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Is there ochratoxin A in "passito" dessert wines from Friuli?

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Abstract. The fungal mycoflora of dried grapes, used in Friuli for the production of "passito" dessert wines, was investigated to detect potential ochratoxin A (OTA)-producers, in particular species of the genera *Penicillium* and *Aspergillus*. From samples of five cultivars of grapevines, grown in different locations, 379 strains of *Penicillium* spp. were isolated, but not one from the genus *Aspergillus*. Four strains produced UV fluorescent metabolites, as detected by thin-layer chromatography (TLC), on grape juice agar and on synthetic liquid media. Three of these were OTA producers, when analyzed by high pressure liquid chromatography (HPLC), following immunoaffinity column purification. On the basis of morphocultural characteristics and ribosomal DNA sequencing, the strains were classified as *P. puberulum* and *P. variabile*. None appeared to belong to the species *P. verrucosum* or *P. nordicum*.

Key-words. *Aspergillus*, fungi, mycotoxins, ochratoxin A, *Penicillium*, *Vitis vinifera.*

Introduction. Some ten years ago a paper appeared in a scientific journal describing the presence of ochratoxins in wine (Zimmerli & Dick 1996). Since then the number of reports on mycotoxin contaminated grapes and grapevine products has progressively increased, involving most of the countries where the crop and the resulting beverages (juice, wines, spirits, etc.) are produced.

In Friuli wine is of major economic interest and consequently an investigation was carried out to determine whether the problem existed in this region and to what extent it could affect the wine industry.

Mycotoxins. In a previous note in this journal (Locci & Gobbi 2002) the history of mycotoxin contamination of agricultural commodities was

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briefly oulined, starting from the first historical records up to the medioeval socio-economic impact of mycotoxicoses, showing that mycotoxicology is not just a discovery of our times. Obviously the increased worldwide trade of agricultural commodities and their bulk storage has increased the hazards.

Mycotoxins are secondary metabolites of moulds and have adverse effects on humans, animals and crops, resulting in illnesses and economic losses. In the report mentioned above, toxins produced by the genera *Aspergillus* (aflatoxins) and *Fusarium* (mainly the fumonisins) were indicated as the most dangerous to animal (man included) health.

They have different biological effects depending on their chemical structure: some are mutagenic, teratogenic, carcinogenic, estrogenic, hemorhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic or neurotoxic. Human ingestion of mycotoxins is due to their consumption in plant-based food and residues and metabolites in animal-derived foods (*e.g.*, aflatoxin M1 in milk). The impact of mycotoxins on health depends on the amount, the toxicity of the compound, acute or chronic (*e.g.*, carcinogenic), the body weight of the individual, the presence of other mycotoxins (synergistic effects) and so on.

However aflatoxins and fumonisins are not the only mycotoxins and the number of dangerous metabolites produced by fungi is increasing steadily. In the present note interest is focussed on another group of mycotoxins, ochratoxins and in particular ochratoxin A, with special reference to its present in grapes and derived beverages.

Ochratoxin A. Ochratoxin A (OTA), a secondary metabolite of *Aspergillus* spp. and *Penicillium* spp., is receiving increasing attention because of the hazard it poses to animal and human health (Abarca et al. 1998). The metabolite (Fig. 1) has nephrotoxic, as well as teratogenic, immunosuppressive and carcinogenic effects in animals and possibly also in man. It has been associated with Balkan Endemic Nephropathy and the development of urinary tract tumors. The International Agency for Research on Cancer

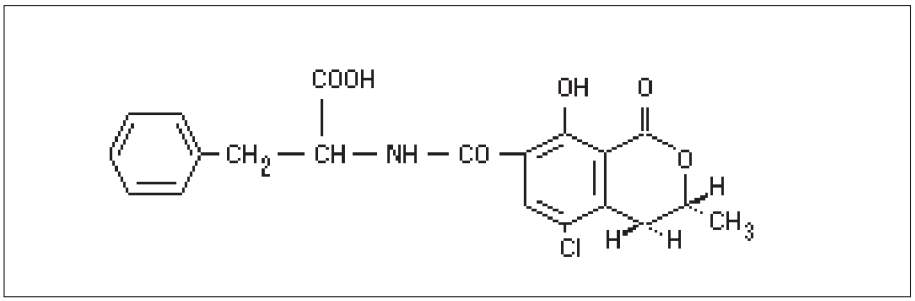

Figure 1. Ochratoxin A (OTA).

classified OTA in group 2B as a possible human carcinogen. The toxin is also immunosuppressive, due to general inhibitory effects on the synthesis of proteins containing phenylalanine and on other enzymes (Mantle 2002).

OTA is produced mainly by isolates of *Aspergillus ochraceus*, *A. carbonarius* and *Penicillium verrucosum* (Abrunhosa et al. 2001). These *Aspergillus* spp. and *Penicillium* spp. are characterized by different temperature requirements.

Penicillium spp. that produce OTA grow well over a wide range of temperatures (4-31 °C) (Zimmerli & Dick 1996), so they are dominant in temperate regions such as northern Europe and Canada (Larsen et al. 2001). *P. verrucosum* is the principal producer of OTA under these conditions. *Aspergillus* spp. producing OTA require higher temperatures (12-39 °C) (Zimmerli & Dick 1996) and they predominate in warmer and tropical regions (Larsen et al. 2001). *A. ochraceus* grows at 8-37 °C, and OTA is produced over the whole temperature range. *A. carbonarius* grows from about 10 to 40 °C but little is known about the conditions favouring toxin production.

Since 1970 the occurrence of OTA has been investigated and its presence identified in various commodities such as cereals, coffee, spices, beer, cocoa, dried fruits and meat; recently OTA has also been detected in milk. Due to its occurrence in such a wide range of food commodities, humans are continuously exposed to OTA, especially in Europe, where the highest frequencies and levels of OTA contamination are found in cereals. European Union regulations regarding maximum admissible limits of OTA in foods and feeds exist for a number of agricultural commodities.

OTA in grapevines and oenological products. Following the first report by Zimmerli and Dick (1996) on the presence of OTA in grape juice and wine, more papers have appeared in recent years (for details see Torelli et al. 2002, 2006; Varga & Kozakiewicz 2006).

Early investigations revealed some interesting facts. The toxin is not synthesized in the wine itself, but is the result of contamination of the starting material (grapes and juice), the relative abundance of OTA is closely related to the climate and geographic origin of the grapes, the agricultural practices and the methods used in wine making.

The first hypothesis is confirmed by the presence of OTA in grapes and in grapevine must. In addition mould growth in wine is strongly inhibited by ethanol and anaerobic conditions (Zimmerli & Dick 1996). Of course cross contamination from poorly cleaned vessels (fermentation tanks, barrels, casks, etc.) cannot be discarded. Both grape and wine analyses show a clear relationship between geographic location of the crop and OTA presence. This is most probably related to fungal contamination of the crop at harvest time, a critical stage when natural defenses of the plant are weakened and the grape is easily colonized by saprotrophic fungi (such as

Aspergillus and *Penicillium* species). Agricultural and phytosanitary practices (Otteneder & Majerus 2000) also contribute. Finally the reason for the different levels of OTA of white, rose and red wines is clearly due to the different wine-making technique, since the longer mash periods needed for red wines leads to a greater transfer of the toxin into the wine (Majerus et al. 2000).

Commercial wines with a high level of contamination may increase the total daily intake of OTA, especially in countries with a high consumption of wine. The Codex Committee on Food Additives and Contaminants estimates that 15% of the total intake of OTA is due to wine consumption.

Experimental work. "Passito" dessert wines are obtained by the fermentation of grapes dried for 90-120 days (raisining) in wooden boxes or on iron grids after harvest. Dehydration reduces the water content by 35% with a consequent increase in sugar (25-40%). During drying, grapes are prone to colonization by moulds such as *Botrytis cinerea*, *Aspergillus* spp., *Penicillium* spp., *Mucor* spp. (Tachis 1988).

Considering the risks to health and the importance of wine production for Friuli, Colli Orientali "passito" wines were investigated. The aim of this work was to assess the presence of ochratoxigenic fungi during the raisining of grapes used for red and white "passito" wine production.

Materials and methods

Sampling. About a hundred grape bunches from grapevine (*Vitis vinifera*) of five cultivars (Picolit, Verduzzo, Riesling Renano, Ribolla nera and Refosco) were randomly collected from wooden boxes during their raisining. The bunches had been kept in storage rooms for 20-25 days after harvesting (September-October) from 8 vineyards, located in Cividale, Nimis and Prepotto, in the Colli Orientali DOC area of Friuli, Italy (Table 1). The storage rooms were not heated and the average outdoor temperatures in the areas were 18 °C in September and 14 °C in October.

Samples were incubated in sterile moist chambers at 25 °C for 7 to 14 days until fungal colonies appeared. Each colony was transferred to PDA plates.

Cultural conditions. All mycelia were isolated and purified on Difco potato

Table 1. Grape cultivars, corresponding wines and sites where grapes were collected.

Grape cultivar	*Type of wine*	*Location*
Picolit	White	Cividale, Nimis, Prepotto
Refosco	Red	Nimis
Ribolla nera	Red	Cividale, Nimis
Riesling renano	White	Cividale
Verduzzo	White	Cividale, Nimis

dextrose agar (PDA) plates. The fungal isolates were grown at 25 °C on PDA and stored at 4 °C on PDA and on Difco white quartz sand at 25 °C. Two OTA-producing reference strains (*Aspergillus niger*, CBS 101697 and *Penicillium verrucosum*, CBS 323.92) were used as controls.

OTA production, quantification and confirmation

Pre-screening. All *Penicillium* spp. strains isolated from the samples and the reference strains were inoculated onto coconut cream agar (CCA) and incubated at 37 °C for 7 days. The fluorescent halo around the colonies visible under UV light was used to select putative OTA-producing isolates.

Thin-layer chromatography (TLC). Penicillium spp. strains showing fluorescence on CCA and the reference strains were grown on grape juice agar (GJA) for 7 days at 25 °C (Abrunhosa et al. 2001). Secondary metabolite production was analyzed by removing the agar from Petri dishes, blending in 20 ml of chloroform / methanol (2:1), and filtering through filter paper (Whatman, no 1001 110). After evaporation of the solvent, 20 µl for each strain were loaded onto 20x20 Silica gel 60 plates without an indicator (Merck, Germany). TLC was carried out in a saturated chamber using TEF (toluene, ethyl acetate and formic acid, 5:4:1). The spots were visualized by spraying the plates with p-anisaldehyde (0.5%) in methanol / sulphuric acid / acetic acid (18:1:1) (Sigma-Aldrich, USA) followed by heating for 8 min at 105 °C. From each lane, the spot corresponding in color and R_f value to those of the reference strains and of the standard was scraped from the plate and suspended in 40 µl of chloroform / methanol (2:1). After 15 min the supernatant was loaded onto a new plate and TLC was carried out as described above. Ochratoxin A (Sigma-Aldrich, USA) used as standard (1 mg/ml of chloroform /methanol 2:1) was stored at 4 °C.

Optimization. To maximize the OTA production, the strains that were positive by TLC were grown on a number of commonly used mycological media and under different conditions. Broths were filtered and concentrated in an oven before testing by TLC as indicated above.

Immunoaffinity chromatography. Penicillium spp. fluorescent on CMA and positive in TLC were analysed for the production of OTA on 10 ml of MEB, inoculated with 200 µl of a conidial suspension (10^7 conidia/ ml). Flasks were incubated for 10 days at 25 °C in the dark without shaking and their contents filtered through Miracloth paper (Calbiochem) in a 0.45 µm syringe (Roth). After evaporation to 6 ml at 40 °C, the broth was applied to an OchraTest immunoaffinity column following the manufacturer's instruction (Vicam, Watertown, MA). The methanol eluate was analysed by HPLC.

High pressure liquid chromatography (HPLC). The HPLC system (Varian Analytical Instruments, USA) was

equipped with a sampling module (loop volume 20 µl), a Prostar 363 fluorescence detector set at 333 nm excitation and 470 nm emission and a Prostar 310 UV/Vis detector. A Nucleosil C18 column (5 µm particle size) (Varian Analytical Instruments, USA) was employed at room temperature with a mobile phase (acetonitrile /water / acetic acid 57:41:2) at a flow rate of 1 ml/min (Abarca et al. 1998). Methanol solutions of OTA from 0.1 to 1.5 µlg/ml were used for calibration.

OTA production was qualitatively confirmed by derivatization and subsequent HPLC analysis of the methyl esters prepared from the extracts.

Identification of fungi. All the fungal colonies isolated from grapes and purified on PDA were microscopically examined in order to identify and select only *Aspergillus* and *Penicillium* spp. isolates. Further morphological observations for identification to species level were performed only for TLC positive *Penicillium* spp. strains. Eleven fungi were cultured on CYA, MEA and G25N and were identified by macro and microscopic examination in accordance with guide lines published for the genus (Pitt 1979). Three OTA producing strains were deposited in the National Culture Bank, Udine (NCB).

Isolation of fungal DNA. To obtain additional information for the identification of the OTA-producing colonies, their genomic DNA was isolated from mycelium scraped from agar plates and suspended in 250 ml of 50 mM EDTA. Lysis was obtained by adding 20 µl lyticase (20 U/µl) and incubating at 37 °C for 1 h. The digested mycelium was centrifuged at 12000 g for 4 min. Further purification of fungal genomic DNA was performed with the Wizard Genomic purification kit (Promega Italia Srl, Italy). After phenol extraction the DNA was precipitated by isopropanol, suspended in water and stored at 20 °C.

DNA sequencing. The rDNAs of two OTA-producing strains, NCB1494 and NCB1495, were amplified by PCR using the general primers NS7 and NS8 and also the primers ITS5 and ITS4 following standard protocols. Two independent PCR reactions were carried out and the amplified DNAs were sequenced directly using standard methods. The nucleotide sequences were compared to those present in the NCBI nucleotide databases employing the Blast-n algorithm.

Results

Isolation. The most prevalent genus found on grapes was *Penicillium*, in addition to species of *Cladosporium*, *Fusarium*, *Alternaria* and *Botrytis*. Three hundred and seventy-nine *Penicillium* spp. isolates were purified from grape bunches used for the production of red and white "passito" wines collected during the raisining at the 3 locations. The Penicillia were isolated from the stems and from the surface of the berries. No *Aspergillus* spp. strains were found.

The 379 *Penicillium* strains were morphologically recorded as biverticil-

late (56%), monoverticillate (24%), biverticillate/ terverticillate (14%), monoverticillate rarely with metulae (4%) and terverticillate (1%) depending on their penicil structures.

Screening and TLC. The production of fluorescence on CCA by mycotoxigenic fungi has been used for the rapid screening for OTA producers. In this study the preliminary screening for OTA producers on CCA revealed the presence of 34 out of 379 (9%) *Penicillium* strains showing fluorescence when grown for 7 days. Their fluorescent halos were identical to that shown by the reference strain *P. verrucosum*, while the reference strain *A. niger* grew more abundantly and produced a larger halo. Expecting the production of toxic metabolites, these 34 isolates were analyzed by TLC. Eleven fungi formed fluorescent spots: 4 strains out of 34 (10%) produced a metabolite generating a spot similar in color and R_f value to that of the OTA standard and of *A. niger* when grown on grape juice medium (Figure 1), while the other 7 strains produced two spots different in color and R_f value from those of the OTA standard and reference strains.

The reference strain *P. verrucosum* produced such a low amount of OTA that analysis by TLC was not possible. Even when cultivated on MEB, its growth was so impaired that the final OTA concentration was too low to be detected by TLC. Nonetheless, its OTA production was confirmed by immunoaffinity column and fluorometric readings.

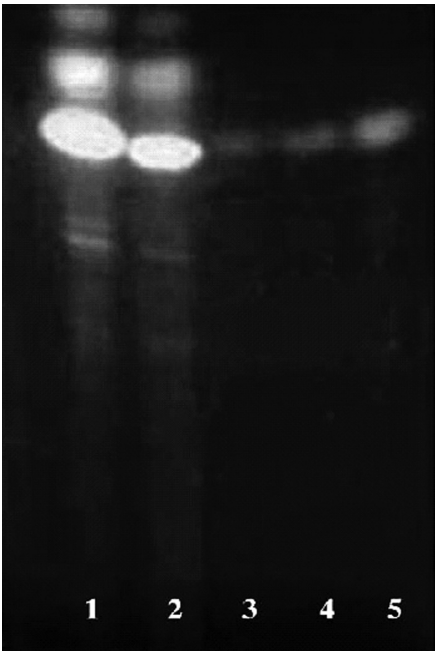

Figure 2. TLC of extracts from silica plate spots generated by different fungal strains. Lane 1, OTA standard solution 1 mg/ml; Lane 2, OTA from *A. niger*; Lane 3, strain NCB1493; Lane 4, strain 3.2.20; and Lane 5, strain NCB1495. Fungal colonies were grown on GJA.

The best synthetic medium for the production of OTA was MEB and the optimal cultural conditions for OTA production by the 4 TLC positive strains consisted in growing them on MEB, pH 5, for 10 days at 25 °C, as confirmed by TLC where a single UV fluorescent spot with the same colour and R_f as that of the OTA control was detected. The addition of KCl did not affect OTA production.

Immunoaffinity chromatography and HPLC. When the four TLC positive

Table 2. Levels of OTA produced by *Penicillium* spp. isolated from grapes in Friuli and by the reference strain *A. niger* as determined by HPLC.

Strain	*OTA (µg/ml MetOH)*
NCB1493	0.07
NCB1494	0.30
NCB1495	0.20
CBS 101697	2.00

Penicillium strains were analyzed by HPLC, following immunoaffinity purification, only the strains NCB1493, 1494 and 1495, isolated from Verduzzo and Picolit grapes, produced detectable amounts of OTA. Strain 3.2.20 did not produce any OTA.

Identification. The TLC positive *Penicillium* isolates were micromorphologically identified according to Pitt (1979) (Table 3). All the strains were biverticillate: despite extensive observations no terverticillate structures were ever detected. Strains 3.2.20, NCB1493 and NCB1495 were morphologically indistinguishable. Because identification at species level is notoriously difficult, strains NCB 1493, NCB1494 and NCB1495 were also sent to the Centraalbureau voor Schimmlecultures (CBS), Utrecht, The Netherlands, for identification.

In addition partial direct sequencings of ribosomal genes of the strains NCB1494 and NCB1495 were carried out. The sequences obtained from amplifying the rDNAs of the two strains with the NS8-NS7 primers had very similar results (> 99% identity). When compared to the other sequences reported in the databases, strain NCB1494 had a DNA sequence 97% identical to *P. radicum*; while strain NCB1495 showed an identical sequence to that of *P. radicum* and *P. rugulosum*.

When the analysed portion of rDNAs was the ITS1-5.8- ITS2 region, the sequence of strain NCB1495 was 99% identical to the sequences of *P. glabrum* (AY373915) and *P. thomii* (AY373934), while the sequence of strain NCB1494 was 93% and 96% identical to that of *P. glabrum* and *P. thomii*. None of the strains isolated from grapes of Friuli had sequences identical to those of the *P. verrucosum* reported in databases.

In conclusion the three strains were identified as *P. puberulum* (NCB 1493 and 1495) and *P. variabile* (NCB 1494).

Discussion. The results of the present investigation show that only 0.8% of the *Penicillium* cultures isolated in

Table 3. Morphological identification of *Penicillium* strains isolated from grapes, fluorescent on CMA and positive after TLC.

Strain	*Subgenus*	*Section*	*Series*
3.2.20	*Penicillium*	*Penicillium*	*Urticicola*
NCB1493	*Penicillium*	*Penicillium*	*Urticicola*
NCB1494	*Biverticillium*	*Simplicium*	
NCB1495	*Penicillium*	*Penicillium*	*Urticicola*

Friuli from grapes destined for the "passito" wine production of were potential OTA-producers when grown on laboratory media. This does not mean that the berries themselves were contaminated by the toxin, as confirmed by independent investigations (De Zan, personal communication) which failed to reveal detectable amounts of OTA in the derived wines. In addition no *Aspergillus* species was isolated.

These results are in contrast with those of investigations carried out in other regions of Italy (Sage et al. 2002; Battilani et al. 2003) on grapes grown for the production of ordinary wine. In this connection some considerations are in order. To start with in the relatively small-sized farms where sampling was carried out, the grapes for "passito" wines are selected and heavily contaminated bunches are automatically discarded.

The predominance of *Penicillium* spp. over *Aspergillus* spp. in the grapes collected in Friuli, though in contrast with the results of Battilani et al. (2003), is not surprizing, considering the relatively cooler climate of the Friuli region, the mean temperatures in August-September and October in the year of sampling were respectively 23-18 and 14 °C, as compared to the regions sampled by those authors (the average temperatures for the same months were 28-24 and 19 °C in Emilia Romagna and 30-27 and 22 °C in Puglia). The geographic region of origin has a strong influence on OTA contamination, both in red and dessert wines. A concentration gradient going from north to south can be observed in the Italian regions on the Adriatic coast, from Triveneto to Puglia, suggesting that climatic differences affect mould contamination and OTA production (Pietri et al. 2001). Similarly in OTA contamination of cereals, *Penicillium* spp. strains are more abundant in temperate regions whereas *Aspergillus* spp. tend to predominate in warmer climates (Zimmerli and Dick 1996). At optimum water activity the temperature range is 4-31 °C for *Penicillium verrucosum* and 12-37 °C for *Aspergillus ochraceus* (Moss 1996).

The *Penicillium* isolates found to produce OTA were identified as *P. puberulum* and *P. variabile*. This unexpected result sheds new light on the production of OTA by fungi belonging to the genus *Penicillium*. Species different from *P. verrucosum* e *P. nordicum* were shown to produce OTA *in vitro*. On the other hand the *P. verrucosum* species appear to be such a complex group that further work at the molecular level is advisable to clarifify its taxonomy.

Finally to our knowledge the only other comparable study on grapes overripening on the vine or subjected to postharvest drying for the preparation of liqueur wines is that carried out by Gomez et al. (2006) in Spain. The authors found that black *Aspergillus* spp. were dominant, but here again, with the possible exception of the Girona region, the grapes were collected in hot and semi-arid districts like those of Cadiz and Cordoba.

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