ORIGINAL ARTICLE

Quantitative evaluation of *Fusarium* species and crop quality traits in wheat varieties of northeastern Poland

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Abstract

This research was conducted to investigate the natural, quantitative composition of the most common *Fusarium* species directly in fields of northeastern Poland. The concentration of *Fusarium* spp. and grain quality traits (yield, 1,000 kernel weight, test weight, grain moisture, ergosterol content, protein content, gluten content and starch content) were compared in four wheat varieties (Mandaryna, Struna, Kandela and Arabella). Obtained results indicated a relation between grain moisture, test weight, ergosterol content, yield and fungi concentration. Protein, starch and gluten content was similar in all wheat varieties. *Fusarium culmorum* was the most common pathogen in Mandaryna and Struna and *F. graminearum* in Kandela and Arabella. *Fusarium avenaceum* and *F. poae* occurred in low amounts in all wheat varieties except Mandaryna. *Fusarium oxysporum* was found in comparable concentrations in Struna, Kandela and Arabella. Struna despite medium *Fusarium* spp. colonization possessed the most desirable grain quality compared to other varieties. We carried out real-time PCR detection of *Fusarium* spp. which is an efficient, cost effective and time saving method in evaluating the development of fungal diseases which are not visible in standard observations.

Keywords: crop quality traits, *Fusarium*, quantitative detection, wheat varieties

Introduction

Fusarium is a genus of filamentous fungi causing the majority of plant diseases. Some of the species in central and eastern Europe (*F. culmorum, F. avenaceum, F. graminearum, F. poae* and *F. oxysporum*) are major crop pathogens which reduce grain yield and quality (Stepien *et al.* 2008; Kuzdralinski *et al.* 2014). Species distribution is related to climatic zones and most frequently occur in temperate climates with high moisture and temperatures ranging from 15 to 30°C (Muthomi *et al.* 2008; Saremi *et al.* 2013; Lenc 2015). Changes in climatic conditions influence the distribution of different species. For instance, in Croatia *F. graminearum* or *F. avenaceum* occur more frequently while *F. oxysporum* or *F. poae* are rarer than in Poland (Spanic *et al.* 2010). In Poland other *Fusarium* spp. have been

identified only during several outbreaks and do not possess a strong impact on cereal contamination or economic losses.

High fungal colonization may be associated with high moisture or ergosterol content (Kuzdralinski *et al.* 2014; Schmidt *et al.* 2017). The best wheat grains for bakery processes are above 72 kg · hl⁻¹ of test weight. Values exceeding 76 kg · hl⁻¹ indicate better endosperm development and flour efficiency. Mean starch content in wheat is 57–65%, gluten content 20–30%, protein content 9.5–14% and grain moisture up to 14.5% (Borght *et al.* 2005).

Fusarium head blight (FHB) is one of the most dangerous diseases of wheat, barley, rye, oat, triticale and corn contributing to economic losses (Fernandez

et al. 2005; Dweba *et al.* 2017). There are methods of molecular qualitative detection of fungal colonies grown on solid media (Divakara *et al.* 2013) including polymerase chain reaction (PCR), random amplified polymorphic DNA-PCR (RAPD-PCR) and restriction fragment length polymorphism-PCR (RFLP--PCR) (Kachuei *et al.* 2015) but it takes a few days to let them grow, delaying the analytical process. However, PCR based methods are also currently being used for pathogen identification from environmental samples. Generally, genetic qualitative identification methods take more time because they require post amplification analysis and allow verification of the presence of a desired species at the end point of the reaction. Real-time PCR enables qualitative or quantitative detection of desired species and track amplification in real time after each cycle. It is more sensitive, timesaving and results are more precise than qualitative methods. Less is known about the quantitative occurrence of *Fusarium* spp. in environmental samples. Results of *quantitative polymerase chain reaction* (qPCR) in Europe indicate that *F. culmorum* and *F. graminearum* are the most common species with concentrations up to 1,500 pg DNA $\cdot$ mg^{-1} dry mass and up to 185 pg DNA $\cdot$ mg^{-1} dry mass, respectively, 15 pg DNA $\cdot$ mg^{-1} plant sample of *F. poae* (Fredlund *et al.* 2008), and *F. avenaceum* up to 8.6 pg DNA $\cdot$ ng^{-1} plant DNA (Nicolaisen *et al.* 2008). In addition to *Fusarium* spp. in northeastern Poland *Puccinia recondita*, *Pyrenophora tritici-repentis*, *Septoria tritici*, *Helgardia herpotrichoide* and *Rhizoctonia cerealis* occur in lower amounts (Nugmanov *et al.* 2018).

The aim of this study was to examine and compare natural quantitative species composition of the most common *Fusarium* spp. directly in the fields of northeastern Poland in four wheat varieties. We also studied grain quality traits in the wheat varieties. The quantitative detection of *Fusarium* spp. which we proposed in our study makes it possible to determine fungi concentration in environmental samples taken from natural conditions.

Materials and Methods

Field experiment

Spring wheat (*Triticum aestivum* L.) seeds of four varieties (Mandaryna, Struna, Kandela and Arabella) were sown on April 1, 2017 (400 grains per 1 m^2) on experimental plots (4 × 5 m) under natural fungal infection conditions in Dobrzyniewo Duże, Podlaskie Voivodeship, northeastern Poland (53°11'43,6"N, 23°01'02,7"E). Each variety was grown in four repetitions under the same conditions. Mean temperature during the crop season was 16.2°C and rainfall, 292 mm. When full maturity was reached, in the BBCH 89 phase, grains from the four wheat varieties were manually collected on August 16, 2017. The ears were cut using sickles, 1 m^2 from the center of each plot. Harvested grains were separated from husks and each repetition was kept separately for use in further analysis.

Previously conducted physicochemical analysis of the soil revealed pH 7.4. The macroelement content of the soil was as follows: 18.9 mg $\cdot$ 100 g^{-1} K$_2$O, 19.3 mg $\cdot$ 100 g^{-1} P$_2$O$_5$ and 7.6 mg $\cdot$ 100 g^{-1} Mg. The concentration of organic carbon was 1.2%.

Yield and grain quality traits assessment

Grain quality was measured using Infratec 1241 Grain Analyzer (Foss, Hilleroed, Denmark), based on NIR/NIT technology. Yield (t $\cdot$ ha^{-1}) and traits which were evaluated included 1,000 kernel weight (g), grain moisture (%), test weight (kg $\cdot$ hl^{-1}), protein content (%), starch content (%), gluten content (%) and ergosterol content (mg $\cdot$ kg^{-1}). Grain traits were assessed randomly from 80 g of grains collected on the same day from 1 m^2 of each repetition separately within a variety. Means and standard deviations for each parameter were then calculated (Fig. 1; Table 1). Infratec 1241 Grain Analyzer is calibrated in its analysis and accuracy of all the mentioned grain quality traits according to Quality Management System (ISO 9001) and Quality Control (QC).

The wheat disease incidence at the milk-dough growth stage (BBCH 79–83) on the first and second leaves and stem was assessed. Visual disease evaluation was made by examining 25 plants collected randomly from plots according to the European and Mediterranean Plant Protection Organization (EPPO) scale (Nugmanov *et al.* 2018). Tan spot, leaf rust, Septoria leaf blotch, eyespot, and sharp eyespot were evaluated. Disease incidence was considered as: low <20%, moderate 20–40% and high >40%.

### DNA isolation from fungal cultures and wheat samples

Fusarium culmorum, *F. avenaceum*, *F. graminearum*, *F. poae* and *F. oxysporum* reference strains, obtained from the Bank of Plant Pathogens of Institute of Plant Protection (Poznan, Poland), were grown for 5 days at 23ºC on potato dextrose agar (PDA). Next, 100 mg of mycelium was scraped from the solid medium and DNA isolation was performed according to the CTAB (cetyl trimethylammonium bromide) method modified for filamentous fungi with NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer.

Grains from all experimental plots (4 g) were ground separately for each repetition in a mortar and 40 mg of flour was taken for DNA isolation. DNA was

Table 1. A comparison of mean grain quality traits in four wheat varieties

Grain quality traits [%]	Mandaryna	Struna	Kandela	Arabella	± SD
Proteins	10.22	9.50	9.62	9.82	0.31
Gluten	19.12	17.45	17.10	18.32	0.90
Starch	69.90	72.95	72.90	72.30	1.43
LSD (0.05)	ns	ns	ns	ns	

ns – no significant difference
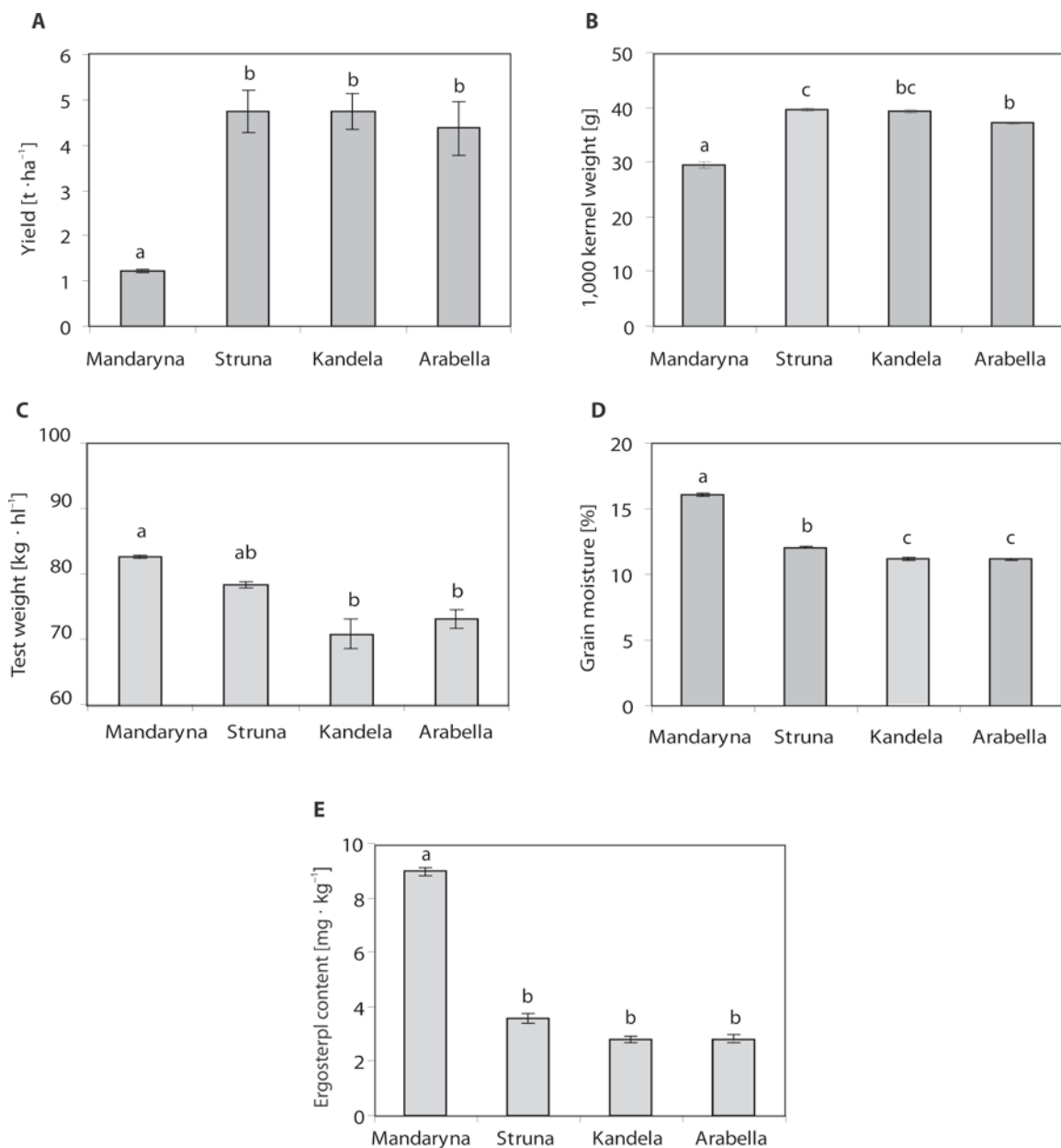
Fig. 1. Comparison of grain quality of Mandaryna, Struna, Kandela and Arabella wheat varieties: (A) yield of wheat varieties, LSD (0.05) = 1.09; (B) 1,000 kernel weight, LSD (0.05) = 2.46; (C) test weight of wheat varieties, LSD (0.05) = 8.32; (D) grain moisture of wheat varieties, LSD (0.05) = 0.652; (E) ergosterol content in wheat varieties [mg · kg^{-1}] LSD (0.05) = 1.002. Bars represent the means and ±SD. Values marked with the same letters do not differ significantly (0.05) according to Fischer's exact test

extracted using a modified CTAB method with Nucle-oSpin Plant II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Concentration and purity of the isolated DNA were measured in a Nanospectrophotometer P300 (Implen, Munich, Germany). Next, DNA wheat samples were diluted to the working concentration of 100 ng · µl^{-1}. Fungal and wheat DNA were kept at −20°C for further analysis.

Real-time PCR

Quantitative analysis of wheat samples was preceded by real-time PCR with serial dilutions of *Fusarium* spp. DNA as positive controls to assess accuracy and specificity in amplifying sequences of the *EF1-alpha* gene with primers (Table 2) designed in Primer3Plus software and with DNA isolated from sterile, *Fusarium* spp. free grains as negative control and wheat grains artificially inoculated. Fungal and plant real-time PCR products were purified with Clean-Up kit (A&A Biotechnology, Gdynia, Poland) and their concentrations were measured in a Nanospectrophotometer P300 (Implen, Munich, Germany). Standard curves in qPCR were achieved by 10-fold serial dilutions of fungal and plant qPCR amplicons in sterile water. After optimization, prepared standards were used in quantifying *Fusarium* spp. concentrations. To exclude the problem of nonspecific *F. cerealis* amplification with *F. culmorum* primers, we initially performed qPCR with primers specific for *F. cerealis* and DNA from wheat samples. However there was not sufficient amplification to determine the presence of this pathogen in grains.

Real-time PCR analysis was performed to detect and quantify the most common and naturally occurring *Fusarium* spp. found in northeastern Poland. Species tested included: *F. culmorum, F. avenaceum, F. graminearum, F. poae* and *F. oxysporum*. Reactions were performed in a Illumina Eco thermal cycler (Illumina, San Diego, USA) using specific primers (Table 2) hybridizing to the sequences of the *EF1--alpha* gene. The reaction mixture contained 10 μl 2× RT HS-PCR SYBR A Mix (A&A Biotechnology, Gdynia, Poland), 0.5 μM of each primer, 10 ng of the sample DNA and the mixture volume was adjusted to 20 μl using sterile water. PCR conditions were as follows: 40 cycles of denaturation at 95°C
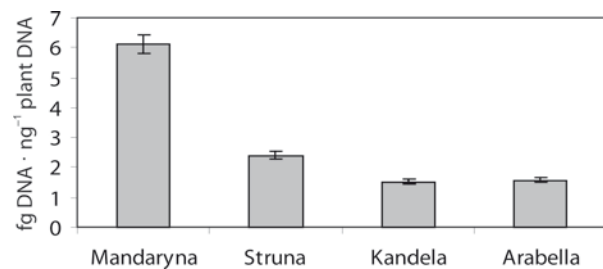
Fig. 2. Total *Fusarium* spp. concentration in Mandaryna, Struna, Kandela and Arabella wheat varieties (fg DNA · ng⁻¹ plant DNA); LSD (0.05) = 0.262. Bars represent the means and ±SD

for 15 s, annealing at 54°C (*F. culmorum, F. avenaceum*), 56°C (*F. oxysporum*), 58°C (*F. graminearum, F. poae*), 60°C (plant assay) for 1 min (for fungi) or 15 s (for plant assay) and elongation at 72°C for 15 s. To achieve reliable results, we analyzed four repetitions of each variety in triplicates separately. Next, obtained concentrations were calculated as fg fungal DNA · ng⁻¹ plant DNA based on plant *EF1--alpha* gene and repetitions were averaged for each wheat variety. Results are shown as total *Fusarium* spp. concentration calculated by finding the mean DNA concentration of five analyzed *Fusarium* spp. within a variety (Fig. 2) or presented separately for varieties (Fig. 4).

Statistical analysis

To assess statistical significance Fischer's exact test was performed. Values used in statistical analysis in tables and figures were calculated as means and standard deviations (±SD) from repetitions in Statistica 9.0 software. Statistical significance was established as p < 0.05. Least significant difference (LSD) and Pearson correlation (r) for some traits were calculated.

Table 2. Primers used in this study

| Species name	Primer name	Sequence (5'–3')	Amplicon size [bp]	References
Fusarium culmorum	cul-F cul-R	GTAATTTTTCTGGTGGGGCT AACTGATTGACACGTGATGG	104	this study
F. avenaceum	ave-F ave-R	ATTCATTACCCCGCTCAAGT TGTGGTAAGGTTTTGTGGGA	124	this study
F. graminearum	gra-F gra-R	TATCATTCGAATCGCCCTCAC GACAGGTGGTTAGTGACTGGT	201	this study
F. poae	poa-F poa-R	GCTAACATGCTTGACAGACC ATGGATCGAGGGAAAGTAGG	128	this study
F. oxysporum	oxy-F oxy-R	CATACTGACATCGTTTCACAG TAGCGGGTACGTTTCGAGT	156	this study
Triticum aestivum	Hor1f Hor2r	TCTCTGGGTTTGAGGGTGAC GGCCCTTGTACCAGTCAAGGT	63	Nicolaisen *et al.* 2008

Results

Of the tested wheat varieties, Mandaryna had the highest grain moisture (16%), the highest ergosterol content (8.98 mg · kg⁻¹) and test weight (82.65 kg · hl⁻¹), however, it had the lowest yield (1.22 t · ha⁻¹) and 1,000 kernel weight (29.49 g); ($p < 0.05$). The content of ergosterol was linked with the fungi concentration (Figs 1 and 2) in four varieties ($p < 0.05$; $r = 0.867$). High grain moisture is an essential factor of wheat crop colonization by *Fusarium* spp. directly in fields, which was confirmed by a high ergosterol level ($p < 0.05$; $r = 0.999$). Test weight (>72 kg · hl⁻¹) is an important feature in the evaluation of desirable crop quality in the baking industry and is associated with grain moisture. It is the highest in Mandaryna and the lowest in Kandela (Fig. 1). Comparing all parameters of the tested varieties, Struna, despite medium concentration of total *Fusarium* spp. (Fig. 2), possessed the most desirable crop quality traits (yield, 1,000 kernel weight, test weight) (Fig. 1). Other varieties had high moisture, ergosterol content, low yield, 1,000 kernel weight or test weight. Protein, gluten and starch contents were similar in the different varieties (Table 1). However, Mandaryna had the highest concentration of these traits except for starch, indicating that higher moisture is a crucial feature for correct synthesis of proteins and gluten.

In our experiment the *Fusarium* spp. concentration was the determinant of general and natural fungal colonization in the field. Our study found a link between grain moisture, ergosterol content, the amount of *Fusarium* spp., yield and test weight of the different varieties (Fig. 3; $p < 0.05$). Mandaryna had the highest grain moisture (16%), ergosterol content (8.98 mg · kg⁻¹), *Fusarium* spp. concentration (6.11 fg DNA · ng⁻¹ plant DNA) and test weight (82.65 kg · hl⁻¹) but the lowest 1,000 kernel weight (29.5 g) and yield (1.22 t · ha⁻¹). Struna had lower grain moisture (12%), ergosterol content (3.58%), *Fusarium* spp. accumulation (2.4 fg DNA · ng⁻¹ plant DNA), test weight (78.33 kg · hl⁻¹) and higher yield (4.75 t · ha⁻¹). A similar situation was observed in Kandela and Arabella with grain moisture (11.2% and 11.15%), ergosterol content (2.8 mg · kg⁻¹ and 2.83 mg · kg⁻¹), *Fusarium* spp. concentration (1.52 fg DNA · ng⁻¹ plant DNA and 1.56 fg DNA · ng⁻¹ plant DNA), test weight (70 kg · hl⁻¹ and 73 kg · hl⁻¹), 1,000 kernel weight (39.28 g and 37.15 g) and yield (4.75 t · ha⁻¹ and 4.38 t · ha⁻¹), respectively. Our results showed that disease incidence on leaves and stems was low and comparable between varieties (<20% for Mandaryna, Struna, Kandela and Arabella) thus did not have an impact on grain quality.

Differences in the susceptibility of varieties to *Fusarium* spp. (Fig. 4) were observed. In Mandaryna *F. culmorum* and *F. poae* (2.94 fg DNA · ng⁻¹ plant DNA and 1.4 fg DNA · ng⁻¹ plant DNA, respectively) occurred in the highest concentrations. Struna had *F. culmorum* (0.87 fg DNA · ng⁻¹ plant DNA) and *F. graminearum* (0.85 fg DNA · ng⁻¹ plant DNA) in the greatest amounts. In Kandela and Arabella *F. graminearum* occurred in the highest accumulation (0.54 fg DNA · ng⁻¹ plant DNA and 0.52 fg DNA · ng⁻¹ plant, respectively). *Fusarium oxysporum* occurred in comparable amounts in all the examined wheat varieties except Mandaryna (Fig. 4). *Fusarium culmorum*, *F. graminearum* and *F. oxysporum* were found in higher amounts in all four varieties. However, *F. avenaceum* and *F. poae* occurred in greater concentrations only in Mandaryna (0.79 fg DNA · ng⁻¹ plant DNA and 1.4 fg DNA · ng⁻¹ plant DNA, respectively).
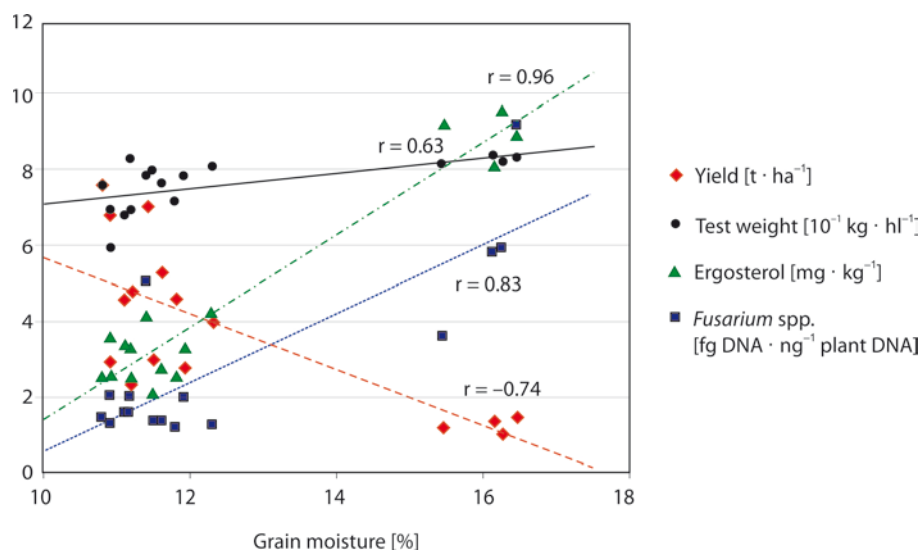
Fig. 3. Correlation diagram of examined traits in Mandaryna, Struna, Kandela and Arabella wheat varieties
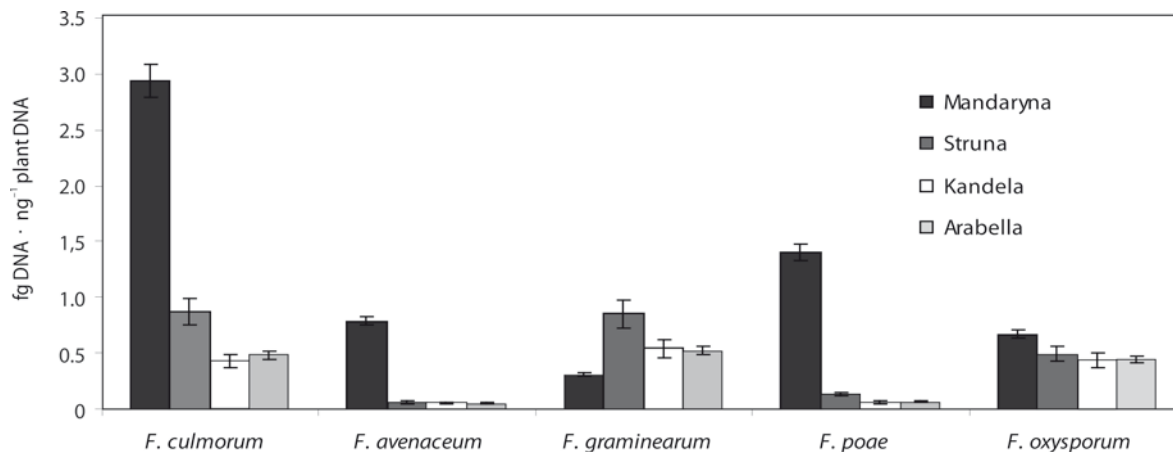
Fig. 4. Concentration of *Fusarium culmorum*, *F. avenaceum*, *F. graminearum*, *F. poae* and *F. oxysporum* (fg DNA · ng^{-1} plant DNA) in Mandaryna, Struna, Kandela and Arabella wheat varieties. Bars represent the means and ±SD

Discussion

In our study *Fusarium* spp. concentration was the determinant of general and natural fungal colonization in the field. Grain moisture is one of the factors which has the most influence on fungal growth (David *et al.* 2016). High grain moisture, ergosterol content and *Fusarium* spp. concentration are reflected in the lowest yield and 1,000 kernel weight indicating negative effects of crop fungi on these traits in four wheat varieties. Typical wheat yield in Poland is 6–8 t · ha^{-1}. In our results the yield for Mandaryna was 1.22 t · ha^{-1}, 4.75 t · ha^{-1} for Struna or Kandela and 4.38 t · ha^{-1} for Arabella. Lower yields are related to poorer soil quality (light, fifth class soil for Podlaskie Voivodeship) compared to western Poland. Poor soil conditions have the greatest effect on the Mandaryna variety as reflected in lower grain quality. Evaluation of fungal content in field samples is more problematic due to irregular crop colonization. However, disease incidence on leaves and stems of the tested varieties was low (<20%). Due to the direct, quantitative evaluation of *Fusarium* spp. naturally occurring in fields, it was not possible to inoculate cereals artificially because of irregularities in natural species composition. There is evidence that the effect of fungi colonizing wheat heads and causing grain yield losses depends on the wheat variety (Vergara-Diaz *et al.* 2015). This is confirmed in our results. In contrast, foliar and stem diseases do not have a strong impact on these traits (disease incidence <20% for all four varieties). The highest DNA concentration of *F. culmorum* and *F. poae* in the Mandaryna variety, *F. culmorum* and *F. graminearum* in Struna and Arabella or *F. graminearum* and *F. oxysporum* in Kandela is reflected by the same changes in grain quality traits indicating that a high concentration of a particular *Fusarium* spp. does

not affect crop quality but total *Fusarium* spp. accumulation causes decreased yield and 1,000 kernel weight. Our study revealed that wheat quality including starch and protein content, but not gluten, were in the range described by other authors (Borght *et al.* 2005) and did not differ significantly between varieties. Furthermore, internal features of wheat varieties or climatic and soil conditions may play important roles in the levels of these compounds (Wang *et al.* 2005). Generally, the amounts of *Fusarium* spp. found directly in the field from natural colonization are quite low and further investigation will be conducted to assess the influence of artificial inoculation of *Fusarium* spp. on the wheat grain quality under controlled conditions.

In different climatic zones distribution of *Fusarium* spp. is diversified. An overview of *Fusarium* spp. in northeastern Poland (Fig. 4) shows differences from other research. In Kenya *F. poae* and *F. oxysporum* (Muthomi *et al.* 2008) occur predominately, while in Denmark *F. poae* and *F. graminearum* (Nicolaisen *et al.* 2009) are predominant. *Fusarium asiaticum* may be nonspecific amplified with *F. graminearum* DNA, however outbreaks of this pathogen have been observed only in Asia, North America and western Europe but not in Poland (Bilska *et al.* 2018). There are even differences in areas with the same climate. Our research showed that *F. culmorum* and *F. graminearum* are predominant in northeastern Poland but in southern Poland *F. graminearum*, *F. avenaceum* and *F. poae* are seen the most often while *F. culmorum* is less frequent (Kuzdralinski *et al.* 2014; Kuzdralinski *et al.* 2017). Some research (Wisniewska *et al.* 2014) presents similar *Fusarium* spp. composition on wheat, indicating that there is a relationship between species and a particular local microclimate or soil conditions. Miscellaneous wheat varieties' susceptibility to *Fusarium* spp. may be the result of irregular natural

crop colonization and differences in grain moisture preferred by a particular species. The *Fusarium* spp. concentration and specific accumulation on wheat varieties may depend on relationships between different fungi species and varieties as well as soil parameters.

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