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# Occurrence of mycotoxins (ochratoxin A, deoxynivalenol) and toxigenic fungi in Moroccan wheat grains: impact of ecological factors on the growth and ochratoxin A production

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The aim of the present work was to evaluate the contamination of some samples, taken from Moroccan wheat grains, by ochratoxin A (OTA), deoxynivalenol (DON) and the associated toxigenic fungi. Moreover, we focused on the influence of environmental factors on both the growth and OTA production by three strains of *Aspergillus*. The results showed that only few samples were contaminated by the two mycotoxins (2 samples for OTA and 7 for DON). The main isolated fungi belong to the *Aspergillus*, *Penicillium* and *Fusarium* genus; 74 *Aspergillus* and 28 *Penicillium* isolates were tested for their ability to produce OTA. Only 2 *A. alliaceus* and 14 *A. niger* were able to synthesize OTA. However, none of *Penicillium* isolates can produce this toxin under the conditions mentioned. In respect of the effects of the temperature and water activity (*aw*), the optimal conditions for the growth and OTA production were different. While the optimal conditions of growth for *A. alliaceus* and *A. terreus* are 30°C and 0.98 *aw, A. niger* prefered 0.93 – 0.95 *aw* at 25°C, whereas the optimal production of OTA was observed at 30°C for both *A. alliaceus* and *A. niger* at 0.93 and 0.99 *aw*, respectively.

**Keywords:** Ochratoxin A / Deoxynivalenol / *Aspergillus* / Water activity / Growth rate

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## 1 Introduction

Fungal growth is one of the main causes of food spoilage. It not only generates great economic losses, but also represents a threat to human and animal health, particularly through the synthesis of mycotoxins. Ochratoxin A (OTA) and deoxynivalenol (DON) seem to be the most important mycotoxins, because of the diversity of products that they contaminate and their large geographic distribution [1 – 4].

OTA is a mycotoxin regularly present in the stored grains [5, 6]. It is produced mainly by *Penicillium verrucosum* in temperate climates [7] and by certain species of *Aspergillus* in warm climates [8]. This toxin has a nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties [9 – 11]. Many studies have shown that cereals and cereal products are the main sources of OTA [1, 12, 13]. It has also been detected in other products such as green coffee, milk, wine and grape juice [14 – 16].

DON is considered as the main contaminant of cereals and their derivatives [17]. It is frequently detected in the high concentrations worldwide [18] and is synthesized mainly by *Fusarium graminearum* and *Fusarium culmorum* [19]. DON presents various toxicological characteristics, such as skin irritability, hemorrhagic syndrome, diarrhea, nausea, feed refusal and vomiting [20], and may be immunosuppressive [21].

The fungal growth and the production of mycotoxins are greatly influenced by temperature, water activity (*aw*) and incubation time [22 – 26]. Pardo *et al.* [23] reported that the optimal conditions for both the growth of *A. ochraceus* on

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**Abbreviation: aw**, water activity ; CYA, Czapek yeast autolysate agar; **DI**, days of incubation; **DON**, deoxynivalenol; **OTA**, ochratoxin A; **WMEA**, wheat meal extract agar

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barley grain and its production of OTA were 0.99 *aw* and 30°C, and a decrease in *aw* level from 0.99 to 0.95 resulted in a sharp reduction of the growth rate and a marked decrease in concentration of OTA produced.

The aim of the present work was to evaluate the contamination of some samples of Moroccan wheat grains by OTA, DON and associated fungal flora, and to study the ecology of two ochratoxigenic strains among the screened strains isolated from wheat grains.

## 2 Material and methods

### 2.1 Analysis of OTA in wheat grain samples

Seventeen samples (1 kg) of wheat grain stored (in bulk or in bugs) in farm warehouses in the region of Beni-Mellal were collected for the analysis of OTA and DON, and for isolation of toxigenic fungi.

The extraction and analysis of OTA from ground samples of wheat grains were conducted with spiked samples as described by Scudamore *et al*. [27]. The extracts were analyzed by HPLC (Waters 600). The mobile phase was (ACN/water/acetic acid, 45 : 54 : 1) pumped at a flow rate of 1 mL/min. The column (Nucleosil 100 C18, 250 mm × 4.6 mm × 5 µm; Alltech) and a fluorescence detector (Shimadzu RF535) with excitation wave length set at 333 nm and emission wavelength at 460 nm; 20-µL samples were injected. The recovery rate was (93%), the detection limit of the method was 0.2 µg/kg (three times the electronic base line noise) and the quantification limit was 0.35 µg/kg.

### 2.2 Analysis of DON in wheat grain samples

DON extraction was done with 10 g ground wheat samples blended with a mixture of ACN/water (80:20) for 3 min using an Ultra Turrax at 9500 tour/min. The mixture was centrifuged at 2000 rpm for 3 min. Then 10 mL supernatant were purified on a Mycosep 227 (Coring system), and 7 mL portion of the eluate were recuperated and evaporated under nitrogen flow N28 at 40°C. Subsequently, a mixture of 1 mL dimethylaminopyridine solution and 50 µL heptafluorobutyric anhydride (both Sigma) was added to the obtained residue. After agitation, the mixture was placed in Duran tubes at 60°C for 20 min, and 1 mL 3% $NaHCO_3$ (Merck) aqueous solution was added. After centrifugation and elimination of the lower aqueous phase, 1 mL ultra pure water was added. After centrifugation, the upper organic phase was recovered, and 0.5 µL were injected into the gas chromatograph system, a Chrompack CP9001 coupled to a $^{63}Ni$ electron capture detector (GC-ECD), and equipped of an split/ splitless injector and an integrator (Shimadzu CR8A). The analysis of DON was carried out on a capillary column CP SIL 5 CB, 45 m × 0.32 mm × 1.2 µm film thickness). The oven temperature was maintained at 170°C for 5 min, and increased of 2°C/min to 200°C and then of 50°C/min to 260°C. The oven was maintained to this temperature for 20 min. The flow rate of nitrogen gas was 1.2 mL/min. Temperatures of the injector and the detector are respectively 260°C and 300°C. All samples were analyzed in duplicate and the injection was done in Split mode. The recovery rate was 88%. The detection limit of the method was 50 µg/kg and quantification method was 85 µg/kg.

### 2.3 Fungal isolation and identification

Each sample (20 g) was surface disinfected for 2 min with 0.2% sodium hypochlorite solution and rinsed three times with sterile distilled water. From each sample, 60 grains were randomly selected and then plated in petri plates (90-mm diameter, 10 grains/dish) containing malt extract agar (MEA) or Czapek agar (CZA) medium. Petri plates were incubated at 25°C for 6 – 10 days. Fungi present in each grain were re-isolated on CZA and MEA medium. *Aspergillus* was identified according to Raper and Fennell [28], *Penicillium* according to Pitt [29] and *Fusarium* according to Burgess *et al*. [30].

### 2.4 Ability of *Aspergillus* and *Penicillium* strains to produce OTA

The isolates belonging to *Aspergillus* (74 isolates) and *Penicillium* (28 isolates) genus were evaluated using a previously described HPLC screening method [31]. Briefly, the isolates were grown on Czapek yeast autolysate agar (CYA) medium and incubated at 25°C for 7 days. Three plugs (7-mm diameter) were removed from the inner, middle and outer areas of each colony. Plugs were weighed and introduced into 3-mL vials; 1 mL methanol was added and the vials were shaken and incubated at 25°C for 60 min. The extracts were centrifuged three times for 10 min at 13 000 tour/min and supernatants were filtered (PVDF hydrophilic filter, 0.45 µm) and then separated by HPLC.

### 2.5 Analysis of OTA in culture extracts by HPLC

OTA was quantified by HPLC (Biotek, Italy) 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), connected to a guard column (4 × 10 mm) and filled with the same stationary phase. The mobile phase was pumped at 1.0 mL/min and the volume of injection was 80 µL. For a first screening and

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to select strains susceptible to produce OTA, an isocratic mobile phase mode (ACN/acetic acid/water, 54 : 2 : 44) was used. The retention time for OTA was 5 min. The confirmation of OTA selection was done by production of OTA methyl ester according to the method described by Zimmerli and Dick [32]. The retention time of the OTA methyl ester was 37 min.

### 2.6 The influence of ecological factors on growth and OTA production

#### 2.6.1 Culture medium preparation and *aw* adjustments

The basic medium used was a 3% wheat meal extract agar (WMEA) prepared according to Ramos *et al.* [22]. The *aw* of the basal medium was 0.99 adjusted for the other media to 0.98, 0.95, 0.93, 0.90 and 0.88 *aw* by adding increasing quantities of glycerol [33]. The *aw* values were verified with an *aw* meter (Aqua-lab, CX-2. Washington, USA).

#### 2.6.2 Inoculation, incubation, and measurement of growth

Three strains of *Aspergillus*: *A. alliaceus* (MUCL 46784), *A. niger* (MUCL 46785) and *A. terreus* (MUCL 46786), deposited in the Catholic University of Louvain culture collection (Mycothèque de l'Université Catholique de Louvain "MUCL", Louvain-La-Neuve Belgium), were incubated at 25°C for 7 – 14 days on CYA medium. Suspensions of spores were prepared in sterile distilled water containing 0.1% Tween 80. A Thoma chamber was used to determine final spore concentrations (about $10^6$ spores/mL).

WMEA medium (20 mL) were poured into petri plates (90-mm diameter), each of which was point-inoculated at the center with $10^4$ spores (approximately 10 µL spore suspension according to the concentration). The petri plates with the same *aw* were sealed in a same polyethylene bag. For the six *aw* values, five temperatures were used: 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 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).

OTA was extracted after 7, 15, and 21 days of incubation (DI) according to the method of Bragulat *et al.* [31], as described previously (see Section 2.4) and then analyzed by HPLC. All analyses were done on duplicate, except at 30°C and 0.93 *aw* for which five replicate were conducted.

The growth of the three tested isolates is very slow at 15°C after 7 and 15 DI, and the colonies diameter remains very small, so the plugs were taken at up to 21 days and 32 DI.

## 3 Results

Seventeen samples of wheat grains, stored in the region of Beni Mellal, were tested for OTA and DON contamination and the presence of fungi producing these toxins. Table 1 shows that only 2 samples (11.76%) of the 17 were contaminated by OTA, at mean concentration of 29.4 µg/kg. However, 7 samples (41.17%) were contaminated by DON at mean concentration of 65.9 µg/kg.

**Table 1.** Percentage of positive samples contaminated by OTA and DON, and the mean concentration of these toxins in 17 wheat grains samples

| Mycotoxins | OTA | DON |
|---|---|---|
| Positive samples[a)] | 2 | 7 |
| Positive samples (%) | 11.76 | 41.17 |
| Concentration (µg/kg) | | |
| Maximum | 30.6 | 128 |
| Mean of all samples | 3.5 | 27.1 |
| Mean of positives | 29.4 | 65.9 |

a) LOD for DON is 50 µg/kg and for OTA is 0.2 µg/kg.

A total of 198 strains were isolated from wheat grains samples, representing seven genera. The most important of them were *Aspergillus* (37.4%), *Penicillium* (14.1%) and *Fusarium* (9%). *Aspergillus* isolates belonged to the species of *A. niger*, *A. flavus*, *A. terreus*, *A. nidulans*, *A. ochraceus*, and *A. alliaceus*. *Fusarium* species were *F. verticilloides*, *F. nygamai* and *F. equiseti*. However, no *Penicillium verrucosum* was isolated. In this study, we have focused only on the study of OTA-producing strains within *Aspergillus* and *Penicillium.* DON-producing strains were not taken into account because the isolated *Fusarium* species are recognized as fumonisins and not DON producers.

Seventy-four *Aspergillus* and 28 *Penicillium* isolates are tested for their ability to produce OTA on CYA medium after 7 DI at 25°C. As shown in Table 2, none of the 28 *Penicillium* produced OTA under these conditions, whereas

**Table 2.** Species of *Aspergillus* and *Penicillium* screened for production of OTA and percentage of ochratoxigenic strains of each species

| Strain | Screened strains | Ochratoxigenic strains (%) | OTA produced (µg/g)[a)] |
|---|---|---|---|
| *A. alliaceus* | 2 | 100 | 1.41 – 9.7 |
| *A. niger* | 30 | 47 | 0.08 – 0.14 |
| *A. ochraceus* | 6 | 17 | 0.16 |
| *A. flavus* | 15 | 0 | ND |
| *A. nidulans* | 7 | 0 | ND |
| *A. terreus* | 14 | 0 | ND |
| *Penicillium sp* | 28 | 0 | ND |
| Total | 102 | 17 | |

a) OTA produced on CYA after 7 DI at 25°C; ND, not detected.

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**Table 3.** Optimum *aw* for growth and OTA production, and the mean of OTA produced at each temperature tested after 7, 15 and 21 DI

| Strains | | *A. alliaceus* | | *A. niger* | | *A. terreus* | |
|---|---|---|---|---|---|---|---|
| Temperature (°C) | Incubation (days) | G/P[a)] | OTA (μg/g)[b)] | G/P | OTA (μg/g) | G/P | OTA (μg/g) |
| 15°C | 21 | 0.98/– | – | 0.98/0.99 | 0.062 | 0.98/– | – |
| | 32 | 0.98/0.9 | 0.14 | 0.98/0.99 | 0.176 | 0.98/– | – |
| 20°C | 7 | 0.98/0.93 | 0.005 | 0.98/0.99 | 0.063 | 0.95–0.98/– | – |
| | 15 | 0.98/0.9 | 0.7 | 0.98/0.99 | 0.17 | 0.95–0.98/– | – |
| | 21 | 0.98/0.88 | 16.52 | 0.98/0.99 | 0.34 | 0.95–0.98/– | – |
| 25°C | 7 | 0.98/0.9 | 0.9 | 0.93–0.95/0.99 | 0.27 | 0.98/– | – |
| | 15 | 0.98/0.9 | 0.2 | 0.93–0.95/0.99 | 0.282 | 0.98/– | – |
| | 21 | 0.98/0.88 | 2.18 | 0.93–0.95/0.99 | 0.532 | 0.98/– | – |
| 30°C | 7 | 0.98/0.9 | 3.34 | 0.93/0.99 | 0.916 | 0.98/– | – |
| | 15 | 0.98/0.93 | 17.77 ± 1.59 | 0.93/0.99 | 0.376 | 0.98/– | – |
| | 21 | 0.98/0.93 | 45.65 ± 3.52 | 0.93/0.99 | 0.051 | 0.98/– | – |
| 37°C | 7 | 0.98 or 0.93/0.88 | 0.36 | 0.98/0.99 | 0.057 | 0.93/– | – |
| | 15 | 0.98 or 0.93/0.9 | 1.15 | 0.98/0.99 | 0.075 | 0.93/– | – |
| | 21 | 0.98 or 0.93/0.93 | 2.96 | 0.98/0.88 | 0.095 | 0.93/– | – |

a) Optimum *aw* for growth (G) and OTA production (P).
b) Average of two determinations (except at 30°C and 0.93 *aw* for which average ± SD was calculated for five replicates).

2 *A. alliaceus* (100%), 14 out of 30 *A. niger* (47%) and 1 out of 6 *A. ochraceus* (17%) tested were able to synthesize this toxin under these conditions. The amounts of OTA varied from 0.08 to 0.14 μg/g for *A. niger* and 1.41 to 9.7 μg/g for *A. alliaceus*, and 0.16 μg/g for *A. ochraceus*.

For the survey of the impact of ecological factors on growth and OTA production, three *Aspergillus* strains were selected: one *A. alliaceus* strain (MUCL 46784) as the highest OTA producer, one *A. niger* strain (MUCL 46785) as a moderate OTA producer and finally a non-toxigenic strain *A. terreus* (MUCL 46786). The growth of the three isolates depends on the interaction of temperature, *aw* and incubation time.

The optimal conditions for growth on WMEA medium are 30°C at 0.98 *aw* for *A. alliaceus*; whereas, *A. niger* prefers 25°C at 0.93–0.95 *aw.*

The growth is slower for the three isolates at 15°C than at 20–37°C whatever the *aw.* At 20°C the increase in *aw* from 0.88 to 0.98 leads to linear increase in growth rate for the three isolates. Similar results are obtained at 25°C for *A. alliaceus* and *A. terreus.* The optimum conditions for growth and OTA production are summarized in Table 3.

The optimal *aw* for growth of non-ochratoxinigenic *A. terreus* was between 0.93 and 0.98, whatever the temperature used; and the optimal temperature was 30°C. The optimum *aw* level for the growth of *A. alliaceus* (MUCL 46784) at 15–30°C was 0.98 *aw* regardless of incubation time. However, at 37°C, this optimum occurs at 0.98 or 0.93 *aw.* Whereas the optimum *aw* level for OTA production was at the range 0.88–0.93, the maximum OTA production (45 μg/g) occurred at 30°C after 21 DI at 0.93 *aw.* An significant amount of OTA (16 μg/g) was also produced at 20°C after the same period at 0.88 *aw.* However, at 15°C the strain did not produce OTA up to 32 DI.

*A. niger* (MUCL 46785) presents some variation concerning optimum *aw* for growth. It preferred 0.98 *aw* only at high (37°C) and low (15 and 20°C) temperatures regardless of the incubation time. However, at moderate temperature, it preferred 0.93–0.95 *aw* at 25°C and 0.93 *aw* at 30°C. For OTA production, *A. niger* (MUCL 46785) always preferred a higher *aw* level (0.99) regardless of the temperature and incubation time. The optimum temperature range for OTA production was between 25 and 30°C. The maximum OTA produced (0.916 μg/g) was at 30°C after 7 DI. At low temperature (15°C), *A. niger* was able to produce OTA after 21 DI.

## 4 Discussion

This study shows, on the one hand, that the mean concentration of OTA in wheat grain samples (29.4 μg/kg) was above the limit of OTA fixed by legislation in cereals (3 μg/kg) [34], and the mean concentration of DON (65.9 μg/kg) was below both to the LOQ and the limit proposed currently by legislation (500 μg/kg) [35], showing that the two toxins could be present in Moroccan wheat and may constitute a risk to human health. On the other hand, the strains that could be responsible for OTA production in Moroccan wheat grain samples are *A. alliaceus*, *A. niger* and *A. ochra-*

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*cesus*. These species have already been reported as ochratoxigenic fungi in cereal grains [13, 36]. In addition, the ecological study showed that the growth rates of the three isolates increase with an increase of *aw*, except for highest *aw* 0.98 – 0.99 at which the growth rates showed a sharp decrease at all temperatures tested. Similar results were obtained by Ramos *et al*. [22]. The optimal *aw* level for growth of *A. alliaceus* (MUCL 46784) and *A. terreus* (MUCL 46786) is similar to that of *A. ochraceus* strains [22, 23]. In contrast to *A. ochraceus* [22], all the three Moroccan strains are able to grow at 37°C whatever the *aw*. A reason could be that our isolates are adapted to high temperature conditions. At 15°C, the minimal *aw* levels required for growth are in the range 0.88 – 0.9 for *A. alliaceus* (MUCL 46784) and *A. terreus* (MUCL 46786) and 0.93 *aw* for *A. niger* (MUCL 46785). This minimal *aw* varies with strains and culture medium [22, 23, 33], and seems to be influenced by nutrient source as suggested by Wearing and Burgess [37].

Our work shows that the optimal conditions for the growth are significantly different from the optimal conditions for OTA production for both *A. alliaceus* and *A. niger* strains. The optimal conditions for OTA production by *A. ochraceus* are often at high *aw* and 30°C in different substrates [22, 23], while *A. alliaceus* (MUCL 46784) strain prefers low *aw* at 30°C. In addition, *A. alliaceus* (MUCL 46784) produces notable quantities of OTA at 0.9 *aw* after 21 DI at 20°C. A similar result was obtained by *A. ochraceus* under the same conditions [23]. Likewise, at 15°C, when the growth rate is very slow, MUCL 46784 and MUCL 46785 are able to produce low concentration of OTA, depending on the *aw* and incubation time. A similar result was reported for *A. ochraceus* at 10°C and 0.95 *aw* [23]. Indeed, different isolates may have different responses to environmental factors regarding their growth and synthesis of OTA. Even though some studies have dealt with the interaction of temperature, *aw*, and time on the growth and OTA production by *A. alliaceus* [38, 39], they were studied only in media with freely available water (0.99 *aw*) at only one temperature. This fact makes direct comparison with our results very difficult. At 25°C, *A. niger* (MUCL 46785) produced maximum of OTA at high *aw* (0.99) after 21 DI. A similar result was reported by Belli *et al*. [25] for Spanish *A. niger* aggregates but after 5 days only. Another study on Spanish dried vine fruits shows that *A. carbonarius* produced more OTA at 30°C after 10 days than at 25°C for the same period. Likewise, 87 isolates out of 88 *A. carbonarius* produced OTA on CYA medium at 30°C [40]. These results are in accordance with those of *A. niger* (MUCL 46785). Under most conditions investigated previously, OTA concentration increased with increasing incubation time, and the amounts of OTA found after 21 DI were higher than those found after 15 DI, which were also higher than those found after 7 DI. This suggests an accumulation of OTA with increasing incubation time, except for *A. niger* at 30°C where OTA concentration decreases to only 0.051 μg/g after 21 DI. A similar conclusion was reported by Belli *et al*. [25]. The reason for the decrease may be the degradation of OTA with increasing time by the strain itself as suggested by Varga *et al*. [41].

Furthermore, the composition of culture medium seems to have a great influence on growth and OTA production. For example, *A. alliaceus* (MUCL 46784) produced more OTA (9.7 μg/g) on CYA medium after 7 days at 25°C than on WMEA medium (up to 0.09 μg/g) under the same conditions. However, a low level of OTA was produced by *A. niger* (MUCL 46785) on both CYA medium (0.087 μg/g) and WMEA medium (0 – 0.27 μg/g) after 7 days at 25°C. It may be this strain is a weak producer of OTA.

This survey shows that *A. niger* and *A. alliaceus* species isolated from Moroccan wheat grains could be considered as the most probable source of OTA in this substrate, because both of them are able to grow and produce OTA at a wide range of *aw*, especially at the high temperatures that characterize the Mediterranean coast.

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