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Helena BIS[1], Krzysztof FRĄCZEK[1]
and Renata BROŻEK[1]

OCCURRENCE OF FUNGI AND INCLUDING TOXICOGENIC FUNGI IN STORED GRAIN SEEDS

WYSTĘPOWANIE GRZYBÓW W TYM TOKSYNOTWÓRCZYCH W MAGAZYNOWYM ZIARNIE ZBÓŻ

Abstract: Analyses for the present paper were carried out from October 2009 to February 2010. Grain seeds for microbiological analyses were sampled from various grain stores, both from granaries and from silos, in Brzeznica, Czernichow and Tomice communes in Malopolskie province, from individual farms and from agricultural schools.

Quantity of microscopic fungi – *Micromycetes* in the stored grain seeds was influenced by the following factors: seed moisture, store type, grain type and length of storage. 10 out of 22 isolated fungi belonged to potentially toxicogenic genera: *Alternaria alternata*, *Aspergillus candidus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium graminearum*, *Fusarium nivale*, *Fusarium oxysporum*, *Fusarium redolens*, *Penicillium citrinum*, *Trichoderma viride*. Metabolites of the following strains showed the strongest phytotoxicity: *Aspergillus flavus* – strain isolated from spring-wheat 'Bombona' cv., *Penicillium citrinum* – strain isolated from spring barley 'Poldek' cv. and *Fusarium graminearum* – strain isolated from winter-wheat 'Tonacja' cv.

Keywords: grain seeds, fungi, phytotoxicity

Grains are the major group of nutritional plants in the world. They are the most important food source for people all over the world due to favourable seed composition that contains almost all components necessary for the proper development of human organism [1, 2]. Grain seeds are also the most comprehensive concentrate for all types of livestock especially for poultry, pigs and horses, mainly due to high carbohydrates' concentration [3]. It needs to be stressed that every year as much as 4 % of the grain seeds production is loss caused by different factors, such as: improper storage, insufficient protection against pests and mould fungi [1, 4]. Danger to human and animal health, effects of mould fungi and mycotoxins depend mostly on grain seeds' storage. Therefore, the only action that allows for minimisation and reduction of this

[1] Department of Microbiology, University of Agriculture in Krakow, al. A. Mickiewicza 24/28, 30–058 Kraków, Poland, phone: +48 12 662 4181, email: rrfracze@cyf-kr.edu.pl

problem is proper agricultural engineering and proper conditions of seeds' storage as well as examination of material samples before its processing [5, 6].

Consequently, the objective of the research was to find the presence and to recognise the mould fungi – *Micromycetes,* with particular emphasis to potentially toxicogenic fungi in the stored grain seeds.

Material and methods

The research was carried out in the laboratory of the Department of Microbiology, University of Agriculture in Krakow, monthly from October 2009 to February 2010. Seeds for microbiological analyses were sampled from various grain stores, both from granaries and silos in the following communes: Brzeznica, Czernichow and Tomice in Malopolskie province from individual farms and from agricultural schools. The Table 1 shows the characteristics of the analysed objects.

Table 1

Characteristics of the analysed objects

Sample number	Form of grain	Cultivar	Sampling point (commune)	Owner of the store	Store type
1	Spring-wheat	Helia	Brzeznica	Individual farmers	Silo
2		Tybalt	Czernichow		Granary (pile)
3		Bombona	Brzeznica		
4	Winter-wheat	Rywalka			
5		Tonacja			
6		Turnia	Tomice	Agricultural school	Silo
7	Spring-barley	Stratus		Individual farmers	Granary (pile)
8		Poldek			
9		Sezam	Czernichow		
10	Oat	Dukat		Agricultural school	Silo
11		Hetman			
12		Krezus	Brzeznica	Individual farmer	

Grain seeds for the analyses were sampled with a cane probe, subject to principles of microbial purity.

During the experiment the samples were taken once in each month. Each grain sample was collected from the seed lot by random collection of small samples in various parts of the lot in different depths. Subsequently, they were integrated. Minimal number of primary samples was calculated as follows: square root was calculated from the number of tones of the loose statistical lot and then the result was divided into two and rounded up to the next integer. The average sample weighed about 1000 g mass and was placed in sterile box. Seed sampling was performed in accordance with the recommendations of Polish Standard PN-EN ISO 13690:2007. Moreover, each time

grain seeds' moisture was measured. The analysis was performed within 24 hours from the seed sampling. Mycological analyses were carried out with the serial dilution method. Fungi were cultured on the wort agar at 28 ^{o}C for 5 days and then pure cultures were isolated and inoculated on the diagnostic media accordingly to the standard requirements [7–10]. Fungi that are known to be potentially toxigenic were tested to check their ability to produce toxins. Consequently these isolates were inoculated on the liquid Eldrige's medium. They were cultured for 14 days at room temperature. The ability to produce toxins was evaluated in a biological test [11], using the testing plant – green peas seeds "Nike" ST93137(115)24G/C. Fungi strains were considered to be toxic when their metabolites inhibited 30 % germination energy of the tested plant in relation to the control plants.

Results and discussion

High quantitative differentiation of microscopic fungi – *Micromycetes* – was proved on the basis of mycological analyses of seeds of 12 grain cultivars. Table 2 presents the abundance of fungi in the tested seed samples.

Table 2

Abundance of microscopic fungi – *Micromycetes* in 1 g of seeds in the performed analyses

Grain cultivar	Number of cfu · g^{-1} of seeds				
	2.10.2009	4.11.2009	3.12.2009	6.01.2010	2.02.2010
Spring-wheat Helia	1200	167	100	100	26
Spring-wheat Tybalt	2000	4620	4237	3383	2413
Spring-wheat Bombona	1900	1047	693	793	580
Winter-wheat Rywalka	2400	1833	2800	1730	2357
Winter-wheat Tonacja	1163	757	617	657	503
Winter-wheat Turnia	650	300	117	150	43
Spring-barley Stratus	2717	1513	2807	1527	2630
Spring-barley Poldek	2440	1680	1547	1683	1024
Spring-barley Sezam	2543	2833	2033	1780	433
Oat Dukat	797	393	213	90	35
Oat Hetman	200	53	67	37	5
Oat Krezus	443	367	700	80	10

Quantity of mould fungi *Micromycetes* in the analysed samples ranged from 5 to 4620 cfu · g^{-1} of seeds, which proves that the admissible concentration of microscopic fungi was not exceeded in any of the tested seed samples. Evaluation of the obtained results was performed on the basis of the microbial contamination limit of the cereal grain proposed by ICC (*International Association of Cereal Science and Technology*). The general number of mould fungi on the seed surface according to the above standards should not exceed $3.0 \cdot 10^4$ cfu · g^{-1} of seeds. Figure 1 presents arithmetic

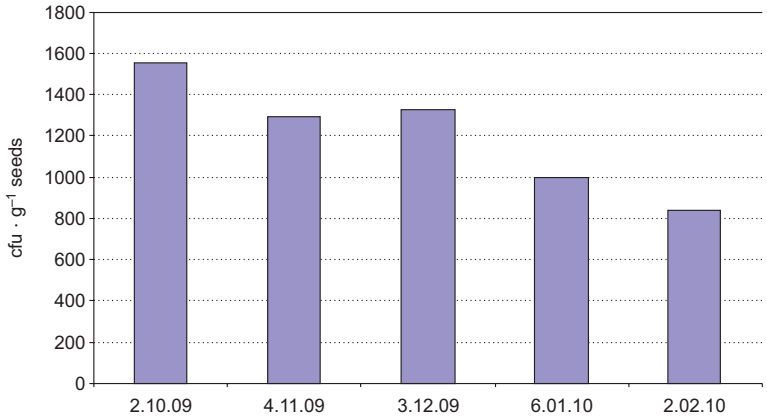

Fig. 1. Arithmetic mean of microscopic fungi – *Micromycetes* in different periods of analyses

mean of the *Micromycetes* fungi in each analysis. In October 2009 the abundance of fungi reached 1558 cfu · g^{-1} of seeds, which constituted 25.6 % of all fungi cultured during the whole test period (from October 2009 to February 2010). In November 1297 cfu · g^{-1} of seeds were found, which constitutes 21.6 % of all fungi from the test period. In December 1328 cfu · g^{-1} of seeds (22.1 %), in January 1001 cfu · g^{-1} of seeds (16.7 %), whereas in February 838 cfu · g^{-1} of seeds were cultured, which constituted 14 %. Based on the presented data it was ascertained that the abundance of fungi decreased with time.

Table 3 presents comparison of arithmetic mean of fungi abundance in 1 g of seeds with seeds' moisture.

Table 3

Comparison of arithmetic mean of fungi count in 1 g of seeds with seed moisture in each grain genus and cultivar

Sample number	Grain cultivar	Seed moisture [%]	Number of fungi in 1 g of seeds	Average
1	Spring-wheat Helia	9.3–13.1	319	
2	Spring-wheat Tybalt	19.5–21.4	3331	1551
3	Spring-wheat Bombona	15.3–16.7	1003	
4	Winter-wheat Rywalka	15.5–17.7	2224	
5	Winter-wheat Tonacja	11.5–13.4	739	1072
6	Winter-wheat Turnia	12.3–13.2	252	
7	Spring-barley Stratus	18.7–19.5	2239	
8	Spring-barley Poldek	16.1–17.9	1675	1946
9	Spring-barley Sezam	15.6–17.2	1924	
10	Oat Dukat	11.4–12.6	306	
11	Oat Hetman	12.3–13.7	72	233
12	Oat Krezus	12.8–13.4	320	

The highest arithmetic mean of fungi abundance in the whole research period – 3331 cfu · g^{-1} of seeds was found in 'Tybalt' spring-wheat sample. On the other hand the lowest abundance was found in oat sample – 'Hetman'. It reached 72 cfu · g^{-1} of seeds. The average abundance of fungi found in all analysed objects was 1200 cfu · g^{-1} of seeds. The results presented in the Table 3 indicate that the most contaminated grain was the spring-barley, and the average abundance of fungi in 1 gram of seeds was 1946 cfu. All cultivars of spring-barley sampled for microbiological analyses were taken from the pile in granaries from individual farmers. Oat was the least contaminated by microscopic fungi – *Micromycetes* – 233 cfu · g^{-1} of seeds. This amount definitely differs from the other amounts. All the oat seeds were stored in silos. In case of the spring-wheat fungi amount reached 1551 cfu · g^{-1} of seeds, whereas in winter-wheat it reached 1072 cfu · g^{-1} of seeds. About 66 % of seed samples from the analysed spring-wheat and winter-wheat were stored in the granaries (piles) that belonged only to individual farmers and the rest was stored in silos. As presented in the Table 2, comparison of arithmetic mean of fungi abundance in 1 g of seeds with seed moisture indicates that: with lower seed moisture, about 9.3–13.7 %, the abundance of microscopic fungi – *Micromycetes* – was lower than 1000 cfu · g^{-1} of seeds. In this moisture range the maximum amount of fungi (739 cfu · g^{-1} of seeds) occurred in winter-wheat 'Tonacja', which moisture ranged from 11.5 to 13.4 %. In case of increased seed moisture (15.3–21.4 %) the amount of fungi was a few to several times higher than in the previous group, reaching the maximum in the spring-wheat 'Tybalt' (3331 cfu · g^{-1} of seeds), which moisture was between 19.5 and 21.4 %.

Mycological analyses of 12 grain cultivars revealed presence of 22 fungi species. They belonged to the following genera: *Actinomucor* sp., *Alternaria* sp., *Aspergillus* sp., *Fusarium* sp., *Fusidium* sp., *Humicola* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *Rhodotorula* sp., *Torulopsis* sp. and *Trichoderma* sp. Table 4 presents the frequency of their occurrence.

Genus *Aspergillus* was definitely the most frequently occurring group of fungi where the predominant species were *Aspergillus niger* which infected 72 % of the analysed grain seeds and *Aspergillus fumigatus* (61 %). Also *Penicillium* genus was numerous with the predominant species *Penicillium citrinum* (39 %), and *Mucor hiemalis*, that infected 42 % of the analysed seeds as well as *Rhizopus nigricans* (47 %). *Actinomucor elegans* (5 %), *Humicola grisea* (5 %) and *Trichoderma viride* (5 %) were the least frequently isolated fungi. Predomination of each mould fungi changed in time. During five months of the research the share of genus *Aspergillus* increased from in about 15 % in October (the beginning of the analyses) to about 75 % in February 2010. The increase of *Penicillium* percentage was definitely less rapid, and was about 18 %. In case of *Fusarium* and *Alternaria* the situation was different and their percentage decreased by about 45 % and 56 %, respectively. As much as 10 out of 22 isolated fungi genera were recognised to be toxigenic. From the analysed seeds 210 potentially toxigenic strains were isolated and all of them were subject to the biological test. Only 55 % were found to be toxigenic. The tested strains inhibited from 3 to 93 % of germination energy of the tested plant and germination ability was reduced by 32 to 93 % in relation to the control plant. Metabolites of the rest of the strains did not show toxic properties. Metabolites of

Table 4

Frequency of each fungi species in the analysed grain cultivars

No.	Fungi species	Spring-wheat			Winter-wheat			Spring-barley			Oat		
		Helia	Tybalt	Bombona	Rywalka	Tonacja	Turnia	Stratus	Poldek	Sezam	Dukat	Hetman	Krezus
1	*Actinomucor elegans*						++						
2	***Alternaria alternata***	+	+++	+	++	++	+					+	+
3	*Alternaria geophila*	+	+++	++	+++	++	++					+	+
4	***Aspergillus candidus***									+++			
5	***Aspergillus flavus***	+		++	+++	+++		++		++			+
6	***Aspergillus fumigatus***	+	++	+++	+++	++	+++	++	+++		++	+	
7	*Aspergillus niger*		++	+++	+++	+++	+++	+	+++	+++	++	++	+
8	***Fusarium graminearum***		++			+		+++			++		++
9	***Fusarium nivale***		++					+			+		+
10	***Fusarium oxysporum***		+++			++		+++			++		++
11	***Fusarium redolens***		+			+		++			+++		+
12	*Fusidium* sp.		+							+++			+++
13	*Humicola grisea*					+						+	
14	*Mucor hiemalis*	+++		++		++	++	+	+	++		+	+
15	***Penicillium citrinum***		+	+	+	+	++	+++	+++	++			
16	*Penicillium frequentans*			+	++		+	+++	+	++			+
17	*Penicillium notatum*					+		++					
18	*Penicillium oxalicum*			+	++			+++	+	+++	+		
19	*Rhizopus nigricans*	+++	+++	++	+++		+	+	+		++		+
20	*Rhodotorula glutinis*				+		+	+	+				
21	***Trichoderma viride***					++							
22	*Torulopsis* sp.				+		+	+	+				

Explanation: +++ very frequent (67–100 %); ++ frequent (34–66 %); + occasional (0–33 %); potentially toxicogenic species were bolded.

the following strains showed the strongest phytotoxic effects: *Aspergillus flavus* – strain isolated from the spring-wheat 'Bombona' (reduced germination energy by 82 % and germination ability by 93 %), *Penicillium citrinum* – strain isolated from the spring-barley 'Poldek' (reduced germination energy by 72 % and germination ability by 81 %) and *Fusarium graminarum* – strain isolated from the winter-wheat 'Tonacja' (reduced germination energy by 69 % and germination ability by 89 %). Metabolites of the following strains proved to have the weakest effect: *Fusarium redolns* – strain isolated from the spring-barley 'Stratus' (reduced germination energy by 39 % and germination ability by 42 %), *Aspergillus fumigatus* – strain isolated from the winter-wheat 'Turnia' (reduced germination energy by 48 % and germination ability by 51 %) and strain *Fusarium oxysporum* isolated from oat 'Kresus' (reduced germination energy by 30 % and germination ability by 35 %). Metabolites of the rest of the strains appeared to be of medium toxicity. Mycotoxins that showed very strong toxicity in many cases not only inhibited but totally destroyed viability of peas seeds.

Parametric Pearson's correlation coefficient (r) and its statistical significance ($p \leq 0.05$) were calculated using Statistica software (v. 5.1 G) for Windows PL in order to evaluate the possible impact of environmental variables: moisture and storage on the number of microscopic fungi isolated from the analysed seeds. The statistical analysis revealed that the abundance of microscopic fungi was impacted by moisture. On the other hand statistically important relevance was not observed for the species of the analysed seeds and abundance of fungi on their surface.

Grain seeds, often collected in unfavourable weather, are kept in different grain stores where together with pollutants, pests and microorganisms they create an ecosystem of conserved and stored seeds. Proper care of such seeds bases on skillful inhibition of the seeds' life processes which are the major cause of the loss and seeds' damage [12]. As given by Trojanowska [13], mould fungi occurrence on seeds causes loss as much as 10–30 % of the whole material depending on the world region. Undoubtedly, seed moisture is one of the most important factors that influence seed storage conditions. The research proved that increased moisture of caryopses favoured the increase of pollution of seed samples with mould fungi. 15 % of seed moisture is the threshold above which mould develops due to fungi spore germination. This causes danger to the collected seed [14]. Mycotoxins produced by mould fungi are very dangerous to human and animal health. They cause damage of the most important organs: lungs, liver, kidneys, heart as well as nervous, circulatory, digestive and endocrine systems. They cause mucous membranes, placenta and skin damage. They perturb reproduction processes [15]. The only protection of seed against mycotoxins is its protection against mould fungi. There are no possibilities to remove mycotoxins from seeds or from feed. This is why the precaution and control of the basic parameters of seeds (especially temperature and moisture) as well as ventilation and seeds' cooling processes throughout the whole period of seed storage is so important [16]. This is the reason why it is so crucial that the seeds are free from unwanted microorganism contamination, especially from mould fungi and mycotoxins.

Conclusions

1. Occurrence of microscopic fungi – *Micromycetes* was found in the samples of the analysed grain material stored in silos and in granaries.

2. The abundance of microscopic fungi – *Micromycetes* in the stored grain seeds was influenced by the following conditions: seed moisture, store type, grain type and storage period.

3. Seed moisture played the crucial role during storage. The higher the moisture was, the higher number of fungi infected the seeds. Moisture of 13.5–14 % was considered the threshold for safe seed storage.

4. Analysis of the seed samples revealed the presence of 22 mould fungi species. They belong to the following genera: *Actinomucor* sp., *Alternaria* sp., *Aspergillus* sp., *Fusarium* sp., *Fusidium* sp., *Humicola* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *Rhodotorula* sp., *Torulopsis* sp. and *Trichoderma* sp.

5. As much as 10 out of 22 isolated fungi species were potentially toxicogenic: *Alternaria alternata*, *Aspergillus candidus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium graminearum*, *Fusarium nivale*, *Fusarium oxysporum*, *Fusarium redolens*, *Penicillium citrinum*, *Trichoderma viride.*

6. The biological test proved that 55 % of the analysed strains were toxigenic.

7. Metabolites of the following strains appeared to show the highest phytotoxic effect: *Aspergillus flavus* – strain isolated from the spring-wheat 'Bambona', *Penicillium citrinum* – strain isolated from the spring-barley 'Poldek' and *Fusarium graminearum* – strain isolated from the winter-wheat 'Tonacja'.

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WYSTĘPOWANIE GRZYBÓW W TYM TOKSYNOTWÓRCZYCH W MAGAZYNOWYM ZIARNIE ZBÓŻ

Katedra Mikrobiologii
Uniwersytet Rolniczy im. Hugona Kołłątaja w Krakowie

Abstrakt: Badania zostały przeprowadzone w okresie od października 2009 do lutego 2010 r. Próbki ziarna przeznaczone do badań mikrobiologicznych pobierano z różnych magazynów zbożowych zarówno podłogowych, jak i silosowych, występujących na terenie gmin: Brzeźnica, Czernichów i Tomice położonych w województwie małopolskim, z gospodarstw indywidualnych i szkół rolniczych.

Na liczebność grzybów mikroskopowych – *Micromycetes* w magazynowanym ziarnie zbóż miały wpływ następujące czynniki: wilgotność ziarna, rodzaj magazynu, rodzaj zboża i długość okresu przechowywania. Spośród 22 wyizolowanych gatunków grzybów, 10 gatunków należało do grzybów potencjalnie toksynotwórczych: *Alternaria alternata*, *Aspergillus candidus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium graminearum*, *Fusarium nivale*, *Fusarium oxysporum*, *Fusarium redolens*, *Penicillium citrinum*, *Trichoderma viride*. Najsilniejszym oddziaływaniem fitotoksycznym odznaczały się metabolity następujących szczepów: *Aspergillus flavus* – szczep wyizolowany z pszenicy jarej 'Bambona', *Penicillium citrinum* – szczep wyizolowany z jęczmienia jarego 'Poldek' oraz *Fusarium graminearum* – szczep wyizolowany z pszenicy ozimej 'Tonacja'.

Słowa kluczowe: ziarno zbóż, grzyby, fitotoksyczność