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Natural mycoflora on olives and risk assessment

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Filamentous fungi from post harvest storage of olives in Morocco were isolated, identified and analysed. During the olive oil production campaigns in Morocco in 2003, 2004 and 2005, 279 samples from spoiled olive and olive husk were collected; analyzed and 504 strains were isolated as pure cultures. Isolates included 332 mesophilic strains belonging to ten genera: *Penicillium, Aspergillus, Geotrichum, Mucor, Rhizopus, Trichoderma, Alternaria, Acremonium, Humicola, Ulocladium* and 172 thermophilic strains mainly belonging to six species: *Aspergillus fumigatus, Paecilomyces variotii, Mucor pusillus, Thermomyces lanuginosus, Humicola grisea*, and *Thermoascus aurantiacus. Penicillium* (34%) and *Aspergillus* (27%) represented the majority among the mesophilic strains. Among the total strains (including thermotolerant strains) Aspergilli were the predominant strains; and hence follow-up studies on mycotoxins were therefore focused primarily on aflatoxins (*AFs*) and ochratoxin A (*OTA*). All the isolated strains of *A flavi* group (16) and *A niger* group (70) were studied in order to evaluate their potency to produce *AFs* and *OTA*, respectively, when grown on starch-based culture media. Thirteen of the sixteen tested A. flavus strains isolated from olive and olive husk produced *AF B1* at concentrations between 48 and 92 $\mu g.kg^{-1}$ of dry rice weight. As for the *A. niger* strains, 53 of the 70 strains produced *OTA*.

1. INTRODUCTION

Olive tree (*Olea europaea* L.) growing surfaces worldwide are estimated to be 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 (Tables 1 and 2). 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 (1). The tree is well adapted to local climatic conditions. The "Picholine" olive variety represents 96% of olive-tree plantations, the remaining comprises varieties introduced from various countries (France, Italy, Spain, Greece, the United States). The olive-tree represents over 50 % of the surface occupied by tree-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 fifty olive canning plants (1). However, olive growing in Morocco does not benefit from suitable farming techniques and the oil extraction process is, for a large part, still traditional (2). 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 (3). Post harvest 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 olives could present a danger for animals because of the preferential concentration of mycotoxins in oil cakes (4).

Table 1. Production of table olives and olive oil for European countries, average values (1998-2002) x 1000 tons.

Country	*Table olive*		*Olive oil*	
	Quantity	*%*	*Quantity*	*%*
Spain	443.2	72.4	962	48.4
France	1.9	0.3	3.5	0.2
Greece	93.8	15.3	421	21.2
Italy	62.5	10.2	564	28.4
Portugal	11.0	1.8	36	1.8
Total Europe	612.4	48.8	1,986	78.5
Total Word	1,337	100	2,531	100

Table 2. Olive oil and table olive production in North Africa countries (average 1998-2001)

Country	*Table olive %*	*Olive oil %*
Tunisia	8	61
Morocco	63	21
Algeria	27	15
Lybia	2	3

Recent studies showed the presence of spores of toxinogenic moulds (*Aspergillus*) in olives (5). Some species, in particular *Aspergillus flavus* and *Aspergillus ochraceus* were able to produce aflatoxin B1 and ochratoxin A (OTA) in olives. The oil extracted from such olives were contaminated by small quantities of such mycotoxins (6). Indeed, the presence of aflatoxin has been reported in Spanish (7) and in Tunisian olive oils (8). As for OTA, it was found in Moroccan olive oils (9; 10) and very recently in olive oils from Greece (11). Concerning Moroccan olives, several studies have shown that black olives "prepared the Greek way" present a potential risk of contamination by moulds and their mycotoxins (12). This is mainly due to their conservation process and preparation method which do not include any heat treatment in order to destroy moulds (6).

Mycotoxins are secondary metabolites secreted by moulds belonging mainly to the genera *Aspergillus, Penicillium* and *Fusarium* (13). 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 (14). The main classes of mycotoxins considered as important in the food industry are: Aflatoxins, Ochratoxin, Patulin, Fumonisin,

Deoxynivalenol and Zearalenone (14). All mycotoxins are dangerous for human and animal health and cause various diseases, of which some are deadly.

1.1. Moulds

Moulds are imperfect fungi. They are ubiquitous in nature as saprophyte, phyto-pathogens or entomopathogens (15). Beneficial forms including *Penicillium roqueforti, Aspergillus oryzae, and Rhizopus oligosporus* are widely used in the agro-industry. However, unwanted or detrimental moulds, producing toxic metabolites (mycotoxins) are often present (*Aspergillus, Fusarium, Penicillium*) among others.

1.2. Mycotoxins

Mycotoxins are fungal secondary metabolites produced by a wide variety of moulds. In small concentrations, they are toxic to vertebrates and other animals, when introduced via feeding. The consumption of mycotoxin-contaminated food and feed products poses an acute risk to human and animal health, as these mycotoxins are carcinogenic and can potentially impair the immune system (16). Recent outbreaks of diseases caused by mycotoxin-producing fungi pose a great problem for the agroindustry and are potentially threatening to the global food supply (17). Nevertheless, the illness caused by mycotoxins and known as mycotoxicosis, remains as "neglected disease". Therefore, there is a need for rapid and accurate identification and rapid implementation of control measures with regards to diseases caused by these fungi. In order to do so, prior knowledge of the fungal strains and the toxins they produce, is necessary. The major classes of mycotoxins present in food and methods used for their analysis are described in the Table 3.

1.2.1. Aflatoxins

Aflatoxins are essentially produced by specific strains of Aspergillus Flavi group as *Aspergillus flavus* and by other *Aspergillus* strains such as *A. parasiticus*, and *A. nomius*. Aflatoxins were "rediscovered" in the 60's as the causal agent of the Turkey X disease that broke out in England wiping out most of the Christmas turkeys as well as ducks. This resulted in stimulated interest on aflatoxins. There are four types of aflatoxins (B1, B2, G1 and G2) according to the blue or green fluorescence produced under UV light. In the products of plant origin, B1 and G1 are the major compounds found to be present (18).

Table 3. Mycotoxins present in food and methods used for their analysis.

Mycotoxin	*Moulds*	*Food*	*Analysis*
Aflatoxin B1	*A. flavus*; *A. parasiticus*	Cereals, peanuts, olives	TLC, HPLC
Aflatoxin M1	*A. flavus*; *A. parasiticus*	Milk Products	TLC, HPLC
Ochratoxin A	*A. ochraceus*; *Penicillium*	Coffee, onions, wheat, Barley, olives	TLC, HPLC
Fumonisin	*Fusarium*	Products from vegetables, olives	HPLC
Patulin	*Penicillium expensum*	Apple Juice, cider	HPLC
Zearalenone	*F. roseum*; *F. moliniformis*	Cereals, corn	TLC, HPLC, GC
Trichothecene	*Fusarium*	Cereals, corn	TLC, HPLC

1.2.2. Ochratoxin A (OTA)

OTA is a mycotoxin produced by *Aspergillus ochraceus* and *Penicillium verrucosum.* It is formed from isocoumarin and phenylalanin, which are bonded together by a peptide. OTA is nephrotoxic and is said to be the causal agent for Balkan Endemic Nephropathy, a human kidney disorder first described in the 50's (19). The toxin has been regularly found in food and feed from Tunisia, since 1983 (16). OTA has also been widely detected in blood samples of rural tunisian populations.

Other identified mycotoxins include fumonisins, zearalenone, trichothecenes, patulin, sterigmatocystin, 3-Nitropropionic acid or verrucosidin (14).

1.3. Factors influencing toxigenesis

Conditions permissive to toxigenesis are more limited than those permitting fungal growth. Among the factors leading to the presence of mycotoxins in foods, there are two main categories; intrinsic factors, which are dependent on the fungal species or strain and extrinsic factors which depend on environmental conditions (13; 20).

1.3.1 Intrinsic factors

Mycotoxins is rarely produced by only one species or closely related ones, as it is the case for aflatoxins by *Aspergillus flavus* or *A. parasiticus,* fumonisins by *Fusarium moniliforme* (and related species), sporidesmins by *Pithomyces chartarum.* More frequently, the same toxin can be synthesized by a diversity of fungal species sometimes belonging to different genera, for example, cyclopiazonic acid by *A. flavus* and *P. camemberti,* ochratoxin A (OTA), patulin and penicillic acid by strains of different species of *Aspergillus* and *Penicillium.* Moreover, one fungal species and strain can produce more or less simultaneously several mycotoxins: OTA and citrinin by *P. verrucosum,* OTA and penicillic acid by *A. ochraceus,* aflatoxins and cyclopiazonic acid by *A. flavus,* PR toxin, roquefortine, mycophenolic acid and patulin by *P. roquefortii.*

The present work describes (i) the distribution and characterization of mesophilic and thermophilic moulds isolated from olives and olive cake from different locations in Morocco, (ii) the study of the toxinogenic capacity of *Aspergillus* isolated during 2003, 2004 and 2005 olive harvest campaigns, (iii) the detection of mycotoxins in olive oil and (iv) the risk assessment and some recommendations in order to prevent mould post harvest proliferation in olive.

2. MATERIAL AND METHODS

2.1. Samples

During 2003, 2004 and 2005 olive harvest campaigns in Morocco, 279 samples from olive and olive husk were sampled directly in maâsra (olive mills) in several Moroccan areas: Sidi Kacem, Meknès, Fès, Taounate, Sefrou, Khenifra, Errachidia, Goulmima, Marrakech. Mâasras and samples were selected randomly. Three types of maasra were sampled, traditional with animal traction; semi-traditional with electrical extraction system and industrial-scale extraction system. Sampling was carried out in all cases from stored spoiled olive samples as well as from the resulting husk after olive-oil extraction.

2.2. Culture media for mould isolation and identification

Potato Dextrose Agar (PDA) from Sigma (St Louis, USA) was used for the isolation, purification and conservation of moulds (16). Three culture media from Sigma were used for the identification of the micro-organisms according to standard conditions: the Malt Extract Agar (MEA) medium, Czapeck Agar medium (CZA) and Potato Dextrose Agar (PDA) medium. These media were sterilized at 121°C for 20 minutes and distributed in Petri dishes.

2.3. Isolation strategy

From each sample an olive was taken randomly, and six fragments were separated using a scalpel. Three fragments were put at three different locations on the surface of Petri dishes containing 20 ml of PDA medium and incubated. Each sample was prepared in duplicate. In order to isolate mesophilic and thermophilic or thermotolerant strains present in olive and olive cake samples, the Petri dishes for mesophilic strain isolation were incubated at 25°C for 72 h and those for thermophilic and/or thermotolerant strains were incubated at 50°C for 48 h (Fig. 1).

2.4. Preservation and identification of strains

PDA, a non-selective medium, was used in the purification steps. In the case of bacterial contamination, chloramphenicol (50 mg. L^{-1}) was added (21). The strains obtained as pure cultures were maintained on PDA at 4°C. For each group of filamentous fungi, identification keys were then used as described by Pitt (22) and Samson et al.. (23) for *Penicillium*, Raper and Fennell (24) and Samson et al. (23) for *Aspergillus*; Schipper (25) for *Rhizopus* and for all the other genera by Cooney and Emerson (26) and Domsch et al. (27). For comparative studies of mycotoxin production by *Aspergilli*, reference strains from the Mycological Collection of Catholic University of Louvain, Belgium (MUCL) were used. They were: *Aspergillus flavus* MUCL 18903; *Aspergillus niger* MUCL44639 and *Aspergillus ochraceus* MUCL 44640.

2.5. Mycotoxins production

2.5.1. Culturing of strains on starch-based substrates (rice/wheat)

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

2.5.2. Aflatoxin production conditions on rice grains

In a 250 ml Erlenmeyer flask, 25 g of rice was weighed and it was moistened with 21 ml of distilled water (to get final moisture-50% w/w). Each flask was sterilized at 121°C for 20 minutes. The flask was then inoculated with 2 ml of spore suspension ($1x10^8$ spores.mL^{-1}) of the strain that was screened. The flask was incubated at 25°C for 7 days. After incubation, the flask was heated at 70°C for 24 hours (in order to destroy mould spores) and then dried at 80°C for 24 hours. Then, aflatoxin was extracted and quantified by HPLC analysis according to the method described by Roussos et al. (5).

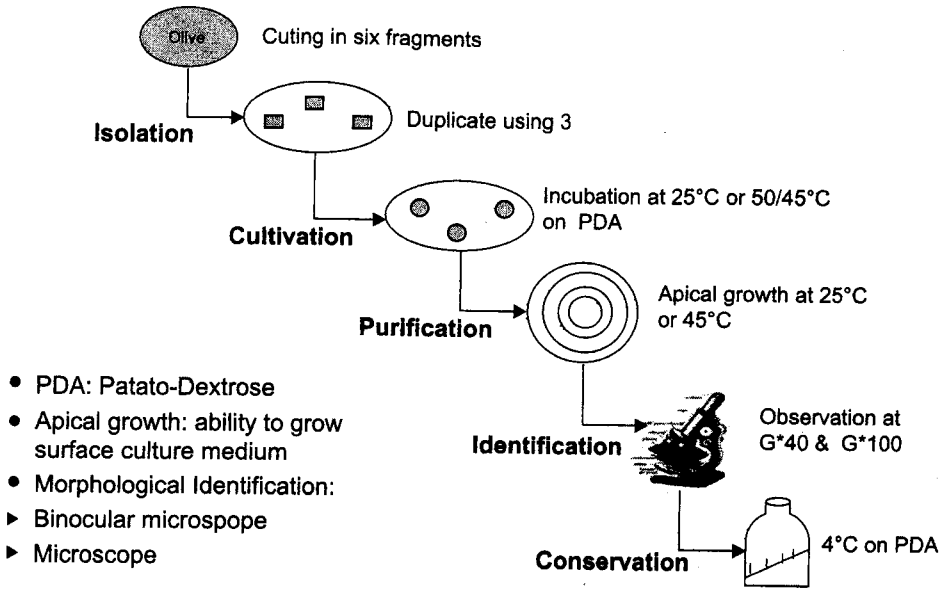

Figure 1. Treatment of the samples for isolation in pure culture of mesophilic ant thermotolerant moulds from olive and olive husk samples.

2.5.3. Ochratoxin A production conditions on wheat grains

A 250 ml Erlenmeyer flask, containing 10 g of wheat (Ebly) was moistened with 6 ml of distilled water (final moisture-50% w/w). After sterilization at 121°C for 20 minutes, it was inoculated with 2 ml of spore suspension ($1x10^8$ spores.mL^{-1}) of the strain to be screened. The flask was incubated at 25°C for 12 to 15 days. After incubation, the flask was heated at 70°C for 24 hours (in order to destroy mould spores) and then dried at 80°C for 24 hours (7).

3. RESULTS

During 2003, 2004 and 2005 olive harvest campaigns, 50, 86 and 143 samples from spoiled olive and olive husk were analysed respectively and 504 strains of mould were isolated in pure culture. Strains included 332 mesophilic filamentous fungi belonging to six principal genera : *Penicillium, Aspergillus, Geotrichum, Rhizomucor, Fusarium* and *Alternaria.* Other genera included: *Trichoderma, Acremonium, Humicola* and *Ulocladium. Penicillium* and *Aspergillus* alone represented 61% (34% *Penicillium* and 27% *Aspergillus*) of the total number of mesophilic fungi which could be isolated (Table 4). The identification of 134 thermophilic or thermotolerant strains isolated in 2004 and 2005 showed that the majority of the strains belonged to six species: *Thermoascus aurantiacus, Aspergillus fumigatus, Paecilomyces variotii, Mucor pusillus, Thermomyces lanuginosus, Humicola grisea* (Table 5). The distribution and number of *Aspergillus* strains isolated from olive and olive husk samples including the mesophilic and thermophilic one are presented in Table 6. *Aspergillus niger* with 54% of the isolated strains represented the majority among them. Similarly, *Aspergillus* species were isolated from olives

and olive fatty cakes by Belaiche (28) and Leondopoulos et al. (29). The previous reports also illustrates that *Aspergillus* species are the dominant filamentous fungi found on olives and olive products (7). Moreover, the ability of toxin-producing fungi to grow on olives was also studied.

Table 4: Mesophilic filamentous fungi isolated from olive and olive husk samples during 2003, 2004 and 2005 campaigns in Morocco.

Genera	*2003*	*2004*	*2005*	*Total*	*General*
	Number of strains			*number*	*distribution %*
Penicillium	26	28	59	113	34
Aspergillus	22	23	45	90	27
Geotrichum	10	22	20	52	16
Rhizomucor	15	12	11	38	11
Fusarium	0	0	22	22	7
Alternaria	1	2	8	11	3
Other	5	1	0	6	2
Total	79	88	165	332	100

Aflatoxin production on olives and olive products has been reported by Toussaint et al. (8), Gracian and Arevalo (7) and Tantaoui-Elaraki et al (4). Olive oil samples originating from Greece, Spain and Morocco have been reported to contain aflatoxin B1 (30, 7). The contamination of commercial olive oils by AFs or OTA represents the fact that, the corresponding moulds were able to grow and release their toxins on olives.

Table 5. Thermophilic and thermotolerant filamentous fungi isolated from olive and olive husk samples during 2004 and 2005 campaign in Morocco.

Genera	*2004*	*2005*	*Total number*	*Distribution %*
	Number of strains			
Thermoascus aurantiacus	23	16	39	29
Aspergillus fumigatus	19	16	35	26
Paecilomyces variotii	17	15	32	24
Mucor pusillus	11	7	18	14
Thermomyces lanuginosus	3	0	3	2
Humicola grisea	3	0	3	2
Other genera	4	0	4	3
Total	80	54	134	100

3.1. Production of Aflatoxin on rice or Ochratoxin on wheat grains by mesophilic *Aspergillus*

In order to demonstrate the toxinogenic capacity of *Aspergillus*, all the mesophilic isolated strains were cultivated on rice for Aflatoxin production and wheat grains for ochratoxin production (5). Three

toxinogenic reference strains (*A. niger* MUCL 44639, *A.flavus* MUCL 18903 and *A.ochraceus* MUCL 44640) were also tested as positive control of aflatoxin (Afs) or ochratoxin (OTA) production. A control sample (not inoculated and free from any trace of OTA) was also added to this experiment. The results are shown in Tables 7 and 8. All the strains isolated during 2003 from olive husk produced aflatoxin when cultured on rice media. Among the 16 isolates of *A. niger* tested (excluding the two reference strains), 14 strains produced OTA on wheat grain. The amounts produced by *A.niger* GS 92 varied from trace to 276 $mg.kg^{-1}$ (Table 8). These contents exceeded in some cases, those produced by reference strains.

Table 6. Species of *Aspergillus* (both mesophilic and thermotolerant) and number of strains isolated from maasra during the 2003, 2004 and 2005 olive oil production campaign in Morocco.

Species	*2003*	*2004*	*2005*	*Total strains*	*Distribution (%)*
A. niger	16	20	34	70	54
A. fumigatus	5	19	16	40	31
A. flavus	6	3	7	16	12
A.ochraceus	0	0	4	4	3
Total	27	42	61	130	100

Table 7. Aflatoxin B1 production by some isolated strains belonging to Aspergillus Flavi group isolated in 2003 and grown on rice for 7 days at 25°C.

Fungi	*Strain number*	*Origin of samples*	*Aflatoxin B1 ($mg.kg^{-1}$)*
A. flavus	GS 2	Olive husk	82
A. flavus	GS 5	Olive husk	60
A. flavus	GS 30	Olive	0
A. flavus	GS 36	Olive husk	48
A. flavus	GS 38	Olive husk	92
A. flavus	GS 43	Olive	0
*A. flavus**	MUCL 18903	Coffee	110
Non inoculated	-	-	0

*Control strain known to produce aflatoxin B1.

The current work confirmed that strains belonging to Aspergillus Nigri group, which correspond to the major mycoflora isolated from olives and olive husk, were able to secrete high amounts of OTA when grown on appropriate culture media.

3.2. Detection of mycotoxins in olive oil

Samples of olive oil extracted with a mobile olive mill unit developed by Ismaili-Alaoui et al. (31), in Tafilalet (Morocco) in December 2003 were analysed for Aflatoxin and ochratoxin presence according to the method described by Daradimos et al. (30). No mycotoxins were found in any olive oil sample (Table 9).

Table 8. Ochratoxin A production by strains belonging to Aspergillus Nigri group isolated in 2004 grown on wheat grain for 7 days at 30°C.

Strain number	*Sample origin*	*Ochratoxin A(mg.kg^{-1})*
A. niger GS 4	Olive husk	84
A. niger GS 25	Olive husk	76
A. niger GS 31	Olive	Trace
A. niger GS 33	Olive husk	158
A. niger GS 34	Olive	210
A. niger GS 39	Olive husk	198
A. niger GS 41	Olive husk	0
A. niger GS 42	Olive	trace
A. niger GS 44	Olive husk	0
A. niger GS 48	Olive mill waste water	82
A. niger GS 74	Olive	113
A. niger GS 75	Olive	91
A. niger GS 76	Olive cake	126
A. niger GS 92	Olive mill waste water	276
A. niger GS 100	Olive mill waste water	47
A. niger GS 101	Olive	52
A. niger MUCL 44639	Coffee	186
A.ochraceus MUCL 44640	Coffee	247
O*	O*	0

* Substrate not inoculated

Table 9. Analysis of mycotoxins (mg.kg^{-1}) in Moroccan olive oil obtained by the mobile unit olive mill.

Samples	*Acidity*	*Aflatoxin*		*Ochratoxin*		
		AFB1	*B2*	*G1*	*G2*	*OTA*
HO 1	0.5	0	0	0	0	0
HO 3	0.96	0	0	0	0	0
HO 5	0.52	0	0	0	0	0
HO 7	0.76	0	0	0	0	0
HO 9	1.03	0	0	0	0	0
HO 14	0.43	0	0	0	0	0
Olive oil Standard*	Extra Virgen	0	0	0	0	0
Olive oil + Aflatoxin**	Extra Virgen	50	11	48	12	0
Olive oil + OTA**	Extra Virgen	0	0	0	0	66

*Negative control; **Positive control

4. DISCUSSION

The results obtained indicate that the mesophilic dominant mycoflora isolated from olives and olive husk belonged to *Aspergillus* and *Penicillium* genera. The capacity of isolated strains belonging to Aspergillus Nigri group and to Aspergillus Flavi group to produce Ochratoxin and Aflatoxin on cereals was confirmed. In addition, these toxins were not expressed when the same strains were cultivated in olives after salting at 25% (data not shown). Other isolated strains which included *Geotrichum, Mucor* and *Rhizopus*. have not been reported to be toxinogenic, but were reported to produce enzymes such as lipases resulting in loss of olive oil quality, in particular by increasing its acidity. Finally, six genera of thermophilic / thermotolerant fungi were isolated and identified (32).

Aspergillus and *Penicillium* were the two major genera found among mesophilic fungi, on both olives and olive husk sampled in Morocco during 2003, 2004 and 2005. Among the thermophilic fungi most representative strains included *Thermoascus aurantiacus*, *A. fumigatus* and *Paecilomyces variotii*. At the species level, *A. niger* represented over half of the genus, followed by *A. flavus*. Of the sixteen *A. flavus* strains tested, fourteen produced aflatoxins when grown on rice. Most of the Aspergillus Nigri group strains tested (53 of the 70 strains) produced ochratoxins when grown on wheat. To conclude with, in relation to contamination problems of olives with moulds, more research should be undertaken on the toxinogenic potential of *Penicillium* strains that are capable of growing on olives more frequently than mesophilic *Aspergillus*, and are known to produce ochratoxin A and patulins.

5. RISK ASSESSMENT

No moulds on harvested olive = no mycotoxins in the extracted olive oil. There is a potential risk of contamination by moulds and mycotoxins during olive storage. Based on the current study following recommendations are made which can significantly stop the mould proliferation during the post harvest storage period:

- Collect only non-damaged olives
- Prevent mould contamination from the soil (use of a net under olive trees during harvest)
- Use aerated boxes for olive storage
- Use shortest possible storage time after harvest
- Wash olives before olive oil extraction
- Modernize traditional olive mills in Morocco
- The mobile olive mill unit is well adapted for the local reality.

6. CONCLUSIONS

Olives and olive oil play an increasingly important nutritional role not only in Mediterranean countries but also in the world and are an essential part of what is now widely known as the « Mediterranean diet ». However, the quality of the product varies greatly from one country to the other and often from one region to the other. There is a need for technological progress through the modernisation of traditional farms or the improvement of the quality of the end products whether for table olives, olive

oil or olive husk (32). In the present study, fungi from post harvest storage of olives in Morocco were isolated, identified and analysed. Data indicated a possible impact on the safety and quality of the end products particularly due to the presence of moulds on samples of spoiled olives and olive husk. *Aspergillus* and *Penicillium* are the two major genera found among mesophilic fungi, on both olives and olive cake sampled in Morocco in 2003, 2004 and 2005. Most of the Aspergillus Flavi group strains tested produced aflatoxin when grown on rice. Most of the Aspergillus Nigri group strains produced ochratoxin when grown on wheat grain.

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