Letters in Applied Microbiology 2003, **37**, 179–184

Mycoflora and ochratoxin-producing strains of *Aspergillus* section Nigri in wine grapes in Argentina

C. Magnoli[1], M. Violante[1], M. Combina[2], G. Palacio[3] and A. Dalcero[1]

[1]Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Córdoba, [2]Instituto Nacional de Tecnología Agropecuaria (INTA), Lujan de Cuyo, Mendoza, and [3]Departamento de Matemáticas, Facultad de Ciencias Exactas, Físico, Químicas y Naturales, Universidad Nacional de Río Cuarto, Córdoba, Argentina

2002/373: received 2 December 2002, revised 23 April 2003 and accepted 15 May 2003

ABSTRACT

C. MAGNOLI, M. VIOLANTE, M. COMBINA, G. PALACIO AND A. DALCERO. 2003.

Aims: The aims of this work were to evaluate the mycoflora and to identify the species of *Aspergillus* with the potential to produce ochratoxin A (OA) from different wine grape varieties from Mendoza, Argentina. Likewise, the capacity to produce OA by *Aspergillus* section Nigri was studied.

Methods and Results: Fifty samples of wine grapes were obtained from a winery of Mendoza province, Argentina. The surface-disinfection method was used for mycoflora determination using the medium dichloran 18% glycerol agar (DG18). *Alternaria*, *Aspergillus* and *Penicillium* were identified at species level. OA production was tested in 63 strains belonging to section Nigri. *Alternaria* genus was the most frequent (80% of the samples) followed by *Aspergillus* (70%). *Alternaria alternata* was the only specie identified from the *Alternaria* genus, followed by *A. niger* var. *niger*, *A. flavus* among others. From *Penicillium* genus, *P. crysogenum* was the most frequent specie. From 63 strains of *Aspergillus* section Nigri, 41·3% were OA producers. The levels of produced toxin ranged from 2 to 24·5 ng ml^{-1} of culture medium.

Conclusions: The presence of ochratoxigenic strains of Nigri section in this substrate suggests that they may be an important source of OA in grapes from tropical and subtropical zones. Therefore, the industry should work further to diminish the growth of these fungi and mycotoxins formation in grapes, with the aim to reduce OA content in wine products.

Significance and Impact of the Study: The wine grape contamination with *A. alternata* and *Aspergillus* section Nigri was significant.

Keywords: *Aspergillus*, *Aspergillus* section Nigri, mycoflora, ochratoxin, *Penicillium*, wine grapes.

INTRODUCTION

In Argentina, the wine grape production regions are situated in Mendoza and San Juan provinces. Grapes are used in wine, juice, dried vine fruits and food (Pearson and Goheen 1994). Rotting and spoilage of grape berries before harvest can be caused by a variety of fungal species. *Botrytis cinerea*, which causes bunch rot, is the main species. Other species include *Penicillium*, *Aspergillus*, *Alternaria* and *Cladosporium*. Grapes that are heavily infected with moulds alter in chemical composition and mould enzymes adversely affect wine flavour and colour and the growth of yeasts during alcoholic fermentation (Fleet 1999; Fleet 2001).

Contamination by different moulds occurs during pre-harvesting, harversting and grape processing. During these periods, temperature and humidity are important factors in mycelium growth and conidia germination (Lozada 1995). The mycotoxins of greatest significance in grapes and wine grapes produced by *Aspergillus* and *Penicillium* spp., include aflatoxins, citrinin and ochratoxin A (OA). The toxicological

Correspondence to: Ana María Dalcero, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional No. 36 Km 601 (5800) Río Cuarto, Córdoba, Argentina (e-mail: adalcero@exa.unrc.edu.ar).

profile of OA includes teratogenesis, nephrotoxicity and immunotoxicity. This toxin is very persistent in human beings due to unfavourable toxicokinetic elimination, that may cause serious damage to kidneys. These facts indicate that human exposure should be kept to minimum concentrations (Walker 2002). Wine is a widely consumed product by adult individuals in both developed and developing countries and, due to its high frequency of contamination with OA, it may represent, after cereals, a major source of daily OA intake for this population. In European countries, provisional estimates of Codex Alimentarius Commission, based on limited data, suggest that 15% of the total intake of this toxin is due to wine (Codex Alimentarius Commission 1998).

Most data on the natural occurrence of OA in dried vine fruits, grapes and wine have been obtained from Europe, especially from Germany, France, Scandinavia and the Balkans (Pittet 1998; MacDonald *et al.* 1999; Otteneder and Majerus 2000; Markaki *et al.* 2001; Sage *et al.* 2002). *Penicillium verrucosum* is the principal species associated with OA production in foods and feeds in temperate climates, while *Aspergillus* spp. predominates in warmer countries (Pitt and Hocking 1997). These species had been previously isolated in low frequencies from poultry feeds in our region (Dalcero *et al.* 1998; Magnoli *et al.* 2002). While a high percentage of *Aspergillus* section Nigri, potential producers of OA, was found in corn-based feeds (Dalcero *et al.* 2002), in grape-growing Argentinean regions, the temperature and humidity conditions are suitable for colonization of *Aspergillus* section Nigri and mycotoxin production. Therefore, it is reasonable to think that these fungi may be an important source of OA in grapes in this zone. In addition, Argentina is the fourth largest producer of wine in the world, having excellent organoleptic characteristics. Consequently, it becomes of utmost importance to guarantee a quality control of the grapes, through a contaminant mycoflora identification.

The aims of this work were to evaluate the mycoflora and to identify the species of *Aspergillus* with the potential to produce OA from different wine grape varieties from Mendoza, Argentina. Likewise, the capacity to produce OA by *Aspergillus* section Nigri was studied.

MATERIALS AND METHODS

Sampling

Fifty samples of wine grapes (*Malbec*, *Chardonnay*, *Merlot*, *Cabernet* and *Bonarda* varieties) were obtained from a winery of Mendoza province, Argentina, during 2001 harvest. When boxes containing fresh grapes arrived at the winery, two bunches were taken at random from each box (10 to 20 boxes of each wine grapes variety). One hundred grape berries were taken from each bunch at random, sent to the laboratory as soon as possible and tested upon arrival.

Isolation of mycoflora

Each sample of 100 grape berries was surface-disinfected with sodium hypochlorite solution (1%) for 1 min; rinsed in sterile distilled water three times and plated onto a dichloran 18% glycerol agar (DG18) (Pitt and Hocking 1997). The plates were incubated for 1 week at 25°C. On the last day of incubation, the colonies of *Aspergillus*, *Penicillium* and *Alternaria* genera were picked and transferred to malt extract agar (MEA) slants, and allowed to grow at room temperature for 7 days for identification to species level.

Identification of *Aspergillus*, *Penicillium* and *Alternaria* species

For *Penicillium* identification, cultures are grown on Czapek yeast extract agar (CYA) at 5, 25 and 37°C, MEA at 25°C, and 25% glycerol nitrate agar (G25N) at 25°C. For *Aspergillus* identification, cultures are grown on CYA (25 and 37°C), MEA (25°C) and Czapek yeast extract with 20% sucrose agar (CY20S) at 25°C. All plates were incubated for 7 days. Each strain was identified according to the methods given by Pitt (1988), Klich and Pitt (1994), Pitt and Hocking (1997) and Samson *et al.* (2000).

For *Alternaria* identification, single conidia were isolated by standard dilution plate methods. A conidial suspension of each *Alternaria* isolate was placed on 1·5% water agar in a Petri dish, and incubated at room temperature. After 16–18 h, single conidium colonies were identified with a dissecting microscope (×30), and transferred to dichloran chloramphenicol malt agar, potato carrot agar, V8 juice medium and 1·5% water agar. After incubation for 7 or 10 days at 28°C, each colony was examined using macroscopic and microscopic criteria according to the classification schemes proposed by Simmons (1993).

The percentage of grain grapes with *Aspergillus*, *Penicillium* and *Alternaria* species, and the occurrence of these moulds in wine grape samples were determined.

Ochratoxin A production

The OA production was tested in 63 strains belonging to section Nigri (*A. niger* var. *niger*, *A. niger* var. *awamori*, *A. foetidus*). OA was determined following the methodology described by Téren *et al.* (1996). The strains were grown in stationary cultures using 25 ml quantities of YES medium (2% yeast extract, 15% sucrose) in 125 ml Erlenmeyer flasks and were inoculated with 0·1 ml of dense conidial suspensions and incubated at 30°C for 10 days in darkness.

After incubation, 1 ml portions of this culture media were then mixed with 1 ml of chloroform and centrifuged at 4000 *g* for 10 min. The chloroform phase was transferred to a clean tube, evaporated to dryness and dissolved in appropriate amounts of the mobile phase (57% acetonitrile, 41% water and 2% acetic acid) and OA was detected on a HPLC isocratic system. The HPLC apparatus used for detection was a Hewlett-Packard chromatograph (Hewlett-Packard Company, Palo Alto, CA, USA) with a loop of 100 μl, equipped with a spectrofluorescence detector (excitation, 330 nm; emission, 460 nm) and a C_{18} column (Supelcosil LC-ABZ, Supelco, Belleforte, PA, USA; 150 × 4,6 mm, 5 μm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20 × 4,6 mm, 5 μm particle size). The mobile phase, was pumped at a rate of 1 ml min^{-1}. Extracts were considered positive if they yielded a peak at retention time identical to that of standard OA. The standard solution was prepared as described by AOAC (1995) using OA obtained from Sigma (St Louis, MO, USA). The lowest limit of detection was 1 ng ml^{-1}.

Statistical analysis

The mean percentage of grape berries contaminated by *A. alternata*, *A. flavus* and section Nigri species were analysed by the General Lineal Models Procedure of SAS (1988; SAS Institute, Cary, NC, USA). Multiple comparison of Dunn was applied to compare the significant differences among the percentages of berries grape contaminated (Berenson and Levine 1996).

RESULTS

The percentage of grape berries contaminated by fungal genera, *Aspergillus* and *Penicillium* species and the occurrence of these moulds in the samples were used to characterize the mycoflora in wine grapes. Mycological survey of the 50 samples of wine grapes indicated the presence of seven genera of filamentous fungi. Among them *Alternaria* spp. was the most frequent mould of the mycoflora that occurred in 80% of samples, followed by *Aspergillus* spp. in 70%. The genera isolated in smaller frequency were *Cladosporium* spp. and *Penicillium* spp. among others (Fig. 1). *Alternata alternata* was the only species identified from the *Alternaria* genus. Five species of *Aspergillus* were identified. The predominant were *A. niger* var. *niger* (61%) and *A. flavus* (17%), followed by *A niger* var. *awamori*, *A. foetidus* and *A. candidus* in smaller frequency. Among *Penicillin* spp., *P. crysogenum* was the most frequent, isolated in 22% of the samples (Fig. 2).

Tables 1 and 2 showed the percentage of grape berries contaminated by fungi genera and *Aspergillus* and *Penicillium* species. *Alternaria* spp. contaminated the major number of grape berries followed by *Aspergillus* spp. Statistical analysis demonstrated that the species *A. alternata* was isolated in higher frequency (9·5%, $P < 0{\cdot}01$).

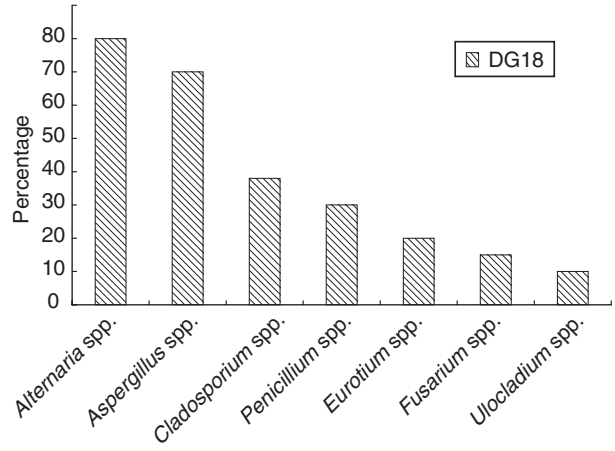

Fig. 1 Percentage of wine grape samples contaminated by fungi genera, harvest 2001

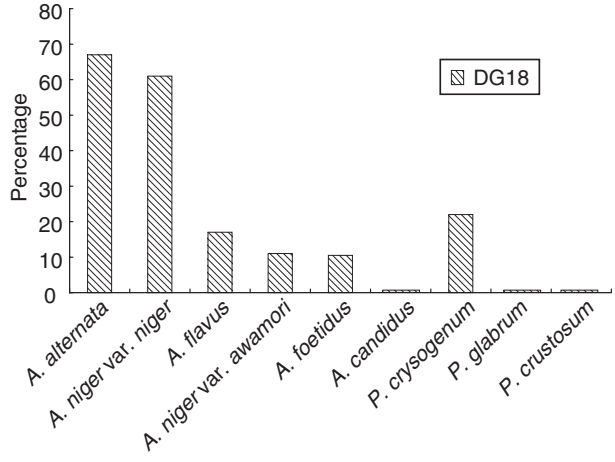

Fig. 2 Percentage of wine grape samples contaminated by *Alternaria*, *Aspergillus* and *Penicillium* species, harvest 2001

Twenty-six strains (41·3%) of 63 *Aspergillus* section Nigri were OA producers. The levels of toxin produced ranged from 2 to 24·5 ng ml^{-1} of culture medium. In the species *A. niger* var. *niger* the highest percentages of ochratoxigenic strains were detected (45·45%), with levels ranging from 2 to 24·5 ng ml^{-1} (mean level 12·7 ng ml^{-1}). While 33% of *A. niger* var. *awamori* was OA producers with mean levels of 14·5 ng ml^{-1} (Table 3).

DISCUSSION

Moulds that contaminate wine grapes include species of *Botrytis*, *Penicillium*, *Aspergillus*, *Mucor*, *Rhizopus*,

Table 1 Percentage of wine grape berries infected by fungi genera in DG18 media

	No. of berries infected ($n = 50$)	
Fungal genera	Range (%)*	Mean (%)†
Alternaria spp.	1·4–26·5	9·25
Aspergillus spp.	1·0–24	7·6
Fusarium spp.	1·0–14	5·5
Ulocladium spp.	2·0–5·0	3·25
Eurotium spp.	1·0–6·0	3·0
Penicillium spp.	1·0–5·5	2·4
Cladosporium spp.	1·0–4·0	2·0

*Minor and major percentage of wine grape berries infected.
†Mean percentage of wine grape berries infected.

Table 2 Percentage of wine grape berries infected by *Aspergillus*, *Alternaria* and *Penicillium* species in DG18 media

	No. of berries infected ($n = 50$)	
Fungal species	Range (%)*	Mean (%)†
A. alternata	1·5–26·5	8·5[a]
A. flavus	1·0–12	8·0[b]
A. niger var. *niger*	1·0–24	6·0[b]
A. niger var. *awamori*	2·0–6·0	4·0[b]
A. foetidus	2·0–6·0	4·0
P. crysogenum	1·0–5·0	2·5
A. candidus	ND	2·0
P. glabrum	ND	1·0
P. crustosum	ND	3·0

*Minor and major percentage of wine grape berries infected.
†Mean percentage of wine grape berries infected.
ND, not detected.
[a,b]Values with no common superscripts are significantly different ($P < 0·01$) according to Dunn test.

Table 3 Ochratoxin A production by species of *Aspergillus* section *Nigri* isolated from wine grapes

Species	Positive strains*	Mean levels (ng ml^{-1})
A. niger var. *niger*	5/44	2·0–3·0
	10/44	13·0–17·0
	4/44	19·5–24·5
A. niger var. *awamori*	1/15	3·0
	1/15	13·45
	3/15	18·0–20·0
A. foetidus	1/4	2·0

*Number of producer strains vs total strains.
Detection limit: 1 ng ml^{-1}.

Cladosporium, *Alternaria*, *Uncinula* and *Plasmopara*. *Botrytis cinerea* is regarded as the most serious cause of spoilage in grapes (Doneche 1993). In relation to the isolated genera, the results obtained in this study showed differences with those obtained from previous work with grapes for wine from Argentina and Brazil. In this sampling period, *B. cinerea* was not isolated and *A. alternata* was the most prevalent specie, while the genera *Aspergillus*, *Penicillium* and *Cladosporium* were detected in high percentage (Da Rocha Rosa *et al.* 2002). *Alternaria* spp. are important fungal contaminants of vegetable fruits and grain products, including *A. alternata*, a contaminant of various fruits (Scott 2001). The toxins produced by this species (alternariol, alternariol monomethyl ether, tenuazonic acid, altertoxin I, II and III) have been reported in various fruits (Tournas and Stack 2001). *Aspergillus* species can infect grapes and *A. niger* is by far the most common *Aspergillus* species responsible for postharvest decay of fresh fruit including grapes (Barkai-Golan 1980; Snowdon 1990). In general the percentage of contaminated grape samples with *A. niger* var. *niger*, *A. flavus* and *P. crysogenum* was higher in this study. While the potentially ochratoxigenics *A. ochraceus* and *A. carbonarius* species were only identified in Brazilian wine grapes in previous work (Da Rocha Rosa *et al.* 2002). These differences are explained because the diversity and population of fungi in wine grapes depend on many factors, including degree of physical damage, viticulture practices, and weather conditions.

The percentage of black *Aspergillus* strains was higher, with levels of OA lower than the one detected in the wine grapes from Argentina and Brazil (Da Rocha Rosa *et al.* 2002). In a previous work, a low percentage of OA producing isolates of *Aspergillus* section Nigri was found in corn-based feeds, with similar levels of OA (Dalcero *et al.* 2002).

In the literature, the reported percentages of OA production by *A. niger* aggregate isolated from cereals and field are quite variable and depend on the number of isolates studied (Téren *et al.* 1996; Nakajima *et al.* 1997; Heenan *et al.* 1998; Abarca *et al.* 2001).

Penicillium species apparently do not attack grapes before harvest (Pitt and Hocking 1997), but are prevalent in stored grapes where *P. expansum* is the most common contaminant species (Snowdon 1990). Other species isolated from stored grapes are *P. aurantiogriseum*, *P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. crustosum*, *P. decumbens* and *P. glabrum* (Barkai-Golan 1980; Benkhemmar *et al.* 1993). In the present study, *Penicillium* spp. isolated were similar to the ones found in grapes in previous work (Da Rocha Rosa *et al.* 2002). The major species responsible for the ochratoxin production in foods from temperate climate, *P. verrucosum* (Pitt and Hocking 1997), has not been isolated from these wine grape samples. In other study, *P. crysogenum* was found at high percentage in table wine grapes in France (Sage *et al.* 2002). This species may produce a very wide range of toxic compounds: roquefortine C, meleagrin

and penicillin. These metabolites could be considered as a potential hazard to human health (Samson *et al.* 2000).

It is reasonable to think that black aspergilli may be an important source of OA in grapes from tropical and subtropical zones. Therefore, the industry should work further to reduce the growth of these fungi and mycotoxin formation in grapes, with the aim to diminish OA content in wine products. The determination of OA in wine is highly desirable in order to fulfil the need to protect consumers health from the risk of exposure to the toxin.

ACKNOWLEDGEMENTS

The authors are grateful to the SECyT (Secretaría de Ciencia y Técnica, Universidad Nacional de Río Cuarto) Res. No. 129/01, 077/03; CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) PIP Res. No. 962/01; FONCyT - PICT Res. No. 08-07197, which supported this study through grants. Dr Carina Magnoli is a fellowship from CONICET.

REFERENCES

Abarca, M.L., Accensi, F., Bragulat, M.R. and Cabañes, F.J. (2001) Current importance of Ochratoxin A-producing *Aspergillus* spp. *Journal of Food Protection* **64**, 903–906.

AOAC, (1995) Official Methods of Analysis, 17th edn, ed. Horwitz, W. Gaithersburg, MA, USA: AOAC.

Barkai-Golan, R. (1980) Species of *Penicillium* causing decay of stored fruits and vegetables in Israel. *Mycopathologia Mycologia Applicada* **54**, 141–145.

Benkhemmar, O., Lahlou, H., Dupont, J., Bompiex, G., Boubekri, C. and EL-Mniai, H. (1993) Identification of different species of *Penicillium* causing deterioration of Moroccan table grapes during storage. *Mycopathologia* **124**, 27–30.

Berenson M.L. and Levine D.M. (1996) *Estadí*stica Básica en Administración: Conceptos y Aplicaciones, 6th edn. Englewood Cliffs, NJ, USA: Prentice Hall.

Codex Alimentarius Commission, *Position Paper on Ochratoxin A* (1998) CX/FAC 99/14.

Da Rocha Rosa, C.A., Palacios, V., Combina, M., Fraga, M.E., De Oliveira Rekson, A., Magnoli, C.E. and Dalcero A.M. (2002) Potential ochratoxin A producers from wine grapes in Argentina and Brazil. *Food Additives and Contaminants* **19**, 408–414.

Dalcero, A., Magnoli, C., Luna, M., Ancasi, G., Reynoso, M.M., Chiacchiera, S., Miazzo, R. and Palacio, G. (1998) Mycoflora and naturally ocurring mycotoxins in poultry feeds in Argentina. *Mycopathologia* **141**, 37–43.

Dalcero A., Magnoli C., Hallak C., Chiacchiera S.M., Palacio G. and Da Rocha Rosa C.A. (2002) Detection of ochratoxin A in animal feeds and capacity to produce this mycotoxin by *Aspergillus* section *Nigri* in Argentina. *Food additives and Contaminants* **19**, 1065–1072.

Doneche, B. (1993) Botrytized wines. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp. 1–26. Chur, Swizerland: Harwood Academic Publishers.

Fleet, G.H. (1999) Microorganisms in food ecosystems. *International Journal Food Microbiology* **50**, 101–117.

Fleet, G.H. (2001) Wine. In *Food Microbiology Fundamentals and Frontiers*, 2nd edn ed. Doyle, M.P., Beuchat, L.R. and Montville, T.J. pp. 747–772. Washington, DC: ASM Press.

Heenan, C.N., Shaw, K.J. and Pitt, J.I. (1998) Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. *Journal of Food Mycology* **1**, 67–72.

Klich, M.A. and Pitt, J.I. (1994) *A Laboratory Guide to Common Aspergillus Species and their Teleomorphs* ed. Klich, M.A. and Pitt, J.I. City?, Australia: Commonwealth Scientific and Industrial Research.

Lozada, A.F. (1995) Isolation and identification of mycotoxigenic fungi in selected foods and feeds. *Food Additives and Contaminants* **3**, 509–514.

MacDonald, S., Wilson, P., Barnes, K., Damant, A., Massey, R., Mortby, E. and Shepherd, M.J. (1999) Ochratoxin A in dried vine fruit: method development and survey. *Food Additives and Contaminants* **16**, 253–260.

Magnoli, C., Chiacchiera, S., Miazzo, R., Palacio, G., Angeletti A., Hallak C. and Dalcero, A. (2002) The mycobiota and toxicity of feedstuffs from South of Córdoba province in Argentina. *Mycotoxin Research* (in press).

Markaki, P., Delpont-Binet, C., Grosso, F. and Dragacci, S. (2001) Determination of ochratoxin A in red wine and vinegar by immunoaffinity high-pressure liquid chromatography. *Journal of Food Protection* **64**, 533–537.

Nakajima, M., Tsubouchi, M., Miyabe and Ueno, I. (1997) Survey of aflatoxin B_1 and ochratoxin A in commercial green coffe beans by high-performance liquid chromatography linked with immunoafinity chromatography. *Food and Agricultural Immunology* **9**, 77–89.

Otteneder, H. and Majerus, P. (2000) Occurrence of ochratoxin A (OTA) in wine: influence of the type of wine and its geographical origin. *Food Additives and Contaminants* **17**, 793–798.

Pearson, R.C. and Goheen, A.C. (1994) Compendium of Grapes Disease. pp. 1–3. St Paul, MN: The American Phytopathological Society.

Pitt, J.I. (1988) *A Laboratory Guide to Common Penicillium Species*, 2nd edn. Sydney: Division of Food Research Sydney.

Pitt, J.I. and Hocking, A.D. (1997) *Fungi and Food Spoilage*, Vol. II ed. Pitt, J.I. and Hocking, A.D. London: Blackie Academic and Professional.

Pittet, A. (1998) Natural occurrence of mycotoxins in foods and feeds: an updated review. *Revuede Médicine Véterinaire* **149**, 479–492.

Sage, L., Krivobok, S., Delbos, E., Seigle-Murandi, F. and Creppy E.E. (2002) Fungal flora and ochratoxin A production in grapes and musts from France. *Journal of Agricultural and Food Chemistry* **50**, 1306–1311.

Samson, R.A., Hoekstra, E.S., Frisvad, J.C. and Filtenborg, O. (2000) *Introduction to Food- and Airborne Fungi*. City?, The Netherlands: Centraalbureau Voorschimmelculturs- utrecht, Ponson & Looyen, Wageningen Press.

Scott P.M. (2001) Analysis of agricultural commodities and food for *Alternaria* mycotoxins. *Journal of AOAC International* **84**, 1809–1817.

Simmons, E.G. (1993) Alternaria themes and variations (63–72). *Mycotaxon* **XLVIII**, 91–140.

Snowdon, A.L. (1990) *A Color Atlas of Post-Harvest Diseases and Disorders of Fruits and Vegetables 1. General Introduction and Fruits*. London: Wolfe.

Tournas, V.H. and Stack, M.E. (2001) Production of alternariol and alternariol monomethil ether by *Alternaria* grown on fruits at various temperatures. *Journal of Food Protection* **64**, 528–532.

Téren, J., Varga, J., Hamari, Z., Rinyu, E. and Kevei, F. (1996) Immunochemical detection of ochratoxin A in black *Aspergillus* strains. *Mycopathologia* **134**, 171–176.

Walker, R. (2002) Risk assessment of ochratoxin: current views of the European Scientific Committee on Food, the JECFA and the Codex Committee on Food Additives and Contaminants. *Advances in Experimental Medicine and Biology* **504**, 249–255.