ROLE OF *ASPERGILLUS* SPECIES IN MYCOTOXIN CONTAMINATION OF AGRICULTURAL PRODUCTS IN CENTRAL EUROPE

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ABSTRACT

Aspergillus species are filamentous fungi which are widespread on agricultural products in subtropical and tropical areas of the world. Aspergilli are able to produce a range of mycotoxins which can be harmful to animals or humans, including aflatoxins, ochratoxins, fumonisins and patulin. According to recent studies, climate change accompanied by global warming affects the occurrence of fungi and their mycotoxins in our foods and feeds. A shift has recently been observed in the occurrence of *Aspergillus* species, especially aflatoxin producers in Europe. Our aim was to examine the occurrence of mycotoxin producing Aspergilli in Hungarian agricultural products to evaluate their importance in food safety. The examined agricultural products included various cereals, onions, nuts and spices. The surface-sterilized products were placed on selective media, and the isolated fungal strains were identified using morphological and sequence-based methods. Regarding cereals, several *Aspergillus flavus* isolates were identified, which are potential aflatoxin producers. This species was identified on various cereal seeds including maize, wheat and barley in different regions of Hungary. Several of the *A. flavus* isolates were found to be able to produce aflatoxins. Onions were found to be infected by *Aspergillus awamori*, a recently described ochratoxin and fumonisin producing species. This species together with other black Aspergilli was also identified on cereal seeds. Besides *A. flavus*, several potential mycotoxin producing species including *A. westerdijkiae, A. melleus, A. terreus, A. awamori* and *A. niger* have also been identified on nuts and spices (chilli, red pepper, spice mixes). Several species including *Aspergillus eucalypticola* and *Aspergillus amoenus* were identified for the first time in Europe. Further studies are in progress to examine the mycotoxin producing abilities and genetic variability of the isolates identified, and to examine the mycotoxin content of the samples.

KEY WORDS: *Aspergillus*, cereals, onions, aflatoxins, fumonisins, climate change

INTRODUCTION

Mycotoxins are secondary metabolites of filamentous fungi which are harmful to animals and humans, and able to provoke various disease symptoms (Varga et al., 2009). Aflatoxins are among the most important mycotoxins, which are produced by species assigned to the *Aspergillus* genus. Among the numerous aflatoxins described, aflatoxin B_1 (*Figure 1*) is the most toxic aflatoxin, being a potent genotoxic carcinogen in laboratory animals and there is strong evidence for its liver carcinogenity in humans. Aflatoxin B_1 exhibits hepatocarcinogenic and hepatotoxic properties, and is referred to as the most potent naturally occurring carcinogen. The International Agency for Research on Cancer has classified aflatoxin B_1 as a group I carcinogen. The most important producer, *Aspergillus flavus* is also an important pathogen of various cultivated plants including maize, cotton and peanut, and cause 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 is not treated as a serious health hazard. However, climate change associated with global warming seems to change the scenario. Based on recent studies, aflatoxin producing fungi and consequently aflatoxins are expected to become more prevalent with climate change in countries with temperate climate (Paterson and Lima, 2010). 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 (for references, see Dobolyi et al., 2011). These observations led us to examine the occurrence of potential mycotoxigenic *Aspergillus* species in Central European agricultural products. The isolates were identified using morphological and molecular methods.

MATERIALS AND METHODS

Sample collection

The cereal samples were collected from various cereal growing regions of Hungary in 2010 and 2011. The cereals examined included wheat, maize and barley. Onion samples, nuts and spices were also collected in this period from various outlets and stores in Central Europe. The samples were surface sterilized using ethanol, 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) and/or Czapek-Yeast Extract agar (CYA) media without antibiotics. Isolates were subcultured as single conidia on MEA, PDA and CYA plates (Samson et al., 2004).

Genotypic studies

The cultures used for the molecular studies were grown on malt peptone (MP) broth for 2 days, and DNA was extracted from the mycelia using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol.) according to the instructions of the manufacturer. Part of the calmodulin gene was amplified and sequenced as described previously (Pildain et al., 2008). 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 expect value of the BLAST output.

RESULTS AND DISCUSSION

Occurrence of Aspergilli on cereals

We examined the occurrence of potential aflatoxin producing fungi in cereals in Hungary. The surface-sterilized cereal seeds were placed on selective media, and the isolated fungal strains were identified using morphological and sequence-based methods. Among the examined samples, several isolates were found to be members of section *Flavi* of the genus *Aspergillus* based on colony morphology and microscopic features (Figure 1). Species assignment of the isolates was carried out using partial sequence analysis of the calmodulin gene. In spite of their high morphological variability, all isolates proved to belong to the *Aspergillus flavus* species based on calmodulin sequence data. The proportion of the positive samples varied: 0.83%, 2.00% and 3.17% rates were observed for maize, barley and wheat, respectively. None of the cereal samples were found to be contaminated by aflatoxins (data not shown). Examination of aflatoxin producing abilities of the isolates is in progress. According to preliminary results, several of the isolates were able to produce aflatoxins B_1 and B_2 (Figure 2).

Figure 1. a. *Aspergillus flavus* and *A. niger* contamination of stored wheat.

Figure 2. HPLC-FLD chromatogram of aflatoxins produced by one of the *Aspergillus flavus* isolates collected from wheat.

Besides *A. flavus*, several other potential mycotoxin producers were identified in the samples. The patulin producer *A. clavatus* and black Aspergilli able to produce both ochratoxins and fumonisins were recovered from several samples.

Occurrence of black Aspergilli on onions

Black mold rot caused by black Aspergilli is often responsible for severe damage of onion bulbs during storage. Infected onion bulbs have a black discoloration at the neck, shallow lesions on the outer scales, streaks of black mycelium and conidia beneath the outer scales and a black discoloration in bruised areas. The disease commonly occurs on onions stored at high ambient temperatures. Contaminated seeds and soil appear to constitute the main inoculum source. The species responsible for black mold rot is usually referred to as *Aspergillus niger*. We examined the mycobiota and fumonisin contamination of mouldy onion bulbs purchased in Hungary. All

except one of the examined mouldy samples were found to be contaminated with black Aspergilli, which could be isolated both from the outer dry and the inner fleshy scales of onion bulbs. Species assignment of the isolates was carried out using sequence analysis of part of the calmodulin gene. Sequence data revealed that all 35 black Aspergilli isolated from onions belong to the *Aspergillus awamori* species. Two of the examined onion samples were found to be contaminated with fumonisins at a low rate (ca. 0.3 mg kg^{-1}; data not shown). This is the first report on fumonisin contamination of onion bulbs.

Occurrence of Aspergilli on spices and nuts

Several spice and nut samples were collected in outlets and stores in Hungary and Serbia. Besides *A. flavus*, several potential mycotoxin producing species including *A. westerdijkiae, A. melleus, A. terreus, A. awamori* and *A. niger* have also been identified on nuts and spices (chilli, red pepper, spice mixes). Several species including *Aspergillus eucalypticola* and *Aspergillus amoenus* were identified for the first time in Europe (data not shown). Further studies are in progress to examine the mycotoxin producing abilities and genetic variability of these species.

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