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Adaptation of the quantitative PCR method for the detection of the main representatives of cereal grain mycobiota

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ABSTRACT

The content of fungal DNA and mycotoxins in cereal crops (31 varieties of wheat, oats, and barley) was quantitatively determined and used for the comparative characterization of grains. The quantitative PCR has been adapted for the analysis of the target DNA of *Alternaria* spp., *Bipolaris sorokiniana (B. sorokiniana)*, *Fusarium graminearum (F. graminearum)*, *F. culmorum*, and *F. sporotrichioides* fungi, which are often present in the mycobiota of small grain cereals. The content of the DNA of the aggressive pathogen *B. sorokiniana* was determined using quantitative PCR for the first time.

The DNA of *Alternaria* fungi was found abundantly in all grain samples, but its content in the oat was significantly higher compared to barley and wheat (5 and 9 times higher, respectively). In barley grain, the content of *B. sorokiniana* DNA was on average significantly higher than in the grains of oats and wheat. All of the analyzed grain samples contained the DNA of *F. graminearum* while *F. culmorum* DNA was found in 70% of the oat samples and in all of the samples of barley and wheat. Mycotoxin *deoxynivalenol* (DON) produced by these fungi was detected in all the analyzed cereal grains in a range from 77 to 4,133 μg/kg. The DNA of *F. sporotrichioides* was detected in 70% of oats and 50% of barley samples but was not found in wheat. The T-2 toxin produced by this fungus was detected in 45% of all samples within a range from 2 to 89 μg/kg.

The statistically significant positive correlation with the Pearson correlation coefficient (r) equal to 0.49 ($p<0.05$) was observed between the content of *F. graminearum* DNA and the amount of DON in the grain samples. Another significant positive correlation ($r=0.72$, $p<0.01$) was found between the DNA contents of *Alternaria* fungi and *F. sporotrichioides* in the grain samples. This leads to the suggestion that conditions for the growth of these fungi in grain substrates are similar.

INTRODUCTION

The microbiological quality of grain is generally characterized by the percentage of infected grains. However, fungi are able to penetrate into the inner tissues of grain to different depths and localize in glumes including the lemma and palea, in the aleurone layer, or completely infect the endosperm and germ. The depth of penetration into the grain depends on various factors (species of cereals, resistance, time of infection, environmental conditions, aggressiveness of fungus, etc.). Therefore, a percentage of infected grain, which is determined as a result of the mycological analysis, is usually not directly related to the grain colonization depth and content of mycotoxins that are produced by fungi [1, 2]. This problem is especially important in case of husked small grain cereals (oats and barley), since their glumes are often abundantly colonized by different fungi, while the aleurone layer and endosperm are only slightly contaminated. In addition, the duration of the analysis of the fungal infection of the grain by the microbiological method (7-14 days) and the dependence of the results of the analysis from the subjective evaluation of the researchers add to the complexity of this method.

The quantitative analysis of the presence of pathogens in grain and the identification of the patterns of infection of the plants in different conditions (annual fluctuations of weather conditions and changes in agriculture technology) are of topical importance. The basic modern method of the quantitative analysis of the content of fungi in grain is a real-time or quantitative PCR (qPCR). This method is based on the use of fungal DNA fragments as molecular markers and allows estimating the quantity of target DNA for a certain pathogen or a group of similar pathogens [1, 3-6].

The use of the qPCR method allows revealing fungi in plant material, even if the pathogen has lost its viability and has not been detected by the traditional microbiological method. The amount of fungal DNA detected in grain, while being dependent on the biomass of the

fungus, is the most correct indicator of the presence of pathogen in grain. This parameter can be used for the reliable predicting of the presence of mycotoxins in grain. In addition, qPCR has some advantages over microbiological methods, such as the objectivity of quantitative assessment, high sensitivity, and speed of analysis as well as the possibility of simultaneous analysis of a large number of samples.

The goal of this study was the adaption of the qPCR method for the estimation of the DNA amount of the most common fungi in grain mycobiota: *Alternaria* Nees, *Bipolaris* Shoemaker and *Fusarium* Link.

MATERIALS AND METHODS

Samples of grain

In 2016, a variety of small grain cereals were sown at the Volosovo State Experimental Station (Leningrad region, Russia). These cereals were represented by 10 varieties of oats: Avatar (Russia), Borrus (Germany), Vsadnik (Russia), Zalp (Russia), KWS Kontender (Germany), Medved (Russia), Ozon (Germany), Privet (Russia), Stipler (Russia) and Yakov (Russia); 9 wheat varieties: Vellamo (Russia), Calixo (France), Leningradskaya 6 (Russia), Leningradskaya 12 (Russia), Leningradskaya 97 (Russia), Likamero (France), Marble (Canada), Sudarynya (Russia) and Trizo (Germany); and 12 varieties of barley: Bente (Germany), Despina (Germany), Inari (Finland), Krinichniy (Belarus), Leningradskiy (Russia), Moscowskiy 86 (Russia), Nord 132523 (Germany), Olympic (France), Salome (Germany), Suzdalets (Russia), Fest (Belarus) and Cherio (Denmark). All the seeds were not treated with any fungicides before the sowing. The cereals were cultivated according to the standard technology.

The 10 g of harvested grain samples of every variety were homogenized in sterile grinding cups using a Tube Mill Control (IKA, Germany) for 30 s at a rate of 20,000 rpm. The obtained flour samples were stored at -20°C. The subsequent isolation of the total DNA and extraction of mycotoxins from these samples were performed using 200 mg and 1 g of the corresponding flour, respectively.

Mycological analysis of grain

In order to identify the main groups of pathogens in grain mycobiota and to determine their DNA content using qPCR, the mycological analysis of six grain samples of different varieties (two of each individual species) was carried out. The 100- 200 grains of each sample were surface sterilized with 5% sodium hypochlorite solution for 1-3 min. Then, the grains were washed with sterile water and put into Petri dishes on potato sucrose agar (PSA) medium containing 1 µl/l of a mixture of antibiotics (HyClone™, GE Healthcare Life Sciences, Austria) that suppressed the growth of bacteria and 0.4 µl/l of Triton X-100 solution (Panreac, Spain) that reduced the linear growth of mycelial fungi [2].

After 7 days of incubation in the dark at 24°C, the species composition of fungi was identified and the number of colonies counted. The taxonomic status of isolated fungi was determined according to the sum of their macro- and micromorphological features [7]. The fungal infection rate of the grain was calculated as the ratio of the number of grains from which these fungi were isolated to the total number of analyzed grains, and expressed as a percentage.

DNA isolation and qPCR

The total DNA from grain flour was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania) according to the modified manufacturer's protocol. Using the same kit, DNA was also isolated from the mycelium of typical strains of *Alternaria tenuissima* (*A. tenuissima*) (Nees et T. Nees: Fr.) Wiltshire (MFP556081), *B. sorokiniana* Shoemaker (MFG59013), *F. graminearum* (MFG58775), *F. culmorum* (Wm.G. Sm.) Sacc. (MFG102100), *F. sporotrichioides* Sherb. (MGF163303) grown on PSA. All the typical strains of fungi are maintained in the Collection of the Laboratory of Mycology and Phytopathology of All-Russian Institute of Plant Protection (St. Petersburg, Russia).

The concentrations of DNA obtained from both the flour samples and typical strains were evaluated using a Qubit 2.0 fluorimeter with a Quant – iT dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). All 31 varieties of small grain cereals were analyzed by qPCR. The concentration of DNA isolated from the flour samples was leveled to 2-50 ng/µl. The native DNA solutions of representative strains of fungi were diluted to a concentration of 10 ng/µl and used to construct a calibration curve.

The DNA amounts of *F. graminearum* and *Alternaria* fungi were evaluated by qPCR with TaqMan probes. The reaction was carried out in a 20 µl volume containing 10 µl of a 2× TaqMan Master Mix (AlkorBio, Russia), 300 nM of each primer, 100 nM of a fluorescent probe (Evrogen, Russia) and 2 µl of the corresponding DNA solution. The DNA amounts of *F. culmorum*, *F. sporotrichioides* and *B. sorokiniana* were determined using qPCR with SYBR Green. The reaction was carried out in a 20 µl volume containing 4 µl of a 5× qPCRmix-HS SYBR Master Mix (Evrogen, Russia), 500 nM of each primer and 2 µl of DNA solution. The primer sequences and amplification protocols are presented in Table 1. The reactions were carried out on a CFX96 Real-Time System thermal cycler (Bio-Rad, USA), and the primary data were processed using Bio-Rad CFX Manager 1.6 software.

The amount of fungal DNA was expressed as a fraction of the total DNA isolated from cereal flour (pg/ng total DNA). The lower limit of detection of the fungal DNA content in the total DNA sample was set at 5×10^{-4} pg/ng of total DNA.

ELISA

The content of two mycotoxins – deoxynivalenol (DON) produced by *F. graminearum* and *F. culmorum*, and T-2 toxin produced mainly by *F. sporotrichioides* – were determined by enzyme linked immuno-sorbent assay (ELISA). Mycotoxins were extracted from 1 g of the flour sample by the addition of 5 ml of the mixture of acetonitrile and

Table 1. Sequences of primers and probes, qPCR amplification protocols used in the study

Target	Name of the primers and probes	Sequences of primers and probes, 5'– 3'	Amplification protocols	References
F. graminearum	TMFg12f	CTCCGGATATGTTGCGTCAA	95°C – 15 min; [95°C – 15 s; 60°C – 60 s]×40	[1]
	TMFg12r	CGAAGCATATCCAGATCATCCA		
	TMFg12p	FAM-GAGAATGTCTTGAGGCAATGCGAACTTT-BHQ1		
F. culmorum	FculC561f	CACCGTCATTGGTATGTTGTCACT	50°C – 2 min, 95°C – 10 s, [95°C – 15 s, 62°C – 60 s]×40	[3]
	FculC614r	CGGGAGCGTCTGATAGTCG		
F. sporotrichioides	PFusf	CCGCGCCCCGTAAAACG	95°C – 3 min, [95°C – 10 s, 60°C – 10 s, 72°C – 20 s]×40	[8]
	PSporR	ACTGTGTTTGCACACAGATC		
Alternaria spp.	DirITSAlt	TGTCTTTTGCGTACTTCTTGTTTCCT	95°C – 3 min; [95°C – 10 s; 60°C – 60 s; 72°C – 3 s]×40	[5, 9]
	InvITSAlt	CGACTTGTGCTGCGCTC		
	AltTM	FAM-AACACCAAGCAAAGCTTGAGGGTACAAAT-TAMRA		
B. sorokiniana	COSA_F	TCAAGCTGACCAAATCACCTTC	95°C – 3 min; [95°C – 10 s; 68°C – 20 s; 72°C – 45 s]×40	[10][a]
	COSA_R	CTTCTCACCAGCATCTGAATATATGA		

[a] The amplification protocol was modified.

water (84:16, v/v), and constantly shaken on the S-3M (ELMI, Latvia) at 300 rpm for 14-16 h. The analysis was performed using two diagnostic test systems for an indirect solid-phase competitive ELISA – "Deoxynivalenol-ELISA" and "T-2 toxin-ELISA" (Institute of Veterinary Sanitation, Hygiene and Ecology (VNIIVSGE), Russia) with a detection limits of 20 and 4 μg/kg, respectively. The standards of mycotoxins diluted with acetonitrile to concentration of 1 μg/ml (VNIIVSGE, Russia) were used to prepare the calibration curves. ELISA was carried out on polystyrene plates (Biomedical, Russia). The optical density of final solutions was determined spectrophotometrically on a LEDETECT 96 (Biomed, Austria) at 492 nm.

Statistical analysis

All of the laboratory tests were performed at least twice. The mean values with the standard error of the mean (M ± SEM) were calculated. The results were processed using the statistical software Microsoft Excel 2010 and STATISTICA 10.0. The relationship between the quantitative traits was evaluated using the linear Pearson correlation coefficient (r) at a significance level of $p<0.01$ or $p<0.05$. The coefficient of variation (CV, %) was defined as the ratio of the standard deviation to the mean.

RESULTS

Mycological analysis showed an abundant presence of *Alternaria* and *Fusarium* fungi in all of the grain samples (Table 2). The *B. sorokiniana* was detected in the oat and barley grain samples but not in wheat. In addition to these representatives of mycobiota, some other fungi were isolated from the grain samples: *Microdochium* Syd. & P.Syd., *Aureobasidium* Viala & G.Boyer, *Epicoccum* Link and *Trichothecium roseum* (Pers) Link.

Table 2. The fungal infection of the grain samples revealed by the mycological analysis

Species of small grain cereals	Variety	Grain infected by fungi, %						
		Fusarium	*Alternaria*	*Bipolaris*	*Epicoccum*	*Microdochium*	*Aureobasidium*	*T. roseum*
Oat	Borrus	66	42	4	4	0	3	0
	Vsadnik	56	31	9	2	0	2	0
Barley	Inari	84	18	10	0	0	7	0
	Krinichniy	89	24	9	0	0	6	0
Wheat	Sudarynya	56	26	0	2	9	0	2
	Trizo	61	18	0	1	4	0	0

The DNA of *Alternaria* fungi was detected in all of the analyzed grain samples. On average, the grains of different varieties of oats contained 5 and 9 times more DNA of *Alternaria* fungi compared with the samples of grain of barley and wheat varieties, respectively (Fig. 1). The smallest amount of *Alternaria* DNA was detected in the Triso wheat grain (184×10^{-4} pg/ng of total DNA), and the highest amount (11160×10^{-4} pg/ng) was found in the grain of oat of Ozon variety.

The quantitative estimation of *B. sorokiniana* infection of grain harvested in Russia was carried out using qPCR (Fig. 2) for the first time. In our study, the molecular primers that originally have been developed for the qualitative detection of DNA of this pathogen in grain [10], were successfully adapted for its quantitative analysis. According to the obtained results, *B. sorokiniana* DNA was detected in 100% of the barley and oats grain samples, but only in 56% of wheat grain samples. On average, the amount of *B. sorokiniana* DNA in the barley samples

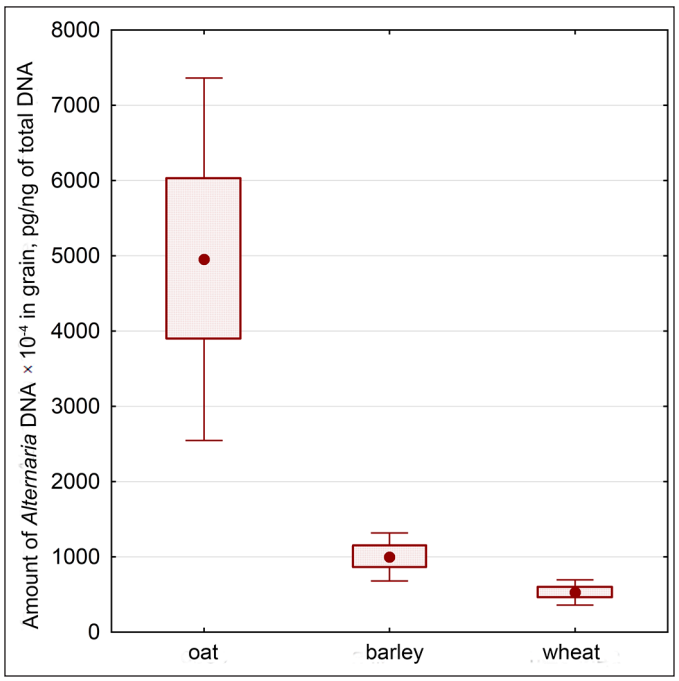

Fig. 1. Amounts of *Alternaria* DNA in oat, barley, and wheat grain samples. Data are presented as the mean value with the standard error of the mean (M ± SEM) and the confidence interval at a significance level of 0.05.

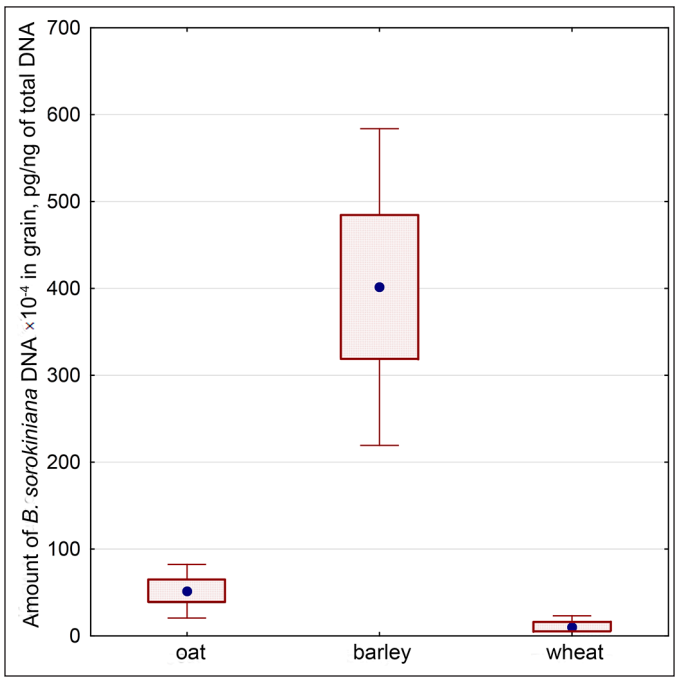

Fig. 2. Amounts of *B. sorokiniana* DNA in oat, barley, and wheat grain samples. Data are presented as the mean value and the standard error of the mean (M ± SEM) and the confidence interval at a significance level of 0.05.

Table 3. Amounts of *Fusarium* fungi DNA and mycotoxins in the grain samples

Analyzed fungus or toxin	Amounts of fungal DNA×10^{-4}, pg/ng of total DNA and mycotoxins, µg/kg					
	Oats		Barley		Wheat	
	n+[a], %	Mean (range)	n+, %	Mean (range)	n+, %	Mean (range)
F. graminearum	100	76 (14-261)	100	170 (22-1,110)	100	203 (55-561)
F. culmorum	70	21 (8-90)	100	17 (9-43)	100	22 (11-39)
F. sporotrichioides	70	17 (5-47)	50	6 (6-26)	0	0
DON	100	357 (9-666)	100	499 (77-2,154)	100	2318 (455-4,471)
T-2 toxin	40	7 (5-12)	67	22 (3-89)	33	18 (2-38)

[a] n+ – number of grain samples containing the DNA of the analyzed fungus or mycotoxin

was significantly higher compared to the grain of oats and wheat. The highest amount of *B. sorokiniana* DNA (648×10^{-4} pg/ng) was detected in the grain of Olympic barley.

DNA of *F. graminearum* was detected in the grain of all the analyzed samples (Table 3). The maximum amount of *F. graminearum* DNA was found in barley grain of Fest variety (1110×10^{-4} pg/ng). *F. culmorum* DNA was detected in 70% of oat samples and 100% of barley and wheat samples. The oats grain of Medved variety contained the highest amount of the DNA of this fungus (90×10^{-4} pg/ng). The DNA of *F. sporotrichioides* was not detected in any wheat grain samples but was found in 70% of the oat samples and in 50% of the barley samples. On average, *F. sporotrichioides* DNA was detected in lower amounts (up to 47×10^{-4} pg/ng) compared with the DNA of other species of *Fusarium* fungi.

The DON mycotoxin was detected in all the analyzed grain samples in the range from 77 to 4,133 µg/kg, while T-2 toxin was found in only 45% of the samples in amounts from 2 to 89 µg/kg.

A comparative analysis of the contamination of the grain with mycotoxins showed that the amount of DON in the samples of wheat was significantly higher than in the samples of oats and barley. The content of T-2 toxin in barley grain samples was 7 and 3 times higher compared with the samples of oats and wheat, respectively.

The minimum quantity of DON was found in Leningradskiy barley grain, while the wheat grain of the Calixo variety contained the highest amount of this mycotoxin. In some grain samples, a strong correlation between the high content of *F. graminearum* and *F. culmorum* DNA and a high amount of DON was observed. In some cases, the revealed levels of DON exceeded the maximum permissible concentration (MPC) of this mycotoxin – 700-1,000 µg/kg – the accepted standard for grain and grain-based food products [11]. The number of grain samples with the amount of DON exceeding the MPC reached 32%

(barley Moscowskiy 86 and Fest as well as all the wheat varieties except Leningradskaya 6). The amount of T-2 toxin in all the grain samples contaminated with this mycotoxin was below the established MPC (100 μg/kg) [11]. The maximum amount of T-2 toxin (89 μg/kg) was found in the grain of Cherio malt barley.

The statistical analysis of the experimental data revealed a significant positive correlation ($r=0.49$, $p<0.05$) between the amount of *F. graminearum* DNA and the amount of DON in grain samples. At the same time, there was not detected any correlation between the amount of *F. culmorum* DNA, which is another DON-producer, and the amount of this mycotoxin. It was noted earlier by Hofgaard *et al*. [12] that *F. culmorum* produced significantly smaller amounts of DON compared to *F. graminearum* [13].

Interestingly, a strong positive correlation ($r=0.72$, $p<0.01$) between the quantities of the DNA of *Alternaria* and *F. sporotrichioides* fungi was revealed, which implies that the conditions for their development on grain substrate are similar. In a previous study, we already showed that there is some symbiotic relationship between aggressive *Fusarium* fungi and relatively weak pathogens as *Alternaria* fungi, which are arising during the colonization of oat grains [14].

DISCUSSION

The fungi of the genus *Alternaria* have a broad host range but, the information about their harmfulness is controversial so far. In our study, we showed that the levels of contamination of studied varieties of small grain cereals with *Alternaria* fungi differ. However, among the same plant species the coefficients of variation (CV) of fungal DNA content were similar (41-68%), which indicates the absence of a differentiated interaction in the system "cereals – *Alternaria* spp.".

The *B. sorokiniana* is a harmful pathogen for cereal crops, especially for barley – it causes a decrease in grain germination, leaf spot, and root rot. According to the data of mycological analysis only oats and barley grains were infected by this pathogen, on the contrary to the analyzed wheat samples. The results obtained by qPCR confirmed a significant DNA amount of *B. sorokiniana* in barley grain that exceeded its content in the grain of oats and wheat 8 and 40 times, respectively.

The analysis of the DNA content of *F. graminearum*, *F. culmorum*, and *F. sporotrichioides* as well as the dangerous mycotoxins produced by these fungi were carried out. It was shown that the amount of *F. sporotrichioides* DNA in grain samples was low as well as the content of T-2 toxin produced by this fungus. Significant contamination of grains with DON, especially of wheat samples, was associated with a high amount of *F. graminearum* DNA detected in these samples. A reliable correlation between the content of the fungal DNA and that of the mycotoxin produced by this fungus enables to use the qPCR method for the quick characterization of breeding material. Therefore, this method can be successfully used when creating new resistant varieties, for assessing the fungicides effectiveness, and for monitoring agricultural commodities as well as foods and feeds for the presence of a fungal contamination. Quantitative PCR is a convenient and reliable method for the analysis of the relationship between plants and diverse representatives of mycobiota. This method allows detecting the content of pathogens in grain and to assess the effects of various conditions on their content over time and, therefore, helps determining the effectiveness of measures focused on plant protection from dangerous pathogens.

CONCLUSION

Quantitative PCR is an appealing technology for the detection of plant pathogens, but the detection of mycotoxin producers is particularly challenging. The adapted qPCR enables quantifying the presence of pathogenic fungi *F. graminearum*, *F. culmorum*, *F. sporotrichioides*, *B. sorokiniana*, and widespread representatives of *Alternaria* spp. in the harvested grain samples of different varieties of wheat, oat, and barley. The abundant content of *Alternaria* fungi, the ecological significance of which is still not precisely known, was found in all of the analyzed grain samples. The *B. sorokiniana* DNA was detected in the grain of all cereals; however, the amount of DNA of this pathogen in the barley grain was significantly higher compared with the oat and wheat grain samples. It was shown that the high DNA amount of *F. graminearum* in grain samples reliably correlated with the significant contamination of the grain with DON.

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CONFLICT OF INTERESTS

The authors declare no commercial or financial conflict of interest.

CITATION

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