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ARTICLE

Identification of potential mycotoxin producing fungi on agricultural products in Hungary and Serbia

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ABSTRACT One of the most important effects of climate change is the occurrence of thermotolerant mycotoxin producing fungi in countries with temperate climate, causing mycotoxin contamination of agricultural products. Indeed, a shift has recently been observed in the occurrence of aflatoxin producing fungi in Europe, with consequent aflatoxin contamination in agricultural commodities including maize and milk in several European countries including Serbia, Croatia, Slovenia, Romania and Ukraine. These observations led us to examine the occurrence of mycotoxin producing Aspergilli in agricultural products in Hungary and Serbia. The samples were collected from cereal fields in Hungary and North-Serbia (Vojvodina) after harvest in 2012. Surface-sterilized cereal seeds were placed on selective media and the isolated fungal strains were identified using morphological methods. The species identification of selected isolates was carried out using sequence-based methods. Several potentially aflatoxigenic *A. flavus* isolates were identified on maize. Further examinations of mycotoxin producing abilities of the isolates, and their occurrence in milk and milk-derived products are in progress.

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Mycotoxins are secondary metabolites of filamentous fungi, which are harmful to animals and humans, and are able to provoke various disease symptoms (Varga et al. 2009). Aflatoxins are the most thoroughly studied mycotoxins, which are produced by species assigned to the *Aspergillus* genus. They were discovered when the toxicity of animal feeds containing contaminated peanut meal led to the death of more than 100,000 turkeys from acute liver necrosis in the early sixties (Turkey-X disease; Blout 1961, Sargeant et al. 1961, van der Zijden et al. 1962). *Aspergillus flavus* was identified as the producing fungus, and aflatoxins were named after the toxic agent. Aflatoxins have both toxic and carcinogenic properties, posing serious threats to both animal and human health (Bennett and Klich 2003). Aflatoxin B_1 is the most toxic aflatoxin, being a potent genotoxic carcinogen in laboratory animals and there is a strong evidence for its liver carcinogenity in humans (Wild and Turner 2002). Comprehensive studies have shown that aflatoxin is a risk factor for human hepatocellular carcinoma, especially in Asia and sub-Saharan Africa (Groopman et al. 2005). Although lethality is an uncommon outcome of aflatoxicosis in humans, several deaths were attributed to that (Nyikal et al. 2004). Because of its toxicity, over 100 countries restrict the content of aflatoxins in the food and feed supplies (van Egmond et al. 2007).

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Aflatoxins are a group of structurally related difuranocoumarins that were named as aflatoxin B_1, B_2, G_1, and G_2 based on their fluorescence under UV light (blue or green) and their relative chromatographic mobilities during thin-layer chromatography. Aflatoxin B_1 is the most potent natural carcinogen known (Squire 1981, IARC 2012), and is usually the major aflatoxin produced by toxigenic strains. Aflatoxin M_1, a hydroxylated metabolite is found primarily in animal tissues and fluids (milk and urine) as a metabolic product of aflatoxin B_1 (Varga et al. 2009).

The most important producer, *A. flavus* is also an important pathogen of various cultivated plants including maize, cotton and peanut, and causes serious yield losses throughout the world. Since aflatoxin production is favoured by moisture and high temperature, *A. flavus* is able to produce aflatoxins in warmer, tropical and subtropical climates (Varga et al. 2009). Consequently, aflatoxin contamination of agricultural products in countries with temperate climate, including Central European countries was not treated as a serious health hazard. However, climate change associated with global warming seems to change the scenario. Recently, several papers have dealt with the effects of climate change on the appearance of aflatoxin producing fungi and aflatoxins in foods (Paterson and Lima 2010, Tirado et al. 2010). Based on these studies, aflatoxin producing fungi and consequently aflatoxins are expected to become more prevalent with climate change

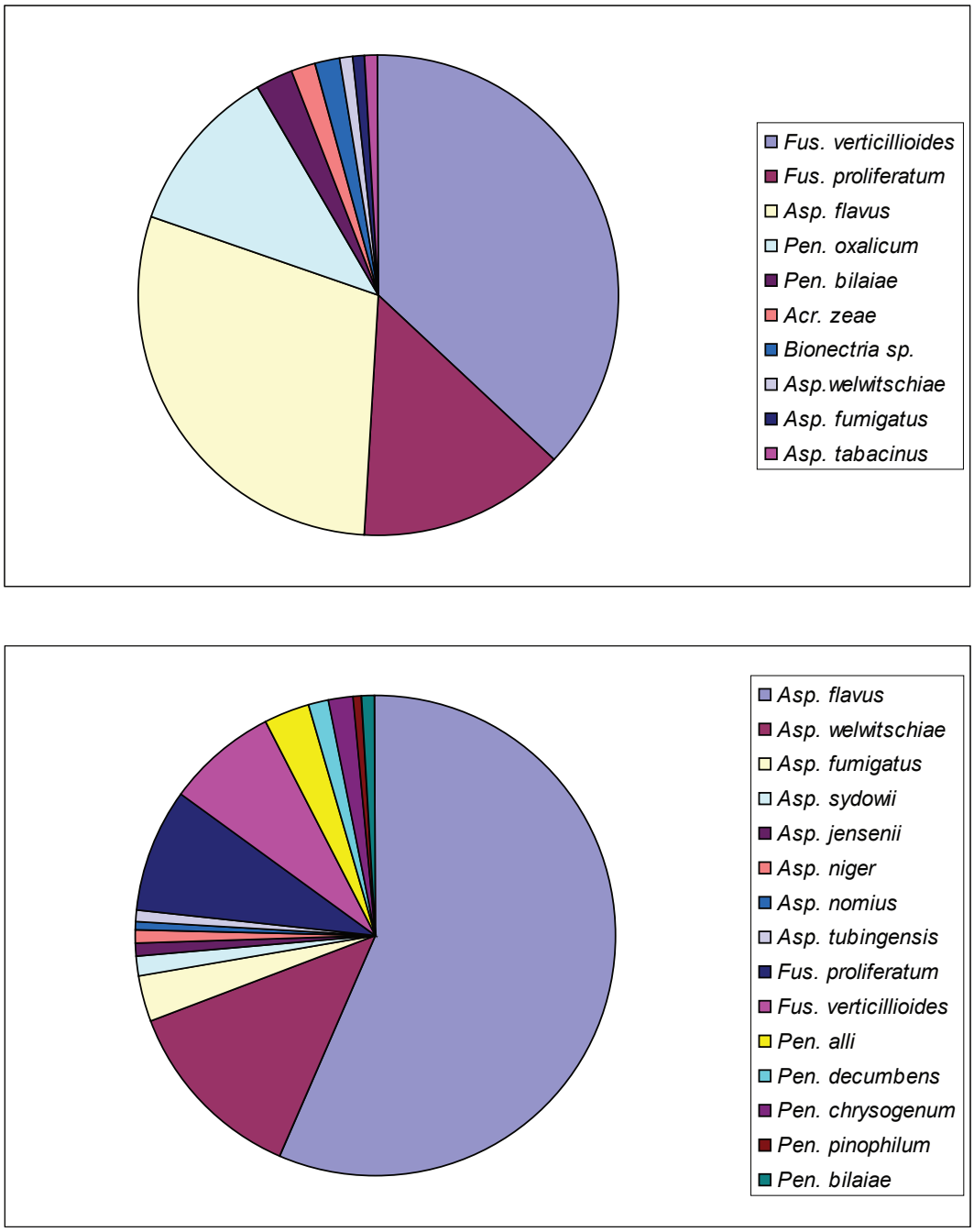

Figure 1. Distribution of potentially mycotoxin producing fungi in maize samples collected after harvest (top), and in samples stored in warehouses (bottom).

in countries with temperate climate. Indeed, several recent reports have indicated the occurrence of aflatoxin producing fungi and consequently aflatoxin contamination in agricultural commodities in several European countries that did not face with this problem before.

Regarding Hungary, Richard et al. (1992) examined the mycotoxin producing abilities of 22 isolates collected from various sources in Hungary, and none of the isolates were found to produce aflatoxins. Besides, Borbély et al. (2010) have examined mycotoxin levels in cereal samples and mixed feed samples collected in eastern Hungary, and detected aflatoxin B_1 levels above the EU limit in 4.8% of the samples. Additionally, more recently, Dobolyi et al. (2013) identified aflatoxin producing *A. flavus* from maize kernel collected in

various parts of Hungary. Furthermore, Tóth et al. (2013) have examined the occurrence of potential mycotoxigenic fungi on Hungarian maize kernels between 2010 and 2012. Several potentially mycotoxigenic *Aspergillus* isolates were identified on maize. In 2010 the weather was very rainy, while in 2011 and 2012 the weather conditions were more hot and dry. In 2010, a large number of *Penicillium* species occurred in the samples, possibly due to the colder weather conditions. In 2011 and 2012, Aspergilli were observed in larger quantities than previously, possibly due to the dry and hot weather conditions. These observations led us to examine the occurrence of potential aflatoxigenic species in maize kernels collected in Hungary and Serbia (Vojvodina).

Materials and Methods

Sample collection

The samples were collected from various maize growing regions of Hungary and Serbia in 2012. The samples were surface sterilized, and plated onto dichloran rose bengal (DRBC) media (King et al. 1979). Plates were incubated at 25 °C in darkness and monitored periodically for characteristic mycelium growing from the kernels. Outgrowing mycelia were purified and transferred to malt extract agar (MEA). Isolates were subcultured as single conidia on MEA, PDA (potato dextrose agar) and CYA (Czapek yeast agar) plates (Samson et al. 2004).

Genotypic studies

The cultures used for the molecular studies were grown on YPD (1% yeast extract, 1% peptone, 1% D-glucose) broth for 5 days, on 25 °C. DNA was extracted from the mycelia using the Masterpure™ yeast DNA purification kit (Epicentre) according to the instructions of the manufacturer. Part of the calmodulin gene was amplified and sequenced as described previously (Pildain et al. 2008). The primers used were: cmd5 (5 -CCGAGTACAAGGAGGCCTTC-3) and cmd6 (5 -CCGATAGAGGTCATAACGTGG-3) (Hong et al. 2005). Calmodulin sequences were compared using nucleotide-nucleotide BLAST (blastn) with default settings (http://blast.ncbi.nlm.nih.gov, Altschul et al. 1990) to the GenBank database, and to our own sequence database. Species identification was determined from the lowest expected value of the BLAST output.

Examination of aflatoxin producing abilities

The *Aspergillus flavus* isolates were grown on 2 ml YES (2% yeast, 20% sucrose) solution. The isolates were incubated for 7 days at 25 °C and at 30 °C in darkness. The aflatoxin extraction were carried out in 2 ml dichloromethane. For the examination of the aflatoxin producing abilities of the isolates thin layer chromatography (TLC) and enzyme-linked immunosorbent assay (ELISA) methods were used.

The used aflatoxin standard included aflatoxin B_1, B_2, G_1 and G_2. Camag Linomat 5 syringe system, Merck Millipore TLC Silica gel 60, and toluene:ethyl-acetate:formic acid 6:3:1 mixture were used for TLC analysis. To quantify the aflatoxin levels, ELISA analysis (AgraQuant® Total Aflatoxin Assay 1/20) was performed according to the instructions of the manufacturer (Romer Labs).

Results and Discussion

Several *Aspergillus* species have been identified recently, which are able to produce aflatoxins (Varga et al. 2009). These species can readily be distinguished using sequence analysis of part of their β-tubulin or calmodulin genes (Varga et al. 2011). In this study, we examined the occurrence of potential aflatoxigenic fungi on maize in several parts of Hungary and Serbia in 2012. *Fusarium* species (mainly *F. proliferatum* and *F. verticillioides*) and *A. flavus* were mostly present in samples collected during harvest in 2012 (Fig. 1). Other *Aspergillus* and *Penicillium* species were also detected. Besides these potentially mycotoxigenic species, *Acremonium zeae*, a potential biocontrol organism against maize pathogens (Wicklow et al. 2005), *Penicillium bilaiae*, which is used as a phosphate-solubizing organism (Leggett et al. 2007), and a *Bionectria* species which was found to be able to degrade zearalenone (Takahashi-Ando et al. 2002) were also identified.

Fusarium species dominated in samples collected after harvest, while *Aspergillus* species were predominant in stored samples. Occurrence of *A. flavus* was 76% in stored samples, and 33% in samples collected after harvest (Fig. 1). Regarding the samples collected during harvest in 2012, some samples were contaminated by aflatoxins. These highly carcinogenic mycotoxins were detected in 2 field samples came from Serbia (3.16 and 0.82 μg/kg). However, these values are below the EU limit (10 μg/kg; http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32010R0165&from=EN). Fumonisins were detected in most of the samples. In two maize samples (from Curug and Pancevo), the amount was above the EU limit (data not shown).

We have also chosen 10-10 *A. flavus* isolates and tested their aflatoxin producing abilities at 25 °C and 30 °C. We have observed some differences in the aflatoxin production at different temperatures (data not shown). We are planning to examine the aflatoxin M_1 content and the aflatoxin producing abilities of *Aspergillus* strains from Hungarian cheese samples too using HPLC and ELISA methods.

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