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Short communication

Isolation of mycotoxins producing black aspergilli in herbal teas available on the Swiss market

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ABSTRACT

Black aspergilli are among the predominant fungal contaminants in herbal teas. Despite the ability of some species in this group to produce the mycotoxins ochratoxin A (OTA) and fumonisins no study had been done to investigate in detail their presence in this commodity. In the present work conventional herbal teas available on the Swiss market were investigated for black aspergilli contamination. Black aspergilli were found in 16 of 22 samples, ranging from 10 to 3500 colony forming units per gram of herbal tea. Recovered isolates were identified to the species level by calmodulin sequencing. The most frequently isolated species were *Aspergillus niger*, *Aspergillus acidus*, *Aspergillus awamori* and *Aspergillus tubingensis*. *Aspergillus carbonarius*, the most important OTA-producing black Aspergillus, could not be recovered. *A. niger* and *A. awamori* isolates were tested for their ability to produce fumonisins and OTA *in vitro*. Fumonisins were produced by 76% of *A. niger* and 37% of *A. awamori* isolates. 7% of *A. niger* and none *A. awamori* isolates could produce OTA. In total, 12 of 22 samples were found to be contaminated with black aspergilli able to produce major mycotoxins. Our results indicate that mycotoxins producing black aspergilli are widespread fungal contaminants of herbal teas. Therefore, their presence as well as the occurrence of their mycotoxins should be further investigated to assess health risks linked with the consumption of this commodity.

1. Introduction

Members of the *Aspergillus* section *Nigri*, also known as black aspergilli, represent one of the most important source of mycotoxins contamination of foods and feeds. Major mycotoxins produced by this group of filamentous fungi are ochratoxin A (OTA) and fumonisins of the B series, in particular FB_2 (Nielsen, Mogensen, Johansen, Larsen, & Frisvad, 2009). OTA is a potent nephrotoxic and carcinogenic toxin produced by different species belonging to the genus *Aspergillus* and *Penicillium* (Bennett & Klich, 2003). Black aspergilli able to produce OTA are *Aspergillus carbonarius*, *Aspergillus sclerotioniger*, and a low percentage of *Aspergillus niger* and *Aspergillus awamori* strains (Frisvad et al., 2011; Varga et al., 2011). Fumonisins are carcinogenic mycotoxins originally described in the genus *Fusarium* (Scott, 2011). Fumonisins production in the *A. niger* species had been reported few years ago following the discovery of a putative fumonisins biosynthesis gene cluster in the *A. niger* genome (Frisvad, Smedsgaard, Samson, Larsen, & Thrane, 2007; Pel et al., 2007). Recent studies showed that a high percentage (>50%) of *A. niger* and *A. awamori* isolated from coffee, grapes and raisins are able to synthetize fumonisins (Logrieco et al., 2009; Noonim, Mahakarnchanakul, Nielsen, Frisvad, & Samson, 2009; Varga et al., 2010).

Since only few species inside the section *Nigri* can produce OTA and fumonisins, a correct identification of black aspergilli isolated from commodities is necessary to evaluate the subsequent risk of mycotoxins contamination (Nielsen et al., 2009). The *Aspergillus* section *Nigri* comprises 26 species that can be distinguished without ambiguity using calmodulin sequencing (Varga et al., 2011). In fact, species recognition based on morphology alone is very difficult, in particular for those species closely related to *A. niger*, which are included in the so-called *A. niger* aggregate (Samson et al., 2007). This group comprises some of the most common species of black aspergilli, including *A. niger*, *A. awamori*, *Aspergillus tubingensis* and *Aspergillus acidus* (Nielsen et al., 2009; Perrone et al., 2011). Of these morphological indistinguishable species, only *A. niger* and its sibling *A. awamori* are known to produce major mycotoxins (Frisvad et al., 2011; Perrone et al., 2011).

Herbal teas, defined as extracts made with plant materials which do not contain *Camellia sinensis*, have been shown to be

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prone to mycotoxin contaminations, including fumonisins and OTA (Martins, Martins, & Bernardo, 2001; Santos, Marín, Sanchis, & Ramos, 2009). Different studies done in the past to assess the microbiological quality of herbal teas reported black aspergilli, and in particular *A. niger*, as one of the most frequently isolated group of filamentous fungi (Bugno, Almodovar, Pereira, Andreoli Pinto, & Sabino, 2006; Halt, 1998; Tournas & Katsoudas, 2008). However, species determination of molds collected in these works was based only on morphological observations. This implies that black aspergilli classified as *A. niger* could also belong to the other atoxigenic species of the *A. niger* aggregate. Moreover, since fumonisins production by *A. niger* became apparent only recently, these studies were mainly focused on the presence of aflatoxin-producing *Aspergillus flavus*/*Aspergillus parasiticus* isolates, and black aspergilli strains were not tested for mycotoxins production. A more focused investigation on black aspergilli in herbal teas is therefore needed to better evaluate their potential role in mycotoxins contamination of this commodity.

The aim of this work is the isolation and characterization of black aspergilli from conventional herbal teas available on the Swiss market. Species identity of recovered aspergilli was determined using calmodulin sequencing and potentially toxigenic isolates were tested for mycotoxins production *in vitro*.

2. Materials and methods

2.1. Counting and isolation of black aspergilli colonies

Twenty-two tea samples (see Table 1) were purchased in various supermarkets and tea shops in Zürich, Switzerland. Tea material from each sample was aseptically pooled and 15 g were mixed with 135 mL (diluted to 10^{-1}) of autoclaved 0.01% Tween-80 solution (AppliChem, Germany) for 3 h on a horizontal shaker (170–180 rpm). The first solution was used to prepare other dilutions. Of each dilution, 200 μL were plated on three plates of dichloran rose bengal agar base (DRBC, Oxoid, England) supplemented with 100 mg L^{-1} chloramphenicol (Sigma–Aldrich, Switzerland). After the first ten samples, plating of 1 mL of solution was preferred instead of 200 μL to increase the number of observed colonies. After 3 days of incubation at 27 °C in the dark, all grown black aspergilli colonies were counted and colony forming units (Cfu) per g of tea were calculated. A maximum of five black aspergilli colonies selected at random from each plate inoculated with the first solution, for a maximum of 15 black aspergilli for each tea sample, were isolated and purified on PDA (PDA, Oxoid, England). Isolates were kept at 3 °C until further analyses.

2.2. DNA extraction and species identification

All isolated black aspergilli were incubated on liquid medium (Potato Dextrose Broth, Difco, USA) for 7 days at 24 °C on a horizontal shaker at 150 rpm. The grown mycelia were lyophilized and used for DNA extraction with DNeasy® Plant Mini Kit (QIAGEN, Germany) following the manufacturer's protocol. Species determination was done by sequencing a portion of the calmoduling gene. Calmodulin amplification was performed using CL1 and CL2A primers (O'Donnell, Nirenberg, Aoki, & Cigelnik, 2000) following Serra et al. (2006) with minor modifications. Sequencing reaction was performed on the purified PCR products using the ABI PRISM BigDye Terminator v3.0 ready reaction sequencing kit (Applied Biosystems, USA) and following the manufacturer's protocol. Sequencing products were purified with sephadex G-50 DNA Grade F (Amersham Biosciences, Switzerland) and loaded into an ABI 3100 automated sequencer (Applied Biosystems). Calmodulin sequences were edited with the Sequencher version 4.2 software

Table 1
Herbal tea samples analyzed in this study: description of the raw materials reported on the box, black aspergilli colony forming units, species identity, FB_2 and OTA production ability of recovered isolates.

Tea sample	Description[a]	Cfu/g	Species (number of isolates)[b]	FB_2	OTA
1	Rosa canina with hibiscus (Europe, Asia, Africa, Sudamerica)	3500	*A. acidus* (6), *A. japonicus* (1), *A. aculeatus* (1), ***A. awamori* (2)**, *A. uvarum* (1)	0/2	0/2
2	*Thymus vulgaris* organic (Europe)	0	none	0/0	0/0
3	Herbal tea mix with Sambucus (Romania, Paraguay, Switzerland)	17	*A. acidus* (1)	0/0	0/0
4	Orange blossom	17	*A. tubingensis* (1)	0/0	0/0
5	Mix rooibos, melissa, orange blossom	567	*A. acidus* (6), ***A. awamori* (4)**, *A. japonicus* (1), ***A. niger* (1)**, *A. tubingensis* (3)	1/5	0/5
6	Verbena with ginger	0	None	0/0	0/0
7*	Rosa canina with hibiscus, organic (Tanzania, Czek rep.)	267	*A. acidus* (3), ***A. awamori* (1)**, ***A. niger* (2)**, *A. tubingensis* (2)	3/3	0/3
8	Rosa canina with hibiscus	0	none	0/0	0/0
9	Rooibos 100% (South Africa)	0	none	0/0	0/0
10	Tea mix ginger	100	***A. awamori* (1)**, *A. tubingensis* (3)	1/1	0/1
11*	Tea mix rose	667	***A. awamori* (4)**, ***A. niger* (4)**, *A. tubingensis* (1)	2/8	0/8
12*	*Urtica dioica* 100%, organic (Czek rep.)	87	***A. niger* (1)**, *A. tubingensis* (1)	1/1	0/1
13	Rosa canina, organic	0	none	0/0	0/0
14*	Rosa canina 100%, organic (Romania)	163	*A. acidus* (1)	0/0	0/0
15	Lime flower 100%	117	*A. acidus* (3), ***A. awamori* (5)**, ***A. niger* (2)**, *A. tubingensis* (3)	4/7	0/7
16	Mix apple cinnamon	1233	*A. acidus* (4), ***A. awamori* (2)**, ***A. niger* (4)**, *A. tubingensis* (3)	3/6	1/6
17*	Mix with rooibos and hibiscus	217	*A. acidus* (3), ***A. awamori* (3)**, ***A. niger* (4)**, *A. tubingensis* (2)	4/7	0/7
18	Rosa canina with hibiscus	10	*A. aculeatus* (1), ***A. niger* (1)**, *A. tubingensis* (1)	1/1	0/1
19*	Mix fruits and flowers, organic	63	*A. acidus* (1), ***A. niger* (13)**	13/13	2/13
20	Mix with *Mentha gentilis*, Verbena and hemp leaves, organic	1200	*A. acidus* (8), *A. aculeatus* (1), ***A. awamori* (2)**, ***A. niger* (3)**, *A. tubingensis* (1)	1/5	0/5
21	Mix fruit tea, organic	1333	*A. acidus* (3), *A. brasiliensis* (1), ***A. awamori* (3)**, ***A. niger* (6)**, *A. tubingensis*(2),	7/9	0/9
22	Rosa canina with hibiscus	0	none	0/0	0/0

*Isolation of black aspergilli limited because of the presence of *Rhizopus*.
[a] The composition and provenience of herbal tea is reported as indicated on the tea box.
[b] Potentially toxigenic black aspergilli are marked in bold.