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Ochratoxin Production by *Aspergillus* Species

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Ochratoxin production was tested in 172 strains representing species in sections *Fumigati*, *Circumdati*, *Candidi*, and *Wentii* of the genus *Aspergillus* by an immunochemical method using a monoclonal antibody preparation against ochratoxin A. Ochratoxin A was detected in *Aspergillus ochraceus*, *A. alliaceus*, *A. sclerotiorum*, *A. sulphureus*, *A. albertensis*, *A. auricomus*, and *A. wentii* strains. This is the first report of production of ochratoxins in the latter three species. Ochratoxin production by these species was confirmed by high-performance thin-layer chromatography and by high-performance liquid chromatography. The chemical methods also indicated the production of ochratoxin B by all of the *Aspergillus* strains mentioned above.

Ochratoxin A (OA) is a mycotoxin which was discovered in 1965 as a secondary metabolite of *Aspergillus ochraceus* strains (21, 27). In subsequent years, several other *Aspergillus* and *Penicillium* species were described as producers of this toxin, including strains of seven species of *Aspergillus* section *Circumdati* (*A. ochraceus*, *A. alliaceus*, *A. ostianus*, *A. sclerotiorum*, *A. sulphureus*, *A. melleus*, and *A. petrakii*) (5, 11), *A. glaucus* (section *Aspergillus*) (4), *A. niger*, *A. awamori*, *A. foetidus*, and *A. carbonarius* (section *Nigri*) (1, 24), and some *Penicillium* species (3, 6, 17, 26).

OA was proved to exhibit nephrotoxic, immunosuppressive, teratogenic, and carcinogenic properties (14, 23). OA contamination of green coffee beans and other plant products such as barley, wheat, and bread is a serious health hazard throughout the world (23, 25); the tolerable daily intake of OA is 5 to 16 ng kg of body weight^{-1} (22). OA was also detected in body fluids of animals and humans (8, 30) and is the causative agent of Danish porcine nephropathy (23). OA was also frequently cited as the possible causative agent of Balkan endemic nephropathy (13), although recently, other nephrotoxic *Penicillium* metabolites have been suggested to be responsible for this toxicosis (15).

Here we describe the results of our survey on the occurrence of OA in several *Aspergillus* species that came from different culture collections or were isolated from OA-contaminated green coffee beans.

MATERIALS AND METHODS

Strains. Sixty-one *A. fumigatus* strains (19), 45 other isolates representing 15 species of section *Fumigati* (29), 60 strains representing the 18 species of section *Circumdati* (Table 1), three *A. wentii* strains, and three *A. candidus* strains were tested for OA production. The identity of these strains was determined by the methods of Raper and Fennell (18) and Kozakiewicz (12). The strains were maintained on malt extract agar slants.

Immunochemical tests. The strains were grown in stationary cultures in 30-ml quantities of YES (2% yeast extract, 15% sucrose) medium at 30°C for 10 days in the dark. For immunochemical screening, the method described by Téren et al. (24) was applied. The Toxiklon OA enzyme immunoassay kit (Agricultural Biotechnology Centre, Gödöllö, Hungary) was used for the detection of OA in our experiments. Direct competitive enzyme-linked immunosorbent assays (ELISAs) were performed as described earlier (9).

TLC. High-performance thin-layer chromatography (TLC) from cleaned concentrated extracts was carried out as described previously (24). OA was identified under UV light (360 nm) as bluish-green fluorescent spots with the same mobility as that of an OA standard (Makor Chemicals, Jerusalem, Israel). The fluorescence of OA spots from both the standard and the extracts changed to deep blue on treatment of chromatograms with $NaHCO_3$ (5% $NaHCO_3$ in 17% ethanol).

HPLC. Benzene extracts used for TLC were evaporated and redissolved in appropriate amounts of methanol. The high-performance liquid chromatography (HPLC) apparatus used was a Hewlett-Packard HP1090 Series II equipped with a binary solvent delivery system, an autoinjector, an autosampler, a temperature-controlled column compartment, a diode array UV detector, and a fluorescence detector (HP1046A; excitation, 333 nm; emission, 450 nm). BST RUTIN C_{18} BD columns (BioSeparation Techniques, Budapest, Hungary; 250 by 4 mm; particle size, 10 μm) were used. For fluorescent detection, the mobile phase, which consisted of an isocratic program of 57% acetonitrile, 41% water, and 2% acetic acid, was pumped at a rate of 1 ml/min (1).

For the confirmation of OA production, diode array detection was used. The analytes were eluted at a constant flow rate (1.5 ml/min) with a gradient containing solvent A (methanol-isopropanol, 9:1), and solvent B (acidified bidistilled water with HCl [pH 2.1]). The program used was the same as that described earlier (30).

RESULTS AND DISCUSSION

ELISAs. Altogether, 172 *Aspergillus* strains were tested for OA production by the immunochemical test. Among these, none of the *Aspergillus* section *Fumigati* strains produced detectable amounts of OA. OA production was also not observed by the ELISA method in two *A. petrakii*, one *A. sepultus*, one *A. robustus*, one *A. quercinus*, one *A. ostianus*, one *A. ochraceoroseus*, two *A. insulicola*, one *A. elegans*, one *A. lanosus*, two *A. bridgeri*, one *A. campestris*, one *A. dimorphicus*, and three *A. candidus* strains (Table 1). Hesseltine et al. (11) reported OA production in some *A. petrakii* and *A. ostianus* strains; the collection strains examined during this study did not produce this mycotoxin. Nine of the 27 *A. ochraceus* strains, including two strains isolated from Ugandan green coffee beans (Table 1), produced OA. The seven *A. ochraceus* strains isolated from Zairean coffee beans did not produce this toxin. The OA contents of Ugandan and Zairean green coffee samples were 4.8 and 9 μg kg^{-1}, respectively. One of five *A. alliaceus*, one of three *A. sclerotiorum*, and one of two *A. sulphureus* strains examined produced OA in YES liquid medium (Table 1); these species were earlier described as OA-producing species (5, 11). In addition, *A. albertensis* strains, one of three *A. auricomus* strains, and two *A. wentii* strains were found to be OA positive (Table 1); these species have not been described previously as producers of this mycotoxin. We also purified a sclerotium-free isolate from *A. albertensis* which produced approximately the same amount of OA as that of the sclerotial strain (Fig. 1). *A. wentii* IMI 017295, which produced OA, has an origin isogenic to that of *A. wentii* ATCC 1023, which did

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TABLE 1. The *Aspergillus* strains examined in this study[a]

Species	OA content (μg ml^{-1})[b]	Strain (source and/or origin)[c]
A. albertensis^{T}[d]	250	ATCC 58745 (ear swab; Edmonton, Canada)
A. albertensis	250	UAMH 2976 (ear swab; Edmonton, Canada; nonsclerotial strain)
A. albertensis	250	UAMH 2976 (ear swab; Edmonton, Canada)
A. alliaceus	−	ATCC 10060 (*Macrobasis albida*; United States)
A. alliaceus	−	FRR 3814 (Great Barrier Reef; Queensland, Australia)
A. alliaceus	−	FRR 4340 (kemiri nuts; Indonesia)
A. alliaceus	−	NRRL 4181 (soil; Australia)
A. alliaceus^{T}	1.2	IMI 126711 (soil; Australia)
A. auricomus	−	FRR 3944 (peanuts; Indonesia)
A. auricomus	−	FRR 3945 (mung beans; Thailand)
A. auricomus^{T}	−	IMI 172277 (P. Biourge)
A. auricomus	5–7	FRR 3819 (peanuts; Queensland, Australia)
A. bridgeri	−	IMI 259060
A. bridgeri^{T}	−	RMF 7745 (soil under *Atriplex gardneri*; Wyoming)
A. campestris^{T}	−	IMI 259099 (soil; North Dakota)
A. candidus	−	SZMC 0565 (soil; Hungary)
A. candidus	−	SZMC 0897 (Agricultural Service of Békés-Csongrád, Szeged, Hungary)
A. candidus^{T}	−	IMI 091889 (C. Thom)
A. dimorphicus^{T}	−	IMI 131553 (garden soil; Bihar, India)
A. elegans^{T}	−	IMI 133962 (A. Blochwitz)
A. insulicola	−	NRRL 6138
A. insulicola^{T}	−	ATCC 26220 (soil; Aves Island, Venezuela)
A. lanosus	−	IMI 226007 (soil; Calicut University, Kerala, India)
A. ochraceoroseus^{T}	−	IMI 223071 (soil; Ivory Coast)
A. ochraceus^{T}	−	IMI 016247
A. ochraceus	240	FRR 3846 (moldy soybeans; Australia)
A. ochraceus	+(ND)	A1 (air-borne contaminant; Szeged, Hungary)
A. ochraceus	+(ND)	A2 (air-borne contaminant; Szeged, Hungary)
A. ochraceus	−	A3 (air-borne contaminant; Szeged, Hungary)
A. ochraceus	+(ND)	FRR 543 (moldy hay; Queensland, Australia)
A. ochraceus	+(ND)	ICMP 939 (insect; Auckland, New Zealand)
A. ochraceus	+(ND)	NRRL 3714
A. ochraceus	+(ND)	306 (D. Bhatnagar)
A. ochraceus	−	FRR 3815 (Great Barrier Reef; Queensland, Australia)
A. ochraceus	−	FRR 60 (high-moisture prunes; New South Wales, Australia)
A. ochraceus	−	ICMP 2043 (rotting bulb, *Caladium* sp.)
A. ochraceus	−	NRRL 405
A. ochraceus	−	O1 (green coffee beans; Uganda)
A. ochraceus	−	O2 (green coffee beans; Uganda)
A. ochraceus	+(ND)	O3 (green coffee beans; Uganda)
A. ochraceus	+(ND)	O4 (green coffee beans; Uganda)
A. ochraceus	−	O5 (green coffee beans; Uganda)
A. ochraceus	−	O6 (green coffee beans; Uganda)
A. ochraceus	−	O7 (green coffee beans; Uganda)
A. ochraceus	−	Z1 (green coffee beans; Zaire)
A. ochraceus	−	Z2 (green coffee beans; Zaire)
A. ochraceus	−	Z3 (green coffee beans; Zaire)
A. ochraceus	−	Z4 (green coffee beans; Zaire)
A. ochraceus	−	Z5 (green coffee beans; Zaire)
A. ochraceus	−	Z6 (green coffee beans; Zaire)
A. ochraceus	−	Z7 (green coffee beans; Zaire)
A. ostianus^{T}	−	IMI 015960 (C. Wehmer; CBS 103.07)
A. petrakii	−	NRRL 416 (D. Hanzawa [*A. melleus*])
A. petrakii^{T}	−	IMI 172291 (*Leptinotarsa decemlineata*; Hungary)
A. quercinus^{T}	−	IMI 235600 (soil; India [*A. melleus*])
A. robustus	−	NRRL 6362
A. sclerotiorum	−	ATCC 16892 (fruit of *Malus sylvestris*; United States)
A. sclerotiorum	0.8–1	FRR 4491 (soil; Chaing Mai, Thailand)
A. sclerotiorum^{T}	−	IMI 056673 (rotting apple; Oregon)
A. sepultus^{T}	−	ATCC 58705 (loess 1 m below surface; Iowa)
A. sepultus^{T}	−	IMI 294498 (loess 1 m below surface; Iowa)
A. sulphureus	−	ICMP 1717 (S. Davidson 6586; from P. Meredith)
A. sulphureus	+(ND)	IMI 211397 (soil; Mysore, India)
A. wentii^{T}	−	ATCC 1023 (soybeans; Indonesia)
A. wentii^{T}	0.05–0.1	IMI 017295 (soybeans; Indonesia)
A. wentii	0.05–0.1	IMI 371128

[a] The *Aspergillus* section *Fumigati* strains, none of which produced detectable amounts of OA, are listed in the tables of references 19 and 29.
[b] Symbols: −, did not produce OA as measured by ELISA; +(ND), OA production was observed in the ELISAs but the amount of OA was not determined by HPLC.
[c] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; FRR, CSIRO Food Research Culture Collection, North Ryde, New South Wales, Australia; ICMP, International Collection of Microorganisms from Plants, Manaaki Whenua, New Zealand; IMI, International Mycological Institute, Egham, Surrey, United Kingdom; NRRL, Agricultural Research Service Culture Collection, Peoria, Ill.; RMF, Rocky Mountain Herbarium, Fungi, University of Wyoming, Laramie; SZMC, Szeged Microbiological Collection, Szeged, Hungary; UAMH, University of Alberta, Microfungus Herbarium and Collection, Edmonton, Alberta, Canada.
[d] T, type strain.

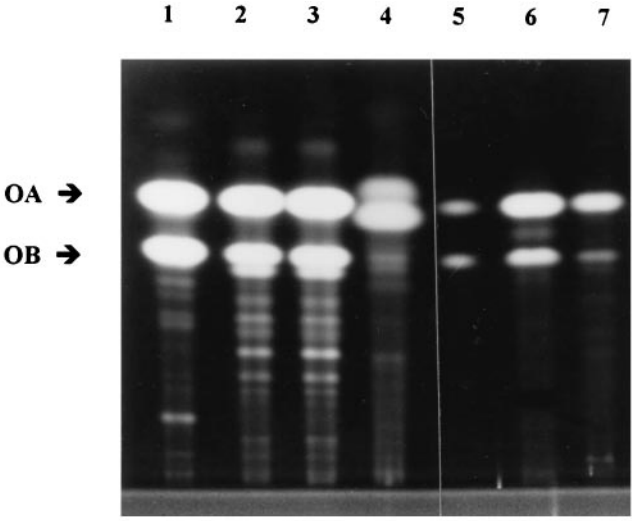

FIG. 1. High-performance TLC of some of the strains examined. Lanes: 1, *A. ochraceus* FRR 3846 (1 μl of extract applied); 2, *A. albertensis* ATCC 58745 (1 μl); 3, *A. albertensis* UAMH 2976 (nonsclerotial strain; 1 μl); 4, *A. insulicola* ATCC 26220 (5 μl); 5, OA plus OB standard; 6, *A. auricomus* FRR 3819 (5 μl); 7, *A. wentii* IMI 017295 (15 μl). *A. insulicola* does not produce OA, as proved by ELISAs. The positions of OA and OB are indicated.

not produce detectable levels of OA. A possible explanation for this observation is that *A. wentii* ATCC 1023 has lost its ability to produce OA during maintenance of the culture by periodic transfer, as observed in the cases of some other *Aspergillus* strains (10). Similar observations were made in two isogenic black *Aspergillus* strains, *A. niger* NRRL 3122 and *A. niger* ATCC 10577, which displayed different double-stranded RNA profiles (28). We should mention that *A. wentii* ATCC 1023 exhibited different growth rates and macromorphologies than *A. wentii* IMI 017295 did on different culture media (data not shown). Consequently, it cannot be ruled out that *A. wentii* ATCC 1023 represents another *A. wentii* isolate, one which replaced the original strain isolated from Indonesian soybeans.

TLC. The ability to produce OA in ELISAs was confirmed by TLC. OA production was observed in all strains which were positive for OA production in ELISAs (Fig. 1). In addition, ochratoxin B (OB) was also observed in all OA-producing *Aspergillus* strains, including those of *A. albertensis*, *A. auricomus*, and *A. wentii* (Fig. 1).

HPLC. For quantification of OA, an HPLC apparatus equipped with a fluorescent detector was used. Extracts were considered positive if they yielded a peak at a retention time identical to that of standard OA (Fig. 2). The amounts of OA observed in *A. albertensis* ATCC 58745, *A. auricomus* FRR 3819, and *A. wentii* IMI 017295 were 250, 5 to 7, and 0.05 to 0.1 μg ml^{-1}, respectively (Table 1). The amount of OA produced by *A. albertensis* strains was comparable to that observed in some *A. ochraceus* strains (Table 1) (10). *A. auricomus* FRR 3819 produced similar amounts of OA as that found in *A. alliaceus* and *A. sclerotiorum* strains earlier (10), while *A. wentii* proved to be a low OA producer, similar to *A. glaucus* and the black *Aspergillus* strains (1, 4, 24).

OA production in *A. albertensis*, *A. auricomus*, and *A. wentii* was also confirmed by HPLC comparing the UV spectra as

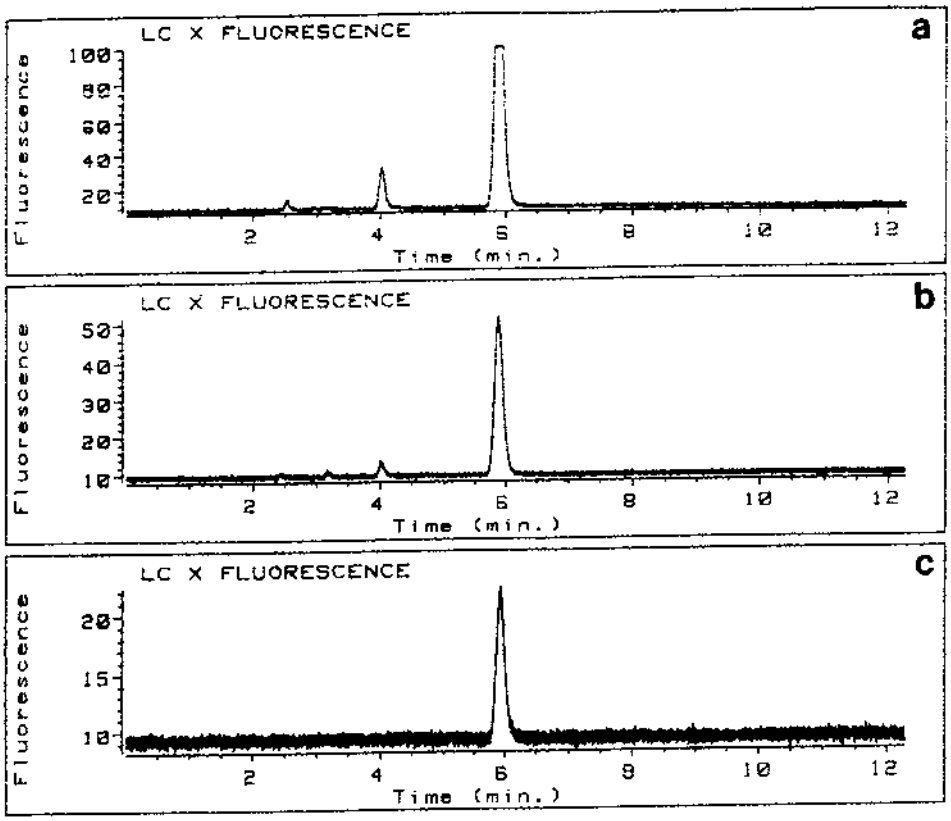

FIG. 2. HPLCs of cleaned, concentrated extracts of *A. albertensis* ATCC 58745 (a), *A. auricomus* FRR 3819 (b), and an OA standard (c). The retention times were 5.91 and 4.01 for OA and OB, respectively.

recorded with a diode array detector. The retention times (18.75 at 330 nm) and UV spectra were similar to that of the OA standard in all strains tested (data not shown).

In conclusion, the immunochemical method based on the application of a monoclonal antibody preparation against OA proved to be a useful tool for the screening of OA production among aspergilli. The sensitivity of the method revealed OA production by seven *Aspergillus* species (Table 1), three of which have not been previously described as OA producers. *A. wentii* strains are often included in the inoculum ("koji") used in the production of different soy products (18) and also used in the fermentation industry for the production of gluconic and malic acids (20), β-glucosidases (2), and pectin-degrading preparations (7). Contamination of these preparations with emodin has recently been observed (7). According to our survey, ochratoxins might also pose problems when such preparations are used in the food industry. *A. wentii* has recently been assigned to *Aspergillus* section *Cremei* on the basis of sequence analysis of the large rRNA subunit (16). It would be worthwhile to test other species in this section for OA production.

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