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Production of ochratoxin A by *Aspergillus ochraceus*

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Abstract

The effects of growth conditions, trace metals, carbon and nitrogen source on ochratoxin A (OTA) production by *Aspergillus ochraceus* NRRL 3174 were investigated in submerged liquid culture medium. No correlation between biomass production and OTA synthesis was observed. OTA production depended on the initial pH and was influenced by changing the pH of the culture medium. This effect was not due to differences in the availability of metal ions. At pH 6.5, 0.2 mg l^{-1} Zn increased biomass and OTA production by 50%. Fe at 0.12 mg l^{-1} resulted in a 40% decrease in the amount of OTA/biomass although the quantity of biomass was not affected. Amounts of OTA produced per biomass increased with decreasing glucose concentrations. Lactose triggered the highest amounts of OTA/biomass despite low yields of biomass. NO_3^- stimulated the amount of OTA/biomass by 25% and NH_4^+ reduced the amount of OTA/biomass by 25% compared with NH_4NO_3 as nitrogen source.

Introduction

Contamination of food and feed with mycotoxins represents a high risk for human and animal health. One of the toxins that has been considered of growing importance in the last decade is ochratoxin A (OTA), a nephrotoxic mycotoxin composed of a polyketide moiety and phenylalanine linked via a peptide bond. OTA is produced by several *Aspergillus* and *Penicillium* species. Although occurrence of OTA in feed and foodstuffs and its toxicity to animals and humans have been extensively studied, relatively little is known about the biosynthesis of this mycotoxin.

Early detection of ochratoxigenic fungi on raw materials, feed and foodstuffs, is vital if OTA is to be eliminated from the food chain. To achieve this, application of molecular diagnostic methods is required. Such methods can best be established when based on special genetic features of OTA producers, i.e. the genes and enzymes involved in the OTA biosynthetic pathway.

In order to elucidate the genes and enzymes involved in OTA biosynthesis, it is necessary first to determine the environmental factors which regulate production of this mycotoxin. Production of secondary metabolites is not essential to the synthesizing organism but it is regulated by several often interwoven environmental signals. For example, aflatoxin synthesis by *Aspergillus flavus* is associated with depletion of nitrogen or trace metals (apart from Zn which has a stimulatory effect on aflatoxin biosynthesis) at high levels of carbon source. This effect is mediated via accumulation of acetylCoA and limited NADPH availability. Under these circumstances, fatty acid synthesis is repressed in favor of polyketide synthesis (Bennett and Christensen, 1983).

OTA production in *Penicillium nordicum* strains appears to be common and relatively stable. OTA producing and non-OTA producing strains were grouped into two distinct clusters by molecular typing methods (Larsen et al., 2001; Castella et al., 2002). On the other hand, OTA producing ability in aspergilli was not correlated with taxonomical groupings obtained from AFLP fingerprints (Schmidt et al., 2003). This might be a sign of heterogeneity occurring in OTA production by

closely related strains. More likely, data concerning OTA production are not reliable because of variability in OTA production.

In *Aspergillus* species, ochratoxin synthesis depends on several environmental factors and has been reported to be sporadic or even lost during cultivation in the laboratory (Varga et al., 1996). Several authors have reported the effect of changes in culture conditions on OTA production by *Aspergillus* strains. The results observed were as diverse as the conditions applied e.g. differences in isolate and composition of culture medium, pH, temperature and degree of aeration. However, most authors observed no correlation between biomass and OTA production.

The enzymatic pathways by which aspergilli and penicillia generate OTA have been studied. Phenylalanine was shown to be incorporated into OTA (Ferreira and Pitout, 1969) and the polyketide residue to be synthesized via the acetate malonate pathway (Steyn and Holzapfel, 1970). Chlorine is incorporated directly (Wei et al., 1971). A possible scheme leading to the biosynthesis of OTA was published by Huff and Hamilton (1979). According to their hypothesis, three distinct steps occur in OTA biosynthesis: the first is polyketide synthesis of OTα via mellein involving a polyketide synthase, followed by chlorination by a chloroperoxidase and carboxyl activation. The second precursor, phenylalanine, is synthesized via the shikimic acid pathway, followed by ethyl ester activation. In the third step, linkage of those activated precursors via a synthetase takes place, generating OTC, an ethyl ester of OTA. De-esterification by an esterase is the last step in this postulated biosynthetic pathway (Moss, 1998). Recently, Harris and Mantle (2001) proposed a different pathway, as they found no evidence for an intermediate role of mellein and OTC in OTA biosynthesis. Instead, they suggested an unspecified pathway leading from OTβ (de-chlorinated form of OTα) via OTα to OTA.

The aim of the current study was to identify culture conditions determining OTA biosynthesis as a prerequisite to elucidation of genes connected to the ochratoxin A biosynthetic pathway, especially a gene coding for the putative 'OTA synthetase'. This enzyme is presumed to catalyze the final step of the OTA biosynthesis, generating ochratoxin A from the proposed precursors, (modified) ochratoxin α and (modified) phenylalanine. Existence of an 'OTA synthetase' was originally postulated by Ferreira and Pitout (1969). The authors observed formation of OTA from its precursors OTα and phenylalanine in cell free extracts of *Aspergillus ochraceus* CSIR804.

More recently, Edwards et al. (2002) employed a heterologous genetic approach to identify the putative polyketide synthase (PKS) involved in OTA biosynthesis. They found two sequences from separate genes in *A. ochraceus* ATCC 22947 with homology to fungal ketosynthases. Yet, in reverse transcription experiments, gene expression was detected under OTA permissive (Yeast Extract Sucrose: YES) and non-permissive (Yeast Extract Peptone) conditions with higher levels of transcription under non-permissive conditions. Therefore, the authors concluded that the *pks* genes are expressed in *A. ochraceus* ATCC 22947, but are not necessarily connected to OTA biosynthesis.

Cultivation of *Aspergillus ochraceus* in YES liquid medium during this study led to considerable variability in OTA production. Therefore, it was necessary to determine the factors which influence OTA biosynthesis. For this purpose, we examined the effects of changes in pH, trace metals, carbon and nitrogen source on OTA production by *Aspergillus ochraceus* NRRL 3174 and other *Aspergillus* spp.

Materials and methods

Organisms and cultivation

All organisms used were obtained from sources as shown in Table 1. Fungi were maintained as glycerol stock cultures at −80 °C (Niessen and Vogel, 1997). Working cultures were inoculated from the stock cultures and grown on 2% malt extract agar for 7–10 days at 25 °C. Spores were harvested by suspension in 5 ml of glycerol water mixture (1:1). Modified Adye–Mateles (AM) synthetic medium (Adye–Mateles, 1964) was used in all experiments. Five hundred ml of AM liquid medium in 1000 ml flasks were inoculated with $\approx 10^7$ spores and shaken for 7–10 days at 120 rpm, 25 °C in the dark. Standard AM medium contained per liter of deionized H_2O: 27.5 g glucose·H_2O, 1.5 g NH_4NO_3, 0.61 mg $Fe_2(SO_4)_3 \cdot 5H_2O$, 0.88 mg $ZnSO_4 \cdot 7H_2O$, 0.15 mg $CuSO_4 \cdot 5H_2O$, 0.35 mg $Na_2B_4O_7 \cdot 10H_2O$, 0.27 mg

Table 1. OTA production by strains of *Aspergillus* and *Penicillium*

Species	Isolate	Source	OTA production as described by supplier	OTA production in this study	
				MEA	AM
A. ochraceus	NRRL 3174[1]	Unknown	+	++	++
A. ochraceus	A8[2]	Unknown	+	+	±
A. ochraceus	CBS 588.86[3]	*Piper* sp., USA	+	−	−
A. ochraceus	CBS 589.86[3]	*Piper* sp., USA	+	−	−
A. ochraceus	CCT 6819[4]	Saõ Paulo	−	−	++
A. awamori	CBS 101704[3]	Unknown	+	+	−
A. carbonarius	M 335[5]	Coffee, Thailand	+	++	−
A. carbonarius	M 336[5]	Coffee, Thailand	+	−	−
A. fresenii	CBS 550.65[3]	Soil, India	+	++	+
A. niger var. *niger*	CBS 101697[3]	Coffee, Kenya	+	++	+
A. usamii var. *shiro-usamii*	CBS 101700[3]	Unknown	+	−	−

MEA: malt extract agar. AM: AM liquid medium.
[1]Obtained as *A. ochraceus* KA 103 from J. Chelkowski (Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland).
[2]F.J. Cabañes (Universitat Autònoma de Barcelona, Spain).
[3]Centraalbureau voor Schimmelcultures (CBS, Fungal Biodiversity Center, Utrecht, The Netherlands).
[4]M.H. Taniwaki (Instituto de Tecnologia de Alimentos, Campinas-SP, Brazil).
[5]H.M.L.J. Joosten (Nestle Research Center, Nestec Ltd., Lausanne, Switzerland).

$NH_4Mo_7O_{24} \cdot 4H_2O$, 0.04 mg $MnSO_4 \cdot H_2O$, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 5.1 g KH_2PO_4, adjusted to pH 6.5.

Assays

Seven to ten days old cultures of *Aspergillus* strains grown on 2% malt extract agar were tested for OTA production by the agar plug technique (Filtenborg et al., 1983). Twenty milliliter of *Aspergillus* cultures grown in AM liquid medium were sampled at specific growth stages. The dry weight of filtered and washed mycelium was determined (mdw). The pH of the filtered medium and amounts of glucose remaining (D-Glucose UV-method Cat. No. 0716251, r-biopharm, Darmstadt, Germany) were measured. The filtered medium was tested for OTA by a rapid detection method: 5 ml of filtered sample were extracted with 5 ml ethyl acetate after acidification with 1 ml 1 M HCl. The water phase was discarded and solvent was evaporated to dryness. The residue was redissolved in 100 µl methanol. Samples were subjected to TLC (mobile phase: toluene: ethyl acetate: formic acid 6:3:1) and OTA was identified by fluorescence at 254 nm and R_f value. The amount of OTA was estimated by comparison with a dilution series of standard OTA (Fluka, Germany).

Furthermore, 2 ml of each filtered medium sample were extracted once with 4 ml dichloromethane. The organic phase was twice extracted with 0.13 M $NaHCO_3$. Water phases were pooled and evaporated to dryness. One milliliter of 43% methanol was added to the dry samples. OTA was quantified by HPLC (isocratic elution with acetonitrile:H_2O:acetic acid 99:99:2 (v:v:v); LiChroCART 250-4 LiChrospher 100 RP 18e, Perkin Elmer; fluorescence detection at 332/465 nm).

Results

pH dependence of OTA production

In AM liquid media adjusted to a pH value within the range from pH 5.5 to 8.5 prior to inoculation, *Aspergillus ochraceus* NRRL 3174 produced up to 2 mg/l OTA after 360 h of incubation at 25 °C, 120 rpm in the dark. There was no significant difference in the amount of OTA produced in cultures adjusted to pH values within this range.

No OTA production was observed outside the range from pH 5.5 to 8.5, whereas growth and

biomass production of *A. ochraceus* NRRL 3174 remained unaffected within a pH range from pH 3.5 to 9.0. In all cultures adjusted to an initial pH 5.0–9.0, the pH slowly dropped to pH 4.2 within 360 h of incubation. Acidification of the culture medium was due to organic acids like citrate and pyruvate produced by the growing fungal mycelium (data not shown).

OTA synthesis by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium initially adjusted to a pH outside the pH range favorable to OTA production was triggered by a pH shift of the culture medium during the exponential growth phase (Figure 1). Initial pH adjustment of the culture medium to pH 6.5 resulted in quantifiable OTA production after 72 h, when the culture was nearly at the end of the exponential growth phase. Initial pH adjustment of the culture medium to pH 5.0 resulted in no quantifiable OTA production within an incubation time of 500 h. A shift in pH from 5.0 to 6.5 after 60 h of incubation triggered onset of measurable OTA production in *A. ochraceus* NRRL 3174 after 40 h of further incubation. However, the amount of OTA produced was lower in cultures with shifted pH compared to cultures with an initial pH of 6.5.

Dependence of OTA production on trace metals

The bioavailability of trace metals in AM medium varies with pH. At pH 6.5, considerable precipitation of Zn and Fe phosphates occurred, which

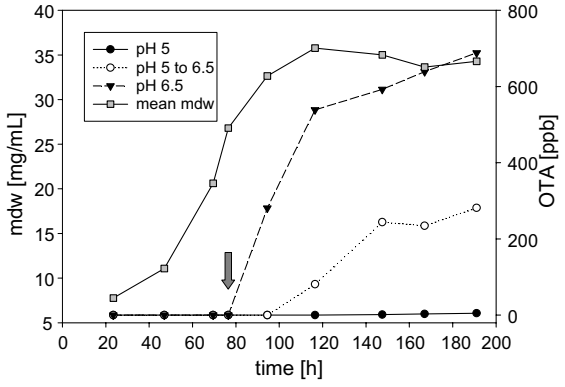

Figure 1. Biomass and OTA production of *Aspergillus ochraceus* NRRL 3174 in AM liquid medium adjusted to different initial pH values. The arrow indicates pH shift in one culture to pH 6.5 after 60 h of incubation at pH 5.0 (○). Mdw = mycelial dry weight.

was not observed at pH 5.0. Therefore, at pH 6.5 the amount of accessible Zn and Fe in the medium must be reduced in comparison to pH 5.0. In order to determine whether the pH effect described above was mediated by different concentrations of those trace metals at different pH values, either Zn or Fe were omitted, or both, from the media at pH 5.0 and 6.5. Omission of trace metals from the culture media had a similar effect on biomass production at initial pH 6.5 and 5.0 (Figure 2a and b). At both pH 6.5 and 5.0, biomass production was not affected by omission of Fe. The final pH settled at pH 4.2 after 360 h of incubation: a value which was independent of the initial pH.

Not supplementing Zn, Zn and Fe or any trace metals to AM media at both initial pH 6.5 and 5.0 reduced biomass of the fungal culture to 25% of the biomass obtained in fully supplemented media. After an initial pH 6.5, the final pH of the cultures not containing Zn, Fe or Zn, or any trace metals only dropped to pH 6 after 360 h of incubation. On the other hand, after an initial pH of 5.0, the final pH of these cultures dropped to < pH 3 after 360 h of incubation (data not shown).

Dependence of OTA production on trace metals at pH 5.0

At pH 5.0, OTA production was not affected by omitting trace metals from the culture media (Figure 2a). No significant OTA production was observed when Zn or Fe or both were omitted from the culture medium (max. 8 μg g^{-1} mdw) after 120 h of incubation. The amount of OTA produced did not increase upon prolonged incubation.

Dependence of OTA production on trace metals at pH 6.5

At pH 6.5, OTA production was sensitive to variations in trace metal supply (Figure 2b). In standard media with 5% glucose, $(NH_4)_2SO_4$ and all trace metals added, 220 μg OTA g^{-1} mdw were produced by cultures of *A. ochraceus* NRRL 3174 after 120 h of incubation at 25 °C. Omitting Fe from the culture media at pH 6.5 resulted in slightly lower biomass production, but more OTA/biomass was produced (310 μg g^{-1} mdw). Omitting Zn or both Zn and Fe, from the culture medium resulted in lower biomass and small

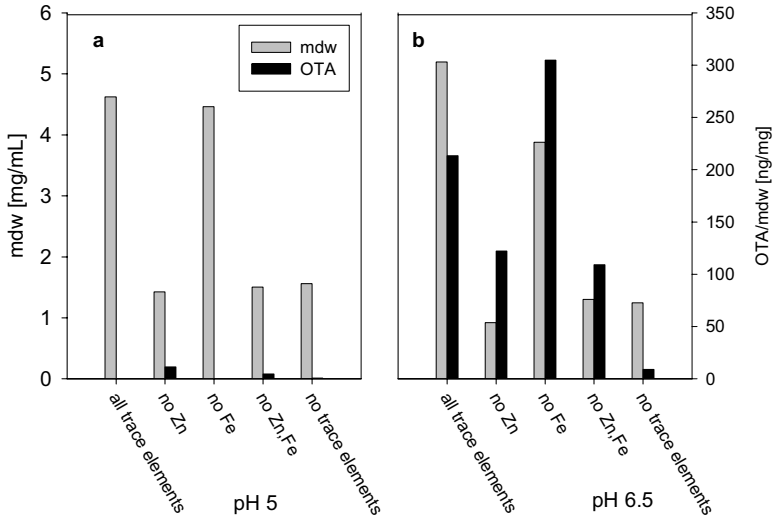

Figure 2. Effect of trace metal supply on biomass and OTA production by *Aspergillus ochraceus* NRRL 3174 after 120 h of incubation in AM liquid medium adjusted to initial pH 5.0 (a) and initial pH 6.5 (b), respectively.

amounts of OTA/biomass (≈120 μg g^{-1} mdw) compared to fully supplemented media. Omitting all trace metals resulted in only minimal OTA production (20 μg g^{-1} mdw). Onset of OTA production was delayed for about 20 h in cultures not containing Zn, Zn and Fe or any trace metals compared to fully supplemented media (data not shown).

Increased amounts of OTA/biomass were also observed with *A. ochraceus* CCT 6819, *A. niger* var. *niger* CBS 101697 and *A. fresenii* CBS 550.65 after an initial pH 6.5 in media that did not contain Fe. There was a decrease in the level of OTA/biomass in media that did not contain Zn or Zn and Fe (data not shown). *Aspergillus ochraceus* CCT 6819 was originally described as a non-OTA producing strain (Schmidt et al., 2003). In our experiments, it did not produce any OTA when cultivated on malt extract agar but up to 300 μg g^{-1} mdw were produced when grown in AM liquid medium which did not contain Fe. OTA production of other strains tested in our experiments was not predictable from data given by the suppliers (Table 1).

Dependence of OTA production on concentration of the carbon source

Compared to standard AM media containing 5% glucose, biomass production after 120 h of incubation was lower in culture media containing 2.5%, 1.25% or 0.625% glucose. Fungal biomass production was also reduced at 10% glucose compared to standard media.

In contrast, OTA production increased with a decreasing supply of glucose (Figure 3a). OTA production was detectable after 72 h in all setups with different glucose concentrations. The amount of OTA increased most rapidly in cultures supplied with 0.625% glucose and continued to increase to 2 mg l^{-1} after glucose in the culture medium became depleted.

Dependence of OTA production on nature of the carbon source

Exchange of the 5% glucose with 5% sucrose, lactose or fructose, respectively, resulted in different amounts of biomass and levels of OTA/biomass after 120 h of incubation depending on the carbon source supplied (Figure 3b). High yields in biomass and OTA/biomass (220 and 230 μg OTA g^{-1} mdw) were found in culture media containing 5% glucose or sucrose. Onset of OTA production was delayed for about 20 h in cultures containing sucrose. In culture media containing 5% fructose, both biomass and OTA production were reduced compared to culture media containing 5% glucose (75 μg OTA g^{-1} mdw). Onset of OTA production was delayed for more than

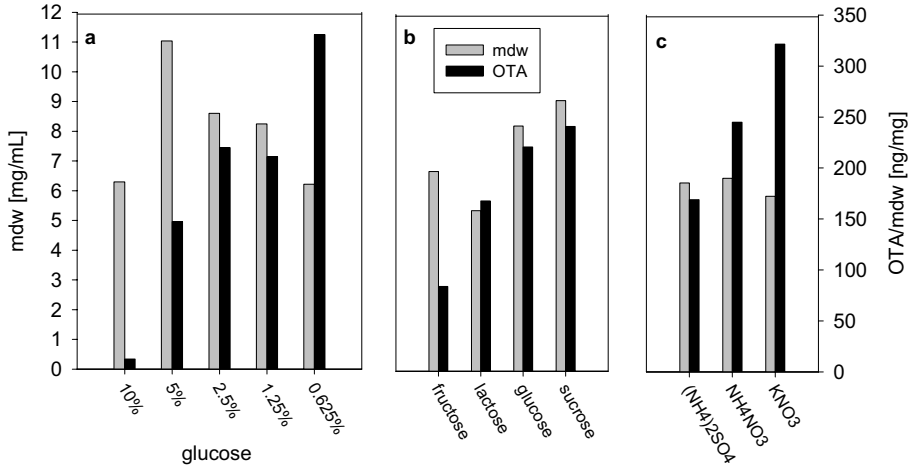

Figure 3. Effect of glucose concentration (a), carbon source (b) and nitrogen source (c) on biomass and OTA production by *Aspergillus ochraceus* NRRL 3174 after 120 h of incubation in AM liquid medium adjusted to initial pH 6.5.

40 h. In culture media containing 5% lactose, biomass production was significantly reduced, but the amounts of OTA/biomass was only slightly reduced compared to glucose (170 μg OTA g^{-1} mdw).

As seen with the supply of different trace metals, good growth in these experiments was accompanied by a drop to pH 4.2 after 360 h of incubation. Effects of trace metals tested in combination with 5% sucrose, 2.5% and 0.625% glucose, respectively, were the same as with 5% glucose. Omitting Fe under these conditions led to a slight decrease in biomass and an increase in amounts of OTA/biomass. Omitting either Zn or Fe and Zn resulted in significantly reduced OTA yields (up to 50% compared to media with all trace metals added) (data not shown).

Dependence of OTA production on the nitrogen source

Using 5% glucose as the carbon source, the supply of different nitrogen sources had no influence on biomass production, but resulted in differences in OTA/biomass production after 120 h of incubation. In standard AM media, containing NH_4NO_3 as nitrogen source, 240 μg OTA g^{-1} mdw were produced. Using $(NH_4)_2SO_4$ instead of NH_4NO_3 reduced the amounts of OTA/biomass to 175 μg g^{-1} mdw. The Addition of KNO_3 increased the levels of OTA/biomass (320 μg g^{-1} mdw) (Figure 3c). When KNO_3 was supplied as the nitrogen source, the pH of the AM medium remained unchanged at 6.5. $(NH_4)_2SO_4$ induced a reduction to pH 3.5 during the incubation period of 360 h (data not shown).

Discussion

Onset of OTA production in AM liquid medium was affected by the initial but not final pH of the culture medium. This regulation mechanism appears to be stringent, as there is no OTA production at <pH 5.5 and uniformly similar levels of OTA production between pH 5.5 and 8.5 (starting pH of the fungal culture). The occurrence of a 'lag time' of about 40 h in the onset of OTA synthesis following a pH shift from pH 5.0 to 6.5 during cultivation indicates that pH affects gene expression rather than enzyme activity. This regulatory effect is not mediated via differences in the availability of trace metals such as Zn and Fe at different pH values. No significant OTA production was observed when Zn, Fe or both were omitted from the culture medium at pH 5.0. Adding up to tenfold more Fe or Zn to the liquid medium at pH 5.0 did not result in increased OTA production. This pH dependent regulation of OTA production was verified with two different species representing two separate taxonomic units within the genus *Aspergillus*: the yellow *A. ochraceus* NRRL 3174

and the black *A. niger* var. *niger* CBS 101697. Other isolates may behave differently: Aziz and Moussa (1997) observed some OTA production by an isolate of *A. ochraceus* at an initial pH of 4.0, with a maximum at pH 5.5–6, but no OTA production at pH 8.0.

The concentration of trace metals had a strong influence on the amount of OTA produced by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium adjusted to pH 6.5. Addition of Zn to the culture medium had a stimulatory effect on both biomass and OTA production. OTA was also produced in the absence of Zn but at a lower rate than media containing 0.2 mg l^{-1} Zn. In contrast, Fe suppressed OTA production, at least at the concentration used (0.12 mg l^{-1}). When both Fe and Zn were absent from the culture media, the 'Zn effect' dominated: biomass and OTA production decreased to 50%. This indicates that OTA production was dependent on a sufficient supply of trace metals. Similar gradual regulation of OTA production has been observed with other *Aspergillus* species.

Another growth factor influencing OTA production was the concentration of carbon source in the culture medium. OTA production increased with decreasing amounts of glucose supplied. In all experiments, OTA production occurred near the end of the exponential growth phase and was dependent on rapid glucose utilization and a steady decrease in pH. However, glucose utilization *per se* did not determine OTA production, as seen in experiments with media at an initial pH of 5.0.

Ferreira (1967) tested the influence of various carbon sources on OTA biosynthesis. He observed the highest yields (100 mg OTA l^{-1}) with sucrose, considerably less with glucose (2 mg OTA l^{-1}), none with fructose and none with lactose. Lai et al. (1970) observed that in AM medium at pH 7.0, highest OTA production by *A. sulphureus* NRRL 4077 occurred with glucose and sucrose as carbon source. In our experiments, *A. ochraceus* NRRL 3174 produced the highest yields of biomass and OTA with glucose and sucrose. Although the mycelial dry weight was reduced by 40% with sucrose compared to glucose, yields of OTA/biomass were only slightly reduced with lactose, by 30% compared to glucose. Biomass production was only slightly reduced with fructose as the single carbon source but OTA production was reduced to 50% compared to glucose. Therefore, with the sugars tested here, the quantity of OTA and biomass produced by *A. ochraceus* NRRL 3174 seems to be influenced by the type of carbon source, but there was no correlation between biomass and OTA production.

In *Penicillium* strains, OTA production was switched on or off depending on the combination of nitrogen and carbon source supplied. OTA production was triggered when the cultures were supplied with NH_4^+ and glycerol, whereas it was suppressed when NO_3^- and glucose were added to solid growth medium (R. Geisen, Federal Research Centre for Nutrition, Germany, personal communication). In contrast, *A. ochraceus* NRRL 3174 produced large amounts of OTA when grown with glucose as the single carbon source and showed fundamental differences in reaction to nitrogen sources supplied. Providing NH_4^+ only, the amount of OTA/biomass was reduced by 25% without any significant change in mycelial dry weight. The amount of OTA produced in standard AM medium seems to be the result of a balance between the influence of ammonia and nitrate on OTA production. Ferreira (1967) observed the best yields (100 mg l^{-1}) using NH_4NO_3 or organic nitrogen sources, such as glutamic acid or proline. Experiments using $NaNO_3$ or $(NH_4)_2SO_4$, respectively, resulted in no OTA production.

The diverging, and in some cases conflicting results reported here as well as in several previous studies, suggest that OTA production by *Aspergillus* is dependent on the interplay of several environmental conditions rather than on a single growth factor. Several strains described as OTA producing by the supplier (incubation conditions not specified) did not produce OTA on solid malt extract or liquid AM medium after up to 15 days of incubation in our laboratory. A*spergillus carbonarius* M335 and M336 both produced OTA when grown on coffee berries and were fluorescence positive on coconut cream agar (Joosten et al., 2001), but no OTA was produced in AM liquid medium. The observed differences reported by several authors could be a result of variable production levels of OTA by different mycelia, even originating from the same spore suspension. Röschenthaler et al. (1984) observed varying amounts of OTA produced in single spore cultures isolated from one *Aspergillus* mycelium. Another explanation for divergent results might be vari-

ability of OTA production in laboratory cultures, or even irreversible loss of ability to produce OTA (Harwig, 1974), a phenomenon also found with aflatoxin production by *A. flavus* and *A. parasiticus* (Mayne et al., 1971; Leiach and Papa, 1974; Lee et al., 1986; Lemke et al., 1989). Furthermore, no correlation was observed between biomass production and OTA synthesis in the current study. OTA production seemed to be completely independent from the growth status of the fungal culture. Conditions favorable to growth did not always correspond with conditions optimal for OTA production or vice versa. Growth rates at pH 5.0 were almost unchanged compared to pH 6.5, but no OTA was produced. Experiments with varying concentrations of trace metals suggest that conditions favorable to growth trigger maximum OTA levels. On the other hand, growth experiments with different amounts of glucose suggest an inverse correlation between OTA and biomass production, whereas results obtained in experiments with different carbon and nitrogen sources showed no correlation between biomass and the amount of OTA produced.

OTA biosynthesis of ochratoxigenic *Aspergillus* spp. is determined more by environmental conditions than by the inherent ability of the organism to produce OTA. If an isolate does not produce OTA under given conditions, this does not justify any conclusion about its general ability to produce OTA. Also, any grouping into 'OTA producers' and 'non-producers' based on such data will be misleading. In contrast, OTA production in the genus *Penicillium* appears to be stable and evenly distributed.

The results obtained from the current study and from the literature suggest that it is necessary to determine the specific conditions determining OTA production for each *Aspergillus* spp. strain. This is important as it indicates that adjusting conditions of food storage or preservation may not be enough to guarantee mycotoxin-free feed and foodstuffs. Clearly, storage or preservation conditions deployed to forestall OTA production by some isolates might not be appropriate to suppress OTA production by other *Aspergillus* isolates. In the absence of methods to predict OTA accumulation, it is essential that molecular tools are developed for the detection of ochratoxigenic species and/or key genes of OTA biosynthesis.

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