Ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from currants of Greek origin

Eleni Kollia[1], Ioanna Pyrri[2], Evangelia Kapsanaki-Gotsi[3], Panagiota Markaki[4]*

[1,4]Laboratory of Food Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, GR-15771 Athens, Greece
Email: elenikollia@chem.uoa.gr/ markaki@chem.uoa.gr

[2,3]Section of Ecology and Systematics, Department of Biology, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, GR-15784 Athens, Greece
Email: ipyrri@biol.uoa.gr/ ekapsan@biol.uoa.gr

*Corresponding author/ tel. 0030-210-7274489, Fax 0030-210-7274476, email: markaki@chem.uoa.gr

Abstract**—***Black aspergilli is an important group of fungi used in biotechnology and food industry. Some species of this group produce hazardous mycotoxins such as ochratoxin A (OTA). During this study, four novel strains of Aspergillus section Nigri isolated from Greek currants (ATHUM 6997, 6998, 6999, 7000) were investigated for OTA production on Yeast Extract Sucrose (YES) medium and currants. As strains control were used both A. carbonarius and A. ochraceus.*

OTA determined using HPLC (Fluorescence Detector). Results revealed that OTA production by A. carbonarius in currants was not significantly different compared to OTA production by the Aspergillus section Nigri strains.

However the maximum amounts of OTA produced by the strains ATHUM 6997 and 6999 in currants were found to be ~2-fold lower than that by A. carbonarius. The maximum OTA levels produced by the strains ATHUM 6997, 6998, 6999 and 7000 were found to be ~7, ~12, ~9 and ~11 fold higher respectively, if compared to the maximum OTA production by A. ochraceus. The novel strains of Aspergillus section Nigri isolated from currants of Greek origin, produced significant amounts of OTA in YES medium and in currants. Along with a previous study, in the present work it is concluded that the four Aspergillus section Nigri strains are able to produce both aflatoxin B_1 *and OTA.*

Keywords—Ochratoxin A, Aspergillus section Nigri, Currants, Yeast extract sucrose

I. INTRODUCTION

Ochratoxin A (OTA) is a polyketide mycotoxin. The chemical structure of OTA consists of a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-methylcoumarin, linked through the 7-carboxy-group to l-β-phenylalanine by an amide bond (van der Merwe et al. 1965) and is produced by some *Penicillium* and *Aspergillus* species in several agricultural products (Pitt, 1987). Ochratoxin A has been classified as "possibly carcinogenic to humans" (group B) by the International Agency for Research on Cancer (IARC, 1993), because of its teratogenic, nephrotoxic, immunosuppressive, carcinogenic and cytotoxic properties. It is produced mainly by *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* in warm climates and by *Penicillium verrucosum* and *P. nordicum* in temperate climates (Magnoli et al. 2006, Pitt et al. 2000).

Strains of *Aspergillus* section *Nigri* are distributed worldwide growing on a great variety of substrates and they are considered as common food spoilage fungi (Pitt and Hocking, 1997, Raper and Fennell, 1965). Moreover, some of them are source of extracellular enzymes and organic acids applied in food industry. For this reason, black aspergilli have always been useful and important in biotechnology and food manufacturing (Samson, 1994).

Many studies showed that OTA contaminates a variety of food commodites such as dried fruits, cereals, coffee etc. Kaya and Tosun (2013) analyzed organic foods produced in Turkey, for aflatoxins, OTA and fumonisin contamination. From a total of 221 samples it was found that 43.43 % of these samples were contaminated with OTA. The concentrations of OTA in dried fruits ranged from zero to 34.35 μg kg^{-1}. Moreover in a survey conducted in United States it was found that from 665 samples (dried fruits, tree nuts, bulk raisins, dates, figs, prunes, almonds, pistachios, walnuts) analyzed, OTA was detected in 48 raisin samples, 4 fig samples, 4 pistachio samples, and 1 date sample (Palumbo et al. 2015). In addition from a total of 56 dried vine fruits samples, collected from Chinese markets analyzed for OTA presence, it was found that 58.9% of the samples examined were contaminated (<0.07 to 12.83 μg kg^{-1} OTA) (Zhang et al. 2014). Another survey showed that OTA occurred in 22% of a total of 228 samples of dates and dried fruits from Tunisian and Spanish markets (Azaiez et al. 2015). Meanwhile in Iran OTA was detected in 10% of dried vine fruit analyzed (Feizy et al. 2012). Ponsone et al. (2010) studied

the natural occurrence of OTA in musts, wines and grape vine fruits from grapes harvested in Argentina and found that 60% of the dried vine fruit samples were contaminated with OTA, ranging from 0.26 to 20.28 ng g^{-1} while red must and red wine samples showed low levels of mean OTA contamination (0.12 ng mL^{-1} and 0.37 ng mL^{-1}, respectively). A survey of aflatoxins and OTA contamination in food products imported in Italy showed that dried vine fruits were the commodities mainly contaminated with OTA, ranged from 2.76 to 9.87 μg kg^{-1} while green coffee was found to be contaminated with OTA 23.7 μg kg^{-1} (Imperato et al. 2011).

Currants (Corinthian type raisins) are dried seedless grapes of *Vitis vinifera* L. cultivated and processed for hundreds of years. This type of dried vine fruits is produced almost exclusively in Southern Greece. Currants together with the other types of dried vine fruits (raisins and sultanas) are important constituents of the Mediterranean diet. They are considered as a rich source of antioxidant compounds (ascorbic acid) and polyphenolic compounds (anthocyanins) (Chiou et al. 2014). Moreover currants contain essential minerals, such as potassium, calcium and magnesium (Nour et al. 2011).

The aim of this work was to investigate the potential of four new strains of *Aspergilli* section *Nigri*, which have been isolated from black currants originating from Crete and Corinth (ATHUM 6998, 6999, 7000 and ATHUM 6997), to produce OTA in black currants. The OTA production by the four strains was also studied in a synthetic microbiological medium, yeast extract sucrose (YES). It is worth mentioning that the same strains of *Aspergillus* section *Nigri* have been previously studied in our laboratory for their ability to produce AFB_1 (Kostarelou et al. 2014, Tavoulari et al. 2014).

II. MATERIAL AND METHODS

2.1 Strains

The four strains of *Aspergillus* section *Nigri* used in this study were isolated from black currants originating from Corinth, Peloponnese (one strain) and Crete (three strains). The fungal isolates were assigned to *Aspergillus* section *Nigri* and have been deposited at the ATHUM Culture Collection of Fungi, in the Mycetotheca of the University of Athens. The four strains isolated from the currants were the following: *Aspergillus* sp. isolated from currants originating from Corinth ATHUM 6997, *Aspergillus* sp. isolated from currants originating from Crete ATHUM 6998, *Aspergillus* sp. isolated from currants originating from Crete ATHUM 6999, *Aspergillus* sp. isolated from currants originating from Crete ATHUM 7000.

The phenotypic examination of *Aspergilli* section *Nigri* mentioned above is previously reported in details by Kostarellou et al. (2014). Cultures were inoculated using Czapek yeast agar (CYA), malt extract agar (MEA), yeast extract sucrose agar (YES) and creatine agar (CREA) (Frisvad and Samson, 2004). The inoculated Petri dishes were incubated in the dark at 25 ^{0}C for 7 days. Moreover CYA plates were inoculated and incubated at 30 ^{0}C in the dark for seven days. After the end of incubation period, the colony diameters, sporulation, the colony colors in the obverse and reverse and the presence of soluble pigments were registered in detail. All the isolates were examined for the production of alkaloids by the Ehrlich test using the filter paper method (Lund, 1995). Microscopic examination was applied by tearing separately a small piece of the colony from MEA in a drop of 60% lactic acid on a glass slide, placing a coverslip. Mounts were observed under a microscope AxioImager.A1 (Zeiss, Germany) with plain light or with Differential Interference Contrast at 1000X magnification. The microscopic characterization of conidia, phialides, metulae, vesicles and stipes were investigated in detail.

The strains of the ochratoxigenic species *A. carbonarius* (ATHUM 2854) and *A. ochraceus* (ATHUM 4781) used throughout this study as control strains, were obtained from the ATHUM Culture Collection of Fungi, Mycetotheca of the University of Athens.

2.2 Apparatus

A laminar flow (Telstar Bio II A, Madrid, Spain), an autoclave, Selecta-Autester-E Dry (PBI Milano, Italy), an incubator WTB Binder (Tuttlinger, Germany) and a centrifuge Sorvall RC-5B (HS-4) (Norwalk, USA) were utilized. The HPLC used was HewlettePackard 1050 (Waldborn, Germany) Liquid Chromatograph (pump and injection system) equipped with a JASCO FP-920 (Co, LTD, Japan) fluorescence detector and an HP integrator 3395. The HPLC column was C_{18} Nova-Pak (60 E, 4 mm 4.6x250 mm) (Waters, Millipore; Milford, MA). The mobile phase was [water:acetonitrile:acetic acid (50:50:2, v:v:v)] which was filtered through Millipore HA-VLP (0.45 mm) filters. OTA was detected at lex 335 nm and lem 465 nm with flow rate 1mL min^{-1} and retention time 6.15 (±0.24) min (**Fig.1**).

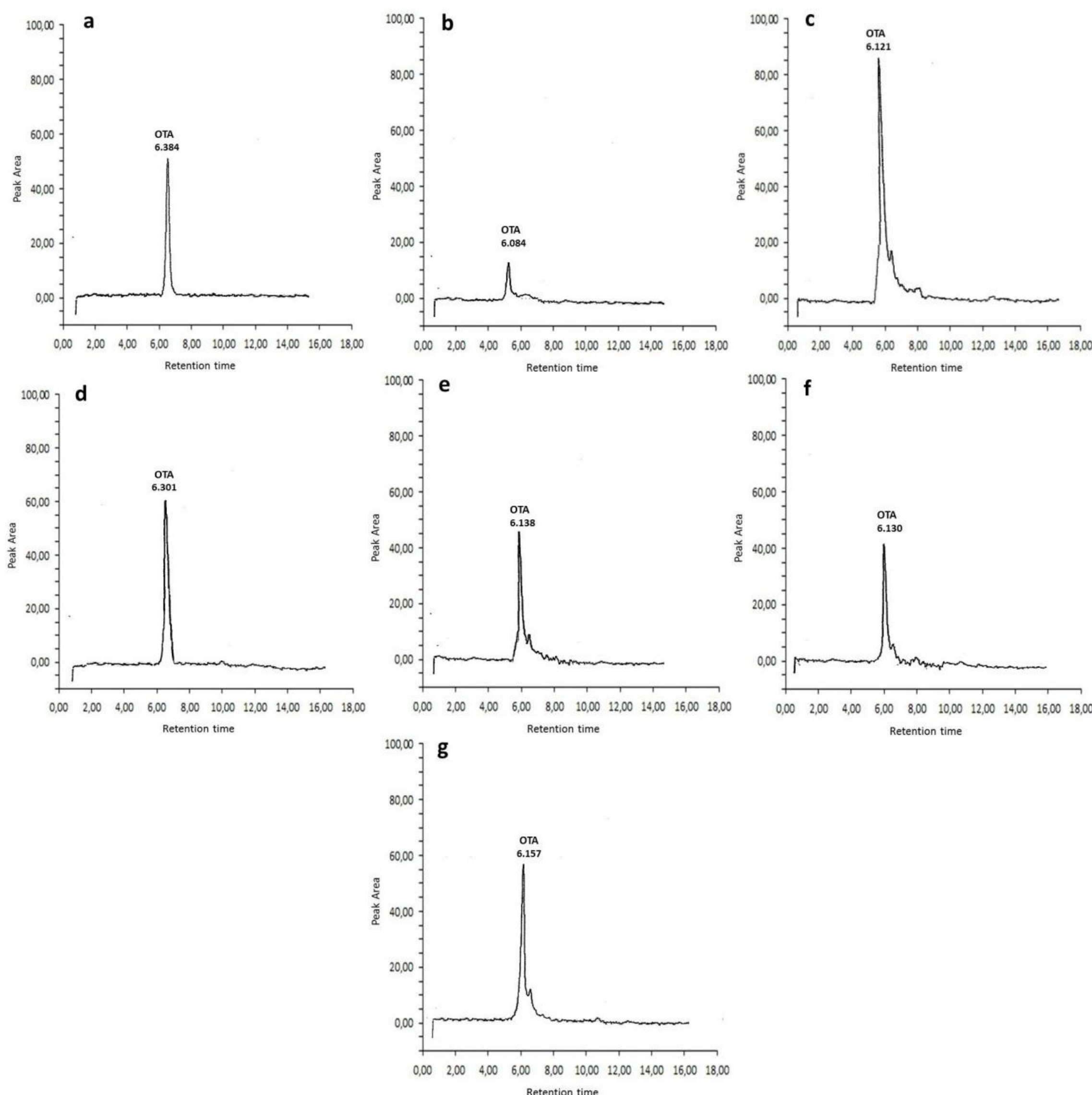

Figure 1: Chromatographs of OTA production: a) standard OTA (10 ng mL^{-1}); b) *Aspergillus ochraceus* inoculated in currants on the 7th day of observation (corresponding to OTA 0.11 ng g^{-1}); c) *Aspergillus carbonarius* inoculated in currants on the 7th day of observation (corresponding to OTA 4.33 ng g^{-1}); d) *Aspergillus* section Nigri 6997 inoculated in currants on the 7th day of observation (corresponding to OTA 2.42 ng g^{-1}); e) *Aspergillus* section Nigri 6998 inoculated in currants on the 12th day of observation (corresponding to OTA 0.96 ng g^{-1}); f) *Aspergillus* section *Nigri* 6999 inoculated in currants on the 12th day of observation (corresponding to OTA 1.03 ng g^{-1}); g) *Aspergillus* section *Nigri* 7000 inoculated in currants on the 12th day of observation (corresponding to OTA 0.87 ng g^{-1}). The injection volume was 40 µL and the retention time 6.15 (±0.24) min.

2.3 Reagents

OTA standard was obtained from Sigma Aldrich (St. Louis, Missouri, USA). The Millipore filters and the C_{18} Nova-Pak HPLC column were from Waters (Millipore; Milford, MA, USA). The Ochratest immunoaffinity columns were purchased from Vicam (Watertown, MA, USA). All analytical grade reagents were from Sigma Aldrich and HPLC grade solvents were from Fisher Chemical (Leicestershire, UK).

2.4 Preparation of phosphate-buffered saline

Phosphate-buffered saline (PBS) was prepared by dissolving 0.2 g potassium chloride, 0.2 g potassium dihydrogen phosphate, 1.16 g anhydrous disodium hydrogen phosphate and 8.0 g of sodium chloride in 900 mL of distilled water. After

adjusting the pH to 7.4 using 0.1M HCl/0.1M NaOH, the solution was made up to 1000 mL (Daradimos et al. 2000) and stored at room temperature.

2.5 Media

Potato Dextrose Agar (PDA) was prepared according to the label directions (BD Difco). Yeast Extract Sucrose (YES) broth was prepared by dissolving 2 g of yeast extract and 15 g of sucrose in 100 mL distilled water, final pH 6.0-6.5 (Pitt, 1986).

2.6 Sampling and treatment

Currants, used in the present work, originating from Corinth, were organic, shade-dried and were purchased from the market of Athens (Greece). For the total experimental requirements, a quantity of 2 kg of Corinthian currants was used. The currants were washed with water, cut into pieces, so that the whole mass of currants could be appropriate to achieve representative sub-samples. They were finally autoclaved at 110 °C for 2 min, in order to eliminate their natural microflora. In our laboratory was also studied an alternative treatment using chlorinated water, but it was absorbed by the currants (Kostarelou et al. 2014, Leontopoulos et al. 2003).

After autoclaving, the whole mass of currants was divided, in order to obtain representative samples, which were distributed aseptically into sterile flasks forming a compact layer. Each flask contained 15 g of currants. Currants were stored at 4 °C until analysis.

2.7 Preparation of spore inoculum

A. carbonarius (ATHUM 2854) and *A. ochraceus* (ATHUM 4781) were used as control strains during this study. An inoculum was obtained from a slant of stock cultures of PDA maintained at 25 °C and fresh colonies of *A. carbonarius* and *A.ochraceus* were obtained on PDA after 7 days at 30 °C. Spore suspensions were prepared aseptically using 10 mL of sterile Tween 80 solution 0.01% v/v.

Then the initial OTA conidia production was minimized by centrifuging the spore suspension and resuspending in 10 mL of sterile Tween 80 solution thrice. Dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) from the initial spore suspension in sterile tubes containing 10 mL of Tween 80 0.05% v/v were made (Vergopoulou et al. 2001). Determination of the spore concentration was performed by the spread plate surface count technique using 0.1 mL of each dilution on PDA plates after incubation at 30 °C for 48 h. PDA plates with 10–100 colony-forming units (CFU) were selected and the preferred 100 spore quantity used in the present study was determined following the method described previously in details (Kostarelou et al. 2014, Vergopoulou et al. 2001). The same procedure was carried out for all the four *Aspergillus* strains used in this study.

2.8 Experimental design

In the present study, OTA production by two control strains *A. carbonarius* and *A. ochraceus* and by the *Aspergillus* section *Nigri* stains (ATHUM 6997, 6998, 6999, 7000) was studied in YES medium. In addition OTA production by *A. carbonarius, A. ochraceus* and *Aspergillus* section *Nigri* strains was also examined in currants (**Table 1**). OTA was assayed on days 0, 3, 7, 9 and 12 of incubation. For each day of observation and for each group, three samples of YES and currants were used. The whole experiment was repeated in triplicate.

TABLE 1
GROUPS OF EXAMINED CULTURES

Substrate	Cultures of strains control [a]	Cultures of strains *Aspergillus* section *Nigri* [a]
YES (10 mL)	YES + 10^2 conidia of *A. carbonarius* YES + 10^2 conidia of *A. ochraceus*	YES + 10^2 conidia of ATHUM 6997
		YES + 10^2 conidia of ATHUM 6998
		YES + 10^2 conidia of ATHUM 6999
		YES + 10^2 conidia of ATHUM 7000
Currants (15g)[b]	Currants + 10^2 conidia of *A. carbonarius* Currants + 10^2 conidia of *A. ochraceus*	Currants + 10^2 conidia of ATHUM 6997
		Currants + 10^2 conidia of ATHUM 6998
		Currants + 10^2 conidia of ATHUM 6999
		Currants + 10^2 conidia of ATHUM 7000

[a] For each day of observation and for each group, three samples of YES and currants were used
[b] samples of currants treated non inoculated were also examined
OTA was assayed on days 0, 3, 7, 9 and 12 of incubation

2.9 Inoculation

All flasks with YES and currants, inoculated and non-inoculated, were incubated under stationary conditions at 30 °C. Immediately after autoclaving for 30 min at 115°C for safety reasons, OTA production in all kind of samples was determined.

2.10 OTA determination and HPLC analysis

Every culture of currants was mixed with 30 mL methanol/water (80:20, v/v) while every culture of YES was mixed with 30 mL methanol and shaken well for 10 min. After filtration, a 5 mL aliquot was used for OTA analysis.

Clean-up: Five milliliters from the filtrate were diluted with 40 mL PBS and mixed for 1 min. The mixture was loaded onto the Ochratest column and washed once with 20 mL of water. The column was dried by passing air. OTA was eluted with 2mL methanol/acetic acid (98:2, v/v). The eluate was then evaporated to dryness under a gentle stream of nitrogen. The residue was re-dissolved in 200mL of mobile phase and analyzed by HPLC with Fluorescence Detector (volume injected=40 μL).

III. RESULTS AND DISCUSSION

A method for OTA determination in dried vine fruits was developed and validated in our laboratory. Mean recovery was found 104% (RSD= 7.56%), while OTA detection limit (DL) and limit of quantification (QL), defined at a signal-to-noise ratio of 3:1, they were found to be 0.26 and 0.51 ng g^{-1}, respectively (Kanapitsas et al. 2016). Moreover the OTA recovery factor in YES medium was found 95% and the DL, defined at a signal-to-noise ratio of 3:1, was 0.01 ng g^{-1} (Markaki unpublished results). In **Fig. 1**, representative chromatograms of OTA production in currants by all strains examined are exposed.

3.1 OTA production by *A. carbonarius*, *A. ochraceus* and *Aspergillus* section *Nigri* strains in YES medium

Both strains of *A. carbonarius* and *A. ochraceus* were used as control strains as mentioned previously, since they belong to major OTA producers. To our knowledge, there is no standard microbiological medium for OTA production by ochratoxigenic fungi. The medium YES was used in the present study for OTA production by the fungal strains, since it is considered as a suitable substrate for mycotoxin production by fungi (Bragulat et al. 2001, Frisvad et al. 1989, Scott et al.1970). It has also been previously used in our laboratory to investigate the AFB_1 production by the same *Aspergillus* strains (Tavoulari et al. 2014) in order to have comparable results.

The maximum amount of OTA produced in YES medium by the *Aspergillus* section *Nigri* strain ATHUM 6997 was observed on the 9th day (0.42 ng g^{-1}). On the other hand, OTA production by the *Aspergillus* section *Nigri* strains ATHUM 6998, 6999 and 7000 in YES medium continued until the last day of incubation (12th day) at levels 0.54 ng mL^{-1}, 0.06 ng mL^{-1} and 0.10 ng mL^{-1} respectively. OTA production by the control strains *A. carbonarius* and *A. ochraceus* achieved their maximum level on the 7th day (2.16 ng mL^{-1}, 120.50 ng mL^{-1} respectively) **(Table 2)**.

TABLE 2: OTA PRODUCTION (ng mL^{-1}) BY *A. CARBONARIUS, A. OCHRACEUS* AND THE FOUR STRAINS OF *ASPERGILLUS* SECTION *NIGRI* IN YES MEDIUM

Cultures of YES						
Days	***A. carbonarius*** (Control)	***A. ochraceus*** (Control)	**6997**	**6998**	**6999**	**7000**
0	0.26 (±0.40)	N.D.[a]	N.D.[a]	N.D.[a]	N.D.[a]	N.D.[a]
3	1.30 (±0.05)	85.35 (±1.18)	0.03 (±0.00)	0.12 (±0.01)	0.01 (±0.00)	0.01 (±0.00)
7	2.16 (±0.04)	120.50 (±4.11)	0.05 (±0.01)	0.36 (±0.01)	0.03 (±0.01)	0.01 (±0.00)
9	1.78 (±0.12)	105.57 (±2.20)	0.42 (±0.02)	0.47 (±0.02)	0.04 (±0.00)	0.08 (±0.01)
12	0.27 (±0.01)	94.71 (±3.13)	0.25 (±0.02)	0.54 (±0.03)	0.06 (±0.02)	0.10 (±0.00)

[a] Non-Detectable

Results from t-test analysis showed that the amounts of OTA produced in YES by the four *Aspergillus* section *Nigri* strains (ATHUM 6997, 6998, 6999 and 7000) was significantly different ($p<0.05$) compared to the production of OTA by *A. carbonarius* and the production of OTA by *A. ochraceus* (**Table 3**).

TABLE 3
STATISTICAL ANALYSIS (T-TEST) OF OTA PRODUCTION IN YES MEDIUM INOCULATED BY *STRAINS A. CARBONARIUS, A.OCHRACEUS* AND STRAINS OF *ASPERGILLUS* SECTION *NIGRI*

Substrate	Paired Samples	Df	T_{exp}	T_{theor}	Statistical significance
YES Medium	*A.carbonarius* + A.section *Nigri* ATHUM 6997	8	2.532	1.860	Yes
	A. carbonarius + A.section *Nigri* ATHUM 6998	8	2.131	1.860	Yes
	A. carbonarius + A.section *Nigri* ATHUM 6999	8	2.902	1.860	Yes
	A. carbonarius + A.section *Nigri* ATHUM 7000	8	2.871	1.860	Yes
	A.ochraceus + A.section *Nigri* ATHUM 6997	8	3.836	1.860	Yes
	A.ochraceus + A.section *Nigri* ATHUM 6998	8	3.829	1.860	Yes
	A.ochraceus + A.section *Nigri* ATHUM 6999	8	3.842	1.860	Yes
	A.ochraceus + A.section *Nigri* ATHUM 7000	8	3.841	1.860	Yes
	A.carbonarius + *A.ochraceus*	8	3.788	1.860	Yes

Specifically, comparing the maximum production by all strains of *Aspergillus* section *Nigri*, regardless the day of appearance (**Table 2**), to the maximum production by *A. carbonarius* and *A. ochraceus* (both on 7th day), it was found that the OTA production by the strain ATHUM 6997 in YES medium, was ~5-fold and ~287-fold lower than that by *A. carbonarius* and *A. ochraceus* respectively. It was revealed that the strains ATHUM 6998, 6999 and 7000 produced maximum amounts of OTA ~4-fold, ~36 and ~22 fold lower in comparison to the maximum OTA production by *A. carbonarius*. Moreover, OTA maximum amounts produced by the strains ATHUM 6998, 6999 and 7000 were ~223, ~2008 and ~1205 fold respectively, lower than the OTA amounts produced by *A. ochraceus.*

One-way ANOVA analysis showed that there was significant difference of OTA production between all strains of *Aspergillus* section *Nigri*, during the whole period of incubation (12 days) (**Table 4**).

TABLE 4
ONE WAY ANOVA STATISTICAL ANALYSIS OF OTA PRODUCTION IN YES MEDIUM INOCULATED BY THE FOUR STRAINS OF *ASPERGILLUS* SECTION *NIGRI*

Substrate	Samples	Df	F_{exp}	F_{theor}	Statistical significance
YES Medium	A.section *Nigri* ATHUM 6997 + A.section *Nigri* ATHUM 6998 + A.section *Nigri* ATHUM 6999 + A.section *Nigri* ATHUM 7000	3, 16, 19	3,572	3.239	Yes

The *Aspergillus* section *Nigri* strain ATHUM 6998 produced higher amounts of OTA from 0.12 to 0.54 ng mL^{-1} (mean: 0.37 ng mL^{-1}) compared to the OTA production by the strains *Aspergillus* section *Nigri* ATHUM 6997 (0.03-0.42 ng mL^{-1}, mean 0.19 ng mL^{-1}), 6999 (0.01-0.06 ng mL^{-1}, mean:0.04 ng mL^{-1}) and 7000 (0.01-0.1 ng mL^{-1}, mean: 0.05 ng mL^{-1}) (**Table 2**).

In addition comparing the OTA production by *A. carbonarius* and *A. ochraceus* in YES medium, it is concluded that there is significant difference ($p<0.05$) between the amounts of OTA produced during the whole period of the observation **(Table 3)**. Consequently, YES medium is a substrate more appropriate for OTA biosynthesis by *A. ochraceus* than the production by *A. carbonarius* and *Aspergilli* section *Nigri* used in the present work.

The strains *Aspergillus* section *Nigri* ATHUM 6997, 6998, 6999 and 7000 have been investigated in our laboratory and showed that they were able to produce AFB_1 in YES medium (Tavoulari et al. 2014). The results of the present work display that the four *Aspergillus* section *Nigri* strains are able to produce both OTA and AFB_1 in YES medium. Nevertheless the maximum AFB_1 amounts produced in YES medium were ~259-fold (ATHUM 6997), ~118-fold (ATHUM 6998), ~107-fold (ATHUM 6999) and ~140-fold (ATHUM 7000) higher than the maximum OTA amounts produced by the same strains in the same medium under the same conditions. Hence YES medium is more favorable substrate for AFB_1 than OTA production by *Aspergillus* section *Nigri* strains (ATHUM 6997, 6998, 6999, 7000) which they could be characterized as aflatoxigenic.

To our knowledge there is no information about the production of both AFB_1 and OTA by *Aspergillus* section *Nigri* strains in microbiological media.

3.2 OTA production by *A. carbonarius*, *A. ochraceus* and *Aspergillus* section *Nigri* strains in currants

In the present work OTA production by *A. carbonarius, A. ochraceus* and *Aspergillus section Nigri strains* was studied in Corinthian type currants and results are shown in **Table 5** and **Fig.2**.

TABLE 5
OTA PRODUCTION (ng g^{-1}) BY *A. CARBONARIUS, A. OCHRACEUS* AND THE FOUR STRAINS OF *ASPERGILLUS* SECTION *NIGRI* IN CURRANTS

Cultures of currants						
Days	***A. carbonarius*** (Control)	***A. ochraceus*** (Control)	**6997**	**6998**	**6999**	**7000**
0	0.20 (±0.05)	0.05 (±0.01)	0.76 (±0.02)	2.84 (±0.17)	1.99 (±0.09)	N.D.[a]
3	0.31 (±0.03)	0.07 (±0.01)	1.19 (±0.02)	3.24 (±0.20)	2.24 (±0.17)	2.01 (±0.05)
7	4.33 (±0.19)	0.11 (±0.02)	2.42 (±0.02)	4.08 (±0.12)	2.68 (±0.07)	3.82 (±0.17)
9	1.33 (±0.33)	0.14 (±0.02)	0.54 (±0.01)	1.50 (±0.17)	1.83 (±0.10)	1.35 (±0.12)
12	0.65 (±0.04)	0.34 (±0.02)	0.02 (±0.00)	0.96 (±0.07)	1.03 (±0.06)	0.87 (±0.07)
[a] **Non-Detectable**						

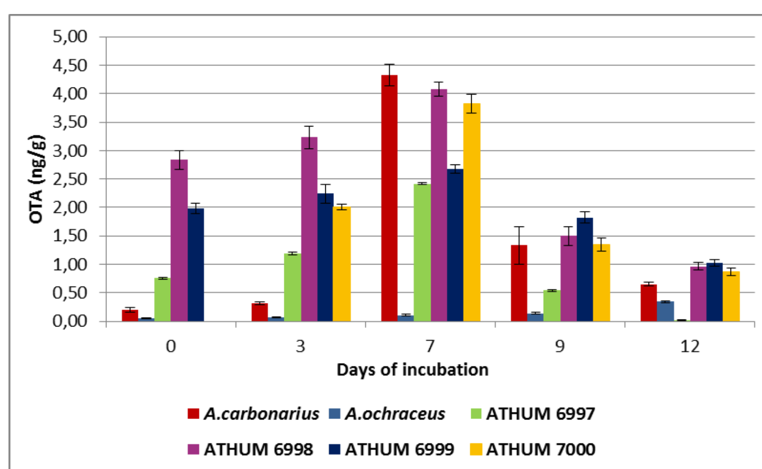

FIGURE 2: COMPARATIVE CHART OF OTA PRODUCTION (ng g^{-1}) BY *A. CARBONARIUS, A. OCHRACEUS* AND THE FOUR STRAINS OF ASPERGILLUS SECTION NIGRI IN CURRANTS. OTA PRODUCTION BY A. CARBONARIUS IN CURRANTS WAS NOT SIGNIFICANTLY DIFFERENT COMPARED TO OTA PRODUCTION BY THE STRAINS ATHUM 6997, 6998, 6999, 7000. MAXIMUM AMOUNTS OF OTA PRODUCED BY THE STRAINS ATHUM 6997, 6998, 6999, 7000 IN CURRANTS WERE FOUND TO BE LOWER THAN THAT BY A. CARBONARIUS (7th day) . THE MAXIMUM OTA LEVELS PRODUCED BY THE STRAINS ATHUM 6997, 6998, 6999 AND 7000 (7th day) WERE FOUND TO BE HIGHER, IF COMPARED TO THE MAXIMUM OTA PRODUCTION BY A. OCHRACEUS (12th day).

The maximum amount of OTA produced in Corinthian currants by the four *Aspergillus* section *Nigri* strains ATHUM 6997, 6998, 6999 and 7000 was observed on the 7th day of incubation and were found to be 2.42 ng g^{-1}, 4.10 ng g^{-1}, 2.68 ng g^{-1} and 3.82 ng g^{-1} respectively. On the other hand the maximum OTA production by *A. carbonarius* (4.33 ng g^{-1}) was observed on the 7th day of incubation while the OTA production by *A. ochraceus* was continued until the 12th day, which was the last day of observation (**Table 5, Fig.2**).

Statistical analysis indicated that OTA production by *A. carbonarius* in Corinthian currants was not significantly different ($p>0.05$) compared to OTA production by the strains ATHUM 6997, 6998, 6999, 7000 (**Table 6**). However the maximum amounts of OTA produced by the strains ATHUM 6997 and 6999 in currants were found to be ~2-fold lower than that by *A. carbonarius.* This is in agreement with Lasram et al., (2012) who showed that *A. carbonarius* is characterized by many OTA-positive strains (97%) in contrast to *A. niger* isolates producing OTA at percentage 3%.

TABLE 6
STATISTICAL ANALYSIS (T-TEST) OF OTA PRODUCTION IN CURRANTS INOCULATED BY STRAINS *A. CARBONARIUS, A.OCHRACEUS* AND STRAINS OF *ASPERGILLUS* SECTION *NIGRI*

Substrate	Paired Samples	Df	T_{exp}	T_{theor}	Statistical significance
Currants	*A.carbonarius* + A.section *Nigri* ATHUM 6997	8	0.438	1.860	No
	A. carbonarius + A.section *Nigri* ATHUM 6998	8	1.212	1.860	No
	A. carbonarius + A.section *Nigri* ATHUM 6999	8	0.722	1.860	No
	A. carbonarius + A.section *Nigri* ATHUM 7000	8	0.246	1.860	No
	A.ochraceus + A.section *Nigri* ATHUM 6997	8	2.070	1.860	Yes
	A.ochraceus + A.section *Nigri* ATHUM 6998	8	4.157	1.860	Yes
	A.ochraceus + A.section *Nigri* ATHUM 6999	8	6.514	1.860	Yes
	A.ochraceus + A.section *Nigri* ATHUM 7000	8	2.277	1.860	Yes
	A.carbonarius + A.ochraceus	8	1.593	1.860	No
	A.carbonarius + A.ochraceus (Only for maximum production)	4	36.653	2.132	Yes

Moreover OTA production by *A. ochraceus* was significantly different ($p<0.05$) in comparison to OTA amounts produced by the four strains *Aspergillus* section *Nigri* for the whole period of observation. Specifically the maximum OTA levels produced by the strains ATHUM 6997, 6998, 6999 and 7000 were found to be ~7, ~12, ~9 and ~11 fold higher respectively, if compared to the maximum OTA production by *A. ochraceus* (**Table 5, Fig.2**).

One way ANOVA analysis showed that there was not significant difference of OTA production between all strains of *Aspergillus* section *Nigri*, during the period of incubation (12 days) in currants (**Table 7**).

TABLE 7
ONE WAY ANOVA STATISTICAL ANALYSIS OF OTA PRODUCTION IN CURRANTS INOCULATED BY THE FOUR STRAINS OF *ASPERGILLUS* SECTION *NIGRI*

Substrate	Samples	Df	F_{exp}	F_{theor}	Statistical significance
Currants	*A.*section *Nigri* ATHUM 6997 + *A.*section *Nigri* ATHUM 6998 + *A.*section *Nigri* ATHUM 6999 + *A.*section *Nigri* ATHUM 7000	3, 16, 19	1.693	3.239	No

Having compared the two control strains *A. carbonarius* and *A. ochraceus* no significant difference was observed between the OTA amounts that produced in currants during the whole period of observation. Although it was shown that the maximum amounts of OTA produced by *A. carbonarius* (7th day) in currants was ~13-fold higher than the maximum production by *A. ochraceus* (12th day). This difference was found to be statistically significant **(Table 5, Table 6)**.

The strains ATHUM 6997, 6998, 6999 and 7000, studied in our laboratory previously, produced AFB_1 in currants (Kostarelou et al. 2014). In the present work it was shown that the *Aspergillus* section *Nigri* strains examined, have the ability to produce OTA in addition to AFB_1 in currants. It is noteworthy that the strain ATHUM 6997 produced maximum OTA amounts ~2-fold lower than the maximum AFB_1 amounts produced in currants. Wheareareas OTA maximum production by the strains ATHUM 6998, 6999 and 7000 were ~13-fold, ~9-fold and ~11-fold higher respectively, than the corresponding maximum AFB_1 amounts reported by Kostarellou et al. (2014).

Although the *Aspergillus* section *Nigri* strains studied in the present work are producers both of AFB_1 and OTA, no information exists in the literature for the production of the two mycotoxins by the same *Aspergillus* section *Nigri* strain. However, it has been reported that 10% of *Aspergillus niger* strains (total n=180) and 26% of industrial strains (total n = 69) of *A. niger* produced both fumonisins and ochratoxin A (Frisvad et al. 2011). In addition, Abrunhosa et al. (2011) examined 681 strains of black aspergilli isolated from Portuguese wine grapes and found that 10 strains produced FB_2 and ochratoxin A simultaneously.

On the other hand, co-occurrence of OTA and AFB_1 is usually observed in several food products. In a survey conducted in Turkey aflatoxins (AFs) and OTA were both present in nine maize flour samples (Kara et al. 2015). Moreover, the co-occurrence of AFB_1 and OTA was detected in 62.5% of red chilli flake, 40.9% of red chilli powder and 4.3% of black pepper powder samples, analyzed during a study in Turkey (Ozbey and Kabak, 2012). Klarić et al. (2009) investigated the presence of aflatoxins (AFs), OTA, fumonisins (FBs) and zearalenone (ZEA) in food commodities and found that aflatoxins and OTA co-occurred in 17% of cereals samples followed by AFs+OTA, OTA+ZEA and ZEA+FBs (12.5%) while the four mycotoxins co-occurred in one sample of maize. In a study from Iran, fourteen barley and nine corn samples intended for animal feed, analyzed for aflatoxins and OTA but only one of the corn samples was co-contaminated with AFs and OTA (Yazdanpanah et al. 2001). In addition Perrone et al. (2013) reported for the first time the co-occurrence of OTA and FB_2 in dried vine fruits originating from Greece, while Kollia et al. (2014) analyzed twenty-six samples of dried vine fruits for AFB_1 and OTA occurrence and found that six of the samples were concomitantly contaminated by the two mycotoxins.

The results of all these studies and the results of the present work, showed that more than one mycotoxin could occur concomitantly in the same substrate. Nevertheless these reports do not clarify that this co-occurrence of the different mycotoxins is the result of the production by one or different mycotoxigenic strains. However, Streit et al. (2012) reported that co-occurrence of mycotoxins is expected for several reasons. The same fungus can produce more than one mycotoxins, while different fungi could contaminate food and agricultural products. Moreover, feeds and foods are usually made from a variety of commodities with the potential of multiple contaminations, contributing to the final mycotoxin activity. However accumulation of more than one toxins produced by a fungus, is affected by several factors. It is important to take into account the interactions that could occur between the microorganism, the substrate and the environmental conditions. Each factor

may affect on a different way the production of mycotoxins. So changes of the temperature or the a_w could influence the production of each toxin (Magan et al. 1984). Regarding the risk to consumers according to Speijers and Speijers (2004) the concomitant occurrence of more than one mycotoxin can induce additive antagonistic and synergistic effects. The risk for consumers by a combination of mycotoxins in agricultural products, is due to modifications to the toxicokinetics, the metabolism and the toxicodynamic properties of mycotoxins. Therefore concerning the risk assessment for consumers needs more attention due to the synergism and interaction of the mycotoxins. Thus from the public health approach, the present study points to an elevated risk for human and animals. On the other hand, in practice, the consequence of combined exposure to mycotoxins might be altered from that which would be expected because the result could be influenced by several factors (Pohland et al., 1992).

Mycotoxin biosynthesis is a complicate process with a lot of parameters involved. The consistency of the results on mycotoxin production is depending on the exact experimental protocols used in each case.

IV. CONCLUSIONS

The novel strains of *Aspergillus* section *Nigri* ATHUM 6997, 6998, 6999 and 7000, of Greek origin, examined during this study, produced significant amounts of OTA under the exact experimental conditions applied in YES medium and in currants. Along with a previous study, in the present work it is concluded that the four *Aspergillus* section *Nigri* strains are able to produce both aflatoxin B_1 and OTA, which is reported for the first time to the best of our knowledge. Therefore, mycotoxin production by strains of *A.* section *Nigri* investigated in the present study is an additional problem for the agricultural products contamination and may raise concerns for human health and economy.

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