Separation and Identification of Volatile Components in the Fermentation Broth of *Trichoderma atroviride* by Solid-Phase Extraction and Gas Chromatography–Mass Spectrometry

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Abstract

A preseparated fermentation broth of *Trichoderma atroviride* strain 11 is analyzed by gas chromatography followed by mass-spectral detection using a Finnigan MAT GCQ apparatus. After preseparation in a C18 and a silica gel column, nineteen pyrone and dioxolane derivatives and two aliphatic esters are obtained, respectively. Among these, the four dioxolane derivatives have not been identified previously. The main component is found to be 5,5'-dimethyl-2*H*-pyran-2-on. The relative standard deviation for the determination of the retention time and the peak area (measured in ion counts) is 0.1% and 4.5%, respectively.

Introduction

Trichoderma (*T.*) species are ubiquitous fungi that can be widely found in soil. They produce volatile (e.g., ethylene, hydrogen cyanide, alcohols, aldehydes, and ketones up to C_4 chain-length) and nonvolatile (e.g., peptides) compounds that are able to inhibit the mycelial growth of fungi. This ability gives the *T.* species an ecological advantage in soil and the rhizosphere of cultivated plants and trees. Antibiosis is one of the main antagonistic interactions between micro-organisms and *T.* species, and this species with adequate antibiotic production can be used as biological control agents (BCAs) for several economically important plant-pathogenic fungi (4). However, the role of antibiosis in biocontrol needs to be deeply explored, because a large number of *T.* species and strains (i.e., homokaryotic mycelium) can yield antibiotics not only in nature, but also in selected laboratory cultures. Metabolite production also depends on whether sporulating or mycelial cultures were examined (5).

The most important BCAs belong to *T. harzianum*, which is a strain aggregate grouped on the basis of morphological features. Recent studies (6) have served to neotypify and redistribute *T. harzianum* (after molecular examination) into two major groups: *T. harzianum* and *T. viride–T. atroviride* complex. According to this new classification, the studied strain 11 has been replaced with the *T. atroviride* species (7). The described molecular diversity can justify the following great array of antimicrobial compounds with respect to structure and function produced by the *T. harzianum* aggregate: pyrones (6-*n*-pentyl-2*H*-pyran-2-one) (8), anthraquinone, butenolide (9), cyclopentyl isocyanide, isonitrine-type compounds, short-chain-length hydrophobic peptides containing a high proportion of amino acid residues, an alkyl *N*-terminus, and a hydroxy amino C-terminus (generic name, peptaibols) (8,10,11). Cyclonerodiol and four octaketide derivatives were found in liquid culture (12). An antibiotic harzianic acid was isolated from a culture filtrate of the SY-307 strain (13) and trichothecene ($C_{23}H_{18}O_6$) on spores of ATCC 90237 (14). Some nonantibiotic-producing strains yield isonitrile-type antibiotics after ultraviolet (UV) mutagenesis (15,16). The pyrone formation can also be stimulated by UV irradiation (16). *T.* species grown on solid media (in the best case ground corn) leads to an excess of pyrone accumulation. The addition of liquid supplements and cultivation time affect the yield (17).

Production of some hydrolytic enzymes and their synergism with the peptaibol antibiotics has been observed in a strain of *T. harzianum* aggregate (18,19).

The Gas Chromatographic (GC)–Mass Spectrometric (MS) method is a modern and widely used technique for the analysis of volatile compounds (e.g., drugs, antibiotics, and toxins) in biological systems (20–24).

In this study, we have analyzed volatile components found in *T. atroviride* strain 11 by mass-spectral detection after GC separation with the aim of establishing in further studies a valid correlation between the production of antibiotic metabolites by this isolate and their effectiveness as BCAs.

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Experimental

Sample preparation

Strain 11 of *T. atroviride* (International Mycological Institute, Egham, U.K., strain IMI 352941) was cultured in a synthetic medium (10) for 14 days at 25°C without shaking. The culture broth was as follows: 0.5% glucose, 0.08% KH_2PO_4, 0.07% KNO_3, 0.02% $Ca(H_2PO_4)_2$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 5H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$, and 0.0001% $FeSO_4 \cdot 7H_2O$ at pH 6.

In vitro antimicrobial susceptibility tests were performed using a panel that included both clinical pathogens and laboratory control strains.

The solid-phase extraction (SPE) was carried out in the following way: 100 mg mycelium of *T. atroviride* (strain 11) was eluted both in 2-g Hypersyl C18 5-µm (Phenomex, Torrance, CA) and Hypersyl silica gel 5-µm (Phenomex, Torrance, CA) columns with 5 mL of methanol. The eluates were concentrated to 1 mL by evaporation in a nitrogen flow.

Chromatographic instrumentation

GC analysis was performed on a Finnigan MAT GCQ type GC–MS. A 30-m long apolar DB-5MS (J&W Scientific, Folsom, CA) fused methyl silicone column (0.25-mm i.d., 0.25-µm film thickness) was used. The column temperature setting was programmed to begin at 60°C for 5 min, then increase at a rate of 20°C/min to 250°C and hold for 10 min with a final injection temperature of 250°C. The linear velocity of the He carrier gas was 35 cm/s. Samples were injected by splitless mode with 0.5 min and 2 min for the close and open time, respectively, of the split valve before and after injection, also respectively.

The ionization in MS detection was performed using the electron impact (EI^+) mode at 70 eV ionization energy in full-scan mode (10–650 amu mass range) at a 0.5-s/scan velocity and an acquisition threshold equal to zero. The temperatures of the ion source and the transfer line were 160°C and 220°C, respectively. The starting time for the acquisition was 5 min after the injection. The detected components were identified by matching the EI^+ spectra against the NIST library (Finnigan Corp. San Jose, CA), which contains approximately 100,000 compounds.

Results and Discussion

The names, retention times, and relative quantities (comparing the peak areas expressed in total ion counts) of the volatile compounds that were analyzed are displayed in Table I. The matching of mass spectra with the representatives of the spectral library was also indicated. For cases in which the matching was less than 50%, only the type of compounds was determined. The total ion chromatograms are shown in Figures 1 and 2. An explanation for the peaks's denoted numbers is found in Table I.

Among the 21 components found, 19 were detected after the preseparation of *T. atroviride* strain 11 in the C18 column. These compounds were mainly pyrone and dioxolane derivatives. After elution on the silica gel column, two aliphatic esters were obtained. The major components were denoted number 11 (31.8%), 9 (17.0%), 2 and 6 (in the same quantity), and 15 (13.6%), and the minor components were 20 (7.0%), 12 (6.0%), and 18 (4.4%). The relative quantities of all the other compounds were below 3%. The reproducibility of the determination of retention time and of the peak area was as follows: average relative standard deviation 0.1% and 4.5%, respectively.

The pyrones are well-known volatile constituents of the *T.* species (12), and dioxolane derivatives were the first identified compounds in this fungus. The major part of the latter components were present in very low quantity, and the 2-ethyl-2-acetyl-1,3-dioxolane was observed at the highest level (7%). Dioxolides were found in another micro-organism (on the culture filtrate of the bacterium *Streptomyces tendae* Tu 4042), but no biological activity was found against other bacteria, yeast, and fungi (25). In general, dioxolanes have various influences in biological systems. 1,3-Dioxolane is known as an antimicrobial agent (26) and 2-(2,4-dichlorophenyl)-1,3-dioxolane has antifungal activity against pathogenic fungi

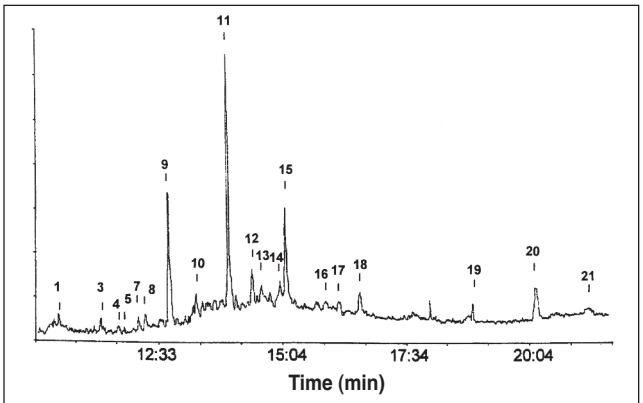

Figure 1. Total ion chromatogram of volatile compounds detected and identified by GC–MS after preconcentration in a C18 column for *Trichoderma atroviride* strain 11. The names of the components (denoted by the peak numbers) are found in Table I.

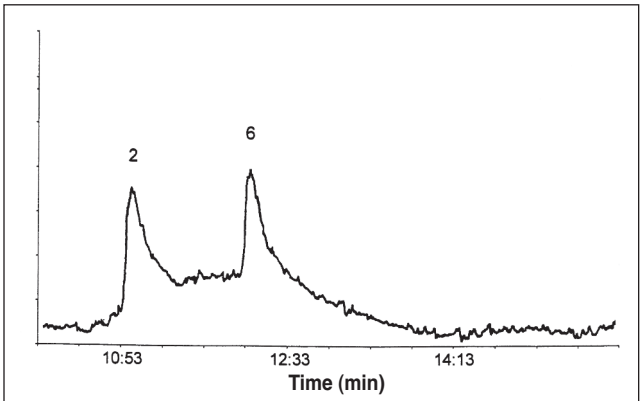

Figure 2. Total ion chromatogram of volatile compounds detected and identified by GC–MS after preconcentration in a silica gel column for *Trichoderma atroviride* strain 11. The names of the components (denoted by the peak numbers) are found in Table I.

Table I. Volatile Compounds in *Trichoderma atroviride* Strain 11 Detected and Identified by GC–MS After Preconcentration on C18 and Silica Gel Columns

Compound number	Retention time (min, *n* = 3) and average (% RSD*)	Relative intensity (%, *n* = 3) and average (% RSD)	Name of identified compound	Matching of spectra (%)
1	10:36 (0.01)	1.0 (2.0)	2-methoxy-1,3-dioxolane	86.1
2	10:57 (0.02)	**	methylacetate	84.6
3	11:28 (0.13)	1.0 (2.3)	dioxolane derivative	< 50
4	11:48 (0.11)	1.0 (4.1)	1,3-dioxolane-2-(1-hydroxyethyl)-methylate	75.7
5	11:56 (0.14)	1.2 (4.1)	dioxolane derivative	< 50
6	12:02 (0.04)	**	methylisopropyonate	64.5
7	12:10 (0.10)	1.0 (5.0)	pyranone derivative	< 50
8	12:18 (0.02)	1.8 (2.8)	pyranone derivative	< 50
9	12:46 (0.21)	17.1 (0.8)	pyranone derivative	< 50
10	13:21 (0.07)	2.2 (11)	pyranone derivative	< 50
11	13:59 (0.01)	31.8 (0.6)	5,5-dimethyl-2*H*-pyran-2-one	85.2
12	14:29 (0.11)	6.0 (1.7)	pyranone derivative	< 50
13	14:37 (0.11)	1.9 (0.6)	6-*n*-butanal-2*H*-pyran-2-one	71.8
14	14:49 (0.13)	1.2 (2.3)	pyranone derivative	< 50
15	15:07 (0.07)	13.6 (0.7)	2-*n*-heptyl-8-hydroxy-2*H*-1-benzopyran-5-one	82.5
16	15:56 (0.21)	1.7 (12)	3-methyl-6-(1,3-dioxo-butan)-2*H*-pyrane-2,4-dione	75.4
17	16:10 (0.10)	2.0 (10)	pyranone derivative	< 50
18	16:34 (0.03)	4.4 (0.8)	3,3',4,4',5,5'-hexamethyl,6-(5-hydroxy)pentyl-2*H*-pyran-2-one	79.5
19	18:44 (0.13)	1.7 (11)	6-*n*-pent-1,2-enyl-2*H*-pyran-2-one	62.0
20	20:06 (0.21)	7.0 (7.1)	2-ethyl-2-acetyl-1,3-dioxolane	72.0
21	21:11 (0.20)	2.7 (7.4)	dioxolane derivative	< 50

* Relative standard deviation.
** Preseparated on silica gel column. The intensity is identical with the value of compound 9.

Table II. Results of the Antibiotical Test of *T. atroviride* Strain 11 on Synthetic Media

Type of isolates	Quantity (%)
Total active	8.7
Antibacterial	25.0
Antifungal	0
Antiyeast	0
Antiphytopathogenic	0

for humans and animals as a result of the oxime group combined with chlorine atoms (27). The halogen (especially iodine-substituated 1,3-dioxolanyluracils) has antiviral activity (28).

Antibiotical tests (made in the University of Salamanca, Salamanca, Spain) have shown that *T. atroviride* strain 11 has slight antibiotical activity. Only an antibacterial effect was observed (Table II).

A good agreement was observed between the low antibiotical activity and the volatile pattern of *T. atroviride* strain 11. Dioxolanes have no presumable biological activity (25) and their toxicity (29) appears to be very low. 6-*n*-pentyl-Pyranone, the main volatile antibiotically active constituent of *T. harzianum* (8), was completely missing, and 6-*n*-pentenyl-2*H*-pyran-2-one was not found in large quantity either.

Because *T. atroviride* had previously been taxonomically included within *T. harzianum*, the description of the metabolite production in that novel species had been cited as part of *T. harzianum*. However, their biological activity as BCAs has demonstrated the difference between these species (7), and the presence of the new metabolites produced by *T. atroviride* is in agreement with the redistribution of both species in a molecular scheme.

Conclusion

In the fermentation broth of *T. atroviride,* nineteen pyrone and dioxolane derivatives, and two aliphatic esters were detected after preseparation in a C18 and a silica gel column, respectively. The major part of them was identified by greater than 50% probability matching with a spectral library.

To the best of our knowledge, four compounds (the dioxolane derivatives) have never been previously found in the fermentation broth of any *T.* species when identified by the GC–MS method.

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