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KINETICS OF OCHRATOXIN A PRODUCTION IN DIFFERENT *ASPERGILLUS* SPECIES

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Kinetics of ochratoxin A production was examined in a number of ochratoxin producing isolates representing different sections of the *Aspergillus* genus. Both weak and high ochratoxin producers were tested using immunochemical or high-performance liquid chromatograhic methods. All isolates were found to produce the highest amounts of ochratoxin A after 7–10 days of incubation. Ochratoxin production varied between 30 – 5×10^5 ng ml^{-1} among the *Aspergillus* isolates tested. The *A. albertensis* and *A. melleus* isolates examined were found to produce ochratoxin A constitutively. *A. albertensis* produced the highest amounts of ochratoxin A at 30 °C after 7 days' incubation in YES liquid medium. Ergosterol content and ochratoxin production of *A. albertensis* cultures were in good correlation.

Keywords: Ochratoxin A – *Aspergillus* – *A. albertensis* – mycotoxin

INTRODUCTION

Ochratoxin A (OA) is a mycotoxin which was discovered in 1965 as a secondary metabolite of *A. ochraceus* isolates [14]. In the subsequent years several other *Aspergillus* and *Penicillium* species were described as producers of this toxin [1, 2, 4, 13, 15, 17, 18]. OA-producing species are especially associated with stored cereals, and may also contaminate coffee beans, spices, cocoa beans, soybeans, groundnuts, rice and maize. OA contamination of cereals, green coffee beans and other food products is a serious health hazard throughout the world, since OA was proved to exhibit nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties [12, 17, 18].

While some *Aspergillus* and *Penicillium* isolates were found to produce large quantities (>10 μg ml^{-1}) of OA in different media (the so-called high producers; e.g. *A. albertensis*, some *P. verrucosum* and *A. ochraceus* isolates), other species produce only small amounts of OA (so-called weak producers; e.g. black Aspergilli, *A. wentii, A. fumigatus, A. versicolor, A. glaucus* [1, 4, 13, 15]. In this study, we wished to

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examine the effect of incubation time on OA production in *Aspergillus* isolates representing both high and weak OA producers. The effect of temperature on OA production, and possible correlations between ergosterol content, mycelial dry weight and OA production have also been examined in an *A. albertensis* isolate.

MATERIALS AND METHODS

Strains

The strains examined are listed in Table 1. The strains were maintained on malt extract agar slants. For kinetic studies, the strains were grown in 5–5 mls of YES media (2% yeast extract, 15% sucrose) at 30 °C for 4, 7, 10 or 13 days.

Immunochemical detection of ochratoxin A

Culture broths of weak OA producers were tested for OA content by an enzyme-linked immunosorbent assay procedure (ELISA; [3]). One ml portions of the culture broths were vortexed with 1.25 ml of 1 N HCl and 2 ml of dichloromethane, and centrifuged (4000×*g,* 10 min). The organic phase was mixed with 2 ml of 1% $NaHCO_3$ and centrifuged again (4000×*g,* 10 min). Fifty µl aliquots of appropriate dilutions of

Table 1
Fungal strains examined

Species	Strain number and origin[a]	OA production[b] (ng ml^{-1})
A. albertensis	ATCC 58745	3×10^5
A. awamori	NRRL 3112	120
A. carbonarius (1)	IMI 041875	100
A. carbonarius (2)	N1 (J. F. Peberdy)	150
A. foetidus	CBS 618.78	100
A. melleus	IMI 257368	3×10^5
A. niger	A 136 (CECT 20157, M. L. Abarca)	590[c]
A. ochraceus	NRRL 3174	1×10^5
A. ochraceus	O4 (from green coffee beans, Uganda)	500
P. verrucosum	ATCC 18411	5×10^5 [d]

[a] *Abbreviations:* ATCC, American Type Culture Collection, Rockville, Maryland, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; IMI, International Mycological Institute, Egham, Surrey, UK; NRRL, Agricultural Research Service Culture Collection, Peoria, Illinois, USA.

[b] The highest amount of OA observed in liquid YES media.

[c] According to Abarca et al. [1].

[d] According to Walbeek et al. [19]

these extracts were tested for OA content with the Toxiklon ochratoxin A enzyme immunoassay kit (Agricultural Biotechnology Centre, Gödöllő, Hungary). Direct competitive ELISA tests were performed as described earlier [3, 13].

Quantitation of ochratoxin A by thin-layer chromatography (TLC)

The OA content of the culture broths of *Aspergillus* isolates producing high amounts of OA was tested by TLC. The culture broths were acidified and vortexed with 5 ml of dichloromethane. Two ml portions of the organic phase were evaporated to dryness, dissolved in acetonitril, and appropriate amounts were spotted onto silica gel plates (HPTLC precoated plates, 10×10 cm, No. 5633, E. Merck, Darmstadt, Germany). Plates were developed in toluene-ethyl acetate-formic acid (5 : 4 : 1), and 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 amount of OA was determined by fluorescence densitometry.

Ochratoxin A detection by high performance liquid chromatography (HPLC)

For HPLC analysis, the acetonitril extracts used for TLC were evaporated, and OA was redissolved in appropriate amounts of the mobile phase (57% acetonitrile, 41% water and 2% acetic acid). The 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 C18 BD columns (BioSeparation Techn., Budapest, Hungary; 250×4 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 [15]. OA was quantified on the basis of HPLC fluorometric response compared to that of an OA standard.

Determination of ergosterol content in A. albertensis *cultures*

A microwave-assisted method was used for ergosterol extraction. Liophylized mycelia of *A. albertensis* were saponified by 2 ml methanol + 0.5 ml 2 M KOH, and mycelia were disrupted by 60 sec microwave treatment in a professional LABOTRON 500 apparatus. After microwave treatment, the mixture was neutralized with 1 ml 1 N HCl, and ergosterol was extracted with 3×2 ml hexane. The extracts were pooled, evaporated to dryness, and dissolved in 1 ml acetonitrile. Five μls of these extracts were used in HPLC analysis. The HPLC apparatus used was a Hewlett-Packard 3395, equipped with a binary solvent delivery system, an autoinjector, an autosampler, a temperature-controlled column compartment and a fluores-

cence detector (Shimadzu SPD-6AV). BST RUTIN C18 BD columns (BioSeparation Techn., Budapest, Hungary; 250×4 mm, particle size: 10 µm) were used. For fluorescent detection, the mobile phase, which consisted of an isocratic program of 80% acetonitrile and 20% methanol, was pumped at a rate of 1 ml/min [20].

RESULTS AND DISCUSSION

Kinetics of ochratoxin A production in different Aspergillus *species*

In previous studies, OA-producing abilities of different *Aspergillus* isolates were found to vary considerably. While some species (mainly members of *Aspergillus* subgenus *Circumdati* section *Circumdati*, and *A. albertensis* [8]) produce large amounts of OA in YES media in the range of 0.1–1 mg ml^{-1} [14, 15], other *Aspergillus* isolates (e.g. black Aspergilli, *A. fumigatus, A. versicolor, A. wentii, A. glaucus*) produce OA in ng ml^{-1} quantities only [2, 4, 13, 15]. Our aim was to examine the kinetics of OA production of both high and weak OA producing *Aspergillus* isolates by HPLC and ELISA techniques as described previously [13, 15]. A simple medium containing only sucrose and yeast extract (YES) was used, since it was found to be the best for testing OA production in most *Aspergillus* and *Penicillium* isolates [6, 11].

The effect of incubation time on OA content of the culture broth was found to vary from isolate to isolate. Among the weak OA producers, relatively high levels of OA were detected after 7 days of incubation in some isolates (e.g. *A. awamori, A. foetidus, A. niger* isolates in Fig. 1), while high OA levels were observed only after 10 days of incubation in other strains (*A. carbonarius* isolates, *A. ochraceus* O4). The OA contents of *A. foetidus*, and *A. carbonarius* isolates together with *A. ochraceus* O4 decreased sharply after incubation for 7 and 10 days, respectively, possibly because the strains removed and assimilated the phenylalanine moiety from the OA molecule, as other nitrogen sources of the culture broth became exhausted. Damoglou et al. [7] earlier observed that some OA producing strains were able to further metabolize OA over time. The OA content of the tested *A. awamori* and *A. niger* isolates decreased slowly after incubation for 10–14 days.

Among the high OA producers, 7-day incubation was optimal for *A. ochraceus* NRRL 3174, while 10 days were necessary for reaching the highest OA levels in *P. verrucosum* isolates (Fig. 2; [19]). The OA content of the culture broths of *A. ochraceus* and *P. verrucosum* isolates decreased after 7 and 10 days of incubation, respectively. For the *A. albertensis* and *A. melleus* isolates examined, the highest OA levels were observed after 7 days' incubation, and the amount of OA did not decrease after prolonged incubation times. In *A. albertensis*, the OA content of the culture broth did not decrease considerably even after 21 days of incubation (data not shown). These isolates can possibly be considered as constitutive OA producers. Since both *A. albertensis* and *A. melleus* isolates produce high amounts of OA (about 300 µg ml^{-1} in YES media), these strains could be used for OA production in large quantities needed for other research purposes (e.g. OA degradation studies; [16]).

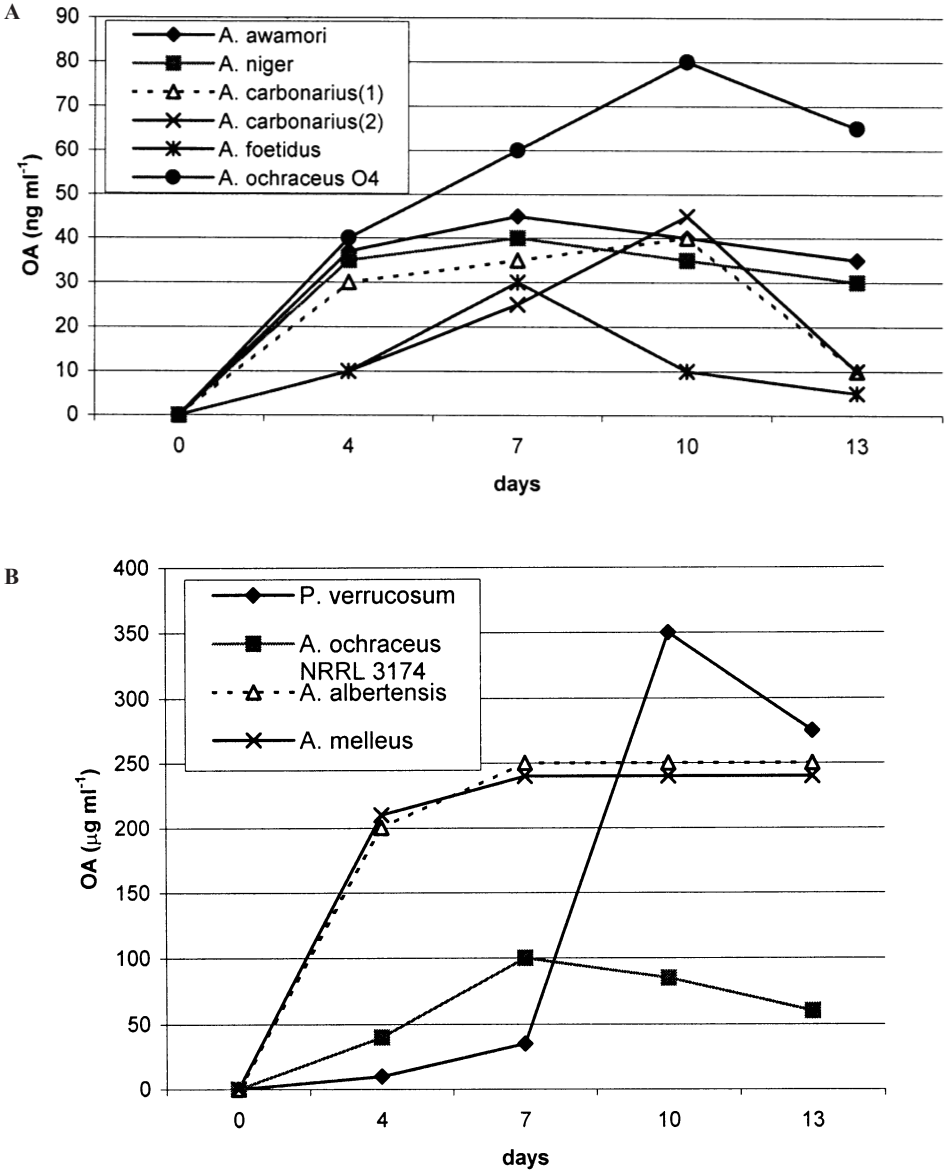

Fig. 1. Kinetics of OA production by different *Aspergillus* species.
A – weak OA producers; B – high OA producers

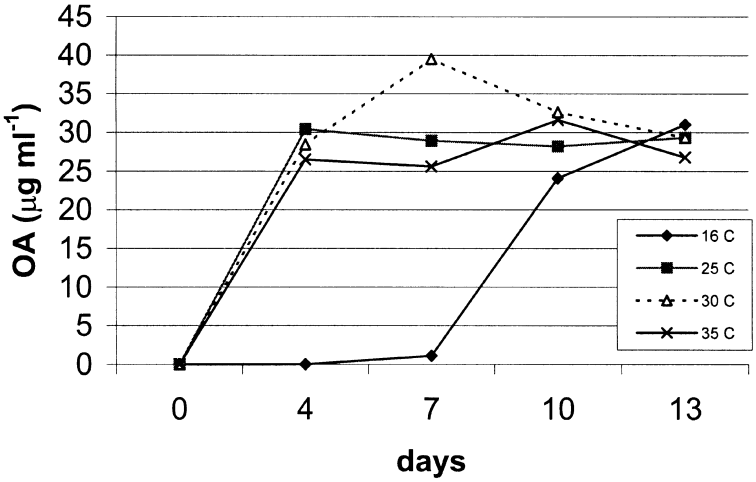

Fig. 2. Effect of incubation temperature on OA production in *A. albertensis*

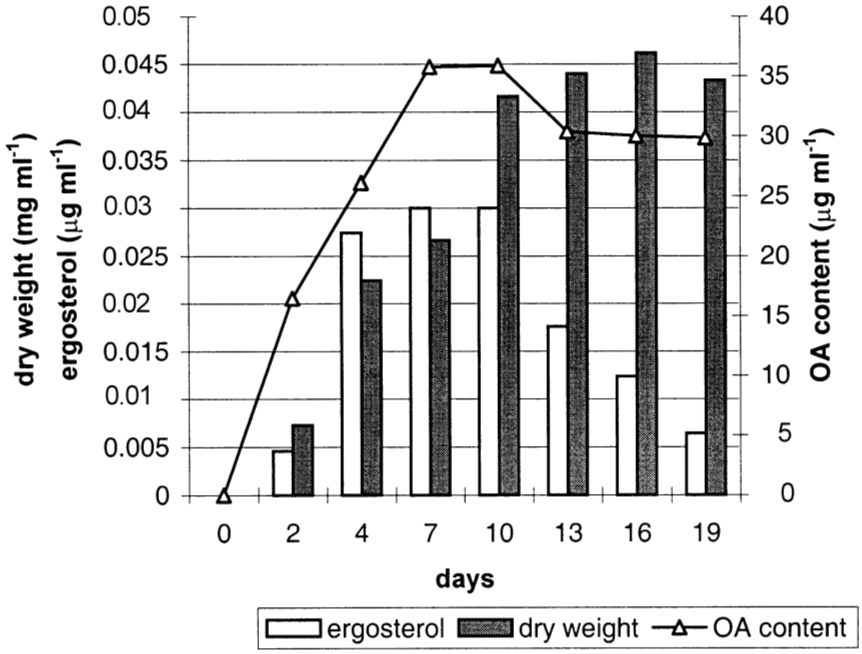

Fig. 3. OA production, ergosterol content and mycelial dry weight of *A. albertensis* cultures at 30 °C

Examination of ochratoxin A production in A. albertensis

A. albertensis was grown in liquid YES medium, and the dry weight of mycelia, ergosterol content and OA content of the cultures were determined (Figs 2 and 3). *A. albertensis* produced the highest amounts of ochratoxin A at 30 °C after 7 days incubation in YES liquid medium. However, the amounts of ochratoxin A produced at different temperatures (16, 25, 30 and 35 °C) were nearly the same after 10 days, and did not decrease significantly even after 19 days (Fig. 2). The dry mycelial mass produced by *A. albertensis* at 30 °C increased for 16 days, then a slight decrease took place (Fig. 3). A good correlation was observed between ergosterol content and OA production of *A. albertensis.* Both values increased for 7 days, than ergosterol content of mycelia decreased sharply, while OA content decreased slowly. Schnürer [10] suggested that ergosterol content of stored plant products (mainly cereals) could be used for predicting mycotoxin contamination. Our data indicate that ergosterol content of agricultural products should be used cautiously for such purposes.

In summary, kinetics of OA production was examined in a number of *Aspergillus* isolates. While OA production showed normal distribution over time in most isolates, an *A. albertensis* and an *A. melleus* isolate produced OA consistently. The *A. albertensis* isolate produced highest amounts of OA at 30 °C after 7 days incubation. Incubation at different temperatures led to altered OA levels, however, after 10 days, the amount of OA produced was nearly the same at all temperatures tested. Ergosterol content and OA production were in good correlation in *A. albertensis* cultures. However, ergosterol content of mycelia dropped, while OA content decreased very slowly after 10 days' incubation.

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