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MYCOLOGICAL AND MYCOTOXICOLOGICAL EVALUATION OF GRAIN

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Abstract: Grain storage conditions affect its quality. In Lithuania, different types of farms have various harvesting, processing and storing conditions. Grain samples were tested from agricultural granaries of 3 different types with different grain storage conditions in Lithuania. During March-April in 2001 the investigation on mycological and mycotoxicological state of stored grain from different types of agricultural granaries were performed. Wheat (*Triticum aestivum* L.) samples (n = 33) were tested from small, medium and large granaries. Barley (*Hordeum distichon* L.) (n = 22) was tested from small and medium granaries. Considering this issue, 31 species of micromycetes ascribed to 8 genera were isolated and identified. The results obtained indicate that highest levels of micromycetes contamination are found in small granaries with good storing and drying equipment. Micromycetes of some species belonging to the *Penicillium* Link, *Aspergillus* Mich. Ex Fr., *Fusarium* Link, and other genera, are able to produce secondary metabolites - mycotoxins of various compositions that are toxic to plants, animals, and humans. The levels of mycotoxins zearalenone and ochratoxin A were established. The highest concentration of zearalenone and ochratoxin A were found in grains from small farm granaries.

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INTRODUCTION

Toxin-producing micromycetes are widespread in nature, and when occurring in grains they often reduce both the yield and the quality of grains. These micromycetes infect grain in the field before harvest or, if storage conditions are suboptimal, grow during storage [13]. Grain quality greatly depends on its storage conditions. Storage requirement violations can result in grain deterioration. Animals fed on such grain can fall sick or become intoxicated [3]. Toxins produced by microorganisms affect both people and animals [5, 6, 15].

High initial contamination of granaries and inadequate grain preparation for storing are the decisive factors of grain deterioration outbursts [25]. However, while storing for long periods, even with properly prepared grain its parameters can change due to a rise in humidity and temperature. These changes can be caused by inadequate ventilation, an increase in water concentration on the walls of the granary, biological heating and changes in the chemical composition of the grain stored [22]. In the case of critical granary conditions so called “vicious circle” of grain deterioration is triggered - accelerating grain moulding and bacterial damage [9]. This process can be eliminated by an improvement of sanitary conditions and proper preparation of the grain for storage. EC recommendations point out that the greatest risk of the grain deterioration arises in the countries with high relative air humidity. Special attention should be paid to safe storage of grain grown on individual farms. Grain deterioration is accompanied by changes in the percental composition of microorganisms, therefore, while evaluating

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fodder quality, its contamination micromycetes and bacteria should be taken into account [10].

Different countries have different grain quality evaluation criteria. They can be related to the amount of spores or mycotoxins and the overall bacteria contamination. Microbiological and mycotoxicological tests of grain are carried out by appropriate methods [2].

About 30–40% of all micromycetes can produce mycotoxins under favourable conditions [16]. *Fusarium*, *Aspergillus*, and *Penicillium* are the main mycotoxin-producing genera [3, 19]. Many species of micromycetes are not only recognised plant pathogens but are also sources of the important mycotoxins of concern in animal and human health [1, 17]. The enzyme-linked immuno-sorbent assay method is applied for the quantitative analysis of mycotoxins. The method is based on the antibody antigene interaction [4, 24]. The immunoassays provide sensitive, rapid and accurate monitoring of mycotoxins and are suitable for screening of large numbers samples [24].

Zearalenone is produced by the *Fusarium* spp. Its highest concentrations in cereal grain occur in Central Europe (Austria, Germany, France) [6]. Approximately 90% of the tested samples of grain from these countries contain zearalenone. Its content depends on the season and the geographical location and ranges from 0.008–0.30 mg/kg. Zearalenone is a phytohormone, possessing both anabolic and estrogenic properties. Its estrogenic properties can cause fertility problems in horses, cows and sheep [6, 12].

Penicillium verrucosum and *Aspergillus ochraceus* fungi produce ochratoxins [22], which is more often found and in higher concentrations in grain in Central and Northern Europe (Denmark, Germany, Poland) [10, 22]. A total of 19 out of 33 samples of fodder tested in Denmark contained ochratoxin A. Its concentration exceeded 0.2 mg/kg. Ochratoxin's concentration in Danish pork is from 0.1 to 1.3 µg/kg [8].

Ochratoxins have been found to be nefrotoxic, carcinogenic and immunosupressive [10, 22]. Acute poisoning is described by non-specific symptoms, such as weight loss, feed refusal, vomiting, and necrosis of epithelium of the stomach and intestines [9, 10].

The purpose of this study was to determine the micromycete species most frequently detected on wheat and barley, and levels of zearalenone and ochratoxin A in grain from small, average and large granaries in Lithuania.

MATERIALS AND METHODS

Samples. In March-April 2001, 33 samples of wheat (*Triticum aestivum* L.) and 22 samples of barley (*Hordeum distichon* L.) were randomly taken from 33 of 3 different types agricultural granaries with different grain storage conditions in Lithuania. Wheat samples (n = 33) were tested from small, medium and large granaries. Barley (n = 22) was tested from small and medium granaries. In Lithuania, different types of farms have various harvesting, processing and storing conditions. There are many 3 ha farms in Lithuania. The grain grown on them is usually used for farm needs and is stored in improper facilities under unsatisfactory sanitary conditions. There is no equipment for the drying of grain and its purification. The grain is fed to the farm animals, and the food product is used by the farmers.

Agricultural cooperatives have better equipped granaries; sanitary requirements, however, are often ignored. The grain produced by such farms is used for fodder.

Grain processing enterprises have good storage conditions and adequate grain processing equipment; the grain is used both for human consumption and fodder. Each sample was randomly taken by sampling methods from the granaries and sent to the Lithuanian Veterinary Academy.

Evaluation of grain quality. To evaluate the general hygienic quality of wheat and barley, sources were determined the number of colony forming units (cfu) per gram of grain. 1 g grain sample in water extract (1:1000) was spread onto standard Czapek agar in Petri dishes according to Kurasova *et al.* [11].

Isolation of micromycetes. Wheat and barley were analysed according to the methods described by Lugauskas *et al.* [14], Rabie *et al.* [18], Samson *et al.* [19], Smirnova *et al.* [21]. The analysis of each sample was performed; direct plating and dilution plating were applied for isolation of micromycetes. In the first case, with a sterile pincers, 10 grains laid onto standard Czapek agar with chloramphenicol (50 mg/l) in Petri dishes. In the second case, 10 g of grain were taken and placed in 100 ml of sterile water, shaken for 15 min. and a series of dilutions prepared from the obtained suspension. From each dilution series, 1 ml of suspension was drawn into Petri dishes and poured over with 15 ml (48ºC) of the same standart Czapek agar enriched with antibiotic. The dishes were kept for 10 days in a thermostat at a temperature 26 ± 2ºC. Pure micromycete cultures were isolated, cultivated in standard Czapek agar at temperature of 28ºC for 5–6 days, according to manuals [3, 14, 19, 20]. Detection frequency (%) of each identified genera was calculated.

Mycotoxins analysis. The wheat and barley samples were analysed by the ELISA (enzyme-linked immuno-sorbent assay) method [4, 27]. The RIDASCREEN® Zearalenone and RIDASCREEN® Ochratoxin A test kits ('R-Biopharm AG', Germany) were used for the analysis. Mycotoxins extraction and tests performed according to manufacturer's instruction.

Statistical analysis. The obtained results were processed using Prism 2.01 programme. Test results are statistically significant at $p < 0.05$.

RESULTS

In March-April the temperature is about 15–25ºC in the granaries and favourable conditions can form for the development of micromycetes.

The results of mycological tests indicate that grain stored in the granaries of small farms is strongly contaminated with micromycetes: wheat contained 31.37×10^3 cfu/g ($p < 0.05$) on average, compared to medium granaries - 50% bigger, compared to large granaries - 71% bigger (Fig. 1). Barley from small granaries contained 32×10^3 cfu/g ($p < 0.05$) on average, compared to medium granaries - 30% bigger (Fig. 1). Contamination of some samples from small farm granaries accounted for 76–79×10^3 cfu/g of wheat and 74–77×10^3 cfu/g of barley.

The wheat samples from medium granaries contained 15.93×10^3 cfu/g ($p < 0.05$) on average, compared to those from large granaries - 71% bigger. Barley from medium granaries contained 22.3×10^3 cfu/g ($p < 0.05$) on average.

The smallest number of micromycetes colonies forming units of 9.25×10^3 cfu/g was found in samples of wheat collected in grain processing enterprises.

The grain grown on different types of farms in Lithuania and stored in granaries were contaminated with *Penicillium* spp., *Mucor* spp., *Rhizopus* spp., *Alternaria* spp., *Fusarium* spp., *Aspergillus* spp. and other genera of micromycetes (Tab. 1). The most dangerous micromycetes belong to the genera *Penicillium*, *Fusarium*, *Aspergillus*. Up to 61% of wheat and 54% of barley from small farm granaries were contaminated with *Penicillium*, 18% of wheat and 17% of barley with *Fusarium*, 3% of wheat and 7% of barley with *Aspergillus*. Wheat from large granaries was less affected. The results for wheat in large granaries were *Penicillium* - 23%, *Fusarium* - 44% and *Aspergillus* - 1% less compared to wheat from small farm granaries. The results of wheat in medium size granaries were: *Penicillium* - 18%, *Fusarium* - 5% less compared to those from large granaries, *Aspergillus* - up to 3% wheat from for medium granaries; and *Penicillium* - 21%, *Fusarium* – 3% less compared to barley from small farm granaries, *Aspergillus* - up to 7% barley.

From wheat and barley 31 micromycete species ascribed to 8 genera were isolated (Tab. 2). The research results revealed that the most active in stored grain and excretion of mycotoxins fungal species were *Penicillium aurantiogriseum*, *P. expansum*, *P. verrucosum*, *P. viridicatum*, *P. oxsalicum*, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Alternaria alternata*. Some grain were contaminated by fungi of the *Fusarium* Lnk genus: *F. graminearum*, *F. moniliforme*, *F. oxysporium*, *F. solani*, *F. sporotrichioides*. Micromycetes of the last named species produce and excrete mycotoxins: deoxynivalenol, nivalenol, zearalenone, toxins T-2, HT-2 and others strongly influencing livestock.

The zearalenone content was 2.89 µg/kg in wheat from medium granaries and 5.01 µg/kg in wheat from small granaries (Tab. 3). Zearalenone content in grain from grain processing enterprises was only 1.77 µg/kg, amounting to 64.6% ($p < 0.025$) and 40% of samples from small and medium sized granaries. In wheat, the ochratoxin A content was 1.13, 1.78 and 3.19 µg/kg in grain from large, medium and small granaries. The number of samples contaminated from small and medium granaries was 63.5% and 34% bigger compared to those from large granaries.

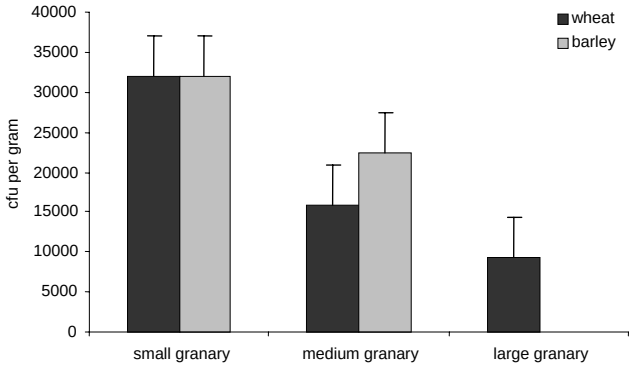

Figure 1. Wheat and barley mycological test.

Small amounts of mycotoxins were found in barley, zearalenone and ochratoxin A were present only in medium and small granaries. The content of zearalenone was 0.58 and 0.34 µg/kg, ochratoxin A was 0.92 and 0.37 µg/kg in medium and small granaries, respectively.

DISCUSSION

Micromycetes are the most important spoilage organisms in cereal grains. Fungi growth leads to reduced nutritional

Table 1. Dominating fungal genera in grain from different types of granaries in Lithuania.

Genera	Small granary	Medium granary	Large granary
	% of grain contaminated*	% of grain contaminated*	% of grain contaminated*
	Wheat		
Penicillium	61.0	43.0	38.0
Fusarium	18.0	13.0	10.0
Aspergillus	3.0	2.8	1.4
Mucor	21.3	19.8	11.3
Rhizopus	34.2	24.1	15.6
Alternaria	7.6	5.4	4.7
Other	5.6	5.1	4.5
	Barley		
Penicillium	54.0	33.4	-
Fusarium	17.2	13.7	-
Aspergillus	6.8	6.3	-
Mucor	18.8	18.6	-
Rhizopus	15.6	13.9	-
Alternaria	7.5	4.8	-
Other	6.1	5.1	-

* Average of 4 replications on the medium.

Table 2. Dominating fungal species isolated from wheat and barley.

Mucor hiemalis	*Fusarium graminearum*
M. racemosus	*F. moniliforme*
Rhizopus oryzae	*F. oxysporium*
R. stolonifer	*F. solani*
Alternaria alternata	*F. sporotrichioides*
A. tenuissima	*Penicillium aurantiogriseum*
Aspergillus flavus	*P. brevicompactum*
A. fumigatus	*P. chrysogenum*
A. niger	*P. digitatum*
A. oryzae	*P expansum*
A. raperi	*P. nalgiovense*
A. restrictus	*P. oxsalicum*
A. versicolor	*P. roquefortii*
A. pulcherus	*P. verrucosum*
Cladosporium herbarum	*P. viridicatum*
Eurotium chevalieri	

Table 3. Content of mycotoxins in wheat and barley.

Grain	% of samples with mycotoxins	Zearalenone µg/kg			Ochratoxin A µg/kg		
		Type of granary			Type of granary		
		small	medium	large	small	medium	large
Wheat	33	5.01	2.89	1.77	3.19	1.78	1.13
Barley	17	0.58	0.34	-	0.92	0.37	-

and technical quality of cereals grains [7]. Cereal grain inevitably leads to the contamination of the final feed by fungi [3]. The highest mycological contamination of wheat and barley were found in the samples from small farm granaries, which are not adequately equipped, have substandard sanitary conditions, and do not dry grain properly before storing it.

Under certain storing conditions, as a low initial content of fungi, low temperature and humidity, efficient ventilation, desinfection, the contamination of grain with micromycetes substantially decreases. This applies to medium and large sized granaries. The mycological contamination of wheat can be considered as good when fungi cfu is within the range 10^3–10^5 per gram [23].

In small farm granaries, however, wheat and barley grain were strongly contaminated *Penicillium* spp., *Fusarium* spp. and *Aspergillus* spp. In the case of their intense development on wheat and barley, micromycetes of certain species of the *Penicillium*, *Fusarium*, *Aspergillus* and other genera can produce mycotoxins that have a range of toxic, carcinogenic, mutagenic and teratogenic effects in animals and humans. Results of our research correspond to the results presented by other authors [1, 6, 8, 14, 15, 19]. Mycotoxins have a significant economic and commercial impact. Both the productivity and nutritive value of the infected cereal and forage is affected.

There are also higher concentrations of mycotoxins: zeralenone and ochratoxin A in small farm granaries. Our investigation showed the differences in the quality of wheat and barley grain stored in small, medium and large granaries.

The grain stored in small farm granaries of Lithuania is therefore unlikely to be hazardous to farm animals.

Proper sanitary conditions are essential in order to protect the stored grain from deterioration. Other determinants of stored grain characteristics are water activity (a_w), pH, gas composition in granaries, as well as insect activity [2, 3].

The most effective and cheapest prevention of fungi formation in grain is protection from moulding. Implementation of advanced grain storing technologies, regular quality inspections and the application of chemical conservants are essential [7, 13, 25].

CONCLUSIONS

1. Grain stored in the granaries of small farms is strongly contaminated by micromycetes: wheat contained 31.37×10^3 cfu/g on average, compared to medium granaries, were 50% bigger, compared to large granaries - 71% bigger; barley - 33×10^3 cfu/g on average compared to medium granaries - 30% bigger.

2. From wheat and barley 31 species of micromycetes were isolated and identified. Micromycetes species ascribed to the *Penicillium* spp., *Mucor* spp., *Rhizopus* spp., *Alternaria* spp., *Fusarium* spp., *Aspergillus* spp genera dominated.

3. The highest amounts of mycotoxins: zeralenone and ochratoxin A, are found in wheat samples from small granaries; zeralenone - 5.01 µg/kg, ochratoxin A - 3.19 µg/kg.

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