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Volatile organic compounds released by the entomopathogenic fungus *Beauveria bassiana*

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Summary

The composition of volatile organic compounds (VOC) released by the entomopathogenic fungus *Beauveria bassiana* (Hyphomycete: Deuteromycotina) utilizing two different carbon sources was investigated. Analyses were performed by solid-phase microextraction (SPME) coupled to capillary gas chromatography (CGC) and CGC-mass spectrometry (MS). Major components in glucose-grown cultures were diisopropyl naphthalenes, ethanol, and sesquiterpenes. Alkane-grown fungal VOC switched to a fingerprint with prevalence of *n*-decane. This is the first report on the volatiles released by entomopathogenic fungi.

Introduction

Volatile organic compounds (VOC) released by fungi can be properly used as markers of fungal presence based on their characteristic capillary gas chromatography (CGC)-mass spectrometry (MS) fingerprints (Fiedler et al., 2001). In the last decade, after headspace-solid-phase microextraction (HS-SPME) development by Pawliszyn (Zhang and Pawliszyn, 1993; Pawliszyn, 1997), a growing number of studies on fungal volatile metabolites either for strain profiling, off-odors detection, or metabolic modifications have been published (Larsen and Frisvad, 1995; Jelen and Wasowicz., 1998; Jelen, 2003).

In insect control strategies, there is growing interest in the utilization of ecological tools to control insect-borne diseases, food spoilage, and crop protection. The development of resistance to chemical insecticides and concerns over the deleterious effects of chemicals on environmental and human safety have provided a strong impetus to develop microbial control agents. Different formulations of mycoinsecticides are currently used and considerable effort has been focused on the

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utilization of the Hyphomycete *Beauveria bassiana*, one of the most promising biocontrol agents in terms of large-scale applications (Inglis et al., 2001). The factors responsible for the initiation and development of mycosis in insects are extremely complex, involving fungal production of biologically active volatile and non-volatile metabolites that could be related to the mechanism of pathogenicity.

Entomopathogenic fungi invade their insect host through the cuticle, covered by a thin layer of lipids, usually composed of a mixture of very-long-chain hydrocarbons together with variable amounts of fatty alcohols and fatty acids. These lipids play a major role in protecting insects from desiccation, penetration of chemical or biological insecticides, as well as in chemical communication events (Juárez 1994). Previous research in this laboratory showed fungal ability to degrade insect host cuticular hydrocarbons, by utilizing them for energy production and incorporation into fungal components (Napolitano and Juárez, 1997). Furthermore, alkane-growth adaptation of fungi was either correlated to an increased insect host mortality (Crespo et al., 2002) or to an enhanced speed to kill their insect host (Pedrini et al., unpublished). In order to set up a fast and simple method to evaluate fungal hydrocarbon degradation ability, the measurement of their fermentation products was evaluated as an adaptation marker. In this study, CGC-MS in combination with HS-SPME was used to investigate VOC released by the entomopathogenic fungus *B. bassiana* (Deuteromycotina: Hyphomycete) and the effect of utilizing an insect-like hydrocarbon as carbon source on their composition.

Materials and methods

Fungal cultivation: Glucose-grown (GG) cultures of *B. bassiana* strain GHA (Mycotech, Butte, MT, USA) were obtained from fungal isolates maintained on complete medium agar (CMA) plates containing 0.4 g KH_2P0_4, 1.4 g Na_2HP0_4, 0.6 g $MgS0_4 \cdot 7H_20$, 1.0 g KCl, 0.7 g $NH_4N0_3 \cdot 7H_20$, 10 g glucose, 5 g yeast extract, and 15 g agar in 1000 ml of distilled water. Alkane-grown (AG) cultures were obtained after growth on CMA deficient in glucose and yeast extract (DMA), and supplemented with an insect-like hydrocarbon, *n*-octacosane (*n*-C28) (Sigma, St. Louis, MO, USA). The hydrocarbon (2.5 ml of a 10% hexane solution, w/v) was layered onto the surface media and evaporated. GG and AG cultures (1×10^8 conidia/vial) were grown in 20-ml headspace vials at 26 °C for 6 d. Control vials contained similar amounts of culture media.

VOC extraction: Samples and controls were maintained at 60 °C for 20 min, and VOC were extracted by HS-SPME after exposure for an additional 20-min period at 60 °C employing a 65-μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (Supelco, Bellefonte, PA, USA).

VOC analysis by CGC-MS: CGC analysis was performed on a Hewlett Packard 6890 gas chromatograph fitted with a non-polar DB-5 capillary column (30 m length, 0.32 mm I.D., 0.25 μm film thickness) (J & W Scientific, Folson, CA, USA). VOC desorption from the SPME fiber was carried out by introducing the SPME needle into the injection port operated in the splitless mode at 250 °C; the oven temperature was programmed at 40 °C for 3 min, 5 °C min^{-1} to 80 °C, 20 °C min^{-1} to 150 °C, and 30 °C min^{-1} to 250 °C, with a holding time of 10 min at the final temperature. The flame ionization detector temperature was set at 280 °C. Blank runs with the SPME fiber were performed prior and after each sample injection according to manufacturer instruction. Volatile analysis by CGC-MS was performed on a Finnigan Polaris Q ion trap equipment, chromatographic conditions were similar to those described for CGC, the ion source was set at 200 °C and the transfer line at 275 °C. After calculating their equivalent chain lengths (ECL) by comparison of their elution time with that of hydrocarbon standards run similarly, straight-chain hydrocarbons were identified by their ECL and their typical mass fragmentation pattern. Sesquiterpenes ($C_{15}H_{24}$ hydrocarbons) exhibited the characteristic molecular ion at *m/z* 204, and typical sesquiterpene fragmentation ions. Diisopropyl naphthalenes were identified by comparison of their spectra to MS libraries (NIST/EPA/NIH, NIST 98), interpretation of their fragmentation pattern, and their respective molecular ions.

Results and discussion

Among VOC released by GG fungi, the major components detected were diisopropyl naphthalene (>57%), ethanol (10.2%), and sesquiterpenes (6.4%) (Table 1). Minor amounts of alkyl benzene derivatives, benzeneacetaldehyde, straight even-chain saturated hydrocarbons of 10–12 and 16 carbons, 1-pentadecene, and methyl-alkyl ketones were also detected. Major quantitative alteration in the VOC profile, together with a lower detector response, were evident when glucose was replaced by *n*-octacosane as the sole carbon source.

Table 1. Analysis by SPME–CGC–MS of the volatile organic compounds released by *Beauveria bassiana* after growth on two different carbon sources.

Compound	Equivalent chain lengths	Diagnostic ions	VOC percentage[a]	
			Glucose-grown cultures	Alkane-grown cultures
Ethanol		31, 45 (M–1)	10.22±2.57	tr
n-Decane	10.00	142 (M+)	tr	84.21±5.47
Sesquiterpene 1	15.23	67, 93, 105, 119, 189, 204 (M+)	5.08±1.19	tr
Sesquiterpene 2	15.48	105, 119, 161, 204 (M+)	1.14±0.12	tr
Diisopropyl naphthalene 1	16.32	141, 155, 197, 212 (M+)	22.18±1.05	6.59±2.39
Diisopropyl naphthalene 2	16.69	141, 155, 197, 212 (M+)	35.55±3.00	9.20±3.15

VOC structures were assigned as described in Materials and methods; tr: trace.
[a]Values are means of 8 replicates±S.E. Minor components complete the total VOC percentage in glucose-grown cultures.

n-Decane was by far the largest component (84.2%) together with diisopropyl naphthalene (>15%), only trace amounts of the remaining volatiles were evident (Table 1). None of the reported peaks were present in the controls. The small amount of VOC collected was estimated close to 150 ng for GG cultures, decreasing more than 2-fold for AG cultures, assuming for each component a response factor similar to that of the corresponding *n*-alkane eluting close to them. Attempts to collect larger amounts by solvent extraction to further identify these compounds failed due to the presence of hydrophobic proteins (Bidochka et al., 1995) hexane-extractable, remaining even after TCA precipitation and partitioning attempts, preventing us from performing a direct CGC injection of the concentrate.

Fungal VOC are produced during both primary and secondary metabolism, some components are indicators of food and feed spoilage, flavor (Schnürer et al., 1999), as well as indoor building contamination (Sunesson et al., 1995). The most probable metabolic pathway leading to the formation of *B. bassiana* volatiles from different carbon sources is shown in Fig. 1, in coincidence with previous studies from this laboratory (Crespo et al., 2000; Juárez et al., 2004) and with established pathways in filamentous fungi (Schnürer et al., 1999). In AG cultures, Very long chain hydrocarbon oxidation provides the appropriate fatty acyl-CoA for complete β-oxidation, thus generating acetyl-CoA units for energy and cellular component production, and releasing CO_2 (Napolitano and Juárez, 1997; Crespo et al., 2000). It has been reported that cultivation on different substrates render a VOC distinctive quantitative pattern; production of volatiles is influenced also by the strain specificity, moisture, temperature, and

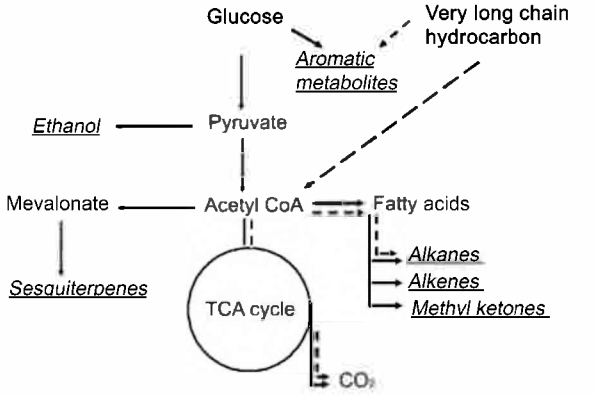

Figure 1. Metabolic pathway for *Beauveria bassiana* volatile organic compounds released after growth on two different carbon sources. End products detected by SPME-CGC-MS are underlined.

incubation period among other factors (Fiedler et al., 2001). Although other filamentous fungi such as Penicillium and Fusarium sp. release large amounts of VOC easily detectable by any of the usual techniques (Sunesson et al., 1995), the sensitivity of SPME-CGC is appropriate to profile the small amount of VOC produced by *B. bassiana* in the assayed conditions, as well as to monitor *B. bassiana* volatiles after adaptation to different carbon-source incubation media. The chromatographic fingerprint of GG fungi switched into a different profile for AG fungi, with a short-chain alkane as the major compound. The ability of entomopathogenic fungi to fully degrade very-long-chain alkanes and to synthesize de novo a variety of hydrocarbons of shorter chain length than the precursor chains was already shown (Napolitano and Juárez, 1997; Juárez et al., 2000). After incubation with labeled C28 hydrocarbon, labeled undecanoic acid was detected as the major fatty

acid product (Crespo et al., 2000). In many systems, hydrocarbon synthesis proceeds via decarboxylation of a fatty acid one-carbon longer (Kolattukudy et al., 1976); hence, undecanoic acid might be the most probable precursor to *n*-decane, the major component detected in AG fungi. Thus, when glucose is replaced by a very-long-chain insect-like hydrocarbon, the major pathway proceeds through complete degradation of the alkane to acetyl-CoA, converted mostly into a shorter chain alkane, probably via synthesis de novo. Hydrocarbons are ubiquitous components of insect cuticles (Blomquist et al. 1987), thus this methodology is potentially useful in biocontrol strategies. Further studies should be addressed employing fungal VOC profiling as a potential diagnostic tool of virulence in other pathogenic fungi. Whether MS identification is fully achieved or not, this technique might be a simple and fast method to detect fungal adaptation to different nutrient sources. This is the first report dealing with volatiles released from entomopathogenic fungi used as biocontrol agents.

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