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# **Biodegradation study of ochratoxin A by Nigri section *Aspergillus* species isolated from French grapes: a hope for OTA decontamination in grapes and musts.**

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## **ABSTRACT**

Ochratoxin A (OTA) is a very dangerous mycotoxin frequently reported in different foods and beverages such as grapes, grape juices and wines. Detoxifying those products is then of prime importance to protect consumer health. Biological approaches are the most promising and the black aspergilli, the principle colonizers of berries could be of interest.

In this report, 40 fungi of black aspergilli species (*Aspergillus carbonarius*, *A. niger* aggregate, *A. japonicus*) isolated on different varieties of French grapes, were assessed for ochratoxin A (OTA) degradation capacities in liquid CZAPEK Yeast extract Medium (CYM) and for the first time in a Simulating Grape juice one (SGM). It was clearly observed that in both media used those fungi had OTA degradation ability regardless of being ochratoxigenic (*A. carbonarius*) or not (isolates of *A. niger* aggregate and *A. japonicus*). The same degradation pathway was observed for the three black aspergilli species. OTA was converted to OT $\alpha$  that was then decreased and gradually transformed to a new compound. However, differences between media used and species tested during OTA degradation were noted. OTA

degradation in SGM was more efficient than that in CYM and *A. niger* could be the first interesting species for OTA detoxification processes followed by *A. japonicus*.

## **INTRODUCTION**

Ochratoxin A is a very dangerous fungal secondary metabolite exhibiting nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties (Smith and Moss, 1985). It is produced by a number of ubiquitous molds of *Penicillium* and *Aspergillus* genera that contaminate agricultural commodities either before harvest or during storage (Moss, 1996; Galtier, 1999). Ochratoxin A is detected in different food products such as cereals (Jorgensen et al., 1996), coffee (Pittet et al., 1996), beer (Visconti et al., 2000), dried vine fruits (McDonald et al., 1999) grapes, grape juices and wines (Zimmerli and Dick, 1995).

According to Codex Alimentarius Commission, 15% of the total intake of OTA is due to wine (Anonyme, 1999), that is also considered as the second major source of OTA intake after cereals (Anonymous 1998). Moreover, grape juices are shown to contain more OTA than some wines and so contribute to OTA intake by children (Zimmerli and Dick, 1996). OTA contamination in grapes and grape products could then be considered as a serious health problem. Efforts to understand this contamination origin were carried out in different countries especially in the European ones in order to strictly control it (Vth PCRD Project). Prevention of OTA production and/or detoxification of those contaminated products are of prime importance to protect consumer health (Anonymous, 1997).

Several physical and chemical methods have been proposed to remove mycotoxins (Sinha, 1998). Although some of those methods could be promising (ozone (McKenzie et al., 1997), alkaline hydrogen peroxide (Fouler et al., 1994), Gamma irradiation (Refai et al., 1996)), others are not recommended for practical decontamination (hypochlorite treatment (

castegnaro et al., 1991), ammoniation (Chelkowski et al., 1982), heat treatment (Boudra et al., 1995).

Biological approaches are now being widely studied (Sweeny and Dobson, 1998), but none of them were especially interested in grape juices decontamination.

According to studies done in French vineyards, Black aspergilli, were the principle microorganisms belonging to the biotop of grapes and colonizing berries from setting to harvest (Bejaoui et al., in press). Our objective in this work was to do a large screening on black aspergilli isolated on French grapes, in order to see their capacity to degrade OTA in a laboratory medium (CYA) and for the first time in a simulating grape juice one (SGM).

Kinetics of OTA and of its degradation metabolites as well as comparisons between OTA degradation profiles by *A. carbonarius*, *A. niger* and *A. japonicus* in SGM were achieved for the first time during this survey.

## **MATERIALS AND METHODS**

### **Strains**

Forty fungi belonging to three species of black aspergilli (*A. carbonarius*, *A. japonicus* and *A. niger*) isolated on five different grape cultivars (Syrah, Carignan, Grenache, Sauvignon, Muscat) from French Mediterranean area were used.

### **Culturing media**

For fungi strains both a modified CZAPEK YEAST EXTRACT LIQUID media (CYA) and Synthetic Grape juice Medium (SGM) were used.

Synthetic grape juice (SGM) was prepared by dissolving: 70g glucose D(+) (Fisher Labosi), 30g fructose(D-) (Sigma), 7g tartaric acid(L-) (Rectapur-Prolabo), 10g malic acid (L-) (Fisher Labosi), 0.67g (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (Prolabo-Rhône Poulenc), 0.67g KH<sub>2</sub>PO<sub>4</sub> (Acros), 1.5g MgSO<sub>4</sub>·7H<sub>2</sub>O (Acros), 0.15g NaCl (Fisher Labosi), 0.15g CaCl<sub>2</sub> (Acros), 0.0015g CuCl<sub>2</sub> (Prolabo), 0.021g FeSO<sub>4</sub>·7H<sub>2</sub>O (Riedel-de Haën), 0.0075g ZnSO<sub>4</sub>·7H<sub>2</sub>O (Fisher Labosi) and

0.05g hydrated catechin (Sigma); in 1 l distilled water and the pH was adjusted at 3.8-4.0 with KOH 2N.

A modified CZAPEK YEST EXTRACT LIQUID media (CYL) was prepared by dissolving: 30g: saccharose; 5g: yeast extract; 50 ml: solution A((for 500ml) (NaNO<sub>3</sub>:20g; KCl:5g; MgSO<sub>4</sub>. 7H<sub>2</sub>O: 5g; FeSO<sub>4</sub>.7H<sub>2</sub>O:0.1g)); 50 ml: solution B((for 500 ml): K<sub>2</sub>HPO<sub>4</sub>:10g); 1 ml: solution C ((100 ml): ZnSO<sub>4</sub>.7H<sub>2</sub>O:1g, CuSO<sub>4</sub>.5H<sub>2</sub>O: 0.5g:)) in one liter distilled water.

These laboratory media were supplemented with ochratoxin A at a concentration of 2µg ml<sup>-1</sup>.

### **Fermentation conditions**

The degradation capacity screening of these strains was first undertaken in screw cap's tubes in 2 ml appropriate medium (CYA or SGM), under agitation (240 rpm/min), at 30°C, during 12 days. Each medium was inoculated at 1% either from a conidial suspension (10<sup>7</sup> conidia/ml). Kinetic studies of OTA degradation and OTA production for three *Aspergillus* strains (*A. carbonarius* SA332, *A. japonicus* AX35 and *A. niger* GX312) were conducted during batch fermentation in 20 ml of SGM media in Erlenmeyer flasks, at 25°C under agitation (240 rpm/min).

A negative control (control 1), an OTA contaminated medium without fungi, was used to calculate OTA removal percentage. A positive control (control 2), a fungi culture in a medium without OTA, was also used.

All assays were performed in triplicates.

### **OTA extraction**

For all samples, after removal of fungal mycelium, 1 ml of supernatant was extracted twice with ethyl acetate (v/v), then evaporated until dry and dissolved in 0.5 ml of methanol before HPLC analysis.

### **Detection and quantification of OTA**

OTA was detected and quantified by reversed-phase high performance liquid chromatography. The HPLC apparatus consisted of a solvent delivery system and fluorescence ( $\lambda_{\text{ex}} = 332 \text{ nm}$ ;  $\lambda_{\text{em}} = 466 \text{ nm}$ ) and UV detectors. The analytical column used was a 150 x 4.6 mm Uptisphere 5  $\mu$  C18 ODB fitted with a guard column of 10 x 4 mm. The mobile phase consisted of a mixture of HPLC grade acetonitrile/water/acetic acid (100/99.8/0.2) at a flow rate of 1 ml min<sup>-1</sup> and the column temperature was at 30°C. Kroma 3000 (Biotek) was the data acquisition system. Injections were made with an autoinjector (BIO-TEK, Milan, Italy) and the injection volume was of 80  $\mu$ l. Ochratoxin A was identified by its retention time (33 min) according to a standard obtained from Sigma (Steinheim, Germany) and quantified by measuring the peak area.

The OTA degradation percentage was calculated according to the following equation:  $100 \times [1 - (\text{peak area of OTA} / \text{peak area of OTA in control 1})]$ .

Standard of ochratoxin  $\alpha$  was prepared by enzymatic hydrolysis of ochratoxin A. This method was performed with carboxypeptidase A (EC3.4.17.1) from bovine pancrea (Sigma, type II-PMSF) 1000 units/L. Ochratoxin A was dissolved in 1 mL of buffered solution (0.025 M Tris-HCl, 0.5M NaCl, pH. 7.5) and 20  $\mu$ L of enzyme solution was added. Total degradation was observed after 2 days of incubation at room temperature.

## **RESULTS**

### **Screening of Nigri section *Aspergillus* isolates for OTA detoxifying activity**

A total of 40 *Aspergillus* isolates representing *Nigri* section (*A. carbonarius*, *A. niger* and *A. japonicus*), were tested for their ability to degrade OTA in either CYA or on Synthetic Grape juice Medium (SGM) (table1). Chosen *A. carbonarius* isolates show low capacities to produce OTA, whereas *A. niger* and *A. japonicus* ones are not ochratoxigenic. All these isolates cultured on either laboratory medium (CYA) or on SGM initially contaminated with OTA (2  $\mu$ g mL<sup>-1</sup>) have shown significant capacities to degrade the mycotoxin after 12 days of

incubation time. However, OTA degradation was more frequently observed in SGM than in CYA medium. Indeed, 77% and 45% of the isolates tested were able to degrade more than 80% of the OTA added to SGM and CYA medium respectively. Moreover, lowest capacities in decreasing OTA were higher than 30% in SGM whereas some isolates were not able to degrade OTA in CYA medium. According to species tested, *A. niger* was the best OTA degrader, followed by *A. japonicus* and *A. carbonarius*. For all those species  $O\alpha$  : an OTA degradation metabolite was produced.

### **Kinetics of ochratoxin A degradation by three isolates of Nigri section *Aspergillus* species on SGM.**

Kinetics of OTA degradation by three Nigri section *Aspergillus* species: *A. carbonarius* SA332, *A. niger* GX312 and *A. japonicus* AX35, have been examined in SGM.

Regardless of the species tested, HPLC analysis indicated that OTA was degraded and converted to  $O\alpha$ . After the 9<sup>th</sup> day of incubation, the amounts of  $O\alpha$  decreased (result not shown) and an unknown compound appeared (Figure 2).

Although the same pathway in OTA degradation by the three black aspergilli species was observed, particularities in kinetics for each species are noteworthy. In fact, during the first three days of incubation, *A. niger* degraded OTA relatively slowly (20%) compared to *A. carbonarius* (55%) and *A. japonicus* (80%). However, after the 3<sup>rd</sup> day, OTA degradation by *A. niger* became the most rapid and reached 99% after 5-day incubation. At this time, *A. carbonarius* and *A. japonicus*, decreased OTA at 83% and 89% respectively.

## DISCUSSION

In this study, we examined the OTA decomposing activities of 40 isolates of black aspergilli. Both in the first screening carried out in CYA and SGM and during kinetics in SGM, decreases in OTA by black aspergilli were observed.

Regardless of the species used, OTA was converted to Ota which still has limited toxicity (Harwig, 1974). This OTA metabolism was previously observed for different microorganisms like protozoa (in gastrointestinal tracts of ruminants) (Galtier and Alvinerie, 1976; Hult et al., 1976), *Acinetobacter calcoaceticus* (Hwang and Draughon, 1994), *Phenylobacter immobile* (Wegst and Lingens, 1994), *A. niger* (Xiao et al., 1996; Varga et al., 2000) and black aspergilli isolated on Portuguese grapes (Abrunhosa et al., 2002).

A carboxypeptidase, previously found to be able to convert OTA to Ota (Deberghes et al., 1995), may be involved in OTA degradation, and was also supposed as an OTA metabolizing enzyme for black aspergilli (Varga et al., 2000; Abrunhosa et al., 2002). A crude lipase from *A. niger*, was also proved to substantially hydrolyse OTA to Ota and phenylalanine (Stander et al., 2000). The three species of black aspergilli were then able to degrade Ota to an unknown compound in SGM. This was also reported by Varga et al. (2000) for *A. niger* in YES medium. This was explained by those authors as probably due to an attack of isocoumarin ring as previously reported by Galtier and Alvinerie (1974). However this pathway is still unknown.

Although the same pathway in metabolising OTA was observed for the three species of black aspergilli, differences between OTA degradation capacities and profiles in media and by species used are noteworthy. Indeed, important capacities to degrade OTA were more frequently observed in SGM than in CYA medium. The nearest composition of SGM to grapes: the natural habitat of black aspergilli used during this study could be an important



factor to fungal development and to OTA catabolism. Moreover, SGM is an acid substrate with a pH of about 4.5 whereas CYA is rather neutral medium (pH. 6.8-7). Previous studies showed that carboxypeptidases from *A. niger* had an optimal activity at pH 4 and were unstable above 7 (Dal Degan et al., 1992).

So, OTA degradation depends on media used. Furthermore, the important capacity to OTA degradation in SGM exhibited by all black aspergilli is of interest as it could be present in grapes and should then be taken into account when trying to relate the OTA presence in berries with their mycoflora (Abrunhosa et al., 2002). However, according to species tested, differences in OTA degradation were noted. *A. niger* had the most important capacity to detoxify OTA, followed by *A. japonicus* and by *A. carbonarius*. Indeed, almost all the isolates of *A. niger* tested were able to degrade OTA in both media used. Kinetics in SGM has also shown a complete degradation of the mycotoxin by this species in shorter times than by *A. japonicus* and *A. carbonarius*.

Although Hwang and Draughon (1994) found that *A. niger* is unable to degrade OTA, Varga et al. (2000) found among isolates belonging to this species either degraders and non degraders of OTA and the totality of *A. niger* isolates tested by Abrunhosa et al. (2002) were able to decrease OTA amounts. Furthermore this species was one of the few fungi which has received the GRAS status from the U.S. Food and Drug administration (due to its low toxigenicity) and could then be interesting to further uses in biological elimination of OTA in foods. However, *A. japonicus* frequently used in fungal biotechnology could also be of interest as it has never been reported ochratoxigenic.

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## Appendix

Table 1: Black aspergilli isolates examined for OTA degradation activities

Black aspergilli isolates	OTA degradation (%)	
	CYA	SGM
<i>A. carbonarius</i> isolates		
Mu243	99	87
Mu644	68	76
Mu441	52	67
A535	6	36
B935	39	72
C234	39	82
C933	14	98
C232	50	86
SA231	70	97
SA434	47	82
SA332	0	97
G731	61	96
G738	0	98
CA332	35	80
CA532	10	57
CA224	0	99
<i>A. niger</i>		
Mu148	100	99

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MuX44	80	97
A433	99	99
B434	99	98
B939	10	99
C432	99	91
C339	99	98
C137	90	99
C535	5	93
CX33	98	99
SA731	72	99
GX312	98	99
G431	79	98
CAX38	99	75
CA932	95	92
<i>A. japonicus</i>		
Mu541	97	82
A437	82	82
AX35	85	80
SA413	90	88
G933	50	99
G231	96	61
CA223	89	71
CAX35	72	85
CN311	38	75

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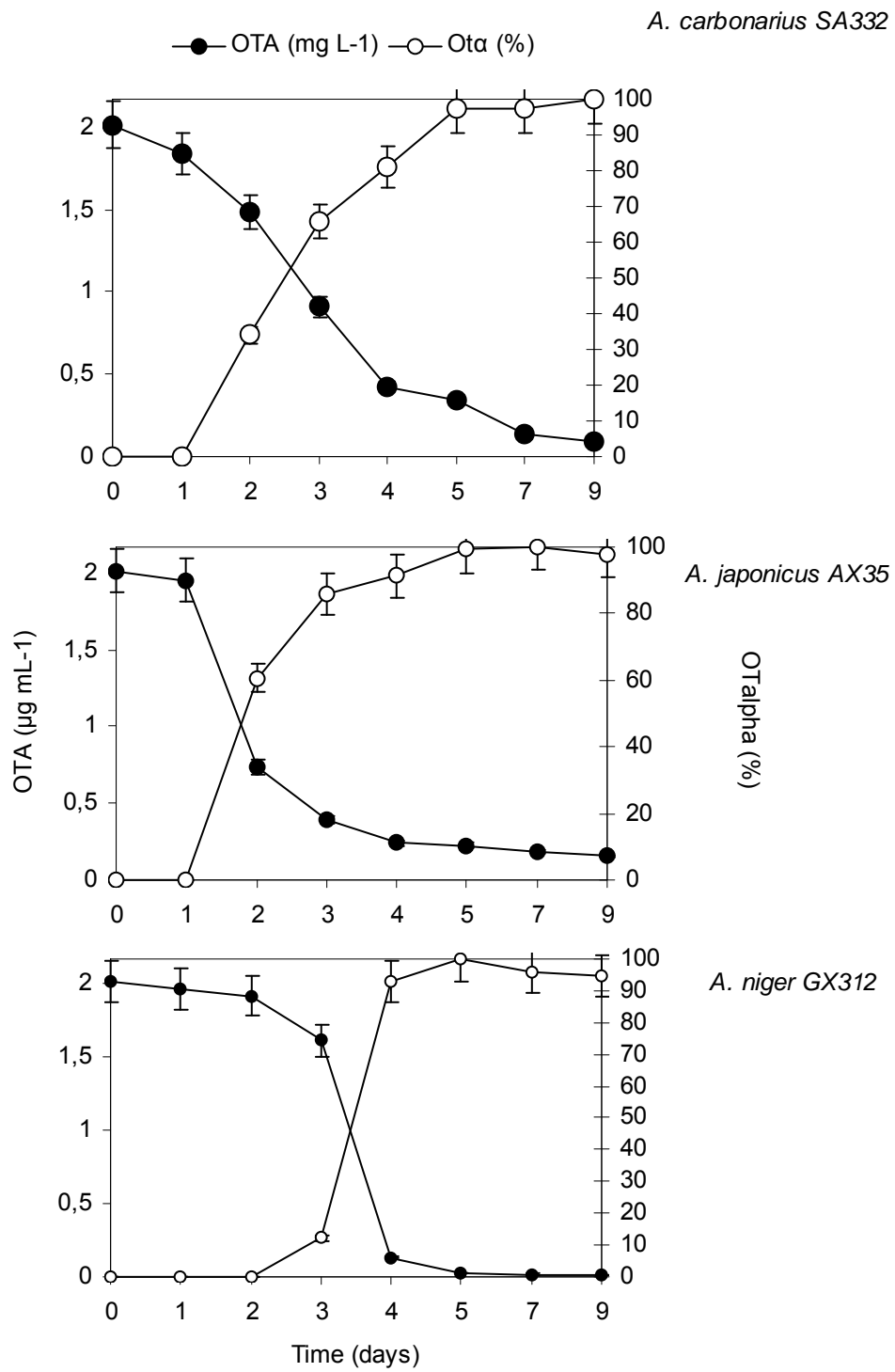


Fig 1: Evolution of OTA and Ota in presence of *Aspergillus* isolates on SGM medium.



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### **List of figures**

*Figure 1.* Evolution of OTA and OT $\alpha$  in presence of *Aspergillus* isolates on SGM medium

*Figure 2.* Compunds resulting from degradation of ochratoxin A (O $\alpha$ ) and O $\alpha$  (HE : unknown compound) in SGM medium by *A. japonicus* after 22 days of incubation (A) and the chromatogram of the control without OTA at the same day.

### **List of tables**

*Table 1.* Black aspergilli isolates examined for OTA degradation activities