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Article

Volatile Organic Compounds from Filamentous Fungi: a Chemotaxonomic Tool of the Botryosphaeriaceae Family

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Volatile organic compounds (VOCs) from ten endophytic fungal species belonging to the Botryosphaeriaceae family were extracted by headspace-solid phase micro-extraction (HS-SPME) and analyzed by gas chromatography-mass spectrometry (GC-MS). Thirty-four VOCs were identified. Most of the compounds are sesquiterpenes (14 non-oxygenated and 10 oxygenated), and two linear ketones and eight alcohols were also identified. Multivariate data analysis (PCA and HCA) allowed the differentiation of all investigated species, and proved to be efficient for the differentiation of *Neofusicocum parvum* and *N. ribis*, which are considered very similar species. α-Bisabolol, α-selinene, α-cedrene epoxide and guaiol acetate were suggested as biomarkers.

Keywords: Botryosphaeriaceae, volatile organic compounds, endophytic fungi, chemotaxonomy, α-bisabolol

Introduction

Botryosphaeriaceae comprises a broad group of cosmopolitan filamentous fungi (endophytes, pathogens or saprobes) that inhabits several hosts.[1,2] Although endophytic fungi are viewed as plant mutualists, under certain circumstances these microorganisms can become phytopathogens.[3] This phenomenon has been reported in some species of Botryosphaeriaceae, which have caused disease in agronomically important plants.[2,4] *Lasiodiplodia theobromae*, *Neofusicoccum luteum*, *N. parvum*, *N. australe*, *Botryosphaeria dothidea*, *Diplodia mutila*, *D. seriata*, *Dothiorella iberica* and *D. viticola* are examples of phytopathogenic Botryosphaeriaceae fungi that cause damage to *Vitis vinifera*,[5] *Eucalyptus globulus*,[4] *E. urophylla*[6] and *Mangifera indica*.[7]

The taxonomy of Botryosphaeriaceae is rather confusing, mainly because of the overlay of the morphological characteristics and species diversity. Several anamorphic species are described for this family belonging to several genera such as *Botryodlplodla*, *Diplodia*, *Dothiorella*, *Fusicoccum*, *Lasiodiplodia*, *Macrophoma* and *Sphaeropsis*.[8] Crous *et al*.[1] reported the existence of anamorphic forms of Botryosphaeria species, which present morphological characteristics of both *Diplodia* and *Fusicoccum* genera. Data from DNA (28S rDNA) sequencing were used to differentiate ten phylogenetic strains and to group some of them of the Botryosphaeriaceae family. According to Phillips *et al*.,[2] studies on morphological characters are inadequate to define or identify Botryosphaeriaceae species, and taxa with no DNA sequencing data should not be grouped in this family. Although phylogenetic studies using data from molecular biology have contributed significantly to the taxonomy of Botryosphaeriaceae,[1,2,9] some problems still exist. For instance, *Neofusicocum parvum* and *N. ribis,* classified as a complex *N. parvum*/*N. ribis,* are closely related, and molecular analysis provided inconsistent results when used to differentiate these species.[10-12]

About 10,000 microbial species have been described in the literature, although the microbial volatile organic

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compound (mVOC) of only a reduced number of them (349 bacteria and 69 fungi species) have been investigated.[13] The mVOC profiles have been used as auxiliary tools for the chemotaxonomic classification of some microorganisms, since the production patterns of these compounds are unique to certain microorganisms under controlled conditions.[13-15] Larsen and Frisvad[16] were the first to demonstrate the use of mVOC for the discrimination of *Penicillium* species. Strains of dermatophytic fungi were also differentiated based on mVOC, suggesting the use of this analytical tool in early diagnosis and treatment of contaminated patients.[17] Volatile profiles of nine root-associated fungal strains (eight species) from three different functional groups (ectomycorrhizal, pathogenic and saprophytic) were successfully used as a chemotyping tool for non-invasive identification of these microorganisms.[18]

Among the different techniques used to obtain volatile and semi-volatile compounds (terpenes and other classes of VOC), the solid phase micro-extraction (SPME) stands out for its practicality in the sample preparation and analyte pre-concentration, under relatively mild conditions.[4,19] Studies on volatile metabolites from fungi have involved the use of headspace-solid phase micro-extraction (HS-SPME), by having polydimethylsiloxane/divinylbenzene (PDMS/DBV) as the most efficient fiber mixture.[19,20] After extraction, the identification and characterization of the VOCs has mostly been performed by gas chromatography coupled to mass spectrometry (GC-MS).[18-21]

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) are among the statistical techniques (multivariate data analysis) most used for the analysis of VOCs obtained by HS-SPME associated with GC-MS. For example, three chemotypes of *Lippia graveolens* HBK, distributed in eight populations from Guatemala, were differentiated by these two techniques.[22]

In this work we describe the use of HS-SPME followed by GC-MS analysis to study VOCs produced by ten species of endophytic fungi from the Botryosphaeriaceae family associated with plants from the Caatinga biome (state of Ceará, Brazil). In addition, the multivariate data analyses PCA and HCA were used to establish differentiation patterns of the investigated species, and to identify biomarkers for the chemotaxonomic classification of these species.

Experimental

Fungal strains

Ten strains of endophytic fungi were isolated from plants collected in Caatinga biome (Ceará, Brazil), and are deposited in the Laboratory of Phytopathology at Embrapa Tropical Agro-business (CNPAT, Fortaleza, Ceará, Brazil). The strains were identified by molecular analysis (DNA sequencing of the regions ITS1/ITS4) as: *Lasiodiplodia theobromae* (strain 71), *L. pseudotheobromae* (strain 277), *L. citricola* (strain 258), *L. gonubiensis* (strain 474), *L. parva* (strain 511), *Neofusicoccum cordaticola* (strain 434), *N. parvum* (strain 600), *N. ribis* (strain 683), *Botryosphaeria mamane* (strain 20), and *Pseudofusicoccum stromaticum* (strain 477).

Culture media and materials

Potato dextrose broth (PDB, 90.9% of potato broth and 9.1% of dextrose) was purchased from Himedia® (Mumbai, India), and prepared according to the manufacturer's instructions (24.0 g L^{-1}, pH 5.1 ± 0.2). Potato dextrose agar (PDA, 84.4% of potato broth, 8.4% of dextrose and 7.2% of bacteriological agar) was obtained from Kasvi® (Roseto degli Abruzzi, Italy), and prepared following the manufacturer's instructions (42.0 g L^{-1}). Disposable sterile Petri dishes (90 × 15 mm) were purchased from J. Prolab® (São José dos Pinhais, Brazil). Glass vials (40 mL) with screw caps and PTFE/silicone septa, and divinylbenzene/polydimethylsiloxane (PDMS/DBV 65 μm) fiber were obtained from Sigma-Aldrich® (St. Louis, USA). The mixture of saturated *n*-alkanes C7-C30 was from Sigma-Aldrich® (St. Louis, USA).

Cultivation of fungi, extraction and analysis of the volatile organic compounds, and multivariate data analysis

All strains were separately inoculated in Petri dishes containing PDA medium, and incubated for 7 days at 25 °C in order to ensure that all of them were of the same age. Then, one pellet (diameter 6 mm) of the strain was transferred to vials (40 mL) containing 10 mL of PDB, and immediately sealed with septa of silicone and threaded caps. After incubation for 14 days at 25 °C under static conditions, the vials were placed in a bath of ethylene glycol at 60 °C, and the VOCs were extracted for 30 min by HS-SPME using a PDMS/DVB fiber placed above (ca. 1 cm) the surface of the fungal culture. After this period, the fiber was removed and inserted in the GC-MS at 250 °C for 4 min for the VOC desorption. A vial containing only PDB (no fungus) was used as the control. For the optimization of the HS-SPME conditions, a 2^2 trial planning with two quantitative variables (temperature and extraction time) was carried out at two levels (50 and 60 °C; 10 and 30 min, all in duplicate), with a central point (60 °C and 20 min, in triplicate). Eleven experiments were

performed, and the statistical analysis was done by using the program Quality Tools: Statistics in Quality Science.[23] Analyses of GC-MS were performed on a QP-2010 gas chromatograph coupled to a mass spectrometer from Shimadzu® (Tokyo, Japan), with a capillary DB-5 column (25 m × 0.32 mm × 0.5 μm) from J & W Scientific® (Folsom, USA). The analysis conditions were as follows: injector temperature 250 °C; GC oven temperature 35 °C for 2 min, from 35 to 195 °C (20 °C min^{-1}), from 195 to 220 °C (10 °C min^{-1}), and from 220 to 280 °C (20 °C min^{-1}); mode of injection 1:5 split; volumetric flow rate of the carrier gas (Helium) 0.59 mL min^{-1}; detector temperature 250 °C. Mass spectra were obtained by electron impact (70 eV) in the range of *m/z* 18 to 400 (intervals 0.5 s). The VOCs were identified by the obtained mass spectra with those from mass spectral libraries (NIST 05, NIST 27, Wiley 229 and Adams[24]), and by the calculated linear retention indexes with literature data.[19,24,25] The GC-MS of the VOCs were subjected to PCA and HCA analyses by using the free software R Project from R Foundation for Statistical Computing® (Vienna, Austria).[23] The analyzed data correspond to the averages of injections in triplicate. The matrix arrangement was made up of ten lines (number of fungi analyzed) and thirty-four columns (compounds identified). The matrix of Pearson correlation was used to perform the PCA. To the HCA, Euclidean distance was used as the coefficient of dissimilarity, and the grouping was done by the method of association average (Ward). The option of automatic truncation was chosen to define the conglomerates and to obtain the dendrogram.

Results and Discussion

The HS-SPME was used on the extraction of the VOCs produced by ten species of endophytic fungi from the Botryosphaeriaceae family. The extraction of volatile compounds was optimized by performing the experimental design 2^2, considering the temperature (50 and 60 °C) and extraction time (10 and 30 min) as variables. The highest number of compounds was detected when the experiment was performed at 60 °C and 30 min.

As already reported by Valente and Augustos,[26] both time and temperature affect the method of extraction by HS-SPME, influencing the kinetics of mass transfer of the volatile compounds between the different phases of the system, and the thermodynamics, which describes the partition equilibrium of the VOCs. In order to verify the significance of these two variables on the extraction of VOCs, all data were analyzed by the application of the Pareto graph method (Figure 1). It was observed that the variable time (B) has greater significance than the variable temperature (A), and that lower significance was observed when the two variables were together.

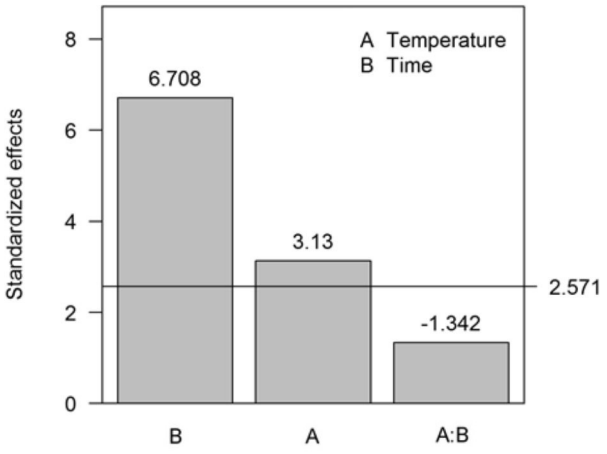

Figure 1. Pareto chart for the effects of temperature and time on the VOCs extraction.

The VOCs from all strains were extracted under the optimized conditions and analyzed by GC-MS. Thirty-four volatile compounds were identified as being produced by the fungal strains (Table 1), and not observed in the control experiments. Most of the compounds are sesquiterpenes (14 non-oxygenated and 10 oxygenated), and only two linear ketones and eight alcohols were identified. Strain 683 (*Neofusicoccum ribis*) produced the greatest number of identified compounds (26), while strain 20 (*Botryosphaeria mamane*) had the lowest number of identified VOCs (6 compounds).

Comparison of the identified compounds with those from the database of microbial volatiles,[13] revealed that only twelve of them were previously reported as fungal VOCs (isobutanol, isopentyl alcohol, 2-methylbutan-1-ol, octan-1-ol, phenylethyl alcohol, β-elemene, calarene, δ-amorphene, germacrene D, valencene, α-selinene and zonarene). However, α-bisabolol and *n*-decanol were already reported as VOCs from two strains of *Phlebia radiata* (Basidiomycetes).[27] As compounds 2-ethyl-decan-1-ol, *n*-decanol, undecan-2-one and α-copaene were reported as VOCs from bacterial origin, half of the identified compounds (17) are being reported for the first time as fungal VOCs. These compounds are: 2-buthyloctan-1-ol, aristolene, eremophylene, aristolochene, γ-cadinene, δ-cadinene, *trans*-cadina-1(2)-4-diene, palustrol, globulol, α-cedrene epoxide, β-cedren-2-one, α-cadinol, juniper camphor, 5-neo-cedranol, guaiol acetate, 13-hydroxyvalencene and hexandecan-3-one. Most of the VOCs are bicyclic sesquiterpenes with eudesmane (2 compounds), guaiane (3 compounds), cadinane (6 compounds), cedrane (3 compounds), aristolane

Table 1. VOCs produced by endophytic fungi from the Botryosphaeriaceae family

Compound	IK1	IK2	*Lasiodiplodia*					*Neofusicoccum*			*Botryosphaeria*	*Pseudofusicoccum*
			71	277	258	474	511	434	600	683	20	477
Isobutanol	–	660	4.50 ± 1.02	13.64 ± 1.16	8.21 ± 1.43	2.85 ± 0.35	16.94 ± 3.53	9.04 ± 0.00	2.95 ± 0.92	1.68 ± 0.72	5.91 ± 2.23	1.12 ± 0.32
Isopentyl alcohol	–	734	15.9 ± 2.67	21.06 ± 1.21	19.56 ± 2.78	10.11 ± 0.41	36.88 ± 2.10	21.24 ± 3.35	13.20 ± 3.62	8.34 ± 3.00	16.72 ± 3.55	3.91 ± 0.33
2-Methylbutan-1-ol	–	739	7.94 ± 1.30	10.29 ± 1.56	11.97 ± 0.94	6.27 ± 0.55	17.63 ± 1.53	15.14 ± 2.40	5.53 ± 1.99	3.57 ± 1.03	11.44 ± 2.83	1.69 ± 0.25
Octan-1-ol	1070	1070	–	8.77 ± 1.35	–	–	–	–	–	–	–	–
Phenylethyl alcohol	1126	1110	5.57 ± 1.89	24.28 ± 4.62	15.98 ± 2.72	4.25 ± 1.21	12.46 ± 1.62	6.93 ± 1.72	5.53 ± 1.27	3.80 ± 1.65	16.5 ± 3.80	1.37 ± 0.49
2-Buthyloctan-1-ol	1194	–	–	2.06 ± 1.20	–	–	–	–	–	–	–	–
2-Ethyldecan-1-ol	1239	–	–	–	–	–	–	0.44 ± 0.15	0.15 ± 0.05	–	–	–
n-Decanol	1274	1269	–	5.40 ± 1.23	–	–	–	–	–	–	–	–
Undecan-2-one	1297	1291	–	–	0.38 ± 0.20	0.12 ± 0.04	–	0.18 ± 0.09	0.06 ± 0.02	0.07 ± 0.04	–	0.17 ± 0.05
α-Copaene	1376	1376	–	–	–	0.12 ± 0.04	0.1 ± 0.00	0.09 ± 0.03	–	–	–	–
β-Elemene	1418	1393	1.29 ± 0.49	–	0.10 ± 0.07	–	0.51 ± 0.10	0.29 ± 0.09	0.35 ± 0.18	0.30 ± 0.27	–	–
Aristolene	1459	1450	0.34 ± 0.32	–	0.57 ± 0.10	1.29 ± 0.25	0.18 ± 0.08	0.18 ± 0.07	–	0.23 ± 0.12	1.57 ± 0.46	–
Calarene	1475	1490	14.99 ± 2.98	3.89 ± 1.29	24.00 ± 1.99	50.50 ± 2.57	–	0.47 ± 0.09	0.09 ± 0.02	0.23 ± 0.07	–	–
Eremophylene	1503	1503	–	–	–	–	–	0.19 ± 0.09	–	0.81 ± 0.33	–	–
δ-Amorfene	1509	1509	0.47 ± 0.33	–	1.01 ± 0.32	2.30 ± 0.57	0.22 ± 0.08	0.07 ± 0.03	–	0.18 ± 0.09	–	–
Germacrene D	1518	1513	–	–	–	–	–	3.40 ± 0.86	2.66 ± 1.49	1.17 ± 0.64	–	0.26 ± 0.06
Aristolochene	1524	1532	31.25 ± 3.89	–	–	0.14 ± 0.03	–	5.65 ± 2.70	3.64 ± 1.39	–	–	–
Valecene	1530	1524	0.23 ± 0.14	–	0.94 ± 0.57	2.18 ± 0.48	0.75 ± 0.09	0.15 ± 0.27	–	1.05 ± 0.18	–	–
α-Selinene	1535	1530	0.37 ± 0.07	–	0.58 ± 0.28	0.81 ± 0.14	0.52 ± 0.10	0.20 ± 0.08	0.10 ± 0.06	0.35 ± 0.04	33.78 ± 3.72	–
γ-Cadinene	1546	1543	–	–	–	0.14 ± 0.13	–	–	0.04 ± 0.02	–	–	–
δ-Cadinene	1552	1548	–	–	0.55 ± 0.32	0.65 ± 0.20	1.36 ± 0.18	0.04 ± 0.07	–	0.62 ± 0.27	–	–
Zonarene	1558	1547	–	–	0.47 ± 0.24	0.33 ± 0.09	0.41 ± 0.09	0.06 ± 0.10	–	0.59 ± 0.14	–	–
Trans-Cadina-1(2)-4-diene	1566	1551	–	–	0.25 ± 0.12	0.51 ± 0.17	0.11 ± 0.06	–	–	0.09 ± 0.06	–	–
Palustrol	1574	1569	6.38 ± 1.53	–	–	–	–	–	–	–	–	–
Globulol	1614	1592	–	–	–	–	0.35 ± 0.09	–	2.26 ± 1.59	1.68 ± 0.69	–	–
α-Cecrene epoxide	1625	1613	–	–	–	–	–	18.61 ± 2.23	38.99 ± 3.58	4.93 ± 1.32	–	–
β-Cedren-9-one	1636	1631	–	–	–	–	–	2.82 ± 1.45	1.24 ± 0.68	1.66 ± 0.08	–	–
α-Cadinol	1678	1670	–	–	–	–	–	3.564 ± 1.04	13.19 ± 3.27	2.03 ± 0.21	–	–
Juniper camphor	1686	1691	–	–	–	–	–	–	0.34 ± 0.23	0.14 ± 0.08	–	–
5-Neo-Cedranol	1693	1685	–	–	–	–	–	–	–	1.88 ± 0.09	–	–
α-Bisabolol	1715	1701	–	–	–	–	–	–	–	–	–	88.83 ± 0.99
Guaiol acetate	1714	1724	0.80 ± 0.36	1.83 ± 0.53	0.48 ± 0.22	1.56 ± 0.36	5.33 ± 1.41	3.39 ± 1.41	0.41 ± 0.34	59.89 ± 5.41	–	–
13-Hydroxyvalencene	1769	1768	4.26 ± 1.20	0.66 ± 0.13	11.61 ± 1.98	12.50 ± 1.90	–	–	–	–	–	–
Hexadecan-3-one	1800	1798	–	1.25 ± 0.47	–	–	–	–	–	–	–	–
Total / %	–	–	94.27 ± 3.48	93.12 ± 2.97	96.69 ± 1.12	96.69 ± 2.78	93.87 ± 6.21	92.14 ± 5.70	90.74 ± 5.10	95.46 ± 0.71	85.91 ± 9.59	97.67 ± 0.10

IK1: Kovats index calculated; IK2: Kovats index from literature; 71: *Lasiodiplodia theobromae*; 277*: L. pseudotheobromae*; 258*: L. citricola*; 474*: L. gonubiensis;* 511*: L. parva*; 434*: Neofusicoccum cordaticola*; 600*: N. parvum*; 683: *N. ribis*; 20: *Botryosphaeria mamane*; 477: *Pseudofusicoccum stromaticum*.

(2 compounds), valencane (3 compounds), copaane (α-copaene) and eremophylane (eremophylene) skeletons. Only three monocyclic sesquiterpenes (β-elemene, α-bisabolol and germacrene D) were found. The non-oxygenated sesquiterpenes belong mostly to the cadinane group while the oxygenated ones possess the guaiane and cedrane skeleton. No monoterpenes were identified in the investigated strains. Seven primary alcohols with different side chain lengths, an aromatic alcohol (phenylethyl alcohol), and two ketones (undecan-2-one and hexadecane-3-one) were also identified.

PCA (Figure 2) followed by HCA (Figure 3) were applied to the identified VOCs in order to investigate the chemical variability patterns. All compounds were considered, and the averages of triplicates were treated as independent variables in a similarity matrix. According to Figure 1, which shows a plot of the scores of the 34 variables from the ten samples, it was possible to differentiate all species. In this case, two main components explain 47.89% of the variance, while five main components explain 77.71% of the total. *P. stromaticum* (strain 474) presented a greater separation from the other strains due to the presence of α-bisabolol as the main component in high concentrations (88.83%). The dendrogram of HCA (Figure 3) corroborates the differentiation of the ten investigated species. With respect to *N. parvum* (strain 600) and *N. ribis* (strain 683), both PCA and HCA clearly differentiated them as two distinct species, and corroborated the use of VOCs analysis as an auxiliary tool for the identification of these species.

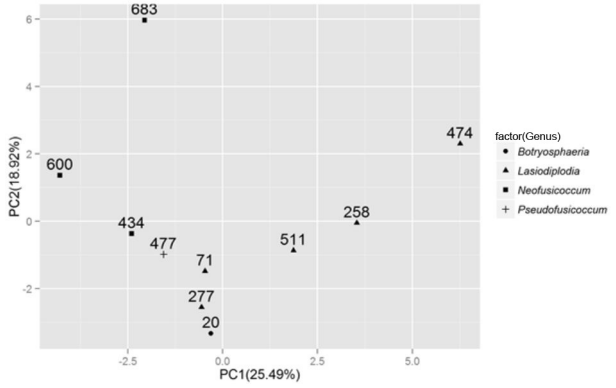

Figure 2. PCA scores plot of 34 variables from samples of HS-SPME-GC-MS of ten fungi from the Botryosphaeriaceae family.

The high content (88.83%) of the sesquiterpene, α-bisabolol, produced exclusively by *P. stromaticum* (strain 477), suggests its potential as a biomarker. This compound is naturally occurring in plants and was first isolated from *Matricaria chamomilla* (Asteraceae).[27] α-Bisabolol has been used in cosmetic formulations and has important biological activities, such as anti-

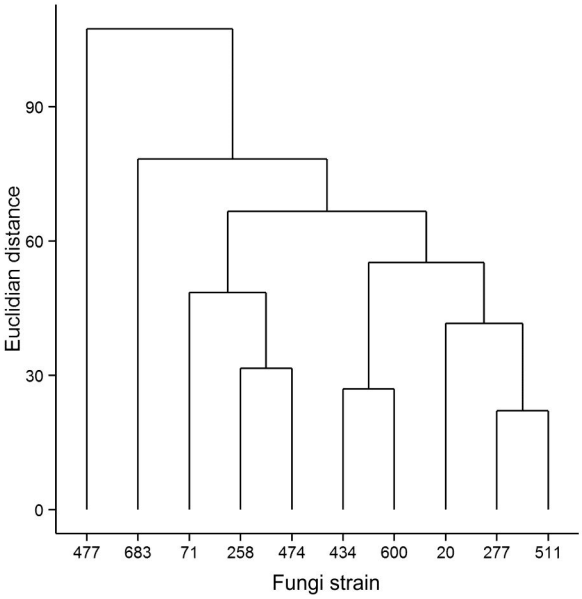

Figure 3. Dendrogram of HCA of the HS-SPME-GC-MS of ten fungi from the Botryosphaeriaceae family.

inflammatory, anti-irritant, antibacterial, antispasmodic, anti-allergic, drug permeation and vermifuge.[27,28] Although α-selinene has been found in most of the investigated strains (except strains 277 and 477), the high content (33.70%) of this sesquiterpene in *B. mamane* (strain 20) may suggest it as a biomarker for this species. As already mentioned, *N. parvum* (strain 600) and *N. ribis* (strain 683) are closely related, and it is not trivial to differentiate them even by molecular analysis. In this study, both species produced α-cecrene epoxide and guaiol acetate, but in different concentrations. α-Cecrene epoxide (38.99%) was produced in high percentage by strain 600, while guaiol acetate (59.89%) was the major constituent of strain 683. Thus, these sesquiterpenes may be considered biomarkers for differentiating these two species.

Conclusions

The HS-SPME in association with GC-MS was successfully used for the study of the VOCs produced by ten filamentous fungi species from the Botryosphaeriaceae family. The VOCs profiles of the investigated strains proved to be adequate to differentiate them by multivariate data analysis (PCA and HCA). In addition, *N. parvum* and *N. ribis*, which were previously considered very similar species, were also differentiated through their volatile chemical profiles. The high content of α-bisabolol in *P. stromaticum* suggested this compound as a potential chemotaxonomic marker for this species. As far as we know, this is the first report on the VOCs profile of the ten investigated Botryosphaeriaceae species.

Supplementary Information

The chromatograms and mass spectra of VOCs produced by ten filamentous fungi species from the Botryosphaeriaceae family are available free of charge at http://jbcs.sbq.org.br as PDF file.

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