Eur J Plant Pathol (2009) 124:163–170
DOI 10.1007/s10658-008-9403-1

Assessment of infection in wheat by *Fusarium* protein equivalent levels

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Received: 8 August 2008 / Accepted: 17 November 2008 / Published online: 9 December 2008

Abstract Determination of the *Fusarium* protein equivalent (FPE) levels in kernels for better characterisation of genotypes showing *Fusarium* head blight (FHB) resistance, and better detection of susceptibility to kernel infection among genotypes with slight symptom expression was carried out. Twelve wheat cultivars and eight hexaploid winter wheat lines derived from a cross of *Triticum aestivum* with related species *T. macha*, *T. polonicum,* and *T. dicoccoides* were evaluated for levels of spike and kernel infection, the content of the mycotoxin deoxynivalenol (DON) and FPE in kernels after artificial inoculation with the fungus *Fusarium culmorum* in the field in 2006–2007. The ELISA immunochemical method was employed for the quantitative analyses of DON and FPE. Three wheat lines had a significantly low infection of spikes and kernels compared to cvs Sumai 3 and Nobeoka Bozu, indicating the presence of specific resistance mechanisms to FHB**.** The significantly low AUDPC (area under the disease progress curve) and the high level of FPE and DON content in kernels indicated a lack of resistance in one wheat line (crossed with *T. polonicum*). The results showed highly significant correlations ($P < 0.01$) between FPE and DON content and between FPE and AUDPC. In addition, correlations between FPE and reductions in yield components were also highly significant. Quantification of *Fusarium* spp. in wheat kernels can be helpful for evaluating wheat genotypes for their levels of resistance to FHB.

Keywords *Fusarium* infection · *Triticum aestivum* · AUDPC · Deoxynivalenol · Yield components

Abbreviations

FPE	*Fusarium* protein equivalent
DON	deoxynivalenol
AUDPC	under disease progress curves
FDK	*Fusarium*-damaged kernels
R-TKW	reduction in 1,000-kernel weight
R-KWS	reduction in kernel weight per spike
R-KNS	reduction in kernel number per spike

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Introduction

Fusarium head blight (FHB), caused by members of the genus *Fusarium*, ranks among the most damaging of spike diseases in wheat. FHB on wheat and other small-grain cereals is caused by five major *Fusarium* species: *F. avenaceum* (teleomorph, *Gibberella ave-*

nacea), *F. culmorum*, *F. graminearum* (teleomorph, *G. zeae*), *F. poae* and *Microdochium nivale* (teleomorph, *Monographella nivalis* (Parry et al. 1995). The influence of factors such as different climatic conditions can lead to a change in the FHB species profile from region to region. In the Slovak Republic (SR), the most common FHB species in wheat are *F. culmorum* and *F. graminearum* (Šrobárová et al. 2008). In infected spikes the kernels can be contaminated by mycotoxins that have proved to be a health risk in humans and animals. There are limits to the amount of *Fusarium* mycotoxins acceptable in unprocessed grain for food and feed production in the EU (European Commission 2006a, b). For the integrated protection of wheat, the best way to control FHB, economically and ecologically, is to grow wheat cultivars resistant to this disease.

FHB resistance in wheat is a complex phenomenon (Leonard and Bushnell 2003) and five types of active resistance mechanisms have been identified: resistance to initial infection (type I), resistance to pathogen spread (type II), resistance to kernel infection (type III), tolerance to infection (type IV) (Schroeder and Christensen 1963; Mesterházy 1995), and resistance to mycotoxin deoxynivalenol (DON) accumulation (type V) further divided into two subtypes (Boutigny et al. 2008). To determine the level and types of FHB resistance in wheat, spike infection, number of infected kernels, reduction in yield components, and amount of mycotoxin accumulation in kernels are usually measured. These evaluations of infected spikes and kernels are indirect methods, whereas molecular (Edwards et al. 2001; Burlakoti et al. 2007) and immunological (Abramson et al. 1998; Chala et al. 2003; Hill et al. 2006) techniques have been developed for direct quantification of *Fusarium* in plant material.

The overwhelming majority of cultivars in commercial production are either susceptible or highly susceptible to FHB (Mesterházy 2003). Worldwide, there are limited numbers of known donors of resistance to FHB; therefore, it is important to find new and effective resistance sources to use in wheat breeding. However, there are no known genetic resources of wheat with full resistance to FHB. Among the best examined sources of FHB resistance are cvs Sumai 3 and its lines (Buerstmayr et al. 2002; Cuthbert et al. 2007), Brazilian cv. Frontana (Steiner et al. 2004), and some European winter wheats (Gervais et al. 2003). Prospective resistance sources are likely in wild and related wheat species (Cai et al. 2005). Recently, spring forms of wheat (Jiang et al. 2006) and the winter wheat line RCATL33 (Tamburic-Ilincic et al. 2006) resistant to FHB have been registered.

In the present study, the objectives were to compare the symptomatic evaluation of spike attack, DON accumulation in kernels, and the reduction of yield components to the Fusarium protein equivalent (FPE) levels accumulated in wheat kernels after artificial inoculation with *F. culmorum*. Additionally, we examined the responses of the tested set to inoculation with *F. culmorum* in the field. The prospective hexaploid wheat lines derived from crossing of *T. aestivum* with related species *Triticum macha*, *T. polonicum*, and *T. dicoccoides* were compared to registered cultivars of the SR, and the wheat lines were also compared to other known sources of FHB resistance (cvs Sumai 3 and Nobeoka Bozu) after artificial inoculation with *F. culmorum*.

Materials and methods

Experimental materials

The examined set included the wheat lines: P-104-1 (Siria/*T. macha*); P-104-2 (Siria/*T. macha*); P-105-2 (HYB93.13/*T. macha*); P-106-1 (KM/*T. dicoccoides*); P-106-2 (KM/*T. dicoccoides*); P-109-2 (2286-70/*T. polonicum* cv. Buitre Cometa); SO-107-05 (Record/3/ZG K 3-82/*T. polonicum* cv. Buitre Cometa//ST 2009); SO-111-05 (Record/3/ZG K 3-82/*T. polonicum* cv. Buitre Cometa//ST 2009); registered SR wheat cvs Estica, Malyska, Manhattan, Mladka, Noah, Rapsodia, Residence, Sana, Vanda, and Zerda; and FHB-resistant cvs Sumai 3 and Nobeoka Bozu. The wheat lines were selected from a large set of F_4 generation wheat hybridised with related species as prospects for utilisation in breeding. These were based on the evaluation of natural incidence of diseases (powdery mildew, leaf rust, and FHB) in field stands and agronomic traits in experimental plots of Agrotest Fyto Ltd, in Kroměříž, Czech Republic.

Inoculum preparation

For multiplication of *F. culmorum*, a monosporic isolate of an aggressive strain was cultivated for

21 days on potato dextrose broth from Sigma (St. Louis, USA), 20 g l^{-1}, at 25°C, and a 12 h light period. The isolate *F. culmorum* (RA/02) from Radošina (SR) was obtained from a microorganism collection held at the Research Institute of Plant Production, Piešťany. Conidial and aerial mycelium, were scraped with a razor blade from 100 mm diam Petri dishes, and homogenised with distilled water using an ETA grinder. The average number of conidia counted in a Bürker chamber was 5×10^5 ml^{-1}.

Design of field experiment

In October 2006 and 2007, plot experiments with two blocks, inoculated (I) and non-inoculated (N), were established under natural conditions in Piešťany. There were five rows per plot, with 1 m length and 150 mm row spacing. At anthesis, 20 spikes from each cultivar were inoculated using a point method (block 1) with *F. culmorum* by injecting inoculum directly into the central floret of each inoculated spike. Spikes of similar size and developmental stage were selected for inoculation. After inoculation, the spikes were covered with plastic bags for 24 h.

The spikes were visually evaluated on a 0–100% scale at 10, 15 and 20 days after inoculation. The area under the disease progress curve (AUDPC) was computed. After ripening, the spikes were harvested by hand from each cultivar and from each plot. The threshed grains were manually cleaned and the percentage of visually *Fusarium*-damaged kernels (FDK) was evaluated. Reduction in 1,000-kernel weight (R-TKW), reduction in kernel weight per spike (R-KWS), and reduction in kernel number per spike (R-KNS) were calculated in comparison with the non-inoculated block as shown in the general form: $R=100–(100\times I/N)$.

Statistical analysis

AUDPC and percentages of FDK and R-KWS (in arcsine) were statistically evaluated by analysis of variance (ANOVA) using SPSS software. The significance of the differences ($P<0.05$; $P<0.01$) between individual genotypes and the mean for all tested genotypes were calculated by the *t*-test. For assessment of the relationship between the traits, Pearson's correlation analysis was applied using Microsoft Excel® 97 SR-2 software.

DON content

A commercial ELISA kit for quantitative analysis of DON in cereals was used to determine the DON concentration in wheat samples (Ridascreen® Fast DON, RBiopharm AG, Darmstadt, Germany). The kernels obtained from inoculated spikes were ground, and subsequently 20 ml of distilled water was added to 1 g of each sample and the mixture filtered. Dilution factors of 5, 10, and 20 were used where necessary because the DON concentrations often exceeded the range of the immunoassay (0.2–6.0 mg kg^{-1}). The absorbances (of the wells) were determined photometrically at 450 nm (MRX II, DYNEX Technologies). DON concentrations were calculated in mg kg^{-1} by Revelation Version 4.25.

Fusarium protein equivalent (FPE) analysis

ELISA was also used to quantify *Fusarium* fungal biomass. Analyses were performed at the University of Göttingen by Dr. J. Weinert according to the method of Chala et al. (2003). Results are given as a value of FPE and expressed as mass of mycelium of *F. culmorum* and *F. graminearum* in mg kg^{-1} of grain.

Results

ANOVA of AUDPC, FDK, and R-KWS in 2006 and 2007 showed significant differences among the genotypes (Table 1). The AUDPC values were lower ($P<0.01$) for cvs Sumai 3, Nobeoka Bozu, and Zerda, and also for P-104-1 and P-109-2 ($P<0.05$) than the mean of AUDPC for all genotypes (Table 2). AUDPC values were high ($P<0.01$) for P-105-2 and SO-111-05 of the wheat lines and for Estica, Mladka, and Vanda ($P<0.01$) of the registered cultivars. FDK was low ($P<0.01$) for cv. Sumai 3 of the cultivars with FHB resistance, for wheat lines P-104-1, P-104-2 and P-109-2 ($P<0.01$), but for none of the registered cultivars. R-KWS was low ($P<0.01$) for cvs Sumai 3 and Nobeoka Bozu and for P-104-1 and P-104-2 ($P<0.05$). R-KWS was high ($P<0.01$) for the registered cvs Estica, Noah, and Vanda. Values of other yield components (R-TKW and R-KNS) were lower in wheat lines than in registered cultivars and higher than in cultivars with FHB resistance. The level of reductions in yield components in the lines P-104-1,

Table 1 ANOVA for area under the disease progress curve (AUDPC), *Fusarium*-damaged kernels (FDK), and reduction in kernel weight per spike (R-KWS) for wheat genotypes inoculated with *F. culmorum* by the point method in two experiments (2006 and 2007)

Parameter	Source of variation	DF	MS	*F*-value	*P*-value
AUDPC	Experiment	1	93303.4	0.777	0.379
	Genotype	19	855539.6	7.122	0.000
	Genotype×Experiment	19	344527.3	2.868	0.000
	Error	340	120133.8		
FDK	Experiment	1	18858.8	35.20	0.000
	Genotype	19	5045.6	9.40	0.000
	Genotype×Experiment	19	3714.4	6.90	0.000
	Error	340	535.3		
R-KWS	Experiment	1	1404.4	3.82	0.051
	Genotype	19	1974.4	5.38	0.000
	Genotype×Experiment	19	2069.8	5.63	0.000
	Error	340	367.2		

DF degrees of freedom, *MS* mean square

Table 2 Mean values of area under the disease progress curve (AUDPC), yield components, deoxynivalenol (DON) and *Fusarium* protein equivalent (FPE) in 20 wheat genotypes (2006; 2007)

Genotype		AUDPC	FDK (arcsin)[a]	R-TKW %[b]	R-KNS[c]	R-KWS (arcsin)[d]	DON (mg kg^{-1})	FPE (mg kg^{-1})
Wheat lines	P-104-1	162.2*	18.7**	27.1	16.4	27.6*	5.2	12.7
	P-104-2	226.6	27.9**	26.9	25.7	26.9*	13.8	17.9
	P-105-2	676.9**	54.9	45.0	50.2	44.6	62.1	47.4
	P-106-1	270.0	43.3	30.5	46.1	32.2	15.1	28.4
	P-106-2	298.8	42.1	35.6	44.3	37.3	22.2	45.7
	P-109-2	192.5*	28.1**	22.4	33.2	28.9	10.7	18.6
	SO-107-05	200.7*	58.3*	37.3	56.9	35.5	31.3	33.0
	SO-111-05	587.6**	40.9	45.1	33.0	41.6	71.7	117.8
Mean for wheat lines		326.9	39.3	33.7	38.2	34.3	29.0	40.2
Registered cvs	Estica	574.2**	51.5	41.5	59.5	50.4**	50.2	89.3
	Malyska	221.9	42.3	27.1	43.4	28.8	10.2	26.4
	Manhattan	512.2	59.3*	34.4	57.3	34.6	53.6	63.8
	Mladka	526.3*	60.3*	41.7	63.7	47.3*	72.4	66.9
	Noah	510.1	52.2	46.0	56.9	52.8**	66.6	66.9
	Rapsodia	342.2	56.1	31.9	59.7	34.1	21.6	36.2
Registered cvs	Residence	468.8	55.0	36.9	58.6	39.5	27.0	38.6
	Sana	394.5	70.7**	40.6	72.2	41.6	52.6	51.8
	Vanda	862.0**	84.3**	53.3	85.5	61.5**	99.5	214.0
	Zerda	114.7**	30.2	24.4	31.1	29.3	16.4	27.7
Mean for registered cvs		452.7	56.2	37.8	58.8	42.0	47.0	68.2
Cvs resistant to FHB	Nobeoka- bozu	101.3**	36.9	30.3	31.5	22.0**	2.1	9.9
	Sumai 3	48.8**	27.7**	4.3	33.2	22.3**	8.9	7.8
Mean for cvs resistant to FHB		75.1	32.3	17.3	32.4	22.2	5.5	8.9
Mean for all genotypes		364.6	47.0	34.1	47.9	36.9	35.7	51.0

Significant at *$P<0.05$; **$P<0.01$

[a] *Fusarium*-damaged kernels

[b] Reduction in 1,000-kernel weight

[c] Reduction in kernel number per spike

[d] Reduction in kernel weight per spike

P-104-2, and P-109-2 were comparable with those in the cultivars with FHB resistance.

The lowest DON content was in grain of FHB-resistant cultivars, and the highest was in the registered cultivars (Table 2). The wheat lines accumulated 18.8% less DON than the mean of the all tested genotypes, and 38.3% less than the mean of registered cultivars. The lowest DON accumulation was in FHB-resistant cv. Nobeoka Bozu. The wheat line P-104-1 had 5.5% less DON than cultivars with FHB resistance. The relatively low DON concentration was found in kernels of P-104-2, P-109-2, and cv. Malyska. The cvs Sumai 3 and Nobeoka Bozu (with low DON accumulation) had low ($P<0.01$) values of AUDPC and R-KWS, and for cv. Sumai 3 also of FDK. There were similar responses for wheat lines P-104-1 and P-109-2. The highest DON concentration was in cv. Vanda, which had highly significant AUDPC values ($P<0.01$); there were similar results for wheat lines P-105-2 and SO-111-05. There was a different reaction for SO-107-05 (from the cross of wheat with *T. polonicum*), which had a relatively high DON concentration and significantly higher kernel infection but significantly lower AUDPC.

On average, the lowest FPE in kernels was in FHB-resistant cultivars (Table 2). The wheat lines had 21.2% lower FPE than the mean of all tested genotypes and 41% less than the registered cultivars. Among wheat lines, the lowest FPE was in P-104-1, of the registered cultivars in Malyska and Zerda, and of FHB-resistant cultivars in Sumai 3. The highest FPE of all genotypes was in cv. Vanda. FPE in kernels of line SO-111-05 was up to 131% higher than the mean of all tested genotypes. Among registered cultivars, there was lower FPE in Malyska and Zerda. There was a lower AUDPC ($P<0.05$) but higher kernel infection ($P<0.05$) and lower FPE and DON accumulation in the kernels of line SO-107-05.

The significant positive correlations were between means combining the 2 years between DON and AUDPC, DON and reduction in yield components, DON and FPE, as well as between FPE and AUDPC (Table 3). In individual years, highly significant correlations were found between FPE and DON (in 2006, $r=0.94$; $P<0.01$; in 2007, $r=0.88$, $P<0.01$). Further, there were highly significant correlations between FPE and R-TKW, between FPE and R-KWS, and between FPE and R-KNS. The correlation between FPE and R-KNS was the weakest. Despite low FPE being generally related to low reductions, this tendency for R-KNS was not clear in SO-111-05. This line had significantly higher AUDPC but R-KNS was comparable to R-KNS in FHB-resistant cultivars, and FDK was also lower than the mean for all tested genotypes (Table 2).

Discussion

Triticum macha, *T. polonicum*, and *T. dicoccoides* are reported to have contributed to the enhancement of wheat FHB resistance, and these species were

Table 3 Correlation coefficients between individual traits, for 20 wheat genotypes (2006; 2007)

Trait	FPE[a]	DON[b]	R-KWS[c]	R-KNS[d]	R-TKW[e]	FDK[f]
AUDPC[g]	0.84**	0.91**	0.86**	0.70**	0.91**	0.75**
FDK	0.68**	0.77**	0.77**	0.97**	0.75**	–
R-TKW	0.73**	0.83**	0.87**	0.66**	–	–
R-KNS	0.64**	0.71**	0.78**	–	–	–
R-KWS	0.83**	0.91**	–	–	–	–
DON	0.86**	–	–	–	–	–

Significant at $^{**}P<0.01$

[a] *Fusarium* protein equivalent

[b] Deoxynivalenol mycotoxin

[c] Reduction in kernel weight per spike

[d] Reduction in kernel number per spike

[e] Reduction in 1,000-kernel weight

[f] *Fusarium*-damaged kernels

[g] Area under the disease progress curve

employed to develop the tested wheat lines. The transfer and identification of the FHB-resistance genes (type I) from *T. macha* to wheat were published by Steed et al. (2005) and Mentewab et al. (2000) respectively. FHB resistance has been identified in a number of related and wild species, for instance, in *T. polonicum* (Cai et al. 2005) and transferred from *T. dicoccoides* to wheat. Oliver et al. (2005) found among wheat-alien species derivatives, lines with comparable resistance to cv. Sumai 3, the most widely used source of FHB resistance.

There was a tendency for increased DON content in kernels in the genotypes with higher infection of spikes (AUDPC) and kernels (FDK). This was confirmed by high correlation coefficients between the traits. In our study, the correlation between AUDPC and DON was higher than that between FDK and DON, similar to results of Culler et al. (2007). These tendencies were clear for lines P-104-1, P-104-2, and P-109-2, as well as for cvs Nobeoka Bozu and Sumai 3 that all had low values of AUDPC, FDK, and DON concentration, typical for FHB-resistant genotypes. The results are in agreement with the conclusions of Mesterházy et al. (2005) that the relationship between seed infection and toxin contamination is not simple, but that a higher resistance level is generally associated with low or a lower toxin contamination. Under our conditions a lower contamination content was confirmed for two genotypes of wheat after infection by Perkowski et al. (2002). Both high DON concentration and high AUDPC and FDK in kernels of some of the registered cvs Vanda, Mladka, and Estica, confirmed that susceptibility to FHB is typical for many commercial cultivars (Mesterházy 2003).

Positive correlations between the infection of spikes and kernels, yield loss and DON contamination of wheat tested in the field was mentioned by Mesterházy et al. (2005). A close relationship between DON and visual disease manifestation (percent damage) was also reported by Gosman et al. (2007), although estimates of disease severity did not always correspond with DON levels in grain samples. Such a response was found for the line SO-107-05 (from a cross of wheat with *T. polonicum*) that accumulated a relatively high amount of DON and had high kernel infection; however, the value of AUDPC was significantly low. A similar response (the lowest percentage of head blight and the highest level of infected seeds and DON content) was found in cv. Kinnan (Liu et al. 1997) among cereals infected with *F. culmorum* and indicated a lack of type III and type V resistances. Culler et al. (2007) found that cv. Wheaton had almost identical DON levels over 2 years; however, the FHB index was three times higher in one year than the other. As the authors explained, there might have been a direct effect of light and temperature on the fungus, indirect effects such as wheat growth stage at the time of infection, or soil water and nutrient availability, which all determine overall plant health and response to infection.

The FPE value in our trials was used to estimate the amount of *F. culmorum* fungal biomass in the grain sample. The FPE value in kernels was closely related to visual infection of spikes and kernels, yield loss, and based on our results it was closely correlated with DON content in kernels. A close correlation (r= 0.74) between *Fusarium* exoantigen and a FHB rating, based on the evaluation of 110 wheat genotypes infected with *F. culmorum* was found by Miedaner et al. (2006). If the pathogen in kernels was quantified using molecular methods, there were good correlations between fungal DNA content and DON content (Burlakoti et al. 2007) and also between DON and the amount of trichothecene-producing *Fusarium* (Edwards et al. 2001). Likewise, a positive correlation between DON production and *Fusarium* content was confirmed if ELISA was used to quantify the pathogen (Cumagun et al. 2004). In our experiment, the lines P-104-1, P-104-2, and P-109-2 had both low FPE values and DON content in kernels, comparable with those for cultivars with FHB resistance, which indicates resistance to *F. culmorum.* Despite the tendency for higher FPE in kernels to be closely correlated with AUDPC (Table 3), the line SO-107-05 had significantly lower AUDPC, but significantly higher kernel infection, FPE, and DON concentration. A similar case was found in a barley line with low infection but high amounts of DON and *Fusarium* pathogen biomass in grain quantified with ELISA (Hill et al. 2006). Gosman et al. (2007) reported that some cultivars with significant levels of DON and *F. culmorum* DNA had relatively low disease scores, suggesting that visual symptoms were not closely correlated with DON levels in some genotypes.

The present results indicate that quantification of FPE in kernels refine the characterisation of geno-

types for FHB resistance, as this enabled detection of infection even with asymptomatic expression of the disease on spikes. The evaluation of genotypes for FHB resistance by the quantification of the pathogen is more reliable than using only the AUDPC or DON content. The FPE and AUDPC is a practical alternative to AUDPC and DON content for use in research-breeding programmes. The wheat lines P-104-1, P-104-2, and P-109-2 had low reductions of yield components, DON accumulation, and FPE values in kernels. Wheat lines might be included in breeding elite cultivars for developing resistant varieties to FHB.

Acknowledgements The results were obtained within Research Projects No. 2006 UO 27/0910501/0910511 supported by the Ministry of Agriculture of the Slovak Republic and No. MSM 2532885901 supported by the Czech Ministry of Education, Youth and Sports.

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