INCIDENCE OF *ASPERGILLUS* STRAINS AND OF AFLATOXIN B1 IN CEREALS IN SOUTH-WESTERN ROMANIA

Cristina TABUC[1], Ciprian STROIA[2] Alina NEACŞU[2]

[1]National Research Development Institute for Animal Biology and Nutrition Balotesti
[2]University of Agricultural Sciences and Veterinary Medicine of Banat, Timisoara
Corresponding author:cristina.tabuc@ibna.ro

Abstract: *Cereals are the substrates most exposed to micromycete attack. The development of moulds determines changes of the physical properties (aspect, taste, smell) and of the chemical properties as well (nutrient degradation and lower food quality). The presence of aflatoxin B1 was rarely reported in Italy and Spain, after extremely hot summer seasons. Romania was not included in these surveys because it became EU member state at a later moment. The investigations conducted on cereal grains coming from south-eastern Romania showed the constant presence of Aspergillus species and of aflatoxins in this part of the country. The purpose of this study was to evaluate the presence of Aspergillus species in the cereal grains samples from various areas of Banat and to determine the concentration of aflatoxin B1 and of ochratoxin A, synthesized by Aspergillus species. We assayed a total of 56 cereal samples (corn, wheat, barley and oats). The level of fungal contamination was determined by direct count of the fungi colonies and mycotoxin concentration was determined by ELISA. Although Aspergillus species are mainly associated to the warm areas, our investigations showed the presence of Aspergillus species in over 80% of the samples. The most frequently identified species was Aspergillus flavus (55%), followed by A. niger (40%) and by other species: A. fumigatus, A. verzicolor si A. parasiticus. The mycological analysis is supported by the results we obtained with the cereals from south-eastern Romania. Aflatoxin B1 has been identified in almost 30% of the samples (corn mainly), but the concentrations were low, below 10 µg/kg, the maximal limit admitted by the EU. The results are similar with the findings of previous research in south-eastern Romania.*

Key words: *cereals, Aspergillus sp., Aflatoxine B1, Ochratoxin A*

INTRODUCTION

The genus *Aspergillus* comprehends 185 species assigned to 18 groups closely related morphophysiologically and genetically. Among them, about 20 are involved in human and animal pathologies (RAPER and FENNELL, 1965; BOTTON *et al*., 1990; ROQUEBERT**,** 1998).

The species of *Aspergillus* genus are widely spread geographically, often been associated to the warm areas (CASTEGNARO and PFOHL-LESZKOWICZ, 2002). They grow on decaying organic matter, soil, compost, foods, cereals. Many species are living in the environment (dust and air) (MORIN, 1994). Some species may be directly pathogenic for humans and animals, where they invade various tissues and produce aspergilloses (MORIN, 1994). Many species are reputed for their capacity to elaborate mycotoxins. Some species are used in the food industry and by biotechnologies to produce enzymes and organic acids (BOTTON *et al*., 1990).

The main species are: *Aspergillus flavus, A. fumigatus, A. niger, A. ochraceus* and *A. oryzae*, next to which are many other species producing mycotoxins: *A. flavus* and *A. parasiticus* are the main producers of aflatoxins, aflatoxin B1 being the top cancerigenic agent to humans and animals (IARC, 1993; 2000); *A. niger* produces oxalic acid, malformins, and some strains produce aflatoxins; *A. ochraceus* is the main producer of ochratoxin (PITT, 2000).

The species of *Aspergillus* genus are opportunist pathogenic whose development requires certain conditions and which produce aspergilloses in humans (BADILLET *et al*., 1987;

MORIN, 1994). The main species responsible for aspergilloses are: *A. fumigatus* the main agent of avian and human aspergilloses (80-90% of the human aspergilloses) (BADILLET *et al.*, 1987; MORIN, 1994); *A. flavus* causes pulmonary aspergilloses and generalised aspergilloses in immunodeficient patients (BACULARD and TOURNIER, 1995); *A. niger* causes otitis, sinusitis and is often involved in cutaneous, pulmonary and generalised infections (MORIN, 1994); *A. terreus* is an important agent of the pulmonary and cerebral aspergilloses in immunodeficient patients (BACULARD and TOURNIER, 1995 ; KHAN *et al.*, 1999).

Few *Aspergillus* species are useful in the agro-food industries and for synthesis purposes: *A. awamori*, lipolytical agent for oleaginous and for alcoholic fermentations; *A. niger* is used in biotechnological processes for the synthesis of acids (citric acid, gluconic acid) and of enzymes, such as alpha-amylase, beta-glucanase, catalase, glucose oxidase, lipase, pectinase, polygalacturonase (BOTTON *et al.*, 1990).

Aflatoxins are produced by three *Aspergillus* species: *A. flavus* which produces mainly aflatoxins B1 and B2; *A. parasiticus* which produces aflatoxins B1, B2, G1 and G2 and *A. nomius*, a more rare species, resembling morphologically to *A. flavus*, which produces aflatoxins B1, B2, G1 and G2 (CASTEGNARO and PFOHL-LESZKOWICZ, 2002). The optimal conditions for the production of aflatoxins are: poor hydric activity of the medium (0.84-0.86) and a high temperature, between 25 and 40^{o}C (PFOHL-LESZKOWICZ, 2001; CASTEGNARO and PFOHL-LESZKOWICZ, 2002).

The family of aflatoxins has 13 different molecules, the most important ones being aflatoxins B1, B2 and G1, G2 and aflatoxin M1.

Taking into account the conditions for synthesis, aflatoxins are generally detected in foods from the warm regions (South America, Africa and Asia). They were observed in cereal grains (corn, wheat, barley, oats, rye, rice) and in cereals, in oleaginous (soybean), nuts and derivates (ground nuts, peanuts butter, pistachio), in legumes (potatoes, lentil, pepper), dry fruits (figs) and in beer.

Aflatoxin B1 (AFB1) from the forages ingested by dairy cows is partially metabolised in the liver and transformed in a 4-hydroxy derivate known as aflatoxin M1, which is excreted in the milk. This substance is stable and can be found in dairy products (yogurt, cheese).

The effects of aflatoxins on animal health vary with the species, age, sex and physiological state of the animal, manner of administration, diet composition. The most toxic is AFB1, followed by AFM1, AFG2, AFB2 and AFG1. The toxicity of aflatoxins G1, B2 and G2 is 50, 80 and 90% of AFB1 toxicity (COLE and COX, 1981). Ingested in large amounts, aflatoxin produces acute intoxications generally characterised by a fast demise of the animals. Upon necropsy the animals display decoloured, larger liver (hepatotoxicity), glomerulonephritis signs on the kidneys, congested lungs. Aflatoxins are teratogenic and this effect is properly described in the chicken embryo which displays delayed development, microcephaly, anophtalmy, split palates (rabbit mouth) and deformed jaws ARORA *et al.*, 1981; VESELY *et al.*, 1983).

The major toxic power of AFB1 is carcinogenesis, this molecule being accountable for the hepatocarcinoma in humans and animals. AFB1 has been included in class I of the cancerigenic substances by IARC or CIRC.

Ochratoxin A or OTA: the family of ochratoxins includes 10 known substances, of which OTA singles out in importance. OTA is produced by species of *Aspergillus (A. ochraceus)* and *Penicillium (P. verrucosum, P. viridicatum)* which allows it to be synthesized in very variable conditions. The optimal temperature for OTA synthesis by *A. ochraceus* is 28°C, the production being strongly limited at 15°C or at 37°C, while *Penicillium viridicatum* developes slowly and can produce OTA at a wide range of temperatures: 4-30^{o}C (POHLAND *et al.*, 1992; VARGA *et al.*, 1996).

OTA is detected in cereal grains (corn, wheat, barley, oats, rye, rice), in soybean, coffee, green beans, peas, ground nuts, dry fruits (figs, raisins). It has also been detected in cereal products (flour, bread, pasta) (MAJERUS *et al*., 1993), in legumes (potatoes, lentil), in beer (EL-DESSOUKI, 1992), wine, and must (ZIMERLI and DICK, 1996). Unlike the aflatoxins, which are present in foods from the warm regions, OTA is present in products from all around the world because it can be produced in the warm countries by *A. ochraceus*, and in the colder regions by *Penicillium*.

OTA has been detected in compound feeds when contaminated raw materials have been used or due to improper storage (DALCERO *et al*, 2002; MAGNOLI *et al.*, 1998). The food cereals generally contain low amounts of OTA. *Aspergillus* is a mould which grows on grain surface so that a significant amount of mycotoxins are removed during the technical processes (NGUNDI *et al.*, 2006).

Ochratoxin A has been detected in slaughterhouses, in the meat of animals fed contaminated feeds (JORGENSEN, 1998); it has been revealed in blood and tissues; OTA builds up in the liver and kidneys (TERPLAN and WENZEL, 1993; MAC DONALD *et al*., 1993; GAREIS, 1996).

Ochratoxin A is nephrotoxic for all tested species of animals (laboratory animals, farm animals), except for the adult ruminants (RIBELIN *et al*., 1978). The key organ for OTA is the kidney (POHLAND *et al*., 1992; MARQUARDT and FRÖHLICH, 1992). OTA toxicity varies with the species, sex, route of administration. Cases of *Aspergillus ochraceus* spores inhalation were described, where they caused renal modifications (oliguria and tubulonecrosis) (POHLAND *et al*., 1992; MARQUARDT and FRÖHLICH, 1992). OTA is rapidly absorbed in the breathing ducts (BREITHOLZ-EMANUELSSON *et al.*, 1995).

Administered experimentally, OTA causes changes in the bone marrow and immune response; it is agent for lymphopenia, thymus regression and immune response suppression (SINGH *et al*., 1990, LEA *et al*., 1989, LUSTER *et al.*, 1987); it is teratogenic for the animals causing morphological abnormalities in the laboratory animals and in farm animals; it produces a high foetal mortality, foetal malformations of the viscera and bone system (PFOHL-LESZKOWICZ, 2001).

In humans, OTA is suspected to be involved in the Balkan endemic nephropathy (ABOUZIED *et al*., 2002; VRABCEVA *et al*., 2000; VRABCEVA *et al*., 2004). OTA is ranked in group 2B of the cancerigenic substances for animals and possibly cancerigenic for humans (VAINIO *et al*., 1992).

MATERIAL AND METHODS

56 cereal grains samples (25 corn samples, 12 wheat samples, 10 barley samples, 9 oats samples) collected from different locations in Banat region, Veratox kits to determine mycotoxin concentration by ELISA.

Isolation and identification of Aspergillus species: The *Aspergillus* species contaminate the cereal grains both in the field and during storage. The cereal grains samples were ground finely, immerged in tween 80. 5% solution and then diluted (10^{-2}, 10^{-3}); 1 ml volumes from these solutions were applied on medium with malt and agar with 6% NaCl. The plates were incubated for 5-7 days at 25^{o}C. By successive repicking on medium with malt and agar with 6% NaCl we obtained pure cultures which were used to determine the species by studying the cultural characteristics of the developed colonies, as well as the macroscopic and microscopic characteristics as described by RAPER and FENNELL, 1965 and BOTTON *et al.*, 1990.

Determination of mycotoxin concentration: The mycotoxin concentration of the studied samples was determined using Veratox Neogen kits by ELISA.

We observed all the stages described in the instructions for each kit. The optical density was read over 10 minutes with a TEKAN microplates reader, at 650 nm wavelength, after which the concentration of mycotoxins was calculated.

RESULTS AND DISCUSSION

Isolation and identification of Aspergillus species: The *Aspergillus* species were present in 45 of the 56 analysed samples (80.35%); the most frequently identified species were *Aspergillus flavus, A. niger, A. parasiticus, A. verzicolor* and *A. fumigatus* (fig. 1). Just one strain of *Aspergillus ochraceus* has been identified in corn. These results are similar in terms of microflora composition with the results of the study on the cereal grains samples from south-eastern Romania.

Aspergillus flavus is the species most widespread in both regions, but its frequency was higher in south-eastern Romania (68%) than in Banat (55.35%) being present mainly in the corn, 25 samples (100%), followed by barley with 6 samples (60%) and wheat, 4 samples (33.33%). The other species (*A. niger, A. fumigatus, A. verzicolor* and *A. parasiticus*) shared almost equal proportions. In the cereal grains from south-eastern Romania we didn't identify any strain of *Aspergillus ochraceus. A. candidus, A. terreus*, and *A. restrictus* were not identified in any sample from Banat region.

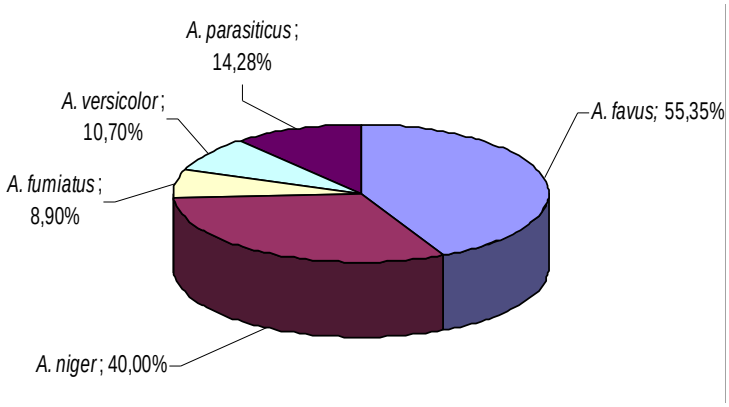

Fig.1. *Aspergillus* species that have been isolated

Determination of mycotoxin concentration:

Corn: aflatoxin B1 has been identified in 5 of the 25 analysed samples (20%). In the 5 samples AFB1 concentration ranged between 0.9 and 6.8 μg/kg, concentrations, within the maximal limits admitted by the EU (10 μg/kg). None of the analysed samples had OTA.

Wheat: of the 12 wheat grain samples that were analysed, 2 samples had AFB1 in low concentrations (2.4 – 3.4 μg/kg). No wheat sample had OTA.

Barley: only 2 samples had AFB1 in concentrations of 3.1 and 6.8 μg/kg. OTA was not identified in any barley sample.

Oats: none of the 9 oats samples had the surveyed mycotoxins (AFB1 and OTA).

CONCLUSIONS

The analyses showed that 80.35% of the studied samples (45 of the 56 cereal grains samples) were contaminated with species of *Aspergillus* genus; the most frequently isolated species were *A, flavus*, 55.35% and *A. niger*, 40%; these results are in agreement with the

findings of previous studies in south-eastern Romania, where these two species were constantly present, mainly in corn (TABUC *et al*., 2004; 2007; 2009).

AFB1 was identified in 20%, 16.66% and 22% of the corn, wheat and barley samples, while no oats sample was contaminated with AFB1, while in the samples from south-eastern Romania, where AFB1 was identified in 29% of the corn samples, with the highest concentration being 45 µg/kg; AFB1 contamination was 7% in the wheat, barley and oats samples (TABUC *et al*., 2009). Ochratoxin A has not been identified in any of the 56 surveyed samples, as supported by the findings of the south-eastern survey.

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