ISSN 1392-3196 / e-ISSN 2335-8947
Zemdirbyste-Agriculture, vol. 101, No. 3 (2014), p. 285–294
DOI 10.13080/z-a.2014.101.037

Fusarium infected grain removal efficacy in cleaning wheat grain prior to milling

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Abstract

Four wheat cultivars with different levels of tolerance to Fusarium head blight (FHB) were grown in small plots and were inoculated with spores of *Fusarium* fungi during the anthesis. The harvested grain was cleaned by a special cleaner which separated it in 4 size fractions: F1 – >2.4 mm, F2 – 2.0–2.4 mm, F3 – 1.8–2.0 mm, F4 – <1.8 mm. These were further divided manually in four sub-fractions according to the rate of FHB symptom expression, i.e. grain without any visible symptoms, grain with changes in colour without changes in shape, grain with changes in colour and moderate changes in shape or size, and heavily infected malformed grain. Deoxynivalenol (DON) content in grain of different fractions was determined by high-performance liquid chromatography with UV detection (HPLC-UV) analysis. Comparisons between initial DON content before cleaning and DON content in individual fractions after cleaning were made. The cleaning efficacy (rate of DON content reduction) due to the cleaning and separating of grain in size fractions was higher in semi-tolerant and susceptible cultivars than in FHB tolerant cultivar. Due to the low cleaning efficiency, cleaned grain of tolerant cultivar (without any symptom of FHB), contained twice more DON (870–1350 μg kg^{-1}) than cleaned and apparently healthy grain of semi-tolerant cultivar (160–570 μg kg^{-1}); its DON content was comparable to that of the susceptible cultivar (905–1140 μg kg^{-1}). Our results indicate that FHB tolerant cultivars may contain a high proportion of grain which is apparently healthy, but contains excessive DON levels. Such tolerant cultivars can contribute a lot to minimisation of yield loss caused by FHB, yet they may present a potential health hazard of organic whole-grain flour produced in non-industrial grain processing systems.

Key words: deoxynivalenol, *Fusarium* spp., grain cleaning, organic whole-grain flour.

Introduction

Fusarium diseases of wheat, especially Fusarium head blight (FHB) caused by several species of fungi from the toxigenic genus *Fusarium*, are of great concern to cereal producers and cereal-processing industry worldwide and also in Lithuania (Mačkinaitė et al., 2006; Mankevičienė et al., 2006; 2007; 2011 a; b). The usual advice given to growers on how to cope with *Fusarium* diseases of cereals is to grow FHB resistant (tolerant) cultivars (Scudmore, 2005; Beev et al., 2011). Grain cleaning is another challenging area in the prevention of *Fusarium* mycotoxin contamination of final cereal products. Different technical solutions are available on a farm level and on the level of grain-processing industry. The efficacy of cleaning depends on the type of machine used, the type of cleaning process, the machine operating parameters, the severity of kernel infection, and the wheat cultivar processed (Dexter, Nowicki, 2003; Draganova et al., 2010).

This research was initiated by boutique millers and growers that farm wheat to produce organic whole-grain flour. The analyses of their cleaned grain that appeared to be visually totally healthy and free of *Fusarium* infection showed high deoxynivalenol (DON) content. When these millers and growers ground such grain into whole grain flour, DON content reached 1300–1600 μg kg^{-1}. This came as a surprise because low DON content is usually expected if healthy grain is processed. This is why we tried to determine the reason(s) for such high DON content.

The market demand for organic wheat grain for producing whole-grain flour at home is continually increasing. Indeed, a number of quite sophisticated mills are available for preparing whole-grain flour at home (e.g., NutriMill, VitalMill, KoMo Mill and the Country Living Grain Mill). Currently, all over Europe it is popular to make fresh wheat whole-grain flour at home, but doing so we may run the risk of processing grain that contains relatively high content of *Fusarium* mycotoxins. Also Lithuanian consumers follow the

trends and Lithuanian organically produced wheat grain is sometimes contaminated with *Fusarium* mycotoxins (Suproniene et al., 2010).

Certain groups of consumers no longer accept industrially prepared wheat grain (whole-grain flour), but prefer to purchase organic grain (whole-grain flour) directly from farms or in shops offering organic foods. However, if farmers do not manage their grain processing according to the high food-safety standards of industries, this could lead to an increase in adverse health effects from *Fusarium* mycotoxins for consumers.

The common belief among farmers and consumers of whole-grain flour is that separating apparently healthy grain of a high 1000-grain weight can assure a low mycotoxin content in whole-grain flour. Occasionally, however, unusually high DON content is found in flour after milling well-cleaned and apparently healthy grains. Is it possible that *Fusarium* fungi develop on or inside grain kernels and do not visibly damage the kernel tissues while still producing large quantities of mycotoxins? If this is true, can the water solubility and volatility of *Fusarium* mycotoxins allow them to be transmitted from infected kernels (or chaff) to healthy kernels in field conditions during rainy periods that occur two to three weeks prior to harvest?

It is expected that *Fusarium*-infected kernels change their shape, density and weight to such an extent that they can be separated by standard cleaning procedures (e.g., screening, aspiration, and on gravity tables) from high-quality uninfected kernels. It is also expected that usual grain cleaning procedures reduce DON content by at least 20–40% (Tkachuk et al., 1991) and standard milling procedures by another 20–30% (Murphy et al., 2006). In the case of whole-grain flour preparation, the reduction of DON content is considerably lower, and thus, the initial content in cleaned grain prior to milling must be considerably lower (<750 μg kg^{-1}) to guarantee a low DON content in whole-grain bakery products (Lešnik et al., 2008).

The objective of our research was to compare the cleaning efficacy for removal of *Fusarium*-infected grain, prior to milling, in the wheat cultivars with different levels and types of resistance to fungi causing FHB.

Materials and methods

Trial procedure and design. During the 2010 season, two field trials were performed in two locations in central (Jablje location) and eastern (Maribor location) Slovenia with four winter wheat cultivars cultivated on small plots (10 m^2) arranged in a randomized plot design. According to the information gathered in several trials aimed at cultivar comparisons, cv. 'Incisif' was found to be susceptible to Fusarium head blight (FHB) infection, cv. 'Zitarka' was moderately susceptible, cv. 'Renan' was moderately tolerant and cv. 'Bastide' was tolerant to FHB. Wheat was inoculated with *Fusarium* spores at anthesis. Grain yield (13% of moisture) was determined after plots were combine-harvested at maturity with a plot harvester "Wintersteiger" (Wintersteiger AG, Austria). Harvested grain from individual plots was packaged in paper bags and later cleaned with a grain cleaner. Before and after cleaning, grain samples were taken from each bag for mycotoxin analysis to obtain data on the initial mycotoxin content of the harvested grain and on the mycotoxin content after cleaning and separating grain into different size classes. Plots were managed according to the standard integrated wheat production practice. Wheat was sown after corn. Average stand density ranged from 470–530 tillers m^{-2} (410–430 heads m^{-2}). Nitrogen fertilizers were applied twice during the vegetative period; the cumulative amount of added nitrogen was 120 kg ha^{-1}.

Wheat inoculation with conidia and evaluation of disease severity. All wheat plots were inoculated during anthesis when 80% of plants had flowered (BBCH 63–65) (Meier, 1997). Fungicides for control of FHB were applied 24 h before inoculation. We applied conidia (2000 ml^{-1}) of two *Fusarium* species (*F. culmorum* (W.G. Sm.) Sacc. KIS 722/07 and *F. graminearum* Schwabe KIS 1220/08) stored in the Myco-Bank of the Agricultural Institute of Slovenia. Conidia were produced on wheat-grain media in Petri dish culture. Plots were sprayed with a plot sprayer powered by an electric pump, which delivered spray 350 L ha^{-1} and was operated at a constant pressure of three bars. Additionally, we scattered *Fusarium*-infected head parts on the soil surface of plots. Infected heads were collected one season before our trial from several locations throughout Slovenia. These heads were stored at ambient conditions outside, hanging in sacks under a roof. In the middle of April, the infected heads were dipped in water for three days and then stored in high humidity conditions at 25°C. Mouldy heads were scattered within each plot (20 g m^{-2}) at the end of the boot stage (BBCH 45), and thus, wheat plants in the trial plots were also infected with several unknown local strains of *Fusarium* fungi. Rating of disease severity (% FHB) was conducted five weeks after inoculation (BBCH 77). Disease severity was determined by visual observation of 300 randomly chosen heads per plot. The percent surface area of each head under fungus attack was determined. From the beginning of flowering until the end of the dough stage, it rained six times, and plants were wet several times for long periods of time (more than 12 hours). Weather conditions were very suitable for disease development.

Fungicide application. Fungicides were applied twice with a plot sprayer (spray volume 350 L ha^{-1}). The first application was performed at mid-boot stage (BBCH 43), three weeks before flowering, and the second application was performed during flowering (BBCH 63–65), 24 hours prior to the flower inoculation with *Fusarium* spores. The 1st treatment (1TR) was performed to control powdery mildew, rust and septoria leaf blotch, and the 2nd treatment (2TR) prevented the infection of flowers by *Fusarium* fungi. At Jablje, 1TR was 0.75 L ha^{-1} Amistar Xtra (a.i. 20% azoxystrobin + 8% cyproconazole), and 2TR was 0.8 L ha^{-1} Prosaro (a.i. 12.5% prothioconazol + 12.5% tebuconazole). At Maribor, 1TR was 1.3 L ha^{-1} Opus Team (a.i. 8.4% epoxiconazole + 25% fenpropimorph), and 2TR was 0.6 L ha^{-1} Falcon (a.i. 25% spiroxamine + 16.7% tebuconazole + 4.3% triadimenol). Half of the plots were treated with fungicides, and half were not.

Grain cleaning procedure. Grain cleaning was performed with a high-quality grain sample cleaner Pfeuffer (Pfeuffer GmbH, Germany), which removes all impurities (aspiration) and separates grain size classes according to the size of screens mounted. Using this machine, we attempted to mimic the grain cleaning process of the cereal grain processing industry. Harvested grain from individual plots was separated into 4 size classes according to the size of the mounted screen slots: kernels >2.4 mm, 2.0–2.4 mm, 1.8–2.0 mm and <1.8 mm grain diameter. After separation with the cleaner, each size class was weighed, and the portion of each size class of the total mass of harvested grain was calculated. Samples were taken from each of the individual size classes obtained during the cleaning process for visual grading of the kernel infection rate by *Fusarium* fungi. Grain from individual size classes was packaged and delivered to the laboratory to analyze the mycotoxin content.

Visual assessment and grading of grain before and after cleaning. Samples were taken from each sack of grain from each plot before and after cleaning to visually assess the presence or absence of symptoms of *Fusarium* infection. In this research, we decided to use the term "symptoms" despite knowing that changes observed in infected kernels can be described as either symptoms or signs of disease. Size classes of seeds with different levels of *Fusarium* damage were separated by visual assessment under 20× magnification. Three hundred kernels were randomly selected from each bag of grain before cleaning and also from each size class separated during the cleaning process. Kernels were visually scored using a 1–4 rating, where 1 indicated healthy kernels with no visually noticeable symptoms of infection, 2 – kernels with altered colour but no visible changes of shape or size, 3 – kernels with changes of colour and moderate changes of grain shape and 4 – seeds that were shrivelled, malformed and had typical pink colouring. Seeds from individual size classes were counted and weighed. Grain from the >2.4-mm size class graded 1 (no symptoms) was gathered (100 g) and analyzed to determine deoxynivalenol (DON) content. We modified a little the method described by Sinha and Savard (1997).

Mycotoxin analysis. The analyses of DON content in the grain samples were performed in the laboratories of QUANTAS (Analytik GmbH, Austria). The method applied was high-performance liquid chromatography (HPLC) with UV detection analysis. A detailed description of the standardized internationally validated diagnostic protocol is presented by Fuchs et al. (2004). Basic samples of wheat grain (0.6–1.0 kg) were ground with a mill Romer Series II (Romer Labs Inc., USA), and homogenization was performed afterward. Homogenized sub-samples weighing 25 g were processed using 100 ml acetonitrile: water (84:16, v/v) for three minutes in a blender Osterizer (Oster®, USA) with 250 ml blender jars. The extracts were then filtered through folded filter paper. Four millilitres of the filtrates were slowly pressed through a Mycosep® 227 first step cleanup column followed by a Mycosep® 216 final step cleanup column (Romer Labs Inc., USA) previously conditioned with five ml acetonitrile/water (84:16, v/v). The eluate from the Mycosep® 216 was evaporated to dryness and then redissolved in 400 µl HPLC running solvent. An HP 1100 Series HPLC device equipped with an HP Hypersil ODS column (2.0 × 125 mm, 5 µm) and HP Hypersil ODS precolumn (2.0 × 4 mm, 5 µm) (Agilent® Technologies, Germany) was used. The gradient mobile phase system consisted of (A) water: acetonitrile: methanol (96:2:2, v/v) and (B) acetonitrile at a flow rate of 0.5 ml min^{-1}. The injection volume was 50 µl. Detection was performed with an 1100 series diode array detector (DAD) at a wavelength of 220 nm. The total recovery of the method was 88–90%, the limit of detection (LOD) was 50 µg kg^{-1} and the limit of quantification (LOQ) was 130 µg kg^{-1}.

Statistical analysis. Analysis of variance was performed with *Statgraphics Plus* for *Windows 5.0* (StatPoint Technologies, USA). To test the significance of treatment differences, the Tukey HSD test ($P < 0.05$) and *t*-test ($P < 0.05$) were applied. Data expressed in percentages were transformed prior to analysis by taking the arcsine of the square root of the percentage values; non-transformed data are presented in tables.

Results and discussion

Grain yield, Fusarium head blight (FHB) rate and deoxynivalenol (DON) content at harvest. Tables 1 and 2 present data on grain yield, FHB and DON content at harvest. Fungicide application significantly increased the yields of three cultivars in the Maribor trial and all four of the studied cultivars in the Jablje trial. The highest yield decrease due to FHB was observed in the cv. 'Incisif' (20% at Jablje and 30% at Maribor, plots not treated with fungicides), which is the most susceptible to FHB disease of all of the studied cultivars. The second highest decrease was found in cv. 'Zitarka' (13.8% at Jablje and 21.4% at Maribor), which is moderately susceptible to FHB. Most of the yield loss was associated with FHB disease because other diseases (powdery mildew, rust and leaf blotch) were present on most of the plots at very low levels due to the high fungicide efficacy and the high level of cultivar resistance to those diseases.

The FHB rate (on average from 29.7–20.3% at Maribor and 19.6–12.7% at Jablje) was decreased significantly in all cultivars at both trial locations due to the application of fungicides (Tables 1–2). The same was true for the FDK rate (decreased from 27.2% to 21.1% at Maribor and 22.4% to 16.2% at Jablje). The efficacy of the applied fungicides was low. In terms of the reduction of DON content at harvest, fungicide performance was slightly better but was still not good. On average, fungicide application reduced DON content by 37% (from 3098 to 1952 µg kg^{-1}) at Maribor and by 36% (from 2282 to 1458 µg kg^{-1}) at Jablje. The conditions for disease development were very suitable, and in suitable conditions, we often cannot control *Fusarium* fungi successfully. Thus, the DON concentrations and FDK rate were quite high in most of the plots. In both trials, we experienced realistic situations that often occur in years with suitable conditions for *Fusarium* disease development across Europe. The results of our trials are similar to those recorded by other researchers (Liu et al., 1997; Simpson et al., 2001; Ioos et al., 2005;

Table 1. Yields, grain size classes obtained during cleaning of harvested grain, percentage of diseased head area Fusarium head blight (FHB), percentage of *Fusarium*-diseased kernels (FDK) and grain deoxynivalenol (DON) content prior to cleaning from fungicide-treated and not treated plots from the Maribor trial

Treated with fungicides					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
Yield kg ha^{-1}	5306 Bc	5049 Bb	5036 Ab	4719 Ba	5028 B
Class <1.8 mm %	3.3 Ab	2.9 Aab	1.4 Aa	4.4 Ab	3.0 A
Class 1.8–2.0 mm %	6.4 Aa	14.7 Ab	3.4 Aa	15.1 Ab	9.9 A
Class 2.0–2.4 mm %	11.7 Aab	7.7 Aa	15.1 Ab	9.3 Aa	10.9 A
Class >2.4 mm %	78.6 Bb	74.7 Ba	80.1 Bb	71.2 Ba	76.1 B
FHB rate %	14.3 Aa	23.7 Ab	12.4 Aa	30.6 Ab	20.3 A
FDK %	16.8 Ab	26.5 Ac	8.4 Aa	30.5 Ac	21.1 A
DON content µg kg^{-1}	933 Aa	2548 Ab	1137 Aa	3189 Ac	1952 A
No fungicide applied					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
Yield kg ha^{-1}	4850 Ac	3969 Ab	4703 Ac	3306 Aa	4207 A
Class <1.8 mm %	4.8 Bbc	6.6 Bc	2.8 Ba	7.8 Bc	5.5 B
Class 1.8–2.0 mm %	10.6 Bb	19.2 Bc	4.9 Aa	21.2 Bc	14.0 B
Class 2.0–2.4 mm %	13.2 Aab	7.1 Aa	17.3 Ab	9.3 Aa	11.7 A
Class >2.4 mm %	71.4 Abc	67.1 Ab	75.0 Ac	61.7 Aa	68.8 A
FHB rate %	23.9 Bb	36.9 Bc	15.2 Ba	42.8 Bd	29.7 B
FDK %	22.5 Bb	34.2 Bc	14.3 Ba	37.7 Ad	27.2 B
DON content µg kg^{-1}	2425 Ba	3065 Bb	2878 Bb	4023 Bc	3098 B

Note. Capital letters serve for comparisons of specific parameters between fungicide-treated and non-treated plots (*t*-test, $P < 0.05$) and lowercase letters for comparisons among tested cultivars (Tukey test, $P < 0.05$).

Gaurilčikienė et al., 2010). We also noticed differences among the studied wheat cultivars in terms of all of the studied parameters. According to the information obtained from cultivar breeders, we chose cultivars with different responses to *Fusarium* infection, and these variable responses were confirmed by our trial results. The most susceptible cv. 'Incisif' had the highest DON content and highest FDK rate, while the tolerant cvs 'Renan' and 'Bastide' had the lowest DON and FDK rates. These results were expected and are in accordance with those of other researchers who found that high FHB and FDK rates lead to high DON content in harvested grain (Perkowski et al., 1991).

Table 2. Yields, grain size classes obtained during cleaning of harvested grain, percentage of diseased head area Fusarium head blight (FHB), percentage of *Fusarium*-diseased kernels (FDK) and grain deoxynivalenol (DON) content prior to cleaning from fungicide-treated and not treated plots from the Jablje trial

Treated with fungicides					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
Yield kg ha^{-1}	6518 Bb	5907 Ba	6959 Bc	6380 Bb	6441 B
Class <1.8 mm %	0.6 Aa	2.7 Ab	1.0 Aa	4.2 Ac	2.1 A
Class 1.8–2.0 mm %	9.7 Ab	9.3 Ab	6.3 Aa	7.1 Aa	8.1 A
Class 2.0–2.4 mm %	4.2 Aa	5.6 Aa	16.5 Ab	18.8 Ab	11.3 A
Class >2.4 mm %	85.5 Bc	82.4 Bc	76.2 Bc	69.9 Aa	78.5 B
FHB rate %	6.9 Aa	20.4 Ab	5.7 Aa	17.9 Ab	12.7 A
FDK %	12.3 Aa	21.5 Ab	10.4 Aa	22.7 Ab	16.2 A
DON content µg kg^{-1}	557 Aa	1289 Ab	1151 Ab	2837 Ac	1458 A
No fungicide applied					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
Yield kg ha^{-1}	6085 Ab	5090 Aa	6553 Ac	5097 Aa	5706 A
Class <1.8 mm %	1.4 Ba	4.4 Bb	1.9 Ba	4.7 Ab	3.1 B
Class 1.8–2.0 mm %	17.4 Bc	12.7 Bb	4.5 Aa	14.3 Bbc	12.2 B
Class 2.0–2.4 mm %	6.6 Aa	14.8 Bb	24.5 Bc	15.1 Ab	15.2 A
Fraction >2.4 mm %	74.6 Ab	68.1 Aa	69.1 Aa	65.9 Aa	69.4 A
FHB rate %	11.8 Ba	29.2 Bb	9.0 Ba	28.5 Ba	19.6 B
FDK %	18.5 Ba	26.7 Bb	14.5 Ba	29.7 Bb	22.4 B
DON content µg kg^{-1}	1855 Ba	2454 Bb	1910 Ba	2908 Ac	2282 B

Explanation under Table 1

Expression of disease symptoms on grain. Comparisons of the rate of expression of symptoms of FHB disease on grain (300 kernels) before and after cleaning are presented in Tables 3 and 4. Here, we focus predominantly on the F1 (>2.4 mm) size class because this grain class is usually processed into whole-grain flour. If grains of class F2 are processed, the mycotoxin contamination level of the flour rapidly increases. In Table 3 (Maribor trial), we can see that before cleaning, there was an average 69.1% of grains declared free of FHB symptoms (no FHB) in plots treated with fungicides and 58.7% from plots not treated with fungicides. Cleaning (separating) of grains in class F1 increased the share of kernels free of symptoms from 69.1% to 71.8% (treated with fungicides) and from 58.7% to 68.5% (no fungicide treatment). Conversely, cleaning the grain decreased the share of kernels that were graded as significantly damaged by fungal infection (CySSsig), in class F1 grain from 4.7% to 1.2% (treated with fungicides) and from 9.7% to 4.3% (not treated with fungicides) (Table 3). We also found out that cleaning efficacy was not equal in all cultivars and was not the same for the F1 and F2 size classes. In cv. 'Renan' grain, the fraction with significant changes (CySSsig) was reduced from 7.3% to 1.6% in plots without fungicide treatment and from 2.8% to 1.1% in plots with fungicide treatment. Cleaning efficacy in class F2 was lower than in class F1, which was expected because the cleaning machine can exclude heavily infected grain much easily than slightly infected grain.

Table 3. Fusarium head blight (FHB) symptom ratings of grain before (BC) and after cleaning (F2 – grain >2.4 mm, F1 – grain 2.0–2.4 mm) for the Maribor trial

Treated with fungicides					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
No FHB % BC	66.5 Ba**a**	74.9 Bb**a**	72.4 Ab**a**	62.4 Aa**a**	69.1 B**a**
CySSn % BC	19.8 Aa**b**	17.2 Aa**b**	17.7 Aa**b**	23.3 Aa**c**	19.5 A**b**
CySSm % BC	6.4 Aa**a**	5.1 Aa**a**	6.6 Aa**a**	9.0 Ab**a**	6.8 A**a**
CySSsig % BC	7.3 Ab**c**	2.8 Aa**b**	3.3 Aa**c**	5.3 Aab**c**	4.7 A**c**
No FHB % F2	75.1 Aa**b**	74.4 Aa**a**	73.3 Aa**a**	70.9 Aa**b**	73.4 A**a**
CySSn % F2	5.5 Aa**a**	8.8 Aab**a**	11.9 Bb**a**	11.2 Bb**a**	9.4 B**a**
CySSm % F2	14.7 Aa**b**	14.0 Aa**b**	13.5 Aa**b**	14.2 Aa**b**	14.1 A**b**
CySSsig % F2	4.7 Ab**b**	2.8 Aa**b**	1.2 Aa**b**	3.7 Aab**b**	3.1 A**b**
No FHB % F1	74.3 Ba**b**	71.1 Aa**a**	72.2 Aa**a**	73.4 Aa**b**	72.7 A**a**
CySSn % F1	17.4 Aa**b**	23.2 Ab**c**	22.1 Bb**b**	17.9 Aa**b**	20.2 A**b**
CySSm % F1	6.7 Aa**a**	4.6 Aa**a**	5.1 Aa**a**	7.4 Aa**a**	6.0 A**a**
CySSsig % F1	1.6 Ab**a**	1.1 Aa**a**	0.6 Aa**a**	1.3 Aab**a**	1.2 A**a**
No fungicide applied					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
No FHB % BC	56.3 Aa**a**	55.7 Aa**a**	66.0 Ab**a**	56.6 Aa**a**	58.7 A**a**
CySSn % BC	21.1 Ab**b**	20.8 Ab**b**	16.1 Aa**b**	23.2 Ab**c**	20.3 A**b**
CySSm % BC	13.8 Bb**a**	11.9 Ba**b**	8.8 Aa**a**	10.9 Aa**a**	11.4 B**ab**
CySSsig % BC	8.8 Aa**c**	11.6 Bb**b**	9.1 Ba**c**	9.3 Ba**b**	9.7 B**b**
No FHB % F2	65.4 Aa**b**	72.2 Aa**b**	81.2 Bb**c**	79.2 Ab**c**	74.5 A**c**
CySSn % F2	6.8 Aa**a**	6.9 Aa**a**	5.7 Aa**a**	5.2 Aa**a**	6.2 A**a**
CySSm % F2	22.6 Bb**b**	14.8 Aa**b**	9.8 Aa**a**	11.9 Aa**a**	14.8 A**b**
CySSsig % F2	5.2 Aa**b**	6.1 Bb**a**	3.3 Ba**b**	3.7 Aa**a**	4.6 B**a**
No FHB % F1	63.0 Aa**ab**	66.4 Aa**b**	76.2 Ab**b**	68.3 Aa**b**	68.5 A**b**
No FHB % F1	23.3 Ab**b**	18.1 Aa**b**	12.9 Aa**ab**	14.2 Aa**b**	17.1 A**b**
CySSm % F1	11.0 Ba**a**	7.6 Aa**a**	8.5 Ba**a**	12.3 Bb**a**	9.9 B**a**
CySSsig % F1	2.7 Aa**a**	7.9 Bc**a**	2.4 Ba**a**	4.2 Bb**a**	4.3 B**a**

Notes. Capital letters serve for comparisons of specific parameters from fungicide-treated and non-treated plots (*t*-test, $P < 0.05$), lowercase letters for comparisons among cultivars and bold lowercase letters for comparisons of symptom ratings before and after cleaning (Tukey test, $P < 0.05$). No FHB – no symptoms observed; CySSn – changes of colour observed, no visible changes of grain shape or size; CySSm – changes of colour and moderate changes of grain shape; CySSsig – significant changes in grain colour, size and shape observed.

In the Jablje trial, our results were similar to those from the Maribor trial. In grain treated with fungicides separated into class F1 (>2.4 mm), there was only a small increase in the proportion of kernels with no FHB symptoms present (from 80.2% to 81.4%) due to cleaning (Table 4). The increase was much higher (from 68.7% to 80.0%) for separated grain from plots that were not treated with fungicides. This leads to the conclusion that fungicide application decreases the level of disease expression, but also decreases the ability of the cleaning machine to exclude infected kernels. Thus, the efficacy of the cleaner is lower in fungicide-treated grain than in non-treated grain. Additionally, we found differences among the studied cultivars in the Jablje trial. The cvs 'Renan' and 'Bastide' are considered less susceptible and tolerant to *Fusarium* infection, respectively. In class F1 grain (>2.4 mm), at the level of individual cultivars, there were small differences in the proportion of significantly

damaged kernels, but the differences in the F2 class and the CySSn symptom grading group (changes in colour and no changes in size and shape) are much larger. In this symptom grading group (CySSn), we can see that there was a significant increase (on average from 7.8% to 14.2% for fungicide-treated and from 13.3% to 20.3% for non-treated plots) in the proportion of all cultivars after cleaning. This type of kernels (CySSn) cannot be separated by the cleaning machine due to the aspiration air flow if they have the same weight as totally healthy kernels. If these kernels contain mycotoxins, the proportion of such grain greatly influences the final content of mycotoxin in flour.

Table 4. Fusarium head blight (FHB) symptom ratings of grain before (BC) and after cleaning (F2 – grain >2.4 mm, F1 – grain 2.0–2.4 mm) for the Jablje trial

Treated with fungicides					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
No FHB % BC	83.1 Aa**a**	79.5 Ba**b**	84.8 Ab**b**	73.3 Ba**a**	80.2 B**b**
CySSn % BC	7.6 Aa**b**	6.1 Aaa	5. 0 Aa**a**	12.4 Ab**b**	7.8 A**a**
CySSm % BC	6.4 Aa**a**	12.4 Ab**a**	7.0 Aa**a**	7.5 Aa**a**	8.3 A**a**
CySSsig % BC	2.9 Aa**b**	2.0 Aa**a**	3.2 Aa**b**	6.8 Ab**b**	4.7 A**b**
No FHB % F2	83.5 Bb**a**	67.2 Aa**a**	77.6 Ab**ab**	68.4 Ba**a**	74.2 A**a**
CySSn % F2	9.3 Aa**b**	21.2 Bc**b**	14.3 Ab**b**	11.9 Aa**b**	14.2 A**b**
CySSm % F2	5.6 Aa**a**	9.3 Aa**a**	6.7 Aa**a**	10.9 Aa**a**	8.1 A**a**
CySSsig % F2	1.5 Aa**a**	2.3 Aa**a**	1.4 Aa**a**	8.8 Ab**b**	3.5 A**b**
No FHB % F1	87.5 Ab**a**	82.6 Aa**c**	75.8 Aa**a**	79.6 Aa**a**	81.4 A**b**
No FHB % F1	4.2 Aa**a**	6.2 Ab**a**	14.8 Ac**b**	6.7 Ab**a**	8.0 A**a**
CySSm % F1	7.2 Ba**a**	9.1 Aa**a**	8.3 Aa**a**	10.8 Aa**a**	8.9 A**a**
CySSsig % F1	1.1 Aa**a**	2.1 Aba	1.1 Aa**a**	2.9 Ab**a**	1.8 A**a**
No fungicide applied					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
No FHB % BC	77.5 Ab**b**	62.3 Aa**a**	76.9 Ab**a**	57.9 Aa**a**	68.7 A**a**
CySSn % BC	10.8 Ba**a**	12.7 Ba**a**	11.0 Ba**a**	14.2 Aa**b**	13.3 B**ab**
CySSm % BC	7.6 Aa**b**	9.6 Aab**a**	9.4 Aab**b**	12.4 Bb**b**	9.7 A**b**
CySSsig % BC	4.1 Bb**b**	10.9 Bc**c**	2.7 Aa**b**	15.7 Bd**c**	8.4 B**c**
No FHB % F2	69.2 Ab**a**	72.8 Ab**ab**	77.1 Ab**a**	56.7 Aa**a**	69.0 A**a**
CySSn % F2	22.4 Bb**b**	14.4 Aa**a**	15.2 Aa**b**	28.6 Bc**c**	20.3 B**b**
CySSm % F2	4.6 Aa**a**	7.9 Ab**a**	4.8 Aa**a**	7.2 Ab**a**	6.1 A**a**
CySSsig % F2	3.8 Ba**b**	4.9 Bab**b**	2.3 Ba**ab**	7.5 Ab**b**	4.6 B**b**
No FHB % F1	85.9 Ab**c**	77.6 Aa**b**	77.4 Aa**a**	78.9 Aa**b**	80.0 A**b**
No FHB % F1	8.9 Ba**a**	12.8 Ba**a**	10.3 Aa**a**	9.4 Ba**a**	10.4 B**a**
CySSm % F1	4.1 Aa**a**	7.0 Ab**a**	10.5 Ac**b**	7.8 Ab**a**	7.4 A**a**
CySSsig % F1	1.1 Aa**a**	2.6 Aab**a**	1.8 Aa**a**	3.9 Bb**a**	2.4 A**a**

Explanations under Table 3

DON content in different grain size and symptom grading classes. Data on DON content in different grain size and grain symptom grading classes are presented in Tables 5 and 6. The cleaning process reduced the mycotoxin content differently with respect to different cultivars and with respect to the application of fungicides. First, we noted that on average, the reduction rate of DON content in the F1 fraction (>2.4 mm) was higher in plots not treated with fungicides (Maribor F1 – 37.7% and Jablje F1 – 53.9%) than in plots treated with fungicides (Maribor F1 – 28.6% and Jablje F1 – 39.2%). In the Maribor trial (Table 5), the highest reduction of mycotoxin content due to cleaning was found in the moderately tolerant cv. 'Renan' (39.7% and 59.7%) and the moderately susceptible cv. 'Zitarka' (40.3% and 45.6%). In the Jablje trial (Table 6), the highest reduction of DON content was again obtained with cv. 'Renan' (66.2% and 71.8%) and second highest with cv. 'Incisif' (49.4–57.7%).

The final DON content in cleaned grain illustrates the differences among cultivars very well. In the Jablje trial, the lowest DON concentration was found in cv. 'Renan' grain treated with fungicides (188 µg kg^{-1} for F1 size class and 472 µg kg^{-1} for F2) (Table 6). The grain of cv. 'Renan' is suitable for milling or grinding into whole-grain flour. The situation was different with the cv. 'Bastide', in which FHB, FDK and yield loss were lower than those of cv. 'Renan', but after cleaning, grain size classes F1 and F2 of this tolerant cultivar contained much more DON (1043 µg kg^{-1} for F1 and 1121 µg kg^{-1} for F2) due to a lower cleaning efficacy (Table 6). The grain of cv. 'Bastide' from this trial was not suitable for processing into whole-grain flour. A similar situation occurred in the Maribor trial (Table 5). Cultivar 'Renan' grain was suitable for processing into whole-grain flour, but the grain of all other cultivars was not. In the case of cv. 'Zitarka', we observed a high *Fusarium* infection rate but quite effective cleaning. In the case of cv. 'Bastide',

Table 5. Deoxynivalenol (DON) content of grain (μg kg^{-1}) before (BC, all size classes together) and after cleaning (AC1, AC2) in two grain size classes from the Maribor trial

Treated with fungicides					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
BC	933 Aa**b**	2548 Ac**b**	1137 Ab**a**	3189 Ad**b**	1952 A**b**
AC1 (grain 2.0–2.4 mm)	988 Aa	2456 Ac**b**	1235 Ab**a**	3420 Ad**c**	2024 A**b**
Relative AC1/BC	+4.5 Aa**b**	−27.5 Bb	+8.7 Ba	+8.8 Aa	−1.4 A
AC2 (grain >2.4 mm)	565 Aa**a**	2.013 Ac**a**	1.045 Ab**a**	2.316 Ac**a**	1.485 A**a**
Relative AC2/BC	−39.7 Ac	−40.3 Ac	−8.0 Aa	−26.5 Ab	−28.6 A
No FHB	490 Aa	930 Ab	970 Ab	1070 ab	940 A
No fungicide applied					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
BC	2425 Ba**c**	3065 Bb**b**	2878 Ba**b**	4023 Bc**b**	2911 B**b**
AC1 (grain 2.0–2.4 mm)	2139 Ba**b**	3918 Bc**c**	2907 Bb**b**	4263 Bd**b**	3307 B**b**
Relative AC1/BC	−12.5 Ba	+4.7 Ab	+1.0 Ab	+4.9 Ab	−0.5 B
AC2 (grain >2.4 mm)	975 Ba**a**	2006 Ab**a**	2207 Bb**a**	3258 Bc**a**	2111 B**a**
Relative AC2/BC	−59.7 Bc	−45.6 Ab	−23.7 Ba	−21.9 Aa	−37.7 B
No FHB	570 Ba	1030 Ab	1350 Bc	1140 Abc	1022 A

Notes. Capital letters serve for comparisons of specific parameters from fungicide-treated and non-treated plots (*t*-test, $P < 0.05$), lowercase letters for comparisons among cultivars, and bold lowercase letters for comparisons of DON content before and after cleaning (Tukey test, $P < 0.05$). Relative DON content AC/BC = 100 – ((AC/BC) × 100) (± %). No FHB – grain from fraction >2.4 mm without any visible FHB symptoms.

Table 6. Deoxynivalenol (DON) content of grain (μg kg^{-1}) before (BC, all size classes together) and after cleaning (AC1, AC2) in two grain size classes from the Jablje trial

Treated with fungicides					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
BC	557 Aa**c**	1289 Ab**b**	1151 Ab**a**	2837 Ac**c**	1458 A**c**
AC1 (grain 2.0–2.4 mm)	472 Aa**b**	1,015 Ab**b**	1,121 Ab**a**	2,216 Ac**b**	1,206 A**b**
Relative AC1/BC	−15.3 Ab	−21.3 Bc	−2.6 Aa	−21.9 Bc	−15.3 B
AC2 (grain >2.4 mm)	188 Aa**a**	881 Ab**a**	1043 Ac**a**	1435 Ad**a**	887 A**a**
Relative AC2/BC	−66.2 Ad	−31.7 Ab	−9.4 Ac	−49.4 Ac	−39.2 A
No FHB	160 Aa	420 Ab	870 Ac	905 Ac	589 A
No fungicide applied					
	'Renan'	'Zitarka'	'Bastide'	'Incisif'	Average
BC	1855 Ba**b**	2454 Bb**c**	1910 Ba**b**	2908 Ac**c**	2282 A**c**
AC1 (grain 2.0–2.4 mm)	1730 Ba**b**	2287 Bb**b**	1560 Ba**a**	2499 Ab**b**	2019 B**b**
Relative AC1/BC	−6.7 Ba	−6.8 Aa	−18.3 Bb	−14.1 Ab	−11.5 A
AC2 (grain >2.4 mm)	524 Ba**a**	1134 Bb**a**	1290 Ab**a**	1231 Ab**a**	1045 B**a**
Relative AC2/BC	−71.8 Ac	−53.8 Bb	−32.5 Ba	−57.7 Bb	−53.9 B
No FHB	220 Aa	570 Bb	920 Ac	980 Bc	672 B

Explanations under Table 5

we found a low *Fusarium* infection rate but also a low cleaning efficacy. The grain of cv. 'Incisif' was infected so heavily that none of the size classes were suitable for milling into whole-grain flour.

During the determination of the rate of *Fusarium* disease expression on kernels, we manually selected 100 g grain from the F1 (>2.4 mm) size class in which no visual symptoms of *Fusarium* infection could be observed (in Tables 3 and 4, marked as No FHB present) and analyzed their DON concentrations. In Tables 5 and 6, these data are presented under code DON No FHB. In the Maribor trial, the lowest DON concentration was determined in cv. 'Renan' grain, and the concentration of DON in cv. 'Bastide' grain (970 and 1350 μg kg^{-1}) was not statistically lower than the DON concentration of cv. 'Incisif' grain (1070 and 1140 μg kg^{-1}). This result was surprising, especially considering the initial concentration of DON before cleaning, which was almost twice as high in the susceptible cv. 'Incisif' as in the tolerant cv. 'Bastide'. Results were similar at the Jablje trial. Again, the lowest concentration was determined in cv. 'Renan' grain and the highest in cv. 'Incisif' grain. The difference among DON concentrations of cv. 'Bastide' (870 and 920 μg kg^{-1}) and cv. 'Incisif' grain (905 and 980 μg kg^{-1}) was not statistically significant. These results support our hypothesis about the existence of cultivars that can tolerate infection by *Fusarium* fungi and do not suffer significant grain tissue damage from infection. The cv. 'Bastide'

had the lowest FHB and FDK rate at field evaluation, but healthy and cleaned cv. 'Bastide' grain had nearly the same DON content as grain of the much more susceptible cv. 'Incisif'.

Significant differences in relations between FHB ratings and DON levels were observed among cultivars in many trials (Bai et al., 2001; Mankevičienė et al., 2011 b). Cultivars can greatly differ in terms of defence mechanisms and in terms of the spread of fungus from primarily infected spikelets throughout the entire head. The correlation between DON levels and field disease severity ratings depends on the type of FHB resistance; different cultivars have different defence mechanisms (Mesterházy, 2002). Type I (resistance to initial penetration) and type II (resistance to pathogen spread within the tissue of host) resistance is the most common (Mesterházy et al., 1999). In certain cultivars, attacked tissues can tolerate high DON levels without being damaged. In such a situation, the grain kernels appear relatively unchanged but contain DON (Mesterházy, 2002). Resistance to toxins is termed type V resistance (Mesterházy, 2003). During the research of Mesterházy (2003), wheat genotypes were found in which DON concentrations were significantly higher than expected according to the correlation functions obtained for describing the relation between DON and FHB ratings. Mesterházy (2002; 2003) provided evidence about wheat genotypes in which correlations between DON content and FHB or FDK rating are low, and his findings are comparable to our own. Similar explanations about inconsistencies between FHB and FDK ratings and DON content were presented by Snijders and Perkowski (1990), Bennett and Richard (1996), Sinha and Savard (1997) and Vrabcheva and Nenov (1997).

Some researchers (Osborne, Stein, 2007; Wagacha, Muthomi, 2007) presented findings about the possible paths of FHB disease development in which the *Fusarium* fungi can colonize grain tissue without directly parasitizing it, and therefore, even in the absence of visually detectable symptoms, it is possible to find mycotoxins in grain at harvest. One of the reasons for DON contamination of visually healthy grains is also translocation of DON from invaded tissues to tissues that were not invaded by *Fusarium* mycelium. DON is water soluble and can be translocated in the wheat phloem tissue. DON has been found in tissues of *F. culmorum*-infected wheat heads that were not directly invaded by the fungus, and toxins produced in the chaff can translocate to the grain (Snijders, Krechting, 1992).

Beyer et al. (2007) demonstrated that very high grain-grading accuracy is required to separate healthy from infected kernels. It was found that, on average, 4.27% of *Fusarium*-diseased kernels are sufficient to reach the 1250 µg kg^{-1} DON grain limit for unprocessed cereals, which is the limit set by European Union regulations. The correlation between percent infected grain and DON content is high in their study (determination coefficient = 0.93–0.99). They concluded that a similar FHB rating on the field level can lead to different levels of FDK and to different levels of DON in kernels. In their opinion, an exact forecast of DON levels in grains on the basis of field disease severity ratings or visual observations of symptom expression on grain is not feasible. This means that the absence of *Fusarium* symptoms on grain is no guarantee that whole-grain flour obtained from symptom-free grain will not contain high levels of DON. According to our previous research on the reduction of DON concentrations during grain cleaning, milling and baking of whole-grain products (Lešnik et al., 2008), we believe that only grain that contains less than 750 µg kg^{-1} DON is suitable for processing into whole-grain flour.

Conclusions

1. Significant differences were established among the four tested wheat cultivars with respect to the cleaning efficacy for removal of *Fusarium*-infected grain prior to milling.

2. The cleaning efficacy of grain originating from the fungicide treated plots was lower than the efficacy established in the grain originating from the plots not treated with fungicides. This proves that cleaning efficacy could also be influenced by the use of fungicides for Fusarium head blight (FHB) control.

3. The cv. 'Bastide', which had the highest level of tolerance to FHB, exhibited the lowest efficacy of cleaning. As a consequence, cv. 'Bastide' grain contained a higher concentration of deoxynivalenol (DON) after cleaning, when compared to the more susceptible cvs 'Renan' and 'Zitarka', despite the fact, that the concentration of DON in their grain at harvest was higher in comparison to the cv. 'Bastide' grain.

4. The cv. 'Bastide' represents those types of FHB tolerant cultivars, where a considerable proportion of grain that appears totally healthy, could contain high levels of *Fusarium* mycotoxins. As a consequence, the expected cleaning efficacy, in terms of the reduction of grain mycotoxin content during cleaning, becomes low.

5. The growing of tolerant cultivars with a similar response to FHB as cv. 'Bastide' does not solve the risk of deoxynivalenol DON contamination in the food chain from field to fork, especially if grain is processed at farm level, without properly determining the DON content. Cultivars with a FHB tolerance similar to the type of tolerance the cv. 'Bastide' displays may not be suitable for organic farmers intending to grow and sell grain directly to consumers, who prepare homemade whole-grain flour.

6. The *Fusarium* removal efficacy in cleaning infected grain prior to milling shall be taken into account, when deciding upon certain suitable wheat cultivars for growing at organic farms and for the processing to whole grain flour.

Received 18 11 2013
Accepted 18 04 2014

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ISSN 1392-3196 / e-ISSN 2335-8947
Zemdirbyste-Agriculture, vol. 101, No. 3 (2014), p. 285–294
DOI 10.13080/z-a.2014.101.037

Fusarium užkrėstų grūdų pašalinimo efektyvumas juos valant prieš malimą

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Santrauka

Keturių veislių kviečiai, pasižymintys nevienoda tolerancija varpų fuzariozei, auginti mažuose laukeliuose ir žydėjimo metu užkrėsti *Fusarium* grybų sporomis. Nukulti grūdai buvo išvalyti specialia valomąja, kuri grūdus suskirstė į keturių dydžių frakcijas: F1 > 2,4 mm, F2 – 2,0–2,4 mm, F3 – 1,8–2,0 mm, F4 < 1,8 mm. Jos rankiniu būdu pagal varpų fuzariozės simptomų pasireiškimą buvo padalintos į keturias subfrakcijas: grūdai be matomų simptomų; pakitusios spalvos, bet nepakitusios formos grūdai; pakitusios spalvos ir vidutiniškai pakitusios formos arba dydžio grūdai; smarkiai užkrėsti deformuoti grūdai. Deoksinivalenolio (DON) kiekis įvairių frakcijų grūduose nustatytas taikant didelio efektyvumo skysčių chromatografiją. Buvo palygintas pradinis DON kiekis prieš grūdų valymą su DON kiekiu atskirose frakcijose po grūdų valymo. Valymo efektyvumas (DON kiekio sumažėjimas) grūdus valant ir suskirstant į frakcijas pagal dydį buvo didesnis pusiau tolerantiškų ir jautrių veislių, palyginus su varpų fuzariozei tolerantiškomis veislėmis. Dėl mažo valymo efektyvumo išvalytuose tolerantiškos (be jokių varpų fuzariozės simptomų) veislės grūduose DON kiekis buvo du kartus didesnis (870–1350 µg kg^{-1}), palyginus su išvalytais ir vizualiai sveikais pusiau tolerantiškos veislės grūdais (160–570 µg kg^{-1}); jos DON kiekis buvo panašus į jautrios veislės DON kiekį (905–1140 µg kg^{-1}).

Tyrimų rezultatai rodo, kad didelė dalis varpų fuzariozei tolerantiškų veislių grūdų gali vizualiai atrodyti sveiki, tačiau būti užteršti dideliu kiekiu DON. Tokios veislės yra labai tinkamos dėl varpų fuzariozės patiriamų grūdų derliaus nuostoliams sumažinti, tačiau jos gali būti potencialiai žalingos sveikatai, naudojant viso grūdo miltus, pagamintus grūdus perdirbant ne pramoniniu būdu.

Reikšminiai žodžiai: deoksinivalenolis, ekologiški viso grūdo miltai, *Fusarium* spp., grūdų valymas.