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PRELIMINARY RESEARCH REGARDING STORED WHEAT GRAINS CONTAMINATION LEVEL

Roxana Dudoiu[1,2*], *Fătu Viorel*[1], *Carmen Lupu*[1], *Stelica Cristea*[2], *Daria Popa*[1]
[1]Research Development Institute for Plant Protection, Bucharest
[2]University of Agronomic Science and Veterinary Medicine, Bucharest

[*]Correspondence address:
Bd. Ion Ionescu de la Brad nr.8,CP 013813,s 1,
Bucharest, ROMANIA
Tel.: 004-021-2693231 (32,34)
Fax.: 004-021-2693239
E mail: roxyanna_21@yahoo.com

Abstract: 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 2014, the wheat 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 evolution /development of specific warehouse micromycetes during the first months of storage. Immediately after being deposited, it has been identified the specific micoflora for this period, respectively species *of Alternaria, Cladosporium, Aurobasidium, Cephalosporium* 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: *stored wheat, phytopathogen fungi, mycotoxin production.*

INTRODUCTION

Contamination of agricultural and food products with various toxigenic fungi is a problem of global concern. 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 (CAST, 1989). Outdating 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.

Micromycetes' development on stored grains is conditioned by temperature and atmospheric humidity present in stored areas and by its fluctuations in time. 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).

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

MATERIALS AND METHODS

Wheat grains production of 2014 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 three stages, immediately after harvest, after 60 days of storage and after 90 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

The pathogens were isolated using Ulster method and identified with optical microscope, after 12 days of growth. Using the Ulster method there can be identified the majority of seed pathogens, regardless thee 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 ºC), with enough space between the grains in order to allow the development of fungi or bacteria. The dishes were kept at room temperature (22-24 ºC) and normal light conditions. After 5-6 days it was examined the development of pathogens in Petri dishes and performed determinations at optical microscope. Potato-dextrose- agar medium was used for further isolation and purification of each fungus. 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 6 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 5 days.

Analysis of deoxynivalenol from wheat grains

Research in this stage was focused also on production of deoxynivalenol mycotoxin, metabolized by species of *Fusarium*. Determinations regarding the presence or absence of this mycotoxin were made by enzyme immunoassay using ELISA kits, namely RIDASCREEN FAST DON. The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with capture antibodies directed against anti-deoxinyvalenol antibodies. DON standards or samples solutions, DON enzyme conjugate anti-DON antibodies are added; Free DON and DON enzyme conjugate compete for the DON antibody binding sites, which represents the competitive enzyme immunoassay. At the same time, the anti-DON 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 chromogen 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 DON concentration from the sample.

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

Test protocol

- Samples preparation – the wheat 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 distilled water 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-DON 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 450nm with a StartFax 2000 spectrophotometer.

RESULTS AND DISCUSSIONS

The micoflora developed on wheat grains from the storages taken into analysis is represented by microorganisms like micromycetes, actinomycetes and bacteria. The researches were focused on micromycetes' study.

From the wheat 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, Aurobasidium, Trichoderma viride* and *Phythophtora infestans* (figures 1-5). *Alternaria* and *Cladosporium* fungi induce in the vegetation period an infection of the grains' apex, developing a "black point". Due to 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 2014 environmental conditions during the vegetation season, characterized by heavy rainfall and high temperatures, especially during kernels development, the wheat grains were not infected by a various micoflora.

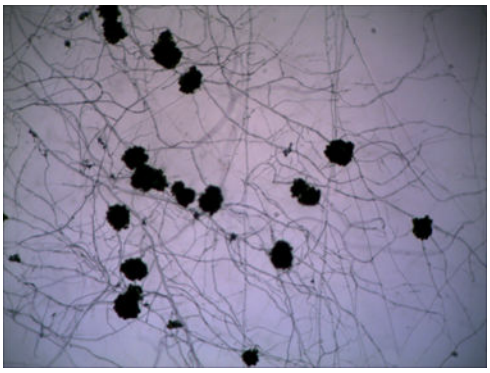

Figure 1. *Cladosporium spp*. (original)

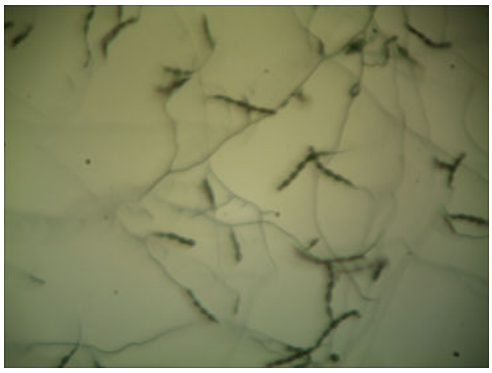

Figure 2. *Alternaria tennuisima (*original)

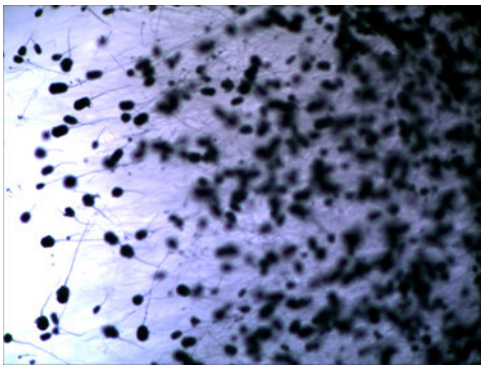

Figure 3. *Penicillium* spp. (original)

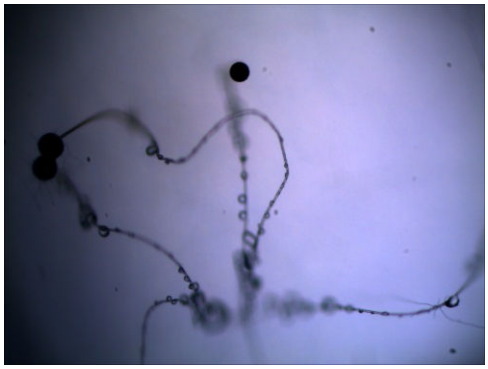

Figure 4. *Mucor pussilus* (original)

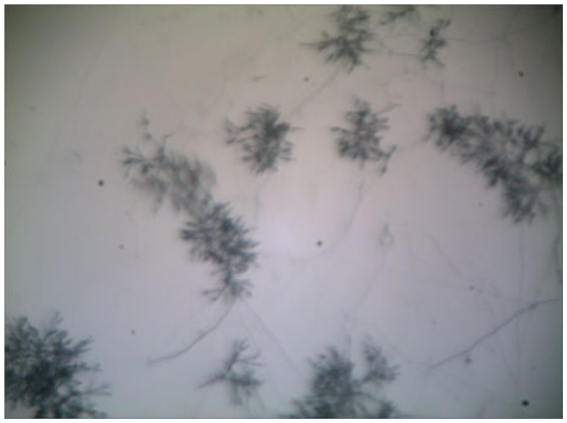

Figure 5. *Fusarium* spp. (original)

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. 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. It was also

shown that bacteria species percent remained constant during the 90 days storage period taken into study.

Though Fusarium species incidence was low, the analysis performed to determine the occurrence of deoxinivalenol toxin revealed that the toxin was produced but in a very small concentration. The wheat grains from several locations shown in figure 6, analyzed at the beginning of storage period 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 wheat of 1,7 ppm (figure 7). Further analysis will be performed during storage period to observe the concentration evolution for DON.

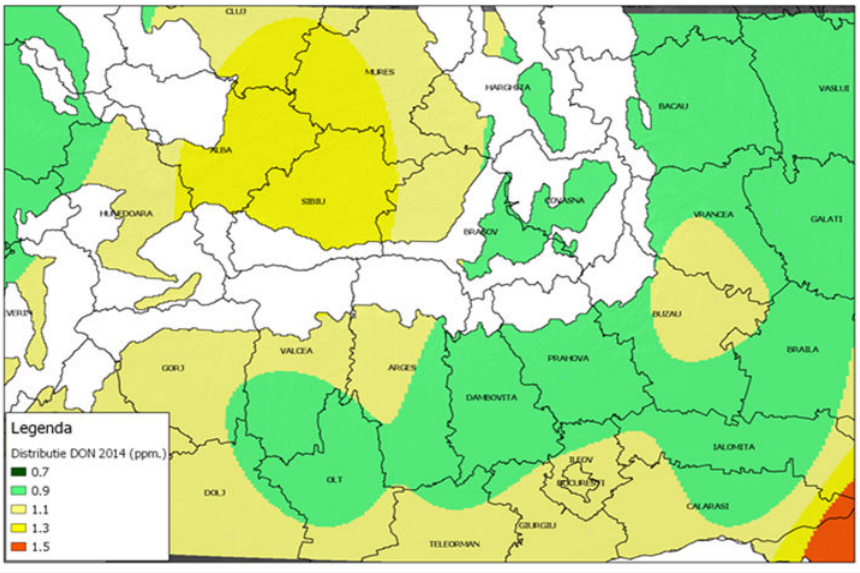

Figure 6. DON occurrence in samples locations

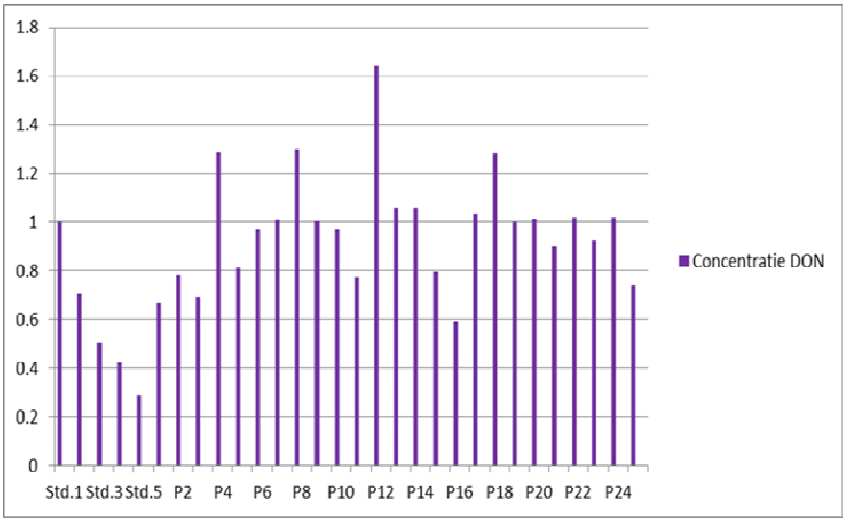

Figure 7. DON samples' concentrations

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 micoflora for this period of time, respectively species of *Alternaria*, followed by species of *Cladosporium, Penicillium, Cephalosporium, Aurobasidium, Trichoderma viride, Phythophtora infestans* and *Fusarium.* The wheat infection started in field, during vegetation period, the grains being already infected before storage with saprophytic fungi such as *Alternaria, Cladosporium, Aurobasidium, Cephalosporium* and a low percent of *Fusarium* species. After a 90 days storage period, the field contaminating micromycetes development decreased, while developing specific storage phytopathogens.

Low percent development of Fusarium species during analyzed period determined deoxynivalenol mycotoxin production in small concentrations, below EU' imposed value limits.

Crops safety and security can be achieved by maintenance of climatic factors in storage areas, thus limiting the contaminating microorganisms' populations' level.

Table 1. Micoflora structure of wheat grains

Fungi	**Contamination level**		
	At harvest	60 days of storage	90 days of storage
Pathogenic			
Fusarium	+	+	++
Cephalosporium	+	++	-
Alternaria tennuissima	+++	++	++++
Cladosporium herbarum	+++	++	+
Trichoderma viride	++	++	-
Phythophthora	++	-	+
Gonatobotrys	-	-	+
Saprophytic			
Mucor pussilum	+++	++	+
Alternaria spp.	++++	+++	++
Penicillium spp.	++	++	++
Rodotorula	+	-	-
Periconia	-	-	+
Aurobasidium	+	+	-
Actinomycetes	++	++	+
Bacteria	++	++	++

Legend: + = low frequency; ++ = moderate frequency; +++ = incresed frequency; ++++ = high frequency.

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