The Natural Production of Organohalogens by Basidiomycetes

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1. Introduction

The occurrence of organohalogens in nature has generally been ascribed to anthropogenic activities[1]. Bulk parameters like adsorbable organic halogen (AOX) are used to monitor the extent of this type of xenobiotic pollution in the environment. However, the pool of AOX detected in pristine environments was recently shown to be at least 300 times greater than that which could be accounted for by anthropogenic sources[2]. Since AOX production was shown to take place during the decay of forest litter[3], microorganisms responsible for degradation of lignocellulosic debris were implicated. Basidiomycetes have been recognized as the most ecologically significant group of organisms responsible for lignocellulose decomposition[4]. Furthermore, several basidiomycetes are known to have the ability to synthesize *de novo* organohalogen metabolites[5-10].

In this study, the ubiquity of organohalogen production among basidiomycetes was investigated. Ecologically significant organohalogen producing species were also tested for AOX production on natural lignocellulosic substrates. New organohalogen producing fungal species were addressed as well as new metabolites were identified.

2. Materials and Methods

Screening for AOX production.

Fungal strains were grown in a liquid medium with a high nitrogen content (50 mM N), supplied as peptone and in a liquid medium with a low nitrogen (2 mM N) content Compositions od the media were described before[11]. Inorganic halide contents of the media were 98 and 70 mg/l, respectively. Medium (10 ml liquid volume) in a 100-ml serum bottle was inoculated with a plug (diameter 5 mm), which was taken from an agar medium covered with mycelium. Duplicate fungal cultures were incubated in the dark at 25°C under an air atmosphere. A duplicate set of sterilew medium containing a sterile agar plug were incubated parallel to the fungal cultures and were harvested as controls at the time of AOX analysis. When the culture fluid was covered by the mycelium (2-6 weeks), the culture fluid was harvested for organohalogen determination, which was conducted as described before[11].

AOX production on natural substrates.

Selected fungal strains were inoculated in media containing beech (*Fagus sylvatica*) wood

30 g/l, pine (*Pinus sylvestris*) 30 g/l and forest litter (beech leaves 10 g/l, beech twigs 5 g/l, oak (*Quercus robur*) leaves 10 g/l, oak twigs 5 g/l), hemp (*Cannabis sativa*) stem wood 30 g/l, or wheat (*Triticum aestivum*) straw 30 g/l (particle sizes less than 0.5 mm). Fungal cultures of 10 ml liquid volume placed in 100-ml sreum bottles were incubated in quadruplicate in the dark at 25° under an air atmosphere. A quadruple set of sterile media were incubated parallel to the fungal cultures and were harvested as controls at the time of organohalogen analysis. After 6 weeks, culture fluids were harvested for AOX determination.

Identification of novel metabolites.

Fungal cultures were grown in a liquid medium with a high nitrogen content, as described above. After 3 to 6 weeks of incubation, the culture fluid was harvested for determination of chlorinated anisyl metabolites (CAM). After filtration of the extracellular fluid, the pH was adjusted to 2 with 4 M H_2SO_4 and the filtrate was extracted three times with freshly distilled ethyl acetate. The combined organic layers were washed with H_2O and concentrated under reduced pressure at ambient temperature. The concentrate was filtered over silica gel 60. After removal of the solvent, the remaining residue was redissolved in 0.5 ml of ethyl acetate containing 4-bromoanisole as internal standard. Identification was performed with GC-MS. Concentrations of CAM were determined with GC analysis using the internal standard quantitation method. All measurements were done in duplicate from duplicate set of cultures.

3. Results

A total of 191 fungal strains were monitored for AOX production when grown on defined liquid media. Approximately 50 % of the strains tested and 55 % of the genera tested produced AOX. A low production of 0.1 to 0.5 mg AOX/l was observed among 25 % of all strains tested, a moderate production of 0.5 to 5.0 mg AOX/l was observed among 16 % of all strains tested and 9 % of the strains produced high levels (5 to 67 mg AOX/l). The highest AOX producing fungal strains are given in Table 1.

Table 1. Highest AOX-producing basidiomycetes grown in high nitrogen (HN) and low nitrogen (LN) liquid media. FC = frequency class in The Netherlands, based on presence in 5-km^2-grid blocks: 0, unknown; 1, <0.2%; 2, 0.2-0.6%; 3, 0.6-2%; 4, 2-5%; 5, 5-10%; 6, 10-25%; 7, 25-45%; 8, 45-75%; 9, 75-100% of 5-km^2-blocks investigated[12]. - no FC given because species name is unknown. N.D. not detectable (detection limit 0.1 mg/l).

Species	Strain	FC	AOX-concentration (mg/l) HN	LN
Hypholoma elongatum	WIJS94.28	6	66.95	32.35
Mycena metata	RHEN93.1	7	54.13	1.91
Hypholoma capnoides	ONO93.7	7	28.53	22.12
Bjerkandera adusta	WAG91.2	9	27.45	9.21
Hypholoma fasciculare	RHEN93.5	9	16.46	10.36
Mycena epipterygia	ITAL94.3	7	15.36	2.51
Hypholoma sublateritium	WIJS94.27	8	14.49	5.05
Phylloporia ribis	IJFM(B7)	4	12.91	N.D.
Bjerkandera sp. BOS55	CIMW91.1	-	12.30	4.79
Peniophora pseudopini	CBS162.65	0	10.87	4.14

The highest producers were dominated by species belonging to the genera *Hypholoma*, *Mycena* and *Bjerkandera*, showing specific AOX productions in the range 1074 to 30893 mg AOX per kg dry weight of mycelial biomass. Many highly ecologically significant fungal species were identified among the high to moderate producers. These species were also able to produce AOX when cultivated on natural lignocellulosic substrates. *Hypholoma fasciculare* and *Mycena metata* produced up to 132 mg and 193 mg AOX per kg dry weight of forest litter substrate in 6 weeks (Table 2).

Table 2. Concentrations of AOX produced by selected fungi in 10 mL of medium containing 300 mg dry weight of natural substrate after incubation for 6 weeks at 25°C.

Substrate	Species	Strain	AOX-concentration[a] (mg/kg substrate[b])
Pine wood	*Hypholoma fasciculare*	RHEN93.5	60.7 ± 3.3
	Hypholoma capnoides	ONO93.7	20.2 ± 3.9
	Peniophora pseudopini	CBS162.65	13.5 ± 1.7
	Mycena epipterygia	ITAL94.3	12.7 ± 2.2
	Gymnopilus sapineus	WIJS94.10	10.0 ± 2.3
	Stropharia aeruginosa	CBS839.87	2.9 ± 2.3
Beech wood	*Hypholoma sublateritium*	WIJS94.27	41.3 ± 2.7
	Hypholoma fasciculare	RHEN93.5	40.8 ± 3.5
	Mycena galericulata	WIJS94.23	5.6 ± 0.9
	Megacollybia platyphilla	WIJS94.11	4.6 ± 1.1
	Gymnopilus sapineus	WIJS94.10	3.9 ± 1.0
Forest litter	*Mycena metata*	RHEN93.1	192.9 ± 17.2
	Hypholoma fasciculare	RHEN93.5	132.4 ± 3.3
	Mycena galopus	ITAL94.4	28.4 ± 5.5
	Collybia butyracea	OUDES93.1	21.9 ± 4.6
	Collybia dryophila	WIJS94.3	17.0 ± 2.0
	Megacollybia platyphilla	WIJS94.11	13.2 ± 3.2
Hemp stem wood	*Hypholoma fasciculare*	RHEN93.5	75.9 ± 8.5
Wheat straw	*Hypholoma fasciculare*	RHEN93.5	115.2 ± 8.6

[a]Net AOX concentration after correction for levels in parallel incubated sterile controls. Control AOX values (mg/kg dry weight): pine wood, 2.5; beech wood, 1.9; forest litter, 6.2; hemp stem wood, 7.7; wheat straw, 7.2.
[b]Calculations on basis of dry weight of substrate at time of inoculation.
Determinations were done in quadruplo.

In the continuation research, four new chlorinated anisyl metabolites (CAM) producing genera and three new CAM producing species were identified (Table 3). In the ethyl acetate extracts from the extracellular fluid of the mycelium of species belonging to seven different genera of basidiomycetes CAM were detected. CAM production by species belonging to the genera *Mycena*, *Peniophora*, *Phellinus* and *Phylloporia* was observed for the first time. Although it is known that some species of the genera *Bjerkandera*, *Hypholoma* and *Pholiota* produce CAM, the ability of *Bjerkandera fumosa*, *Hypholoma elongatum* and *Pholiota adiposa* to do this was not reported before. The selective and high-yield production of 3,5-dichloro-*p*-anisyl alcohol by *Hypholoma elongatum* was remarkable.

Table 3. Production of chlorinated anisyl metabolites by pure cultures of basidiomycetes grown in liquid peptone medium.

Species	CAld (mg/l)	DCAld (mg/l)	DCAlc (mg/l)
Bjerkandera fumosa	2.0	trace[1]	-[2]
Hypholoma elongatum	-	0.4	108.0
Mycena epipterygia	2.4	11.5	-
Peniophora pseudopini	13.0	0.5	-
Phellinus torulosus	-	-	2.4
Pholiota adiposa	-	0.2	5.3
Phylloporia ribis	-	-	11.1

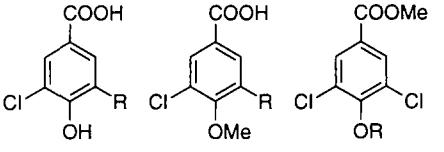
CAld= 3-chloro-*p*-anisyl-aldehyde; DCAld= 3,5-dichloro-*p*-anisyl-aldehyde; DCAlc= 3,5-dichloro-*p*-anisyl-alcohol
[1]trace: only detected with ECD,
[2]not detected with both FID and ECD.
Values are means of duplicate determinations.

Moreover, in the culture fluids of *Bjerkandera* spp. novel chlorinated aromatic derivatives could be demonstrated (Table 4). The EtOAc extract from the extracellular fluid of the mycelium of *Bjerkandera* sp. BOS55 contained four new chlorinated benzoic acid derivatives, i.e. 3-chloro-4-hydroxybenzoic acid, 3,5-dichloro-4-hydroxybenzoic acid, methyl 3,5-dichloro-4-hydroxybenzoate and methyl 3,5-dichloro-*p*-anisate. 3-Chloro-4-hydroxybenzoic acid was also produced by *Bjerkandera adusta*.

Table 4. Novel chlorinated compounds produced by *Bjerkandera* spp. in defined liquid media.

	Bjerkandera sp.	
	BOS55	BEUK47
compound 1	+	+
compound 2	+	-
compound 3 + 4[1]	+++	+++++
compound 5	++	-
compound 6	+	-
CAld[2]	+++	++
DCAld[2]	+	+

CAld= 3-chloro-*p*-anisyl-aldehyde;
DCAld= 3,5-dichloro-*p*-anisyl-aldehyde.
Symbols: -, not detected; +, ≤1%; ++, ≤5%; +++, ≤10%; +++++, >50%. Percentages refer to total detected chlorinated and non-chlorinated metabolites.
[1]Partly overlapping peaks in GC-MS, compound [3]>> compound [4].
[2]*Known compounds from Bjerkandera* spp.



1: R = H
2: R = Cl
3: R = H
4: R = Cl
5: R = H
6: R = Me

4. Conclusion

Our results clearly indicate that organohalogen production is a ubiquitous capacity among commonly occurring basidiomycetous fungi. Many of the high and moderate AOX-producing species are highly ecologically significant fungi. Therefore, basidiomycetes probably are a major source of natural organohalogens in forest ecosystems.

5. References

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