhinina et al. 2011), which makes them highly suitable for use in biological control. Mechanisms that have been described as the basis for biocontrol activity include competition for nutrients and space, antibiosis and mycoparasitism, stimulation of plant growth, and elicitation of plant defense reactions against pathogens (Papavizas 1985; Howell 1998; Benítez et al. 2004; Harman et al. 2004, Harman and Kubicek 1998). It has been shown that the ability of *Trichoderma* species to antagonize is reflected in their capacity to secrete a spectrum of biochemicals, such as cell wall-degrading enzymes, siderophores, chelating iron, and volatile and non-volatile metabolites (Harman et al. 2004; Reino et al. 2008; Vinale et al. 2008; Stoppacher et al. 2010; Druzhinina et al. 2011).

Among the volatile antifungal compounds produced by *Trichoderma* strains, the most important and well-documented is 6-n-pentyl-2H-pyran-2-one (6-PAP), a polyketide with a characteristic sweet coconut-like aroma. 6-PAP and other α-pyrone analogs have been detected in cultures of several *Trichoderma* strains, such as *T. viride* (Collins and Halim 1972), *T. harzianum* (Claydon et al. 1987; Bonnarme et al. 1997), *T. koningii* (Simon et al. 1988), and *T. atroviride* (Reithner et al. 2005, 2007). However, it should be noted that the identification of most of these strains was based only on morphological characters, which are known to be prone to misidentification. As the *Trichoderma* species names reported prior to the introduction of advanced molecular and bioinformatics methods have been found to be questionable, it has been suggested to interpret rather cautiously the results of previous studies (Samuels 2006; Kubicek et al. 2008).

6-PAP and its analogs have been demonstrated to inhibit growth of several plant-pathogenic fungi: *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *lycopersici*, *Fusarium verticilioides* (*moniliforme*), *Phytophthora megasperma*, *Rhizoctonia solani*, and *Armillaria mellea* (Al-Heeti and Sinclair 1988; Scarselletti and Faull 1994; Worasatit et al. 1994; Poole et al. 1998; Tarus et al. 2003). In addition, it has been shown that 6-n-pentyl-2H-pyran-2-one can reduce the production of deoxynivalenol by *Fusarium graminearum* on agar medium (Cooney et al. 2001). Recent work has been undertaken to disclose the role of 6-PAP in plant growth regulation and activation of plant defense responses (Vinale et al. 2008; El-Hassan and Buchennauer 2009).

Other volatile metabolites derived from *Trichoderma* spp., which are involved in complex *Trichoderma*–plant pathogen interactions, have been mainly assigned to alcohols, ketones, alkanes, furans, and mono- and sesquiterpenes (Ghisalberti et al. 1992; Mannina et al. 1997; Wheatley et al. 1997; Fiedler et al. 2001; Tarus et al. 2003; Lloyd et al. 2005; Nemčovič et al. 2008; Stoppacher et al. 2010; Polizzi et al. 2011). These volatiles have been described for *T. atroviride*, *T. aureoviride*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii*, and *T. viride*, and their spectrum is characteristic for each strain as well as dependent on growth phase and nutritional, biological, and environmental conditions (Wheatley et al. 1997; Bruce et al. 2000; Tarus et al. 2003; Nemčovič et al. 2008; Stoppacher et al. 2010; Polizzi et al. 2011).

It is noteworthy that studies on the identification and profiling of volatile metabolites of *Trichoderma* included only a few species (individual strains) of this genus—*T. atroviride*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii*, and *T. viride*—which are well known for their potential in biological control. Furthermore, there is only limited information on inter- and intraspecific variability in the production of 6-PAP and other volatile compounds by strains of various *Trichoderma* species.

Therefore, the aim of this paper was to examine the ability of 77 isolates belonging to eight different *Trichoderma* species, including species that are not yet fully recognized, as important biological control agents (BCA) to form 6-n-pentyl-2H-pyran-2-one and other volatiles. In addition, an evaluation was conducted on the dynamics of 6-PAP production by *T. atroviride* AN35. Furthermore, the inhibitory effect of this metabolite on six toxigenic *Fusarium* species, considered to be the most important plant pathogens worldwide, was studied.

## Materials and methods

### Fungal collection

The 77 *Trichoderma* strains investigated in this study are listed in Table 1. Fifty-eight *Trichoderma* strains sourced from decaying wood, soil and mushroom farms in northern, eastern and central Poland had previously been identified by Błaszczyk et al. (2011). Ninety *Trichoderma* isolates were collected from pieces of decaying wood in the forests of southern Poland (mountains) and isolated as described by Błaszczyk et al. (2011). All the studied *Trichoderma* strains are deposited in the collection of the Institute of Plant Genetics, Polish Academy of Science, Poznań, Poland, and are available to the scientific community. Ten selected *Trichoderma* strains (Table 1) are deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS).

### Morphological analysis

Ninety isolates of *Trichoderma* sourced from decaying wood in the forests of the Karkonosze Mountains, Tatra Mountains, Sudety Mountains, and Gorce Mountains were identified morphologically following Gams and Bissett (1998). Colony characteristics were examined in cultures grown on potato dextrose agar (PDA; Oxoid) and Synthetischer Nährstoffarmer Agar (SNA; Nirenberg 1976) after 3–7 days at a temperature of 25 °C. Microscopic observations were performed in cultures grown on SNA.

### Molecular analysis

Molecular species identification was based on the sequencing of two different phylogenetic markers: the internal transcribed spacer region 1 and 2 (ITS1 and ITS2) of the rRNA gene cluster and a fragment of the translation-elongation factor 1-alpha (*tef1*) gene. Mycelium for DNA extraction was obtained as described previously (Błaszczyk et al. 2011). Isolation of total DNA was performed using the CTAB method (Doohan

**Table 1** *Trichoderma* isolates analyzed for 6-PAP production by SPME-GC-MS

| Culture code/CBS no. | Origin | | NCBI GenBank Assession no.[a] | | 6-PAP production[b] (μg per culture) |
|---|---|---|---|---|---|
| | Locality | Source | ITS | *tef1* | |
| *T. atroviride* | | | | | |
| AN 19 | Central Poland | Forest soil | HQ292786 | HQ292963 | 37.5±1.1 |
| AN 35/ CBS136452 | | Maize kernels | HQ292787 | HQ292953 | 1426.0±1.6 |
| AN 90 | | Garden soil | HQ292788 | HQ292954 | 53.5±21.5 |
| AN 95/ CBS136451 | | Compost | HQ292789 | HQ292955 | 1174.3±13.5 |
| AN 111 | | Forest wood | HQ292791 | HQ292964 | 291.9±23.2 |
| AN 152/ CBS136453 | | Triticale kernel | HQ292792 | HQ292957 | 904.9±107.4 |
| AN 182/ CBS136454 | | Forest wood | HQ292794 | HQ292965 | 854.9±105.9 |
| AN 206/ CBS136455 | | Mushroom compost | HQ292804 | HQ292960 | 980.5±44.6 |
| AN 212 | | Mushroom compost | HQ292795 | HQ292966 | 33.4±19.5 |
| AN 215 | | Mushroom compost | HQ292796 | HQ292967 | 243.7±0.2 |
| *T. citrinoviride* | | | | | |
| AN 89 | Central Poland | Garden soil | HQ292841 | - | 200.4±11.8 |
| AN 98 | | Forest wood | HQ292843 | - | ND |
| AN 99 | | Forest wood | HQ292848 | - | 3.7±1.7 |
| AN 198 | | Mushroom factory | HQ292845 | - | 0.6±0.9 |
| AN 199 | | Mushroom factory | HQ292846 | - | ND |
| AN 201 | | Mushroom factory | HQ292849 | - | ND |
| AN 262 | | Forest wood | JX184109 | - | ND |
| AN 303 | Tatra Mt, SP | Forest wood | JX184109 | - | 2.3±0.2 |
| AN 393 | Gorce Mts, SP | Forest wood | JX184109 | - | ND |
| AN 500 | Sudety Mts, SP | Forest wood | JX184109 | - | ND |
| *T. hamatum* | | | | | |
| AN 118 | Central Poland | Forest wood | HQ292854 | - | ND |
| AN 119 | | Forest wood | HQ292855 | - | 10.3±2.7 |
| AN 120 | | Forest wood | HQ292855 | - | 87.6±6.6 |
| AN 155 | Eastern Poland | Rye rhizosphere | HQ292851 | - | 155±5.5 |
| AN 175 | Central Poland | Forest wood | HQ292854 | - | 2.5±0.1 |
| AN 225 | | Forest soil | HQ292856 | - | 14.6±0.8 |
| AN 238 | | Forest soil | HQ292853 | - | ND |
| AN 277 | | Forest wood | HQ292853 | - | 0.4±0.6 |
| AN 279 | | Forest wood | HQ292853 | - | ND |
| AN 521 | Northern Poland | Forest wood | HQ292856 | - | 26.3±2.1 |
| *T. harzianum* | | | | | |
| AN 91 | Central Poland | Compost | HQ292860 | - | ND |
| AN 94 | | Forest soil | HQ292873 | - | |
| AN 108 | | Forest wood | HQ292869 | - | |
| AN 132 | | Forest wood | HQ292867 | - | |
| AN 133 | | Forest wood | HQ292874 | - | |
| AN 150 | | Forest wood | HQ292878 | - | |
| AN 181 | | Forest wood | HQ292875 | - | |
| AN 203 | | Mushroom compost | HQ292879 | - | |
| AN 207 | | Mushroom compost | HQ292881 | - | |
| *T. koningii* | | | | | |
| AN 100 | Central Poland | Forest wood | HQ292903 | HQ292975 | ND |
| AN 105 | | Forest wood | HQ292905 | HQ292977 | |
| AN 106 | | Forest wood | HQ292906 | HQ292978 | |

**Table 1** (continued)

| Culture code/CBS no. | Origin | | NCBI GenBank Assession no.[a] | | 6-PAP production[b] (μg per culture) |
|---|---|---|---|---|---|
| | Locality | Source | ITS | *tef1* | |
| AN 113 | | Forest wood | HQ292908 | HQ292980 | |
| AN 116 | | Forest wood | HQ292911 | HQ292983 | |
| AN 121 | | Forest wood | HQ292913 | HQ292985 | |
| AN 124 | | Forest wood | HQ292914 | HQ292986 | |
| AN 144 | | Forest wood | HQ292919 | HQ292990 | |
| *T. viride* | | | | | |
| AN 141 | Central Poland | Forest wood | HQ292922 | HQ293008 | 1.9±0.1 |
| AN 176 | | Forest wood | HQ292923 | HQ293010 | 2.0±0.1 |
| AN 179 | | Forest wood | HQ292924 | HQ293011 | 5.5±3.0 |
| AN 235 | | Forest soil | HQ292921 | HQ293013 | ND |
| AN 242 | Karkonosze Mts, SP | Forest wood | JX184121 | JX184098 | ND |
| AN 250 | | Forest wood | JX184121 | JX184098 | ND |
| AN 249 | | Forest wood | JX184122 | JX184099 | ND |
| AN 255 | | Forest wood | JX184121 | JX184098 | ND |
| AN 371 | Tatra Mts, SP | Forest wood | JX184124 | JX184100 | 8.8±0.8 |
| AN 402 | | Forest wood | JX184121 | JX184098 | ND |
| *T. viridescens* | | | | | |
| AN 93 | Central Poland | Forest wood | HQ292927 | HQ292995 | 264.7±11.5 |
| AN 122 | | Forest wood | HQ292928 | HQ292994 | 200.1±7.1 |
| AN 145/ CBS136456 | | Forest wood | HQ292930 | HQ292996 | 526.3±1.5 |
| AN 148/ CBS136457 | | Forest wood | HQ292933 | HQ292999 | 378.0±10.6 |
| AN 231/ CBS136458 | | Forest wood | HQ292938 | HQ293003 | 501.6±9.3 |
| AN 245 | Karkonosze Mts, SP | Forest wood | JX184127 | JX184103 | 372.7±17.1 |
| AN 248 | | Forest wood | JX184128 | JX184104 | 256.0±3.9 |
| AN 288/ CBS136459 | Central Poland | Forest wood | HQ292941 | HQ293006 | 394.6±20.6 |
| AN 323 | Tatra Mts, SP | Forest wood | JX184127 | JX184103 | 290.5±3.9 |
| AN 366 | | Forest wood | JX184128 | JX184104 | 1.4±0.1 |
| AN 492 | Gorce Mts, SP | Forest wood | JX184127 | JX184103 | 289.6±16.1 |
| AN 405/ CBS136460 | | Forest wood | JX184127 | JX184103 | 472.2±22.7 |
| *T. virens* | | | | | |
| AN 68 | Eastern Poland | Compost | HQ292943 | - | ND |
| AN 69 | | Compost | HQ292944 | - | |
| AN 70 | | Compost | HQ292947 | - | |
| AN 73 | | Compost | HQ292945 | - | |
| AN 74 | | Compost | HQ292946 | - | |
| AN 75 | | Compost | HQ292948 | - | |
| AN160 | | Grass root | HQ292945 | - | |
| AN 185 | Central Poland | Mushroom compost | HQ292947 | - | ND |

*Mts* mountains, *SP* Southern Poland, *ND* Not detected

[a] Identical accession numbers refer to identical sequences

[b] Amount of 6-PAP formed by the *Trichoderma* isolates grown by 6 days on PDA medium at 20 °C

et al. 1998). PCR amplification, DNA sequencing, and sequence analysis was carried out under the conditions described by Błaszczyk et al. (2011). The sequences were identified by BLASTn (http://blast.ncbi.nlm.nih.gov/) as well as *Trich*OKEY and *Tricho*BLAST (http://www.isth.info; Druzhinina et al. 2005; Kopchinskiy et al. 2005). The sequences were deposited in the NCBI GenBank and listed in Table 1.

Volatile metabolite analyses by SPME GC-MS

*Trichoderma isolate preparation for solid-phase microextraction (SPME) sampling*

Solid-phase microextraction (SPME) was used for the determination of 6-PAP and other volatiles by gas chromatography–mass spectrometry (GC/MS). *Trichoderma* isolates examined for the production of volatile compounds were grown on PDA at 28 °C for 7 days. Spores were suspended in sterile distilled water, diluted to a final concentration of $5\times10^5$ ml and used for inoculation of 10 ml PDS slants in 44-ml headspace vials, capped with sterile cotton plugs. The cultures were incubated in darkness at 20 °C for 6 days. For each of the 77 *Trichoderma* isolates, three biological replicates were performed. For SPME sampling, the cotton plugs were replaced with a cap with a hole and silicone membrane to facilitate the piercing of the septum with an SPME needle. A manual version of SPME was used for sampling.

*Sampling volatile compounds using SPME*

For the elaboration of optimal SPME extraction conditions, three different fibers and various sampling times were tested. All SPME sampling was performed at room temperature (20 °C±1 °C) to facilitate non-invasive sampling during fungal growth. As the SPME fiber after desorption in the GC injection port (260 °C) can be regarded as sterile, it was possible to sample the same vial several times during the process of monitoring volatile compound formation. The following fibers were tested: Carboxene/Divinylbenzene/Polydimethylsiloxane (CAR/DVB/PDMS), Divinylbenzene/Polydimethylsiloxane (DVB/PDMS) and Polydimethylsiloxane (PDMS). All fibers were manufactured by Supelco, Bellefonte, PA., USA. To choose the optimal extraction time, the best fiber, showing the highest extraction efficiency for 6-PAP in the tested conditions, was chosen, and sampling at 20 °C was performed for 5, 15, 30, and 60 min. Extraction was performed for 30 min on the 6th day of culture growth at 20 °C. All samples were run in triplicate.

To quantify the amount of 6-PAP formed by the examined cultures, a standard curve was prepared using spiking medium with 1–20 μL of an ethanolic solution of 6-PAP (Sigma-Aldrich, Poznań, Poland). The 6-PAP concentration range used in the standard curve was 20–400 μg of 6-PAP in the 10 ml of medium. The medium was spiked with 6-PAP ethanolic standard 1 day before SPME sampling. Results for 6-PAP concentration were expressed as μg of 6-PAP per culture.

*Volatile compound GC-MS analysis*

Volatile compounds were resolved on a Supelcowax-10 column (30 m×0.25 mm×0.25 μm; Supelco). An Agilent Technologies 7890A gas chromatograph with a single quadrupole (5975C VL MSD) mass spectrometer was used for volatile analysis. Compounds were resolved in the following conditions: helium flow 0.8 mL/min; oven temperature 40 °C (1 min), then 5 °C/min to 200 °C (0 min), then 20 °C/min to 240 °C (3 min); splitless injection (1 min, 260 °C); and mass spectrometer monitoring in full scan mode (*m*/*z* 33–330). Compounds were tentatively identified by their mass spectra using NIST 05 mass spectra library, or by comparison of retention times and mass spectra of authentic standards (in cases of 2-pentylfuran, 3-octanone, toluene, isoamyl alcohol, 2-octanone, 2-butanone, 2-heptanone, 2-nonanone, 1-octene-3-ol, phenylethyl alcohol, D-limonene, 1-propanol, and ethyl decanoate). All standards were purchased from Sigma-Aldrich.

*Examination of Fusarium species growth reduction caused by 6-PAP*

Seven *Fusarium* strains: KF 2818 *F. avenaceum*, KF 1157 *F. cerealis*, KF 846 and KF 350 *F. culmorum*, KF 2870 *F. graminearum*, KF 925 *F. proliferatum*, and KF 506 *F. subglutinans* used in the analysis of the antifungal activity of 6-PAP were obtained from the collection of the Institute of Plant Genetics, Polish Academy of Science, Poznań, Poland. These strains were identified on the basis of morphological, molecular, and biochemical (mycotoxin production) analyses by Stępień et al. (2008, 2011).

To determine the inhibitory effect of 6-PAP on mycelial growth of the *Fusarium* species, the fungal isolates were grown on PDA medium in 9-cm Petri dishes at 20 °C. The agar discs about 4 mm in length from the center of 6-day-old cultures were transferred to the center of new PDA-Petri dishes. 6-PAP (0.1–2 μl) was applied by a micropipette to the surface of each transferred agar disc of *Fusarium* culture at concentrations ranging from 0.2 to 40 μg/plug. Control treatments contained sterilized distilled water. The PDA-Petri dishes were immediately closed and incubated at 20 °C for up to 3 weeks. The percentage of inhibition of mycelial growth was determined by measurement the colony diameter (mm) after 5, 6, 7, and 18 days of incubation. Each treatment consisted of three replicates.

## Results

Evaluation of SPME suitability for 6-PAP quantification analysis

SPME was the method of choice for volatile sampling, based on earlier experience in the analysis of fungal metabolites (Jeleń 2002, 2003). SPME allows the monitoring of volatile compounds emitted by living systems on-line, and, for all

experiments, extraction was performed at room temperature to facilitate the monitoring of the dynamics of volatile formation in *Trichoderma* inoculated onto slants in headspace vials, where the sampling was performed from the same vial on each day of sampling. The high desorption temperature in the GC injection port ensured its sterility for the subsequent analysis. Three fibers were tested for their affinity towards *Trichoderma* volatiles. PDMS, due to the absorption of volatiles into the fiber coating, often offers better linear response than fibers in which adsorption takes place on the fiber surface, as is the case with polymer based fibers (DVB/PDMS or CAR/DVB/PDMS). The latter usually provide better sensitivity than PDMS. The affinity of the tested fibers, checked for 6-PAP in a 6-day-old culture of *T. atroviride* strain AN35, is shown in Fig. 1a. The 2-cm CAR/DVB/PDMS fiber developed for off-odor analysis performed better than DVB/PDMS and PDMS fibers and was chosen for further experiments. Although SPME is an equilibrium extraction method, sampling can be performed before the equilibrium is reached, providing there is sufficient sensitivity and accurate sampling time. As can be seen in Fig. 1b, the amount of 6-PAP adsorbed on the fiber surface increased significantly during the whole investigated time range. Therefore, long extraction times should be applied to reach the lower detection limits when using CAR/DVB/PDMS fiber. For screening isolates for 6-PAP production, detection limits were not a priority, and to achieve a compromise between separation (GC run) time and extraction time, 30 min was chosen to provide a sufficient sample throughput for further experiments. To provide quantitative results for the main compound of interest in *Trichoderma* cultures, a 6-PAP standard curve was prepared by spiking 6-PAP ethanolic standard onto the agar slant surface and the linearity of 6-PAP extracted from cultures in the range of 20–400 μg was satisfactory (Fig. 2c). When sampling was performed from the same vial (to avoid variations related to fungal growth and metabolism) relative standard deviation values for 6-PAP did not exceed 10 % and decreased significantly with the increase in extraction time (from 8.7 % for 5 min sampling to <1 % for 60 min sampling). The detection limit for 6-PAP using SCAN mode was estimated at 0.1 μg per culture.

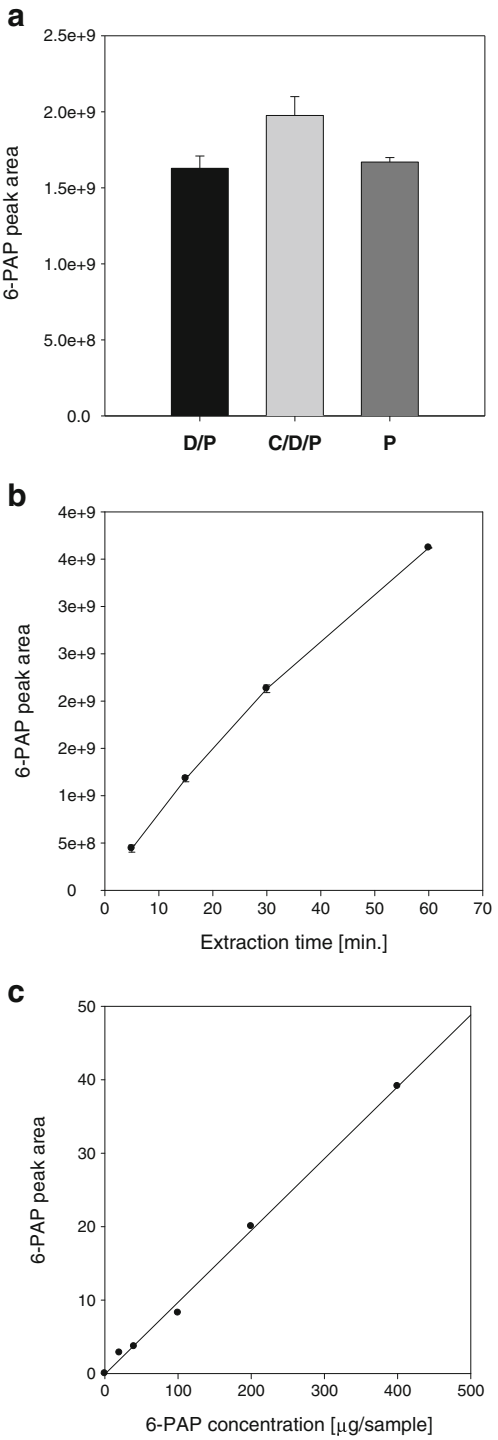


**Fig. 1** Selected parameters for SPME method optimization: **a** comparison of fiber responses to 6-PAP in *T. atroviride* AN35 strain; *D/P* Divinylbenzene/Polydimethylsiloxane fiber; *C/D/P* Carboxene/Divinylbenzene/Polydimethylsiloxane fiber; *P* Polydimethylsiloxane fiber; **b** extraction time profile for 6-PAP on C/D/P fiber; **c** calibration curve used for quantitation of 6-PAP in *Trichoderma* cultures

## Dynamics of the formation of 6-PAP and selected volatiles in *T. atroviride* AN35

*Trichoderma atroviride* AN35 was selected as a reference strain to observe the dynamics of the formation of 6-PAP, as well as 2-methylketones and $C_8$ compounds, during 6 days of growth on PDA at 20 °C. The results of this study are shown in Fig. 2. The highest emission of 6-PAP was found between the 4th and 6th days of incubation. When recalculated using a standard curve, it was 7.2±3.4 μg on day 1, 25.5±16.2 μg on day 2, 5.3±2.7 μg on day 3, 3.6±1.1 μg on day 4 and, increased to 366.6±176 μg on day 5, reaching a maximum of 1,458±200 μg on day 6. The high standard deviation values are a consequence of variation in three biological

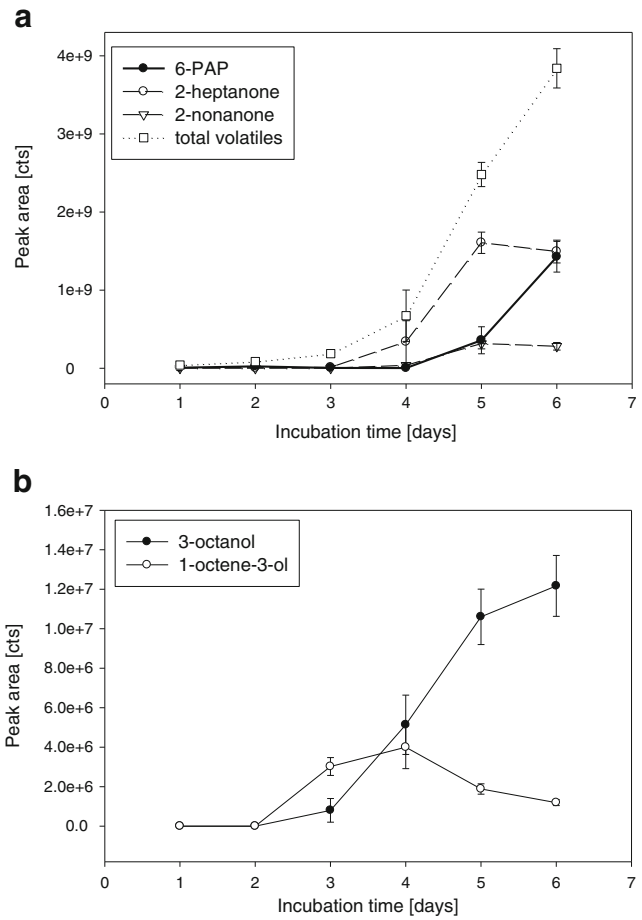


**Fig. 2** Dynamics of the formation of **a** 6-PAP, 2-heptanone and 2-nonanone, and **b** 3-octanol and 1-octene-3-ol by *T. atroviride* AN35 grown for 6 days on PDA medium

replicates (three separated cultures of *T. atroviride* AN35 strains) monitored in subsequent days.

The vast amounts of 6-PAP in the *T. atroviride* AN35 culture were accompanied by high amounts of 2-heptanone and to a lesser extent 2-nonanone (Fig. 2a). Both of these metabolites peaked after 4–5 days of incubation. Towards the end of the cultivation period of 6 days, the production of these metabolites slowly decreased. The formation of 3-octanone and 1-octene-3-ol were detected at the beginning of the cultivation period, after 2 days of incubation, and for the former increased throughout the whole incubation period. 1-octene-3-ol showed maximum amounts after 2–4 days of growth and thereafter exhibited a slow decrease in production (Fig. 2b).

## Production of 6-PAP by examined *Trichoderma* isolates

A total of 77 *Trichoderma* isolates, belonging to eight species, were examined for their ability to form 6-PAP. As shown in Table 1, *T. atroviride* was the most efficient species in this respect. The amounts of 6-PAP produced by 5 out of 10 strains exceeded 800 μg per culture. However, the variation in 6-PAP synthesis differed significantly among the *T. atroviride* strains, the lowest value being 33.4±19.5 μg (AN212 strain), whereas the highest reached 14,26±1.6 μg per culture for strain AN35. Also, *T. viridescens* was found to be an efficient 6-PAP producer (200.1±7.1–526.3±1.5 μg for 11 out of 12 strains). However, 1 of the 12 isolates of *T. viridescens* (AN366) produced very low amounts of 6-PAP. In the set of *T. citrinoviride* and *T. hamatum* strains, there were isolates that did not produce 6-PAP (AN98, AN199, AN201, AN262, AN393, AN500, AN118, AN238, AN279), but there were also isolates producing over 100 μg (*T. hamatum*, AN155 strain) and 200 μg (*T. citrinoviride*, AN89 strain) per culture. *Trichoderma viride* isolates showed very little production of 6-PAP on PDA. No 6-PAP formation was detected in cultures of the 25 isolates belonging to three species: *T. koningii*, *T. harzianum*, and *T. virens*.

Formation of 6-PAP was correlated with total volatiles for all species at $R = 0.9521$ ($p < 0.05$). The relationship of 6-PAP produced to the total volatile compounds is shown in Fig. 3. Efficient producers of volatile compounds (*T. atroviride* and *T. viridescens*) were also efficient in 6-PAP production. However, the levels of volatiles and 6-PAP within a particular species vary significantly (error bars).

## Fusarium species growth reduction by 6 PAP under laboratory conditions

The analysis of the antifungal activity of 6-PAP towards six *Fusarium* species indicated the inhibitory effect of all *Fusarium* strains used in this study on mycelial growth. The addition of 0.2 μg/plug of 6-PAP caused a 39 % reduction of

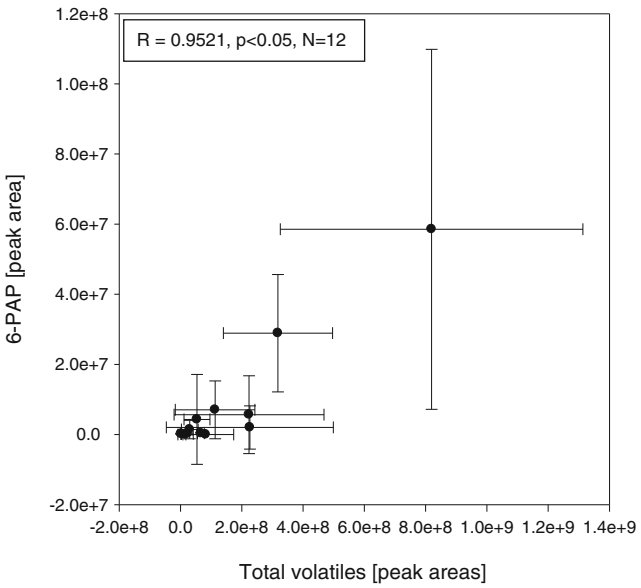


**Fig. 3** Correlation between average amount of total volatile compounds produced by investigated *Trichoderma* species and amount of produced 6-PAP expressed as peak areas

growth of *F. culmorum* KF 846 strain on day 5 after inoculation. However, after 6 days of incubation, retardation of the mycelial growth of *F. culmorum* KF 846 was 15 %, and after 7 days of incubation, no inhibitory effect was observed—the activity of 6-PAP decreased during the incubation period. When 40 μg/plug of 6-PAP was applied, mycelial growth of KF 846 strain was inhibited by 100 % on day 5 after inoculation. This effect remained constant after up to 18 days of incubation. Similar results were obtained for the other *Fusarium* strains (KF 2818 *F. avenaceum*, KF 1157 *F. cerealis*, and KF 350, KF 2870 *F. graminearum*, KF 925 *F. proliferatum*, and KF 506 *F. subglutinans*). At a concentration of 40 μg/plug of 6-PAP, the mycelial growth of the investigated *Fusarium* strains was inhibited by 100 % from days 5 to 18 of incubation. The inhibition of the mycelial growth of *F. culmorum* KF 846 strain by different concentrations of 6-PAP are shown in Table 2.

## Production of other volatile compounds by the examined *Trichoderma* isolates

Apart from 6-PAP, 77 *Trichoderma* isolates investigated in this study produced various numbers and amounts of volatile compounds. Using SPME extraction methods, it was possible to identify over 40 volatile metabolites in the headspace of their cultures on PDA. However, the profile of metabolites was found to be different in eight of the examined species (Table 3). The highest number (30 metabolites) and the highest amount (up to $819.3\times10^6$ when measured as relative peak area) of volatile metabolites were identified in cultures of *T. atroviride*. As shown in Table 3, most of the *T. atroviride* isolates produced 2-pentylfuran, 3-octanone, toluene, α-bergamotene, linalol isobutyrate, 2-methyl-1-propenylbenzene, β-cymene, isoamyl alcohol, 2-octanone, and *trans*-2-(1-pentenyl)-furan. Isolates of *T. viridescens* were also found to be effective producers of volatile compounds ($318.1\times10^6$, relative peak area), but in their cultures only 21 metabolites were detected. Of these, toluene, D-limonene, 2-pentylfuran, and 1-octene-3-ol were emitted as major compounds.

*Trichoderma citrinoviride* produced medium amounts of volatiles ($226.4\times10^6$, 26 metabolites). The dominant products of this species were: toluene, 2-butanone, pyridine, isoamyl alcohol, 3-octanone, linalol isobutyrate, 1-octene-3-ol, and phenylethyl alcohol.

*Trichoderma harzianum, T. hamatum, T. koningii*, and *T. viride* showed very little production on PDA ($82.6\times10^6$, $30.2\times10^6$, $53.9\times10^6$, and $22.6\times10^6$, relative peak area, respectively); however, the number of identified metabolites was different between these species. *Trichoderma harzianum* produced mainly D-limonene, toluene, linalol isobutyrate, cyclohept-3-en-1-one, and 1-octene-3-ol. Most of the *T. hamatum* isolates formed toluene, D-limonene, and 1-propanol. Of the 30 volatiles produced by *T. viride*, 2-hexanone, isoamyl alcohol, geranyl acetone, 1-propanol, and linalol isobutyrate were observed as major compound. In cultures of *T. koningii* were mainly identified toluene, 1-octene-3-ol, 2-hexanone, 1-pentanol, cyclohept-3-en-on, 1-propanol, and α-bergamotene. Most of the *T. virens* isolates produced D-limonene, isoamyl alcohol, toluene, pyridine, cyclohept-3-en-1-one, and 2-ethylhexanol. However, *T. virens* was observed as the least productive species (14 metabolites and $12.8\times10^6$ relative peak area).

Of the over 40 volatile metabolites detected in this study, 9 compounds were formed in cultures of individual strains: 2-pentanone (*T. harzianum* AN91), α-pinene (*T. atroviride* AN19), β-pinene (*T. atroviride* AN19 and AN35), *p*-xylene (*T. koningii* AN121, *T. viridescens* AN288), 2-heptanol (*T. atroviride* AN19 and AN35), ethyl octanoate (*T. atroviride* AN19 and AN35), ethyl decanoate (*T. atroviride* AN19, AN35, AN215 and *T. koningii* AN121, AN124), methyl benzoate (*T. citrinoviride* AN199 and AN500, *T. viridescens* AN148), α-curcumene (*T. atroviride* AN35, *T. harzianum* AN207), and β-farnesene (*T. atroviride* AN35, *T. koningii* AN144).

**Table 2** The inhibition of the mycelia growth of *Fusarium culmorum* KF 846 strain by different concentrations of 6-PAP after 5, 6, and 7 days at 20 °C on PDA medium

| 6 -PAP concentration (μg per plug) | 5 days | | 6 days | | 7 days | |
|---|---|---|---|---|---|---|
| | mm | % | mm | % | mm | % |
| 0.1 | 52 | 39 | 72 | 15 | 85 | 0 |
| 0.2 | 50 | 41 | 70 | 18 | 85 | 0 |
| 0.5 | 39 | 54 | 60 | 30 | 79 | 7 |
| 1.0 | 32 | 63 | 51 | 40 | 78 | 8 |
| 1.5 | 16 | 81 | 23 | 73 | 30 | 65 |
| 2.0 | 0 | 100 | 0 | 100 | 0 | 100 |

## Discussion

This study has reported the screening of 77 *Trichoderma* isolates, representing eight species, for 6-PAP and other volatile metabolite formation activities. Intra- and interspecies variability in 6-PAP synthesis have been demonstrated. The most efficient producer of 6-PAP on PDA medium was *T. atroviride* AN35 strain. In previous studies, *T. atroviride* AN35 strain exhibited the most considerable antagonistic potential towards *Fusarium* species (Buśko et al. 2008; Popiel et al. 2008). The results obtained from the dual culture assay indicated that the *T. atroviride* AN35 strain significantly reduced both the mycelial growth of *F. culmorum*, *F.*

**Table 3** Volatile metabolites profiles of eight *Trichoderma* species grown on PDA

| Species | No. of isolates | Volatile metabolites profiles[a] |
|---|---|---|
| *T. atroviride* | 10 | 2-pentylfuran (10), toluene (10), 3-octanone (10), α-bergamotene (10), **linalol isobutyrate** (10), **2-methyl-1-propenyl benzene** (9), β-cymene (9), isoamyl alcohol (8), 2-octanone (7), ***trans*-2-(1-pentenyl)-furan** (6), 2-butanone (5), pentyl acetate (4), 2-heptanone (4), 3-octanol (4), 2-nonanone (4), β-bisabolene (4), ***cis*-2-(1-pentenyl)-furan** (4), **ethyl decanoated** (3), **ethyl octanoate** (2), D-limonene (2), **2-heptanol** (2), **β-pinene** (2), 2-methylbutyl acetate (1), **1-pentanol** (1), phenylethyl alcohol (1), **cyclohept-3-en-1-one** (1), α-curcumene (1), β-farnesene (1), α-cedrene (1), **α-pinene** (1) |
| *T. citrinoviride* | 10 | toluene (10), isoamyl alcohol (9), 1-propanol (9), 3-octanone (9), **linalol isobutyrate** (8), 2-butanone (7), **pyridine** (7), 1-octene-3-ol (6), phenylethyl alcohol (6), **cyclohept-3-en-1-on** (4), **methyl benzoate** (2), **2-hexanone** (2), **2-methyl-1-propenyl benzene** (2), 2-methylbutyl acetate (1), pentyl acetate (1), D-limonene (1), 2-pentylfuran (1), **1-pentanol** (1/10), 3-octanol (1), β-cymene (1) |
| *T. hamatum* | 10 | toluene (10), D-limonene (9), **linalol isobutyrate** (6), 2-pentylfuran (4), 1-octene-3-ol (3), **cyclohept-3-en-1-one** (2), **pyridine** (2), α-bergamotene (2), isoamyl alcohol (1), 2-ethylhexanol (1), **2-methyl-1-propenyl benzene** (1), **geranyl acetone** (1) |
| *T. harzianum* | 9 | D-limonene (9), toluene (9), **linalol isobutyrate** (7), **cyclohept-3-en-1-one** (6), 1-octene-3-ol (5), **pyridine** (4), α-cedrene (4), **3-ethyl-5-methylphenol** (4), **2-pentanone** (1), α-curcumene (1), isoamyl alcohol (1), 3-octanone (3), 2-ethylhexanol (1), 3-octanol (1), α-bergamotene (1), phenylethyl alcohol (1) |
| *T. koningii* | 8 | toluene (8), 1-octene-3-ol (7), **2-hexanone** (6), **1-pentanol** (6), **cyclohept-3-en-on** (6), 1-propanol (6), α-bergamotene (5), 2-butanone (4), 2-pentylfuran (4), 3-octanone (3), ethyl decanoate (2/8), isoamyl alcohol (2), 2-nonanone (2), **pyridine** (2), D-limonene (1), ***cis*-2-(1-pentenyl)-furan** (1), ***trans*-2-(1-pentenyl)-furan** (1), **linalol isobutyrate** (1), pentyl acetate (1/8), α-cedrene (1), β-farnesene (1), *p*-xylene (1) |
| *T. viride* | 10 | **2-hexanone** (10), isoamyl alkohol (10), 1-propanol (9), **geranyl acetone** (8), **linalol isobutyrate** (6), 2-methylbutyl acetate (5), 2-pentylfuran (4), β-cymene (4), **cyclohept-3-en-1-one** (3), α-bergamotene (3), 2-ethylhexanol (2), **2-methyl-1-propenyl benzene** (1), 1-octene-3-ol (1) |
| *T. viridescens* | 12 | toluene (11), D-limonene (10), 2-pentylfuran (10), 1-octene-3-ol (7), **geranyl acetone** (4/12), isoamyl alcohol (3), ***trans*-2-(1-pentenyl)-furan** (3), α-bergamotene (3), 2-nonanone (2), **2-hexanone** (1), 2-octanone (1), **cyclohept-3-en-1-one** (1), ***cis*-2-(1-pentenyl)-furan** (1), *p*-xylene (1), **pyridine** (1), **methyl benzoate** (1) |
| *T. virens* | 8 | D-limonene (8), toluene (8), isoamyl alcohol (8), **pyridine** (7), **cyclohept-3-en-1-one** (5), 2-ethylhexanol (5), 3-octanone (3), 1-octene-3-ol (3) |

[a] Normal text indicates compounds, that have been previously identified as *Trichoderma* metabolites; numbers in parentheses indicate the number of strains for which this specific compound was identified; bold indicates volatiles that have never been reported previously as a secondary metabolites of *Trichoderma*; underline indicates compounds that were formed only in cultures of several strains

*graminearum*, and *F. avenaceum* isolates, as well as moniliformin, zearalenone, and five trichothecene mycotoxin (DON, 3AcDON, 15AcDON, NIV, FUS) production (over 95 %) by *Fusarium* isolates (Buśko et al. 2008; Popiel et al. 2008). As shown here, the mycelial growth retardation of *Fusarium* isolates (*F. culmorum*, *F. graminearum*, and *F. avenaceum*) was also observed in the presence of the pure form of 6-PAP. Furthermore, the present study investigated the antagonistic potential of purified 6-PAP towards *F. cerealis F. proliferatum*, and *F. subglutinans* isolates. The addition of 40 μg/plug of 6-PAP caused a 100 % inhibition of the growth of all targeted *Fusarium* isolates from days 5–18 of incubation. A similar study by Cooney et al. (2001) demonstrated that a 6-PAP-producing *Trichoderma* isolate grown in a competition assay system with *F. graminearum* isolate, as well as 6-PAP applied in pure form in culture medium displayed an inhibitory effect on mycelial growth and trichothecene mycotoxin (DON) production by *Fusarium*. The activity of 6-PAP in the mycelial growth retardation of *F. oxysporum* f. sp. *lycopersici* and *F. moniliforme* was also described by Scarselletti and Faull (1994) and El-Hasan et al. (2007). The antibiotic assay disc test performed by Scarselletti and Faull (1994) showed that the addition of 0.3 mg/ml 6-PAP caused a 31.7 % reduction in the growth of *F. oxysporum* f. sp. *lycopersici* after 2 dpi. El-Hasan et al. (2007) found a 93.5 % inhibition of *F. moniliforme* growth at a concentration of 250 μg/ml of 6-PAP 9 dpi. Thus, the ability of *T. atroviride* AN35 strain to produce high amounts of 6-PAP, and their confirmed antagonistic activity towards *Fusarium* species, make this strains potential biological control agent.

As observed in this study, the presence and the concentration of 6-PAP in cultures of *Trichoderma* isolates varied

significantly. This characteristic appeared to be isolate-specific and not species-specific. 6-PAP was detected in the headspace of all *T. atroviride* and *T. viridescens* isolates, as well as in individual cultures of *T. citrinoviride* (4 isolates), *T. hamatum* (8 isolates), and *T. viride* (3 isolates). Previously, these compounds had been described as being produced by *T. viride* (Collins and Halim 1972; Bonnarme et al. 1997), *T. harzianum* (Claydon et al. 1987; Serrano-Carreon et al. 1992; Scarselletti and Faull 1994; Bonnarme et al. 1997; Whitaker et al. 1998; El-Hasan et al. 2007, Souza Ramos et al. 2008; Vinale et al. 2008; Siddiquee et al. 2012), *T. koningii* (Cutler et al. 1986; Simon et al. 1988), and *T. atroviride* (Reithner et al. 2005, 2007; Stoppacher et al. 2010; Polizzi et al. 2011). To the best of our knowledge, 6-PAP has never been reported to be formed by *T. viridescens, T. citrinoviride*, and *T. hamatum*.

No 6-PAP emission was observed in cultures of *T. koningii*, *T. harzianum*, and *T. virens* isolates. The finding that *T. harzianum* was incapable of 6-PAP formation is not consistent with several earlier studies (Claydon et al. 1987; Serrano-Carreon et al. 1992; Scarselletti and Faull 1994; Bonnarme et al. 1997; Whitaker et al. 1998; El-Hasan et al. 2007; Vinale et al. 2008, Souza Ramos et al. 2008; Siddiquee et al. 2012). However, in the opinion of Samuels (2006), Dodd et al. (2003), and Polizzi et al. (2011), *T. harzianum* strains used in mentioned works were misclassified strains of *T. atroviride* and *T. viride*. Moreover, these authors contend that the coconut-like odor characteristic for 6-PAP has been only detected in *Trichoderma* species belonging to the *Trichoderma* section (Samuels 2006; Dodd et al. 2003; Polizzi et al. 2011). To exclude the risk of misidentification based on morphological features, the *T. harzianum* isolates investigated here were identified at the species level by morphological characteristics and analysis of their ITS1 and ITS2 rDNA as well as the fragment of the translation-elongation factor 1-alpha (*tef1*) gene sequences. In addition, the mycelium odor analysis (data not shown) did not prove the coconut aroma associated with *T. harzianum* cultures on PDA medium. On the other hand, Ghisalberty et al. (1990) reported that not all strains of *T. harzianum* used in their study produced 6-PAP. El-Hasan et al. (2007) have shown that, among two *T. harzianum* isolates (T16 and T23)—identified at the species level using the PCR technique—only the T23 isolate was able to produce this pyrone. Similarly, Vinale et al. (2008), analyzing the effect of *Trichoderma* secondary metabolites on plant growth and the induction of defense mechanisms, did not identify 6-PAP in cultures of *T. harzianum* (T22, T39, A6) strains. This finding could suggest the existence of chemotypes within a *T. harzianum* species. In addition, the production of volatiles and their type proved to be dependent on the content of the growth medium (Cooney et al. 1997a, b; Wheatley et al. 1997; Polizzi et al. 2011). In this connection, it is interesting to remark that the emission of 6-PAP by *T. harzianum* was analyzed here on PDA medium, whereas in the other studies, *T. harzianum* for 6-PAP production was cultivated in potato dextrose broth (PDB) or malt extract agar (MEA) medium (Scarselletti and Faull 1994; El-Hasan et al. 2007; Siddiquee et al. 2009, 2012). Therefore, the inability of *T. harzianum* to produce 6-PAP could also be due to the difference in substrate composition used in present study.

As shown in Fig. 3, the 6-PAP formation by *T. atroviride* AN35 strain were accompanied mainly by production of lipid derived volatiles—2-heptanone, 2-nonanone, 3-octanone, and 1-octene-3-ol. These compounds, with the exception of 2-heptanone, were also found in several cultures of *T. atroviride*, *T. citrinoviride*, *T. hamatum*, *T. harzianum*, *T. koningii*, *T. viride*, *T. viridescens*, and *T. virens* (Table 3). Previously, 2-heptanone was reported for *T. viride* (isolate T60), *T. atroviride*, and *T. harzianum* (Wheatley et al. 1997; Fiedler et al. 2001; Stoppacher et al. 2010; Polizzi et al. 2011; Siddiquee et al. 2012). 2-nonanone has been described to be produced by *T. aureoviride*, *T. atroviride*, and *T. harzianum* (Bruce et al. 2000; Stoppacher et al. 2010; Polizzi et al. 2011; Siddiquee et al. 2012). 1-octen-3-ol and 3-octanone have been found before in culture samples of *T. harzianum* and *T. atroviride* (Fiedler et al. 2001; Nemčovič et al. 2008; Stoppacher et al. 2010; Polizzi et al. 2011; Siddiquee et al. 2012).

Many other compounds detected here have also been identified as *Trichoderma* metabolites in previous studies, but their production had only been found for *T. atroviride*, *T. aureoviride*, *T. harzianum*, *T. pseudokoningii*, or *T. viride* (Wheatley et al. 1997; Bruce et al. 2000; Nemčovič et al. 2008; Stoppacher et al. 2010; Citron et al. 2011; Polizzi et al. 2011; Siddiquee et al. 2012), whereas for the first time in the present investigation, these compounds have been annotated as metabolites of *T. hamatum* and *T. citrinoviride* (Table 3). It is interesting to remark that no metabolite common to all the investigated *Trichoderma* species was found, but on the other hand, neither was any species-specific metabolite found. In general, the profiles of the volatiles identified here were differentiated both within strains of the same species, as well as between species. This finding is consistent with the observations from approximated studies on the chemotaxonomic classification of *Penicillium*, *Aspergillus*, and other fungal species (Fiedler et al. 2001; Sunesson et al. 1995; Polizzi et al. 2012).

Of the over 40 volatiles detected in this study, 17 have so far not been ascribed to *Trichoderma* (the names of these metabolites are shown in bold in Table 3). However, the fungal origin of some these compounds, for example, 1-pentanol, 3-ethyl-5-methylphenol, 2-pentanone, 2-hexanone, cyclohept-3-en-1-one, geranyl acetone, methyl benzoate, α-pinene, and β-pinene, has previously been corroborated (Sunesson et al. 1995; Mauriello et al. 2004; Korpi et al. 2009; Polizzi et al. 2012; Müller et al. 2013).

## Conclusion

This is the first comprehensive survey of 6-PAP and other volatile metabolites produced by eight *Trichoderma* species originating from different matrices which has been conducted under laboratory conditions. The application of the SPME-GC-MS methods resulted in the identification of over 40 volatile metabolites from their culture samples. Seventeen of the detected compounds have never before been reported as being produced by *Trichoderma*. To our knowledge, this is also the first study revealing the ability of *T. hamatum* and *T. citrinoviride* towards 6-PAP emission. Furthermore, among the 77 investigated *Trichoderma* isolates, the *T. atroviride* AN35 strain was observed to be the most efficient producer of 6-PAP and other metabolites on PDA medium. Considering the production of 6-PAP by this strain and its antagonistic ability, it can be concluded that *T. atroviride* AN35 is a candidate fungus for the biological control of toxigenic *Fusarium* species (such as *F. culmorum*, *F. graminearum*, and *F. avenaceum*) by reducing their inoculum, as well as preventing mycotoxin accumulation in plant tissues.

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## References

Al-Heeti MB, Sinclair JB (1988) Antagonism between *Gliocladium roseum*, *Trichoderma harzianum*, or *Trichothecium roseum* and *Phytophthora megasperma* f. sp. *glycinea*. Mycopathologia 103: 135–140

Benítez T, Rincón AM, Limón MC, Codón AC (2004) Biocontrol mechanisms of *Trichoderma* strains. Int Microbiol 7:249–260

Błaszczyk L, Popiel D, Chełkowski J, Koczyk G, Samuels GJ, Sobieralski K, Siwulski M (2011) Species diversity of *Trichoderma* in Poland. J Appl Genet 52:233–243

Bonnarme P, Djian A, Latrasse A, Feron G, Ginies C, Durand A, Le Quéré JL (1997) Production of 6-pentyl-a-pyrone by *Trichoderma* sp. from vegetable oils. J Biotechnol 156:143–150

Bruce A, Wheatley RE, Humphris SN, Hackett CA, Florence MEJ (2000) Production of volatile organic compounds by *Trichoderma* in media containing different amino acids and their effect on selected wood decay fungi. Holzforschung 54:481–486

Buśko M, Chełkowski J, Popiel D, Perkowski J (2008) Solid substrate bioassay to evaluate impact of *Trichoderma* on trichothecene mycotoxin production by *Fusarium* species. J Sci Food Agric 87:536–541

Citron CA, Riclea R, Brock NL, Dickschat JS (2011) Biosynthesis of acorane sesquiterpenes by *Trichoderma*. RSC Adv 1:290–297

Claydon N, Allan M, Hanson JR, Avent AG (1987) Antifungal alkyl pyrones of *Trichoderma harzianum*. Trans Br Mycol Soc 88:503–513

Collins RP, Halim AF (1972) Characterization of the major aroma constituent of the fungus *Trichoderma viride* (Pers.). J Agric Food Chem 20:437–438

Cooney JM, Lauren DR, Jensen DJ, Perry-Meyer LJ (1997a) Effect of harvest time, temperature, light, and spore inoculums concentration on 6-n-pentyl-2H-pyran-2-one production by *Trichoderma spp*. J Agric Food Chem 45:2802–2806

Cooney JM, Lauren DR, Jensen DJ, Perry-Meyer LJ (1997b) Effect of solid substrate, liquid supplement, and harvest time on 6-n-pentyl-2H-pyran-2-one (6PAP) production by *Trichoderma* spp. J Agric Food Chem 45:531–534

Cooney JM, Laurent DR, Di Menna ME (2001) Impact of competitive fungi on trichothecene production by *Fusarium graminearum*. J Agric Food Chem 49:522–526

Cutler HG, Cox RH, Crumley FG, Cole PD (1986) 6-pentylapyrone from *Trichoderma harzianum*: its plant growth inhibitory and antimicrobial properties. Agric Biol Chem 50:2943–2945

Dodd S, Lieckfeldt E, Samuels G (2003) *Hypocrea atroviridis* sp. nov., the teleomorph of *Trichoderma atroviride*. Mycologia 95:27–40

Doohan FM, Parry DW, Jenkinson P, Nicholson P (1998) The use of species-specific PCR-based assays to analyse *Fusarium* ear blight of wheat. Plant Pathol 47:197–205

Druzhinina IS, Kopchinskiy AG, Komoń M, Bissett J, Szakacs G, Kubicek CP (2005) An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet Biol 42:813–828

Druzhinina IS, Seidl-Seiboth V, Herrera-Estrella A, Horwitz BA, Kenerley CM, Monte E, Mukherjee PK, Zeilinger S, Grigoriev IV, Kubicek CP (2011) *Trichoderma*: the genomics of opportunistic success. Nat Rev Microbiol 16:749–759

El-Hasan A, Walker F, Schoene J, Buchenauer H (2007) Antagonistic effect of 6-pentyl-alpha-pyrone produced by *Trichoderma harzianum* toward *Fusarium moniliforme*. J Plant Dis Prot 114:62–68

El-Hassan A, Buchennauer H (2009) Action of 6-penthyl-alpha pyrone in controlling seedling blight incited by *Fusarium moniliforme* and inducing defence responces in maize. J Phytopathol 157:697–707

Fiedler K, Schütz E, Geh S (2001) Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. Int J Hyg Environ Health 204:111–121

Friedl MA, Druzhinina IS (2012) Taxon-specific metagenomics of *Trichoderma* reveals a narrow community of opportunistic species that regulate each other's development. Microbiology 158:69–83

Gams W, Bisset J (1998) Morphology and identification of *Trichoderma*. In: Harman GE, Kubicek CP (eds) *Trichoderma* and *Gliocladium*, vol 1. Taylor and Francis, London, pp 3–34

Ghisalberti EL, Narbey MJ, Dewan MM, Sivasithamparam K (1990) Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all and to produce pyrones. Plant Soil 121:287–291

Ghisalberti EL, Hockless DCR, Rowland C, White AH (1992) Harziandione, a new class of diterpene from *Trichoderma harzianum*. J Nat Prod 55:1690–1694

Harman G, Kubicek C (1998) *Gliocladium* and *Trichoderma* Vol 1. Basic biology, taxonomy and genetics. Taylor and Francis, London

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* species –opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2:43–56

Howell CR (1998) The role of antibiosis in biocontrol. In: Harman GE, Kubicek CP (eds) *Trichoderma* and *Gliocladium*, vol 1. Taylor and Francis, London, pp 173–184

Jaklitsch WM (2009) European species of *Hypocrea* Part I. The green-spored species. Stud Mycol 63:1–91

Jeleń H (2002) Volatile sesquiterpene hydrocarbons characteristic for *Penicillium roqueforti* strains producing PR toxin. J Agric Food Chem 50:6569–6574

Jeleń H (2003) Use of solid phase microextraction (SPME) for profiling fungal volatile metabolites. Lett Appl Microbiol 36:263–267

Klein D, Eveleigh DE (1998) *Tichoderma* and *Gliocladium*. In: Harman GE, Kubicek CP (eds) *Trichoderma* and *Gliocladium*, vol 1. Taylor and Francis, London, pp 57–69

Kopchinskiy A, Komon M, Kubicek CP, Druzhinina IS (2005) *Tricho*BLAST: a multilocus database for *Trichoderma* and *Hypocrea* identifications. Mycol Res 109:657–660

Korpi A, Järnberg J, Pasanen AL (2009) Microbial volatile organic compounds. Crit Rev Toxicol 39:139–193

Kubicek CP, Komon-Zelazowska M, Druzhinina IS (2008) Fungal genus *Hypocrea/Trichoderma*: from barcodes to biodiversity. J Zhejiang Univ Sci B 9:753–763

Lloyd SW, Grimm CC, Klich MA, Beltz SB (2005) Fungal infections of fresh-cut fruit can be detected by the gas chromatographye mass spectrometric identification of microbial volatile organic compounds. J Food Prot 68:1211–1216

Mannina L, Segre AL, Ritieni A, Fogliano V, Vinale F, Randazzo G, Maddau L, Bottalico A (1997) A new fungal growth inhibitor from *Trichoderma viride*. Tetrahedron 53:3135–3144

Mauriello G, Marino R, D'Auria M, Cerone G, Rana GL (2004) Determination of volatile organic compounds from truffles via SPME-GC-MS. J Chromatogr Sci 42:299–305

Müller A, Faubert P, Hagen M, Zu Castell W, Polle A, Schnitzler JP, Rosenkranz M (2013) Volatile profiles of fungi – Chemotyping of species and ecological functions. Fungal Genet Biol 54:25–33

Nemčovič M, Jakubíková L, Víden I, Vladimír F (2008) Induction of conidiation by endogenous volatile compounds in *Trichoderma* spp. FEMS Microbiol Lett 284:231–236

Nirenberg HI (1976) Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion *Liseola*. Mitt Biol Bund Land-Forst Berlin-Dahlem 169:1–117

Papavizas GC (1985) *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. Annu Rev Phytopathol 23:23–54

Polizzi V, Adams A, Picco AM, Adriaens E, Lenoir J, Van Peteghem C, De Saeger S, De Kimpe N (2011) Influence of environmental conditions on production of volatiles by *Trichoderma atroviride* in relation with the sick building syndrome. Build Environ 46: 945–954

Polizzi V, Adams A, Malysheva SV, De Saeger S, Van Peteghem C, Moretti A, Picco AM, De Kimpe N (2012) Identification of volatile markers for indoor fungal growth and chemotaxonomic classification of *Aspergillus* species. Fungal Biol 116:941–953

Poole PR, Ward BG, Whitaker G (1998) The effects of topical treatment with 6-pentyl-2-pyrone and structural analogues on stem and postharvest rots in kiwifruit due to *Botrytis cinerea*. J Sci Food Agric 77:81–86

Popiel D, Kwaśna H, Chełkowski J, Stępień S, Laskowska M (2008) Impact of selected antagonistic fungi on *Fusarium* species – toxigenic cereal pathogens. Acta Mycol 43:29–40

Ramos AS, Fiaux SB, Leite SGF (2008) Production of 6-pentyl-α-pyrone by *Trichoderma harzianum* in solid-state fermentation. Braz J Microbiol 39:712–717

Reino JL, Guerrero RF, Galan RH, Collado IG (2008) Secondary metabolites from species of the biocontrol agent *Trichoderma*. Phytochem Rev 7:89–123

Reithner B, Brunner K, Schuhmacher R, Peissl P, Seidl V, Krska R, Zeilinger S (2005) The G protein α subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites. Fungal Genet Biol 42:749–760

Reithner B, Schuhmacher R, Stoppacher N, Pucher M, Brunner K, Zeilinger S (2007) Signaling via the *Trichoderma atroviride* mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection. Fungal Genet Biol 44: 1123–1133

Samuels GJ (2006) *Trichoderma*: systematics, the sexual state, and ecology. Phytopathology 96:195–206

Scarselletti R, Faull JL (1994) In vitro activity of 6-pentyl-a-pyrone, a metabolite of *Trichoderma harzianum*, in the inhibition of *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *lycopersici*. Mycol Res 98:1207–1209

Serrano-Carreon L, Hathout Y, Bensoussan M, Belin JM (1992) Production of 6-pentyl-α-pyrone by *Trichoderma harzianum* from 18:n fatty acids methyl esters. Biotechnol Lett 14:1019–1024

Siddiquee S, Umi KY, Kausar H, Sarwar J (2009) In vitro studies on the potential *Trichoderma harzianum* for antagonistic properties against *Ganoderma boninense*. J Food Agric Environ 7:970–976

Siddiquee S, Cheong BE, Taslima K, Kausar H, Hasan MM (2012) Separation and identification of volatile compounds from liquid cultures of *Trichoderma harzianum* by GC-MS using three different capillary columns. J Chromatogr Sci 50:358–367

Simon A, Dunlop RW, Ghisalberti EL, Sivasithamparam K (1988) *Trichoderma koningii* produces a pyrone compound with antibiotic properties. Soil Biol Chem 20:263–264

Stępień Ł, Popiel D, Koczyk G, Chełkowski J (2008) Wheat-infecting *Fusarium* species in Poland-their chemotypes and frequencies revealed by PCR assay. J Appl Genet 49:433–441

Stępień Ł, Koczyk G, Waśkiewicz A (2011) Genetic variation of *Fusarium proliferatum* isolates from different host species – analysis of growth rate and fumonisin production. J Appl Genet 52:487–496

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. J. Microbiol Methods 81:187–193

Sunesson AL, Vaes WHJ, Nilson CA, Blomquist G, Anderson B, Carlson R (1995) Identification of volatile metabolites from five fungal species cultivated on two media. Appl Environ Microbiol 61: 2911–2918

Tarus PK, Lang'at-Thoruwa CC, Wanyonyi AW, Chhabra SC (2003) Bioactive metabolites from *Trichoderma harzianum* and *Trichoderma longibrachiatum*. Bull Chem Soc Ethiop 17:185–190

Verma M, Brar SK, Tyagi RD, Surampalli RY, Valéro JR (2007) Antagonistic fungi, *Trichoderma* spp.: panoply of biological control. Biochem Eng J 37:1–20

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M (2008) *Trichoderma*- plant-pathogen interactions. Soil Biol Biochem 40:1–10

Wheatley R, Hackett C, Bruce A, Kundzewicz A (1997) Effect of substrate composition on production of volatile organic compounds from *Trichoderma* spp. Inhibitory to wood decay fungi. Int Biodeterior Biodegrad 39:199–205

Whitaker G, Poole PR, Cooney JM, Lauren DR (1998) Production of [$^{14}$C]-6-pentyl-2-pyrone in liquid cultures of *Trichoderma harzianum*. J Agric Food Chem 46:3747–3749

Worasatit N, Sivasithamparam K, Ghisalberti EL, Rowland C (1994) Variation in pyrone production, lytic enzymes and control of *Rhizoctonia* root rot of wheat among single-spore isolates of *Trichoderma koningii*. Mycol Res 98:1357–1363