

Mycoflora of fresh chokeberry (*Aronia melanocarpa*) and ochratoxin-producing ability of *Penicillium* isolates

R. Genchev, G. Angelova, I. Laskova, V. Gotcheva and A. Angelov*

University of Food Technologies, Department of Biotechnology, 26 Maritza, 4002 Plovdiv, Bulgaria; angelov@uft-bio.com

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RESEARCH ARTICLE

Abstract

Black chokeberry (*Aronia melanocarpa*) is a plant with unique health-promoting properties due to the high content of vitamins and polyphenols in its fruits. Despite their high resistance to spoilage, chokeberry fruits may get contaminated with mycotoxin-producing fungi. In the present study, samples of chokeberry fruits were collected from seven different sites in Bulgaria and their fungal profile was investigated. The most common moulds in fresh chokeberry were *Penicillium* sp. (77.5% out of all isolates), followed by *Alternaria* sp. (14.5%) and *Botrytis* sp. (8%). Eighteen randomly chosen *Penicillium* isolates were further screened by enzyme-linked immuno sorbent assay (ELISA) for their ability to produce ochratoxin A (OTA) in liquid yeast extract sucrose medium. Four of the tested isolates (no. 11 from the region of Panagurishte, no. 2 and 10 from the region of Plovdiv and no. 47 from the region of Koprivshitsa) showed ability to produce OTA in concentrations 15.42, 14.62, 19.75 and 20.97 ng/ml, respectively (ELISA screening). High-performance liquid chromatography with post column fluorescence derivatisation analysis was also performed for all OTA-positive isolates. The maximum estimated OTA concentration was 27.47 ng/ml for isolate 47 from the region of Koprivshitsa.

Keywords: ELISA, HPLC, moulds, mycotoxins

1. Introduction

The genus *Aronia* (*Rosaceae* family, *Maloideae* subfamily) includes two species of native North American shrubs: *Aronia melanocarpa* (Michx.) Ell. (black chokeberry) and *Aronia arbutifolia* (L.) Pers. (red chokeberry) (Kokotkiewicz *et al.*, 2010). Black chokeberry is a plant grown in several European and Asian countries, with unique health-promoting properties due to the high content of vitamins and polyphenols in its fruits, and used for the production of various functional foods (Kulling and Rawel, 2008; Trinklein, 2010).

The high content of sugars and other nutrients in the fruits, and the favourable pH and water activity are preconditions for fungal growth. Fruits get contaminated with fungi during cultivation, harvesting, handling in the field, transportation, post-harvest storage and at the market (Tournas and Katsoudas, 2005). During the past 2-3

decades, a variety of fruits and vegetables that form part of our daily diet have been added to the list of products exposed to mycotoxin contamination. Factors affecting mycotoxin production by fungi include fruit or vegetable type and cultivar, geographical location, climate, pre-harvest treatments, harvest methods, post-harvest treatments and storage conditions (Barkai-Golan and Paster, 2008a). Since *Aspergillus*, *Penicillium* and *Alternaria* species are the major mycotoxigenic fungi that attack harvested fruits and vegetables, a variety of mycotoxins produced by these fungal genera during pathogenesis may contaminate fruit and vegetable tissues. The mycotoxins most commonly associated with fruits and vegetables and their products are ochratoxin A (OTA), patulin, aflatoxins and alternaria toxins. Some of them are very stable during processing, therefore, although consumers will reject a visibly rotten fruit, processed fruit products may still form a significant source of these toxins (Barkai-Golan and Paster, 2008a,b; Sekar *et al.*, 2008). It has been estimated that 25% of all crops

worldwide are contaminated with moulds that produce mycotoxins. Consumption of foods with such origin results in accumulation of mycotoxins in the animal and human systems and possible cause of mycotoxicoses, which are manifested through carcinogenic, hepatotoxic, mutagenic, teratogenic, cytotoxic, immunosuppressive, estrogenic and anabolic effects (Barkai-Golan and Paster, 2008a,b; Fernández-Cruz *et al.*, 2010; Galvano *et al.*, 2005).

Aspergillus species are widespread in nature. They are saprophytic on a wide range of substrates, including foods and feeds, and several species are among the typical pathogens of harvested fruits and vegetables (Barkai-Golan, 2001). The major mycotoxins associated with *Aspergillus* species in fruits and vegetables are aflatoxins, produced mainly by strains of *Aspergillus flavus* and *Aspergillus parasiticus*, and OTA produced by *Aspergillus carbonarius* and other ochratoxigenic aspergilli (Barkai-Golan, 2001; Doster *et al.*, 1996; Roze *et al.*, 2004). *Penicillium* species are among the most common airborne fungi and are frequently associated with spoilage of foods and feeds. Several *Penicillium* species are highly prevalent agents of postharvest diseases, and they attack a wide range of fruits and vegetables. Some of them are capable of producing several potent mycotoxins in harvested fruits and vegetables, including patulin, OTA, citrinin, penicillic acid, cyclopiazonic acid, chaetoglobosin, communesin B, roquefortine C and expansolides (Andersen *et al.*, 2004; Barkai-Golan and Paster, 2008b). Contamination of food with OTA in warm and tropical climates is caused by *Aspergillus* species, whereas its occurrence in food in cool climates is associated with *Penicillium* species (Abrunhosa *et al.*, 2001; Drush and Ragab, 2003; Khoury and Atoui, 2010; Pitt, 1987; Torielli *et al.*, 2006). OTA is one of the most important mycotoxins, with a number of negative effects on animals such as nephrotoxic, hepatotoxic, neurotoxic, immunotoxic, teratogenic and carcinogenic effect. Carcinogenicity of OTA has been evaluated by IARC (1993) and it has been classified as a possible group 2B human carcinogen (Al-Anati and Petzinger, 2006; Khoury and Atoui, 2010; Pardo *et al.*, 2006). OTA has been associated with the Balkan endemic nephropathy and the development of urinary tract tumors (Khoury and Atoui, 2010; Pfohl-Leszkowicz *et al.*, 2002). Various *Alternaria* species are plant pathogens that cause damage in the field, and several are postharvest pathogens of a wide range of fruits and vegetables. *Alternaria* mycotoxins, including alternariol, alternariol monomethyl ether, tenuazonic acid, altenuene, and altertoxins, have been detected in a variety of fruits and vegetables, such as tomatoes, peppers, melons, citrus fruits, apples and olives (Jackson and Al-Taher, 2008). Since *Alternaria* mycotoxins are produced naturally in a variety of infected fruits and vegetables, and since infection may occur even under refrigeration, these mycotoxins may be considered as toxic contaminants in our everyday food (Barkai-Golan and Paster, 2008b).

With regards to fungal microflora and mycotoxin contaminations, the most studied fruits are different types of berries (blueberry, blackberry, strawberry) and grapes, which differ significantly as matrices for fungal growth compared to chokeberry. Although black chokeberry fruits are highly valued for their health effects and increasingly used for the production of numerous food products, we could not find publications on the mycology of black chokeberry and the potential risk of mycotoxin contaminations of these fruits and their products. Therefore, the aim of the present work was to explore the fungal profiles of black chokeberry fruits from different regions in Bulgaria and to estimate the potential ability of the isolated fungi to produce OTA.

2. Materials and methods

Samples

Ten samples of black chokeberry (harvested in 2010) were collected from seven chokeberry fields in Bulgaria, located in the regions of Plovdiv, Kazanlak, Suhozem, Koprivshitsa, Klisura, Panaguirishte and Karlovo (Figure 1). Samples were homogenised and stored at 4-6 °C.

Culture media

Three culture media were used for the mycological analysis of the chokeberry samples:

- Dichloran rose bengal chloramphenicol agar (DRBC; Merck Millipore, Darmstadt, Germany) for isolation of mycoflora from fruits.
- Malt extract agar (MEA) for identification of the isolated fungi. MEA was prepared from 20 g/l malt extract (Bacto[®]; Becton and Dickinson, Sparks, MD, USA), 1g/l yeast extract (Difco[®]; Difco, Lawrence, KS, USA), and 20 g/l agar and distilled water to 1l, with pH adjusted to 5.8-6.0 with 20% NaOH. The medium was autoclaved for 15 min at 121 °C.
- Yeast extract sucrose (YES) broth used as a standard medium to study the OTA production ability of *Penicillium*. The medium was prepared from 20 g yeast extract, 150 g sucrose, 1 g MgSO₄·4H₂O and distilled water to 1l, with pH adjusted to 6.5 with 20% NaOH. The medium was sterilised at 121 °C for 15 min.

Reagents and standards

Pure standards of OTA were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dichloromethane and chloroform (p.a. reagents; Sigma Aldrich Co.) were used for OTA extraction. Solvents used were high-performance liquid chromatography (HPLC)-grade methanol and acetonitrile (Merck Millipore).

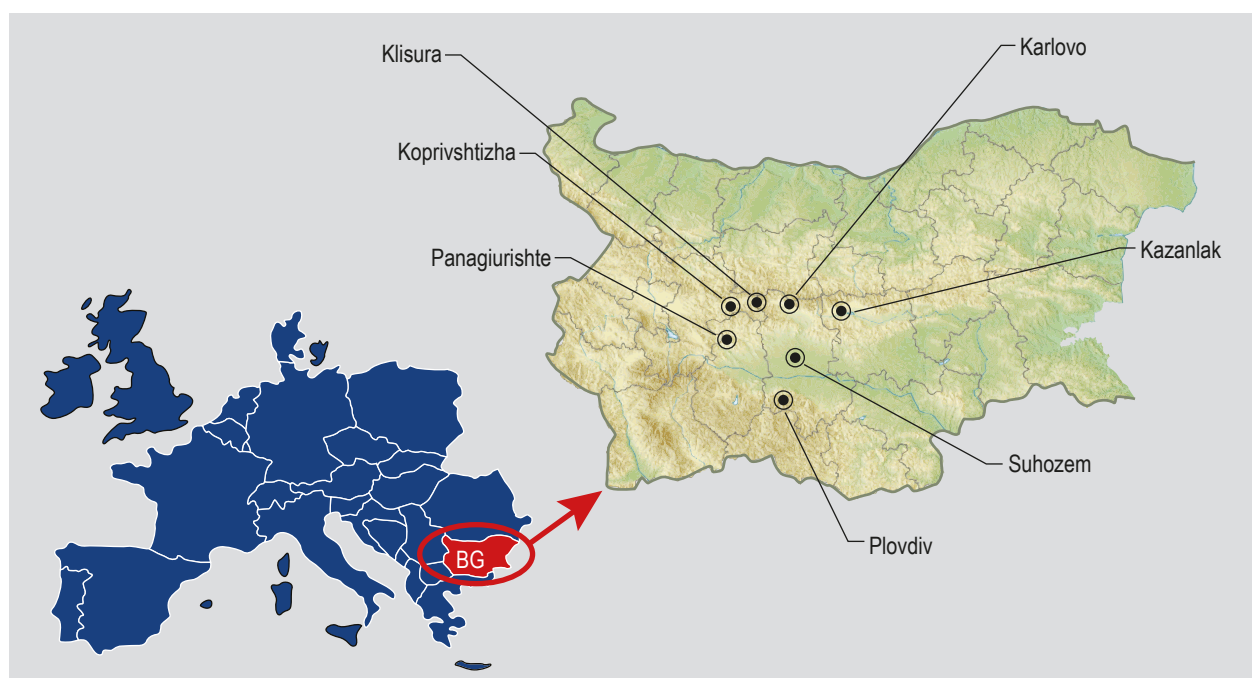


Figure 1. Sites of chokeberry fields in Bulgaria (BG).

Isolation and identification of moulds

Fungal cultures were isolated from berries by direct plating after surface disinfection with 3.5% (v/v) sodium hypochloride for 2 min and duplicate washing with sterile distilled water (Barkai-Golan and Paster, 2008a; Pitt and Hocking, 2009; Serra *et al.*, 2005). The disinfected berries were cut under aseptic conditions and plated onto DRBC. All plates were incubated at 25 °C for 7 days in dark conditions.

Pure fungal cultures from black chokeberry were obtained by the streak plate method on MEA. The isolates were identified to the genus level by macroscopic and microscopic criteria in accordance to appropriate keys (Pitt and Hocking, 2009).

The isolation frequency (F_r ; %) and the relative density (RD; %) of the genera were calculated according to Castillio *et al.* (2004) (Equations 1 and 2):

$$Fr = \left(\frac{ns}{N} \right) \times 100 \quad (1)$$

$$RD = \left(\frac{ni}{Ni} \right) \times 100 \quad (2)$$

Where ns was the number of samples where a genus occurred, N the total number of samples, ni the number of isolates of a genus, and Ni is the total number of fungal isolates obtained.

Ochratoxigenic capacity of the isolates

Seventeen *Penicillium* isolates were tested for OTA-producing ability. The strains were cultivated in 100 ml YES medium in 500 ml Erlenmeyer flasks inoculated with 0.2 ml of dense (4.3×10^9 - 1.5×10^{10} spores per ml) conidial suspension and incubated at 25 °C for 14 days at stationary conditions.

Enzyme-linked immuno sorbent assay analysis

After cultivation, filtrates from the culture media were collected. Extraction and purification of OTA were carried out by the method of Botton *et al.* (2008). The samples were analysed using the RIDASCREEN®FAST Ochratoxin A kit for quantitative enzyme-linked immuno sorbent assay (ELISA) (R-Biopharm AG, Darmstadt, Germany), following the manufacturer's instructions. OTA concentration was calculated comparing the absorptions of samples and standards by the RIDASoft WIN 1.55 software (R-Biopharm AG). Absorption was determined at 450 nm with microplate reader PlatePCread (BGT BioGenTechnologies GmbH, Steinfurt, Germany). Calibration solutions (0-40 ng/ml) were provided with the commercial kit. The limit of detection (LOD) of the method was 5 ng/ml. Each sample was analysed in duplicate.

HPLC-FLD analysis

HPLC analysis of OTA was carried out according to the methodology of Teren *et al.* (1996) with some modifications. Ten ml of filtrate were mixed with 10 ml chlorophorm in a flask and shaken for 15 min. After separating the phases, the lower organic phase containing OTA was collected. The chlorophorm phase was evaporated to dryness with vacuum rotary evaporator and dissolved in 2 ml HPLC-grade methanol. Extracts were analysed using a Thermo Surveyor HPLC-FLD (fluorescence derivatisation) system composed of a quaternary pump Surveyor LC pump plus, Surveyor Autosampler Plus Lite, fluorescence detector Surveyor FL Plus and data acquisition system ChromQuest 5.0 (Thermo Fisher Scientific, San Jose, CA, USA). Separation of compounds was performed on a C18 column (4.6×250 mm, 5 µm particle size; Nucleodur, Machenrey-Nagel, Germany) with acetonitrile, methanol and acetic acid (51:47:2, v/v/v) as mobile phase at a flow rate of 1.0 ml/min at isocratic conditions. The fluorescence detector was set at 333 nm excitation and 460 nm emission wavelengths. Calibration solutions were prepared in HPLC mobile phase. Calibration curves were prepared in the range of 5 to 80 ng/ml. Samples were considered positive if they yielded a peak at retention time identical to that of the OTA standard. The LOD of the protocol was 2.5 ng/ml.

3. Results and discussion

Isolation and identification of fungi

Fungal flora was detected in all tested samples using direct plating on a selective medium (DRBC) with preliminary surface disinfection of the berries. The presence of dichloran in the selective medium limited the fast-growing moulds such as *Mucor* and *Rhizopus*, whereas chloramphenicol inhibited the bacterial growth. Surface treatment removed the sporadic microbial contamination and allowed the isolation only of moulds strongly attached to the berry skins. The fungal strains grew on the edge of the berries first and further affected the entire surface of the berries (Figure 2).

Direct plating resulted in the isolation of 124 mould cultures. Identification to the genus level was based on the macroscopic and microscopic features of the fungal isolates, taking into consideration their cultural characteristics, morphology of conidial apparatus and morphology of conidia (Figure 3).

Ninety-six isolates had cultural characteristics and conidial morphology typical for the *Penicillium* genus, 18 were identified as *Alternaria* spp., and 10 belonged to the *Botrytis* genus. No isolates of the *Aspergillus* genus were observed. The distribution of the most common mould genera found on black chokeberry from different regions in Bulgaria is presented in Table 1.

The most frequently isolated moulds were *Penicillium*, *Alternaria* and *Botrytis*. Frequency of distribution (Fr) of these fungi was 100, 90 and 40%, and the density of distribution (RD) for each genus was 77.4, 14.5 and 8%, respectively. Moulds of the genus *Penicillium* were identified as the most common cause of chokeberry spoilage (77.4% of all isolates). *Penicillium* spp. has dominant presence in all samples from different sites (RD>60%), which is most significant in the samples from Karlovo (RD=95%). The site of Suhozem was the only one where *Penicillium* isolates were of equal presence with those of the genera *Alternaria* and *Botrytis* (RD=33%). The dominance of *Penicillium* is probably due to the fact that *Penicillium* species are among the most common airborne fungi that attack a wide range of fruits and vegetables. The *Penicillium* fungus can also penetrate the fruit through damaged spots of the skin. Numerous studies have shown that species from the *Aspergillus* and *Penicillium* genera form the predominant flora of most berries (Battilani *et al.*, 2003; Sage *et al.*, 2004; Serra *et al.*, 2005; Stefanaki *et al.*, 2003; Varga *et al.*, 2007). *Penicillium* spp. is the major causal agent of the blue mould rot of apples, pears, plums, peaches, apricots, cherries, blackberries, melons and strawberries (Snowdon, 1990) and it is commonly found on vegetables such as onions, garlic and cabbages (Lugauskas *et al.*, 2006). The absence of *Aspergillus* spp. in the chokeberry samples analysed could be attributed to the fact that Bulgaria is located in a temperate climatic region, while *Aspergillus* spp. tend to predominate in warmer climates. Our results agree with the findings of other authors that *Penicillium* spp. are more abundant in temperate climatic regions, whereas *Aspergillus* are the predominant isolated genus in warmer and dry climate (Fernández-Cruz *et al.*, 2010; Khoury and Atoui, 2010; Magan and Olsen, 2004; Pitt, 1987).



Figure 2. Mould growth on fresh black chokeberries on dichloran rose bengal chloramphenicol agar.

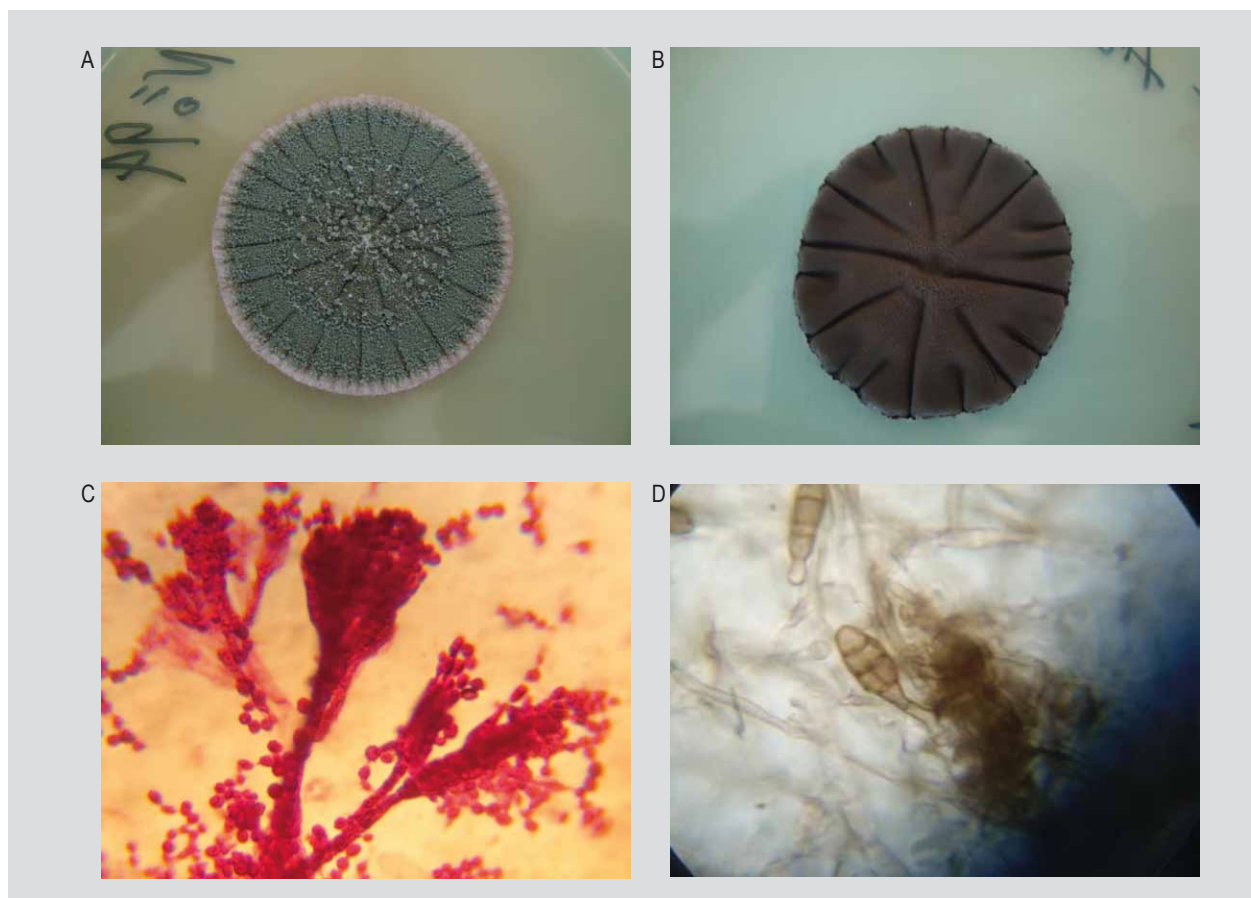


Figure 3. Colonies of (A) *Penicillium* spp. and (B) *Alternaria* spp. on malt extract agar; (C) morphology of conidia of *Penicillium* spp. and (D) *Alternaria* spp. (magnification 100×).

Table 1. Distribution of fungal genera found on *Aronia melanocarpa* fruits from various regions in Bulgaria.

Geographic region	No. of isolates	Fungal genera					
		<i>Penicillium</i>		<i>Alternaria</i>		<i>Botrytis</i>	
		No. of isolates	RD (%)	No. of isolates	RD (%)	No. of isolates	RD (%)
Koprivshitsa	9	8	89	1	11	-	-
Suhozem	9	3	33	3	33	3	33
Klisura	6	4	67	2	33	-	-
Plovdiv	43	33	77	7	16	3	7
Panaguirishte	26	19	73	4	15	3	12
Karlovo	22	21	95	1	5	-	-
Kazanlak	9	8	89	-	-	1	11
General count	124	96	77.4	18	14.5	10	8
		$F_r=100\%$		$Fr=90\%$		$Fr=40\%$	

F_r = frequency of distribution of the mould genera; RD = density of distribution of different mould genera.

Alternaria spp. was found in nine of the tested samples (Fr=90%). Fungi of this genus represented 14.5% of all isolates and were most common for the samples from Klisura (RD=33%) and Suhozem (RD=33%), followed by the ones from Plovdiv, Panagurishte and Koprivshitsa regions. The comparatively high *Alternaria* contamination of chokeberries could be explained by the lack of faster-growing moulds and, therefore, competition and prevalence of the latter organisms was not possible. *Alternaria* is one of the common pathogens of pome and stone fruits, grapes, blueberries, raspberries, persimmons, mangoes, and other fruits (Barkai-Golan and Paster, 2008b). In a study of Tournas and Katsoudas (2005), *Alternaria* was found in 46% of blueberry and 8% of strawberry samples. The presence of *Alternaria* in chokeberry is of concern since these fungi can grow and produce toxins at refrigeration temperatures (Barkai-Golan and Paster 2008b; Kralova *et al.*, 2006).

The genus *Botrytis* was less common on the tested chokeberry samples. *Botrytis* strains represented 8% of all fungal isolates. This result differs significantly from the outcomes of a study of Tournas and Katsoudas (2005), who found *Botrytis cinerea* to be the most common spoiler of berries, contaminating 78% of blackberry, 55% of blueberry, 75% of raspberry and 77% of strawberry samples. The low *Botrytis* contamination level of the chokeberry samples in our study could be explained by the application of preliminary surface disinfection and the use of a selective medium that did not allow the fast growth of these fungi. Our results also differ from those of some other authors who found *B. cinerea*, followed by *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium* and *Rhizopus* to be the most common moulds in the mycoflora of fresh berries (Flaj *et al.*, 2009; Tournas and Katsoudas, 2005). The differences in the genus composition of the fungal cultures isolated from chokeberry could be attributed mainly to the climate variability. However, the geographic origin also has a strong influence on the fungal mycoflora. The limited

variety of fungi found on chokeberry might be due to the fact that these berries have a hard skin which is too difficult for most fungi to attach and penetrate in the pulp tissue.

In the present study, fungi of the *Penicillium* genus were found to be predominant in chokeberry fruits, representing a potential consumer risk since these fungi can produce OTA. Therefore, our next task was to evaluate the ability of the *Penicillium* isolates to produce OTA in liquid YES medium.

Ochratoxigenic capacity of the isolates

Preliminary TLC screening showed that 17 of the 96 *Penicillium* isolates were able to produce OTA (data not shown). Therefore, our further analyses were conducted for those 17 isolates. ELISA is considered a reliable and fast method for OTA detection and was applied to assess the OTA-producing capacity of *Penicillium* spp. Results showed that only 23.5% of the tested isolates were able to produce OTA in liquid YES medium (Figure 4), which confirms that the presence of fungal flora on berries is not necessarily associated with mycotoxins formation. It depends on the physical and chemical composition of the matrix, the growth conditions for the fungi and other environmental factors (Garcia *et al.*, 2009).

The isolates synthesizing OTA in levels detectable by the ELISA method originated from three chokeberry fields: Plovdiv (isolates 2 and 10), Koprivshitsa (isolate 47) and Panagiurishte (isolate 11). The isolate from Koprivshitsa gave the highest OTA concentration (20.97 µg/kg), followed by isolates 10 and 2 from Plovdiv (19.75 and 15.42 ng/ml, respectively), and Panagiurishte isolate (11-14.62 ng/ml). All samples with absorbance values corresponding to concentrations lower than the LOD of 5 ng/ml were not considered positive.

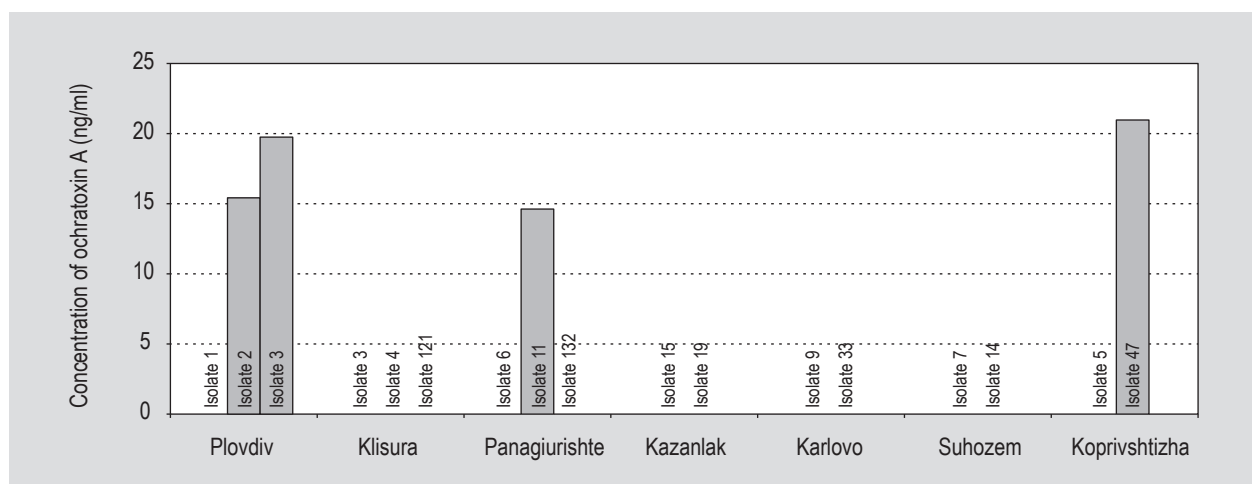


Figure 4. Ochratoxin A production by *Penicillium* isolates in liquid yeast extract sucrose broth medium.

The OTA-positive samples were further analysed by HPLC-FLD and results were compared with those from the ELISA test (Table 2). The HPLC analysis confirmed that OTA was present in all samples with positive results from the ELISA test. Maximum OTA concentration (27.47 ng/ml) was found in the extract of isolate 47 from the Koprivshtitsa site. This value is the only one that is comparable with the result obtained by ELISA (20.97 ng/ml). OTA concentration in the sample from isolate 11, was lower than the LOD of the HPLC-FLD method (2.5 ng/ml), and in samples 2 and 10 the values estimated by HPLC-FLD are much lower than those obtained by ELISA. For these isolates, results obtained by the two methods differed by more than 10 times, which shows a serious discrepancy in the accuracy of the methods.

The comparative analysis showed that the applied ELISA method significantly overestimated the OTA content compared to the HPLC-FLD method. This could probably be explained with the fact that although ELISA is based on the very sensitive and specific antigen-antibody reaction, antibodies often show cross-activity to compounds similar to the mycotoxins analysed, giving false positive results. Similar observations were reported by other authors as well (Flaj *et al.*, 2009; Fujii *et al.*, 2007; Varga and Kokakiewicz, 2006, 2005; Zheng *et al.*, 2006).

Despite the demonstrated capability of some of the *Penicillium* strains isolated from chokeberry fruits to produce OTA in liquid YES medium, the presence of these toxigenic fungi does not necessarily result in OTA occurrence in the berries and their products. OTA contamination depends on a number of endogenous factors such as pH, chemical composition and ripening state of the matrix, as well as exogenous factors (ambient temperature, relative humidity and competitive fungi). However, the presence of toxigenic fungi on black chokeberry fruits always holds a risk of potential mycotoxin contamination of the fruits and products from them.

Table 2. Ochratoxin A content analysis by ELISA and HPLC with post column fluorescence derivatisation.

Isolate no.	Concentration of ochratoxin A (ng/ml)	
	ELISA	HPLC
2	15.42	4.84
10	19.75	3.25
11	14.62	not defined
47	20.97	27.47

4. Conclusions

The present work explores the fungal flora and potential OTA producers on black chokeberry in Bulgaria. Such information is necessary in order to address the potential risk of mycotoxin contaminations related to these fruits and their products.

Our results show that the mycological profile of black chokeberry is quite different from the other berry fruits. The most common fungal genera found on fresh chokeberry collected from different regions in Bulgaria were *Penicillium*, *Alternaria* and *Botrytis*. *Penicillium* spp. gave the highest isolation frequency, constituting 77.5% of all isolated mould strains. *Penicillium* spp. presents a serious risk for human health related to the ability of these fungi to produce OTA under certain conditions. The ELISA screening on the ochratoxigenic potential of 17 *Penicillium* isolates revealed that 23.5% of them were able to produce OTA in a liquid culture medium. These results were confirmed by HPLC-FLD analysis, with a maximum OTA concentration of 27.47 ng/ml found in one of the isolates. However, the values for OTA concentrations obtained by the two methods differed significantly, proving the HPLC-FLD method as the one of preference for OTA analysis.

The presence of fungi able to produce OTA under *in vitro* conditions does not necessarily reflect the presence of OTA in chokeberry. Therefore, the levels and frequency of OTA occurrence in fruits and chokeberry products should be assessed. Also, further studies are needed to get an insight of the factors affecting the potential OTA synthesis by fungi in black chokeberry fruits and products.

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