FULL PAPER

Study of the Vapor Phase Over *Fusarium* Fungi Cultured on Various Substrates

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The compositions of volatile organic compounds (VOCs) emitted by *Fusarium* fungi (*F. langsethiae*, *F. sibiricum*, *F. poae*, and *F. sporotrichioides*) grown on two nutritive substrates: potato sucrose agar (PSA) and autoclaved wheat kernels (WK) were investigated. The culturing of fungi and study of their VOC emissions were performed in chromatographic vials at room temperature (23 – 24 °C) and the VOCs were sampled by a solid-phase microextraction on a 85 μm carboxen/polydimethylsiloxane fiber. GC/MS was performed using a *60-m HP-5* capillary column. Components of the VOC mixture were identified by electron impact mass spectra and chromatographic retention indices (*RI*s). The most abundant components of the VOC mixture emitted by *Fusarium* fungi are EtOH, AcOH, ^{i}BuOH, 3-methylbutan-1-ol, 2-methylbutan-1-ol, ethyl 3-methylbutanoate, terpenes with M 136, sesquiterpenes with M 204 (a total of about 25), and trichodiene. It was found that the strains grown on PSA emit a wider spectrum and larger amount of VOCs compared with those grown on wheat kernels. *F. langsethiae* strain is the most active VOC producer on both substrates. The use of SPME and GC/MS also offers the potential for differentiation of fungal species and strains.

Keywords: *Fusarium* fungi, Gas chromatography/mass spectrometry, Headspace solid-phase microextraction, Retention indices, Volatile organic compounds.

Introduction

Fusarium fungi are some of the most devastating plant pathogens of small-grain cereals (*e.g.*, oats, rye, wheat, barley) and maize worldwide. Among the resultant diseases are *Fusarium* head or ear blight (FHB) as well as root and stem rots. In addition to the adverse economic effect due to large crop losses, contamination of grain with *Fusarium* produced mycotoxins can create significant health concerns for livestock, poultry, humans, and pets. In particular, these are the *Fusarium* trichothecene type A mycotoxins (*e.g.*, T-2 toxin, HT-2 toxin, diacetoxyscirpenol) formed by biosynthesis of the sesquiterpene metabolite trichodiene. The trichothecene type B group (*e.g.*, deoxynivalenol (DON), nivalenol (NIV), and the acetyldeoxynivalenols) are generally less toxic than the type A mycotoxins.

Living systems are known to interact with each other mostly by the emission and recognition of semiochemical volatile organic compounds (VOCs). The interaction of plants**,** filamentous fungi, and insects plays a vital role in ecosystem function, but little is known about the biological mechanisms of their interactions. However, it is clear that VOC chemical messengers are responsible for semiochemical interactions between them.

F. langsethiae, *F. poae*, *F. sibiricum*, and *F. sporotrichioides* are the objects of numerous modern studies due to their ability to produce trichothecenes. At the same time, the chemical composition and biological properties of volatile secondary metabolites of these four have scarcely been studied [1][2][3]. The GC/MS determination of the VOC emissions of various *Fusarium* fungi strains utilizing SPME potentially offers a new view for differentiation of these species.

The indicative role of VOCs in bioanalytical research is hard to overestimate. Investigations into the profile of VOCs emitted by filamentous fungi on toxin biosynthesis, determination of VOC markers, and development of techniques for their fast and highly sensitive detection allow reliable identification of toxin-producing fungi at early stages of plant infection and safety control of raw materials and products.

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In view of the fact that marker VOCs are present in the air together with a great variety of other volatile components emitted not only by the fungi in itself, but also by the substrate, any technique for the detection, identification, and quantitation of marker VOCs should necessarily include separation of the VOC mixture. Collection of VOCs emitted by a sample to be analyzed can be performed in different ways. There are two techniques of VOC sampling from the equilibrium vapor phase: active and passive sampling. Active sampling involves the use of an air sampling pump to actively pull VOCs through a collection device such as a filter or a sorbent tube. In the case of passive sampling, VOCs are collected at a rate controlled by a physical process such as diffusion, without the use of an air sampling pump. Many publications are available, where VOCs emitted by microorganisms were collected by active sampling. *Jelen et al.* [4] collected volatile emissions of *Fusarium* fungi onto tubes with a sorbent (*Tenax*) with subsequent thermal desorption of the collected components into a chromatographic column. In *Larsen*'s work [5], volatile fungal metabolites were isolated by diffusive sampling from the surface of a substrate in a *Petri* dish which allowed differentiation of two taxonomically closely related species, *Penicillium clavigerum* and *Penicillium vulpinum*. Further, it has been established that VOC emissions of damaged and undamaged living systems have different compositions [6]. The metabolic profile of microorganisms and the composition of VOC emissions should strongly alter not only in case of stress or death, but also under any external factors. The most rational approach to metabolic profiling of VOCs *in vivo* is gas chromatography/mass spectrometry (GC/MS) in combination with passive sampling, as such sampling techniques minimize any disturbance of a living system. Among such techniques, solid-phase microextraction (SPME) has gained the widest acceptance. The advantages and peculiar features of SPME *in vivo* as a nondestructive method for research on living system have been reviewed in [7]. SPME consists in the collection of volatile analytes by adsorption on fibers coated with a sorbent stationary phase. Such construction allows all stages of sample preparation to be joined in a single procedure. SPME makes it possible to save time for sample preparation, exclude the use of solvents, and reduce the cost of analysis and provides lower detection limits. A combination of SPME with GC/MS has been used to success in the determination of a wide range of compounds in gaseous, liquid, and solid samples, and especially in the extraction of VOCs from natural and biological samples. SPME can also be combined with liquid chromatography [8] or capillary electrophoresis [9] for the determination of nonvolatile or thermally unstable compounds which are impossible to analyze by GC and GC/MS.

In SPME, exhaustive adsorption does not occur; instead equilibrium is established between the substrate and the stationary phase. In terms of the possibility to reach a high degree of concentration of the analytes, it might be considered as a disadvantage. However, in this case, one can repeat analysis of the same sample after a certain time lapse required for equilibration of the system. This feature allows tracing changes in the metabolic profile with the age of the culture. For collection of VOCs produced by microorganisms, and, in particular, fungi *in vivo*, microfiber should not be brought in direct contact with the substrate. The most convenient approach is collection of VOCs on a microfiber from an equilibrium vapor phase (HS-SPME). Unfortunately, in many works where HS-SPME has been used no rationalization for the choice of microfiber was given. Thus, *Girotti et al.* [2] in their study of the composition of VOCs emitted by *Fusarium* fungi made use of a polydimethylsiloxane/divinylbenzene microfiber, but the cited article did not explain the choice of this microfiber and provided no comparisons with other data. According to our experience, a carbon fiber (carboxen) makes it possible to analyze the widest range of VOCs but, on the other hand, shows the strongest memory effect, which requires several blank runs to be performed after each analysis and, as a result, increases the total analysis time.

Among VOC emissions of toxin-producing fungi, most emphasis has been put on sesquiterpenes. The latter are genetically related to trichodiene which is considered as a volatile marker of toxin formation [10]. The fact that the compositions of sesquiterpenes vary over a wide range suggests their use for revealing genetic diversity and species differentiation of toxin-producing fungi.

Results

The detected volatile products of the secondary fungal metabolism can be divided into two groups. The first group includes low-boiling compounds whose retention indices (*RI*s) on the weakly polar stationary phase *HP-5* are not higher than 1100: linear and branched alkanes and alkenes, alcohols, ethers, furans, and terpenes. These compounds are generally easy to identify, because their reference samples are available and the mass spectra and *RI*s can be found in numerous sources. Compounds belonging to the first group are biosynthesis products characteristic of many living systems. Some researchers consider VOCs in this group as species-specific signatures of fungi, including *Fusarium* fungi [11]. Compounds with *RI*s not higher than 1100 and detected in emissions of *F. langsethiae*, *F. sibiricum*, *F. poae*, and *F. sporotrichioides* are listed in *Table 1*. If a compound was detected at least once in the equilibrium vapor over a fungal culture, it is marked by '+' in *Table 1*, otherwise, the corresponding cell is left empty.

Table 2 includes VOCs whose *RI*s fall in the range 1100 – 1550. This group primarily includes isomeric sesquiterpenes with the formula $C_{15}H_{24}$. These compounds, which present undeniable interest in terms of chemotaxonomy [12], are fairly difficult to identify because their reference samples are often commercially

Table 1. Highly volatile *Fusarium fungi* emissions, growing on two nutrient substrates: potato sucrose agar (PSA) and autoclaved wheat kernels (WK)

Entry	t_R[a]) [min]	$RI_{exp.}$[b])	$RI_{lit.}$[c])	*RI* (Ref.)	Compound	*F. langsethiae*		*F. sibiricum*		*F. poae*		*F. sporotrichioides*	
						PSA	WK	PSA	WK	PSA	WK	PSA	WK
1	4.570		427	[15]	EtOH [23]	+	+	+		+	+	+	
2	4.775		501	[15]	Acetone [23]	+	+	+	+	+	+	+	+
3	4.806		500	[15]	Pentane [23][26]	+	+	+	+	+	+	+	+
4	5.109		521 – 523	[15]	Methyl acetate [23]					+			
5	5.428		521 – 557	[15]	1-Propanol [26]		+	+					
6	5.871		567	[15]	3-Methylpentane [23]		+	+	+		+	+	+
7	6.060	600	600	[15]	Hexane [23][26]		+	+	+	+	+	+	+
8	6.395	607	603	[15]	2-Methylfuran [23][26]			+	+	+		+	
9	6.433	613	614	[15]	3-Methylfuran [23]	+	+	+	+	+	+	+	+
10	6.417	615	611	[18]	AcOEt [23][26]	+	+	+	+	+	+	+	+
11	6.703	624	625	[15]	^{i}BuOH [23][26]	+	+	+	+	+	+	+	+
12	7.071	636	636	[15]	4-Methyl-1,3-pentadiene	+							
13	7.477	651	651	[17]	3-Methylbutanal [23][26]		+	+		+	+		
14	7.791	659	660	[18]	2-Methylbutanal [26]			+		+			
15	9.181	700	700	[15]	Heptane [23][26]	+	+		+	+	+		
16	9.311	702	702 – 704	[15]	2-Ethylfuran [23][26]		+		+				
17	10.424	722	726	[15]	Methylcyclohexane [23][26]				+				
18	11.178	736	736	[15]	3-Methyl-1-butanol [23][26]	+	+	+		+		+	
19	11.385	739	742	[15]	2-Methyl-1-butanol [26]	+	+	+		+		+	
20	11.717	745	747	[15]	1-Methyl-1*H*-pyrrole [23]						+		
21	12.908	767	767	[15]	4-Methylheptane [23]	+	+		+				
22	13.013	767	765	[15]	1,3,5-Cycloheptatriene	+							
23	13.467	777	772 – 779	[15]	2-Methylpropyl acetate [22]	+	+	+		+	+		
24	14.809	800	800	[15]	Octane [22][23][26]	+	+	+	+	+	+		+
25	15.069	804	798 – 804	[15]	Ethyl butanoate [26]	+							
26	16.020	822	818 – 823	[15]	2,4-Dimethylheptane [23]		+		+				
27	16.505	831	834	[15]	1,3,5-Trimethylcyclohexane				+				
28	17.100	842	840 – 844	[15]	2,4-Dimethyl-1-heptene [23]	+	+	+	+				
29	17.689	854	845 – 856	[15]	Ethyl 2-methylbutanoate	+	+	+				+	
30	17.781	856	844 – 859	[15]	Ethyl 3-methylbutanoate	+		+				+	
31	18.236	864	862 – 865	[15]	4-Methyloctane [26]				+				
32	18.962	878	879 – 883	[15]	3-Methylbutyl acetate [22]					+			
33	19.063	880	884	[15]	2-Methylbutyl acetate [22]					+			
34	19.620	890	891	[15]	1-Nonene [26]				+			+	
35	20.024	900	900	[15]	Nonane [22][26]	+		+	+				
36	21.705	936	936	[18]	α-Pinene	+		+	+				
37	23.486	977	977	[18]	β-Pinene	+							
38	24.010	991	989	[18]	β-Myrcene	+							
39	24.225	998	993	[15]	2-Pentylfuran [23][26]		+						
40	24.691	1005	–		Unidentified *m/z* 43 (100), 57 (75), 71 (94), 85 (18), 113 (16)	+	+	+	+				

Table 1. (cont.)

Entry	t_R[a]) [min]	$RI_{exp.}$[b])	$RI_{lit.}$[c])	*RI* (Ref.)	Compound	*F. langsethiae*		*F. sibiricum*		*F. poae*		*F. sporotrichioides*	
						PSA	WK	PSA	WK	PSA	WK	PSA	WK
41	24.852	1010	–		Unidentified *m/z* 43 (100), 57 (77), 71 (94), 85 (23), 113 (15)	+	+	+	+				
42	25.554	1030	1032	[15]	Limonene [23]	+	+	+	+	+	+	+	
43	26.517	1054	–		Unidentified *m/z* 43 (100), 57 (91), 71 (77), 85 (48), 113 (8), 127 (8)		+		+				
44	26.723	1060	–		Unidentified *m/z* 43 (77), 57 (100), 71 (59), 85 (35), 113 (8), 127 (6)				+				
45	27.309	1076	–		Unidentified *m/z* 43 (100), 55 (57), 57 (76), 69 (97), 70 (67), 71 (56), 83 (41), 111 (25), 125 (10)	+	+		+				
46	27.457	1078	–		Unidentified *m/z* 43 (100), 55 (58), 57 (78), 69 (92), 70 (77), 71 (59), 83 (40), 111 (23), 125 (7)	+	+		+				
47	27.586	1083	–		Unidentified *m/z* 43 (46), 54 (100), 67 (96), 81 (65), 95 (23), 110 (10), 124 (9), 152 (7)							+	
48	27.718	1087	1088	[15]	1-Undecene [26]							+	+
49	27.835	1089	1087	[18]	α-Terpinolene	+	+						
50	27.996	1098	1100	[15]	Undecane [26]							+	

The results were obtained by GC/MS combined with HS-SPME. The table includes low-boiling compounds (*RI* exp < 1100). Mass spectra are given for unidentified components; *m/z* – mass-to-charge ratio; '+' – compound at least once was detected in the equilibrium vapor over a fungal culture. [a]) t_R – experimental retention time of a compound on the weakly polar stationary phase *HP-5*, 60 m. Retention times (t_R) precision ±0.050 min. [b]) *RI* exp – experimental retention index of a compound on the weakly polar stationary phase *HP-5*, 60 m, calculated using an *n*-alkane homologous series. [c]) *RI* lit – retention index of a compound from published data [15][17][18].

Table 2. Semivolatile *Fusarium fungi* emissions growing on two nutrient substrates: potato sucrose agar (PSA) and autoclaved wheat kernels (WK)

Entry	t_R[a]) [min]	$RI_{exp.}$[b])	$RI_{lit.}$[c])	Compound	m/z[d]) [%]	*F. langsethiae*		*F. sibiricum*		*F. poae*		*F. sporotrichioides*	
						PSA	WK	PSA	WK	PSA	WK	PSA	WK
1	31.512	1184	–	Unknown	53 (19), 79 (46), 81 (76), 109 (100), 152 (18)						+		
2	34.421	1289	–	Unknown	44 (20), 91 (20), 137 (100), 151 (36), 152 (40), 166 (19)	+	+		+				
3	34.439	1293	–	Unidentified sesquiterpene	95 (100), 107 (58), 146 (11), 202	+							
4	35.202	1318	1320	4-Ethylveratrol	77 (18), 91 (17), 95 (22), 108 (12), 151 (100), 166 (54)		+						
5	35.666	1335	1335	δ-Elemene	55 (19), 79 (20), 93 (83), 105 (23), 121 (100), 136 (62), 161 (28)					+			
6	35.752	1340	–	Unidentified sesquiterpene	91 (38), 107 (13), 131 (25), 145 (100), 161 (82), 119 (41), 202 (45)	+							
7	36.299	1360	–	Unidentified sesquiterpene	79 (21), 93 (37), 105 (70), 119 (66), 123 (100), 161 (43), 189 (13), 204 (36)					+			
8	36.408	1362	–	Unidentified isoalkane	43 (52), 57 (100), 71 (87), 85 (34), 113 (11), 127 (10), 267 (6)		+						
9	36.752	1374	1376	Isoledene [26]	93 (33), 119 (45), 105 (75), 161 (100), 204 (31)	+	+	+	+	+	+		
10	36.832	1379	1380	Daucene [26]	93 (31), 121 (52), 161 (100), 204	+							
11	37.008	1383	–	Unidentified sesquiterpene	69 (41), 93 (84), 105 (23), 119 (100), 120 (15), 204 (2)	+	+						
12	37.017	1384	–	Unidentified sesquiterpene	69 (25), 93 (51), 105 (32), 119 (100), 204 (10)	+		+					
13	37.603	1394	–	Unidentified sesquiterpene	69 (100), 91 (53), 117 (42), 118 (90), 120 (39), 132 (26), 202 (16)	+				+			
14	37.650	1410	–	Unidentified sesquiterpene	55 (17), 79 (17), 93 (22), 105 (76), 119 (48), 133 (28), 161 (100), 189 (18), 204 (30)					+			
15	37.684	1410	–	Unidentified sesquiterpene	69 (100), 93 (60), 111 (45), 119 (52), 105 (29), 204 (7)	+		+					
16	37.696	1410	–	Unidentified sesquiterpene	69 (78), 93 (59), 105 (46), 119 (100), 161 (20), 147 (15), 204 (14)					+	+		
17	37.757	1413	–	Unresolved peaks of sesquiterpenes	69 (53), 77 (39), 79 (43), 81 (50), 90 (60), 93 (99), 105 (100), 107 (69), 119 (87), 121 (89), 136 (90), 161 (67), 204 (33)			+		+			
18	37.787	1414	–	Unidentified sesquiterpene	93 (58), 107 (58), 121 (92), 136 (100), 204 (22)	+		+	+	+	+	+	+
19	37.891	1419	1420	α-Santalene	55 (22), 93 (100), 105 (43), 107 (43), 108 (77), 119 (43), 133 (9), 204 (14)	+	+	+					+
20	37.902	1420	–	Unidentified sesquiterpene	79 (18), 93 (27), 105 (91), 119 (100), 121 (46), 136 (21), 161 (38), 204 (12)					+	+	+	

Table 2. (cont.)

Entry	t_R[a]) [min]	$RI_{exp.}$[b])	$RI_{lit.}$[c])	Compound	m/z[d]) [%]	*F. langsethiae*		*F. sibiricum*		*F. poae*		*F. sporotrichioides*	
						PSA	WK	PSA	WK	PSA	WK	PSA	WK
21	37.968	1420	–	Unidentified sesquiterpene	81 (41), 93 (60), 105 (94), 119 (100), 136 (19), 161 (36), 204 (15)			+	+				
22	37.983	1421	–	Unidentified sesquiterpene	69 (100), 92 (31), 119 (48)	+							
23	38.053	1424	1421	*β*-Duprezianene	91 (21), 105 (80), 119 (100), 161 (38), 204 (15)			+	+	+	+		
24	38.184	1430	–	Unresolved peaks of sesquiterpenes	69 (38), 81 (38), 93 (54), 105 (85), 119 (85), 133 (29), 161 (100), 204 (28)					+	+	+	
25	38.230	1431	–	Unidentified sesquiterpene	67 (45), 79 (56), 91 (46), 93 (100), 105 (40), 107 (61), 121 (76), 204 (14)	+	+	+	+				
26	38.285	1433	–	Unidentified sesquiterpene	55 (7), 81 (19), 93 (16), 105 (44), 121 (8), 161 (100), 204 (5)	+	+	+	+	+	+	+	+
27	38.384	1439	1439	Widdrene (Thujopsene)	69 (34), 79 (25), 93 (37), 91 (30), 105 (65), 119 (100), 123 (42), 133 (20), 204 (15)	+	+	+					
28	38.453	1442	–	Unidentified sesquiterpene	93 (48), 121 (100), 136 (44), 189 (25), 204	+	+						
29	38.663	1448	1446	*cis*-*β*-Farnesene	57 (77), 69 (100), 93 (47), 120 (20), 133 (24), 204 (2)	+	+			+	+		
30	38.801	1454	–	Unknown	55 (24), 81 (70), 93 (75), 95 (73), 96 (100), 108 (87), 111 (35)	+		+				+	+
31	38.819	1456	1451	(*E*)-3,5-Muuroladiene (tent)	79 (20), 93 (28), 105 (37), 119 (27), 121 (31), 161 (100), 189 (4), 204 (4)			+	+	+	+	+	+
32	38.910	1460	1463	*β*-Santalene	55 (21), 79 (25), 94 (100), 122 (34), 133 (4), 204 (4)	+	+						
33	38.954	1461	–	Unidentified sesquiterpene	79 (48), 93 (73), 108 (27), 119 (100), 121 (77), 147 (25), 162 (25), 189 (70), 204 (15)	+				+	+		
34	39.060	1464		Contaminant		+	+	+	+	+	+	+	+
35	39.224	1470	1464	α-Acoradiene	57 (51), 93 (81), 105 (92), 119 (100), 121 (63), 147 (55), 189 (19), 204 (7)	+	+	+	+	+	+	+	+
36	39.292	1473	1469	*β*-Acoradiene	69 (42), 79 (40), 93 (57), 105 (50), 119 (100), 121 (45), 147 (20), 161 (16), 204 (11)	+	+						
37	39.374	1476	–	Unidentified sesquiterpene	93 (73), 107 (34), 119 (41), 121 (100), 136 (40), 204 (34)	+	+						
38	39.433	1480	–	Unidentified sesquiterpene	67 (36), 91 (84), 93 (49), 105 (98), 119 (100), 133 (51), 161 (56), 189 (21), 204 (28)					+	+		

Table 2. (cont.)

Entry	t_R[a]) [min]	$RI_{exp.}$[b])	$RI_{lit.}$[c])	Compound	m/z[d]) [%]	*F. langsethiae*		*F. sibiricum*		*F. poae*		*F. sporotrichioides*	
						PSA	WK	PSA	WK	PSA	WK	PSA	WK
39	39.440	1479	1479	ar-Curcumene	55 (27), 91 (22), 105 (63), 119 (86), 132 (100), 202 (23)	+	+						
40	39.442	1479	1480	*β*-Chamigrene	77 (27), 93 (82), 105 (59), 107 (79), 121 (74), 133 (50), 189 (100), 204 (19)	+	+	+			+	+	+
41	39.448	1479	–	Unresolved peaks of sesquiterpenes	93 (79), 105 (70), 119 (100), 121 (60), 133 (22), 189 (44)				+				
42	39.542	1482	1481	*γ*-Curcumene	79 (34), 91 (58), 93 (47), 105 (73), 119 (100), 132 (47), 147 (17), 161 (39), 202 (10), 204 (15)					+			
43	39.620	1487	–	Unidentified sesquiterpene	93 (77), 105 (59), 107 (75), 121 (47), 189 (100), 204 (21)	+		+					
44	39.680	1488	1484	Germacrene D	91 (67), 105 (85), 133 (30), 161 (100), 204 (16)				+				
45	39.759	1491	1489	*β*-Selinene	79 (96), 93 (100), 107 (82), 119 (67), 135 (30), 161 (74), 189 (30), 204 (33)		+						
46	39.842	1496	1503	*cis*-*α*-Bisabolene	67 (24), 93 (100), 105 (24), 119 (27), 121 (20), 133 (15), 161 (11), 204 (19)	+	+				+	+	
47	39.977	1500	1506	Valencene	91 (49), 105 (60), 121 (44), 131 (30), 161 (100), 204 (20)				+	+	+		
48	40.026	1504	1505	*cis*-*β*-Bisabolene	67 (40), 69 (100), 93 (84), 94 (38), 109 (27), 161 (22), 204 (7)	+	+		+	+	+		
49	40.169	1507		Contaminant		+	+	+	+	+	+	+	+
50	40.259	1514	1504 – 1514/1507	*α*-Chamigrene M 204 + Cuparene M 202	91 (26), 93, 121 (41), 132 (100), 136 (39), 202 (19), 204 (23)	+	+	+		+		+	+
51	40.658	1527	–	Unresolved peaks of sesquiterpenes	55 (67), 93 (100), 105 (56), 119 (95), 121 (77), 133 (43), 161 (9), 204 (12)	+	+						
52	40.644	1530	1529	(*E*)-*γ*-Bisabolene	93 (100), 107 (92), 119 (34), 135 (32), 204 (15)	+					+		
53	40.726	1533	1533	Trichodiene	67 (50), 81 (21), 93 (19), 109 (100)	+	+	+	+	+	+	+	+

The results were obtained by GC/MS combined with HS-SPME. Table includes compounds with *RI* exp 1100 – 1550. Mass spectra: m/z – mass-to-charge ratio; '+' – compound at least once was detected in the equilibrium vapor over a fungal culture. [a]) t_R – experimental retention time of a compound on the weakly polar stationary phase *HP-5*, 60 m. Retention times (t_R) precision ±0.050 min, potato sucrose agar (PSA) and autoclaved wheat kernels (WK). [b]) *RI* exp. – experimental retention index of a compound on the weakly polar stationary phase HP-5, 60 m, calculated using an *n*-alkane homologous series. [c]) *RI* lit. – retention index of a compound from published data [17]. [d]) m/z – mass-to-charge ratio from mass spectra of a compound.

unavailable and reference mass spectra and *RI*s can most commonly be found in original articles rather than in databases. Moreover, the published data are not infrequently contradictory. In our research, we faced the problem that the *RI*s of these compounds depended on chromatographic conditions. The same observation was previously reported for polyaromatic hydrocarbons [13]. According to [13], the use a long column (60 m) and step temperature program can sometimes cause inaccuracy in the *RI*s of polycyclic compounds, and the probability of errors increases with increasing absolute value of *RI* [13]. To check the correctness of the experimental *RI*s of sesquiterpenes, we analyzed under the same conditions a mixture of deuterated polyaromatic hydrocarbons. The *RI* of naphthalene (1200) falls in the middle of the reference *RI* range, whereas *RI*s of acenaphthene (1507), phenanthrene (1820), and, especially, chrysene (2534) proved to be overestimated. So *RI*s values for semivolatile compounds (*Table 2*) were corrected according to [13]. *Leffingwell et al.* [14] in their study on the composition of a tobacco extract used the same chromatographic column as we used in the present work. The *RI*s of phenanthrene and chrysene, reported in [14], are higher as compared to the respective values in the official NIST database [15]. Note that corrected *RI* of trichodiene is 1533, which well agrees with published data [16]. Thus, our estimates for the *RI*s of components eluted from the chromatographic column earlier than trichodiene can be considered as correct values, whereas those for later eluted components may involve certain errors. In the present work, we consider VOCs whose *RI*s are not higher than the *RI* of trichodiene. In the *RI* NIST database [15], one mass spectrum is provided by several *RI* values. As reference, we took the *RI* value measured in experimental conditions closest to those in our work. If such conditions were unavailable, data from [17] or [18] were used.

Fig. 1 and *2* show the TIC chromatograms of the VOC mixtures characteristic of *F. langsethiae* grown on two substrates; the elution regions of the highest boiling components listed in *Table 2* are shown in inserts presented on a magnified scale. As seen from the figures, the fractions of the highest boiling components in the VOC mixtures are not too high, but their indicative role in early diagnosis of the poisoning of raw materials and products by toxin-producing fungi is quite significant. Sesquiterpenes $C_{15}H_{24}$ are emitted both by *Fusarium* fungi [19] and certain plants [11], and, therefore, it seems reasonable to search for key compounds responsible for the interaction between *Fusarium* fungi and plants**,** specifically among the sesquiterpenes. Analysis of sesquiterpenes (*M* 204) reveals 13 and 9 compounds, respectively, emitted by *Fusarium* fungi growth on potato sucrose agar (PSA) and autoclaved wheat kernels (WK).

Study of the Composition of VOCs Emitted by F. langsethiae *Strain MFG93001 with Growing on Two Substrates*

The most abundant components of VOC emissions of the *F. langsethiae* strain cultured on PSA are EtOH, AcOEt, ^{i}BuOH, 3-methylbutan-1-ol, 2-methylbutan-1-ol, ethyl 3-methylbutanoate, limonene, α-terpinolene, sesquiterpenes with M 204 (a total of 25 compounds), sesquiterpene with M 202, as well as trichodiene (*Fig. 1*). In the VOC mixture emitted by *F. langsethiae* cultured on PSA, we detected a little of terpene hydrocarbons (*M* 136).

The VOC emissions of *F. langsethiae* cultured on autoclaved WK, too, contain EtOH as the major component; a poorly resolved pair acetone/pentane was identified, as well as methyl-, ethyl, and pentylfurans, 3-methylbutanal, 2-methyl- and 3-methylbutan-1-ols, a

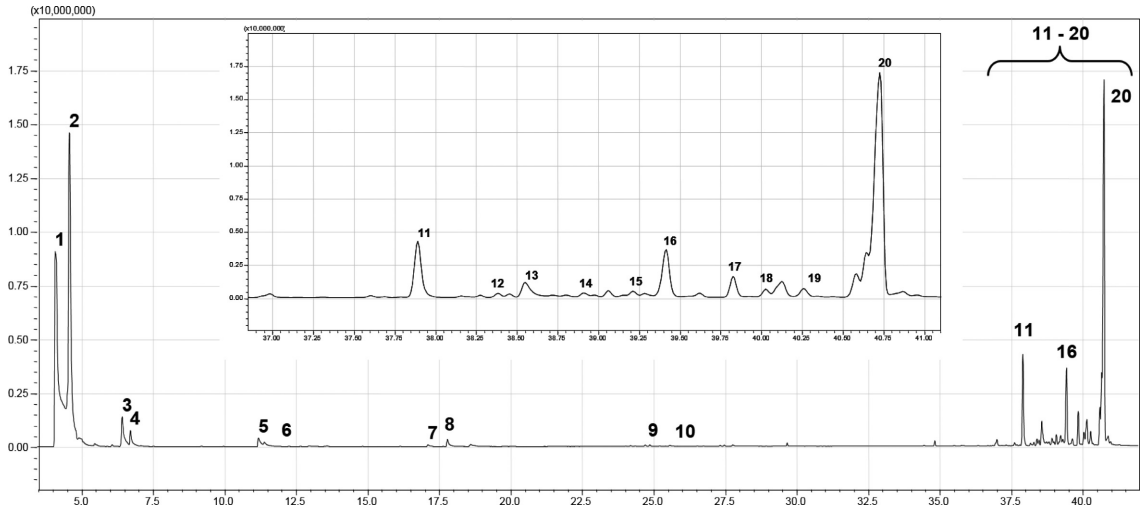

Fig. 1. GC/MS-chromatogram (total ion current) of the vapor phase of *Fusarium langsethiae*, cultured on potato sucrose agar after 9th day of incubation. 1 – CO_2; 2 – EtOH; 3 – AcOEt + 3-Methylfuran; 4 – ^{i}BuOH; 5 – 3-Methylbutan-1-ol; 6 – 2-Methylbutan-1-ol; 7 – 2,4-Dimethyl-1-heptene; 8 – Ethyl 3-methylbutanoate; 9 – Limonene; 10 – α-Terpinolene; 11 – α-Santalene; 12 – Widdrene; 13 – cis-β-Farnesene; 14 – β-Santalene; 15 – α-Acoradiene; 16 – β-Chamigrene; 17 – cis-α-Bisabolene; 18 – cis-β-Bisabolene; 19 – Cuparene + α-Chamigrene; 20 – Trichodiene.

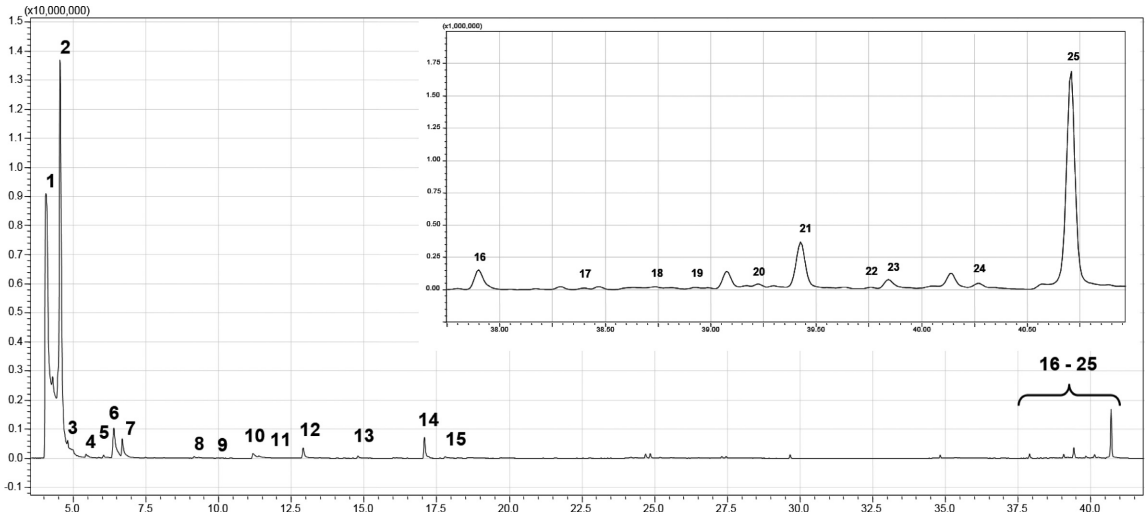

Fig. 2. GC/MS-chromatogram (total ion current) of the vapor phase of *Fusarium langsethiae*, cultured on wheat kernels after 9th day of incubation. 1 – CO_2; 2 – EtOH; 3 – Pentane + Acetone; 4 – PrOH; 5 – Hexane; 6 – AcOEt + 3-Methylfuran; 7 – ^{i}BuOH; 8 – Heptane; 9 – 2-Ethylfuran; 10 – 3-Methylbutan-1-ol; 11 – 2-Methylbutan-1-ol; 12 – 4-Methylheptane; 13 – Octane; 14 – 2,4-Dimethylhept-1-ene; 15 – Ethyl 3-methylbutanoate; 16 – α-Santalene; 17 – Widdrene; 18 – cis-*β*-Farnesene; 19 – *β*-Santalene; 20 – α-Acoradiene; 21 – *β*-Chamigrene; 22 –*β*-Selinene; 23 – cis-α-Bisabolene; 24 – Cuparene; 25 – Trichodiene.

group of aliphatic hydrocarbons, limonene; sesquiterpenes (*M* 204 and 202), and trichodiene (*Fig. 2*).

A distinctive feature of the VOC mixture emitted by *F. langsethiae* grown on WK is that it contains a wide range of aliphatic hydrocarbons: hexane, heptane, octane, as well as branched alkanes C_7H_{16}, C_8H_{18}, and $C_{11}H_{24}$, and alkenes C_9H_{18} and $C_{11}H_{22}$. In the VOC emissions of *F. langsethiae* cultured on WK, we detected ethyl- and pentylfurans, which are absent in the case of fungi growing on PSA.

The range of sesquiterpenes in the emissions of fungal strains cultured on PSA is much wider and they are more abundant, especially trichodiene.

Study of the Composition of VOCs Emitted by F. sibiricum *Strain MFG11016 with Growth on Two Substrates*

Here, we observe the same regularities as described previously; EtOH is the most abundant component but only for culturing on PSA and only until the 12th day, while by the 16th day its production sharply decreases. The following components were identified in VOC mixture emitted by *F. sibiricum* cultured on PSA: acetone and AcOEt (early days of culturing), pentane (late days of culturing), 3- and 2-methylbutan-1-ols and their corresponding aldehydes, linear (hexane, octane) and branched alkanes, and methylfuran.

The VOC mixture emitted by *F. sibiricum* cultured on WK contains a wider range of furans (2-methyl-, 3-methyl-, and 2-ethylfurans) and branched aliphatic hydrocarbons.

The equilibrium vapor over *F. sibiricum* cultured on PSA contains about an order of magnitude more trichodiene than that over *F. sibiricum* cultured on kernels. In the first case, the trichodiene content decreases about two times in going from the 9th to 16th day of culturing, while in the second, it remains almost invariable. The spectrum of sesquiterpenes (*M* 204) on PSA is slightly wider than on WK. In both mixtures, we tentatively identified isoledene and α-acoradiene.

Study of the Composition of VOCs Emitted by F. poae *Strain MFG11046 with Growth on Two Substrates*

The most abundant component of the VOC emissions of *F. poae* on PSA between the 9th and 12th day of growing is EtOH, and its quantity sharply decreases by 16th; by contrast, with growth on WK EtOH was not detected over the entire observation period (from 9th to 16th day). The same picture is observed with AcOEt: it is very abundant in the case of PSA (even though its quantity decreases almost 30 times by 16th day) and is not detected at all with growth on WK. It should be noted that the total concentration of VOCs much decreases by 16th day of culturing with both substrates.

Aliphatic hydrocarbons and methylfuran are identified in both cases. Sesquiterpenes (*M* 204) are more diverse with PSA, but not too abundant. A sesquiterpene with *RI* 1456, most probably (*E*)-3,5-muuroladiene is no less than 10 times more abundant than other sesquiterpenes. Its chromatographic peak is fairly intense already on the first observation day (9th day of culturing), on 11 – 12th days this peak area doubles and then only slightly decreases by 16th day. With WK, this component, too, is detected in the VOC emissions of *F. poae*, but its abundance compares with the abundances of other sesquiterpenes (*M* 204) and tends to continuously increase from the 9th to 16th day of culturing. The concentrations of all the

sesquiterpenes emitted by *F. poae* cultured on WK decrease with time. *F. poae* cultured on PSA emits about two times more trichodiene than in the case of the wheat medium. The tendency of the trichodiene concentration to decrease from the 9th to 16th day of culturing is observed with both substrates.

Study of the Composition of VOCs Emitted by the F. sporotrichioides *Strain MFG163101 with Growth on Two Substrates*

EtOH is the most abundant component among VOCs emitted on PSA: its concentration is especially high on 9th and 10th day of culturing and gradually decreases by the 16th day. Abundant components are also acetone, EtOH, and ^{i}BuOH, and less abundant are 3-methylbutan-1-ol, 2-methylbutan-1-ol, ethyl esters of 2- and 3-methylbutanoic and 2-methylpropanoic acids, limonene, as well as a series of aliphatic saturated and unsaturated hydrocarbons.

Analyzing the composition of the VOC mixture emitted by *F. sporotrichioides* growth on WK, it was first observed that no ethanol was detected throughout the entire observation period. In general, gas emissions on WK are much weaker than on PSA. The group of highly volatile components comprises aliphatic hydrocarbons (pentane, 3-methylpentane, hexane, octane, 3-methylfuran, and AcOEt).

The quantities of trichodiene in the equilibrium vapor over a 9-day *F. sporotrichioides* culture (1st observation day) on both substrates compare with each other; then, starting with the 12th day and up to the 16th day of culturing on WK the quantity of trichodiene decreases about two times, whereas by the 16th day of culturing on PSA the quantity of trichodiene more than doubles.

Discussion

The results of our research show that the composition of VOC emissions of *Fusarium* fungi depends both on the fungal species and cultivation conditions. Potato sucrose agar (PSA) is the most widely used medium for growing fungi. *Fusarium* fungi flourish on PSA, so that abundant mycelial growth and numerous sporulation are obtained. Wheat kernels (WK) are a common substrate for fungi growth and mycotoxin contamination. The studied *Fusarium* fungi all characteristically emit more VOCs, including sesquiterpenes and trichodiene, when cultured on a PSA. The main reason for this fact is the higher bioavailability of nutrients from the agar medium compared to autoclaved kernels. These findings are consistent with our previous data showing that these fungal strains produce much more trichothecene mycotoxins (T-2 toxin and diacetoxyscirpenol), when cultured on PSA compared to nutrient media on the basis of wheat flour [20].

The studied fungal strains belong to phylogenetically closely related fungi which are quite difficult to differentiate by morphological features [21]. Analysis of VOC emissions can be used as an additional tool for differentiation and correct identification of such fungi. The highly volatile components (*Table 1*) are mostly well-known compounds. Of 50 components, only 42 could be identified. At the same time, almost all of the identified compounds are typical of most microorganisms. In particular, 24 out of 42 volatiles are quite typical of bacteria [22][23]. Semivolatile compounds (*Table 2*) have a good indicative potential, but they are primarily represented by sesquiterpenes (*M* 204) and are difficult to identify. Reference samples for them are hardly accessible. No commercial reference sample is available even for trichodiene, a volatile precursor of trichothecene mycotoxins in their biosynthesis and a commonly recognized volatile marker of toxin-producing *Fusarium* fungi [10]. The fungi studied in the present work differ from each other by bioenvironmental requirements and are capable of producing large amounts of trichothecene mycotoxins. Thus, *F. poae* and *F. sporotrichioides* occur widely, and can be found in many plants; they are also of medical interest in view of the ability to cause opportunistic mycoses in humans. The relatively recently described species *F. langsethiae* and *F. sibiricum* were found exclusively in cereals and have limited areals [24]. However, the actual distribution of *F. sibiricum* and *F. langsethiae* may be much larger than what is presently known.

At the same time, it is not excluded that the inhomogeneity in the metabolite spectra is associated with the different growth rates of the fungal strains. It can be suggested that the volume of the agar nutrient substrate (4 ml) is insufficient for such rapidly growing fungi as *F. poae*, *F. sibiricum,* and *F. sporotrichioides* to grow at 24 °C for 16 days: the culture starts to degrade, as evidenced by the sharply decreased VOC production. *F. langsethiae* are slowly growing fungi and, consequently, their most intensive metabolism occurs later compared to rapidly growing fungi. The formation of various organic compounds is associated with synthesis and degradation processes, and, consequently, the possibility to analyze the components formed at the same time by strains growing at different rates on a limited volume of nutrient media has certain limitations.

The full range of VOCs identified over the entire observation periods for all the fungal strains cultured on two substrates includes 117 compounds; some of which could not be identified in this work. In the case of the PSA substrate, 48 light VOCs and 49 terpenes were found in all the emissions studied. In the case of the WK substrate, the number of light VOCs was the same, whereas the number of terpenes was lower (40). The emissions of the *F. langsethiae* strain characteristically contain the widest spectrum of both biologically active compounds and semivolatile components of the sesquiterpene series.

There are large findings in chemical ecology concerning the role of VOCs in the interaction of plants, insects, and fungi. The detected VOCs emitted by *Fusarium* fungi most

probably include metabolites which act as attractants or repellents for insects coexisting with then and, probably, favor their dispersal and adaptation to the environment. In the experiments aimed at revealing semiochemical interactions between various *Fusarium* species and the rice weevil, associated with their development with cereals, the beetles showed different reactions on the studied strains [25]. The low pathogenic strains *F. langsethiae* MFG93001 and *F. poae* MFG103403, grown both on PSA and on autoclaved wheat kernels acted as attractants on insects. The *F. sibiricum* MFG11016 and *F. sporotrichioides* MFG163101 strains grown in the same conditions acted as repellents. The revealed difference in the olfactory reaction of insects on different fungal strains may be associated with different compositions of their VOC emissions.

Twenty of the volatile compounds (*Table 1*) and two of semivolatiles (*Table 2*) are contained in the semiochemicals database [26] and can be considered as potential insect repellents or attractants. However, the observed biological effects could not be related to any of the 22 semiochemicals or their combination in this study. Therefore, we will have to search for chemical attractants or repellents among minor or still unidentified components. First, it will be necessary to find culturing conditions favoring the manifestation of these effects.

Artificial simulation of attractive or repellent properties using synthetic VOC mixtures would allow control of interactions in living systems for agricultural purposes.

Conclusions

In this research, we found that the VOC emissions of *F. langsethiae*, *F. sibiricum*, *F. poae*, and *F. sporotrichioides* fungi grown on two different substrates have different compositions and different component concentration dynamics. The resulting data suggest the possibility for creating a cumulative VOC database for identification of fungal species. However, a serious impediment on the way to developing such a database is the lack of agreement between different researchers concerning concrete conditions (temperature, substrate, culturing time, *etc.*) for VOC screening [27], which is likely to explain the controversy in the results of different studies. Even though there are many publications on the use of VOCs in taxonomic studies, they have scarcely been used for identification of *Fusarium* fungi.

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Experimental Part

Strains of Fusarium *Fungi and Their Culturing Conditions*

Four strains of trichothecene-producing *Fusarium* species and common causal organisms of plant diseases *F. langsethiae* Torp and Nirenberg (#MFG93001), *F. poae* (Peck.) Wollenw. (#MFG11046), *F. sibiricum* Gagkaeva, Burkin, Kononenko, Gavrilova, O'Donnell, Aoki, and Yli-Mattila (#MFG11016), and *F. sporotrichioides* Sherb. (#MFG163101) were used in the study (*Fig. 3*). All the strains were isolated from cereal kernels and as single spore cultures stored in the collection of the Laboratory of Mycology and Phytopathology, All-Russian Institute of Plant Protection (MFG) and in the Russian Collection of Agricultural Microorganisms (RCAM) (RCAM) in the automated *Tube Store* (*Liconic Instruments*, Mauren, Lichtenstein) at −75 °C in 25% glycerol. The species identification of the strains was performed by morphological features and confirmed by a positive DNA amplification test with species-specific primers [19][20][28].

The strains were grown on two nutrient substrates: potato sucrose agar (PSA) and autoclaved wheat kernels of cultivar Leningradka (WK). Culturing on nutrient substrates was performed directly in 20 ml chromatographic vials for headspace analysis (*Supelco* Cat. No SU860097, *Sigma–Aldrich*, St. Louis, MO, USA).

In sterile conditions, 4 ml of PSA was distributed into preliminarily autoclaved vials (30 min, 1 atm). Wheat kernels (moisture content 40%) were autoclaved for 30 min at 1 atm, cooled, sterile distributed in 4 g portions into the vials, and autoclaved under the same conditions. After cooling to r.t., the kernels and PSA were inoculated with *Fusarium* strains. The inoculum contained 10 µl of a suspension of spores and fungal mycelium with a concentration of 10^7 CFU, prefiltered through a capron fine-mesh filter. After inoculation, vials were incubated for 16 days at 24 °C in a thermostat. In control samples, 10 µl of sterile H_2O was added instead of suspension.

Analysis of VOCs by GC/MS Combined with HS-SPME

The profile of VOCs emitted by each strain on each type of substrate was studied in dynamics. Headspace sampling with SPME was performed at r.t. (23 – 24 °C) once a day from the 9th through 16th day after inoculation.

A *QP-2010 Plus* GC/MS system (*Shimadzu*, Kyoto, Japan) was used for GC/MS analysis. HS-SPME was performed using an *AOC-5000* autosampler (*PAL System*, Zwingen, Switzerland). The heating, shaking, and stirring functions were shut down to exclude disturbance of cultures. Once a day, after the analysis cycle was complete, vials were unsealed to expose cultures to air. The daily analysis cycle included analysis of a blank sample (air in an empty vial) and uninoculated substrates. The sequence of operations was the following: A 85-µm Carboxen/Polydimethylsiloxane fiber (*Supelco* Cat. No 57334-U) was conditioned under N_2 at 200 °C for 60 min and exposed to the equilibrium vapor over culture for 30 min, after which collected components were thermodesorbed to a chromatographic column at 250 °C for 1 min.

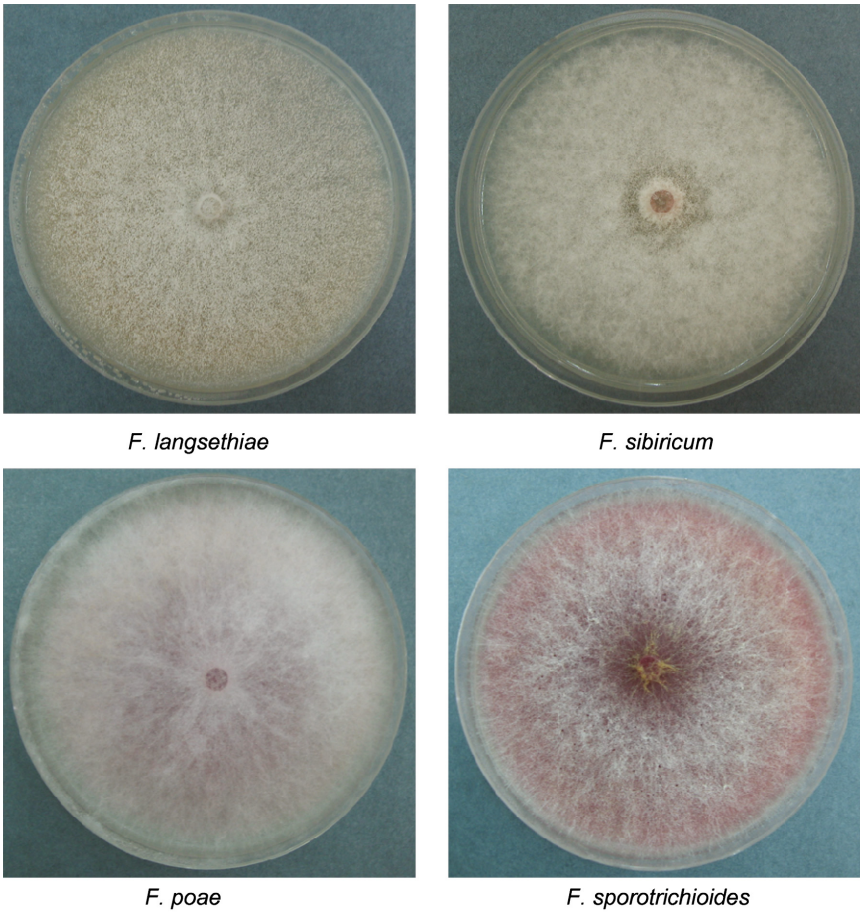

Fig. 3. *Fusarium* strains used in the study.

Gas Chromatography

Injector temp. 250 °C, splitless injection (purge time 1 min); column *HP-5 MS*, 60 m × 0.25 mm × 0.25 μm (*Agilent*; Part No. 19091S-436, Santa Clara, CA, USA); oven temp. program: initial 40 °C (hold 10 min), ramp to 250 °C at 5 °C/min, ramp to 280 °C at 20 °C/min, hold at the final temp. 25 min; carrier gas He, flow rate 1.0 cm^3/min.

Mass Spectrometry

Ionizing energy 70 eV, interface temp. 270 °C, ion source temp. 200 °C, scan mode TIC, *m*/*z* range 40 – 500.

The VOCs emitted by fungal strains were identified by comparing their mass spectra and retention indices (*RI*s) with published data. The experimental *RI*s were calculated using an *n*-alkane homologous series (*Supelco* Cat. No 500631) as standard. The correctness of the *RI*s of the components of the VOC mixtures was checked by determining the *RI*s of the components of a mixture of deuterated polyaromatic hydrocarbons (*Supelco* Cat. No 48230-U).

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