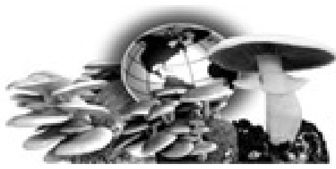

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Production of fumagillin by *Aspergillus fumigatus* isolated from traditional trituration units, "Maasra", in Morocco

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ABSTRACT

Olive oil production in Morocco is carried out at traditional trituration units called "maasras". The thermophilic mycobiota present in these places was studied, from which strains of *Aspergillus fumigatus* were identified by classical and molecular methods (ITS region from the rDNA). Selected strains of *A. fumigatus* were tested for fumagillin production using a quantitative analysis by HPLC. More than 700 thermophilic strains were obtained from "maasras" studied. The predominant species was *A. fumigatus* (31%). Eleven strains (50%), out of 22, of *A. fumigatus* were capable of producing fumagillin ranging from 4.55-46.48 ng/g of wheat dry weight.

Key words: ***Aspergillus fumigatus*, fumagillin, Morocco, thermophillic mycobiota.**

INTRODUCTION

About 50% of the olive oil production in Morocco is carried out at traditional trituration units called "maasras"[17]. This traditional method, as well as storage conditions, allow the development of many microorganisms including fungi. Several fungal species produce mycotoxins, which may be harmful to humans or animals.

Aspergillus fumigatus is an ubiquitous saprophytic fungus which plays an important role in recycling environmental carbon and nitrogen, but it may also be an opportunistic pathogen[13]. Humans constantly inhale high amounts of conidia from this fungus, which may affect their respiratory tract after long exposure. *A. fumigatus* is also capable of producing secondary metabolites, which can be harmful (*e.g.*, gliotoxin, helvoic acid) or of medical importance (*e.g.*, fumagillin as an antibiotic)[4].

Fumagillin, isolated in 1951 by Eble and Hanson[6] from *A. fumigatus*, was originally described as an anti-microbial agent. However, in 1995, Folkman[9] discovered that fumagillin potentially and selectively inhibited the growth of new blood vessels from pre-existing vessels, which is considered a fundamental step in the transition of tumors from a dormant state to a malignat state. This kind of inhibitors are now having great potential therapeutic use[14]. Therefore considerable effort has been made to explore the biological activity of fumagillin and its synthetic analogues, which are expected to have better application and lower toxicity[2,7].

The secondary metabolite production of *A. fumigatus* isolated from natural hot and dry habitats has been poorly studied. At present, there are no studies on strains from *A. fumigatus* isolated from olive products, involving their characterization in terms of growth and the production of fumagillin and mycotoxins. In this work, we studied the thermophilic mycobiota present in traditional "maasras". Strains of *A. fumigatus* were then selected and tested for fumagillin production using a quantitative analysis by HPLC.

MATERIALS AND METHODS

Sampling. Several samples of different sources (soil, leaves, olive seeds, olive press cakes, waste water) were collected from "maasras" (traditional olive mills) during seven successive olive campaigns. "Maasras" are located in several areas of strong olive production throughout Morocco (**Table 1**). Random samples (*ca*. 1 g) of similar size were cultured on several media[12] whose composition (g/L) was: 1) OPC: olive press cake 40, agar 15, chloramphenicol 0.25; and 2) PDA: Potato dextrose agar (Sigma-Aldrich, France) 39, chloramphenicol 0.25. Inoculated Petri dishes were incubated at 50 C to isolate thermophilic fungi. All strains isolated were maintained at 4 C on standard PDA, and deposited at the culture collection of the Laboratoire Biotrans-IRD, Marseille, France[12,17].

Strains of Aspergillus fumigatus

Identification. Conventional keys from Raper and Fennel[16] for *Aspergillus*, as well as that from Cooney and Emerson[5] for thermophilic fungi, were used for tentative identification. This was followed by a molecular identification, which was carried out as follows: 1) Production of fungal biomass in culture medium (malt extract agar, MEA); 2) DNA extraction in Chelex® 100 Resin following a protocol previously described by Bernal *et al*.[3]; and

Table 1. Origin and sources of samples collected at different "maasras" (traditional olive mills) in Morroco.

Region	Leaves	Olive press cakes	Olive seeds	Soil	Waste water	Total
Gharb	3	11	5	0	0	19
Haouz	0	1	31	0	0	32
Haut Atlas oriental	18	79	69	35	0	201
Moyen Sebou	11	71	76	9	8	175
Moyen Atlas	0	7	20	0	0	27
Rabat-Maamoura	1	9	37	1	2	50
Rif	0	28	0	0	0	28
Tadla	6	71	10	12	0	99
Total	39	277	248	57	10	631

3) PCR amplification of the ITS region from the rDNA [primers: ITS-1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC 3')], according to the protocol described by Attili *et al.*[1]. Amplification conditions were as follows: an initial denaturing step of 3 min at 95 C, followed by 45 cycles of 95 C for 60 s, 54 C for 60 s, and 72 C for 2 min, and then a final step at 72 C for 7 min. PCR reaction products were checked by a 2% (w/v) agarose gel electrophoresis. Sequencing of all PCR products was carried out at the Faculty of Pharmacy, University of Lyon, and at the private company Biofidal, France. Data were analysed using the software Chromas, and were compared to the reference sequence data from the GenBank, NCBI (U.S.A.), using BLAST (www.ncbi.nlm.nih.gov).

Production of fumagillin

Origin of strains. Among the isolates obtained, 22 strains of *A. fumigatus* Fresen. were selected according to the year of collection, source and origin of samples. These strains were tested for their potency to produce fumagillin on wheat (**Table 2**).

Substrate preparation. Erlenmeyer flasks (100 ml) containing 20 g of milled wheat (Ebly Casino, France), adjusted to 60% moisture with distilled water, were sterilized at 121 C for 20 min[17]. Inocula were prepared by suspending spores harvested from a 3-day-old PDA cultures. Spores were suspended in 5 ml of sterile water containing 0.1% of Tween 80. Sterilized Erlenmeyer flasks were aseptically inoculated with spore suspensions up to a final concentration of 1 x 10^7 spores/g of wheat. Flasks were incubated at 37 C for 7 days.

Extraction. After incubation, 40 ml of an acetonitrile-distilled water solution (60:40, v:v) was added to the flasks. This substrate-solvent mixture was allowed to become thoroughly wet at room temperature for 2 h, followed by 15 min of mechanical agitation, and then it was filtered using filter paper (Whatman no. 4). The filtrate extract (5 ml) was dried under a flow of N_2 gas at 60 C,

Table 2. Strains of *Aspergillus fumigatus* tested for fumagillin production.

Strain code	Origin	Source or code	Year of collection
Afu 01	Mexique	C6B25	2002-2003
Afu 02	Rabat	Greek style black olive	2005-2006
Afu 03	France*	-	2001
Afu 04	Ouazzane	Grignon	2002-2003
Afu 05	Meknès	Grignon d'olive	2005-2006
Afu 06	Errich	"Maasra", soil	2000-2001
Afu 07	Marrakech	Olive	2005-2006
Afu 08	Errich	Olive	2000-2001
Afu 09	Fkih ben Saleh	Olive	1999-2000
Afu 10	Rabat	Olive	2004-2005
Afu 11	Khemissat	Olive	2003-2004
Afu 12	Marrakech	Olive press cake	2002-2003
Afu 13	Temara	Greek style black olive	2005-2006
Afu 14	Marrakech	Olive	2004-2005
Afu 15	Errachidia	Olive press cake	2000-2001
Afu 16	Fkih ben Saleh	"Maasra", soil	2000-2001
Afu 17	Fkih ben Saleh	Olive press cake	2000-2001
Afu 18	Meknès	Olive	2003-2004
Afu 19	Sefrou	Olive	2005-2006
Afu 20	Marrakech	"Maasra", wall remains	2002-2003
Afu 21	Taourirt	Olive press cake	2005-2006
Afu 22	Fkih ben Saleh	Olive press cake	1999-2000

* Culture collection from the National Museum of Natural History.

and dissolved again in 500 µl of methanol-distilled water solution (1:1, v:v) to be used for mycotoxin analysis.

Analysis. Mycotoxin content was determined by HPLC according to the method of Frisvad[10]. Detection of fumagillin was carried out at room temperature on a Waters C18 reversed-phase column (150 mm×4.6 mm, 5µm). The mobile phase was acetonitrile-water-acetic acid (50:50: 10 ml/L, v/v/v), pumped at a flow-rate of 1 ml/min. Detection was performed using a photodiode at 350 nm. The time of retention of fumagillin was detected by injecting a standard solution of fumagillin of known concentration (Sigma-Aldrich, France). Analysis of the peaks corresponding to fumagillin was performed by the method of enrichment[8] using three chromatograms.

RESULTS AND DISCUSSION

A fungal collection of about 700 thermophilic strains was obtained from "maasras" of Morocco. Tentative identification based on

morphology indicated nine different species, as can be seen in **Fig. 1**. Most previous mycological studies in Morocco were focused on mycotoxicology of mesophilic filamentous fungi[11,17].

The predominant species was *Aspergillus fumigatus* (31%), which was expected due to its tolerance to high temperatures, and abundance in composts, seed containers, haystacks, and soil[13]. A representative sample of strains from this species was characterized on PDA at 25 C.

Macroscopic morphology. Mycelial colonies were smoky gray-green and slightly yellowish at the bottom. Some isolates showed a diffusive pigment of lavender colour. Colonies exhibited rapid growth and, once matured, they turned into slate grey. The texture was woolly to cottony or somewhat hairy. Atypical isolates remained white showing poor development of conidia.

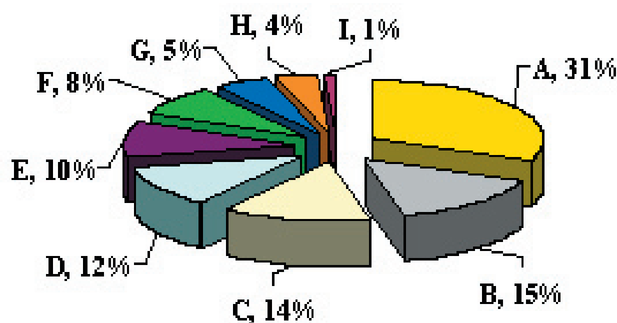

Fig. 1. Proportions (%) of different fungal species isolated from "maasras" in Morocco. A: *Aspergillus fumigatus* Fresen. B: *Rhizopus microsporus* var. *rhizopodiformis* (Cohn) Schipper & Stalpers. C: *Rhizopus* sp. D: *Thermoascus aurantiacus* Miehe. E: *Paecilomyces variotii* Bainier. F: *Myceliophthora thermophila* (Apinis)Oorschot. G: *Thermomyces lanuginosus* Tsikl. H: *Humicola grisea* Traaen. I: *Malbranchea cinnamomea* (Lib.)Oorschot & de Hoog.

Microscopic morphology. Hyphae were septate and hyaline. Conidial heads were strongly columnar on undisturbed culture. Conidiophores were smooth-walled, uncoloured, up to 300 µm long, and terminated in a swollen apex of 20-30 µm diameter. Conidiophores were uniseriate with closely compacted phialides (5-10 x 2-3 µm) occurring only on the upper portion of the swollen apex. Conidia were smooth to finely rugulose, subglobose, 2-3.5 µm in diameter (**Fig. 2**).

Molecular identification. Three strains identified on the basis of morphological and physiological features were confirmed to belong to *A. fumigatus*. Their ITS1-ITS4 sequences were up to 98% homologous to reference strains of *A. fumigatus* Fresen. from the GenBank.

Fumagillin production. Quantitative analysis of fumagillin in 22 selected strains of *A. fumigatus* showed a retention time of 11 min. The production of fumagillin by all strains studied is shown in **Table 3**. Eleven strains (50%) of *A. fumigatus* were capable of producing fumagillin ranging from 4.55 ng/g (Afu 16) to 46.48 ng/g (Afu 22) of wheat dry weight. This producion is relatively low in comparison with that reported of 25 µg mL^{-1} from *A. fumigatus* grown in submerged fermentation[4]. The rest of strains either produced undetectable quantities or did not produced fumagillin at all. The wild origin and substrate source used for strain isolation in this study could have contributed to the heterogeneity of fumagillin production. Fumagillin is one among various other mycotoxins (gliotoxin, helvoic acid, verruculogen) produced by *A. fumigatus*, and it is a sesquiterpene with a remarkable antiangiogenic activity[4,15]. In fact, it has been reported that fumagillin

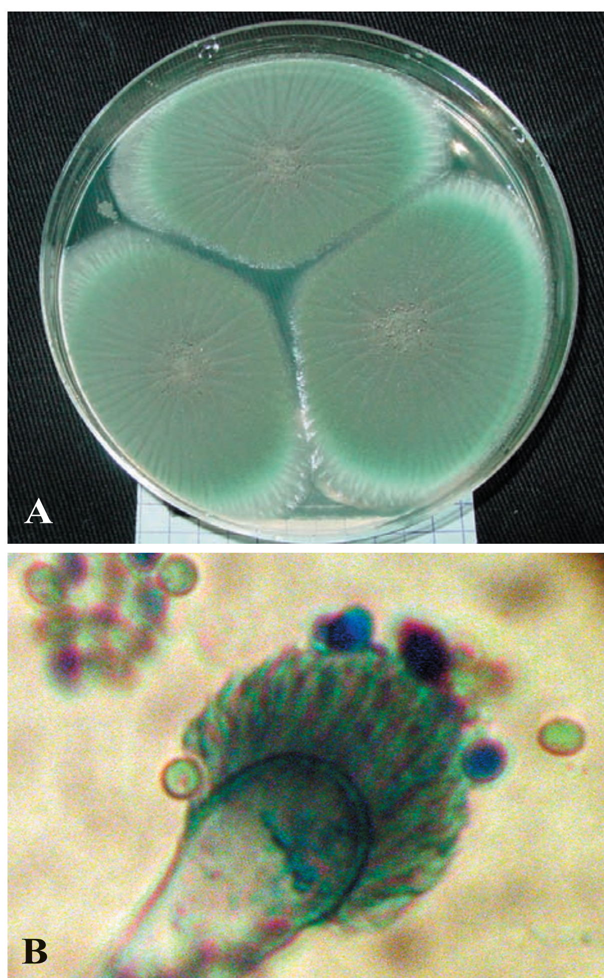

Fig. 2. *Aspergillus fumigatus*. A: Mycelial colony of a typical strain that sporulated heavily, grown on PDA at 45 C. B: A single condial head, x100.

Table 3. Production of fumagillin by *Aspergillus fumigatus* on wheat substrate.

Strains	Fumagillin (ng/g wheat dry weight)
Afu 01	0
Afu 02	0
Afu 03	<1*
Afu 04	<1*
Afu 05	5.86
Afu 06	6.18
Afu 07	0
Afu 08	7.57
Afu 09	<1*
Afu 10	Traces
Afu 11	24.97
Afu 12	13.09
Afu 13	17.32
Afu 14	0
Afu 15	0
Afu 16	4.55
Afu 17	30.27
Afu 18	0
Afu 19	23.32
Afu 20	0
Afu 21	9.67
Afu 22	46.48

* Quantity not detectable by HPLC.

inhibits the vascularization of solid tumours, which is promising to treat certain types of cancer[15].

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