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Characterization of filamentous fungi isolated from Moroccan olive and olive cake: Toxinogenic potential of *Aspergillus* strains

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During the 2003 and 2004 olive oil production campaigns in Morocco, 136 samples from spoiled olive and olive cake were analyzed and 285 strains were isolated in pure culture. Strains included 167 mesophilic strains belonging to ten genera: *Penicillium, Aspergillus, Geotrichum, Mucor, Rhizopus, Trichoderma, Alternaria, Acremonium, Humicola, Ulocladium* as well as 118 thermophilic strains isolated in 2003 and 2004, mainly belonging to six species: *Aspergillus fumigatus, Paecilomyces variotii, Mucor pusillus, Thermomyces lanuginosus, Humicola grisea*, and *Thermoascus aurantiacus*. *Penicillium* and *Aspergillus*, respectively, 32.3 and 26.9% of total isolates represented the majority of mesophilic fungi isolated. When considering total strains (including thermotolerant strains) *Aspergillus* were the predominant strains isolated; follow-up studies on mycotoxins therefore focused primarily on aflatoxins (AFs) and ochratoxin A (OTA) from the latter strains. All isolated *Aspergillus flavus* strains (9) and *Aspergillus niger* strains (36) were studied in order to evaluate their capacity to produce AFs and OTA, respectively, when grown on starch-based culture media. Seven of the nine tested *A. flavus* strains isolated from olive and olive cake produced AF B1 at concentrations between 48 and 95 μg/kg of dry rice weight. As for the *A. niger* strains, 27 of the 36 strains produced OTA.

Keywords: Aflatoxin / *Aspergillus* / Immunoaffinity / Moulds / Ochratoxin

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1 Introduction

Olive trees (*Olea europaea* L.) growing surfaces worldwide are estimated at 8 600 000 hectares, of which 95% are in the Mediterranean area. The average annual olive production is 10 million tons of which 92% are used for oil extraction, the 8% remaining being consumed as table olives. In Morocco, the varietal profile of the olive tree is primarily that of the so-called "Moroccan Picholine," used both for olive oil and table olive production. The tree is well adapted to local climatic conditions. The "Picholine" olive variety represents 96% of olive-tree plantations, the remaining comprising varieties introduced from various countries (France, Italy, Spain, Greece, and the United States).

The olive tree represents over 50% of the surface occupied by trees growing in Morocco. Its cultivation mobilizes an intense agricultural activity with more than 11 million working days *per* year (55 000 employed). In addition, it creates an intense industrial activity providing for 16 000 traditional mills (maâsras), 260 modern units of olive mill, and around 50 olive canning plants. However, olive growing in Morocco does not benefit from suitable farming techniques and the oil extraction process is, for most part, still traditional [1]. Olive harvesting methods are also still traditional, using sticks and a beating technique to drop olives to the ground. Moreover, the inadequate storage of olives in the traditional units before milling decreases olive oil quality [2]. Postharvest storage conditions could result in the production of olive oil with a high risk of contamination by mycotoxins. Moreover, the olive cake resulting from such

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Abbreviations: **AF,** aflatoxins; **CZA,** czapeck agar medium; **OTA,** ochratoxin A; **MUCL,** mycological collection of Catholic University of Louvain, Belgium; **PDA,** potato dextrose agar

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olives could present a danger for animals because of the preferential concentration of mycotoxins in oil cakes [3].

Recent studies showed the presence of spores of toxinogenic moulds (*Aspergillus*) in olives [4]. Some species, in particular, *Aspergillus flavus* and *Aspergillus ochraceus* were able to produce aflatoxin (AF) B1 and ochratoxin A (OTA), respectively, in olives. The oil resulting from such olives contained small quantities of such mycotoxins [3]. Indeed, the presence of AF has been reported by Gracian and Arevalo [5] in Spanish olive oils. As for OTA, it was found in Moroccan olive oils [3, 6] and very recently in olive oils from Greece [7].

Concerning Moroccan olives, several studies [8] have shown that black olives "prepared the Greek way" present a potential risk of contamination by moulds and their mycotoxins. This is mainly due to their conservation process and preparation method which does not include any heat treatment in order to destroy moulds [3].

Mycotoxins are secondary metabolites secreted by moulds belonging mainly to the genera *Aspergillus*, *Penicillium*, and *Fusarium* [9]. They can be produced on a wide range of foods and under varied conditions. The presence of mycotoxins in food for human or animal consumption is potentially dangerous because of the diversity of their toxic effects and their high thermal stability [10]. The main classes of mycotoxins considered as important in the food industry are: aflatoxins (AFs), ochratoxin, patulin, fumonisin, deoxynivalenol, and zearalenone [11]. All mycotoxins are dangerous for human and animal health and cause various diseases, of which some are deadly. The work presented here describes (i) the distribution and characterization of mesophilic and thermophilic moulds isolated from olives and olive cake from different Moroccan areas, and (ii) the study of the toxinogenic capacity of *Aspergillus* spp. isolated during the 2003 and 2004 olive harvest campaigns.

This study had a triple objective: first, to identify natural mycoflora present in olives and olive cake; then, to demonstrate the toxinogenic capacity of wild strains isolated from these specific biotopes; and last, the results obtained from harmful moulds present in spoiled olives contribute toward the evaluation and prevention of their proliferation in order to obtain quality olive oil for a sustainable development of the olive sector in Morocco.

2 Material and methods

2.1 Samples origin

One hundred thirty-six samples from olive and olive cake were sampled directly in maâsras in several Moroccan areas: Sidi Kacem, Meknès, Fès, Taounate, Sefrou, Khenifra, Errachidia, Goulmima, and Marrakech. Mâasras and samples were selected randomly (Table 1). Three types of mâasra (olive mills) were sampled: (A) modern, industrial-scale extraction system, (B) semimodern with electrical extraction system, and (C) traditional with animal traction. Sampling was carried out in all cases from stored spoiled olive samples as well as from the resulting cakes after olive oil extraction.

Table 1. Origin of samples during 2003 and 2004 olive production campaigns

Area	Olives	Olive cake	Samples *per* region
Taounate	6	3	9
Meknes	7	5	12
Fez	3	3	6
Sidi Kacem	18	4	22
Sefrou	14	6	20
Khnifra	2	0	2
Rabat	7	0	7
Khemisat	9	4	13
Marrakech	17	1	18
Goulmima	5	0	5
Errachidia	12	10	22
Total samples	100	36	136

2.2 Microorganisms

2.2.1 Culture medium for strain isolation

Potato dextrose agar (PDA) from Sigma (St. Louis, USA) was used for the isolation, purification, and conservation of moulds [12].

2.2.2 Culture media for the identification strains

Three culture media from Sigma were used according to standard conditions: the malt extract agar (MEA) medium, czapeck agar medium (CZA), and PDA medium. These media were sterilized at 121°C for 20 min and distributed in Petri dishes.

2.2.3 Inoculation of medium and incubation conditions

Two olives were selected randomly from each sample. Each olive was sectioned into six fragments distributed on the surface of two Petri dishes (three in each) containing 20 mL of PDA medium. From each olive sample, one dish was incubated at 25°C for 72 h and the other was incubated at 50°C for 48 h [13, 14]. For olive cake samples, 12 samples were distributed in four PDA-containing Petri dishes, half of which were incubated at 25°C for 72 h and the other half at 50°C for 48 h [13].

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2.2.4 Isolation and conservation of strains

PDA, a nonselective medium, was used in the purification steps. In the case of bacterial contamination, chloramphenicol (50 mg/L) was added [12]. The strains obtained in pure culture were kept on PDA at 4°C.

2.2.5 Identification of the strains

For each group of filamentous fungi, the strategy for identification was as follows:

1. *Penicillium*: For the standard microscopic description and *Penicillium* identification, classical references by Pitt [15] and Samson *et al*. [16] were used. The techniques used by those authors consisted in inoculating the strains in three points on Petri dishes containing CZA or MEA at 2%. Then, cultures are incubated at 25°C. Identification keys were then used.
2. *Aspergillus*: Identification keys proposed by Rapper and Fennell [17] and by Samson *et al.* [16] were used. The culture of *Aspergillus* was carried out on CZA with an incubation at 25°C.
3. *Rhizopus*: The determination of the *Rhizopus* species was carried out according to the key of Schipper [18].
4. For other genera identification, keys by Cooney and Emerson [19], Domsch *et al.* [20], and Mouchacca [21] were used. When possible, reference strains from the IRD collection were used to corroborate identification.

For comparative studies of mycotoxin production by *Aspergillus*, reference strains from the Mycological Collection of Catholic University of Louvain, Belgium (MUCL) were used. They were: *A. flavus* MUCL 18903, *Aspergillus niger* MUCL 44639, and *A. ochraceus* MUCL 44640.

2.3 Mycotoxins

2.3.1 Culture of strains on starch-based substrates (rice or wheat)

Two types of cereals were used as substrate: (i) the wheat grains (Ebly, Casino, France) for the production of OTA by the *A. niger* strains and (ii) rice (Riz de Camargues Perliz, France) as substrate for the production of AFs by the *A. flavus* strains.

2.3.2 AF production conditions on rice grains

In a 250 mL flask, 21 mL of distilled water was added to 25 g of rice (final moisture 50%). Each flask was sterilized at 121°C for 20 min. Rice was then inoculated with 2 mL of a spore suspension (1×10^8 spores/mL) of the strain tested [22]. Cultures were incubated at 25°C for 7 days. After incubation, the flasks were heated at 70°C for 24 h (in order to destroy mould spores) and then dried at 80°C for 24 h [23]. Then, AF was extracted and quantified according to the method described below.

2.3.3 OTA production conditions on wheat grains

In a 250 mL flask, 6 mL of distilled water was added to 10 g of wheat (Ebly). After sterilization at 121°C for 20 min, wheat was inoculated with 2 mL of a suspension of spores (1×10^8 spores/mL) of the strain to be tested, prepared according to the method described above (final moisture 50%). Cultures were incubated at 25°C for 12–15 days. After incubation, the flasks were heated at 70°C for 24 h (in order to destroy mould spores) and then dried at 80°C for 24 h [23].

2.3.4 Extraction and purification of mycotoxins (AF and OTA)

From fungal cultures on cereals, 10 g of the substrate (pasteurized and dried rice or wheat) were ground to a fine powder in an Ika blender (Ika, Germany) and mixed with a stir bar at a high speed for 2 min with 40 mL of an ACN-water solution (60/40 v/v). Then, the mixture was centrifuged at $2800 \times g$ for 10 min. The supernatant was collected and stored at −20°C for 24 h [4]. From the clarified supernatant, 4 mL was taken and diluted with 44 mL of a PBS buffer solution. The extract was finally filtered through a Whatman no.4 filter paper before passing through an immunoaffinity kit Ochraprep or Aflaprep columns provided by R-Biopharm Lyon, France.

In order to calibrate the extraction and purification procedure, known amounts of each mycotoxin, 40 µg of AF B1, G1, or 10 µg of AF B2, G2 or 40 µg of ocratoxin were added to 1 L of olive oil. The extraction and purification procedures were followed as described above and 80 ± 2% recovery was observed for each of the mycotoxins tested.

2.3.5 Preparation of the PBS solution

The PBS solution was prepared by dissolution in 1 L of distilled water of the following compounds: KCl 0.2 g; KH_2PO_4 0.2 g; Na_2HPO_4 1.16 g; NaCl 8 g. pH was adjusted to 7.4 with HCl (0.1 M) or NaOH (0.1 M) [24].

2.3.6 Mycotoxin purification by immunoaffinity

The Ochraprep and Aflaprep kits (specific to OTA and AFs B1, B2, G1, and G2, respectively) were used. The dilute extract (40 mL) was passed through the column (flow rate 2 mL/min). The column was then washed with 20 mL of PBS (flow rate 5 mL/min) in order to eliminate the compounds not fixed to the column. Then, the toxin bound to the column was eluted by the addition of 2 mL of methanol at a flow rate of 1.2 mL/min. This was achieved by reversing the direction of flow through the column for maximum

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mycotoxin recovery. The eluant was then analyzed by HPLC.

2.3.7 Concentration of the extract

The extract was evaporated under a flow of nitrogen. Each AF-containing sample thus obtained was maintained at –20°C prior to derivatization. The sample-containing OTA was dissolved in 200 μL of methanol and maintained at –20°C until HPLC analysis.

2.3.8 Derivatization of AFs

In order to detect AFs B1 and G1 during HPLC analysis, it was necessary to reinforce their fluorescence by transforming them into their fluorescent derivative [24]. Derivatization of AFs B1 and G1 was achieved using TFA thereby transforming them into their corresponding hemiacetal derivatives B_{2a} and G_{2a}. In brief, 200 μL of TFA and 200 μL of hexane were added to each nitrogen-dried sample. The flasks were then heated in a waterbath at 40°C for 10 min. The derivatized extract was evaporated under nitrogen, and then dissolved in 200 μL methanol [24]. Twenty microliters of this solution was injected in the HPLC.

2.3.9 Data calculation

The concentration of OTA or AF in each analyzed sample was obtained with the following expression:

$$\text{OTA or AF}(\mu g/kg) = \frac{Q \times V_{\text{ext}} \times V_{\text{f}}) \times 1000}{(V_{\text{s}} \times V_{\text{i}} \times m)} \quad (1)$$

where Q, quantity (μg) of OTA or AF calculated from the calibration line; V_f, volume of methanol used to dissolve the dry extract (200 μL); V_i, volume of the final extract injected (20 μL); V_{ext}, volume of the solution used for the extraction of sample (40 mL); V_s, volume of the supernatant used after centrifugation for the purification (1 mL); and m, mass of sample (10 g).

2.3.10 Mycotoxin analysis by HPLC

HPLC characteristics: The HPLC used for mycotoxin analysis was a Waters unit equipped with the following accessories: (a) a Waters pump 600^E; (b) manual Rheodyne 7725i injector with an injection volume of 20 μL (c) a Waters RP C_{18} column, 150 mm by 4.6 mm; (d) a mobile phase for the analysis of OTA containing a mixture of ACN-water-acetic acid (50 : 50 : 2 v/v/v) and for the analysis of AFs, the mixture contained water-methanol-ACN (60 : 20 : 20 v/v/v); (e) visible ultra detector: Waters 490E (programmable multi-wavelength detector). The wavelength necessary for the detection of ochratoxin was 335 nm and for AF was 362 nm; (f) a Waters 746 injector.

2.3.11 Preparation of standard solutions

OTA and AFs from Sigma chemicals were used as standard (1 mg/mL) in a mixture of benzene-ACN (98 : 2 v/v) for AF and in benzene-acetic acid (99 : 1 v/v) for ochratoxin.

2.3.12 Injection of samples in HPLC

Twenty microliters of the different purified and concentrated extracts were injected in the HPLC for the detection and quantification of OTA and AFs.

3 Results

3.1 Origin of the samples

During the 2003 and 2004 olive oil production campaign, respectively, 50 and 86 olive and olive cake samples were taken from maâsras (small olive oil production units) located in several areas of Morocco (Table 1). Mycological analysis of the 136 samples resulted in the isolation of 285 strains including 167 mesophilic strains and 118 thermophilic and thermotolerant ones.

3.2 Mesophilic mould strain distribution in maâsras

The identification of mesophilic strains showed a predominance of *Penicillium* (32.3%), *Aspergillus* (26.9%), and *Geotrichum* (19.2%). The remainder were related to the following genera: *Mucor* (8.4%), *Rhizopus* (7.8%), *Alternaria* (1.8%), and *Humicola* (0.6%). The distribution of mesophilic strains according to their genera is given in Table 2. The first year (2003) the *Aspergillus* species was selected for in-depth study regarding mycotoxin production. When taking into consideration mesophilic and thermophilic strains, *Aspergillus* was predominant. It should be noted, however, during the 2004 olive oil campaign, *Penicillium* was found

Table 2. Mesophilic filamentous fungi isolated from olive and olive cake samples during 2003 and 2004 campaigns in Morocco

Genera	2003		2004		Total	General distribution (%)
	Number of strains	%	Number of strains	%		
Penicillium	26	32.9	28	31.8	54	32.3
Aspergillus	22	27.8	23	26.1	45	26.9
Geotrichum	10	12.6	22	25.0	32	19.2
Mucor	9	11.5	5	5.7	14	8.4
Rhizopus	6	7.6	7	7.9	13	7.8
Trichoderma	3	3.8	0	0	3	1.8
Alternaria	1	1.2	2	2.3	3	1.8
Acremonium	1	1.2	0	0	1	0.6
Humicola	0	0	1	1.2	1	0.6
Ulocladium	1	1.2	0	0	1	0.6
Total	79	100	88	100	167	100

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to be slightly predominant. It is to be noted that *Geotrichum* isolates were much higher in 2004 as compared to 2003.

3.3 Distribution of thermophilic and thermotolerant strains isolated from maâsras

The identification of thermophilic or thermotolerant strains isolated in 2004 showed that the majority of the strains belonged to the species: *Thermoascus aurantiacus* (28.7%), *Aspergillus fumigatus* (23.8%), and *Paecilomyces variotii* (21.3%). Among zygomycetes, *Mucor pusillus* (13.8%) were often present in olives (Table 3).

Table 3. Thermophilic and thermotolerant filamentous fungi isolated from olive and olive cake samples during 2004 campaign in Morocco

Genera	Number of strains	Thermophilic mycoflora distribution (%)
T. aurantiacus	23	28.7
A. fumigatus	19	23.8
P. variotii	17	21.3
M. pusillus	11	13.8
Thermomyces lanuginosus	3	3.7
Humicola grisea	3	3.7
Other genera	4	5.0
Total	80	100

3.4 Distribution of *Aspergillus* strains isolated from olive and olive cake samples

Data presenting number and distribution are presented in Table 4. *A. niger* (52.2%) was present in most of the samples and was most widely distributed. In addition, *A. fumigatus* represented 34.8%. To confirm identification, reference strains from MUCL culture collection were used.

Table 4. Species of *Aspergillus* isolated during the 2003 and 2004 olive oil production campaigns in Morocco

Species	2003		2004		Total (2003 and 2004)	
	Number	Distribution (%)	Number	Distribution (%)	Number	Distribution (%)
A. niger	16	59.3	20	47.7	36	52.2
A. fumigatus	5	18.5	19	45.2	24	34.8
A. flavus	6	22.2	3	7.1	9	13.0
Total	27	100	42	100	69	100

3.5 Toxinogenic potential of *Aspergillus* strains cultivated on cereals

Works reporting the contamination of commercial olive oils by AFs or OTA represent evidence that the corresponding moulds were able to develop and release their toxins on olives. Moreover, the ability of olives to allow toxin-producing fungi to grow was checked. The strains isolated, namely *A. flavus* (9 strains) and *A. niger* (36 strains) coming from different samples were studied to verify their capacity to produce AFs and OTA, respectively, on starch-based media.

3.5.1 Production of AFs by *A. flavus* strains cultivated on rice

Seven of the nine *A. flavus* strains proved to be AF B1 producing with quantities from 60 to 95 μg/kg of dry rice weight (Table 5). All strains isolated from olive cake produced AFs. As for the strains isolated on olives, those isolated in 2003 (GS coded) do not produce AFs whereas the three strains isolated in 2004 (ZNM coded) produced AFs. An *A. flavus* strain was added as positive control (MUCL 18903). Finally the noninoculated substrate was added as a negative control.

Table 5. Production of AFs by *A. flavus* strains cultivated on rice at 25°C for 7 days. All GS-coded strains were isolated in 2003, and all ZNM-coded strains were isolated in 2004. Control strain: MUCL 18903

Strains	Strains code	City/(maâsra)[a]	Origin	AF B1 on rice (μg/kg)
A. flavus	GS2	Errachidia/B	Cake	82
A. flavus	GS5	Errachidia/B	Cake	60
A. flavus	GS30	Errachidia/B	Olives	0
A. flavus	GS36	Errachidia/B	Cake	48
A. flavus	GS38	Errachidia/B	Cake	92
A. flavus	GS 43	Errachidia/B	Olives	0
A. flavus	ZNM1	Goulmima/B	Olives	95
A. flavus	ZNM102	Errachidia/B	Olives	60
A. flavus	ZNM108	Goulmima/C	Olives	71
A. flavus	MUCL 18903	Mexico	Coffee	110
Noninoculated	–	–	–	0

a) Nature of the olive oil extraction units: with B, semimodern mâasra and C, traditional unit mâasra.

3.5.2 OTA production in wheat grains

In order to demonstrate the toxinogenic capacity of *A. niger*, 36 strains were cultivated on cereal grains, on a solid medium. Two toxinogenic reference strains (A. *niger* MUCL 44639 and *A. ochraceus* MUCL 44640) were also tested as positive controls of ochratoxin production. A control sample (not inoculated and free from any trace of OTA) was also added to this experiment. The results are shown in Table 6. Among the 36 isolates tested (excluding the two reference strains), 27 strains produced OTA on a wheat-containing medium. Quantities produced vary from traces to 360 μg/kg produced by *Aspergillus* sp. (Table 6). These contents exceeded, in some cases, those produced by reference strains (190 μg/kg for MUCL 44639 and 250 μg/kg for MUCL 44640). This work confirmed that *A. niger* strains which correspond to the major mesophilic myco-

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Table 6. Production of ochratoxin by *A. niger* strains cultivated on wheat grains (Ebly) at 25°C for 15 days. All GS-coded strains were isolated in 2003, and all ZNM strains were isolated in 2004. Control strains were MUCL 44639 and MUCL 44640

Strains	Strain code	Maâsra[a)]	City	Origin	OTA in wheat (µg/kg)
A. niger	GS4	B	Errachidia	Cake	84
A. niger	GS25	B	Fez	Cake	76
A. niger	GS31	C	Khémisat	Olive	Trace
A. niger	GS33	B	Fez	Cake	158
A. niger	GS34	B	Errachidia	Olives	210
A. niger	GS39	B	Errachidia	Cake	198
A. niger	GS41	B	Errachidia	Cake	0
A. niger	GS42	B	Errachidia	Olive	Trace
A. niger	GS44	B	Errachidia	Cake	0
A. niger	GS48	C	Khémisat	Cake	82
A. niger	GS74	C	Meknes	Olives	113
A. niger	GS75	C	Khémisat	Olive	91
A. niger	GS76	A	Errachidia	Cake	126
A. niger	GS92	B	Fez	Cake	276
A. niger	GS100	C	Khémisat	Cake	47
A. niger	GS101	C	Meknes	Olives	52
A. niger	ZNM_9	B	Marrakech	Olives	170
A. niger	ZNM_{10}	B	Sidi Kacem	Cake	31
A. niger	ZNM_{12}	B	Marrakech	Olives	200
A. niger	ZNM_{13}	B	Marrakech	Olives	140
A. niger	ZNM_{16}	B	Marrakech	Olives	220
A. niger	ZNM_{17}	B	Marrakech	Olives	0
A. niger	ZNM_{20}	B	Marrakech	Olives	0
A. niger	ZNM_{22}	B	Marrakech	Olives	171
A. niger	ZNM_{23}	B	Sidi Kacem	Cake	0
A. niger	ZNM_{28}	B	Sefrou/B	Cake	215
A. niger	ZNM_{29}	B	Sidi Kacem	Cake	0
A. niger	ZNM_{32}	B	Sefrou	Olives	280
A. niger	ZNM_{47}	B	Marrakech	Olives	0
A. niger	ZNM_{57}	D	Rabat	Olives	0
A. niger	ZNM_{83}	B	Sidi Kacem	Olives	155
A. niger	ZNM_{84}	B	Sidi Kacem	Olives	Trace
A. niger	ZNM_{85}	A	Meknes	Cake	126
A. niger	ZNM_{90}	B	SidiKacem	Olives	0
A. niger	ZNM_{91}	B	Sidi Kacem	Olives	195
Aspergillus sp.[b)]	ZNM_{101}	B	Sidi Kacem	Olives	360
A. niger	MUCL 44639		Mexico	Coffee	190
A. ochraceus	MUCL 44640		Mexico	Coffee	250

a) Nature of the olive oil extraction units (maâsra): with A, modern; B, semimodern; C, traditional unit; D, olive from public market.
b) *Aspergillus* spp. was an unidentified strain belonging to *Aspergillus* section Nigri.

flora isolated from olives and olive cake (21.6%) were able to secrete high quantities of OTA when grown on appropriate media.

4 Discussion

In Morocco olive oil mills are called maâsras. There are more than 30 000 traditional maâsras, with small units of a maximum capacity of 700 kg olives/day. In addition to those units, there are also more modern units with a much larger capacity (several tons *per* day). However, the storage of olives before oil extraction is a current practice, in particular for the small units. Moreover, the shelf life is a function of the abundance of olive harvest. Often, salt is added but olives are stored in heaps, and can damage [1]. Most of the samples were taken in semimodern maâsras, having an extraction capacity of several tons of olives *per* day. Olive sampling was carried out preferentially from stored olives, in front of maâsras. The olives or olive cake were already invaded by mycelia. However, this technique of sampling does not result in a quantitative analysis. On the contrary, it does generate valuable information regarding the ecological distribution of dominant mould populations in olives and olive cake. For sampling carried out in 2003 and 2004 in the same area, a similar distribution of the main genera of moulds was found.

The incubation temperature of the culture was maintained either at 25°C to isolate mesophilic filamentous fungi or at 50°C to isolate thermophilic or thermotolerant ones [21]. Among thermotolerant strains, *A. fumigatus*, well documented for its toxinogenic potential, represent an important population (more than 23%). These strains are ubiquitous and were frequently isolated in 2003 and 2004. Among mesophilic *Aspergillus* the following species were major producers of mycotoxins: *A. flavus, A. parasiticus, A. ochraceus, A. clavatus, A. versicolor,* and *A. nidulans* [25]. The isolated strains belonged mainly to *A. niger*. No strain of *Fusarium* was isolated from analyzed samples. Furthermore, this study showed clearly that most *A. niger* strains isolated, when cultivated on cereals, were able to produce ochratoxins. One strain, not identified but belonging to the *Aspergillus* section Nigri, produced very high concentrations of OTA (360 µg/kg), much higher than the reference strains tested. In addition, the use of R-Ochraprep immunoaffinity kits, on the one hand, made it possible to purify OTA's and, on the other hand, facilitated specific analyses to confirm that the majority of the strains of *A. niger* isolated in 2003 and 2004 were toxin producers on cereals. The mycotoxin purification method by immunoaffinity is very powerful, simple, and fast in comparison with traditional purification methods. However, it presents the disadvantage of being expensive for systematic mycotoxin detection.

AFs are primarily produced by strains of *A. flavus* and other *Aspergillus* spp. such as *A. parasiticus* and *A. nomius*. There are four types of AFs (B1, B2, G1, and G2) according to their fluorescence. In vegetable products, AFs B1 and G1 are primarily found to be present [11]. Seven of the nine tested *A. flavus* strains isolated from olive and olive cake produced AF B1 when grown on rice. This result confirmed previous studies obtained in olives showing that black olives, having undergone mechanical surface damage, and inoculated, respectively, with conidiospores of *A. flavus* and *A. ochraceus* contained AF B1 or OTA [3, 6].

In the present, the toxinogenic capacity of *Penicillium* strains was not carried out. However, among *Penicillium* strains, the following strains do produce mycotoxins: *Penicillium cyclopium, P. verrucosum or viridicatum, P. citri-*

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num, P. expansum, and *P. granulatum* [9]. Last, the present study highlighted the absence of *Fusarium*, known to contaminate crop products through the synthesis of fumonisins and zearalenone, two carcinogenic and genotoxic mycotoxins [10, 26].

The results obtained indicate that mesophilic dominant mycoflora isolated from olives and olive cake belong to *Aspergillus* and *Penicillium* genera. The capacity of *A. niger* and *A. flavus* strains to produce ochratoxin and AF on cereals was confirmed. In addition, these toxins are not expressed when the same strains are cultivated in olives after salting at 25% (data not shown). Other isolated strains include *Geotrichum*, *Mucor*, and *Rhizopus*. Those species have not been reported to be toxinogenic, but can produce enzymes such as lipases resulting in loss of olive oil quality, in particular by increasing its acidity. Finally, six genera of thermophilic and thermotolerant fungi were isolated and identified.

In conclusion, *Aspergillus* and *Penicillium* were the two major genera found among mesophilic fungi, on both olives and olive cake sampled in Morocco in 2003 and 2004. Most representavive thermophilic fungi include *T. aurantiacus, A. fumigatus*, and *P. variotii*. At the species level, *A. niger* represented over half the genus, followed by *A. flavus*. Of the nine *A. flavus* strains tested, seven produced AFs when grown on rice. Most of the *A. niger* strains tested (27 of the 36 strains) produced ochratoxins when grown on wheat.

Finally, in relation to contamination problems of olives with moulds, research should be undertaken on the toxinogenic potential of *Penicillium* strains which develop in olives more frequently than mesophilic *Aspergillus*; some of the former are known to produce OTA and patulin.

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