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Occurrence of Ochratoxin A–Producing Fungi in Grapes Grown in Italy

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ABSTRACT

A study was carried out to investigate fungi present on grapes grown in Italy. *Aspergillus* and *Penicillium* spp. isolates were identified and studied in vitro, and their ability to produce ochratoxin A (OA) was investigated. The survey involved nine vineyards, three located in northern Italy and six located in southern Italy. In 1999 and 2000, bunches of grapes at different growth stages were collected from all nine vineyards, and berry samples were placed in moist chambers and incubated. The resultant fungal colonies were then transferred to petri dishes containing Czapek yeast agar and incubated at 25°C for 7 days; the fungal isolates were identified and then cultivated in liquid Czapek yeast medium and evaluated for their ability to produce OA. During the survey, 508 isolates were collected, with 477 belonging to *Aspergillus* spp. and 31 belonging to *Penicillium* spp. Among the aspergilli, species of the *Fumigati, Circumdati,* and *Nigri* sections were identified, with species of the *Nigri* section (464 isolates) largely predominating; for species of the *Nigri* section, 108 isolates were uniseriate, 270 were biseriate, and 86 were identified as *Aspergillus carbonarius.* Black aspergilli isolated over the 2 years of the study showed a very similar pattern. On average, the biseriates represented about 60% of the isolates collected in both years and were followed by uniseriates (21%) and *A. carbonarius* (19%). The most toxigenic strains proved to be those of *A. carbonarius*; about 60% of these isolates were OA producers and produced the highest levels of OA. *A. carbonarius* was more frequent in the south, but in both areas the percentages of OA-producing isolates remained the same.

The mycotoxin ochratoxin A (OA) has been shown to be a potent nephrotoxin in all animal species tested with the exception of mature ruminants. OA is carcinogenic to rodents and possesses teratogenic, immunotoxic, and possibly neurotoxic and genotoxic properties. Furthermore, it may be implicated in the human disease Balkan endemic nephropathy and in the development of urinary tract tumors in humans. In 1993, the International Agency for Research on Cancer classified OA as a possible human carcinogen (group 2B) *(9).*

The worldwide occurrence of OA contamination of raw agricultural products has been amply documented; such contamination occurs in a variety of plant products, such as cereals, coffee beans, beans, and pulses *(14, 15, 21, 28, 31).* OA has also been detected in beverages such as beer, and since 1996 it has also been detected in wine and grape juice *(18, 36).* Several surveys carried out in different European countries as well as Morocco, Japan, and Australia have confirmed the frequent presence of OA on grape products and wine at levels ranging from 0.01 to 3.4 μg/liter *(8).* A gradient that was dependent on region and wine color was observed; both the incidence and the concentrations of OA were higher in products from southern regions of Europe and were highest for red grapes, followed by rosè and white grapes, in that order *(18, 19, 23, 24, 26).* The latter gradient was hypothesized to be relevant to the postharvest period prior to alcoholic fermentation, particularly in connection with the length of mash standing.

The results of a survey of Italian wine concurred with these reports and stressed the strong influence of geographic region of provenance on OA contamination; thus, wines produced in southern Italy were markedly more contaminated than those produced in northern Italy *(26).* Most studies conducted to date have been aimed primarily at quantifying OA content in wine, and little information on the origin of the contamination is available.

Fungi responsible for the presence of OA have been studied, especially for cereals, on which *Penicillium verrucosum* and *Aspergillus ochraceus* are considered the main producers. Until 1998, these fungi were also believed to be responsible for the presence of the toxin in grapes *(23).* OA-producing *Aspergillus carbonarius* and *Aspergillus niger* on dried vine fruits were reported in 1999 *(10),* but the first description of OA production by *A. niger* was published in 1994 *(2)* and reviewed in 2001 *(1).*

The objective of this study was to investigate fungi present in grapes grown in two regions, northern and southern Italy; only isolates belonging to the genera *Aspergillus* and *Penicillium* were identified and studied in vitro to assess their ability to produce OA.

MATERIALS AND METHODS

Grape-sampling locations. The survey was carried out in 1999 and 2000 and involved nine farms, three located in Emilia-

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TABLE 1. *Vineyards and grape varieties involved in the research*

Vineyard location		Grape variety	Color of berries
Area of Italy	Province		
South	Lecce	Negro amaro	Red
	Lecce	Malvasia nero	Red
	Lecce	Negro amaro	Red
	Lecce	Sangiovese	Red
	Taranto	Primitivo	Red
	Brindisi	Verdeca	White
North	Ravenna	Trebbiano	White
	Ravenna	Trebbiano	White
	Ravenna	Sangiovese	Red

Romagna (northern Italy) and six located in Puglia (southern Italy). The vineyards chosen were representative of the grape-growing areas with regard to grape variety and farming methods (Table 1). Five plants along diagonal transects were chosen in each vineyard, and two bunches of grapes were taken from each plant. When the training system consisted of two levels of bunches above the ground, both levels were sampled. In 1999, grapes at two stages of growth (early veraison and ripening) were chosen for sampling; in 2000, samples were collected for two additional (earlier) growth stages (setting and berry increase). The bunches collected were put in cooled bags and delivered to our laboratory within 48 h.

Isolation and identification of fungi. Five berry halves and three ~1-cm-long rachis portions were taken from each bunch and placed in moist chambers comprising petri dishes (9 cm in diameter) containing disks of blotting paper (8 cm in diameter) wetted with 2 ml of sterilized double-distilled water. The moist chambers were sealed with Parafilm and incubated at 25 ± 2°C. After 7 days, growing fungal colonies were transferred to Czapek yeast agar *(27)* and incubated at 25 ± 2°C in the dark for 7 days, and then the fungal isolates were identified to genus level.

The genus identification of *Aspergillus* and *Penicillium* isolates was based on the shape of the conidiophores as observed with a binocular microscope (×40). Identification to species level was carried out according to Raper and Fennell *(29)* and Pitt *(27).* After isolates were identified, they were transferred to 6-ml test tubes containing Czapek solution agar *(29)* and stored in a refrigerator at 4 ± 2°C, with periodic transfer *(22),* until they were used.

Production of OA by fungal isolates. Fungal isolates were cultivated in a liquid medium, Czapek yeast broth *(3, 6)* enriched with 40 g of sucrose (10 g more than is normally used) and 20 g of yeast extract (15 g more than is normally used), to evaluate their ability to produce OA. Conical flasks containing 100 ml of culture broth were autoclaved at 121°C for 20 min and were then inoculated with 0.25-cm^2 plugs taken from colonies incubated for 7 days.

The conical flasks were incubated for 14 days at 25 ± 2°C, and then their contents were homogenized for 30 s (Ultra-Turrax, IKA, Staufen, Germany) and filtered through a folded filter paper (S&S 595½, Schleicher & Schuell, Dassel, Germany). One hundred microliters of the filtrate was diluted with high-performance liquid chromatography (HPLC) mobile phase (900 μl), and the solution was then filtered with a Cameo 13N 0.45-μm nylon syringe filter (Micron Separations Inc., Westborough, Mass.) prior to HPLC analysis.

Ochratoxin standard. OA was purchased from Sigma (St. Louis, Mo.). An OA solution (40 mg/ml in benzene/acetic acid [99:1, vol/vol]) was calibrated spectrophotometrically (Lambda 2, Perkin-Elmer Corp., Norwalk, Conn.) at 333 nm with an extinction coefficient of 5,550 *(5)* and stored at −20°C when not in use; after the OA solution had been calibrated, working standards were prepared by evaporating an exact volume under a stream of nitrogen and redissolving the residue in the mobile phase.

Chromatography. The HPLC system consisted of a Perkin-Elmer 200 instrument equipped with an ISS 200 sampling system (loop volume, 150 μl) and a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan) set at 333-nm excitation and 470-nm emission. The system was controlled by Perkin-Elmer Turbochrom PC software. A Select B RP-8 column (5 μm particle size, 150 by 4 mm inside diameter; Merck, Darmstadt, Germany) was employed at room temperature with a mobile phase of acetonitrile/2% acetic acid (at 41:59 for OA and at 55:45 for OA methyl ester) at 1.2 ml/min. The injection volume was 30 μl.

Quantification and confirmation. OA standards of 2 to 60 pg were injected. Turbochrom PC software was used for quantification on the basis of peak areas. For qualitative confirmation of positive samples, OA was derivatized by methylation of the extracts, and HPLC analysis was subsequently carried out *(35).* The detection limit for OA production was 0.7 μg/kg of medium.

RESULTS AND DISCUSSION

Isolation and identification of fungi. During the 2-year survey, 508 isolates were collected, with 477 belonging to *Aspergillus* spp. and 31 belonging to *Penicillium* spp. Species from the *Fumigati* section (formerly the *A. fumigatus* group), the *Circumdati* section (formerly the *A. ochraceus* group), and the *Nigri* section (formerly the *A. niger* group) were identified, with the *Nigri* section largely predominating; 11, 2, and 464 isolates from these sections, respectively, were isolated. From the *Fumigati* and *Circumdati* sections, only the species *A. fumigatus* and *A. ochraceus,* respectively, were identified.

The identification of species from the *Nigri* section, based on morphology, is notoriously difficult. Three uniseriate members, namely, *Aspergillus japonicus, Aspergillus foetidus,* and *Aspergillus aculeatus,* can be identified with ease, as can certain biseriate species, namely, *A. carbonarius, Aspergillus heteromophus,* and *Aspergillus ellipticus,* with the remainder forming the *A. niger* aggregate. On the basis of restriction fragment polymorphism analysis, Kusters van Someren et al. *(16)* suggested a division of the *A. niger* aggregate into two morphologically indistinguishable species, *A. niger* and *Aspergillus tubingensis.* Studies involving a molecular approach followed and substantially confirmed these results and the diversity of *A. carbonarius (4, 17, 20, 25, 33, 34).* Because of the large number of isolates involved, one of us (Z. Kozakiewicz, who is an expert in *Aspergillus* taxonomy) suggested a separation of the black aspergilli into three groups, namely, those with uniseriate conidial heads, those with biseriate heads, and *A. carbonarius.* Thus, section *Nigri* isolates will be referred to hereinafter as either uniseriate, biseriate, or *A. carbonarius.* Representative isolates from these three groups, together with any penicillia isolated, were sent to CABI Bioscience

TABLE 2. Aspergillus *spp. isolated from bunches of grapes collected during the 2-year survey (1999 to 2000) in the nine vineyards*

Section of isolates	Species of isolates	No. of isolates (% of total no.) obtained in survey year	
		1999	2000
Nigri	*A. uniseriate*	21 (17)	87 (25)
	A. biseriate	71 (58)	199 (56)
	A. carbonarius	27 (22)	59 (17)
Fumigati	*A. fumigatus*	2 (2)	9 (3)
Circumdati	*A. ochraceus*	1 (1)	1 (0)

TABLE 3. *Distribution of* Aspergillus *spp. and percentages of ochratoxin A–positive fungi (%OA+) isolated from grapes collected in the northern and southern vineyards during the 2-year survey*

Section of isolates	Species of isolates	% of species (%OA+) isolated in Italian region	
		North	South
Nigri	*A. uniseriate*	44 (0)	19 (16)
	A. biseriate	27 (21)	62 (8)
	A. carbonarius	19 (62)	18 (56)
Fumigati	*A. fumigatus*	7 (40)	1 (67)
Circumdati	*A. ochraceus*	3 (100)	— (—)

(Egham, UK) for authentication and identification to better define species found on grapes in the Italian territory.

Of the isolates from section *Nigri,* 108 were uniseriate, 270 were biseriate, and 86 were identified as *A. carbonarius. A. aculeatus* and *A. japonicus* were the uniseriate species, and *A. niger* and *A. tubingensis* were among the biseriate species. Fungi isolated during the 2-year period showed a very similar patterns (Table 2). On average, the biseriate species represented about 60% of the isolates collected in both years and were followed by the uniseriate species (21%) and *A. carbonarius* (19%). Species not belonging to the *Nigri* section represented <3% of the *Aspergillus* isolates (*Aspergillus fumigatus* [2%] and *A. ochraceus* [0.6%]). Species identified among the penicillia collected included *Penicillium thomii, Penicillium glabrum, Penicillium spinulosum,* and *Penicillium funiculosum.* All of these species are common soil species. No terverticillate penicillia were isolated.

Production of OA by fungal isolates. A preliminary trial was conducted to define the optimal incubation time, with 7, 14, and 21 days being considered initially. The 14-day option proved to be the best (unpublished data) and was used in this study. The most toxigenic strains proved to be those of *A. carbonarius* (Table 3). About 60% of *A. carbonarius* isolates tested positive for OA production. The percentages of positive strains for uniseriate and biseriate *A. niger* isolates were 3 and 5%, respectively. In addition, some strains of *A. fumigatus* (50%) and all strains of *A. ochraceus* were found to be OA producers.

The geographic distribution of strains was considered (Table 3). It is evident that the incidence of uniseriate isolates was higher in northern of Italy than in southern Italy, but none of the strains collected in northern Italy tested positive for OA production, while 16% of those collected in southern Italy did. All positive uniseriate strains were *A. japonicus* strains, and this species had not previously been found to be able to produce OA *(2, 32).* In contrast, a large number of biseriate strains were collected in southern Italy, with fewer being collected in northern Italy, but a larger percentage of the northern strains were OA producers (21 versus 8%). However, the mean percentages of OA producers in northern and southern Italy were very similar (6 and 8%, respectively, of the total number collected in the survey). The incidence of *A. carbonarius* was higher in the southern Italy, but the percentages of OA-producing isolates remained around 60% in both southern and northern Italy. *A. ochraceus* was found only in the north, while *A. fumigatus* was isolated with almost the same frequency in both areas, with a higher percentage of OA-producing isolates being found in the south (67 versus 40%).

The amount of OA produced in vitro in these studies (Fig. 1) depended on the fungi involved. *A. carbonarius* was the only species that was able to produce >100 μg of OA per kg of medium. Uniseriate species produced <1 μg/kg, while a large number of biseriate species produced <10 μg/kg.

OA-producing penicillium strains, comprising *P. funiculosum* and *P. spinulosum* only, represented 35% of the total number of penicillia collected. There are no reports in the literature regarding the production of OA by these two species. The terverticillate penicillia are considered the main mycotoxin producers *(12).*

The data collected in this study cover 2 years and two grape-growing areas of Italy. These data appear to be homogeneous between years; in fact, the distributions of fungi in the vineyards and the percentages of isolates testing positive for OA production were very similar for 1999 and 2000. Variability in weather conditions and growing systems between the northern and southern areas of Italy influenced both the relative incidence of species and the percentage of OA-producing isolates the pattern of fungi, which differed for the two areas.

Since our results are virtually identical for both years, the information reported in this paper can be considered

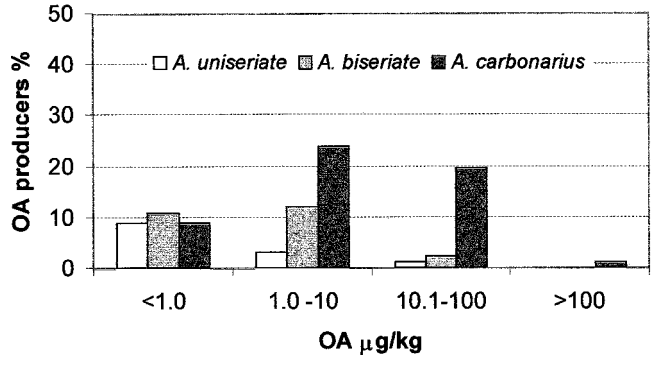

FIGURE 1. *Distribution of OA-producing isolates in the* A. niger *group (*A. niger *uniseriate,* A. niger *biseriate, and* A. carbonarius*) among different OA production classes (in vitro trials).*

reasonably representative of the situation for northern and southern Italian grape-growing areas. These results indicate that *Aspergillus* spp. in general, and those in the *Nigri* section in particular, play the main role in the production of OA on grapes. *P. verrucosum* was not isolated, and *A. ochraceus* was isolated only occasionally. It appears that the OA-producing fungi affecting grapes are different from those affecting cereals. *A. niger* has also been found to be relevant to OA contamination of grapes in France *(30)* and in Argentina and Brazil *(11)*.

The species belonging to the *Nigri* section generally exhibited low percentages of OA-producing isolates. As reported in the literature, *A. carbonarius* is the exception, since it includes higher percentages (41.7% *(32)* to 90.9% *(13)*) of positive strains; in our study, the percentage of positive strains for this species was found to be 57%. In addition, *A. carbonarius* has shown a significant ability to colonize and produce OA in grape berries artificially inoculated in vitro *(7)*.

In conclusion, the results of this first Italian survey of OA-producing fungi stresses that *A. carbonarius* plays a large role in the OA contamination of grapes.

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