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Research Note

Aspergillus carbonarius as the Main Source of Ochratoxin A Contamination in Dried Vine Fruits from the Spanish Market

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ABSTRACT

Ochratoxin A (OTA) can occur in a wide range of foods, but unexpectedly high concentrations have been detected in dried vine fruits of various origins. The European Union has recently established a maximum OTA limit of 10 μg/kg for these foodstuffs. In order to determine the likely origin of OTA, a mycological study of 50 dried fruit samples (currants, raisins, and sultanas) representative of the Spanish market was conducted. Fungal contamination was detected in 49 of 50 (98%) samples. Black aspergilli were isolated from all of the positive samples. *Aspergillus niger* var. *niger* was isolated from 98% of the samples, and *Aspergillus carbonarius* was found in 58% of the samples. One hundred sixty-eight *A. niger* var. *niger* isolates and 91 *A. carbonarius* isolates were screened for their ability to produce OTA. Eighty-eight (96.7%) *A.carbonarius* isolates and one (0.6%) *A. niger* var. *niger* isolate were found to be OTA producers. Black aspergilli were the dominant fungi. Among black aspergilli, *A. carbonarius* has shown a consistent ability to produce OTA and is the most probable source of this mycotoxin in these substrates.

Ochratoxin A (OTA) is a mycotoxin of considerable concern with regard to human and animal health. It is nephrotoxic in all species that have been tested and is classified as a human renal carcinogen (group 2B). It is also teratogenic and can affect the immune system *(10, 17, 18).* Furthermore, it may be implicated in the human disease Balkan endemic nephropathy and the development of urinary tract tumors and renal diseases in humans *(10, 24).*

There is much evidence of human exposure to OTA. Surveys carried out in several countries have clearly demonstrated the presence of OTA in human blood and breast milk *(10, 17, 24).* These findings are consistent with those obtained from the surveillance of food commodities, which confirms that OTA can occur in a wide range of foods *(10, 17).* High concentrations of OTA have recently been detected in dried vine fruits of various origins *(13, 19–21, 28).* Dried vine fruits are healthy foods and are also ingredients in muesli, muesli bars, biscuits, and cakes, among other foods. Although it is estimated that at least 50% of the dietary OTA intake in Europe comes from cereals and cereal products, dried vine fruits can also be an important source of OTA for people who consume large amounts, particularly children *(8).* The European Union (EU) has recently established maximum OTA limits of 5 μg/kg for cereals including rice and buckwheat, 3 μg/kg for derived cereal products and for cereal grains for direct human consumption, and 10 μg/kg for dried vine fruits (currants, raisins, and sultanas) *(9).*

OTA was until recently believed to be produced only by *Penicillium verrucosum (25)* and *Aspergillus ochraceus* and related species *(16, 30). P. verrucosum* is the most important OTA producer in countries with cold and temperate climates, whereas *A. ochraceus* is more commonly associated with warmer and tropical climates *(26).* Nevertheless, these two species are not always the source of OTA contamination. Although some other *Aspergillus* species have been reported to be ochratoxigenic *(1),* the production of OTA by species in *Aspergillus* section *Nigri* (black aspergilli) has received considerable attention since the first description of OTA production by *Aspergillus niger* var. *niger (2).* In this section, the reported OTA-producing species are those now included in the so-called *A. niger* aggregate and *Aspergillus carbonarius (1).*

Because of the unexpectedly high incidence and concentrations of OTA reported in dried vine fruits *(13, 19–21, 28),* we conducted a mycological study of these commodities to determine the likely origin of this mycotoxin.

MATERIALS AND METHODS

Samples. Fifty dried vine fruit samples (currants, raisins, and sultanas) were purchased from different types of retail outlets in Barcelona, Spain. These samples were selected to include a wide range of stores encompassing supermarkets, smaller shops, and market stalls and were representative of the Spanish market. The samples were stored at 4°C and analyzed the day after collection.

Mycological study. Three to five pieces of each sample were plated directly onto different culture media (malt extract agar and dichloran rose bengal chloramphenicol agar) *(26)* in triplicate. Plates were incubated at 28°C. All fungi, which appeared to be distinct from each other, were subcultured and grown on special-

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TABLE 1. *Occurrence of fungal species in dried vine fruits*

Fungi	No. (%) of positive samples (n = 50)	No. (%) of positive plated pieces[a]		
		MEA (n = 539)	DRBC (n = 563)	Total (n = 1,102)
Black aspergilli	49 (98)	433 (80.3)	447 (79.4)	880 (79.8)
A. niger var. *niger*	49 (98)	427 (79.2)	440 (78.1)	867 (78.7)
A. carbonarius	29 (58)	112 (20.8)	202 (35.9)	314 (28.5)
Mucorales	38 (76)	149 (27.6)	105 (18.6)	254 (23.0)
Other fungi[b]	24 (48)	65 (12.1)	91 (16.2)	156 (14.2)

[a] MEA, malt extract agar; DRBC, dichloran rose bengal cloramphenicol agar.

[b] Isolated from <9% of plated pieces: yeasts, *Apergillus flavus, Apergillus versicolor, Aspergillus* sp., *Eurotium* sp., *Alternaria alternata, Monilia* sp., *Trichoderma* sp., *Penicillium digitatum, Penicillium purpurogenum, Penicillium* spp. Some isolates could not be identified to species level because of the overgrowth of *Mucorales*.

ized media for identification in accordance with guidelines published for each genus.

OTA production ability. A representative number of isolates belonging to species that are potential producers of OTA were evaluated by a previously described high-pressure liquid chromatography (HPLC) screening method *(6)*. Briefly, the isolates were grown on yeast extract agar and Czapek yeast extract agar (CYA) *(26)* and incubated at 25°C for 7 days. Isolates identified as *A. carbonarius* were grown on CYA for 10 days at 30 and 35°C. For each isolate, three agar plugs were removed from the central area of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC *(6)*. The OTA-positive isolates belonging to the *A. niger* aggregate were also classified on the basis of their ITS-5.8S rDNA restriction fragment length polymorphism (RFLP) patterns according to the DNA technique previously described *(5)*.

RESULTS AND DISCUSSION

Mycological study. Fungal contamination was detected in 49 of 50 (98%) samples (Table 1). Black aspergilli were the dominant fungi, as they were detected in all of the positive samples and in the 79.8% (880 of 1,102) of the plated pieces. *A. niger* var. *niger* (*A. niger* aggregate) was isolated from all of the positive samples and from 78.7% of the plated pieces. *A. carbonarius* was found in 58% of the samples and in 28.5% of the plated pieces. With the exception of *Mucorales* (found in 76% of the samples and in 23% of the examined pieces), other fungi were relatively rare, being present in <9% of all plated pieces, and could not always be identified because of the overgrowth of *Mucorales*. As shown in Table 1, dichloran rose bengal chloramphenicol agar allowed more extensive recovery of fungal species other than *Mucorales*.

In accord with our results, some other authors have also reported *A. niger* to be dominant fungi in dried vine fruits *(12, 14, 26, 31)*. Black aspergilli can grow to some extent during drying and are presumably highly resistant to drying conditions *(26)*. *A. niger* and *A. carbonarius* have been also isolated from dried fruit wash water *(15)*. It is difficult to know the extent of the natural occurrence of *A. carbonarius* in foods because all of the black aspergilli are very commonly regarded as "*A. niger*." *A. niger* has been found on the surfaces of healthy grapes at all stages *(32)* and was found to be one of the most frequently occurring species in a recent survey of the mycoflora of wine grapes from Argentina and Brazil *(11)*, while it has not been reported in other studies involving grapes *(3)*. *A. carbonarius* was the main species isolated from grapes used to obtain an OTA-contaminated wine during a microvinification trial *(7)*. *A. niger* is also considered the *Aspergillus* species most commonly responsible for the postharvest decay of fresh fruit *(26)*.

Ochratoxin A production. One hundred sixty-eight *A. niger* var. *niger* isolates and 91 *A. carbonarius* isolates were screened for their ability to produce OTA. Eighty-eight (96.7%) *A. carbonarius* isolates and one (0.6%) *A. niger* var. *niger* isolate were found to be OTA producers. No other fungal species were screened because no typical OTA-producing species, (e.g., *A. ochraceus* and *P. verrucosum*) were isolated in this study.

The reported percentages of ochratoxigenic isolates in the *A. niger* aggregate range from 1.7 to 30% *(2, 4, 11, 15, 22, 23, 27, 29)*. For *A. carbonarius*, this percentage is much higher, ranging from 25 to 100% *(7, 11, 15, 27)*. The only OTA-producing isolate from the *A. niger* aggregate (*A. niger* var. *niger*) was classified as type N on the basis of its ITS-5.8S rDNA RFLP pattern *(5)*. To date, all of the OTA-positive isolates belonging to this aggregate whose RFLP patterns are known are of type N *(4)*.

Because of the extremely high percentage of ochratoxigenic isolates, *A. carbonarius* is without any doubt the major producer of OTA within *Aspergillus* section *Nigri*. Strong evidence of the contribution of this species to the OTA contamination in wine has recently been reported *(7)*. Although further studies to assess the optimal conditions for the expression of OTA biosynthesis are in progress, in preliminary studies some *A. carbonarius* isolates were found to produce larger amounts of OTA after 10 days of incubation at 30 and/or 35°C than after the same incubation period at 25°C. In our study, 87 of the 88 positive isolates produced OTA in CYA at 30°C, but one isolate produced OTA only when incubated at 35°C. This feature, which had recently been observed in our laboratory *(7)*, could be related to the origins of the isolates. The methods used to

remove water from grapes, mainly sun drying, involve high temperatures and strong sunlight. Black spores provide protection from sunlight and UV light, providing a competitive advantage in such habitats *(26)*. Since black aspergilli (mainly *A. carbonarius*) are the dominant fungi detected in dried vine fruits and have shown a consistent ability to produce OTA, they are the most probable source of this mycotoxin in these substrates.

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