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Volatile profiles of fungi – Chemotyping of species and ecological functions

Anna Müller [a,2], Patrick Faubert [b,1,2], Michael Hagen [c], Wolfgang zu Castell [c], Andrea Polle [a], Jörg-Peter Schnitzler [b], Maaria Rosenkranz [b,*]

[a] Büsgen Institute, Forest Botany and Tree Physiology, University of Göttingen, Büsgenweg 2, 37077 Göttingen, Germany
[b] Research Unit Environmental Simulation, Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Ingolstädter Landstraße 1, 85764 Neuherberg, Germany
[c] Research Unit Scientific Computing, Institute of Biomathematics and Biometry, Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

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ABSTRACT

Fungi emit a large spectrum of volatile organic compounds (VOCs). In the present study, we characterized and compared the odor profiles of ectomycorrhizal (EM), pathogenic and saprophytic fungal species with the aim to use these patterns as a chemotyping tool. Volatiles were collected from the headspace of eight fungal species including nine strains (four EM, three pathogens and two saprophytes) using the stir bar sorptive extraction method and analyzed by gas chromatography – mass spectrometry (GC–MS). After removal of VOCs released from the growth system, 54 VOCs were detected including 15 novel compounds not reported in fungi before. Principle component and cluster analyses revealed that fungal species differ in their odor profiles, particularly in the pattern of sesquiterpenes. The functional groups and species could be chemotyped by using their specific emission patterns. The different ecological groups could be predicted with probabilities of 90–99%, whereas for the individual species the probabilities varied between 55% and 83%. This study strongly supports the concept that the profiling of volatile compounds can be used for non-invasive identification of different functional fungal groups.

1. Introduction

Volatile organic compound (VOC) emissions play important ecological and physiological roles for many organisms. Also fungi release a large spectrum of VOCs (Splivallo et al., 2011; Kramer and Abraham, 2012). Fungal VOC emissions belong to several chemical groups with different biochemical origins such as monoterpenes, sesquiterpenes, alcohols, aldehydes, aromatic compounds, esters, furans, hydrocarbons, ketones, as well as nitrogen- and sulfur-containing compounds (Splivallo et al., 2007a; Campos et al., 2010; Kramer and Abraham, 2012).

So far, most of the studies on fungal VOCs focused on the emissions from single species, and the odor profiles of different fungal or microbial species were rarely compared. If the VOC patterns are species or ecological group specific, these might be used as ecological indicators or biomarkers to characterize different fungal groups/species. Two studies have demonstrated the applicability of such approach. McNeal and Herbert (2009) succeeded to efficiently distinguish the microbial groups (bacteria and fungi) and characterize the shift in microbial activity by using VOC patterns as ecological indicators of the microbial community composition. More recently, Polizzi et al. (2012) tested the use of fungal VOCs as biomarkers of indoor fungal growth in water-damaged buildings, showing that sesquiterpenes could be used as biomarkers of specific fungi, and the fungal VOC emission patterns classified strains of *Aspergillus*, *Chaetomium* and *Epicoccum* species. Thus, there is a real potential to use VOCs as non-destructive markers to differentiate and identify a fungal species from an unknown VOC mixture in nature. Chemotyping based on VOC emissions could also be an efficient method to identify the fungal pathogens in the early infection stages, which is still not possible (Schwarze, 2000).

Thus, the present study focuses on the diversity of the fungal volatile profile (analysis of all measurable VOCs) released by three functional groups: ectomycorrhizal (EM), pathogenic and saprophytic fungi. We first aimed to characterize the global VOC emission patterns released by eight fungi including nine strains (four EM, three pathogens and two saprophytes) and use these patterns to distinguish the fungi at the functional group and species levels.

Abbreviations: EM, ectomycorrhizal fungi; VOC, volatile organic compound; SQT, sesquiterpene; MT, monoterpene; OVOC, other volatile organic compound; SBSE, stir bar sorptive extraction; HSSE, headspace sorptive extraction; TIC, total ion count; PDMS, polydimethylsiloxane.

* Corresponding author. Fax: +49 (0) 89 3187 4431.
E-mail address: maaria.rosenkranz@helmholtz-muenchen.de (M. Rosenkranz).
[1] Present address: Département des Sciences Fondamentales, Chaire en Éco-conseil, Université du Québec à Chicoutimi, 555, boul. de l'Université, Chicoutimi, Qc, Canada G7H 2B1.
[2] These authors contributed equally to this work.

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Our second aim consisted of chemotyping the fungal groups and species by using the most relevant volatile compounds as biomarkers for characterization. We tested the hypothesis that the nine fungal strains have distinct VOC emission patterns, whose specific composition may serve as non-invasive biomarkers.

2. Materials and methods

2.1. Fungal species and growth

The measurements in this study were performed on the mycelia of nine fungal strains, four of which belonged to the EM group (*Cenococcum geophilum* Fries, *Laccaria bicolor* (Maire) P.D. Orton and the *Paxillus involutus* (Batsch) Fr. strains MAJ and NAU), three to pathogens (*Armillaria mellea* (Vahl) P. Kumm., *Pholiota squarrosa* (Fries) Kummer and *Verticillium longisporum* (C. Stark) Karapapa, strain VL43) and two to saprophytes (*Stropharia rugosoannulata* Farlow ex Murrill and *Trichoderma viride* Pers).

Fungi were grown in glass Petri dishes (diameter 10 cm) on a sterilized cellophane membrane on top of a modified Melin-Norkrans synthetic medium containing (L^{-1}) 10 g glucose, 2.5 g NH_4-Tartrat, 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2SO_4$, 0.15 g $MgSO_4 \times 7H_20$, 0.05 g $CaCl_2$, 0.025 g NaCl, 1 ml $FeCl_3$ (1% (w/v)), 100 μl Thiamine HCl (0.1% (w/v)), and 1% (w/v) Gelrite, pH 5.2. The mycelia were harvested without media after a culture of 24 days in permanent darkness and used to test fungal identity by ITS (internal transcribed spacer) sequencing.

The fungal material (50 mg) was ground in liquid nitrogen with a ball mill (Typ MM 2, Retsch, Haan, Germany), and the genomic DNA was extracted (innuPREP Plant DNA Kit, Analytik Jena AG, AJ Innuscreen GmbH, Jena, Germany). The ITS region of the fungal ribosomal DNA was amplified by PCR using the primers ITS1F (5′-TCC GTA GGT GAA CCT GCG G-3′; Gardes and Bruns, 1993) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; White et al., 1990). PCR, cloning and sequencing were performed as described previously (Druebert et al., 2009). Sequencing was conducted with the Big Dye® Terminator cycle sequencing kit (Applied Biosystems, Darmstadt, Germany) and run on an ABI Prism 3100 Genetic Analyzer (36 cm capillary, Matrix Pop 6, Applied Biosystems, Foster City, CA, USA). The sequences of the rRNA ITS region were aligned using Staden Package 4.10 (http://staden.sourceforge.net), and the consensus sequences were compared to previously published sequences with a BLAST search in the NCBI database (http://www.ncbi.nlm.nih.gov). Sequence alignments of the fungal ITS regions were constructed with ClustalW (Larkin et al., 2007). An unrooted phylogenetic tree was generated with the TreeView software (Page, 1996) based on the alignments using the neighbor-joining method (Saitou and Nei, 1987).

2.2. Sampling of fungal VOCs

Volatile compounds were collected in the headspace from all fungi grown in glass Petri dishes (diameter 10 cm) containing 30 ml of the modified Melin-Norkrans synthetic medium described above. Each Petri dish was inoculated in the center with a single fungal plug (diameter 1 cm) and incubated at 24 °C in permanent darkness (except for the short moment the cultures were taken to light for measuring the diameter of the colonies). Control plates without fungal inocula were incubated under the same conditions. The fungal diameter was measured every six days.

The headspace samples were collected in two independent experiments (22 August 2011 and 7 November 2011) after twenty-four days of growth with the exception of *T. viride*, which displayed high growth rate and, therefore, was measured after three days. In each of the two experiments, five replicates of each fungus were studied. Control plates with the growth medium without fungal mycelia were also sampled. VOCs were collected from gas-tight sealed Petri dishes by headspace sorptive extraction (HSSE) using the stir bar sorptive extraction (SBSE) method with Gerstel Twisters (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany); (Baltussen et al., 1999). The Twisters had a polydimethylsiloxane (PDMS) film thickness of 0.5 mm and a length of 10 mm. The twisters were chosen as a method because they were previously shown to have higher sensitivity than solid-phase micro extraction (SPME) (Bicchi et al., 2002), which in principle is a similar technique. SPME is, however, coated only with 2% of the amount of PDMS compared to twisters (Wihlborg et al., 2008) and has been shown to give less recovery to 16 compounds as tested by Bicchi et al. (2002). To avoid direct contact with mycelium and medium, the Twisters were placed on a 15 mm × 15 mm piece of aluminum foil on the mycelium surface. VOCs from the Petri dish headspace were passively adsorbed on the PDMS film for a period of 6 h. The time was chosen after testing different sampling durations (6 h, 24 h, 48 h) and had also been demonstrated to be an optimum sampling time for trace analysis (Bicchi et al., 2005). Twisters were sealed in their original storage vials after the sampling period and analyzed within two weeks.

2.3. Analyses of VOCs

The samples were analyzed with a thermo-desorption unit (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) coupled to a gas chromatograph-mass spectrometer (GC–MS; GC model: 7890A; MS model: 5975C; Agilent Technologies, Santa Clara, CA, USA). The fungal volatiles in the samples were desorbed from 30 °C to 250 °C at a rate of 400 °C min^{-1}, followed by a holding time of 2 min. The compounds were refocused on Tenax (cryo-cooling technique) at −50 °C and desorbed to 250 °C at a rate of 12 °C s^{-1}, followed by a holding time of 3 min. Compounds were separated using a capillary GC column ((14%-Cyanopropyl-phenyl)-methylpolysiloxane; 70 m × 250 μm, film thickness 0.25 μm; Agilent J&W 122-5562G, DB-5MS + 10 m DG). The carrier gas was helium with a constant flow rate of 1.2 ml min^{-1}. The GC oven temperature was held at 40 °C for 2 min, then increased to 80 °C at a rate of 6 °C min^{-1}, then to 170 °C at a rate of 3.4 °C min^{-1} and finally to 300 °C at a rate of 12 °C min^{-1}.

The chromatograms were analyzed in two steps using the Enhanced ChemStation software (MSD ChemStation E.02.01.1177, 1989–2010 Agilent Technologies, Santa Clara, CA, USA). The first step consisted of building a preliminary dataset in which the compounds were identified according to the total ion counts (TIC) and Wiley data library. The second step consisted of building a final dataset from the preliminary dataset by removing the compounds that were already released by the sampling system (i.e. control plates and conditioned Twisters) itself and those for which the identification was uncertain based on representative abundances of mass to charge ratios (m/z).

In the first step, an automated screening of the TICs of all chromatograms was performed using an in-house function that extracted the VOCs with an identification quality above 90% in the Wiley data library. At this stage, compounds with a low frequency were discarded (low frequency: present less than three times in the whole dataset if the presence was dispersed over more than one fungus). A manual screening was also performed in which a total of approximately 400 volatile compounds that were reported in the literature (Agger et al., 2009; Bäck et al., 2010; Bellesia et al., 2001; review of Campos et al., 2010; Demyttenaere et al., 2000, 2003, 2004; Díaz et al., 2003; Dickschat et al., 2011; Drawert and Barton, 1978; Gioacchini et al., 2008; review of Kramer and Abraham, 2012; Larsen and Frisvad, 1994; Leeder et al., 2011; Mauriello et al., 2004; Menotta et al., 2004; Splivallo et al.,