Fungal biodiversity colonized olives in post collects

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SUMMARY: Penicillium species are well represented among the isolates found in the various olive analyzed samples from two región in Morocco. The olives picked at the crushing units of the Marrakech-Safi region are characterized by a high fungal diversity: 17 species were isolated from olives belonging to this zone. Only 7 among 17 species were isolated from olives belonging to the Guelmim-Oued Noun región, 6 of which belong to the genre Penicillium (P.). Common moulds identified in both regions are *P.verrucosum, P.crustosum, P.cyclopium, P.chrysogenum, P.expansum, P.digitatum* and *Phytophthora sp*. The presence of these species in the two regions demonstrate that they are not dependent on neither the region nor the climate. Their presence is probably due to the picking conditions, transportation, storage time of olives before the crushing or storage conditions in the crushing units. Quantifying OTA by HPLC revealed that all olive oil samples exhibit no contamination.

***KEYWORDS**: Biodiversity; Fungi; Mycotoxins; Olives; Ochratoxin A; Post collect*

INTRODUCTION

The olive trees are growing on all the Moroccan territory except on the Atlantic coastal band, due to their adaptation capacity on all the bioclimatic floors. Culture of the olive's tree is currently in a great expansion, with a rise of the surface devoted, which increased from 840.000 ha in 2011 to 1.000 000 ha in 2014. The olive-growing sector takes part to a total value of 15% of agro-alimentary exports. Approximately 75% of olives produced in Morocco are intended for the production of olive oil, mainly for the national market and 25% for olives of table, mostly intended for export. Total production capacity of crushing units in Morocco achieved 22.976 ton/hour (IOC, 2012). Improper olives transport and storage in the traditional units are responsible of the poor technological quality of extracted oil (IOC, 2012). A mycotoxicologic analysis is necessary, because these traditional units offer a suitable environment for the development of the external fungal aerosols found in the ground and in the indoor air (Andrews, 1996; Adebajo, 1992; Adams and Moss, 2000; Braendlin, 1996; Filtenborg *et al.*, 1996; Andersen and Frisvad, 2002; Hajjaji *et al*., 2006).

In addition to these environmental conditions, ripe olives would be an appropriate substrate for fungi, because it is rich in cellulose (12% in mesocarp and 2,4% in epicarp) (Richard-Molard *et al.*, 1985) and contains 55,2% water. A mycological analysis can isolate and identify mesophilic mould found in olives stored in different crushing units in the southern region of Morocco. A mycotoxicological analysis determined if the identified isolates have the ability to produce a mycotoxin, in olives and oil products. Pitt (1988), Moss (2002), El Adlouni *et al.*(2006), Roussos *et al.* (2006), Pitt (2007), Bragulat *et al.* (2008), Bennett and Klich (2009) reported natural contamination of these products by aflatoxins and ochratoxin A (OTA).

1. MATERIALS AND METHODS

1.1. Sampling

In November, sixteen simple of 5 kg of olives have been selected randomly directly from nine traditional units in three provinces (Essaouira, Amizmiz and El Kalaa of Sraghnas) of the *Marrakech-Safi* region and two provinces (Timoulay and Tagant) of the *Guelmim* region. The olive samples were

chosen during the final period of the crushing olive crop year 2011/2012, which means the period when the olives are mostly in advanced stages of maturity.

1.2. Moulds testing

Isolation

Samples are examined by Direct Plating (Pitt, 1988; El-banna *et al*., 1987). Ten olives per sample were surface disinfected in a sodium hypochlorite solution at 0.4% (Coppeti *et al*., 2011) in a volume 10 times larger than olives, to eliminate external fungal flora existing in the olive surface and isolate moulds inside olives (Pitt *et al.,* 1994; Nguyen *et al*., 2007).

Identification

It's carried out using a taxonomic schema based on morphological characters. Cultures were obtained after 7 days at 25 ° C on three standard CYA environments, MEA and G25N (Samson *et al*., 1986; Pitt *et al*., 1994).

1.3. Toxigenic power analysis

The detection and determination of OTA from 16 samples of olives used for crushing in the southern region of Morocco, and the oil products from the same samples is carried out by TLC and HPLC with fluorescence.

OTA extraction

30mL of methanol-water mixture is added to 15g of each homogenate sample of extracted oil (v: 80-20). The solution is stirred for 10 minutes and decanted for 3 min. 5mL of filtrate is added to 40mL PBS. Immunoaffinity column purification used is specific for OTA (NeoColumn Neogen Europe Ltd) and has a capacity of 3 mL (Castegnaro *et al.,* 2006).

Qualitative detection of OTA by TLC

Plates used were silica gel plates (20cm × 20cm) deposited in a thin layer on aluminum sheets (Sigma-Aldrich P-code: 21311P05) with fluorescence indicator. The migration solvent used is a mixture of 50 % toluene, 40 % ethyl acetate and 10 % formic (Lin *et al.*, 1998).

Quantification by HPLC (Table 1)

HPLC apparatus (Perkin Elmer) in isocratic phase and mobile phase adequate OTA, with fluorimetric detector of variable length wave. The mobile phase for OTA composed of 495mL ultrapure water, 495mL acetonitrile and 10mL acetic acid. The exact concentration of the standard solution of OTA is spectrophotometrically determined between 300nm and 370nm. The toluene/acetic acid is used as negative control. Positive control is an olive oil containing OTA (Martinez, 1998). The maximum absorption is identified and the concentration of OTA (µg/mL) is calculated using the following formula: C=A max*M*100/(E*l)

A max = specific absorption maximum wavelength (333nm); M = Molecular weight of OTA = 403,8g / mol;
E = molar extinction coefficient of the OTA in the mixture toluene / acetic acid = 544 mol; l = optical path of the tank = 1cm

1.4. Statistical analysis

The averages of three replicates is compared by analysis of variance (ANOVA), followed by a comparison of means using a Duncan test at 5% probability (SPSS 22 software).

2. RESULTS

2.1. Climatic conditions of the studied regions (Table 2)

No significant difference between the climates of the regions studied ($p > 0.05$). High moisture and high temperature are factors favoring the growth of mould producing mycotoxins. Thus the risk of mycotoxin contamination is real. Ecological distribution of the various kinds of mesophilic fungi isolated from crushing units showed that Penicillium is the dominant kind found in olives for crushing with 81.90% of all isolates obtained. Geotrichum kind comes in second place with 6.90% of the

isolates; the kinds Fusarium, Alternaria and Phytophthora are each 2.59%; Aspergillus 1.72%; Cladosporium and Paecylomyces account only 0.86% each one.

2.2. Frequency of fungal species (Table 3)

Highly significant difference is observed between fungal strains $p < 0.0001$. The macroscopic and microscopic morphological study has identified several species of Penicillium, including *P. verrucosum* (Figures 1 and 3) and *P. cyclopium* (Figures 2 and 3) which are widely represented in olives for crushing.

2.3. Relationship between fungal strains isolated and climate (Table 4)

Strains-climates interaction is highly significant $p < 0.0001$ (Table 3). Mediterranean climate (Cs) promotes sporulation of eight species of Penicillium isolated: *P.verrucosum, P.cyclopium, P.chrysogenum, P.expansum, P.digitatum, P.citrinum, P.griseofulvum* and *P.glabrum* except *P.nalgiovense* which is found only in the semi-arid climate (BSk), characterized by low humidity and low temperature. *P.verrucosum* and *P.cyclopium* are widely represented in semi-arid climates and desert and weakly represented in temperate climates. Difference between the strains is highly significant $p<0.0001$ for each climate.

2.4. Detection of OTA by TLC

TLC under UV at 365nm of extracted oil showed no band which may correspond to the OTA by its fluorescence or even it's Rf.

2.5.Quantification of OTA by HPLC

Quantifying OTA by HPLC revealed that all olive oil samples showed no contamination (Figure 4a), compared to a positive control (2.5011 mg/Kg of OTA) (Figure 4b). The chromatograms of the various olive oil samples show no peak corresponding to the OTA which is normally detected between 12.5 min and 13.5 min. OTA recovery rate in olive oil samples is higher than 60%, therefore

it can be deduced that purification at this matrix is made in good condition and that the result is significant (Figure 5).

3. DISCUSSION

Penicillium are very commonly found in soil, on decaying plants and compost as well as wood, dry food products, spices, cereals, fresh fruits and vegetables (Coppeti *et al.*, 2011). OTA's lack in oils products can be explained by different possibilities ; the isolated *P.verrucosum* strains of those samples are not toxigenic or they are toxigenic but the conditions of temperature and humidity had not favored the production of OTA, or that rates of OTA were below this method detection limit which is 0.5 ppb (Majerus and Zakaria, 1992). The presence of several species on same sample has an inhibitory effect on the production of toxins. This will be explained on the one hand, by competition for the substrate, and secondly, that some strains can degrade the toxin (Pitt *et al.*, 1994). Lamrani (2009) found 50 and 25% of olive samples in Morocco coming from the retailers and supermarkets contained OTA higher rate than 0.65 mg/kg. Virgin olive oils from the south of Italy and Morocco have been analyzed and it has been reported that 80% of tested samples (30 samples) were contaminated with OTA at levels of around 0.1 to 17ppb (Pitt, 2002; Lamrani, 2009; Roussos *et al*., 2006; Takahashi et Yazaki, 2007). Although the presence of *P. verrucosum* has been detected in different olives samples, none secreted OTA in olives. Some samples of virgin oils from olives stored too long in the small artisan oil mills, and intended for local consumption, contained concentrations about 40µg/kg for OTA (Eltem, 1996; Roussos *et al*., 2006; Ghitakou *et al.*, 2006; Lamrani, 2009). *P. verrucosum* grows slowly at a low water activity (Aw less than 0.80) and low temperature, between 0°C and 31°C with an optimum at 20°C, which is a confined distribution to temperate or cold areas. The optimum temperature for OTA production is 15°C with an optimum Aw of 0.93 to 0.98. The formation of OTA

depends on the power of mold strain's toxin-producing, substrate type and geographic location. OTA production conditions specific to each mold and depends essentially on the good practices of drying and storage of grains and dry fruits.

4. CONCLUSIONS

The isolation and identification of fungi and their distribution showed a significant diversity of mycoflora contaminating the olives used for crushing, with a predominance of the genus *Penicillium.* *P.verrucosum* isolates have been identified in the majority of analyzed olives samples; Those species may produce OTA in toxinogenesis conditions. Hence, the risk of ending this mycotoxin in oils extracted from these olives samples from these traditional crushing units. Although several *P.verrucosum* isolates were isolated, which haven't been found producing this toxin.

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Table 1. Operating conditions

Injected volume	Temperature	Excitation λ	Emission λ	Débit	Analysis Time
50µL	36°C	333nm	420nm	1mL/min	20min

Table 2. Climatic conditions of the sampling regions

Area	Climate	Geographical co-ordinates (classification of Köppen)	Temperature (°C)	Moisture (%)	Altitude (m)
Essaouira	Mediterranean	Csb	12	80	7
Amizmiz	semi-arid	BSk	9	45	900
Kelaa des Sraghnas		BSh	20	75	470
Guelmim	Desert	BWh	17	60	301

Table 3. Comparing the frequency of isolates, all confused climate

P.verrucosum	*P.cyclopium*	*P.chrysogenum*	*P.expansum*	*P.digitatum*	*P. nalgiovense*	*P. citrinum, P. griseofulvum, P. glabrum, Alternaria sp., Cladosporium sp., A.niger*	*Paecylomyces variotii*	*Geotrichum sp.*	*Fusarium sp*	*Phytophtora sp.*
22.47±9.40^{f}	30.04±14.02^{g}	12.74±8.68^{e}	13.38±9.49^{e}	5.36±3.38^{d}	0.89±1.61^{a}	0.91±1.72^{a}	1.79±3.23^{a}	2.34±4.68^{ab}	4.17±7.54^{cd}	.45±3.61^{bc}

Values followed by the same letter are not significantly different according to Duncan test at 5% probability.

Table 4. Isolated fungal strains and Climate (*Values in the same column followed by the same letter are not significantly different according to Duncan test at 5% probability.)*

	Climate			
Strains	**Csb**	**BSk**	**BSh**	**BWh**
P. nalgiovense	0.00 ± 0.00[a]	3.57 ± 0.00[b]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
Fusarium sp	0.00 ± 0.00[a]	0.00 ± 0.00[a]	16.67 ± 0.00 [b]	0.00 ± 0.00[a]
Paecylomyces variotii	0.00 ± 0.00[a]	7.14 ± 0.00[c]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
Phytophtora sp.	0.00 ± 0.00[a]	7.14 ± 0.00[c]	0.00 ± 0.00[a]	6.67 ± 0.00 [b]
P. citrinum	3.65 ± 1.17 [ab]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
P. griseofulvum	3.65 ± 1.17 [ab]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
P. glabrum	3.65 ± 1.17 [ab]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
Alternaria sp.	3.65 ± 1.17 [ab]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
Cladosporium sp.	3.65 ± 1.17 [ab]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
A.niger	3.65 ± 1.17 [ab]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
P.digitatum	7.63 ± 2.13 [bc]	7.14 ± 0.00[c]	0.00 ± 0.00[a]	6.67 ± 0.00 [b]
Geotrichum sp	9.37 ± 4.67 [bc]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
P. verrucosum	11.31 ± 3.65 [cd]	38.58 ± 0.00 [f]	16.67 ± 0.00 [b]	33.34 ± 0.00 [d]
P. cyclopium	15.38 ± 5.74 [de]	21.34 ± 0.00 [e]	50.00 ± 0.00[c]	33.34 ± 0.00 [d]
P. expansum	18.74 ± 9.34 [ef]	21.42 ± 0.02 [d]	0.00 ± 0.00[a]	13.34 ± 0.00 [c]
P. chrysogenum	24.04 ± 4.31 [f]	3.57 ± 0.00 [b]	16.67 ± 0.00 [b]	6.67 ± 0.00 [b]

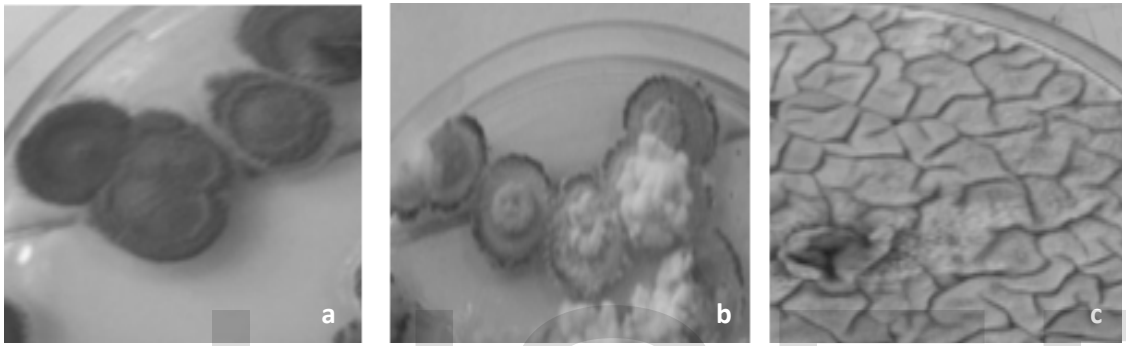


Figure 1. Colonies of *P.verrucosum* on medium CYA (a), MEA (b) and G25N

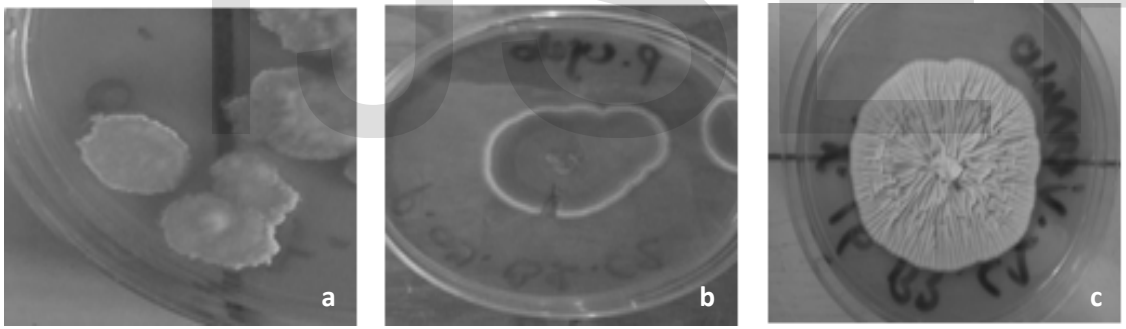


Figure 2. Colonies of *P.cyclopium* on medium CYA (a), MEA (b) and G25N (c)

Predominant Penicillium species in olives for crushing are *P. verrucosum* and *P. cyclopium.* Colony characters and diameters on specific media are important features for species identification. Czapek Yeast Autolysate agar (CYA), Malt Extract agar (MEA, Oxoid) and G25N are recommended as standard media for Penicillium.

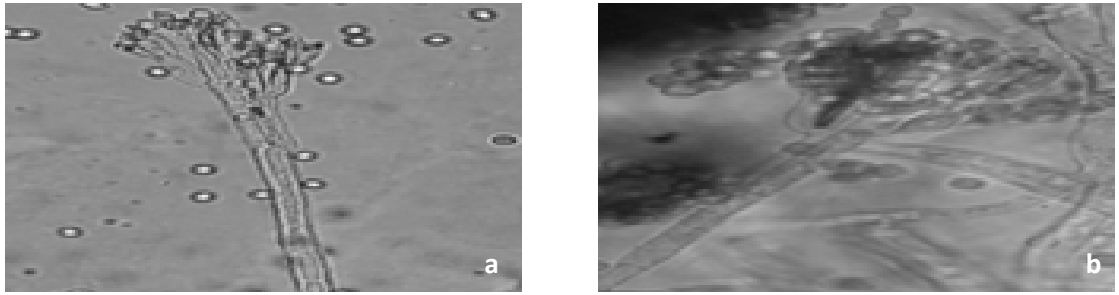
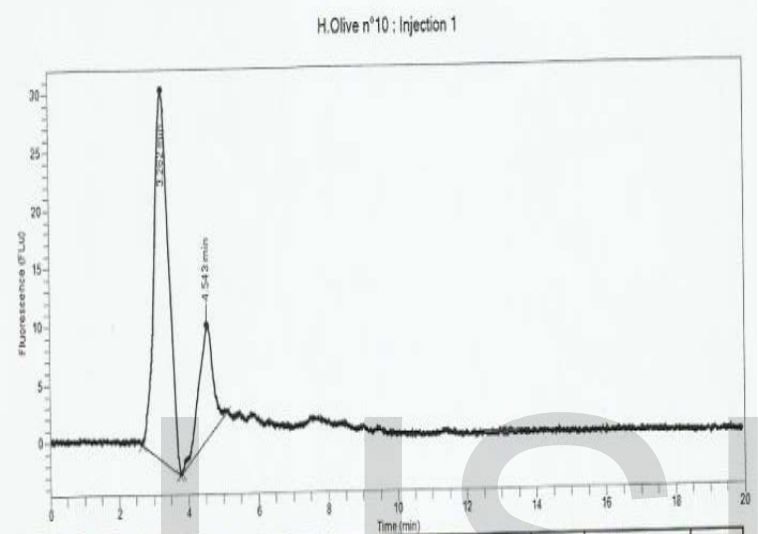
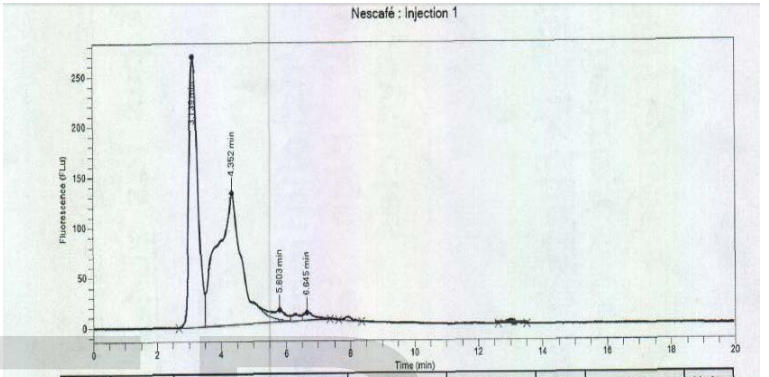


Figure 3. Microscopy (Gx40) of *P.verrucosum* (a) and *P.cyclopium* (b)

Conidiophore characters of Penicillium are of great taxonomic importance. Conidiophore branching patterns were traditionally used in the classification of Penicillium.



Peak #	RT (min)	Component Name	Area	Height	BL	Final Amount	Units
1	3.262		909,013.0	32,007.4	BB		
2	4.543		276,168.4	9,888.1	BB		
Total			1,185,181.5				

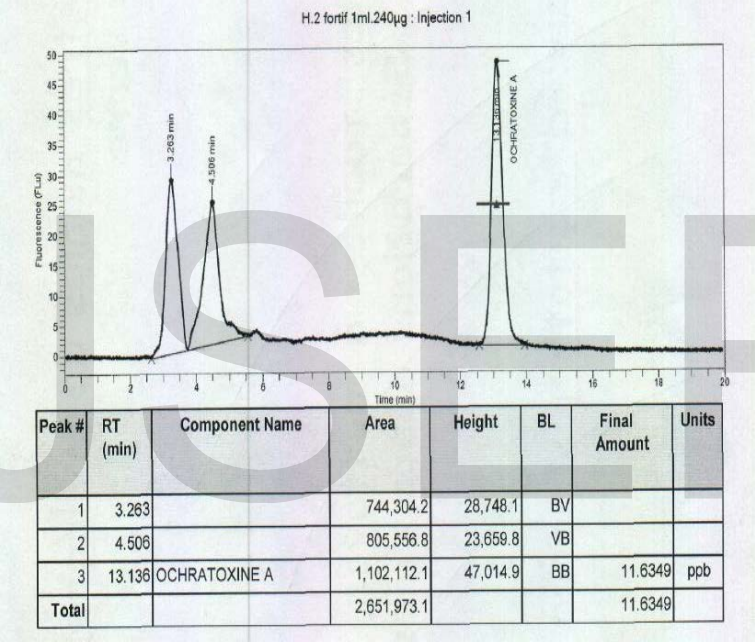


Peak #	RT (min)	Component Name	Area	Height	BL	Final Amount	Units
1	3.139		4,979,231.1	269,378.1	BV		
2	4.352		7,043,858.9	131,483.3	VE		
3	5.803		313,700.6	9,713.1	EV		
4	6.315		117,800.1	6,228.4	VV		
5	6.645		172,580.4	7,659.1	VB		
6	7.928		65,396.3	4,100.5	BB		
7	13.027	OCHRATOXINE A	91,678.7	4,154.0	BB	0.8337	ppb
Total			12,784,246.1			0.8337	

a **b**

Figure 4. Determination of OTA in olive oil (a); positive control (b)

Quantifying OTA by HPLC in olive oil sample, compared to a positive control (2.5011 mg/Kg of OTA). Olive oil samples (a) showed no contamination.



Peak #	RT (min)	Component Name	Area	Height	BL	Final Amount	Units
1	3.263		744,304.2	28,748.1	BV		
2	4.506		805,556.8	23,659.8	VB		
3	13.136	OCHRATOXINE A	1,102,112.1	47,014.9	BB	11.6349	ppb
Total			2,651,973.1			11.6349	

Figure 5. OTA recovery rate in olive oil simple

Purification of OTA recovery rate in olive oil samples is higher than 60% ; matrix used is made in good condition