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Production of mycotoxins by *Aspergillus lentulus* and other medically important and closely related species in section *Fumigati*

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The production of mycotoxins and other secondary metabolites have been studied by LC-DAD-MS from six species in *Aspergillus* section *Fumigati*. This includes the three new species *Aspergillus lentulus*, *A. novofumigatus* and *A. fumigatiaffinis* as well as *A. fumigatus*, *Neosartoria fisheri* and *N. pseudofisheri*. A major finding was detection of gliotoxin from *N. pseudofisheri*, a species not previously reported to produce this mycotoxin. Gliotoxin was also detected from *A. fumigatus* together with fumagillin, fumigaclavine C, fumitremorgin C, fumiquinazolines, trypacidin, methyl-sulochrin, TR-2, verruculogen, helvolic acid and pyripyropenes. Major compounds from *A. lentulus* were cyclopiazonic acid, terrein, neosartorin, auranthine and pyripyropenes A, E and O. Thus in the present study *A. fumigatus* and *A. lentulus* did not produce any of the same metabolites except for pyripyropenes. The fact that *A. lentulus* apparently does not produce gliotoxin supports the idea that other compounds than gliotoxin might play an important role in the effective invasiveness of *A. lentulus*. An overall comparison of secondary metabolite production by strains of the six species was achieved by analysis of fungal extracts by direct injection mass spectrometry and cluster analysis. Separate groupings were seen for all the six species even though only one isolate was included in this study for the two species *A. novofumigatus* and *A. fumigatiaffinis*.

Keywords *Aspergillus* species, *Fumigati*, mycotoxins, fungal metabolites

Introduction

Aspergillus fumigatus is the most important opportunistic human pathogenic *Aspergillus* species, and can cause severe disease in immuno-compromised humans and animals if they inhale many conidia of this fungus [1]. On the other hand it has been shown that other fungi from section *Fumigati* may cause fungal infections, including *Neosartorya pseudofischeri* [2] and *A. lentulus* [3,4]. This raises the question whether other species from section *Fumigati* may infect humans and animals. Recently some fungi previously identified as *A. fumigatus*, were shown to be new species: *A. novofumigatus* and *A. fumigatiaffinis* [5]. Many new *Neosartorya* species have also been described [6]. The taxonomy of *Aspergillus* section *Fumigati* was treated by Raper and Fennell [7], but has since been treated by Kozakiewicz [8], Frisvad *et al*. [9] and Horie *et al*. [10].

Aspergillus fumigatus has been reported to produce a large number of extrolites (secondary metabolites and other secreted metabolites) [11–17] including the very toxic metabolite gliotoxin, which seems to be implicated in the *A. fumigatus* infection process [18–21]. It is less clear whether other extrolites are involved in pathogenesis [22]. In this study we wanted to use LC-

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DAD-MS to clarify in detail which major metabolites are produced in *Aspergillus* section *Fumigati,* comprising *A. fumigatus*, *A. lentulus*, *N. fisheri*, *N. pseudofischeri* and the two newly described species *A. novofumigatus* and *A. fumigatiaffinis.* We also demonstrate that these species can easily be identified based solely on their mass profile of secondary metabolites.

Material and methods

The isolates examined are listed in Table 1. The fungi were inoculated on the following agar media: CYA agar, YES agar and YE agar at 25°C in the dark for one week. The composition of the first two media can be found in Frisvad and Samson [23]. YE agar had the same composition as YES agar, except that sucrose was not added. The cultures were extracted by the plug extraction method [24], adapted to a two-step procedure where three 6 mm plugs cut from the cultures were extracted with 0.5 ml ethyl acetate (with 0.5% V/V formic acid) in the first step and re-extracted with 0.5 ml 2-propanol in the second step. The combined extracts were evaporated to dryness in a rotary vacuum concentrator, re-dissolved in 0.3 ml methanol, and filtered through a 0.45 µm filter before analysis.

Direct infusion mass spectrometric analysis (DiMS) of raw fungal extracts was done on a Waters (Micromass) Q-TOF quadrupole time-of-flight mass spectrometer with a z-spray electrospray source (Manchester, UK). The instrument was tuned in positive electrospray mode to a resolution better than 8500 FWHM (on leucineenkphaline, 0.5 µg/ml in 50% (V/V) acetonitrile with 0.1% (V/V) formic acid) and calibrated on a polyethylene-glycol solution (PEG, mixture of PEG400 and PEG600 in 50% (V/V) acetonitrile with 0.1% (V/V) formic acid). One µl extract was injected by a FAMOS autosampler (LCPackings, The Netherlands) into a carrier stream of methanol delivered at a rate of 15 µl/min from an Agilent 1100 micro-flow HPLC pump (Walbron, Germany). Just prior to the source, water with 2% (V/V) formic acid, was added to the stream at a rate of 5 µl/min from a Harvard syringe pump (Harvard Instruments, USA). The combined flow of 20 µl/min was feed to the electrospray source. The source was optimized for minimal fragmentation and maximal sensitivity: temperature 60°C, needle voltage 3200V, cone 30V, desolvation gas at 300 l/h, nebulizer gas at 15 l/h. Data was collected in continuum mode from m/z 100 to m/z 1000 at a rate of one spectrum per second with 0.1 sec interscan time. Data was collected for 3 min after injection and samples were analyzed at a rate of one sample every 4 min. From each raw continuum data file a centroid mass spectrum was calculated by combining the scans during the elution of the extract. These centroid spectra were then used for statistical analysis. A classification was done by binning each of the spectra into a 0.5 m/z grid. Group specific bins were selected by analysis of variance (ANOVA) for which bins having a P-value less than 0.01 were removed. From the remaining bins pair wise Euclidian distances were calculated ending up with a distance matrix. From the dissimilarity matrix dendrograms were then calculated using UPGMA linkages.

The samples were analyzed by liquid chromatography mass spectrometry (LCMS) on a Waters LCT time-of-flight mass spectrometer equipped with a z-spray source including a Lockspray interface for internal mass correction and connected to an Agilent 1100 HPLC with diode array detection (Walbron, Germany). The mass spectrometer was tuned in positive electrospray to a resolution better than 5000 FWHM and calibrated on the same solutions as for DiMS analysis (see above). Samples (3 µl) were separated on a Luna C18(II) column (Phenomenox, USA), 50 mm × 2 mm id, packed with 3 µm particles and equipped with a SecuriGuard pre-column (2 × 4 mm) (Phenomenex, USA). A gradient of water with 20 mM formic acid and acetonitrile with 20 mM formic acid was used as mobile phase going from 15% acetonitrile to 100% acetonitrile in 20 min and then maintained for 5 min before returning to the starting conditions. The source was operated in positive electrospray mode: desolvation gas flow 600 l/h, desolvation temperature 450°C, nebulizer gas flow 14 l/h, source temperature 120°C, needle voltage 3200V, cone voltage 25V, other parameters are optimized for resolution and maximal sensitivity. The protonated molecular ion at 556.2771 Da/e from leucineenkphaline (1 µg/ml in 50% (V/V) with 0.1% (V/V) formic acid) was used for internal mass correction. Data was collected at a rate of one spectrum every 0.4 second with 0.1 second interscan time, one mass reference scan was collected every third second. Data was stored in mass corrected centroid format. The UV and MS data as well as retention time and index data of individual peaks were

Table 1 Isolates from *Aspergillus* section *Fumigati* examined

A. fumigatiaffinis: CBS 117186 = IBT 12703
A. fumigatus: ATCC 32722 = IBT 14904, NRRL 1979 = IBT 15720, AF 293 = IBT 24699, IMI 376380 = IBT 23720
A. lentulus: FH5 = IBT 27210, FH7 = IBT 27209, IBT 20466, IMI 376377 = CBS 117180 = IBT 23719, IMI 381888 = IBT 26436
A. novofumigatus: CBS 117520 = IBT 16806
N. fischeri: IBT 25695, CBS 317.89 = IBT 24757, IBT 3009
N. pseudofischeri: CBS 404.67 = IBT 3019, CBS 117074 = IBT 27802, CBS 117073 = IBT 3002, CBS 117063 = IBT 6472

compared to data from standards [25,26] and databases such as Chemical Abstracts.

Results

A number of known compounds could readily be detected by comparison of LC-DAD-MS data generated from fungal extracts of *A. fumigatus* (Fig. 1) and *A. lentulus* (Fig. 2) and the more than 600 authentic standards available at Center for Microbial Biotechnology. These included cyclopiazonic acid, trypacidin, helvolic acid, fumigaclavine C, fumagillin, fumitremorgin A and B, verruculogen, neosartorin, auranthine, terrein, *epi*-aszonalenins A and C [27] and gliotoxin (Table 2). The detection of the latter compound in extracts of both *A. fumigatus* and *N. pseudofisheri* was confirmed by ion trace analysis for *m/z* 263.103, which corresponds to protonated gliotoxin having lost two sulfur atoms as also shown for a standard of gliotoxin.

Other compounds such as fumitremorgin C, TR-2, methyl-sulochrin and pyripyropenes could only be tentatively identified since no authentic standards were available (Table 2). Thus the major metabolites produced by the four investigated strains of *A. fumigatus* were tentatively identified (data not shown) to be fumiquinazolines due to their characteristic UV spectra [28]. The MS data strongly indicated the major compound to be fumiquinazoline C or D since the dominant ions seen in the mass spectrum indicated the molecular formula $C_{24}H_{23}N_5O_4$. However, since the spectrum clearly indicated loss of water due to the presence of the fragments $[M-H_2O+H]^+$, $[M+Na]^+$ the major compound is most likely to be fumiquinazoline D since this compounds has a free hydroxy group likely to easily be lost by fragmentation.

A dominant biosynthetic family of metabolites tentatively identified were four pyripyropenes detected from three (*A. lentulus*, *A. fumigatiaffinis*, *N. pseudofisheri*) of the six species studied (Table 2). The MS data for three of the four compounds matched the molecular mass data for pyripyropenes A, E and O respectively (Fig. 3), since a characteristic $[M+H]^+$ and $[M+CH_3CN+H]^+$ ion peak pattern could be seen for the three compounds (Table 2). The MS data for the fourth and most polar pyripyropene indicated an elementary composition of $C_{27}H_{35}NO_5$ not matching any of the pyripyropenes (A-R) known today. Based on the present data we hypothesize that this new 'pyripyropene S' is one of the three possible de-acetoxy forms of pyripyropene A since the difference in mass between these two compounds matches the loss of a CH_3CO- group.

All species produced a characteristic profile of secondary metabolites (Table 2). Thus the five isolates of *A. lentulus* all produced the following and unique combination of metabolites: cyclopiazonic acid, terrein, neosartorin, auranthine and pyripyropenes A, E and O (Fig. 4). The four isolates of *A. fumigatus* all produced helvolic acid, fumiquinazoline D, F (or G), one of the fumigaclavines, trypacidin, methyl-sulochrin and pseurotin A and D. Three isolates (ATCC 32722, NRRL 1979, IMI 376380) produced TR-2, verruculogen, fumitremorgin B and C. None of the strains produced fumitremorgin A. Apparently IBT 24699 (=AF 293), the first *Aspergillus fumigatus* that has been fully genome sequenced [29] (Nierman *et al*., 2005), is not

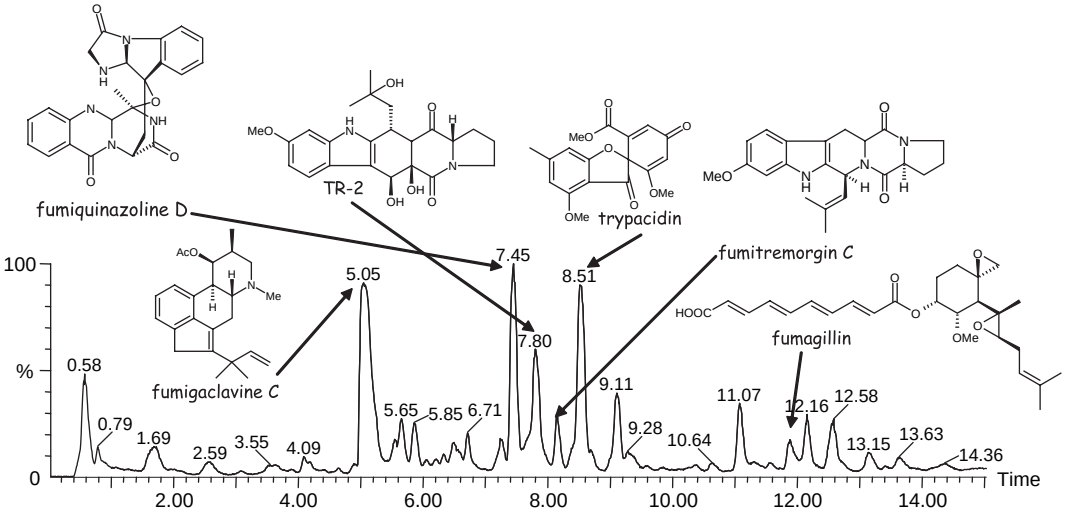

Fig. 1 Total Ion Chromatogram (0–15 mins) from *Aspergillus fumigatus* (IBT 14904), grown on YES for 7 days in the dark, with structures of the compounds identified from some of the major peaks.

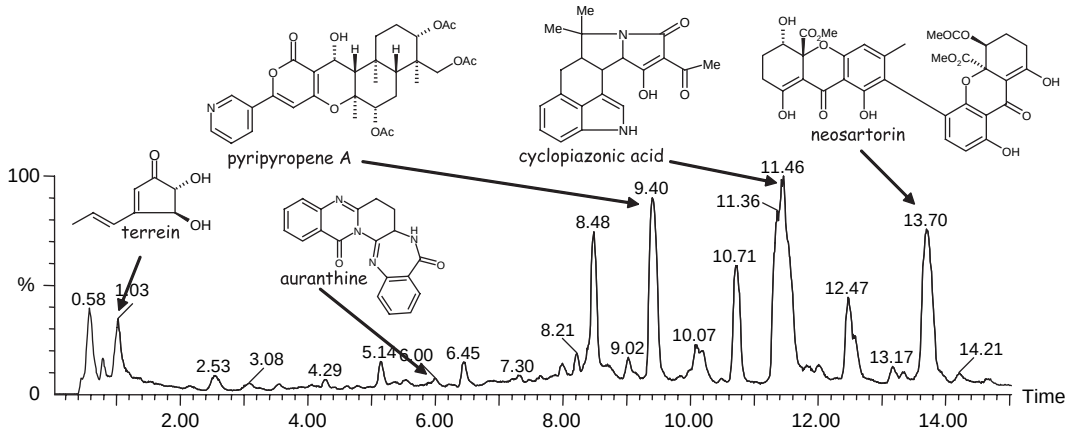

Fig. 2 Total Ion Chromatogram (0–15 mins) from *Aspergillus lentulus* (IBT 27209), grown on YES for 7 days in the dark, with structures of the compounds identified from some of the major peaks.

capable of producing any of the typical end products (TR-2, fumitremorgin A or B or verruculogen) of the fumitremorgin biosynthetic pathway, but based on UV spectrum evidence the strain produces a precursor of that biosynthetic pathway. In these experiments we did not detect gliotoxin in NRRL 1979, but this strain has formerly been reported to produce gliotoxin [30]. Gliotoxin was detected on YE agar for the three other isolates tested. Three isolates produced fumagillin (AF 293, ATCC 32722 and NRRL 1979). Two isolates produced fumigatin derivatives (ATCC 32722 and NRRL 1979), but only one isolate produced 'pyripyropene S' (NRRL 1979). Based on UV spectrum evidence all four *A. fumigatus* strains produced anthraquinones, probably the chloroanthraquinones reported by Yamamoto *et al*. [31]. All the three isolates of *N. fisheri* produced TR-2, fumitremorgin B, fumitremorgin A and verruculogen in large amounts, but none of the strains produced fumitremorgin C, fumigaclavine C, gliotoxin, fumagillin, helvolic acid, or pseurotins,, clearly differentiating *N. fisheri* from *A. fumigatus* (Table 2). Two of the strains of *N. fischeri* (IBT 3009 and CBS 317.89) produced neosartorin, trypacidin and 'pyripyropene S'. All strains of *N. fischeri* produced compounds with UV spectra like those of fischerin and of the tryptoquivalins (or fiscalins), but this was not confirmed by mass spectrometry. *N. pseudofischeri* produced a specific profile of mostly unknown secondary metabolites, but produced compounds with tryptoquivalin-like UV spectra different from those of *N. fischeri*, some pyripyropenes and two isolates (CBS 404.67 and CBS 117073) produced gliotoxin on CYA agar, unlike *A. fumigatus* that produces gliotoxin on YE agar.

Analysis of the raw fungal extracts by direct injection mass spectrometry resulted in species specific mass profiles as illustrated by comparison of profiles from two isolates of *A. lentulus* and two isolates of *A. fumigatus* (Fig. 4). Similarly species specific mass profiles were obtained for *N. fisheri* and *N. pseudofisheri* (data not shown). A cluster analysis based on comparison of all the obtained mass profiles (based on extracts made from YES medium), gave a clear separation of the six species even though we only included one isolate of two species *A. novofumigatus* and *A. fumigatiaffinis* in this study (Fig. 5).

Discussion

Morphologically the six species studied here are all very similar, especially concerning the morphology of the conidial heads, temperature reactions and colony morphology. In contrast to the heavily sporulating *A. fumigatus* and to a certain extent *N. fisheri,* the newly described species *A. lentulus*, *A. fumigatiaffinis* and *A. novofumigatus* as well as *Neosartorya pseudofischeri* sporulated poorly. The difficulties in identifying this and other important groups of *Aspergilli* correctly based solely on morphology and growth characteristics can be solved by using a polyphasic approach also including extrolite profiles and molecular data as argued by Samson *et al*. [32]. In the present study previously correctly identified strains from our culture collection have been separated into species based solely on comparison of mass profiles of raw extracts generated by electrospray MS (Fig. 5), altogether demonstrating the scope of metabolic (or extrolite) profiling as a strong method for differentiation of closely related species as also demonstrated for Penicillia [33–35].

Looking at single metabolite production a number of already known compounds have been identified from

Table 2 Secondary metabolites detected from species in section *Fumigati*. Std. indicates that a standard of the metabolites has been available

Compound	RI	Std.	Formula	Mw calc.	Major ions detected	*A. fumigatus*	*A. lentulus*	*A. novofumigatus*	*A. fumigatiaffinis*	*N. fisheri*	*N. pseudofisheri*
Auranthine	869	+	$C_{19}H_{14}O_2N_4$	330.1117	$M+H^+$, $M++CH_3CN+H^+$		+		+		
Cyclopiazonic acid	1097	+	$C_{20}H_{20}O_3N_2$	336.1473	$M+H^+$		+				
Eepi-aszonalenin A	944	+	$C_{25}H_{25}O_3N_3$	415.1896	$M+H^+$; $2\times M+Na^+$			+			
Eepi-aszonalenin C	1017	+	$C_{23}H_{24}O_2N_3$	373.1790	$M+H^+$			+			
Fumagillin	1117	+	$C_{26}H_{34}O_7$	458.2305	$M+H^+$, $M+Na^+$	+					
Fumigaclavine C	745	+	$C_{23}H_{30}O_2N_2$	366.2307	$M+H^+$	+					
Fumiquinazoline D	868		$C_{24}H_{21}O_4N_5$	443.1594	$M+H^+$, $M-H_2O+H^+$	+					
Fumiquinazoline F/G	938		$C_{21}H_{18}O_2N_5$	358.1430	$M+H^+$	+					
Fumitremorgin A	1342	+	$C_{32}H_{41}O_7N_3$	579.2943	$M+Na^++CH_3CN$; $M-H_2O+H^+$					+	
Fumitremorgin B	1146	+	$C_{27}H_{33}O_5N_3$	479.2420	$M+Na^++CH_3CN$; $M-H_2O+H^+$	+				+	
Fumitremorgin C	894		$C_{22}H_{25}O_3N_3$	379.1895	$M+H^+$	+					
Gliotoxin	807	+	$C_{24}H_{21}O_4N_5S$	326.0395	$M-2\times S+H^+$	+					+
Helvolic acid	1165	+	$C_{31}H_{44}O_4$	568.3036	m/z 509 observed as base peak	+		+	+	+	
Methyl-sulochrin	956		$C_{18}H_{18}O_7$	346.1053	$M+Na^+$, $M+Na^++CH_3CN$	+					
Neosartorin	1263	+	$C_{34}H_{32}O_{15}$	680.1741	$M+H^+$, $M+Na^+$		+	+	+	+	
Pseurotin A or D	761		$C_{22}H_{23}O_9N$	431.1342	$M+Na^+$; $2\times M+Na^+$	+					
Pseurotin A or D	802		$C_{22}H_{23}O_9N$	431.1342	$M+Na^+$; $2\times M+Na^+$	+					
Pyripyropene A	951		$C_{31}H_{37}O_{10}N$	583.2417	$M+H^+$, $M++CH_3CN+H^+$		+		+		+
Pyripyropene E	1133		$C_{27}H_{33}O_5N$	451.2359	$M+H^+$, $M++CH_3CN+H^+$		+		+		+
Pyripyropene O	1025		$C_{29}H_{35}O_7N$	509.2414	$M+H^+$, $M++CH_3CN+H^+$		+		+		+
'Pyripyropene S'	820		$C_{29}H_{35}O_9N$	541.2312	$M+H^+$, $M++CH_3CN+H^+$	+	+		+	+	+
Terrein	578	+	$C_8H_{10}O_3$	154.0630	$M+H^+$, $M-H_2O+H^+$		+	+			
Trypacidin	1033	+	$C_{18}H_{16}O_7$	344.0896	$M+H^+$	+				+	
TR-2	884		$C_{22}H_{27}O_6N_3$	429,1900	$M-2\times H_2O+H^+$	+				+	
Verruculogen	1071	+	$C_{27}H_{33}O_7N_3$	511.2318	$M+CH_3CN+Na^+$	+				+	

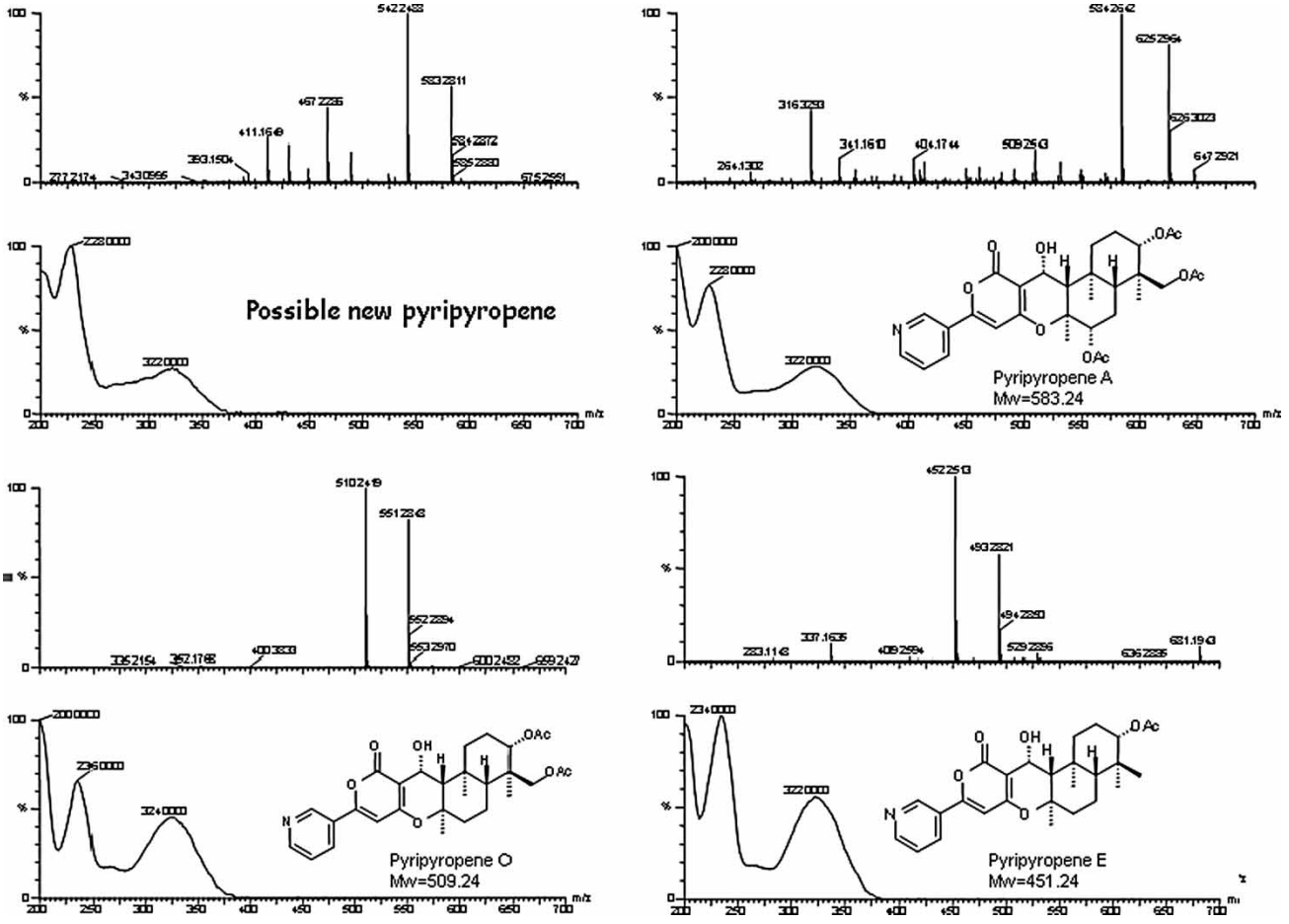

Fig. 3 Tentative identification of pyripyropene A, O, and E in extracts of *Aspergillus lentulus* by comparison of UV data and MS spectra with literature data, and indication of a new pyripyropene.

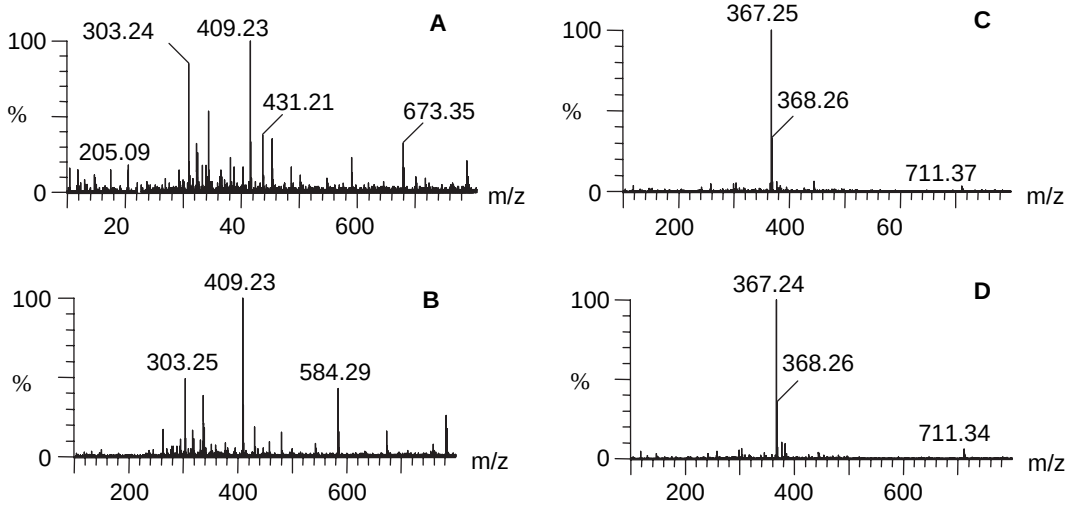

Fig. 4 Mass profiles of *Aspergillus lentulus*, IBT 26436 (A) and IBT 23719 (B) and *Aspergillus fumigatus* IBT 14904 (C) and IBT 24699 (D).

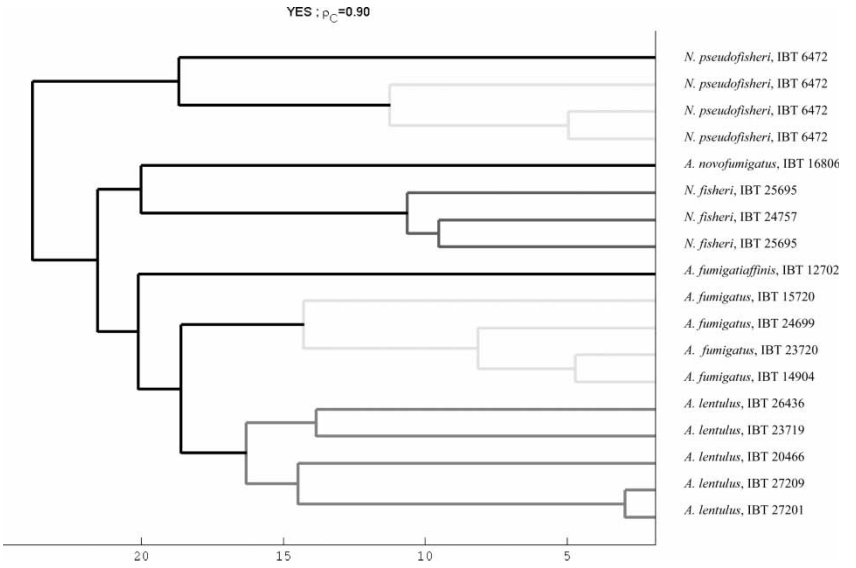

Fig. 5 Cluster analysis based on DIMS from the fungi investigated. Clear groupings can be seen for all studied isolates (Table 1) of the six species investigated in this study.

the six investigated species (Table 2). Most interestingly the present study, for the first time documents that *N. pseudofisheri* is also a gliotoxin producer. As gliotoxin was the sole metabolite that was identified in the lungs of infected mice [20], the high protein and low carbohydrate medium YE medium was included to complement the results from the two carbohydrate rich media YES and CYA. The latter two media yield high amounts of metabolites for species in section *Fumigati*, except for gliotoxin. The fact that we did not detect gliotoxin production from any of the three media by *A. lentulus*, *A. novofumigatus*, *A. fumigatiaffinis* and *N. fisheri* does not prove that these four species are incapable of producing this metabolite at other conditions. Further experiments using other media and growth conditions along with comparison of genomic data will establish whether these latter four species are also potential gliotoxin producers.

There are some deviations in our results as compared to earlier reports. Yamazaki *et al*. [36], Frisvad and Samson [23], Tepsic *et al*. [37], Geiser *et al*. [38] (1998) and Hong *et al*. [5] all reported that *A. fumigatus* produces tryptoquivalins. This could not be confirmed in the present study. Even though one isolate reported to be *A. fumigatus* (CBS 154.89) actually produces tryptoquivalins, we could find no other strains producing these chemical compounds. We think the fumiquinazolins, consistently produced by *A. fumigatus*, may have been misidentified as tryptoquivalins, as they have quite similar UV spectra. CBS 154.89 appears to be an isolate not closely related to *A. fumigatus* (unpublished data). Another compound reported from *A. fumigatus*, ruakuric acid [39], could not be detected in any isolate of this species in any of our chemical analyses. Tryptoquivalins were also reported from *N. fisheri* by Nielsen and Samson [40], but these compounds appear to be other compounds with UV spectra like those of the tryptoquivalins or the fiscalins [41].

According to Geiser *et al*. [38], *A. fumigatus* is closely related to *N. fischeri*, but *N. pseudofischeri* is less closely related to the two former species. Yet *N. pseudofischeri* is a known human pathogenic species [2], while there are no data that indicates that *N. fischeri* is human pathogenic. The gliotoxin production by these two species indicates that gliotoxin really has a role in pathogenesis as indicated by Rementeria *et al*. [17] and Tsitsigiannis *et al*. [20], even though gliotoxin is apparently only one of several virulence factors [22]. On the other hand *A. lentulus* is another human pathogenic species [3], and we could not detect gliotoxin in that species. According to our results *A. lentulus* cannot be called a sibling species to *A. fumigatus*, since the two species apparently only share the production of pyripyropenes at least under the conditions used for growth and metabolite production in this study.

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