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Mycobiota of Czech wine grapes and occurrence of ochratoxin A and *Alternaria* mycotoxins in fresh grape juice, must and wine

VLADIMÍR OSTRÝ[1], JARMILA ŠKARKOVÁ[1], IVANA PROCHÁZKOVÁ[1], ALENA KUBÁTOVÁ[2], FRANTIŠEK MALÍŘ[3] and JIŘÍ RUPRICH[1]

[1]National Institute of Public Health in Prague, Centre for the Hygiene of Food Chains, CZ 612 42 Brno, Czech Republic
ostry@chpr.szu.cz
[2]Department of Botany, Faculty of Natural Sciences, Charles University, Praha, CZ 128 01, Czech Republic
[3]Institute of Public Health Hradec Králové, Department of Xenobiochemistry, CZ 500 02 Hradec Králové, Czech Republic

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The aim of this study was to monitor the mycobiota of wine grapes, occurrence of ochratoxigenic microfungi in wine grapes and occurrence of ochratoxin A and *Alternaria* mycotoxins in fresh grape juice, must and wine from domestic crops in the year 2004. Thirteen samples of wine grapes (white /nine samples/ and red /four samples/) were collected during harvesting in the Znojmo wine region, SE Moravia. One sample of a wine grape variety was represented by three subsamples of wine grapes, which were sampled in left, middle and right part of the vineyard. Five wine grape berries per bunch were randomly selected, plated onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar, and incubated for 5–7 days at 25 °C. *Alternaria alternata*, *Cladosporium herbarum*, *C. cladosporioides*, *Penicillium expansum*, *P. aurantiogriseum*, *P. spinulosum* and *Rhizopus nigricans* were isolated from the samples. Ochratoxigenic microfungi, e. g. *Aspergillus carbonarius*, and other species of section *Nigri*, *A. ochraceus*, *Penicillium verrucosum* and *P. nordicum*, were not found in the samples. The HPTLC method for quantification of ochratoxin A (OTA) and *Alternaria* mycotoxins (alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), and tenuazonic acid (TeA)) in fresh grape juice (13 samples), must (13 samples) and wine (13 samples) was used. The limit of quantification (LoQ) was 8 ng/l for OTA, 1.5 µg/l for AOH, 1.5 µg/l for AME, 1.5 µg/l for ALT and 7.5 µg/l for TeA. Occurrence of OTA and *Alternaria* mycotoxins in fresh grape juice, must and wine was not proved.

Key words: mycobiota, grapes, grape juice, wine, ochratoxin A, *Alternaria* mycotoxins, HPTLC

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Ve studii bylo zkoumáno osídlení hroznů vinné révy vláknitými mikroskopickými houbami se zaměřením na producenty mykotoxinu ochratoxinu A (OTA) a výskyt OTA a alternáriových mykotoxinů v hroznové šťávě, moštu a víně. Třináct vzorků hroznů révy vinné (bílé odrůdy /9 vzorků/ a červené od-

růdy /4 vzorky/) bylo odebráno ve Znojemské vinařské podoblasti během sklizně v roce 2004. Jeden vzorek tvořily vždy tři subvzorky odebrané v levé části, uprostřed a v pravé části vinohradu. Pět bobulí z každého subvzorku hroznu révy vinné bylo umístěno na Dichloran Rose Bengal Chloramphenicol (DRBC) agar a inkubováno při teplotě 25 °C po dobu 5–7 dní. Po inkubaci byly ze vzorků révy vinné izolovány vláknité mikroskopické houby *Alternaria alternata*, *Cladosporium* (*C. herbarum*, *C. cladosporioides*), *Penicillium* (*P. expansum*, *P. aurantiogriseum*, *P. spinulosum*) a *Rhizopus nigricans*. Ochratoxinogenní vláknité mikroskopické houby (*Aspergillus carbonarius*, další druhy *Aspergillus* sekce *Nigri*, *A. ochraceus*, *Penicillium verrucosum* a *P. nordicum*) ve vzorcích révy vinné nebyly zjištěny. Pro stanovení OTA a alternáriových mykotoxinů (alternariolu (AOH), alternariol monometyléteru (AME), altenuenu (ALT) a tenuazonové kyseliny (TeA)) ve 13 vzorcích hroznové šťávy, moštu (13 vzorků) a víně (13 vzorků) byla použita metoda HPTLC. Mez stanovitelnosti metody (LoQ) pro OTA byla 8 ng/l, pro AOH 1,5 µg/l, pro AME 1,5 µg/l, pro ALT 1,5 µg/l a pro TeA 7,5 µg/l. Výskyt OTA a alternáriových mykotoxinů v hroznové šťávě, moštu a víně nebyl zjištěn.

INTRODUCTION

Ochratoxin A (OTA) is the most important mycotoxin produced by several microfungi of the *Penicillium* and *Aspergillus* genera (Abarca et al. 1994, Abarca et al. 2003, Battilani et al. 2003, Bau et al. 2005, Pitt and Hocking 1997). OTA is both acutely and chronically toxic. It has been found to possess carcinogenic, nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic properties. OTA has been linked to nephropathy in humans (Dirheimer 1996, Schlatter et al. 1996, Petzinger and Weidenbach 2002). Investigations of the frequency and levels of occurrence of OTA in foodstuffs, human blood samples and human urine indicate that foodstuffs are frequently contaminated (Gilbert et al. 2001, Jonsyn-Ellis 2001, Mac Donald et al. 2001, Malíř et al. 2001, Malíř et al. 2006, Pascale and Visconti 2001, Ruprich and Ostrý 1993, Zimmerli and Dick 1995). It occurs naturally in a variety of plant products, such as cereals, coffee beans, cocoa beans, and dried fruit, all over the world. It has been detected in products such as cereal products, coffee, spices, beer and grape juice, grape must and wine, but also in products of animal origin, namely pig kidneys (Buttinger et al. 2004, Cholmakov-Bodechtel et al. 2000, Gauchi and Leblanc 2002, Zimmerli and Dick 1995).

A number of surveys has been conducted to monitor OTA levels in wine and must. Most of them have been concentrated on quantification of OTA levels in wine, without providing information on the origin of the contamination (Bellí et al. 2002, Festas et al. 2000, Filali et al. 2001, Ng et al. 2004, Otteneder and Majerus 2000, Pietri 2001, Rosa et al. 2004, Tateo et al. 1999, Zimmerli and Dick 1996).

„Wine-ochra risk“, a 4-year multidisciplinary project, was initiated within the 5th Framework Programme of the European Union in 2001. The project aimed at assessing the risk of OTA in wine in Europe with the purpose of reducing toxin levels through an integrated management of production and processing. All wine-producing countries of the Mediterranean basin were involved (Italy, France,

Spain, Portugal, Greece and Israel). Trans-European populations of OTA producing microfungi in grapes were isolated, identified and characterised for OTA production. Species of *Aspergillus* section *Nigri* were dominant as potential OTA producing fungi in the vineyard; in particular, *A. carbonarius* seems to be confirmed as the target pathogen, primarily because most of its strains are able to produce large amounts of OTA (Abarca et al. 1994, Abarca et al. 2003, Battilani et al. 2003, Bau et al. 2005, Sage et al. 2004, Serra el al. 2003, Tjamos et al. 2004). Ochratoxin A was regulated firstly in wine, wine grape juice and must (2 µg/l) from harvest 2005 according to European Commission Regulation (EC) No. 123/2005. Ochratoxin A is currently regulated in wine, wine grape juice and must under European Commission Regulation (EC) No. 1881/2006.

Alternaria alternata is a frequently occurring species and probably the most important mycotoxin-producing species occur in cereals, sunflower seeds, oilseed rape, lentils, olives, various fruits etc. (Bottalico and Logrieco 1998, Delgado and Gomez-Cordoves 1998, Kosiak et al. 2004, Lau et al. 2003, Logrieco et al. 1990, Logrieco et al. 2003, Motta et al. 2001, Scott 2001, Solfrizzo et al. 2004). *A. alternata* produces a number of mycotoxins, including alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxins I, II, III (ATX-I, –II, –III), L-tenuazonic acid (TeA) and other less toxic metabolites. A large number of *Alternaria* metabolites has been reported to occur naturally in food commodities and to possess toxicological significance. This situation is similar to the *Fusarium* mycotoxins (trichothecenes, e.g. deoxynivalenol, T-2 toxin, and zearalenone) (Brandwagt et al. 2001, Ito et al. 2004, Liu et al. 1992, Wild and Hall 1997).

Contamination with AOH, AME, TeA, and, in some cases, ALT and ATX-I, is normally associated with fruits, vegetables and oilseeds visibly infected by *Alternaria* rot, including tomatoes, olives, mandarins, peppers, and apples. Occurrence of *Alternaria* spp. and their mycotoxins in oilseeds has been reported by a number of workers (e.g. discoloured pecan nuts, sunflower seed, sunflower seed meal and oil seed rape) (Bottalico and Logrieco 1998, Logrieco et al. 1990, Logrieco et al. 2003, Pitt and Hocking 1997). Mycotoxins produced by *Alternaria* spp. have also been reported in cereals such as sorghum, wheat, rye, diseased rice, but also in tobacco (Bottalico and Logrieco 1998, Scott 2001) and lentils (Ostrý et al. 2004). Newly AOH and AME have been detected in red and white wines and cranberry juice (Scott et al. 2006).

As a response to the „Wine-ochra risk“ project, a pilot study in the Czech Republic was prepared. The aim of this study was to monitor the mycobiota of wine grapes, occurrence of ochratoxigenic microfungi in wine grapes and occurrence of ochratoxin A and *Alternaria* mycotoxins in fresh grape juice, must and wine from domestic crops in the year 2004. The pilot study was designed to answer questions about these data for risk assessment of OTA in wine in the Czech Republic.

MATERIAL AND METHODS

Sample characterisation

Five vineyards with thirteen wine grape varieties (Tab. 1) were selected for the pilot study in the south of Moravia, the Znojmo wine region (Fig. 1). Its centre, the historical town of Znojmo, lies near the Austrian border and is not far from Vienna. One sample of wine grapes varieties was represented by three subsamples of wine grapes, which were sampled in the left, middle and right parts of the vineyard. In total thirteen samples of wine grapes were sampled to sterile plastic bags during harvesting at the beginning of October 2004 and transported to a mycological laboratory for immediate processing.

Fig. 1. Znojmo wine region in the Czech Republic. Numbers 1–5 indicate the 5 vineyards studied.

Tab. 1. Wine grape varieties used in the study.

Vineyard	Wine grape variety	Type of grape
Hostěradice	Saint Laurent	Red
	Rhine Riesling	White
	Rulander (Pinot Blanc)	White
	Sauvignon Blanc	White
Miroslav	Frankovka (Limberger)	Red
	Pálava	White
Podmolí	Rhine Riesling	White
	Rulander (Pinot Blanc)	White
	Rulander (Pinot Gris)	White
	Welschriesling	White
	Rulander (Pinot Noir)	Red
Dobšice	Sauvignon Blanc	White
Horní Dunajovice	Saint Laurent	Red

Fresh grape juice (thirteen samples) was prepared from some wine grapes in laboratory conditions by wringing. Must (thirteen samples) and young wine (thirteen samples) of each wine grape variety were acquired and sampled in a winery.

Mycological examination

Five wine grape berries per bunch were randomly selected, plated onto Dichloran Rose Bengal Chloramphenicol (DRBC, HiMedia, Mumbai, India) agar, and incubated for 5–7 days at 25 °C. *Penicillium* spp. and *Aspergillus* spp. were picked off onto Czapek Yeast Extract (CYA, HiMedia, Mumbai, India) agar, *Alternaria*, *Cladosporium*, *Rhizopus* and *Epicoccum* onto Potato Dextrose (PDA, HiMedia, Mumbai, India) agar to obtain pure cultures and identify further species, and *Alternaria* onto Dichloran Malt Extract agar (DCMA, HiMedia, Mumbai, India) for a description of morphological criteria. The identification of isolated strains was carried out according to special mycological literature (Anderson and Thrane 1996, Gravesen et al. 1994, Pitt and Hocking 1997, Powell et al. 1994, Samson and Pitt 2000, Samson et al. 1988, Samson et al. 1992, Smith 1994, Simmons 1986, 1999).

Ochratoxin A determination

The OTA levels were determined in fresh juice pressed from wine grapes, must and wine (Škarková et al. 2005). A sensitive HPTLC (high-performance thin-layer chromatography) method for quantification of ochratoxin A in fresh grape juice, must and wine was developed for the requirement of this study. Fresh grape juice, must and wine samples were filtrated, centrifuged, purified in commercial immunoaffinity columns (VICAM, Watertown, USA) and analysed by instrumental HPTLC on silica gel layers with fluorescence detection. Chromatography was performed on 20 × 10 cm large silica gel 60 HPTLC plates (Merck No. 5641, Darmstadt, Germany). Diluted calibration standard – different volumes for calibration (1, 5, and 10 µl corresponding to 100, 500, and 1000 pg OTA) and purified samples (20 µl) were applied to the plates with the spot technique using a Camag model III automatic TLC sampler (Camag, Muttenz, Switzerland) 1 cm from the edge of the plate. The distance between the samples was 6 mm. The plates were developed with benzene-methanol-acetic acid, 18:1:1 (v/v), in the dark, in a saturated 20 ×10 cm large vertical development chamber. After drying in a flow of cold air, OTA was measured with fluorescence densitometry by means of a Camag TLC Scanner II with a mercury lamp and K 400 secondary filter. The excitation wavelength was 333 nm, the emission wavelength 420 nm, and the SENS and SPAN parameters were 195 and 60, respectively. The R_f of OTA under these conditions was 0.24. The detection limit was 4 ng/l, the limit of quantification was 8 ng/l of fresh grape juice, must and wine. Average recovery of OTA from spiked samples was 95 % in a range of 25–100 ng/l wine. The average relative standard deviation of repeatability (RSDr) was 6.7 %. Validation of the method was performed according to the principles used for HPTLC methods (ICH Guideline for planar chromatography) (ICH Guideline 1996). The method is accredited according to CSN EN ISO/IEC 17025.

Determination of *Alternaria* mycotoxins

An HPTLC method for quantification of *Alternaria* mycotoxins [alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), and L-tenuazonic acid (TeA)] in fresh grape juice, must and wine was used. The combination of solid phase extraction (SPE) and HPTLC increased the selectivity and sensitivity of the method used (Škarková et al. 2001, Ostrý et al. 2005). Validation of the methods was performed according to the principles of ICH Guideline for planar chromatography (ICH Guideline 1996). The limit of quantification for AOH, AME and ALT was 1.5 µg/l and for TeA 7.5 µg/l of fresh grape juice, must and wine. The method is prepared for accreditation according to CSN EN ISO/IEC 17025.

RESULTS

The results of microfungi detection in wine grape samples are presented in Tab. 2.

Tab. 2. Wine grape mycobiota.

Vineyard	**Grape variety**	**Mycological profile**
Hostěradice	Saint Laurent	*Alternaria alternata* biotype 1 *Alternaria alternata* biotype 2 *Cladosporium herbarum* *Rhizopus nigricans*
	Rhine Riesling	*Alternaria alternata* biotype 2 *Cladosporium herbarum* *Rhizopus nigricans*
	Rulander (Pinot Blanc)	*Alternaria alternata* biotype 1 *Alternaria alternata* biotype 2 *Cladosporium herbarum*
	Sauvignon Blanc	*Alternaria alternata* biotype 1 *Alternaria alternata* biotype 2 *Cladosporium herbarum*
Miroslav	Frankovka (Limberger)	*Alternaria alternata* biotype 2
	Pálava	*Alternaria alternata* biotype 2 *Penicillium aurantiogriseum*
Podmolí	Rhine Riesling	*Alternaria alternata* biotype 1 *Alternaria alternata* biotype 2 *Penicillium expansum*
	Rulander (Pinot Blanc)	*Cladosporium cladosporioides* *Penicillium expansum*
	Rulander (Pinot Gris)	*Alternaria alternata* biotype 1 *Alternaria alternata* biotype 2 *Cladosporium cladosporioides*
	Welschriesling	*Alternaria alternata* biotype 1 *Alternaria alternata* biotype 2 *Penicillium spinulosum*
	Rulander (Pinot Noir)	*Alternaria alternata* biotype 1 *Alternaria alternata* biotype 2 *Cladosporium cladosporioides*
Dobšice	Sauvignon Blanc	*Alternaria alternata* biotype 1 *Alternaria alternata* biotype 2 *Cladosporium herbarum*
Horní Dunajovice	Saint Laurent	*Alternaria alternata* biotype 1 *Penicillium aurantiogriseum* *Rhizopus nigricans*

Alternaria alternata, *Cladosporium herbarum*, *C. cladosporioides*, *Penicillium expansum*, *P. aurantiogriseum*, *P. spinulosum* and *Rhizopus nigricans* were isolated from the grape samples. The *Alternaria alternata* strains were divided to biotypes 1 and 2 according to the descriptions of morphological criteria (growth on Potato Dextrose agar – PDA) and Dichloran Malt Extract agar (DCMA) and microscopy of conidia (conidial length). The conidial length of *Alternaria alternata* biotype 1 was greater than that of biotype 2. The identification of *Alternaria alternata* was independently confirmed according to Simmons (1986, 1999) and Anderson and Thrane (1996). We would like to test toxigenity and *Alternaria* mycotoxin production in isolated biotypes of *A. alternata* in further research work.

Frequencies of microfungi in grapes are given in Tab. 3.

Tab. 3. Frequencies of microfungi in grapes.

Microfungi	**Frequency (%) (strains/total samples)**
Alternaria alternata biotype 1	69 (9/13)
Alternaria alternata biotype 2	85 (11/13)
Cladosporium herbarum	39 (5/13)
Cladosporium cladosporioides	23 (3/13)
Rhizopus nigricans	23 (3/13)
Penicillium expansum	15 (2/13)
Penicillium aurantiogriseum	15 (2/13)
Penicillium spinulosum	8 (1/13)

Ochratoxigenic microfungi (*Aspergillus carbonarius*, other species of *Aspergillus* section *Nigri*, *Aspergillus ochraceus*, *Penicillium verrucosum* and *Penicillium nordicum*) were not found in the grape samples.

Occurrence of OTA and *Alternaria* mycotoxins in fresh grape juice, must and wine was not proven.

DISCUSSION

The results of the pilot study indicate that OTA incidence and concentration is lower in wine products (white, rosé and red) from northern regions in Europe and higher in wine products from more southern (warmer) regions (Mediterranean) (Bau et al. 2005, Bellí et al. 2002, Festas et al. 2000, Otteneder and Majerus 2000, Pietri 2001). In a review by Otteneder and Majerus (2000) the results of more than 450 samples taken from the literature and 400 samples tested by themselves were analysed to describe the situation of OTA contamination of wine rather extensively. The wines from the southern and northern regions were compared according to these data. Here, the red wine samples from the northern cultivation area showed a contamination of 12 % in contrast to those from the southern area, which showed a contamination of about 95 %. The results of more than two thousand wine samples taken from the literature were treated in an extensive description of the present situation of OTA contamination of wine in the study by Ballí et al. (2002). When comparing wines from northern and southern regions, the latter showed a higher contamination than those from the northern area. It has been suggested that OTA accumulation could be due to fungi belonging to the genus *Aspergillus* in wines from southern European countries because the crops are exposed to higher temperatures, which favour growth of OTA-producing *Aspergillus* spp. over *Penicillium* spp. This latter point has been interpreted as being due to an absence of the ochratoxigenic microfungi *Aspergillus carbonarius* and other species of section *Nigri* in wine grapes from northern regions. A similar preliminary survey titled „Ochratoxin contamination of wine and grape juices" was carried out in Hungary. Ochratoxigenic microfungi belonging to *Aspergillus carbonarius* and other species of section *Nigri* were not isolated in vineyards, only *Penicillium* spp. were. In another study, OTA-producing microfungi of *Aspergillus* section *Nigri* were found only in vineyards in southern parts of Hungary (Varga, personal communication 2005).

Comparison of our results with results from Spain shows a higher occurrence of *Aspergillus* spp., while the percentage of berries contaminated by non-ochratoxin A (OTA) producing species such as *Alternaria* spp. and *Cladosporium* spp. is lower. *Penicillium verrucosum* and *P. nordicum*, the only confirmed *Penicillium* species that are able to produce OTA, were not isolated (Bau et al. 2005).

Alternaria, *Aspergillus* and *Penicillium* were identified at species level in different wine grape varieties from Mendoza, Argentina. *Alternaria* spp. was the most frequent (80 % of the samples) followed by *Aspergillus* spp. (70 %), including *A. niger* var. *niger* and *A. flavus*, among others. Among *Penicillium* spp., *P. chrysogenum* was the most frequent species (Magnoli et al. 2003).

Fifty wine grapes samples from the Malbec and Chardonnay varieties were collected in Argentina and Brazil during the 1997–1998 harvest in the study by Da et al. (2002). The aim of the study was to identify the natural mycobiota in wine grapes. Yeasts were a major component of the fungal population, and the most frequent mycobiota isolated were: *Aspergillus* (including *A. niger*), *Penicillium* spp. and *Botrytis* spp. Other genera identified (in decreasing order) were *Phytophthora*, *Moniliella*, *Alternaria* and *Cladosporium*. From grapes, the mean total count of microfungi ranged from 1.3×10^4 to 5.4×10^6 CFU/g (CFU – colony forming unit). The results indicate that similar mycobiota were isolated from Argentinian and Brazilian wine grapes and that ochratoxin A could have been produced in this substrate.

A number of mycological surveys from Spain, France, Portugal, Italy and Greece have been conducted to monitor in particular the ochratoxigenic microfungi *Aspergillus carbonarius* and other species of section *Nigri* in wine grapes. Members of *Aspergillus* belonging to section *Nigri* resulted the main responsible for the ochratoxin A accumulation in grapes and wine, particularly in Southern Europe. The main responsible of OTA presence in grapes is *Aspergillus carbonarius* (99 % of strains produce OTA) (Abarca et al. 2003, Battilani et al. 2003, Bau et al. 2005, Sage et al. 2004, Serra el al. 2003, Tjamos et al. 2004).

In our research, ochratoxigenic microfungi (*Aspergillus carbonarius*, other species of *Aspergillus* section *Nigri*, *Aspergillus ochraceus*) were not found in grape samples and Czech wine is probably not an important contributor to the dietary intake of OTA. This opinion has to be confirmed by further epidemiological study.

The main contributors to the dietary intake of OTA in the Czech Republic are cereals and cereal products, meat products, dried wine fruit (e. g. raisins, sultanas), coffee, beer, imported wine, spices and legumes (Ostrý et al. 2001, Ostrý et al. 2002).

The risk of acute toxic effects of OTA was, according to the amount of OTA found in foodstuffs and in human blood serum in the Czech Republic, usually considered to be minimal (Ruprich and Ostrý 1993, Malíř et al. 2006). The risk of late toxic effects (particularly carcinogenic risk) after ingestion of very low single or repeated OTA doses from foodstuffs is however very important and similar in most European countries (EFSA 2006).

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