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INFLUENCE OF HUSK ON GRAIN CONTAMINATION BY *Fusarium* spp. AND *Alternaria* spp. IN HULLED SPELT (*Triticum spelta L.*)

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Abstract

Fusarium Head Blight is caused by several *Fusarium* species. Infections can result in mycotoxin contamination on cereals and associated foods. Harvested products are contaminated due to its secondary metabolites. The aim was to analyse the occurrence of spike *Fusarium* and *Alternaria* spp. in hulled *Triticum spelta* L. wheat species via polymerase chain reaction (PCR) method and the deoxynivalenol (DON) content analysis. Three varieties of spelt were used (Ceralio and Rubiota – winter and one spring form variety from genetics resources). Grains were sown in a randomized complete block design on organic certified experimental parcels during the years of 2011 and 2013. During the vegetation period plants were artificially inoculated with *Fusarium* spp. The occurrence of spike *Fusarium* and *Alternaria* spp. was assessed by the PCR method - DNA extracting and determination of *Fusarium* species and *Alternaria* spp. by the DNA markers and PCR method. DON content was analysed by ROSA®-DON Quantitative test. Strong infestation of grains with *Fusarium* spp. led to low contamination of grains with *Alternaria* spp. The technological operation of grain dehulling was performed and it was highly efficient there. The grain contamination by *Fusarium* spp. and *Alternaria* spp. decreased. Hulls protect grains to a certain point because of protection against *Fusarium* spp. and *Alternaria* spp. occurrence which produce harmful secondary metabolites. On the other hand the protection of grain by hulls only partly works. It is also important to pay attention to chemism of secondary metabolites in grain.

Key words: Alternaria spp., contamination, *Fusarium* spp., husk, wheat

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1. Introduction

Microorganisms commonly found in grain include filamentous fungi, primarily those from the genera *Aspergillus*, *Fusarium* and *Penicillium* (Ostrowska-Kołodziejczak et al., 2016). They are responsible for the accumulation of mycotoxins in grain (Zain, 2011). Currently, over 31,000 known metabolites produced by fungi occur in cereals. Some of these chemicals called mycotoxins are harmful to both humans and animals (AntiBase, 2005; Kuzdralinski et al., 2013). Mycotoxins produced by those pathogens lead to several health problems in humans and animals, and high mycotoxin concentrations in grain cause mycotoxicoses (Richard, 2007). Currently, there have been more than 400

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known mycotoxins (Kuzdralinski et al., 2013). The most studied mycotoxins are aflatoxins, trichothecenes, zearalenone, and ochratoxins (Binder et al., 2007; Filtenborg et al., 2000). Biological and chemical properties of mycotoxins vary, as well as their toxicity (Salem and Ahmad, 2010). Many of these compounds have oestrogenic, teratogenic, mutagenic and carcinogenic effects (Binder et al., 2007; D'Mello et al., 1999; Prelusky et al., 1993). Mycotoxins can enter the human body in different ways, mainly through the consumption of contaminated food (Berthiller et al., 2005; Salem and Ahmad, 2010).

Due to the great economic significance of wheat, particular attention should be paid to its health management as well as to the threat posed by fungal pathogens, including species of the genus *Fusarium* causing Fusarium head blight (Ostrowska-Kołodziejczak et al., 2016). *Fusarium* head blight is one of the most severe diseases of small grain cereals. Four *Fusarium* species, *F. graminearum* Schwabe, *F. culmorum* (W.G. Smith) Sacc., *F. avenaceum* (Fr.) Sacc and *F. poae* (Peck) Wollenw, have been identified as important toxigenic pathogens that affect the heads of small grain cereals (Miedaner et al., 2008; Pasquali and Migheli, 2014).

Cereals produced in temperate zone climatic conditions can be frequently contaminated with mycotoxins (Zachariasova et al., 2014). Due to the fact that in the Central Europe climatic zone the most toxigenic fungi are *Fusarium culmorum* and *F. graminearum*, the presence of these fungi and also of chemotypes 3-acetyl-deoxynivalenol (3-AcDON) and 15-acetyl-deoxynivalenol (15-AcDON) in grain is increasingly often analysed (Ostrowska-Kołodziejczak et al., 2016). In particular *F. culmorum* has been a prevalent species in several countries in Europe and the species has been predominating especially in regions that have cooler climatic conditions while *F. graminearum* has been predominant in relatively warmer regions. However, generalisation about geographic region distribution of *Fusarium* spp. has been interrupted by several investigations in Europe (Yli-Mattila et al., 2013; Yörük et al., 2016). Formally in Central and Central-Eastern Europe, Fusarium head blight was caused primarily by *Fusarium culmorum* (W.G.Smith) Sacc. (Parry et al., 1995). Nowadays *Fusarium graminearum* predominates (Yli-Mattila et al., 2013) which attacks wheat spikes in the flowering stage. As a result, the grain may be shrivelled and discoloured, with a high toxin content, especially trichothecenes of group B (Chelkowski, 1989; Perkowski et al., 2002).

Organic farming is an alternative to the conventional cultivation system providing farm products of high quality, referred to as organic (Jelínková et al., 2016; Maeder et al., 2002). In organic farming, fungicides are not used as prevention against fungal diseases (Janovská et al., 2015). The FAO report (FAO, 2000) concerning the content of mycotoxins in agricultural crops showed no clear differences between organic and conventional farming systems. It was stated however that in certain circumstances, such differences might occur. Most studies published after the FAO report (FAO, 2000) also failed to identify significant differences between organic and conventional cropping systems (Champeil et al., 2004; Cirillo et al., 2003; Jestoi et al., 2004). In some cases, the differences of mycotoxin levels between organic and conventional production systems, nevertheless, were reported (Knudsen et al., 1995; Kuzdralinski et al., 2013; Skaug, 1999; Woese et al., 1997).

The growing of resistant varieties is the best way to reduce *Fusarium* infection (Scholten et al., 2007). Bread wheat (*Triticum aestivum* L.) is the most frequent cereal species grown within the Czech organic farming system. Because in the Czech Republic are only available varieties bred in conventional breeding programmes, organic farmers use different species and crops (Konvalina et al., 2014). The information about the reaction of spikes of ancient wheat species to *Fusarium* spp. infection and toxin accumulation in grain is very important for growing systems limiting chemical plant protection. Hulled spelt wheat (*Triticum spelta* L.) is one from less bred among the *Triticeae* grown by farmers (Suchowilska et al., 2010). Information on the response of spelt cultivars to the infection by pathogens causing FHB is scant. Previous results (Wiwart et al., 2004) show that the response of this cereal to spike infection is slightly stronger than that of common wheat. Probably it is caused because of coverage of grain by hulls. Hulls can create more favourable conditions for *Fusarium* growth. But husks are removed from the grain before use and also an important part of contamination could be solved (Konvalina et al., 2011). There are many conflicting claims related to the role of husk as a protective factor against the secondary metabolites of *Fusarium* contamination. The example of *Alternaria* spp. contamination indicated significantly higher concentrations of Alternariatoxins in hulls than in dehulled kernels which implicate the possible protective effect of spelt wheat hulls (Vuckovic et al., 2013). Therefore, the contamination of the grain product, hulled wheat, by *Fusarium* and *Alternaria* toxins is an important question because of the safety of food and feed.

This study aimed to analyse the occurence of *Fusarium* and *Alternaria* species in organic and conventionally produced hulled and dehulled grain of spelt wheat by DNA markers and PCR (polymerase chain reaction) method and deoxynivalenol (DON) content analysis by ROSA®-DON Quantitative test. The second aim was to analyse the role of the hull factor as potential protection against *Fusarium* spp. and *Alternaria* spp. contamination.

2. Material and methods

2.1. Plant material and field experiments

There were used two varieties of winter spelt (Rubiota and Ceralio) and one variety (spring form)

from the collection of genetic resources. The organic field trials were carried out from 2011 to 2013 as randomized complete block designs with three replications. The seeding rate was adjusted to a density of 350 germinating seeds per 1 m^2. Plot size was 12 m^2. Crop stands were treated in compliance with European Council regulations EC No. 834/2007 and EC No. 889/2008. The weather conditions of the years 2011 and 2012 were favourable for the *Fusarium* spp. development due to high temperatures and precipitation in comparison to the long term mean. The year 2013 was deficient in precipitation. All materials were harvested at maturation stage from small-scale field plots at the Experimental and Research Station of the Department of Crop Production, Czech University of Life Sciences, Prague and at the experimental areas of the University of South Bohemia in České Budějovice.

2.2. Artificial inoculation

The isolates of *F. culmorum* and *F. graminearum* used for the artificial inoculation were obtained from the mycological collection of the Crop Research Institute in Prague and cultivated on sterile wheat grains. More detailed information on isolates can be found in Leišová et al. (2006). The preparation of inoculums for the application: wheat grains with the cultures of *F. culmorum* and *F. graminearum* were put into a vessel with water and shaken for 15 minutes in a laboratory shaker to release the spores into the water. The obtained suspension was filtered through the gauze. Then artificial inoculation was done with the suspension of *F. culmorum* and *F. graminearum* spores in the ratio of 1:1, 10^7 of spores/mL (Bürkerchamber was used for the verification of inoculum density), 2 litres of suspension per experimental plot (12 m^2). The suspension was dosed according to the list of variants with a hand sprayer at the beginning and at the end of the wheat flowering.

During experiments we also inoculated spikes by inoculum contained *Alternaria* spp. The ability of *Alternaria* spp. isolates to produce mycotoxine was proved before. Inoculation was made during milky rippeness, but was not sucessful. There were no differences between inoculated plots and plots only after natural infection.

2.3. Processing of samples

For the dehulling of hulled grain, samples were used Wintersteiger LG 180 (Wintersteiger, Ried, Austria) laboratory thresher. For the analysis were milled both – grain covered by hulls and dehulled (naked) grain. For the milling was used laboratory mill PSY MP 40 (holes in the sieve 0.8 mm).

2.4. Evaluation of spike Fusarium occurence by the PCR method

DNA both from a mycelium of all tested fungi and from infected grain samples was extracted using DNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer instructions. The quality and the concentration of extracted DNA were verified electrophoretically in 0.8% agarose gel. DNA was visualized by ethidium-bromide and detected under a UV lamp. DNA extracted from infected seed samples was diluted to a concentration of 50 ng/μL using a GeneQuantPro spectrophotometer (Amersham, Cambridge, UK).

2.5. Species specific amplification

The markers specific to the species: *F. culmorum* (Fc), *F. graminearum* (Fg), *F. pseudograminearum* (Fpse), *F. poae* (Fp), *F. sporotrichioides* (Fsp), *F. equiseti* (Fe) and *F. avenaceum* (Fa) were borrowed from the literature (Aoki and O´Donnell, 1999; Demeke et al., 2005; Doohan et al., 1998; Leišová et al., 2006; Parry and Nicholson, 1996). Primers were designed based on the sequence of the ITS region of about 5 isolates of *Alternaria* spp. The sequence of primers is in Table 1.

PCR reactions were performed in a 15 μL reaction mixture (0.3 μM of each primer, 170 μM dNTP, 1x PCR buffer, 2 mM $MgCl_2$, 1U *Tth* DNA polymerase Biotools (DYNEX) and 50 ng of template DNA) in the cycler SensoQuest (Goettingen, Germany). The amplification products were separated in 1.6% agarose gel, stained with ethidium bromide and visualised under UV light. The size of the product was verified by comparing it with the size standard GeneRulerTM 100bp DNA Ladder (Thermo Fisher Scientific, USA).

Fusarium culmorum assay was used from a previous project (Leišová et al., 2006). *Fusarium graminearum* and *Alternaria* spp. specific primers for Real-time PCR were designed on the base of the sequences for elongation factor obtained from public databases using the Primer Express for Windows NT 1.5 software (Applied Biosystems, Foster City, CA, USA).

Table 1. The primer list

Name	***Forward***	***Reverse***
Fc92s1	TTCACTAGATCGTCCGGCAG	GAGCCCTCCAAGCGAGAAG
Fg	TTCCCTGGGCGCTCATC	GGCTTCCTATTGACAGGTGGTT
Fpse	CGGGGTAGTTTCACATTTCYG	GAGAATGTGATGAGGACAATA
Fp	CAAGCAAACAGGCTCTTCACC	TGTTCCACCTCAGTGACAGGTT
Fsp	AAAAGCCCAAATTGCTGATG	TGGCATGTTCATTGTCACCT
Fe	CATACCTATACGTTGCCTCG	TTACCAGTAACGAGGTGTATG
Fa	CAAGCATTGTCGCCACTCTC	GTTTGGCTCTACCGGGACTG
Asp	TGGTGTTGGGCGTCTTGTC	TAGGCCGGCTGCCAATTAC

In case of *F. graminearum* the assay contained also a MGB (minor groove binder) probe; in *Alternaria* spp. SYBR Green detection system was used because all *Alternaria* species should have been detected in one reaction without the necessity of species determination. The specificity of all primers and probes was verified in silico by blast analysis. After optimization, PCR reactions were carried out in a 25 μL volume consisting of either 1x TaqMan Universal PCR Master Mix (Life Technologies, Foster City, CA, USA; in case MGB probe was used) or SYBR Green master mix (Life Technologies, Foster City, CA, USA; for *Alternaria* spp. detection), 0.3 μM of each primer, 0.3 μM Taq Man MGB probe and 250 ng of template DNA. Real-time quantitative PCR was performed using the cycler ABI PRISM 7900 (Life Technologies, Foster City, CA, USA) in MicroAmp optical 96-well plates. Reaction consisted of 2 min at 50°C, 10 min at 95°C and 40 cycles of 95°C for 15 s and 60°C for 1 min. followed only in SYBR Green detection system by dissociation stage (95°C for 15 s, 60°C for 15 s and then a slow increase to 95°C). The Sequence Detection Software (Life Technologies, Foster City, USA) collected data for the reported dye every 7 seconds from each well, generating a fluorescence profile for each amplification. The threshold cycle (Ct) was recorded for each dye as the cycle at which the fluorescent signal, associated with an exponential growth of PCR product, exceeded the background fluorescence. Dilution series of *F. culmorum*, *F. graminearum* and *A. alternata* isolates - DNA (from 0.1 pg to 100 ng) was included in triplicate as standard in every real-time PCR experiment. Standard curves for all assayed fungi were generated by plotting the known DNA amounts against the Ct values calculated by the SDS software. Unknown samples were quantified from measured Ct values by interpolation using the regression equation derived from standard curves. Final results of fungal content in samples were expressed in micrograms per 100 mg of groats.

2.6. Deoxynivalenol (DON) content

At first, toxin was extracted from the sample (deionized water was used as a solvent). 100 μL of the extract was diluted in 1 mL of buffer. 300 μL of the diluted extract was applied on the strip (ROSA®-DON Quantitative test). Incubation of the strip - 10 minutes at the temperature of 45°C (ROSA®-M Incubator). Assessment of the test – by ROSA®-M Reader (results in ppb).

2.7. Statistical analysis

In case of evaluation of the presence of DNA of pathogen – based on quantification of the intensity of the luminous band in relation to the marker for the statistical analysis, we used the following scale: 0 = no infection, 1 = weak infection, 2 = medium infection, 3 = strong infection.

The results were statistically evaluated by the Mann-Whitney Test (by variable organic system vs. conventional system, winter variety vs. spring variety, *Alternaria*+natural infection vs. *Fusarium* spp. inoculation, dehulled grain vs. hulled spikelets) and Kruskal-Wallis ANOVA evaluation of factor of variety. The selected factors are displayed in figures with the statistical significance expression on the level $p \leq 0.05$. The calculation was done by the software STATISTICA 12.0 CZ (StatSoft, Inc. USA).

3. Results and discussion

Degree of grain contamination (qualitative) with various *Fusarium* spp. (F.) and relationships between the contamination degree and other evaluated factors were evaluated at first (Tables 2 and 3). Farming system (organic or conventional one) is a statistically non-significant factor there. *Fusarium avenaceae*, *culmorum*, *equiseti* and *poae* infected the grains but they were statistically non-significant in our research. *Alternaria* spp. also infected the grains (Table 3) grown under both farming systems. The difference in DNA of *F. culmorum*, *F. graminearum* and *Alternaria* spp. was also statistically non-significant. Results of the Mann-Whitney test are shown in Table 4.

The difference in the degree of grain contamination with *Fusarium* spp. and *Alternaria* spp. toxins between the organic and conventional farming systems has already been noticed and discussed by many authors. Numerous surveys comparing mycotoxin content in organic and conventional production systems have already been conducted. The mycotoxin contamination of organic food products was reported to be either more or less equal to that of conventional systems (Magkos et al., 2006). Birzele et al. (2000) showed that contamination of conventional samples of winter wheat with DON and ochratoxin A (OTA) was comparable to that of the organic samples. In another study in Germany, comparable results for DON were obtained (Lücke et al., 2003). A study conducted in France revealed that organic cereals were contaminated with a higher level of mycotoxins but less frequently than cereals from conventional production (Malmauret et al., 2002). However, findings are not unequivocal in general. Works giving evidence of organic plants being as infected with Fusarium Head Blight as the conventional ones prevail.

The factor of variety has already been studied and evaluated with various methods. Table 6 (results of Kruskal-Wallis ANOVA) shows the factor of variety did not have any impact on the grain contamination with toxins in our research. Results were statistically non-significant. There was a minimum difference in the degree of grain contamination with toxins between winter and spring varieties (Tables 2 and 3). The same result was shown in the analysis of DON content in grain (Table 5).

Table 2. Grain contamination by different *Fusarium* species related to different factors

Factor		***Fusarium***			
		avenaceae	***culmorum***	***equiseti***	***graminearum***
System	Organic	1.9 ± 0.9	1.7 ± 1.0	0.7 ± 1.0	1.2 ± 1.2
	Conventional	2.4 ± 0.7	1.8 ± 1.0	0.8 ± 1.1	1.2 ± 1.2
Type of variety	Spring	1.5 ± 0.8	1.3 ± 1.1	0.3 ± 0.7	0.8 ± 0.9
	Winter	2.4 ± 0.7	2.0 ± 0.8	1.0 ± 1.1	1.4 ± 1.2
Year	2011	2.3 ± 0.5	1.8 ± 0.7	0.8 ± 1.0	1.3 ± 1.4
	2012	2.5 ± 0.8	1.9 ± 1.1	0.8 ± 1.1	1.5 ± 1.1
	2013	1.7 ± 1.0	1.7 ± 1.1	0.8 ± 1.1	0.9 ± 0.9
Processing	Dehulled	1.9 ± 0.8	1.7 ± 1.0	0.4 ± 0.8	1.1 ± 1.2
	Hulled	2.7 ± 0.6	2.0 ± 0.8	1.6 ± 1.1	1.4 ± 1.3
Inoculation	Natural	2.0 ± 0.9	1.2 ± 0.8	0.4 ± 0.8	0.5 ± 0.7
	Fusarium spp.	2.0 ± 0.8	2.5 ± 0.8	1.0 ± 1.2	2.1 ± 1.0
	Alternaria spp.	2.5 ± 0.7	1.5 ± 0.5	1.0 ± 1.1	0.9 ± 1.1

Note: 0 = no infection, 1 = weak infection, 2 = medium infection, 3 = strong infection

Table 3. Grain contamination by different *Fusarium* species and *Alternaria* spp. related to different factors

Factor		***Fusarium***			***Alternaria spp.***
		poae	***pseudo-graminearum***	***sporotrichioides***	
System	Organic	1.9±0.9	0.0±0.0	1.1±1.1	2.4±0.5
	Conventional	2.1±1.0	0.0±0.0	0.9±0.9	2.4±0.5
Type of variety	Spring	2.3±0.9	0.0±0.0	0.9±0.9	2.3±0.5
	Winter	1.9±0.9	0.0±0.0	1.0±1.1	2.4±0.5
Year	2011	2.3±0.6	0.0±0.0	1.4±1.0	2.0±0.2
	2012	2.0±1.2	0.0±0.0	0.5±0.8	2.0±0.0
	2013	1.7±1.0	0.0±0.0	0.8±1.0	3.0±0.0
Processing	Dehulled	1.8±1.0	0.0±0.0	0.6±0.8	2.3±0.5
	Hulled	2.5±0.7	0.0±0.0	1.8±1.0	2.5±0.6
Inoculation	Natural	1.9±1.0	0.0±0.0	0.6±0.8	2.3±0.5
	Fusarium spp.	2.1±0.9	0.0±0.0	1.2±1.1	2.4±0.5
	Alternaria spp.	2.0±1.0	0.0±0.0	1.1±1.1	2.4±0.5

Note: 0=no infection, 1=weak infection, 2 = medium infection, 3 = strong infection

Table 4. Mann-Whitney Test by variable Organic system vs. Conventional system of growing (concentration of DNA of pathogen in µg/100mg of grain)

Variable	***Rank sum***	***Valid N***	***Rank sum***	***Valid N***	***U***	***Z***	***p-value***	***Z adjus-ted***	***2*1 sided exact p***
	ORGANIC		***CONVENTIONAL***						
F. culmorum	919	30	911	30	446	0.052	0.959	0.052	0.959
F. graminearum	922	30	908	30	443	0.096	0.923	0.096	0.923
Alternaria spp.	906	30	924	30	441	-0.126	0.900	-0.126	0.900

Note: marked tests are significant at p<0.05; U = U value, Z = Z value

Table 5. Concentration of DNA of *F. culmorum*, *graminearum* and *Alternaria* spp. and DON content in grain

Factor		***Fusarium culmorum (µg Fc DNA /100 mg of grain)***	***Fusarium graminearum (µg DNA Fg/100 mg of grain)***	***Alternaria spp. (µg DNA Asp/ 100 mg of grain)***	***DON (ppb)***
System	Organic	5.39±16.68	0.54±1.61	0.04±0.05	2327±2774
	Conventional	3.69±8.66	0.72±2.21	0.04±0.05	2070±2606
Processing	Dehulled	2.07±4.85	0.29±0.87	0.02±0.02	2170±2780
	Hulled	10.72±22.72	1.47±3.23	0.09±0.06	2210±2660
Year	2011	6.72 ±12.09	0.73±1.67	0.06±0.04	2268±2459
	2012	2.05±2.60	0.31 ±0.52	0.01 ±0.01	2514±2924
	2013	3.92±17.59	0.73±2.62	0.03±0.06	1933±2788
Type of variety	Spring	1.47±2.39	0.05±0.09	0.02±0.02	2196±2614
	Winter	5.77±15.46	0.86±2.24	0.05±0.05	2199±2725
Inoculation	Natural	0.036±0.07	0.01±0.02	0.04 ±0.05	2660 ±358
	Fusarium spp.	11.801±19.53	1.63±2.87	0.03±0.05	5337±1613
	Alternaria spp.	0.136±0.23	0.02±0.03	0.05±0.06	2700 ±285

Note: Mean ± Standard deviation

There was practically the same mean but within the samples were differences (high standard deviation). *F. avenaceae*, *culmorum*, *equiseti and graminearum* infected more winter varieties (statistically non-significant occurrence). Winter wheat varieties were more infected by Fusarium spp. than spring wheat ones – it was registered by Etzeriod et al. (2016), for instance. Table 3 shows winter varieties were more seriously infected with *Fusarium culmorum* and *Fusarium graminearum* in our case (they contained more DNA of these two toxins). As there was a high variability between tested samples, it was a statistically non-significant result and finding (Mann-Whitney Test – Table 7).

All spikes were seriously infected with Fusarium Head Blight during every performed inoculation in our research. On the other hand, the tested inoculation with *Alternaria* spp. was not successful under field conditions. The inoculation with *Alternaria* spp. was also tested on the other crops (cumin or colza) (Khan et al., 2012; Özer and Bayraktar, 2015). It was tested on wheat with success – under laboratory conditions (Vergnes et al., 2006). Therefore, it was tested statistically with the natural infection together. All these results are shown in Table 8. A statistically significant difference between *F. culmorum* and *F. graminearum* DNA infestation rate was confirmed via Mann-Whitney test.

Testing and evaluation of the degree of grain contamination with pathogen produced the following results – a middle strong infestation with *F. graminearum* and a strong infestation with *F. culmorum* (Table 1). The artificial inoculation had a minimum impact on the degree of grain contamination with *Alternaria* spp. DNA. It caused neither higher contamination of grains with that pathogen (Table 3), nor a higher concentration of *Alternaria* spp. DNA in grains (Table 4). It brought about an inverse effect (Table 4). Compared to the natural conditions (0.039 μg DNA Asp/ 100mg of grain), the inoculation of grains with *Fusarium* spp. produced a slight decrease of *Alternaria* spp. DNA level in grains (0.033 μg DNA Asp/ 100 mg of grain). Such a decrease of the grain contamination with *Alternaria* spp. was provoked by a competitive interaction of *F. graminearum*. They occurred on wheat plants in the field, and therefore competed for the same resources. In the experiment done by Saß et al. (2007), *Alternaria alternata* was clearly suppressed when growing together with *F. graminearum*.

Table 6. Kruskal-Wallis ANOVA – evaluation of factor of variety of spelt (*Triticum spelta* L.)

***Fusarium culmorum* (μg DNA Fc/100mg of grain)**			
Variety	*N*	*Sum of scores*	*Mean score*
Ceralio	18	633.0000	35.16667
Spring spelt	24	648.0000	27.00000
Rubiota	18	549.0000	30.50000
H (2, N = 60) =2.249430 p=0.3247			
***Fusarium graminearum* (μg DNA Fg/100mg of grain)**			
Variety	*N*	*Sum of scores*	*Mean score*
Ceralio	18	661.0000	36.72222
Spring spelt	24	602.0000	25.08333
Rubiota	18	567.0000	31.50000
H (2, N= 60) =4.652641 p =0.0977			
***Alternaria* spp. (μg DNA Asp/ 100mg of grain)**			
Variety	*N*	*Sum of scores*	*Mean score*
Ceralio	18	605.0000	33.61111
Spring spelt	24	614.0000	25.58333
Rubiota	18	611.0000	33.94444
H (2, N= 60) =3.173588 p =0.2046			

Table 7. Mann-Whitney Test by variable Winter variety vs. Spring variety (concentration of DNA of pathogen in μg/100mg of grain)

Variable	***Rank sum***	***Valid N***	***Rank sum***	***Valid N***	***U***	***Z***	***p-value***	***Z adjusted***	***2*1 sided exact p***
	WINTER		***SPRING***						
F. culmorum	1182	36	648	24	348	1.260	0.208	1.260	0.208
F. graminearum	1228	36	602	24	302	1.954	0.050	1.954	0.051
Alternaria spp.	1216	36	614	24	314	1.773	0.076	1.773	0.076

Note: marked tests are significant at p<0.050; U = U value, Z = Z value

Table 8. Mann-Whitney Test by variable *Alternaria* natural infection vs. *Fusarium* spp. inoculation (concentration of DNA of pathogen in µg/100mg of grain)

Variable	*Rank sum*	*Valid N*	*Rank sum*	*Valid N*	*U*	*Z*	*p-value*	*Z adjusted*	*2*1 sided exact p*
	NATURAL		**INOCULATION**						
F. culmorum	792	36	1038	24	126	-4.610	0.001	-4.610	0.000
F. graminearum	864	36	966	24	198	-3.523	0.001	-3.523	0.001
Alternaria spp.	1192	36	638	24	338	1.411	0.158	1.411	0.159

Note: marked tests are significant at p<0.05000; U = U value, Z = Z value

Table 9. Mann-Whitney Test by variable DEHULLED/HULLED (concentration of of DNA of pathogen in µg/100mg of grain)

Variable	*Rank sum*	*Valid N*	*Rank sum*	*Valid N*	*U*	*Z*	*p-value*	*Z adjusted*	*2*1 sided exact p*
	DEHULLED		***HULLED***						
F. culmorum	565	24	611	24	265	-0.464	0.643	-0.464	0.646
F. graminearum	552	24	624	24	252	-0.732	0.464	-0.732	0.468
Alternaria spp.	423	24	753	24	123	-3.392	0.001	-3.391	0.001

Note: marked tests are significant at p<0.05000; U = U value, Z = Z value

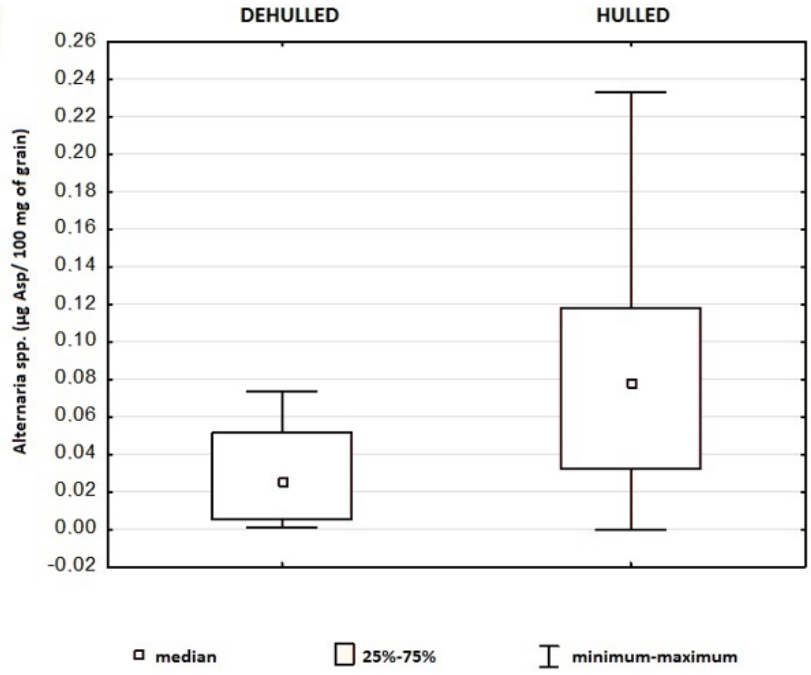

Fig. 1. Differences of grain contamination by the DNA of *Alternaria* spp. before and after dehulling (all the samples)

The factor of dehulling of the grain was studied and analysed in depth (when hulls were removed from spelt wheat spikes). It had the strongest and statistically significant effect (Mann-Whitney test – Table 9) and caused the contamination of grain with *Alternaria* spp. DNA to decrease (Table 4). Dehulled (peeled) grains were less contaminated with *Alternaria* spp. DNA (reduction from 0.09 µg DNA Asp/ 100mg of grain to 0.02 µg DNA Asp/ 100 mg of grain). Such a considerable reduction in the grain contamination with *Alternaria* spp. is shown in Fig. 1; the degree of contamination even decreased to zero in some tested samples. The dehulling of grain had a positive effect on individual years and inoculation options as well (Fig. 2). Released results of the research showed that hulls protected spelt kernels inside of *Alternaria* spp. from infection up to 50 %. The above-mentioned findings indicate that hulls efficiently protect spelt kernels from *Alternaria* spp. infestation and their toxicological metabolites (Vuckovic et al., 2013).

The technological operation of grain dehulling had a strong impact on the degree of grain contamination with *Fusarium* spp. DNA; it was, nevertheless, non-significant (Table 9), as there was a wide range and high variability of tested samples (Table 4). There was an evident lower degree of grain contamination with *Fusarium avenaceae*, *equiseti* (Table 1), *poae*, and *sporotrichioides* (Table 3). The degree of grain contamination with *Fusarium culmorum* DNA decreased from 10.72 µg DNA Fc/100 mg of grain to 2.07 µg DNA Fc/100mg of grain (Table 4).

The reduction in contamination with *Fusarium culmorum* DNA is shown in Fig. 3. Our research produced similar results with *Fusarium graminearum*;

the degree of grain contamination with *Fusarium graminearum* decreased from 1.47 µg DNA Fg/100 mg of grain to 0.29 µg DNA Fg/100 mg of grain (Table 5). The degree of grain contamination with *Fusarium graminearum* DNA decreased dramatically in 2013 (Fig. 4). Previous research had indicated that hulls acted efficiently as barriers to *Fusarium* mycotoxins in hulled *Triticum* species (Castoria et al., 2005; Suchowilska et al., 2010; Wiwart et al., 2004). Wiwart et al. (2011) compared *Fusarium* toxins in spelt and common wheat indicated that the concentrations of fungal metabolites were lower in spelt than in common wheat cultivars. There is a conclusion – dehulling can reduce contamination of fungi and toxins on spelt kernels. More surveys need to be conducted, considering the lack of knowledge on *Alternaria* toxins in food and feed in Europe and worldwide.

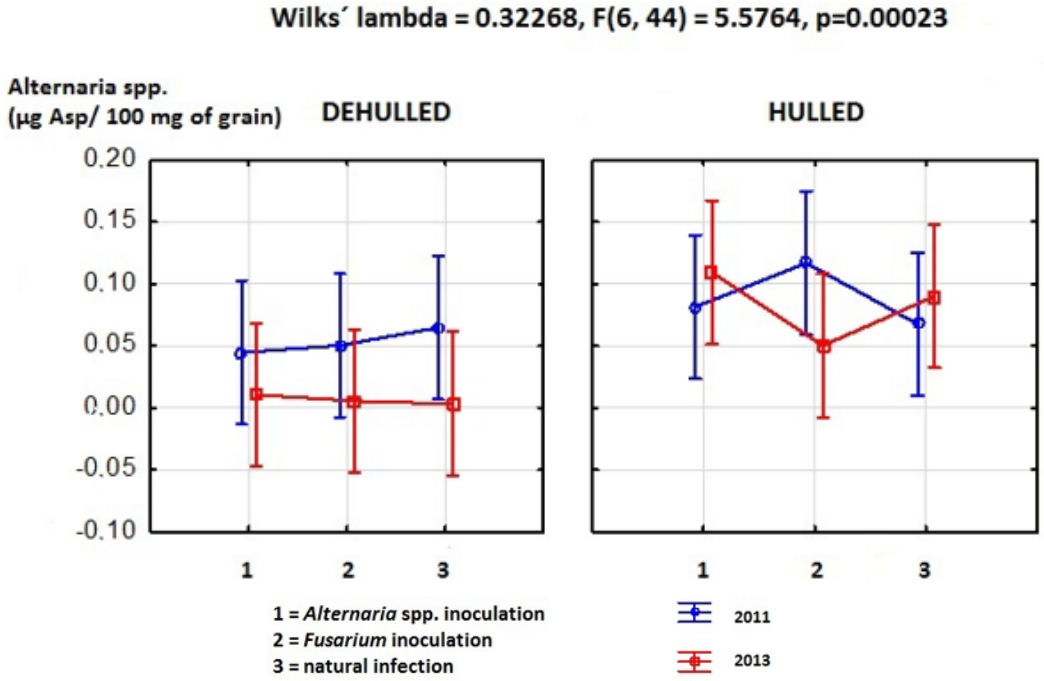

Fig. 2. Differences in grain contamination by DNA of *Alternaria* spp. in different years and grain treatment (results in 2011 were simmilar to 2012)

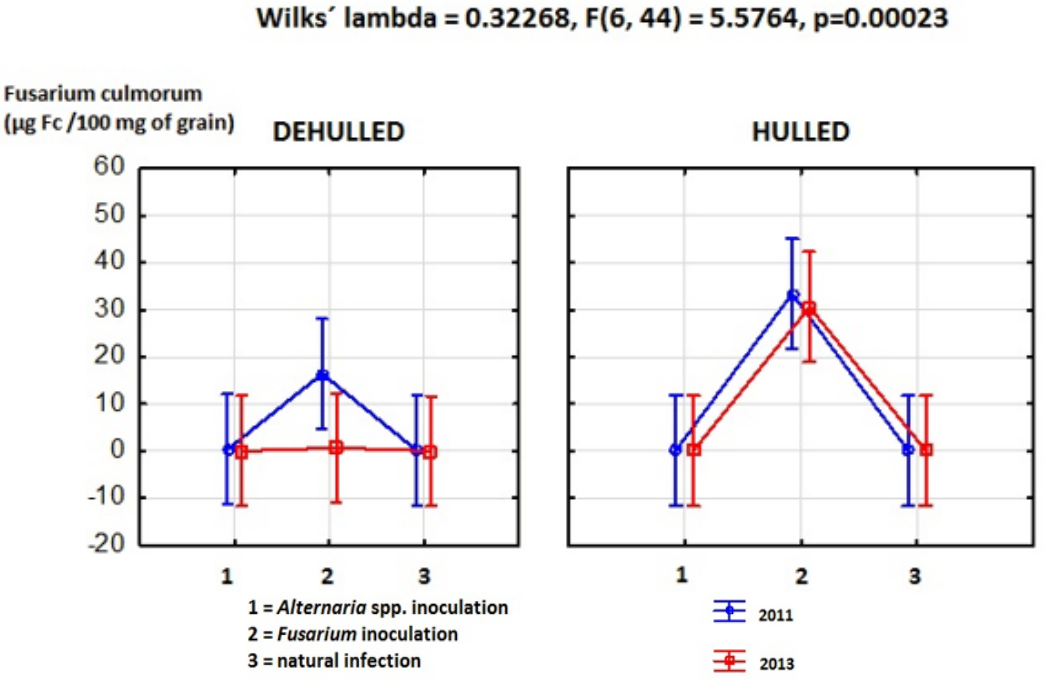

Fig. 3. Differences in grain contamination by DNA of *Fusarium culmorum* in different years and grain treatment

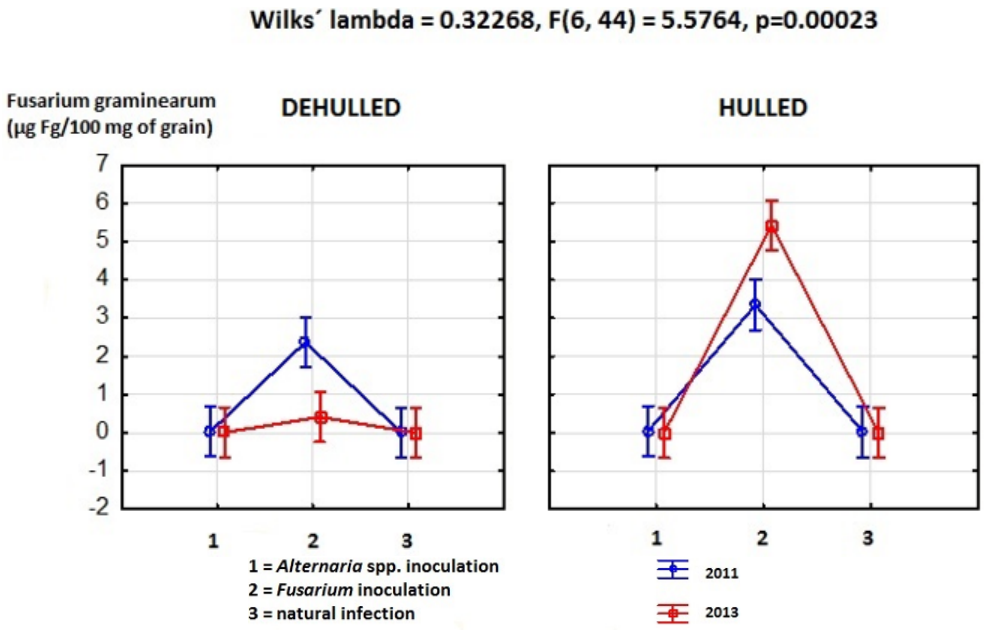

Fig. 4. Differences of grain contamination by DNA of *Fusarium graminearum* in different years and grain treatment

4. Conclusions

The degree of grain contamination with *Fusarium* spp. and *Alternaria* spp. was not influenced by the farming system. There were differences in the degree of grain contamination within individual years. Growing form (winter or spring) did not influence the degree of grain contamination either.

The artificial inoculation of grains with *Fusarium culmorum* and *graminearum* was successful. The natural infestation with *Alternaria* spp. was also studied and evaluated. The artificial inoculation with *Alternaria* spp. was not successful. Strong infestation of grains with *Fusarium* spp. led to low natural contamination of grains with *Alternaria* spp.

The technological operation of grain dehulling was performed and it was highly efficient there – the grain contamination with secondary metabolites and DNA of *Fusarium* spp. and DNA of *Alternaria* spp. decreased. The grain dehulling had a positive effect and reduced the degree of contamination of strongly infested varieties (that were infested artificially – by artificial inoculation). Therefore, it might be dangerous and risky to feed animals with whole spelt wheat spikes.

There are a lot of ambiguities about the biology of *Fusarium* ssp. – how they develop in hulls and grains. Much more experiments with *Fusarium* spp. and *Alternaria* spp. involved have to be carried out in order to make the protecting role of hulls clear – if and how they protect spelt wheat and other hulled wheat species from *Fusarium* spp. and *Alternaria* spp. and possible contamination.

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