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AFLATOXIN B1 CONTAMINATION OF CORN IN REPUBLIC OF SRPSKA

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Abstract

The natural occurrence of fungal contamination was evaluated in samples of corn (*Zea mays* L.) grains. Mycological survey was carried out by direct plating method on PDA and focusing on the mycotoxigenic fungi *Aspergillus* because of the ability of this genus to produce mycotoxin. A total of 83 samples of corn grains from domestic production were analyzed. The most frequent isolated fungi were *Aspergillus* spp. and *Fusarium* spp. while *Penicillium* spp. and *Alternaria* spp. were less frequently isolated genera. Samples are also analyzed for aflatoxin B1 contamination and only two samples were contaminated and had aflatoxin B1 content higher than defined by regulations. The composition of mycotoxigenic fungi in corn grain showed also the presence of other toxigenic fungi and these results indicate possible health hazards for human and animal consumption of such contaminated food grain by mycotoxigenic fungi.

Key words: *mycotoxigenic fungi, Aspergillus, aflatoxin B1, Republic of Srpska*

Introduction

The increasing worldwide concern about food has enhanced interest in fungal contamination and subsequent production of mycotoxins in food products. In this regard, attention is continuously focused on corn (*Zea mays* L.) because it is one of the most important food and feed in different regions of the world (Anon, 2004). Corn is the world's third most important crop after rice and wheat and in Republic of Srpska corn grown for grain accounts for almost one quarter of the harvested crop hectares in the country. Mycotoxins and mycotoxicises have been problems of the past and the present, but scientific attention for mycotoxins did not start until the early 1960's. Nowadays, many mycotoxins are known, and their occurrence in food and feed may cause various adverse effects on human and animal health, including carcinogenic, hepatotoxic, immunotoxic, nephrotoxic, neurotoxic, oestrogenic and teratogenic effects (Egmond, 2013). Mycotoxins are secondary metabolic products from fungi which can grow on the plant either in the field or during storage and are potentially toxic for humans and animals. More than 300 secondary metabolites have been identified although only around 30 have true toxic properties which raise concerns. These toxins are found as natural contaminants in many foodstuffs of plant origin, particularly cereals but also fruits, hazelnuts, almonds, seeds, fodder and foods consisting of or manufactured from these products and intended for human or animal consumption (Anon, 2006).

Mycotoxins are secondary metabolites produced by toxigenic fungi that contaminate food, feed chain, and represent a risk for human and animal health (Bennett *et al*., 2003). They are responsible for many different toxic effects including the induction of cancer, and digestive, blood, kidney and nerve defects. The mycotoxin problem is particularly relevant for human health in tropical areas, such as Sub-Saharan Africa, where crops are quite susceptible to contamination by the carcinogenic aflatoxins and fumonisins. In Europe, the main concern is related to *Fusarium* and *Aspergillus* diseases that have assumed a great relevance for both

health and economic implications (Battilani *et al*., 2006; Magan, 2006; Logrieco, 2001; Logrieco *et al*., 2002; loc. cit. Fanelli & Logrieco, 2012). Aflatoxin B1 is produced by many species of *Aspergillus*, most notably *A. flavus*, *A. parasiticus* and *A. nomius*; it is a proven carcinogen for humans (Castegnaro and Wild, 1995), immunotoxic, and it causes stunted growth in children and growth retardation in animals. High-level of aflatoxin exposure produces acute hepatic necrosis and later it can result in cirrhosis, and/or carcinoma of the liver.

The presence of aflatoxin affects grain quality and marketability, as well as livestock health if the grain is consumed. *Aspergillus* ear rot is commonly observed during hot, dry years on stressed plants (such as those exhibiting symptoms of nutrient deficiency or drought stress). Feeding damage from ear-invading insects also contributes to disease development and aflatoxin contamination. In these cases, accurate mold identification is critical for making the right feeding and management decisions.

Attention of the scientific and professional community was drawn to very intensive appearance of *Aspergillus* ear rot in 2012 in Republic of Srpska, as well as in the region. Specific environmental conditions, especially drought and high temperatures during the period from fertilisation to harvest favored the intensive development of the genus *Aspergillus* on corn ear and grain. In addition, ear damage caused by the larvae of the second generation of European corn borer (*Ostrinia nubilalis* Hbn.) that appeared in 2012 in extremely high intensity is another factor that caused intensive emergence of olive-green powdery mold. The result of intensive development of the disease in 2012 was unusually high contamination of corn grain with aflatoxin B1, that is produced by species of the genus *Aspergillus* (mostly *A. flavus* Link and *A. parasiticus* Speare). Therefore, there was the question of the use of such corn for food and feed, and a consequence were also large economic losses in the production of milk and dairy products so many producers had to spill milk contaminated with aflatoxin M1, which led to huge losses in the economy. Above-mentioned draw a great deal of public attention and caused major concern to consumers and producers.

Considering the situation from previous year as well as extreme drought in July and August 2013, the Ministry of Agriculture, Forestry and Water Management of the Republic of Srpska appointed expert team tasked to continuously monitor the impact of drought on plants yield and quality with special reference to the possible production of mycotoxins. This expert team has assessed the risk of re-occurrence of aflatoxin and proposed a survey that is "Monitoring of the presence of aflatoxin B1 in corn in the Republic of Srpska". Samples were taken by the representatives of the Agricultural Extension Service of Republic of Srpska in regions of Banja Luka, Prijedor, Doboj, Bijeljina and Gradiška, where corn is mostly grown.

Material and Methods

Prior to isolation of toxigenic fungi samples were pre-treated, that is sterilized and dried. Sterilization is done in order to destroy saprophytic micropopulations on sample surface. Surface sterilization of the samples was carried out by immersing the grains in 1% sodium hypochlorite solution (1 part NaOCl: 3 parts of distilled water) and after that grains were rinsed three times in sterile distilled water. Sterilized grains were dried between two layers of paper towels to remove excess water. 50 grains of each sample (10 grains in a Petri dish) were plated on potato dextrose agar (PDA) media with streptomycin sulfate in order to prevent growth of bacteria. Inoculated plates were incubated for seven days at 25°C prior to visual differentiation and counting of colonies. The different fungal colonies on the plates were subcultured on PDA media for identification of species. In order to determine if *Aspergillus flavus* isolates are toxigenic or atoxigenic, isolates were incubated at 30°C in order to form sclerotia, and then based on the size of sclerotia determined whether it is toxigenic or atoxigenic strain.

Furthermore, 250 g of each sample were dried at 60°C for 72 h and in that way prepared for analysis for aflatoxin B1 contamination. Approximately 50-100 gram of sample is ground and pulverised into a fine homogenous compound. After that, to 3 gram of ground sample 9 ml of 80% methanol is added and shook thoroughly at room temperature for 10 minutes. Samples are than centrifuged for 10 minutes at 2000 x g. An aliquot of 50 µl of the supernatant obtained after centrifugation is diluted with 150 µl of dilution buffer to obtain a solution containing 20% methanol. Contamination of corn samples with aflatoxin B1 is done by competitive enzyme immunoassay (ELISA) using a kit from EuroProxima, the Netherlands. ELISA test is highly specific and sensitive immunological reaction that allows detection and quantification of aflatoxin B1 in very low concentrations. The test is based on antibodies against aflatoxin B1. The microtiter plate based ELISA kit consists of 12 strips, each 8 wells, precoated with rabbit antibodies to mouse IgG. In one incubation step, specific antibodies (mouse anti-aflatoxin), enzyme labelled aflatoxin (enzyme conjugate) and aflatoxin B1 standards or sample are added to the precoated wells. The specific antibodies are bound by the immobilised rabbit antibodies and at the same time free aflatoxins (in the standard solution or in the sample) and enzyme labelled aflatoxin compete for the specific antibody binding sites (competitive enzyme immunoassay). After an incubation time of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound enzyme conjugate is visualised by the addition of chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme transforms the chromogen into a coloured product. The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity (O.D.) is measured photometrically at 450 nm and is inversely proportional to the aflatoxin B1 concentration in the sample. Photometric measurement is performed on Thermo Scientific™ Multiskan™ FC Microplate Photometer. The mean optical density (O.D.) of the blank well is subtracted from the individual O.D. of the wells containing the standards and the samples. The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance. The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the aflatoxin B1 equivalent concentration (ng/ml) on a logarithmic X axis. The amount of aflatoxin in the samples is expressed as aflatoxin B1 equivalents. Calculated aflatoxin B1 equivalents are then multiplied by a factor 16. All these calculations are done by Thermo Scientific™ SkanIt™ Software.

Results and Discussion

Eighty three samples of maize grain intended for human or animal consumption and sampled before harvest from domestic production were analyzed for fungal and aflatoxin B1 contamination. Identification of fungal strains revealed that *Aspergillus* was the most frequent genus, found in almost all maize samples. In *Aspergillus* genus (fig. 1), two species were identified, *A. flavus* and *A. parasiticus*, but *A. flavus* was the most frequent contaminant, observed in more than 80% of the samples. Most *A. flavus* isolates were atoxigenic, that is they formed sclerotia bigger than 400 µm in size (fig. 2). Other fungal strains found in maize samples belonged to *Fusarium, Penicillium* and *Alternaria* species and were found in up to 33, 23 and 18% of the samples, respectively. The frequencies of four fungi genera differed from sample to sample. The general means of fungi showed that *Aspergillus* spp. and *Fusarium* spp., were the most frequently isolated genera; while *Penicillium* spp. and *Alternaria* spp. were less frequently isolated genera. Only two maize samples from Doboj and Gradiška were contaminated with aflatoxin B1 at a level of 3,24 and 7,29 ppb, respectively. Other 81 samples had aflatoxin B1 content below the limits defined by the "Regulation on

undesirable substances in feed" (Official Gazette No. 72/11) and the "Regulation on the maximum allowable amount for certain contaminants in food "(Official Gazette, No. 37/09).
These results indicate that the type of fungal contamination of the corn grains in Republic of Srpska was qualitatively similar to that found in other corn producing countries such as the United States (Wu *et al*., 2011), Italy (Covarelli *et al*., 2011), Switzerland (Dorn *et al*., 2011), Malaysia (Reddy & Sallah, 2011), Pakistan (Saleem *et al*., 2012) and Saudi Arabia (Mahmoud *et al.*, 2013).

Fig. 1. *Aspergillus* spp. isolated from corn grains.

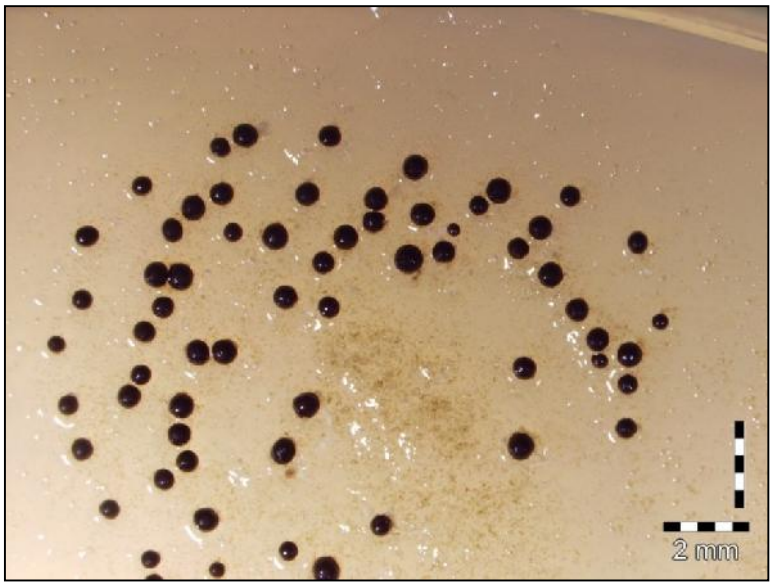

Fig. 1. *Aspergillus flavus* sclerotia on PDA media.

Mycotoxins are among the food-borne risks that are dependent upon climatic conditions. Indeed, the ability of fungi to produce mycotoxins is largely influenced by temperature, relative humidity, insect attack, and stress conditions of the plants (Miraglia *et al*., 2009). Therefore studies on frequency and relative percentage of mycotoxigenic fungi are highly useful and required for further studies on toxin producing fungi and their epidemiological significance in corn crops. *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* genera are mycotoxigenic fungi responsible for the majority of agricultural mycotoxin contamination.

These fungi are common components of the microbial flora associated with many agronomic crops, including corn (Palumbo *et al.*, 2008). Previous studies identified genus *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* as mycotoxigenic fungi that are a natural contaminant in corn crops and also in many other agricultural commodities (Lino *et al*., 2007; Logrieco *et al*., 2007, Pacin *et al*., 2009; Cunha *et al*., 2009) and all these were isolated from the samples in this study.

The use of good agricultural practices that would discourage fungal growth and mycotoxin production would be necessary to reduce mycotoxin levels in the corn and corn products. Contact of the corn with the soil should be avoided during harvest and drying to avoid contamination with the fungal inoculum present in the soil. Drying of corn to safe moisture levels of less than 13% and cleaning of stores at the end of each season would reduce chances of infection and mould growth. If the earth's surface temperatures continue in a warming trend, and other associated climate patterns may be changing, then farmers, food industries, and policymakers should be concerned about changing mycotoxin risks both in the short term and in the long term. In the short term, from year to year, temperature and precipitation may favour or discourage growth of mycotoxigenic fungi and mycotoxin contamination of agricultural products. In the long term, climatic trends may pose longer-term impacts on distribution of fungi, their mycotoxins, and host crop plants (Wu *et al*., 2011).

Conclusions

Based on the results, we can conclude that in this phase of the survey there was no excessive production of aflatoxin B1 in tested corn samples. However, these fungi do not always produce mycotoxin but only under certain circumstances, such as when exposed to adverse environmental conditions, competition with other species of fungi, etc., and amount of produced mycotoxins depends on the environmental conditions, storage conditions, air humidity and other factors. Because of all that facts it should be taken into consideration that there is a possibility of mycotoxins production in later stages, so these analysis should also be perfomed in other phases of corn production, drying, storage and handling.

Also, the composition of mycotoxigenic fungi in corn grain showed the presence of other toxigenic fungi. These results indicate possible health hazards for humans and animals consumption of such contaminated food grain by mycotoxigenic fungi so these analysis should include other mycotoxins such are zearalenone and deoxynivalenol. This study should also include certain number of samples of wheat, barley and other cereals in order to analyze other food and feed components. Besides, it is necessary to carry out permanent control of feed and feed components from import.

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