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Impact of Some Environmental Factors on Growth and Production of Ochratoxin A of/by *Aspergillus tubingensis, A. niger*, and *A. carbonarius* Isolated from Moroccan Grapes

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The effects of temperature, water activity (a_w), incubation time, and their combinations on radial growth and ochratoxin A (OTA) production of/by eight *Aspergillus niger* aggregate strains (six *A. tubingensis* and two *A. niger*) and four *A. carbonarius* isolated from Moroccan grapes were studied. Optimal conditions for the growth of most studied strains were shown to be at 25°C and 0.95 a_w. No growth was observed at 10°C regardless of the water activity and isolates. The optimal temperature for OTA production was in the range of 25°C~30°C for *A. carbonarius* and 30°C~37°C for *A. niger* aggregate. The optimal a_w for toxin production was 0.95~0.99 for *A. carbonarius* and 0.90~0.95 for *A. niger* aggregate. Mean OTA concentration produced by all the isolates of *A. niger* aggregate tested at all sampling times shows that maximum amount of OTA (0.24 μg/g) was produced at 37°C and 0.90 a_w. However, for *A. carbonarius*, mean maximum amounts of OTA (0.22 μg/g) were observed at 25°C and 0.99 a_w. Analysis of variance showed that the effects of all single factors (a_w, isolate, temperature and incubation time) and their interactions on growth and OTA production were highly significant.

***Keywords*:** *Aspergillus tubingensis*, *A. niger*, *A. carbonarius*, ochratoxin A, temperature, water activity

Ochratoxin A (OTA) is a mycotoxin produced by several moulds of *Aspergillus* and *Penicillium* genera as a secondary metabolite highly toxic to animals and humans. While OTA is mainly nephrotoxic to animals (Creppy, 1999), it additionally induces immunodepressive, tetrogenic, genotoxic, and carcinogenic effects in humans (IARC, 1993; MacDonald *et al*., 1999; Otteneder and Majerus, 2000; Walker, 2002; O'Brien and Dietrich, 2005). The frequent detection of OTA in the blood serum of healthy humans suggests widespread and continuous exposure of consumers to this mycotoxin through consumption of contaminated foods (Breitholtz-Emanuelsson *et al*., 1993; IARC, 1993). In effect, the occurrence of OTA has been extensively reported in foods including those of plant origin such as cereal products, coffee, spices, beer, wine, grapes, grape juice, dried fruits, and medicinal plants (Zimmerli and Dick, 1996; Bresch *et al*., 2000; Filali *et al*., 2001; Lombaert *et al*. 2002; Belli *et al*., 2005a; Pardo *et al*., 2005; Trucksess and Scott, 2007), which is regarded as a potential hazard to public health even when the contamination level is low. Therefore, the level of mycotoxins in foods has been controlled by regulations and standards at the national, regional or international level to insure the highest possible degree of protection to consumers. In Europe, the level of mycotoxins in foods and feeds is regulated by the Commission of the European Communities (CEC) which has first set the maximum tolerable levels of some mycotoxins among other contaminants in foodstuffs in commission regulation (EC) No 466/2001 of 8 March 2001 (CEC, 2001). However, OTA was not among the mycotoxins specified in this regulation. Subsequent amendments have been made to include OTA, to revise the maximum tolerable level for certain mycotoxins towards more restrictive levels, and to add other commodities to the list of products to be controlled (CEC, 2002, 2004, 2005). In this regard, the maximum tolerable levels of OTA in cereals and their derivatives, and dried vine fruits (currants, raisins, and sultanas) was set to 5, 3, and 10 μg/kg, respectively (CEC, 2002).

In view of the potential hazard due to the occurrence of OTA in foods, preventive measures have been recommended to reduce its incidence and hence the exposure of consumers to it. These include the implementation of policies such as good agriculture, manufacturing, and post-harvest practices to control the growth of mycotoxin-producing moulds and consequently reduce food contamination with mycotoxin to "as low as reasonably achievable" (ALARA) levels (de Koe, 1999). Such measures have indeed been shown to minimize the growth of moulds (Trucksess and Scott, 2007); however, the complex interaction of ecological factors affecting growth and OTA production by toxinogenic moulds makes it difficult to efficiently achieve such a control of exposure. Yet, as

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water activity, temperature, and substrate availability are the primary environmental factors that influence mycotoxin production (Pitt and Hocking, 1997; Ramos *et al*., 1998; Belli *et al*., 2004a, 2004b, 2005b; Esteban *et al*., 2004, 2006a, 2006b; Pardo *et al*., 2004), better knowledge of their effects separately or in combination should help attaining such a goal.

OTA was originally described to be produced by *Aspergillus ochraceus* (Van der Merwe, 1965); however, it was subsequently demonstrated that other species of *Aspergillus* or *Trichocomaceae* (*Penicillium* spp.) may produce also the toxin, under different environmental conditions. While *A. ochraceus* is known to produce OTA in stored foods under warm and tropical climates, *Penicillium verrucosum* has also been reported to produce the toxin under temperate climates, and exclusively in cereals and cereal products. Furthermore, OTA production by *Aspergillus* section *Nigri* was first demonstrated by Abarca *et al*. (1994), and *Aspergillus carbonarius* of this section has been predominantly associated with the production of OTA in grapes, wine, and dried vine fruits (Sage *et al*., 2002; Abarca *et al*., 2003; Battilani *et al*., 2003a, 2003b; Serra *et al*., 2003; Bau *et al*., 2005; Bejaoui *et al*., 2006).

In Morocco, there are no data available, to our knowledge, on the production of OTA by toxigenic fungi in grapes. Such information is important in developing realistic systems or models to evaluate the risk associated to mycotoxin contamination of grapes and derivatives. Nonetheless, Moroccan wines have been reported to be contaminated with OTA at levels ranging between 0.028 and 3.24 μg/L (Filali *et al*., 2001). These results suggest that Moroccan grapes might be contaminated with ochratoxin-A-producing moulds responsible of the subsequent contamination of wine and other derivatives widely consumed in Morocco when the environmental conditions are favourable to mycotoxin production. Recently, ochratoxigenic fungi and ochratoxin A were detected in Moroccan grapes by Selouane *et al*. (2006, 2009). Therefore, the present study aimed to determine the influence of water activity, temperature, and incubation time on growth and OTA production by a total of 12 strains of *Aspergillus* section *Nigri*, viz. *A. tubingensis* (six), *A. niger* (two), and *A. carbonarius* (four) selected among 140 ochratoxigenic isolates of Moroccan grapes.

Materials and Methods

Strains and culture media

All isolates used in this study were deposited at BCCM/MUCL (Mycothèque de l'Université catholique de Louvain, Belgium). Four ochratoxigenic strains of *A. carbonarius* (MUCL 49223, MUCL 49232, MUCL 49233, and MUCL 49345), six of *A. tubingensis* (MUCL 49224, MUCL 49225, MUCL 49228, MUCL 49229, MUCL 49230, and MUCL 49231) and two of *A. niger* (MUCL 49226, MUCL 49227) isolated from Moroccan grapes or Moroccan raisins (one isolate of *A. niger* MUCL 49226) were used in this study. Molecular fungal identification was performed at BCCM/MUCL. The β-tubulin and calmodulin genes were partially sequenced for all strains (Samson *et al.*, 2007). DNA was extracted from freshly collected mycelium grown in liquid malt extract at 25°C in the dark. Extractions were carried out using the QIAGEN Dneasy Plant Mini kit (QIAGEN Inc., Germany), and later purified with GeneclеanÒ III kit (Q-Biogene, USA), following the manufacturer's recommendations. The primer pairs cmd5-cmd6 (Hong *et al*., 2006) and Bt2a-Bt2b (Glass and Donaldson, 1995) were used to amplify a segment of the Calmodulin gene and the 5' end of the β-Tubulin gene, respectively. Successful PCR reactions resulted in a single band observed on a 0.8% agarose gel, corresponding to approximately 550 bp (β-Tub) and 600 bp (Calmodulin). Polymerase chain reaction products were cleaned using the QIAquickÒ PCR Purification kit (250) (QIAGEN Inc., Germany), following the manufacturer's protocol. Sequencing reactions were performed using CEQ DTCS Quick Start KitÒ (Beckman Coulter Inc., USA), according to the manufacturer's recommendations, with the primers cmd5-cmd6 for Calmodulin (Hong *et al*., 2006) and Bt2a and Bt2b for β-tubulin. Nucleotide sequences were determined with a CEQ 2000 XL capillary automated sequencer (Beckman Coulter Inc., USA). Identifications of our isolates were obtained using the BLAST search at GenBank (Altschul *et al*., 1990).

Prior to the study, OTA production by all strains was confirmed *in vitro* on Czapek Yeast Autolysate agar (CYA). The strains (suspensions of spores) were maintained in 25% glycerol-0.01% Tween 80 at -20°C.

Medium and water activity adjustments

Studies were carried out in vitro using a synthetic nutrient medium (SNM) that has a gross chemical composition close to that of grapes between veraison and ripeness as suggested by Belli *et al*. (2004a), and water activity of 0.99. It had the following composition: D(+) glucose, 70 g; D(–) fructose, 30 g; L(+) tartaric acid, 7 g; L(–) malic acid, 10 g; $(NH4)_2SO_4$, 0.67 g; $(NH_4)_2HPO_4$, 0.67 g; KH_2PO_4, 1.5 g; $MgSO_4$-$7H_2O$, 0.75 g; NaCl, 0.15 g; $CaCl_2$, 0.15 g; $CuCl_2$, 0.0015 g; $FeSO_4$-$7H_2O$, 0.021 g; $ZnSO_4$, 0.0075 g; (+) catechin, 0.05 g; agar, 20 g; distilled water, 1 L; pH adjusted to 4.2 with KOH (2 N).

During experiments, the water activity of SNM was adjusted to 0.90 and 0.95 by glycerol according to Belli *et al*. (2004a). The a_w values were, then, verified with an Aqua-lab CX-2 a_w-meter (USA).

Inoculation and incubation

Inocula were prepared by growing each strain on Czapek Yeast Autolysate agar medium (CYA) at 25°C for seven days. Suspensions of spores ($\sim 10^6$ spores/ml) were prepared in sterile distilled water containing 5‰ Tween 80. A Thoma chamber was used to determine the final spore concentrations.

SNM medium (20 ml) was poured into Petri plates, each of which was inoculated with 10 μl of the suspension of the fungal spores (approximately 10^4 spores) as a drop at the centre of the plate. The inoculated plates were into lots of the same a_w and each lot was sealed in the same polyethylene bag. For the three a_w values (0.90, 0.95, and 0.99), six temperatures were used: 10, 15, 20, 25, 30, and 37°C. All the experiments were carried out in triplicate for the growth survey. The petri plates were examined daily, and diameter

Table 1. Optimal temperatures for growth and OTA production, and the mean of OTA produced by *A. tubingensis*, *A. niger*, and *A. carbonarius* isolates on CYA and SNM media

Isolates	Species	Source	OTA (μg/g)		G/P		
			CYA[a]	SNM[b]	0.90 a_w[c]	0.95 a_w[c]	0.99 a_w[c]
MUCL 49224	*A. tubingensis*	grape fruits	0.0524	ND - 0.0458	20-30/30	25-30/30	30-37/25
MUCL 49225	*A. tubingensis*	grape fruits	0.0226	ND - 0.0007	37/37	25/37	37/30
MUCL 49226	*A. niger*	raisins	2.2905	0.0001 - 0.0003	37/37	25/20	37/30
MUCL 49227	*A. niger*	grape fruits	0.1782	ND - 0.0001	20-30/30	25/30	37/30
MUCL 49228	*A. tubingensis*	grape fruits	0.1069	ND - 0.0001	30/37	25-30/37	30-37/37
MUCL 49229	*A. tubingensis*	grape fruits	0.0053	ND - 0.71	20-30/20	25/30	37/25
MUCL 49230	*A. tubingensis*	grape fruits	0.0930	ND - 0.205	20-30/30	25/37	37/25
MUCL 49231	*A. tubingensis*	grape fruits	0.0352	ND - 0.007	20-37/37	25/30-37	37/15
MUCL 49223	*A. carbonarius*	grape fruits	0.3371	0.029 - 0.705	20/30	25/37	37/25
MUCL 49232	*A. carbonarius*	grape fruits	0.0773	0.0002 - 0.0017	20-25/30	25/30-37	15-20/20
MUCL 49233	*A. carbonarius*	grape fruits	0.1376	0.030 - 0.710	20/30	25-37/37	30/30
MUCL 49345	*A. carbonarius*	grape fruits	0.0508	ND - 0.113	20-25/30	25/30	20/25-30

[a] Mean OTA produced on CYA medium at 25°C after 7 days of incubation.
[b] Mean range of OTA produced on SNM at 25°C, 0.99 a_w and after 5~10 days of incubation; ND, not detected.
[c] Optimum temperature for growth (G) and OTA production (P) at different water activity (0.90, 0.95, 0.99).

of colonies was measured in two perpendicular directions. The linear regression of the radius of the colony against time (days) was used to obtain the growth rates (mm/day).

Extraction of OTA from cultures

Ochratoxin A was extracted according to the method of Bragulat *et al*. (2001). Briefly, three agar plugs (diameter=7 mm) were removed from the inner, middle, and outer areas of each colony at regular intervals during incubation (i.e., at 5, 10, 15, and 20 days). Plugs were weighed and dispensed into 3-ml vials before adding 1 ml of methanol. The vials were shaken for 5 sec with an autovortex and incubated standing at 25°C for 60 min. The extracts were centrifuged three times for 10 min at 13,000 rpm; the supernatant filter-sterilized through a PVDF hydrophilic filter (0.22 μm) and then analysed by HPLC.

HPLC analysis of OTA in culture extracts

OTA was quantified by HPLC (Agilent Technologies, USA) with fluorescence detection (excitation 330 nm, emission 460 nm; calibration with commercial OTA). The separation of metabolites was performed on a C18 RP column (Zorbax SB, 4.6 × 150 mm × 5 μm particle size). The mobile phase was pumped at 1.0 ml/min and the injection volume was 20 μl.

During HPLC analysis, OTA was eluted with a mobile phase consisting of a mixture of HPLC grade acetic acid in water 0.2% (A) and acetonitrile (B) at a flow rate of 1 ml/min. The analysis of OTA was done over 45 min with a linear gradient from 10% to 50% of B over the first 30 min followed by a linear gradient to 90% of B from 30 to 35 min,

Table 2. Analysis of variance of the effects of water activity (a_w), different isolates (I), temperature (T), incubation time on days (d), and their interactions on growth rates and OTA production by *A. niger* aggregate (8 isolates) and *A. carbonarius* (4 isolates) on synthetic nutrient medium

Source of variation	Growth study				OTA production study			
	DF[a]	MS[b]	F	P	DF[a]	MS[b]	F	P
Water activity (a_w)	2	60.2061	1106.50	0.0000	2	0.02984	7.68	0.0005
Isolate (I)	11	1.57331	28.92	0.0000	11	0.15887	40.88	0.0006
Temperature (T)	5	88.7330	1630.79	0.0000	5	0.39903	102.68	0.0013
Incubation time on days (d)					3	0.10359	26.66	0.0000
a_w×I	22	2.06325	37.92	0.0000	22	0.19255	49.55	0.0000
a_w×T	10	11.0508	203.10	0.0000	10	0.40507	104.24	0.0000
a_w×d					6	0.12975	33.39	0.0000
I×T	55	1.09550	20.13	0.0000	55	0.19649	50.56	0.0000
I×d					33	0.20720	53.32	0.0000
T×d					15	0.17730	45.63	0.0013
a_w×I×T	110	0.62974	11.57	0.0000	110	0.19025	48.96	0.0000
a_w×I×d					66	0.16487	42.43	0.0000
a_w×T×d					30	0.18041	46.42	0.0000
I×T×d					165	0.16845	43.35	0.0000
a_w×I×T×d					330	0.17356	44.66	0.0000

[a] DF, degree of freedom
[b] MS, mean square

then a steady flow of 90% of B for 8 min finally reduced to 10% for 2 min. Ochratoxin A was identified by its retention time (35∼36 min) according to a standard (Sigma Aldrich, Germany) and by methylation according to Zimmerli and Dick (1995).

Quantification of OTA was done by measuring peak area

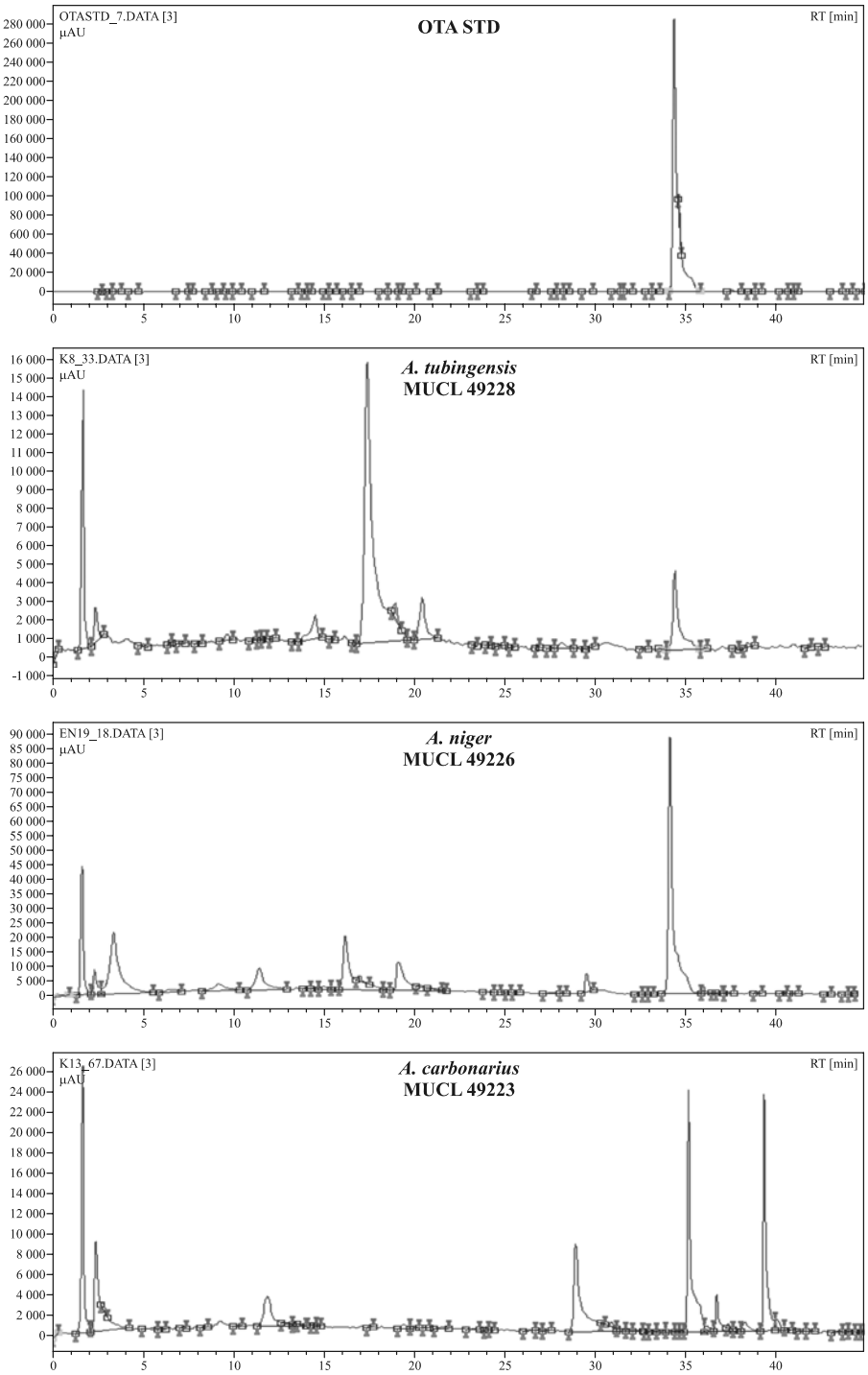

Fig. 1. Typical chromatograms of OTA standard (0.5 µg/ml), OTA produced by *A. carbonarius* MUCL49223 (0.38 µg/g CYA), *A. niger* MUCL49226 (2.29 µg/g CYA) and *A. tubingensis* MUCL 49228 (0.10 µg/g CYA) (isolates grown on CYA medium at 25°C after 7 days) using HPLC-gradient program: 90% A (water/acetic acid 98/2, v/v) and 10% B (acetonitrile), changed to 50% A and 50% B at 30 min, to 10% A and 90% B at 35 min, and then changed after another 7 min to 90% A and 10% B, this last eluent being maintained for 10 min; flow rate: 1 ml/min; Column: C18 reversed-phase column (Zorbax SB, 4.6×150 mm, 5 µm particle size); detection: fluorescence at 330 nm excitation and 460 nm emission. Retention time of OTA was 35∼36 min.

according to a linear standard curve. All analyses were done on duplicate.

Statistical analysis

Linear regression of the radius of the colony against time (days) to determine the growth rates (mm/day) under each set of conditions, was obtained with the program Microsoft Excel version 2003. Analysis of variance for the different sets of growth results and for the amounts of OTA detected were carried out using STATISTIX, version 7.0, for windows.

Results

Identification and mycotoxigenic capacity of fungal isolates

The search with the Blast option at GenBank using the β-tubulin or calmodulin sequences demonstrated homology only with members of the section *Nigri* of *Aspergillus*. In comparison with various sequences of references stains of *A. niger*, *A. carbonarius*, or *A. tubingensis* retrieved from GenBank, the similarity of all our isolates ranged from 100 to 99%, differing then by one to 3 positions.

The ability of *Aspergillus* isolates to produce OTA was evaluated on a solid laboratory medium (CYA) after seven days of incubation and at 25°C. The extracts of culture media from all isolates produced peaks corresponding to the retention time of OTA (35~36 min). Typical chromatograms of OTA standard, OTA produced by *A. tubingensis* (MUCL 49228), *A. niger* (MUCL 49226), and *A. carbonarius* (MUCL 49223) were shown in Fig. 1. The identity of the OTA in each of these extracts was also confirmed by methylation and comparison with retention time of methylated-OTA standard. The OTA producing capacity of the twelve strains in both CYA and SNM media were presented in Table 1.

Effects of water activity and temperature on the growth rate

Combined effects of water activity (a_w) and temperature (T) on radial growth were investigated for three toxigenic species (twelve isolates: I) on SNM. Statistical analysis of variance (ANOVA) showed that all single factor (water activity, isolate and temperature) as well as two- and three-way interactions had a high significant influence on the growth rates of *A. tubingensis*, *A. niger*, and *A. carbonarius* (Table 2).

The interaction of a_w and temperature on the growth rate (mm/day) of the assayed *A. tubingensis*, *A. niger*, and *A. carbonarius* isolates on SNM agar revealed that optimal conditions for the growth of most studied strains were shown to be at 25°C and 0.95 a_w. Under these conditions the maximum growth rate varied between 4.7~5.3, 4.8~5.2, and 4.7~5.1 mm/day for species *A. tubingensis*, *A. niger*, and *A. carbonarius*, respectively (data not shown). No growth was observed at 10°C, regardless of the a_w and isolates. For *A. tubingensis* MUCL 49224, the maximum growth rate was also observed at 0.95 a_w and 30°C (5.0 mm/day). Three isolates MUCL 49228 (*A. tubingensis*), MUCL 49233 (*A. carbonarius*), and MUCL 49223 (*A. carbonarius*) had an other maximal growth rate at 0.99 a_w and 30°C~37°C, 30°C, and 25°C~37°C range of temperature, respectively. All twelve strains grew significantly slower at 15°C than in the 20°C~37°C range, regardless of the water activity. Mean growth rates obtained by all the strains in the species of *A. niger* aggregate (8 isolates) and *A. carbonarius* (4 isolates) at each a_w value and temperature tested (Fig. 2) showed that the optimum a_w for fungal growth was 0.95. The increase of temperature in the range of 15°C~25°C increased the growth rate for most isolates of three species (*A. tubingensis*, *A. niger*, and *A. carbonarius*).

Environmental factors affecting OTA production *in vitro* on SNM

Statistical analysis of variance (ANOVA) showed that the effects of all single factors, water activity (a_w), temperature (T), and incubation time (d), and their two, three- or four-way interactions were highly significant for OTA production (Table 2).

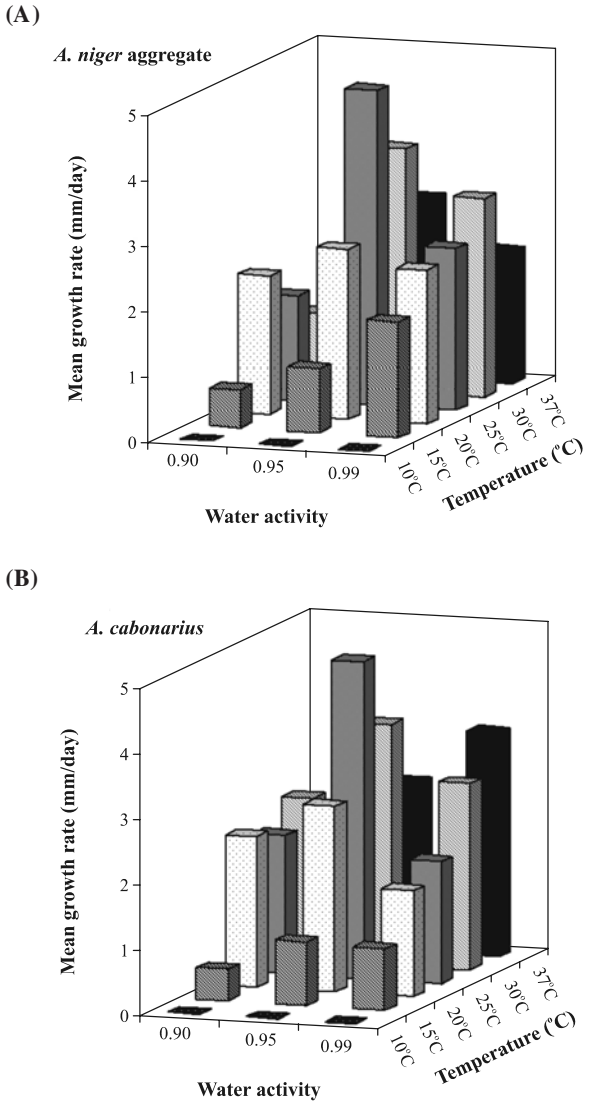

Fig. 2. Mean growth rates obtained by all the strains in the species of *A. niger* aggregate (6 isolates of *A. tubingensis* and 2 isolates of *A. niger*) and *A. carbonarius* (4 isolates) at each a_w value and temperature tested.

The results of OTA production by each of the fungal strains tested in this study showed that optimal conditions for OTA production as well as the maximum attainable level varied according to isolates (data not shown). For *A. niger* aggregate isolates, OTA production was maximal at temperatures ranging between 30°C and 37°C, and a_w values of 0.90~0.95. For *A. carbonarius* a_w values of 0.95~0.99 were optimal. Two isolates, however *A. tubingensis* (MUCL 49229) and *A. carbonarius* (MUCL 49223) produced maximum OTA at 25°C and an a_w of 0.99. The maximum OTA produced was 3.7 μg/g, by *A. tubingensis* (MUCL 49231) at 0.90 a_w and 37°C after 5 days, whereas the maximum OTA produced by *A. carbonarius* (MUCL 49233) was 1.3 μg/g at 0.99 a_w and 30°C after 20 days.

Mean OTA concentration produced by all the isolates of *A. niger* aggregate at all sampling times (Fig. 3) shows that maximum OTA (0.24 μg/g) was produced at the higher temperature of incubation (37°C) and lower water activities (0.90 a_w). At 30°C~37°C, the amount of OTA produced decreased at higher water activity (0.99); the opposite was observed at 20°C~25°C. However, for the four *A. carbonarius*, mean maximum amount of OTA (0.22 μg/g) was produced at higher water activity (0.99) and 25°C (Fig. 3). High concentrations of OTA were also favoured by low water activity (0.95) at 30°C~37°C. For the most isolates, the optimal conditions for growth significantly differ from the optimal conditions for OTA production (Table 1). For example, at 0.90 a_w, the optimal growth rates of *A. carbonarius* were obtained at 20°C or 20°C~25°C, whilst OTA production at this water activity was optimal at 30°C. *A. niger* aggregate showed optimal conditions of OTA production at high temperature 30 or 37°C (except for *A. tubingensis* MUCL 49229: 20°C) and optimal growth rates were obtained at 20°C~30°C (4 isolates), 20°C~37°C (one isolate), 37°C (2 isolates), and 30°C (one isolate).

The results of Table 1 show the strong impact of the composition of the culture medium. The maximum level of OTA production by *A. niger* (MUCL 49226) was 2.3 μg/g on CYA (25°C at day 7). Nonetheless, the same strain produced significantly smaller amounts (0.07 to 8.7 ng/g) of OTA on SNM medium under the same temperature and during 5 to 10 days of incubation, regardless of the water activity value. Similar results were obtained for all strains of *A. niger* aggregate and *A. carbonarius* studied herein.

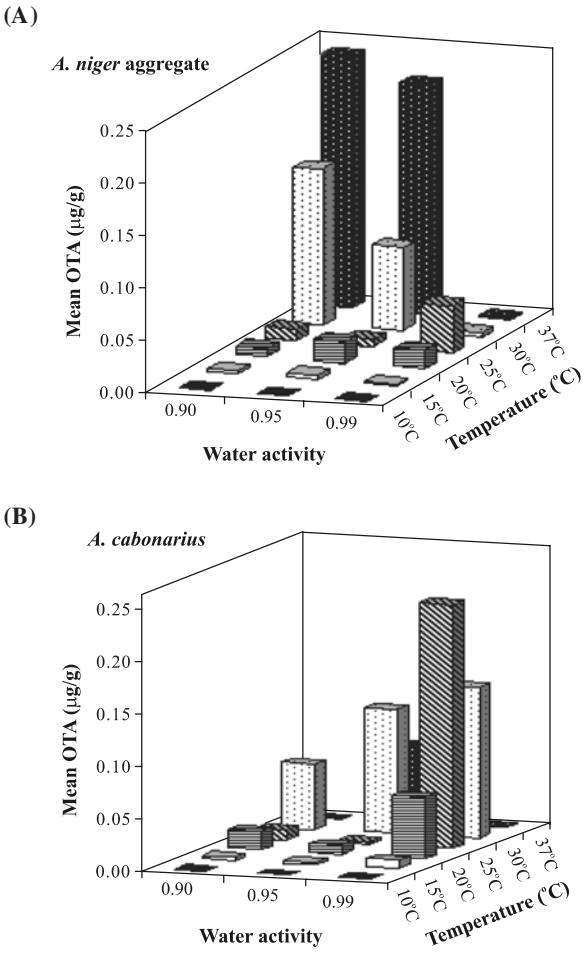

Fig. 3. Mean OTA concentration produced at all sampling times by all the strains in the species of *A. niger* aggregate (6 isolates of *A. tubingensis* and 2 isolates of *A. niger*) and *A. carbonarius* (4 isolates) at each a_w value and temperature tested.

Discussion

The results of the present study demonstrate that the water activity, temperature and their combinations have a significant influence on the growth and ochratoxin A production by strains of *A. tubingensis* (six), *A. niger* (two), and *A. carbonarius* (four) grown on Synthetic Nutrient Medium (SNM). The optimum conditions for the growth and OTA production by Moroccan isolates compared to those of the same species isolated from different regions from Europe, Australia and Argentine are summarised in Table 3. In this study, at most combinations of temperature and water activity tested, all isolates of *A. niger* aggregate and *A. carbonarius* have a similar growth rate with a maximum (4.7~5.3 mm/day) at an a_w of 0.95 and a temperature of incubation of 25°C. The growth by Australian isolates of *A. niger* was significantly faster than growth by *A. carbonarius* isolates at 30°C and 35°C whereas at 20°C and 15°C, growth achieved by some isolates of both species was similar at most of water activities (Leong, 2005). An optimal a_w of 0.95 for the growth of nine Moroccan isolates was observed at 25°C and in the range of 25°C~30°C or 25°C~37°C for the others. Such behaviour of these isolates regarding water activity and temperature requirements was in accordance to that reported on Argentinean strains of *A. carbonarius* isolated from dried vine fruit, showing maximal growth rates of 15~17.46 mm/day at 0.95 a_w and 30°C (Romero *et al.*, 2007). The same optimal conditions were reported by Hajjaji *et al.* (2006) for *A. niger* isolated from Moroccan cereal grains. Whereas, the growth by European and Australian isolates of *A. niger* aggregate was favoured by higher water activities and temperatures (0.98 aw, 30°C~37°C). At optimal temperature (Fig. 2), reduction of water activity from 0.95 to 0.90 has resulted in a 56% decrease in the growth rate for *A. niger* aggregate, as compared with a 67% decrease for *A. carbonarius*. Such a greater tolerance of *A. niger* than *A. carbonarius* for re-

Table 3. Optimal conditions for growth and OTA produced by Moroccan isolates compared to the literature

Isolates[a]	Optimal conditions (OC) for growth (or maximum growth)	Optimal conditions for OTA production	References
Moroccan isolates - *A. tubinegensis* (6) - *A. niger* (2) - *A. carbonarius* (3) Medium : SNM	OC (three species): 0.95 a_w; 25-30°C Maximum growth rate ***A. niger*** : 4.8-5.2 mm/day ***A. tubingensis*** : 4.7-5.3 mm/day ***A. carbonarius*** : 4.7-5.1 mm/day	***A. tubingensis & A. niger*** OC: 0.90-0.95 a_w; 30-37°C Mean Max OTA at 0.90 a_w; 37°C ***A. carbonarius*** OC: 0.95-0.99 a_w; 25-30°C Mean Max OTA at 0.99 a_w; 25°C	Present study
European isolates - *A. niger* aggregate: (8)[b,c], (2)[d,e] - *A. carbonarius*: (6)[b,c], (2)[d,e], (8)[b,f] Medium : SNM	***A. niger* aggregate**[c,e] : 0C : 0.98 a_w ; 30-37°C ; Max growth rate : 6.14-9.34 mm/day ***A. carbonarius***[c,e] : 0C : 0.98 a_w ; 30-37°C; Max growth rate : 3.45-9.11 mm/day ***A. carbonarius***[f] : Optimal temperature: 30°C; Max growth rate at 0.95-0.99 a_w: 6.83-10.1 mm/day	***A. niger* aggregate**[e] Optimal a_w: 0.95-0.98; Max OTA at 0.98-0.995 a_w after 7-13 days ***A. carbonarius***[e] Optimal a_w: 0.95-0.995; Max OTA at 0.96 after 5 days ***A. carbonarius***[f] OC: 20°C; 0.95 or 0.99 depending on the isolates	Belli *et al.* (2004a, 2004b, 2005b)
- *A. niger* aggregate (12) - *A. carbonarius* (6) Medium: CYA, YES	**Not reported** **Conditions for growth** ***A. niger aggregate*** : at 25°C a_w range: 0.86-0.99 ***A. carbonarius*** : 30°C/0.82-0.99 a_w; 15°C/0.94-0.99 a_w	Each strain differed in its optimum conditions ***A. niger* aggregate** Max OTA production at 20-25°C; 0.98-0.99 a_w ***A. carbonarius*** Max OTA production at 15-20°C; 0.98-0.99 a_w	Esteban *et al.* (2004, 2006a, 2006b)
- *A. carbonarius* (8)[g] Medium : SGM	0C: 30-35°C; optimum a_w varied form 0.93 to 0.987 depending on the strain; Max growth rate: 5-10 mm/day	OC for OTA production varied with strain; for some strains: 15-20°C; 0.95-0.98 a_w	Mitchell *et al.* (2004)
Australian isolates - *A. niger* (2) - *A. carbonarius* (5) Medium: SGM	***A. niger*** OC : 0.98 a_w; 35°C; Max growth rate: 9.76-11.35 mm/day ***A. carbonarius*** OC: 0.965 a_w; 30°C; Max growth rate: 5.87-7.92 mm/day	***A. niger*** OC : 0.95 a_w; 15°C; Max OTA at 0.95 a_w; 15°C ***A. carbonarius*** OC : 0.95-0.98 a_w; 15°C; Max OTA at 0.965 a_w; 15°C	Leong *et al.* (2006) Leong (2005)
Argentinean isolates - *A. carbonarius* (4); Medium : CYA	Conditions for maximum growth rate: 0.95 aw; 30°C Max growth rate: 17.46 mm/day	**OTA not analyzed**	Romero *et al.* (2007)

[a] Isolates of *A. carbonarius* and *A. niger* aggregate (number of isolates) tested for effect of one or different parameters (water activity, temperature and incubation time) on growth and/or OTA production; [b] isolates from Italy, France, Portugal and Spain; [c] Belli *et al.* (2004a); [d] isolates from Italy, Portugal and Spain. [e] Belli *et al.* (2004b); [f] Belli *et al.* (2005b); [g] Isolates from Italy, Portugal, Israel and Greece. CYA: Czapek Yeast Autolysate agar; SNM: Synthetic Nutrient Medium; SGM: Synthetic Grape juice Medium; YES: Yeast Extract Sucrose agar.

duced water activity has been reported by some authors (Belli *et al.*, 2004a; Mitchell *et al.*, 2004; Leong *et al.*, 2006). In general, according to the results of published data, the strains from Argentine, Europe, and Australia appear to grow at faster rates than the Moroccan isolates (Table 3).

Furthermore, our results showed that the optimal conditions for the growth of fungal strains differ from those for OTA production (Table 1). The similar conclusions were also reported by Mitchell *et al*. (2004). As shown in Fig. 3, *A. niger* aggregate strains produced maximum OTA at 37°C and an a_w lower than 0.95, while its growth rate was highest at 25°C and an a_w of 0.95 (Fig. 2). *A. carbonarius* produced a maximum OTA at high water activity (0.99) and 25°C while its maximum growth rate was achieved at 0.95 a_w and 25°C. At 0.95 a_w, the mean maximum concentration of OTA was produced at 30°C. In contrast, according to Belli *et al.* (2004b) *A. niger* aggregates, isolated from both Spanish and Portuguese grapes, produces maximum amounts of OTA at higher a_w values (0.98 to 0.995) after 7∼13 days while maximum OTA accumulation produces by *A. carbonarius* isolated from Italian and Spanish grapes were shown to be at 0.96 a_w combined with an incubation time of 5 days (estimated response surface). Moreover, the Moroccan isolates tested in this study yielded very different results as regards their temperature requirements. The optimal temperature for OTA production ranged between 25°C and 30°C for *A. carbonarius* and 30°C and 37°C for *A. niger* aggregate (Fig. 3). However, other studies have reported that optimal temperature for OTA production was in the range of 15°C to 25°C (Table 3). According to Esteban *et al.* (2004) and Mitchell *et al.* (2004) the optimal temperature range for OTA production by *A. niger* (from grapes in Spain) and *A. carbonarius* (from grapes in Europe and Israel) were 20°C∼25°C and 15°C∼20°C, respectively. The increase in the level of OTA production at relatively low temperatures was also reported by Battilani *et al.* (2004) who found that 20°C was more favourable to OTA production than 25°C in Italian grapes artificially inoculated with *A. carbonarius*. Leong *et al.* (2006) also

noted that the same species produced significantly higher amounts of OTA at 15°C than at temperatures above 25°C. At 15°C and growth rates in the range of 0~2.1 mm/day, some Moroccan strains were able to produce OTA, though at low levels (0~37 ng/g), depending on the water activity and incubation time.

The incubation time was another critical factor influencing OTA production. Table 2 shows that the effects of the incubation time alone; the two-factor combinations of temperature, isolate and incubation time (aw*I, aw*d and T*d) or the three-factor combinations (a_w*I*d, a_w*T*d) on the production of OTA were highly significant ($P<0.0013$). Under most of our conditions, OTA levels appeared to increase with increasing incubation time. Yet, in some instances, the amount of OTA detected decreased markedly between 5 and 20 days of incubation. Such a decrease could be explained by a partial loss of the toxin upon degradation by the producer strain itself, as suggested by Varga *et al.* (2002), who has demonstrated that aspergilli transform OTA of the growth medium into other derivatives, such as ochratoxin α, upon extended period of incubation. A similar decrease in the OTA concentration with increasing incubation time was reported for strains of *A. carbonarius* and *A. niger* aggregate by Belli *et al.* (2004b). These authors have also demonstrated that the effect of incubation time was shown to affect OTA production in a strain-dependant manner: *A. carbonarius* has produced maximum levels of OTA after 5 days of incubation, while *A. niger* aggregate needed 7~13 days to achieve such a level when the same temperature (25°C) and culture medium (synthetic nutrient medium) were used. Similarly, Esteban *et al.* (2004) also studied the effect of incubation time (5~30 days) at different temperature (5°C~45°C), by six *A. niger* aggregate and four *A. carbonarius* isolates on YES and CYA. The maximum OTA accumulation in their study varied depending on the strain, and 5 days of incubation were sufficient for OTA detection, but some isolates required 10~30 days to reach maximum accumulation.

This study confirms the influence of water activity, temperature, and incubation time and their interaction on growth and ochratoxin A production. Due to the ability to produce OTA at a wide range of temperature and water activities, *A. tubingensis*, *A. niger*, and *A. carbonarius* could be considered as the most probable source of OTA in Moroccan grapes. No growth was observed at 10°C, regardless of the water activity and isolates. At 15°C~25°C, the growth rates and the amounts of OTA produced by all isolates were low. The most efficient way to protect consumers against OTA health hazards is therefore to minimize grapes accumulation at high temperature levels between harvest grapes and making process of derived products and implement good agricultural and post harvest practices.

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