Plant Impact Volatiles from Higher Fungi: A Biotechnological Perspective

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For a long time, essential oils of higher plants were the sole sources of natural flavors. Today, due to consumer preference for natural food additives, the demand for natural flavors exceeds the supply of flavors produced by higher plants.[3,6] Because that supply is strongly dependent on factors which are difficult to control—factors such as influence of weather, plant diseases, fluctuating qualities, sociopolitical instabilities of major supplying areas and trade restrictions—biotechnology represents a promising alternative. Since progress in the field of production of volatiles by plant cell cultures is still slow, cell cultures of higher fungi were investigated in more detail.

Twenty strains of basidiomycetes were submerged cultured, and the volatile compounds generated were isolated at two different phases of growth by solvent extraction. Two hundred twenty-nine compounds were characterized by gas liquid chromatography (GLC), gas liquid chromatography-mass spectrometry (GLC/MS) and gas liquid chromatography-olfactometry (GLC/O). Many of them are known as character impact compounds of higher plants with interesting odor impressions, representing important industrially used flavor compounds.

Materials and Methods

Microorganisms: The 20 examined strains of basidiomycetes and their origins are shown in Table I.

Cultivation: Strains were inoculated (homogenized mycelium) into 150 mL of a medium of glucose (30 g/L), asparagine (4.5 g/L) and yeast extract (3 g/L), and grown aerobically at 25°C in 300 mL shake flasks on a rotary shaker (INFORS, Multitron, Switzerland). The culture period was 20 days.

Table I. Origin of basidiomycete strains used in this study

No.	Basidiomycete strains		Origin
1	*Clitocybe lignatilis*	Karsten	FSU C 31-2
2	*Clitocybe odora*	Kummer	FSU C 36-5
3	*Collybia peronata*	Kummer	CAS 353
4	*Cortinarius percomis*	Fries	CBS 130.42
5	*Cystostereum murrai*	Pouzar	CBS 257.73
6	*Datronia scutellata*	Domanski	CBS 459.66
7	*Gloephyllum odoratum*	Imazeki	FSU A 40-2
8	*Gloephyllum odoratum*	Imazeki	FSU A 40-3
9	*Ischnoderma benzoinum*	Karsten	CBS 311.49
10	*Lepista irina*	Bigelow	CBS 366.47
11	*Micromphale perforans*	Gray	CBS 209.47
12	*Mycena pura*	Kummer	CAS 817
13	*Nidularia confluens*	Fries	CBS 744.68
14	*Piptoporus betulinus*	Karsten	CAS 583
15	*Piptoporus betulinus*	Karsten	CAS 584
16	*Pleurotus cornucopiae*	Rolland	FSU P 125-7
17	*Serpula lacrymans*	CBS	CBS 751.97
18	*Pleurotus sajor-caju*	FSU	FSU P 225-3
19	*Psilocybe cubensis*	Singer	CBS 324.58
20	*Wolfiporia cocos*	Ryvarden	CBS 279.55

Legend
FSU = Friedrich Schiller Universität, Pilzkulturensammlung, Weimar, Germany
CAS = Institute of Mycology, Culture Collection of Basidiomycetes, Department of Experimental Mycology, Prague, Czech Republic
CBS = Centraalbureau voor Schimmelcultures, AG Baarn, The Netherlands

0272-2666/95/0005-0079$04.00/0

On days 10 and 20, 50 mL of each culture broth was centrifuged, stored in the refrigerator at –30°C, and the volatile compounds of each strain were isolated and analyzed in the combined culture media. This double isolation procedure enables one to obtain information on intermittently changing odor profiles over the entire cultivation period.[2]

Isolation of volatile compounds: GLC extracts were prepared from the centrifuged and combined culture media by solvent extraction. The culture media were adjusted to pH 7.2 with $NaHCO_3$ solution. Methyl decanoate was added as an internal standard (200 µL of a solution of 100 µg methyl decanoate in methanol). Then culture media were extracted three times with 40 mL of a mixture of pentane/dichloro methane (2:1, v/v). The solutions were dried over anhydrous sodium sulfate and concentrated to a volume of 1 mL using a Vigreux column.

Gas chromatographic conditions: The concentrates were analyzed by means of capillary GLC using a Carlo Erba Fractovap Series 2150 gas chromatograph coupled to a Shimadzu CR 5A integrator. Chromatographic conditions were: CW 20M capillary column (25 m x 0.32 mm i.d., Leupold, Germany), injection volume 1.5 µL, splitless, injection port 225°C, flame ionization detector 240°C, temperature program 40°C for 3 minutes and then to 210°C with a rate of 3°C per minute, carrier gas hydrogen, flow rate 4.8 mL per minute. Identification of the odorous compounds was carried out by GLC/O and GLC/MS. The retention indices, odors and the mass spectra of the sample components were compared to those of reference samples. GLC/O was carried out using a Carlo Erba Fractovap Series 2150 gas chromatograph, a CW 20M mega bore capillary column (25 m x 0.53 mm i.d., Leupold, Germany), with an outlet splitter to a heated (230°C) sniffing port. Injection volume was 1.5 µL in the splitless mode (for 1 minute). The same chromatographic conditions were used. Mass spectrometry was performed on a Hewlett-Packard mass spectrometer 5989A (Quadrupole), coupled to an HP 5890 Series II gas chromatograph. The system was equipped with a 25 m x 0.32 mm CW 20M capillary column (Leupold, Germany). Chromatographic conditions were the same as above, with helium as carrier gas. The ionization energy was 70 eV.

Results and Discussion

Table II lists 109 of the 229 volatile compounds identified from the submerged cultures of the 20 basidiomycetes examined. Concentration levels and odor assessments of the compounds are given.[7]

In plants, the majority of aroma compounds are generated along pathways of the secondary metabolism. Many of the aliphatic volatiles derive from the isoprene pathway, whereas aromatic compounds are generated through derivatization of cinnamic acid formed via the shikimic acid pathway. Other aliphatic aldehydes, ketones and lactones are products of lipoxygenase-catalyzed or (α- or β-) oxidative degradation of fatty acids.[10,14,15]

In terms of isoprenoid compounds, head-to-tail condensation of isopentenyl diphosphate leads to terpenoids in plants. Terpene compounds derived from isopentenyl diphosphate were found in the majority of culture broths examined. With this as an indicator for an active isoprenoid metabolism in higher fungi, it is not surprising that typical compounds of essential plant oils were generated by basidiomycetes as well. These compounds include monoterpenes, such as limonene and α-terpinene. They also include the terpene alcohols, such as linalool, geraniol and citronellol, which are widespread in many essential oils from flowers and herbs. Finally, the occurrence of volatile sesquiterpenes (such as farnesene, farnesol and nerol) demonstrates that these fungi have a metabolic diversity of terpenoid anabolism comparable to that of plants.

In plants, elimination of ammonia from phenylalanine and tyrosine by phenyl or tyrosine ammonia-lyase yields cinnamic and hydroxycinnamic acid, respectively. Further transformation of the cinnamic acids leads to derivatives of benzophenone, benzylalcohol and benzaldehyde which include important aroma compounds. Among the aromatic compounds identified in the culture broths, one can find these key compounds: benzaldehyde (bitter almond and cherries), anisaldehyde (anise), 2-phenyl-1-ethanol (rose

Table II. Selected volatile compounds in cultures of 20 basidiomycetes after solvent extraction

Compounds	Odor impression*	Strain number (Table I)																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Hydrocarbons																					
beta-bisabolene	balsamic, flowery	❑																			
trans-beta-farnesene	warm				❑																
alpha-guaiene	wood, balsamic											❑									
alpha-gurjunene	fatty						❑														
limonene	lemon, sweet										❑										❑
alpha-muurolene	fruity									❑											
beta-pinene	resin, wood			❑																	
alpha-terpinene	lemon							❑	❑												
Alcohols																					
3-methyl thiopropanol	pungent, sulfurous				❑			❑		❑											
1-butanol	amylalcohol		❑		❑	❑	❑	❑	❑	❑	❑	❑					❑	❑		❑	
2, 3-butanediol	fatty				❑		❑	▲	❑	❑	❑	■		❑	❑	❑	❑	▲	▲	❑	
2-methyl-1-butanol	alcoholic, pungent		❑		❑							❑			❑	❑					❑
3-methyl-1-butanol	pungent, sour		❑	❑	❑	❑	❑	❑	❑	▲				❑			❑	❑		❑	
2-methyl-3-buten-2-ol	spicy, oily									❑											
3-methyl-2-buten-1-ol	pungent				❑				❑		❑										
3-methyl-3-buten-1-ol	pungent				❑				❑		❑										
1-pentanol	sweet		❑		❑		❑	❑	❑	❑	❑						❑	❑			
2-pentanol	rotgut, green	❑		❑		❑		❑	❑								❑			❑	
3-methyl-1-pentanol	pungent, sweet, wine		❑		❑						❑							❑			
2-(2-methoxyethoxy)-ethanol	musty		❑											❑		❑					
1-hexanol	sweet	❑	❑		❑		❑	❑		❑	❑						❑	❑			
2(E)-hexen-1-ol	fruity, wine		❑					❑	❑											❑	
1,2-hexanediol	spicy, laurel																❑				
2(E),4(E)-hexadien-1-ol	fresh, green, herbal		❑																		
1-heptanol	fatty		❑		❑					❑	❑							❑			
1-octanol	fruity, sweet				❑			❑	❑		❑							❑			
2-octanol	fatty, oily							❑		❑											
3-octanol	peanuts		❑					❑	❑		❑				❑	❑	❑	❑		❑	❑
1-octenol-3-ol	mushrooms, sweet				❑			❑	❑		❑			❑			❑	❑	❑		
7-octen-4-ol	cauliflower									❑											
1-nonanol	rose, fatty, orange		❑		❑		❑	❑	❑								❑			❑	
2-nonanol	fatty, fruity, green										❑										
4-nonanol	sweet, plastic																			❑	
1-decanol	flowery, sweet							❑			❑						❑	❑			
alpha-citronellol	rose, perfume, citrus										❑		❑								
beta-citronellol	rose, perfume, citrus						❑				❑		❑								
(E,Z)-farnesol	flowery																	❑			
geraniol	flowery, rose, sweet			▲													❑				❑
linalool	flowery	❑					❑			❑	❑						❑	❑			❑

Table II. Continued

Compounds	Odor impression*	Strain number (Table I) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alcohols continued																					
nerol	sweet, rose						❑														
(E)-nerolidol	sweet		❑					❑	❑												
phenol	phenolic		❑			❑								❑				❑		❑	
2-methylphenol	phenolic, musty														❑	❑	❑	❑		❑	
3-methylphenol	medical, woody									❑											
4-methylphenol	smoky, phenolic								❑									❑			
2-methoxyphenol	sweet, phenolic			❑					❑												
4-methoxyphenol	sweet							❑	❑								❑	❑			
2-methoxy-4-methylphenol	sweet, phenolic			❑							❑										
3,5-dimethoxyphenol	smoky	❑	❑		❑	❑	❑	❑	❑									❑			
2-furanmethanol	caramel, sweet		❑		❑					❑							❑	❑		❑	❑
phenylmethanol	aromatic		❑		❑			❑		❑		❑					❑	❑			
1-phenylethanol	sharp, gardenia		❑		❑				❑				❑				❑				
2-phenyl-1-ethanol	rose, perfume	❑			❑	❑	❑	❑	❑				❑		❑	❑		❑	❑	❑	
3-phenyl-1-propanol	flowery				❑	❑	❑	❑	❑			❑		❑			❑				
3-phenyl-2-propen-1-ol	sweet, balsamic						❑						❑								
2-phenoxyethanol	hay							❑			❑									❑	❑
3-hydroxyphenylmethanol	fruity																	❑			
4-hydroxyphenylmethanol	sweet		❑					❑	❑								❑	❑			
4-hydroxyphenylethanol	fruity							❑													
4-methoxyphenylmethanol	flowery, sweet				❑			❑	❑	❑							❑	❑			
3,4-dimethoxyphenylmethanol	sweet, milk, vanilla			❑			❑		❑								❑				
Aldehydes																					
hexanol	green			❑		❑															
2(E),4(E)-hexadienal	green, fresh, flowery						❑														
heptanal	fatty, sweet					❑															
nonanal	flowery, citrus									❑	❑										
2(E)-decenal	orange, fatty, flowery									❑											
2(Z), 4(E)-decadienal	fatty, green, sweet				❑					❑											
neral	lemon								❑												
benzaldehyde	bitter almond		❑		❑	❑	❑	❑	❑	■	❑	❑	❑				❑	❑	❑		
4-methylbenzaldehyde	bitter almond		❑																		
4-ethylbenzaldehyde	sweet, bitter almond		❑																		
4-methoxybenzaldehyde	anise				❑		❑	❑	❑		❑	❑	■				❑	❑		▲	
2-phenylacetaldehyde	sweet, honey							❑	❑						❑	❑	❑	❑			
3-phenyl-2-propenal	pungent, spicy						❑						❑								
Ketones																					
3-hydroxy-2-butanone	butter		❑		❑		❑	❑	❑	❑	❑	❑					❑	❑	▲	❑	
2-pentanone	sweet, fruity, wine													❑							
3-penten-2-one	wine, sweet													❑							

Table II. Continued

Compounds	Odor impression*	Strain number (Table I) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Ketones continued																					
4-methyl-3-penten-2-one	green							❑	❑	❑								❑			
6-methyl-5-hepten-2-one	fatty, green, citrus			❑											❑			❑			
2-octanone	flowery, green, fruity									❑											
3-octanone	sweet, flowery		❑		❑			❑	❑		❑				❑	❑	❑	❑		❑	❑
1-octen-3-one	mushroom		❑		❑				❑		❑						❑	❑			
2-nonanone	fruity, flowery															❑					
1-phenylethanone	meal		❑			❑		❑	❑		❑							❑			
4-methyl-1-phenylethanone	flowery, fruity					❑															
Esters																					
methyl butanoate	sweet	❑									❑			❑				❑		❑	
hexyl butanoate	sweet, pineapple		❑																	❑	
ethyl 2-methylbutanoate	sweet, apple														❑	❑	❑			❑	
methyl 3-hydroxybutanoate	fruity														❑	❑	❑				
ethyl hexanoate	apple, sweet			❑							❑		❑				❑				
4-butanolide	fatty, sweet		❑																		
4-pentanolide	rubber									❑					❑	❑				❑	❑
4-hexanolide	sweet, warm						❑			❑	❑				❑	❑	❑				
4-heptanolide	sweet, cocoa						❑			❑					❑						
4-octanolide	cocoa									▲	❑						❑			❑	
2-octen-4-olide	sweet, fruity						❑										❑				
4-decanolide	peach, sweet						❑			▲					❑	❑	❑				
4-dodecanolide	fruity, fatty, butter						❑			❑			❑			❑	❑			❑	
methyl benzoate	fruity, sweet		❑		❑			❑	❑		❑				❑	❑	❑	❑		❑	
methyl 2-methoxybenzoate	sweet, flowery							❑	❑	❑											
methyl 4-methoxybenzoate	flowery				❑		❑	❑	❑						❑	❑	❑	❑			
methyl 4-hydroxybenzoate	sweet														❑	❑	❑				❑
ethyl benzoate	flowery, fruity		❑														❑	❑		❑	
ethyl 4-methoxybenzoate	sweet, fruity, anise							❑	❑						❑	❑	❑	❑	❑	❑	
methyl 4-aminobenzoate	sweet								❑						❑	❑				❑	
methyl 2-phenylacetate	honey		❑					❑		▲	❑				❑	❑	❑			❑	❑
ethyl 2-phenylacetate	sweet, honey, rose		❑					❑			❑									❑	
methyl 2-furancarboxylate	mushroom, sweet		❑		❑			❑	❑						❑	❑		❑		❑	
methyl 3-furancarboxylate	mushroom, sweet				❑				❑						❑	❑	❑	❑		❑	

* Odor impression by GLC/O or according to Furia and Bellanca[7]
Concentrations ❑ = < 1 mg/L
▲ = 1-5 mg/L
■ = 6-50 mg/L

oil), 3-phenyl-2-propen-1-ol (Ceylon cinnamon) or 4-hydroxy phenylmethanol (vanilla).

Similar to catabolism in plants, the oxidative degradation of fatty acids by basidiomycetes yielded very odorous compounds such as 2,4(E,E)-hexadienal with a fresh green odor, or the fruity and mushroom-like impressions of octanols and octenols. Lactones derive from the intramolecular cyclization of hydroxy fatty acids. The character impact compounds of coconut (4-heptanolide and 4-octanolide) and peach (4-decanolide) were generated by several basidiomycetes, again indicating high metabolic capabilities compared to plants.

Knowledge of metabolic pathways used by higher fungi is already being used to advantage. Simple and easily available natural components may serve as precursors to improve the yield of more valuable and complex natural flavor compounds which are generated only in low amounts via normal metabolism (without using specific precursors). For example, phenylalanine and tyrosine are transformed by *Ischnoderma benzoinum* to benzaldehyde and anisaldehyde, respectively.[5] Other precursor applications are the biotransformation of ferulic acid to vanillin by *Pycnoporus cinnabarinus*[11] and the degradation of castor oil to 4-decanolide by *Tyromyces sambuceus*.[13]

Conclusion

Basidiomycetes currently play a major role in food agroindustry as edible mushrooms. This study's data confirm prior data[1,4,13] that show basidiomycetes are also suitable for the biotechnological production of volatile flavors. Compared to secondary metabolism in plants, basidiomycetes possess a significant potential. They are suitable for biotechnological processes because of their excellent cultural behavior (growth, mechanical stability, simple media).[1] Therefore, they may represent an alternative to traditional plant sources. Until now, only a small number of basidiomycetes have been characterized with regard to flavor production. However, more than 30,000 basidiomycete species are known worldwide, so the potential for flavor production can hardly be overestimated, and it appears reasonable to focus further research on the superior biosynthetic capabilities of this class of organisms.[4,8]

Among the broad spectrum of compounds identified in this study were some natural flavor compounds (such as α-citronellol, benzaldehyde, 4-decanolide, 2-phenyl acetaldehyde) which, because of the limited synthetic productivities of many higher plants,[9] command high prices. At present, only the low stationary concentrations of synthesized microbial aroma compounds generated via normal metabolism (without using specific precursors) prevent an immediate industrial application. Extended screenings of other basidiomycete strains, optimization of culture conditions, the continuous in situ removal of volatiles from the fungal biomass, the development of bioreactor design and the addition of suitable metabolic precursors may lead to improvement in the yield of flavor compounds.

There may be further opportunities to exploit the remarkable metabolic diversity of basidiomycetes other than the use of the whole fungal cell for aroma production. The endo- and exoenzyme system may serve as a convenient pool for the isolation of useful enzymes for biotransformations of low-cost natural substrates to flavor compounds.[4,9] Genetic engineering offers an additional approach: cloning fungal genes for a target metabolite in order to transport this genetic information to a suitable food-grade host and express it there. In this way, the cloning of fungal genes may facilitate and improve a biotechnological process for flavor generation.

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