

MICOFLORA ASSOCIATED WITH MAIZE GRAINS DURING STORAGE PERIOD

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Abstract

Agricultural crops are vulnerable to infections by a wide spectrum of plant pathogens. In today's marketplace, the increasing complexity and wide distribution chain represent enormous challenges for food production. The increased fungal infection and cross-contamination hazards are associated with the globalization of cereal trade (Waage et al., 2006). Cereals are one of the most important sources of food (FAO, 2002), which have contributed to human nutrition for millennia. However, cereals are exposed to numerous biotic and abiotic stress factors, from cultivation and throughout their life cycle to processing. The grain losses recorded during storage period on worldwide scale according to FAO estimations are between 5-10% of total production. In developing countries, due to reduced possibilities of implementing appropriate technologies, the reported damages during storage period may increase up to 30%.

*In 2015, the maize grains which are to be stored on an indefinite period of time is affected by the presence of various pests specific to warehouse ecosystem. The paper work presents a study regarding the occurrence and development of specific warehouse micromycetes during the first months of maize storage. Immediately after being deposited, it has been identified the specific microflora for this period, respectively species of *Alternaria*, *Trichoderma*, *Cladosporium*, *Aureobasidium*, *Cephalosporium*, *Aspergillus* and *Fusarium* genera. Crop safety and security can be achieved by maintaining climatic factors in stored spaces, thus limiting the populations' level of contaminating microorganisms.*

Key words: maize grains, storage fungi, aflatoxin production.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important sources of food for human and animal nutrition and raw materials for industrial processing. The nutritional value of stored maize grains could vary significantly due to the interaction between the physical, chemical and biological factors.

The contamination of maize with fungi (moulds) and mycotoxins represents a major problem for its use in human and animal nutrition (Krnjaja, 2013). Infection of grains in the field by fungi could result in the production of mycotoxins during cultivation, harvesting, storage, transport and processing. The most important species of fungi and mycotoxins that could contaminate maize grains are *Aspergillus flavus* and aflatoxins, *Fusarium verticillioides*, *F. proliferatum* and fumonisins and *F. graminearum* and trichothecenes and zearalenone (Chulze, 2010).

Despite decades of intense research, the molds infection is still a major challenge for scientists (Munkvold, 2003). It has been estimated by FAO that approximately 25% of the worldwide cultivated surface is contaminated with phytopathogenic fungi and affected by mycotoxins (CAST 2003, Rice and Ross, 1994), generating economic losses of billions of dollars (Trail et al., 1995). Molds are considered to be the second largest pest agent after insects that cause losses in stored grains. Outdated harvesting agricultural practices, improper drying, handling, storage and transport conditions, all together contribute to fungi development and increase the risk of mycotoxin production.

Mycotoxins are dangerous by their presence in food and agricultural products, as even in low concentrations endanger animal and human health, affecting their immune response. Since the discovery of these toxins, the research were focused on detection and determination

pathways, on the induced toxicity towards humans and animals, on establishing the favorable conditions of toxigenic fungi development and on studies regarding maximum limits of mycotoxins in food and feed (Matei et al., 2011).

Micromycetes' development on stored grains is conditioned by temperature and atmospheric humidity present in stored areas and by its fluctuations in time (Cristea et al., 2004). Toxigenic molds are present, due to various climatic factors, in different stages of food and feed production, including crop growth, harvesting, transport, storage and handling (Beyer et al., 2006).

The most common genera of fungi identified in stored maize grains are *Aspergillus*, *Penicillium* and *Fusarium*. Researches regarding seed microflora were performed on several cultures like wheat, barley, sunflower, mustard and rape (Gheorghies et al., 2001; Berca and Cristea 2015; Berca et al., 2015; Cristea et al, 2004; Cristea et al., 2015; Pana et al., 2014; Radu et al., 2011; Mardare et al., 2014).

The proliferation of these fungi are stimulated with higher grains moisture content, higher temperature during storage, long storage period, intensive infection by fungi before storage and by higher activity of insects and mites. Therefore, it is important to identify the species of fungi in stored maize grains with special emphasis on mycotoxigenic species, which pose a potential risk to human and animal health (Castellari et al., 2010).

This study presents the micromycetes' evolution during the first period of storage.

MATERIALS AND METHODS

The research aimed to identify the present microflora on maize grains, its timeline evolution during 120 day of storage and possible aflatoxin contamination of cereals samples.

Biological material consisted in maize grains samples from warehouses in various locations in Romania, such as Sibiu, Dalga, Ramnicu Sarat, Traian, Tecuci, Targul Secuiesc, Inand, Neresti, Portaresti, Paulesti, Targoviste, Giurgiu, Braila, Calarasi, was stored in horizontal deposits, bags or bulk, from which were taken samples in order to analyze the

contamination of grains. The samples were taken in four stages, immediately after harvest and after 60, 90 and 120 days of storage. The batch sampling was performed on three levels, respectively base, middle and top surface, also from doors and windows area.

Isolation and identification of contaminating fungi

Maize contaminating fungi were isolated using Ulster method (Hulea, 1969) and identified with optical microscope, after 12 days of growth. Using the Ulster method there can be identified the majority of seed pathogens, regardless the species and type of seed. In separate Petri dishes of 10 mm diameter, were placed 15 wheat seeds on growth solid media (water-agar, 20 g/l, autoclaved 20' at 121 °C), with enough space between the grains in order to allow the development of fungi or bacteria. The water agar media was preferred due to its low nutrients composition which allows the fungi growth but not its abundant sporulation. This is an important step in order to be able to isolate each fungus from the Petri dish multitude of pathogens. The dishes were kept at room temperature (22-24 °C) and normal light conditions. After 7 days there were performed macroscopic observations regarding the mycelia growth in Petri dishes, followed by optical microscope determinations. For further isolation and purification of each fungus was used the Potato-Dextrose-Agar medium, prepared after the classic recipe (Hulea, 1969). The isolation method was performed for both epiphytic and endogenous fungi. For the isolation and identification of pathogenic and saprophytic epiphytic flora, the experiments were carried out using unsterilized seed placed directly into water agar Petri dishes.

Afterwards, it was studied the development of endogenous flora during two experiments. The first consisted in placing the seeds on water-agar medium, after being sterilized in a 4% sodium hypochlorite solution and washed three times with sterile distilled water. The microscopical examinations were made after 7 days. In the second experiment, the seeds after being sterilized, washed and dried were ground using a small laboratory mill, and an amount of flour was spread on the growth medium surface. Observations were performed after 6 days.

Analysis of aflatoxin from maize samples

Research in this stage was focused also on production of aflatoxin, metabolized by species of *Aspergillus*. Determinations regarding the presence or absence of this mycotoxin were made by enzyme immunoassay using ELISA kits, namely RIDASCREEN FAST AFLA (Matei et al., 2011). The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with capture antibodies directed against anti-aflatoxin antibodies. Aflatoxin standards or samples solutions, AFLA enzyme conjugate anti-AFLA antibodies are added; Free AFLA and AFLA enzyme conjugate compete for the AFLA antibody binding sites, which represents the competitive enzyme immunoassay. At the same time, the anti-AFLA antibodies are also bound by the immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step. When the chromogenic substrate is added into wells, bound enzyme conjugate converts the chromogenic substrate into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm, the absorbance being inversely proportional to the aflatoxin concentration from the sample.

Each kit contains: microtiter plate with 96 or 48 wells (coated with capture antibodies), 5 different concentrations aflatoxin standard solutions, 1 conjugate peroxidase, anti-AFLA antibody solution, chromogenic substrate solution, stop solution, washing buffer solution.

Test protocol

- Samples preparation - the maize grains were ground with a small laboratory mill. A quantity of 5 g ground sample is placed in a suitable container and 100 ml of 70% methanol was added. The samples were shaken vigorously for three minutes. The samples were filtered through Whatman No. 1 filter and we recovered the extracts.
- Immuno-enzymatic procedure - a sufficient number of wells were inserted into the microtiter plate for all the standards and the samples needed to be run. 50 µl of each standard and sample were pipetted into separate wells, using a new pipette tip for each standard or sample; we added 50 µl enzyme conjugate to each well and then 50 µl anti-AFLA antibody

solution. Plate mixed gently by shaking and was incubated 5 minutes at room temperature. The liquid was dumped afterwards from the wells into a sink, and it was performed a washing step with 200 µl washing buffer, three times. 100 µl of chromogen substrate were added to each well, followed by 3 minutes incubation time, in the dark at room temperature.

At the end were added to each well 100 µl stop solution, the plate was mixed gently and the absorbance was determined at 450 nm with a StartFax 2000 spectrophotometer.

RESULTS AND DISCUSSIONS

The micoflora associated with maize grains from the storages taken into analysis is represented by microorganisms like micromycetes, actinomycetes and bacteria. The study was focused on micromycetes' analysis.

From the maize samples taken immediately after being deposited, there were isolated specific fungi belonging to the field ecosystem, respectively with predominance species of *Alternaria*, followed by species of *Cladosporium*, *Penicillium*, *Mucor*, *Cephalosporium*, *Aureobasidium*, *Trichoderma viride* (Figures 1-5).

Alternaria and *Cladosporium* fungi induced in the vegetation period an infection of the grains' apex, developing a "black point".

In terms of unfavorable climatic conditions a reduced percent of *Fusarium* species was identified, its evolution remaining to be studied in time for evidence of mycotoxin secretion. Due to 2015 environmental conditions during the vegetation season, characterized by heavy rainfall and high temperatures, especially during kernels development, the maize grains were not infected by a various micoflora.



Figure 1. *A. versicolor*



Figure 2. *Fusarium* sp.

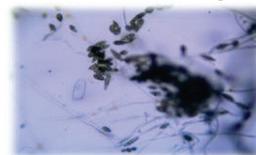


Figure 3. *Alternaria* sp.

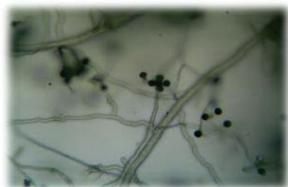


Figure 4. *N. sphaerica*



Figure 5. *A. niger*

Analyzing the data presented in table 1, it is concluded that after 60 days of storage, the percent of the field fungi, respectively species of *Alternaria* and *Cladosporium* decreased, and it was observed the development of stored grain specific fungi such as *Fusarium* spp., *Aspergillus* spp. and *S. sclerotiorum*. Gradually, over a storage period of 90 days, the

grains' micoflora enriched by the incidence and growth of the phytopathogen fungi, due to storage conditions like high atmospheric humidity. Saprophytic fungi such as *Cladosporium* spp., *Periconia* spp., *Aureobasidium* spp. and *S. botryosum* completely disappeared from the storage ecosystem. After 120 days of storage, the maize grains associated micoflora consisted in various species of fungi belonging with predominance to *Fusarium*, *Aspegillus* and *Penicillum* genera. Also, there have been isolated, with a lower incidence, species of *M. pussilum*, *R. stolonifer*, *N. sphaerica* and *Alternaria* spp.

Table 1. Micoflora structure of maize grains

Fungi	Contamination level			
	At harvest	60 days of storage	90 days of storage	120 days of storage
<i>Fusarium moniliforme</i>	+	+	++	+++
<i>Fusarium tricinctum</i>	-	++	+++	+++
<i>Fusarium graminearum</i>	-	++	+++	+++
<i>Cephalosporium</i> spp.	+	++	++	+
<i>Aspergillus</i> spp.	+	++	++	+++
<i>Alternaria tenuissima</i>	+++	++	++	+
<i>Cladosporium herbarum</i>	++	+	-	-
<i>Trichoderma viride</i>	++	+	+	-
<i>Nigrospora sphaerica</i>	+	++	++	+
<i>Sclerotinia sclerotiorum</i>	-	++	+++	++
<i>Mucor pussilum</i>	++	+++	+++	++
<i>Aspergillus niger</i>	-	++	++	++
<i>Alternaria</i> spp.	++++	++++	+++	++
<i>Penicillium frequentans</i>	++	++	+++	+++
<i>Periconia</i> spp.	+	-	-	-
<i>Aureobasidium</i> spp.	+	+	-	-
<i>Stemphillium botryosum</i>	+	+	-	-
<i>Rhizopus stolonifer</i>	-	++	+	+

Legend: + = low frequency; ++ = moderate frequency; +++ = increased frequency; ++++ = high frequency

Though *Aspergillus* species incidence was detected, the analysis performed to determine the occurrence of aflatoxin revealed that the toxin was produced, but in a very small concentration.

The maize samples which were analyzed through Elisa method using specific kits, have been classified as "negative" regarding mycotoxin production, all the samples concentrations being below the minimum legal value permitted for maize of 20 ppb (Figure 6).



Figure 6. Aflatoxin samples' concentrations (in ppb)

CONCLUSIONS

During storage period on wheat grains develop a series of microorganism, mostly represented by micromycetes.

When entering into storages there was identified the specific microflora for this period of time, respectively species of *Alternaria*, followed by species of *Cladosporium*, *Penicillium*, *Cephalosporium*, *Aureobasidium*, *Trichoderma viride*, and *Fusarium*. The maize infection started in field, during vegetation period, the grains being already infected before storage with saprophytic fungi such as *Alternaria*, *Cladosporium*, *Aureobasidium*, *Cephalosporium* and a low percent of *Fusarium* species. During the storage period, the field contaminating micromycetes development decreased, while developing specific storage phytopathogens such as *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp.

Incidence of *Aspergillus* species in stored maize samples determined aflatoxin mycotoxin production in small concentrations, below EU' imposed value limits.

Preventive measures, such as fast drying of maize for the medium and long-term storage in hygiene maintained warehouses, without the presence of insects and microorganisms, and proper regulation of grains moisture content, could significantly reduce the fungi contamination of maize grains.

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