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Conidia of Black Aspergilli as New Biological Adsorbents for Ochratoxin A in Grape Juices and Musts

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Biological removal of ochratoxin A (OTA) by living and heat-treated dead conidia of black *Aspergillus* isolates representing the species *Aspergillus niger*, *Aspergillus carbonarius*, and *Aspergillus japonicus* in synthetic and natural grape juices was found to be a two-stage phenomenon. Several lines of evidence suggest that the first observed stage was passive, metabolism was not required, and OTA adsorption on conidia of black aspergilli could be involved. This removal was fast, without delay just after conidial inoculation both in synthetic and natural grape juices. Moreover, even nonviable, heat-treated conidia were capable of removing OTA. Finally, no OTA degradation products were detected. In the second observed stage, removal of OTA was linked to degradation by live conidia only. Ochratoxin alpha, a degradation product of OTA, was detected in the medium after incubation for 30 and 14 h for biseriate (*A. niger* and *A. carbonarius*) and uniseriate (*A. japonicus*) black aspergilli, respectively, when well-developed mycelium appeared. Comparisons between the three black *Aspergillus* isolates tested showed that *A. carbonarius* detoxified grape juice most effectively. However, this species often produces OTA. *A. niger* and *A. japonicus* isolates were also effective and because those species are not systematically OTA producers, they could be interesting for further OTA detoxification processes in grape juices and musts.

KEYWORDS: Ochratoxin A; conidia of black aspergilli; *Aspergillus niger*; *Aspergillus japonicus*; *Aspergillus carbonarius*; adsorption; detoxification; grape juices; musts

INTRODUCTION

Ochratoxin A (OTA) is a naturally occurring secondary metabolite of mold fungi of *Aspergillus* and *Penicillium* genera (*1*, *2*). It is affecting agricultural products all over the world and is causing harmful effects on human and animal health because of its highly toxic properties (mutagenic, teratogenic, carcinogenic) (*3*, *4*). The presence of OTA in grapes and grape products was reported for the first time in 1995 (*5*). Since then, different studies were undertaken to understand this contamination origin over the world and also in France (*6*). Several studies (*6*−*9*) showed that *Aspergillus* section Nigri isolates (*Aspergillus carbonarius*, *Aspergillus niger*, and *Aspergillus japonicus*) are thought to be the primary source of OTA in grapes, especially *A. carbonarius*. Wines are considered as the second major source of OTA intake after cereals (*10*), and grape juices are shown to contain more OTA than some wines and so contribute to OTA intake by children (*8*). Recently, the OTA limit in wine (2 μg/L) was defined by the Commission Regulation (EC) N° 123/2005, of January 2005, amending Regulation (EC) N° 466/2001 as regards ochratoxin A. So, methods for OTA detoxification are highly needed for compliance with tolerances and guidelines to protect consumer health. Different attempts have been made to find measures to combat this mycotoxin. Physical, chemical, and biological methods were studied (*12*), but few of these have practical applications. Some are targeting to degrade OTA in different laboratory media, while some others tried to adsorb it. Different microorganisms belonging to grapes microbiota such as *Aspergillus* section Nigri isolates were reported as able to degrade OTA (*13*−*15*), whereas both chemical (fining agents (*16*)) and biological agents (heat treated yeasts (*17*)) had the capacity to adsorb it. Although some of those adsorbents could be of interest (*17*), some others have little effect on the removal of OTA and in many cases the quality of wines is severely damaged (*16*, *18*).

For the first time, the capacity of conidia of black *Aspergillus* isolates to adsorb ochratoxin A in both synthetic and natural red grape juices was evaluated. Kinetics of OTA removal and OTα production were done during this survey to clarify the mechanism of OTA removal/degradation by conidia of black aspergilli. Grape juice quality related to color after conidial inoculation was also assessed.

MATERIALS AND METHODS

Strains. Three *Aspergillus* section Nigri isolates, *A. carbonarius SA332* (IMI388497), *A. japonicus* AX35 (IMI389197), and *A. niger*

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GX312 (IMI388497), isolated on French grapes were used. Identification of different strains of black aspergilli was made using macroscopic and microscopic morphological criteria in accordance with appropriate keys (*19*−*22*). They were preserved at the Culture Collection of CABI BIOscience (London). According to a previous study, *A. carbonarius* SA332 is a weak OTA producer, while *A. niger* GX312 and *A. japonicus* AX35 are not.

Culturing Media. A synthetic grape juice medium (SGM) (*23*, *24*) and a natural commercial red one were used.

Synthetic grape juice (SGM) was prepared by dissolving 70 g glucose D(+) (Fisher Labosi (Elancourt cedex, France)), 30 g fructose (D−) (Sigma (Saint Quentin Fallavier, France)), 7 g tartaric acid (L−) (Rectapur-Prolabo (Paris, France)), 10 g malic acid (L−) (Fisher Labosi), 0.67 g $(NH_4)_2HPO_4$ (Prolabo-Rhône Poulenc (Paris, France)), 0.67 g KH_2PO_4 (Acros (Geel, Belgium)), 1.5 g $MgSO_4,7H_2O$ (Acros), 0.15 g NaCl (Fisher Labosi), 0.15 g $CaCl_2$ (Acros), 0.0015 g $CuCl_2$ (Prolabo (Paris, France)), 0.021 g $FeSO_4,7H_2O$ (Riedel-de Haën (Seelze, Germany)), 0.0075 g $ZnSO_4,7H_2O$ (Fisher Labosi), and 0.05 g hydrated catechin (Sigma) in 1 L distilled water, and the pH was adjusted at 4 with KOH 2N.

SGM was supplemented with OTA at 2 mg/L, while grape juice was contaminated at two concentrations of 2 mg/L and at 10 μg/L.

Preparation of Spore Suspensions. Living spores of the three *Aspergillus* section Nigri isolates were obtained from mycelium grown on CZAPEK Yeast extract Agar (CYA) medium, at 25 °C, aged 7 days. Before use, spores were carefully washed with physiological water to remove any contaminants. Dead conidia were obtained through boiling living spores in distilled water for 15 min.

Adsorption and Degradation Conditions. Kinetic studies of OTA adsorption, OTA degradation, and OTα production were performed for the three *Aspergillus* section Nigri isolates, *A. carbonarius SA332*, *A. japonicus* AX35, and *A. niger GX312*, in 20 mL of SGM containing 2 mg/L of OTA, at 25 °C, under agitation (240 rpm). As control to calculate OTA removal percentage, we used SGM containing 2 mg/L of OTA and inoculated with a water blank without conidia. So, this dilution was not significant. All assays were performed in triplicates.

Effect of initial conidial concentration on OTA removal was conducted with *A. japonicus* in SGM containing 2 mg/L of OTA. Four different conidial concentrations 10^4, 10^6, 10^7, and 10^8 conidia/mL were chosen.

The OTA removal capacity of the three *Aspergillus* section Nigri isolates was undertaken in 20 mL of synthetic and natural red grape juices. Each medium was inoculated with conidial suspensions of each isolate to give a concentration of 10^7 conidia/mL.

For all samples, after removal of conidia or fungal mycelium, 1 mL from each supernatant was filtered (0.22 μm) and without a previous cleanup step was analyzed by HPLC.

Conidial Germination. During adsorption studies in SGM inoculated with the three *Aspergillus* section Nigri isolates, conidial germination was followed (NIKON Eclipse Microscope E600), and photos were taken at 7, 11, 14, 20, and 24 h of incubation time to determine the physiological state of spores. Photos were analyzed by the image program VISILOG 5 (NOESIS, Quebec, Canada) to follow conidial germination.

Color of Red Grape Juice. To assess the tint of the red grape juice before and 5 min after introducing fungal conidia, the optical density (OD) was measured at 420 nm (OD_{420}) and at 520 nm (OD_{520}). The tint (T) was determined by the ratio of OD_{420} to OD_{520} ($T = OD_{420}/OD_{520}$) (*25*).

Detection and Quantification of OTA and OTα. OTA and OTα were detected and quantified by reversed-phase high-performance liquid chromatography. The HPLC apparatus consisted of a solvent delivery system and fluorescence (λ_{ex} = 332 nm; λ_{em} = 466 nm) and UV detectors. The analytical column used was a 150 × 4.6 mm Uptisphere 5 μ C18 ODB fitted with a guard column of 10 × 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, 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 80 μL. Ochratoxin A was identified by its retention time (33 min) according to a standard obtained from Sigma (Steinheim, Germany) and was quantified by measuring the peak area. The detection limit was 0.025 μg/L.

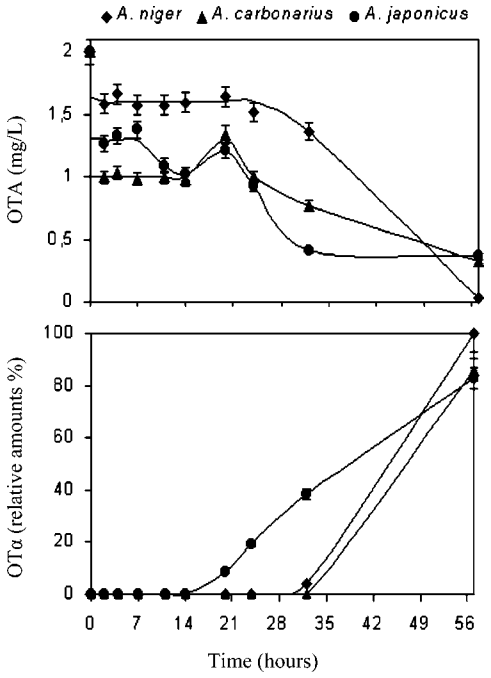

Figure 1. Ochratoxin A removal and ochratoxin α appearance during incubation of the three *Aspergillus* section Nigri isolates *A. niger GX312*, *A. carbonarius SA332*, and *A. japonicus AX35* in SGM medium contaminated with OTA at 2 mg/L at a concentration of 10^7 conidia/mL. The amounts of OTα are relative to initial OTA concentrations.

The OTA removal percentages were calculated according to the following equation: 100 × [1 − (peak area of OTA/peak area of OTA in control)]. OTα was identified at 17 min according to a standard prepared by total degradation of OTA by carboxypeptidase A (EC3.4.17.1) from bovine pancreas (Sigma, type II-PMSF). OTA and OTα were quantified by measuring the peak area and by using standard solutions.

Statistical Analysis. All analysis were done in triplicate. SPSS (Headquarters (Chicago, IL)), version 11.5.1, for windows was used for the statistical analysis of the data. Significant differences in the mean values were reported at P values of <0.05.

RESULTS

Ochratoxin A Removal in Synthetic Grape Juice (SGM). A synthetic grape juice, initially contaminated with OTA at 2 mg/L, was inoculated with three *Aspergillus* section Nigri isolates, *Aspergillus niger*, *A. japonicus*, and *A. carbonarius*, at a concentration of 10^7 conidia/mL. It was incubated at 25 °C under agitation, and samples from SGM were taken over 57 h. **Figure 1** represents OTA removal and OTα production versus time for each species tested. Just after conidia of black aspergilli introduction in SGM and well mixing, decreases in OTA amounts were instantly observed for the three *Aspergillus* section Nigri isolates. Those decreases were about 10% for *A. niger*, 28% for *A. japonicus*, and 45% for *A. carbonarius*. Until 30 h for *A. niger* and *A. carbonarius* and 14 h for *A. japonicus*, no OTA degradation products were observed. OTα was only detected beyond those times. For two *Aspergillus* section Nigri isolates, *A. japonicus* and *A. carbonarius*, slight increases in OTA amounts were also sometimes observed which could indicate a partial release of this mycotoxin in the medium.

Among the three *Aspergillus* section Nigri isolates tested in this study, *A. japonicus* was selected to test the effect of initial conidial concentration (10^4, 10^6, 10^7, and 10^8 conidia/mL) on

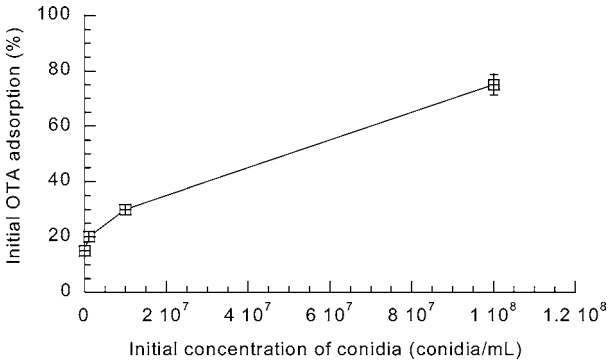

Figure 2. OTA removal by *A. japonicus AX35* in SGM (contaminated with OTA at 2 mg/L) inoculated at different concentrations of live conidia (10^4, 10^6, 10^7, and 10^8 conidia/mL).

OTA removal from SGM (**Figure 2**). We noted that the higher the concentration of conidia introduced in the medium was, the greater the OTA removal. Thus, with 10^4 conidia/mL, OTA adsorption was of 15% to reach 75% with 10^8 conidia/mL.

Conidial Germination. OTA adsorption surveys in SGM permit us to observe the physiological states of conidia of *A. carbonarius*, *A. niger*, and *A. japonicus* (photos not shown). At 14 h, conidia of *A. carbonarius* and *A. niger* could be in a dormant sate, swollen, or have a germ tube. For conidia of *A. japonicus*, swelling and formation of germ tubes earlier began at around 11 h. So, beyond 11 h for *A. japonicus* and 14 h for *A. niger* and *A. carbonarius*, long hyphae were developed and aggregated forming dense fungal mycelium.

Ochratoxin A Removal in a Natural Grape Juice. The red natural grape juice used in this assay was contaminated at two different concentrations of OTA: 2 mg/L and 10 μg/L. Living and dead spores from three *Aspergillus* section Nigri isolates were inoculated in the grape juice at the concentration of 10^7 conidia/mL. Samples from grape juice were taken over 2 h and were analyzed for OTA content.

According to the initial OTA concentration in the grape juice (2 mg/L or 10 μg/L) and to black *Aspergillus* isolates tested, differences between OTA adsorption patterns were noted (**Figure 3** and **Figure 4**). As soon as conidia of black aspergilli were added to the grape juice contaminated at 2 mg/L (**Figure 3**), OTA was immediately adsorbed on living conidia. So, a few seconds later, removal of OTA was already around 30% by conidia of both *A. japonicus* and *A. niger* and reached 55% by conidia of *A. carbonarius*. After this instantaneous adsorption, amounts of OTA were kept constant until 120 min. For heat-treated conidia, the same removal pattern was observed, but OTA removal was improved and was about 41.5% for *A. japonicus*, 47.5% *for A. niger*, and 66.5% for *A. carbonarius*.

For the grape juice contaminated with OTA at 10 μg/L (**Figure 4**), OTA removal by living or heat-treated conidia of *A. japonicus* and *A. carbonarius* was instantly observed and reached around 80%. Then, OTA removal was kept constant till 120 min, except with heat-treated conidia of *A. carbonarius* on which OTA adsorption reached 96% after 75 min. A particular behavior was observed with both living and heat-treated conidia of *A. niger*, especially in the first minutes. No immediate adsorption occurred on living conidia and only 15% occurred on dead conidia. However, after 40 min, OTA adsorption on living conidia was around 60% and reached 70% after 60 min. With heat-treated conidia, OTA adsorption was improved to 90% after 120 min.

We also determined the impact of conidia of black *Aspergillus* isolates on grape juice color. The tint of the grape juice was assessed before and after introducing living fungal conidia. The tint of the control was about 1.18, whereas it was 1.46, 1.47, and 2.19 after addition of conidia of *A. japonicus*, *A. niger*, and *A. carbonarius*, respectively, indicating a darker color of the red juice after addition of black conidia.

DISCUSSION

OTA adsorption studies on conidia of black aspergilli, in both synthetic and natural red grape juices contaminated at 2 mg/L and 10 μg/L, showed that this is an instantaneous removal of OTA in many cases for the three *Aspergillus* section Nigri isolates, except for *A. niger* introduced in grape juice contaminated at 10 μg/L. No degradation product like OTα was detected during stages of conidial dormancy, swelling, and germ tube but occurred over 30 h for *A. niger* and *A. carbonarius* and 14 h for *A. japonicus*. Heat-treated conidia were also able to remove OTA from the natural red grape juice. Those observations suggest that OTA removal is an adsorption phenomenon. The OTA adsorption was a little higher in natural red grape juice than in SGM. This could be related to the different composition of the two media. SGM is closed to grape juice at early veraison and hence would contain less sugar and more acid. Two different concentrations were tested, 10 μg/L and 2 mg/L. The first one is very closed to a concentration naturally present in some musts that needed biological detoxification. The second, 2 mg/L, was obviously 1000-times greater than the 2 μg/L limit in wine and allowed, in vitro, to better understand the mechanism of OTA removal/degradation (i.e., easier detection of eventual degradation products) and to determine the importance of OTA/conidia ratio. Results showed that the efficacy of OTA adsorption was better with a small OTA/conidia ratio even in the case of the biggest conidia of *A. carbonarius*. OTA adsorption seems to be related both to the size and to the amounts of conidia. For *A. carbonarius* conidia, the biggest one, 10^7 conidia/mL, adsorbed 50% of OTA versus only 30% with 10^7 conidia/mL of *A. japonicus*, the smallest. To increase the efficacy of *A. japonicus* conidia, it is necessary to increase the concentration to 10^8 conidia/mL.

To understand interactions involved in this adsorption, properties of both OTA and conidia surfaces should be described. According to the literature, mycotoxin binding was reported as a function of their structural characteristics (*26*), their molecular size, and their physicochemical properties (*16*). OTA is a complex organic compound, consisting of chlorine-containing dehydroisocoumarin linked through the 7-carboxyl group to 1-β-phenylalanine (*27*). Phenol and carboxyl are the main functional groups of this molecule (*28*) that could be involved in different adsorption mechanisms. First, OTA is considered with zearalenone as the less polar mycotoxins and could then be bound on hydrophobic surfaces (*29*) through the phenol group and via interactions of two-π-electron orbital (*30*). This was already shown for OTA adsorbents such as hydrophobic transformed zeolites (*31*). Second, OTA acidic function is a weak acid with a pK_a value for the carboxyl group of the phenylalanine moiety of 4.4 (*32*). OTA is then partially dissociated at pH 4.0 of grape juices and carries a positive charge introduced by the amine function (NH_3^+).

For conidia, and particularly the airborne types, a hydrophobic nature was reported (*33*). This hydrophobicity could be due to proteins called hydrophobins (*34*, *35*) or to lipids previously found on *Botrytis fabae* conidia (*33*). Negatively charged carbohydrates were also found on the surface of *Aspergillus fumigatus* conidia (*36*). Those properties have intervened in adhesion phenomena of living (*37*) or dead (*38*) conidia on different natural surfaces (roots (*37*), fruit cuticules (*38*))

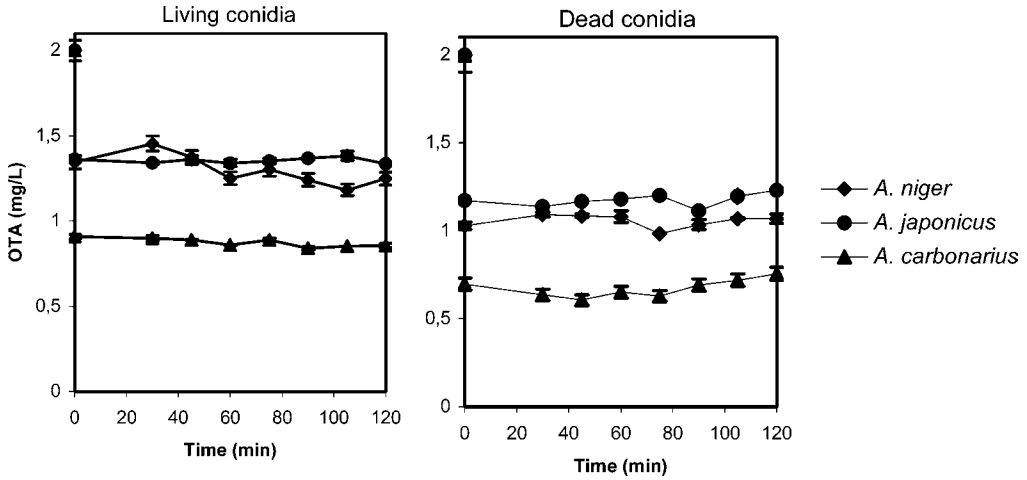

Figure 3. OTA removal by living and dead conidia of the black aspergilli tested at 10^7 conidia/mL (*A. niger*, *A. japonicus*, and *A. carbonarius*) in a natural red grape juice contaminated with OTA at 2 mg/L.

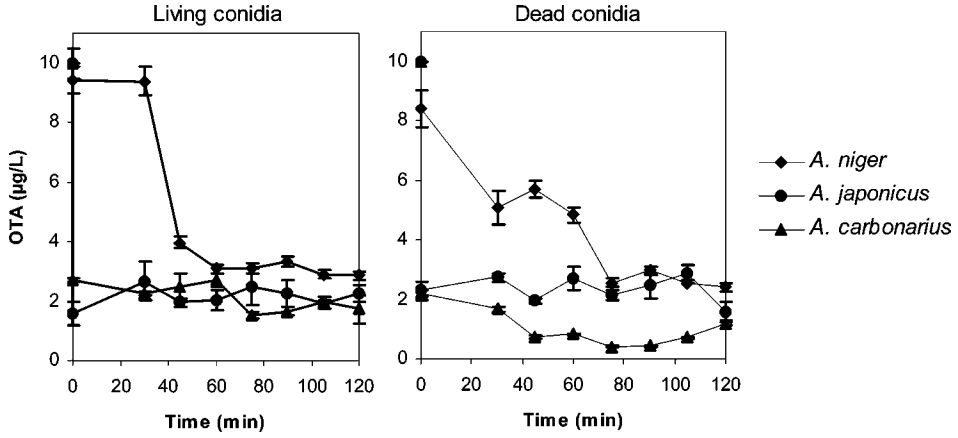

Figure 4. OTA removal by living and dead conidia of the black aspergilli tested at 10^7 conidia/mL (*A. niger*, *A. japonicus*, and *A. carbonarius*) in a natural red grape juice contaminated with OTA at 10 μg/L.

According to all the above properties reported in the literature, adsorption phenomenon of OTA on conidia of black aspergilli could be related to hydrophobic interactions. The global positive charge of OTA molecule in acid media (grape juices) could also interact with negatively charged molecules found on fungal conidia. This interaction is likely to be nonspecific, as many conidia are able to bind nonspecifically or without requiring chemical compounds.

According to some of our results, increases in OTA amounts were observed during the adsorption phenomenon. This could be a partial release of the initially bound mycotoxin. This release was also observed for *Fusarium oxysporum* conidia initially adsorbed on root surfaces (*37*) and during the adsorption of different metal toxicants on fungal conidia (*39*). Perhaps conidial interactions were easily broken, as conidia adhesion was previously reported as a relatively weak attachment for *Botrytis cinerea* (*38*).

After OTA adsorption by conidia of black aspergilli, OTA degradation took place by well-developed mycelium after 14 h for *A. japonicus* and 30 h for both *A. niger* and *A. carbonarius*. Thus, OTα, an OTA degradation product, also observed in different previous studies (*13*, *15*), increased while OTA disappeared from the grape juice. So, two stages were involved in OTA detoxification by *Aspergillus* section Nigri isolates: adsorption and then degradation. Commercially, the only stage very interesting is adsorption because it is fast, avoiding long time contact between fungi and grape juice. So, potentially commercial OTA detoxification by using conidia of black aspergilli must be completed before the mycelium is well-developed and degradation starts. Indeed, production of a degradation product like OTα that is even reported as less toxic (*40*) would still be harmful for consumer health. Moreover, degradation is useless for detoxification of grape juice insofar as by this stage, the fungus is actually using up all the grape sugars for its own growth. We also noted that even the difference between dead and live conidia was not so important as dead conidia consistently bound more OTA at 2 mg/L. In view of commercial detoxification, dead conidia could also only be used to completely prevent germ tub formation and degradation phenomenon.

Among three *Aspergillus* section Nigri isolates tested, conidia of *A. carbonarius* were in many cases the most efficient in adsorbing OTA, regardless of the media used and the OTA concentration tested. In fact, conidia of *A. carbonarius* had the largest diameter (7−10 μm) compared to conidia of *A. niger* (3.5−4 μm) (*41*) and those of *A. japonicus* (5 μm) (*19*) and so presented a higher surface to bind OTA. However, *A. carbonarius* could not be so interesting for practical detoxification, as it hardly severely deteriorated grape juice color and because it is particularly significant in the production of OTA on French grapes (*6*, *42*) and elsewhere (*43*−*45*). We noted that *A. japonicus* and *A. niger* conidia deteriorated the grape juice color less than the *A. carbonarius* conidia. For *A. niger* and *A. japonicus*, OTA adsorption patterns were different according to the media used and to OTA concentrations tested. However, those two species could be interesting for OTA removal in grape

juices and musts as the first has the GRAS label, and the second, frequently used in fungal biotechnology, has been reported as ochratoxigenic only a few times (*7*, *46*, *47*) and both of them deteriorated grape juice color less than *A. carbonarius*.

Finally, the risk of using conidia of OTA producing species in OTA detoxification process (i.e., having both OTA production and degradation products such as OTα in the medium to be detoxified) could be drastically eliminated by using OTA nonproducing species and dead conidia allowing dormancy state to be prolonged.

For a routine use of conidia of black aspergilli for lowering OTA levels in grape juices, two points remain to be improved. The first is to do a treatment of spores to eliminate their black color which affects final juice color, and the second is to think about an immobilization of the spores to facilitate their use in industrial scale. OTA decontamination of wine by this process could be promising insofar as the efficiency of conidia of black aspergilli is demonstrated.

SAFETY

Ochratoxin A is a toxic compound that needs to be manipulated with care and with appropriate safety precautions. Decontamination procedures for laboratory wastes have been reported by the International Agency for Research on Cancer (IARC) (*48*).

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