Effects of Genotype, Environment and Fungicide Treatment on Development of Fusarium Head Blight and Accumulation of DON in Winter Wheat Grain

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Abstract: Reactions to artificial infection with *Fusarium culmorum* and (metconazole- or tebuconazole-based) fungicides were studied in nine winter wheat cultivars that were evaluated in field experiments at the location Prague-Ruzyně for four years (2001–2004) for deoxynivalenol (DON) content in grain, pathogen DNA content (Ct) by real-time quantitative PCR, percentage of Fusarium damaged grains (FDG), symptom scores and reductions in grain yield components. All examined traits were highly affected by conditions of experimental years and interactions with cultivars and treatments. Moderately resistant cultivars Arina and Petrus were included in the first homogeneous group in all traits, including the pathogen DNA content. To predict cultivar resistance to Fusarium head blight and accumulation of DON, the examination of the percentage of FDG in different environments appeared to be useful from practical aspects. The pathogen DNA content was significantly related to the content of DON under different conditions, however, the correlation coefficients ranged between 0.42 and 0.92. Different levels of DON could be detected at similar pathogen contents. The higher colonization of grain by the fungus was mostly connected with a strongly reduced amount of DON per pathogen unit (DON/Ct ratio). The fungicide treatment had a significant effect on a reduction in all traits except DON/Ct, but the effects on different traits were not often proportional and they were highly variable in the particular years (range 10–69%) and cultivars (range < 0–60%). While the application of fungicide caused a reduction in DON content in all cultivars, an increase in pathogen content after the application of fungicides was not exceptional. The low fungicide effect on a reduction in pathogen content was connected with higher temperatures (temperature extremes) in a 30-day period of disease development. The efficacy of fungicide treatment for DON was low at high pathogen content and late heading. The use of the collected data to improve control measures is discussed.

Keywords: *Fusarium culmorum*; deoxynivalenol content; pathogen DNA content; real-time PCR assay; disease severity traits; disease control; cultivar resistance

Fusarium head blight (FHB) is a devastating disease in most wheat-growing countries resulting in yield and quality losses, and contamination by mycotoxins. The disease is predominantly caused by *Fusarium graminearum* (Schwabe) and *Fusarium culmorum* (W.G. Smith) Sacc. The mycotoxin contamination of human food and animal feed has become a more important feature than direct yield losses that often occur irregularly. In the conditions of Central Europe deoxynivalenol (DON) is also reported to be the most frequent toxin reaching the highest concentration levels (Golinski *et al.* 1996).

There is enough evidence that currently grown wheat cultivars, fungicide applications and other protective precautions cannot not separately guar-

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antee sufficient protection against the disease. Mielke and Weinert (1996) showed that it was only possible to combat head blight completely through the cultivation of less susceptible winter wheat cultivars and additional application of fungicides containing tebuconazole. Generally, the fungicides with triazole chemistry (tebuconazole, metconazole, and bromuconazole) are the most effective compounds. The efficacy of the best fungicides may exceed 70%, but it is known that the efficacy of fungicides is highly variable, influenced mainly by cultivar resistance, fungus isolate aggressiveness and weather conditions (Mesterházy & Bartók 2002). Most authors agree that temperature and humidity during pathogenesis are critical for natural Fusarium infections of small grains (Parry *et al.* 1995). However, Magan *et al.* (2002) pointed out that very few experiments had considered interactions between the efficacy of fungicides and key environmental factors, particularly water availability and temperature. The impact of different interacting water availability and temperature regimes and fungicides on growth and DON production of *F. graminearum* isolates was evaluated by Ramirez *et al.* (2004). To predict FHB and mycotoxin contamination of grain under natural conditions, requirements of different *Fusarium* species associated with FHB for temperature and humidity (water activity) in the decisive time period should be taken into consideration. Recent studies (Doohan *et al.* 2003; Brennan *et al.* 2005; Hope *et al.* 2005) revealed different ecological requirements for growth and mycotoxin production by *Fusarium* species. This type of information is without doubt essential for developing climate-based risk models to determine the potential for contamination of cereal grain by mycotoxins (Hope *et al.* 2005).

The mechanisms of plant resistance to FHB are very complex, and now it is generally agreed that FHB resistance is controlled by a polygenic system. Wisniewska *et al.* (2004) pointed to at least three different resistance components: resistance to pathogen spread, to kernel colonization and to toxin accumulation. Resistance of studied wheat accessions could be described by disease score on head, per cent of Fusarium damaged grains, kernel weight per head and DON content. Evaluation of disease incidence in practice and breeding may therefore be complicated because many characters are needed to fully describe the state. Different studies revealed significant correlations between DON content and characters measuring the severity of FHB infection (Arseniuk *et al.* 1999; Mesterházy *et al.* 1999; Miedaner *et al.* 2001; Šíp *et al.* 2002a; Lemmens *et al.* 2003), but it is often reported that these relationships are highly influenced by environmental conditions, genotype, fungus isolate aggressiveness and other factors. It is advantageous that the developed advanced molecular tools now enable to determine the content of causal agent in plant tissues. The determination of pathogen DNA content is expected to significantly contribute to better identification of resistance level and understanding factors that influence FHB, which is important for eliminating the risk of mycotoxin contamination of grains and foodstuffs (Nicholson *et al.* 2003). With the developed real-time PCR system it was possible to clearly specify cultivar reactions to FHB and effects of fungicide treatment on DON content in spring barley (Šíp *et al.* 2004).

The aim of the present study was to analyse the relationship between pathogen DNA content, DON content and the other FHB traits, and to evaluate the effects of genotype, fungicide treatment and environmental conditions on the severity of FHB infection and accumulation of DON in grains of wheat cultivars.

MATERIAL AND METHODS

Plant materials. Reactions to artificial infection with *F. culmorum* and fungicide treatment were studied in nine winter wheat cultivars with varying levels of resistance. Brief characteristics of selected cultivars are given in Table 1. The cultivars Saskia and Šárka could be classified as early in heading, Nela, Bona and Sepstra as medium early and Petrus, Siria, Arina and Ebi as late in heading. The latter group of cultivars also exhibited relatively greater plant height.

Description of field experiments and treatments. Experiments at the location Prague-Ruzyně lasted four years (2001–2004). Winter wheat plots were planted after mustard (*Sinapis arvensis* L.) to minimize the inoculum of *Fusarium* spp. from debris. Each genotype was sown at 450–500 seeds per m^2 on 2.5 m^2 plots in three replications of four treatments: (1) I – inoculation by the pathogen, no fungicide, (2) IF – inoculation by *F. culmorum* and application of fungicide (Caramba or Horizon 250 EW), (3) C – no inoculation, no fungicide and (4) CF – no inoculation, application of fungicide.

Table 1. Characteristics of the examined winter wheat cultivars

Cultivar	Country of origin	Average days to heading*	Average plant height (cm)	Resistance to FHB**
Arina	CH	160	102	MR
Petrus	DE	159	100	MR
Bona	CZ	155	84	MR–MS
Nela	CZ	155	84	MR–MS
Ebi	DE	160	97	MR–MS
Šárka	CZ	153	85	MS
Saskia	CZ	151	88	MS
Sepstra	CZ	156	83	S
Siria	CZ	159	94	S

*Days after the 1st of January (average of years 2001, 2002, 2003 and 2004)

**MR = medium resistant; MS = medium susceptible; S = susceptible (long-term examination)

A randomized complete block design was used for cultivars and treatments. Inoculated (I) and control (C) plots formed two separate blocks isolated by the five meters wide wheat stand that was kept free from diseases by protective chemicals. A highly pathogenic isolate (B) of *F. culmorum* (Šíp *et al.* 2002a) was used for inoculation. The spore mixture (0.8×10^7 per ml) was applied at a rate of approximately 150 ml/m^2 onto the heads with a hand sprayer on two dates: for the first time at mid-flowering (GS 64: anthesis half-way) (Zadoks *et al.* 1974) and then one week later. Inoculation started on 13 June in 2001, 4 June in 2002, 3 June in 2003 and 8 June in 2004. Inoculation dates for individual cultivars differed according to their flowering time. The period of inoculation at GS 64 lasted 6–8 days. Fungal infection was promoted by mist irrigation of plots (applied in all treatments). In 2001 the fungicide Caramba (active ingredient Metconazole; supplied by BASF AG, Agricultural Products, Ludwigshafen, Germany) and in 2002, 2003 and 2004 the fungicide Horizon 250 EW (active ingredient Tebuconazole; supplied by Bayer, Aktivengesellschaft, Leverkusen, Germany) were applied (treatments IF and CF) according to the manufacturer's instructions (application rate: 1.0 l/ha). The inoculation with Fusarium conidial suspension followed (IF) after 24 hours, when the positive occurrence of fungicide in the plant tissue was assured.

Disease evaluation and examined yield components. Head blight symptoms (VSS) were evaluated usually 28 days after inoculation on a 1–9 scale. The rating classes were: 1 < 5%, 2 = 5–17%, 3 = 18–30%, 4 = 31–43%, 5 = 44–56%, 6 = 57 to 69%, 7 = 70–82%, 8 = 83–95% and 9 > 95% of the spikelets with FHB symptoms. Determination of other resistance traits was based on seed samples obtained in each plot from randomly selected 50 spikes, which were threshed at a low wind not to lose light infected scabby grains. *Fusarium* damaged (scabby) grains (FDG) were calculated as percentage of the total seed number. Tolerance to the infection was expressed as percent reduction (R) against the non-inoculated control (C) in the components of thousand grain weight (TGW) and grain weight per head (GWH).

Separate experiments at the location Prague-Ruzyně enabled to obtain average VSS data on 9 cultivars under study on 5 dates (7, 14, 21, 28 and 35 days after inoculation) and illustrate differences in FHB development between years (Figure 1). Each year the cultivars were grown on 1m^2 plots replicated twice. The spore mixture (0.8×10^7 per ml) of isolate B of *F. culmorum* was applied with a hand sprayer onto 20 selected heads from all sides at GS 65. Following the inoculation, each group of heads was covered with a polythene bag; bags were removed after 24 hours. The described 1–9 scale was used to evaluate head blight symptoms.

Chemical analyses. The content of deoxynivalenol (DON) was determined by ELISA on RIDASCREEN® FAST DON kits (R-Biopharm GmbH, Darmstadt, Germany). A representative

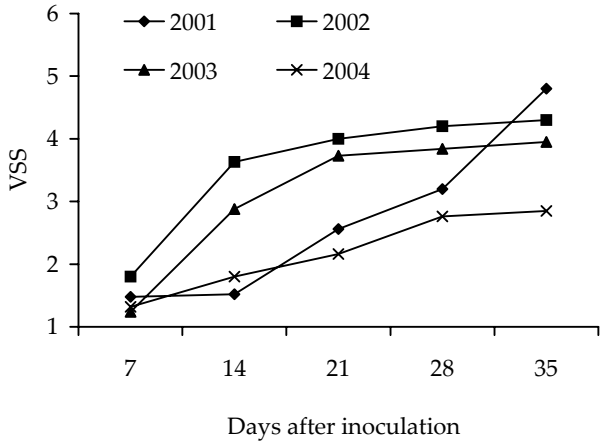

Figure 1. Development of Fusarium head blight in the years 2001, 2002, 2003 and 2004; average visual symptom scores (VSS) of nine winter wheat cultivars (Table 1) on 5 dates after the inoculation of selected heads

sample was ground and thoroughly mixed. Afterwards 5 g of the ground sample was shaken with 100 ml of distilled water and filtered. 50 µl of the filtrate was used for the assay. Samples and standards were applied according to the manufacturer's instructions. The absorption of final solution was measured at 450 nm with a SUNRISE spectrophotometer. RIDAWIN® software was used for data processing.

DNA isolation. DNAs were isolated by DNeasy Plant Mini Kit (QIAGEN) from mycelia and from infected/uninfected plant tissues. DNA concentrations and qualities were checked spectrophotometrically using a Gene Quant Pro spectrophotometer. DNA samples were diluted to a fixed concentration of 50 ng/µl before PCR.

Real-time (RT) quantitative PCR. Real-time PCR were carried out in 25-µl volumes consisting of 1× PCR buffer, 4mM $MgCl_2$, 100µM (each) dATP, dGTPand dCTP, 200 µM dUTP, 2.5 U Ampli Taq Gold polymerase, 0.3µM each of primer FC92s1-F and FC92s1-R, 0.3µM Taq Man MGB probe FC92s1 and 250 ng of template DNA according to the optimised protocol (Leišová *et al.* 2006). Real-time quantitative PCR was performed using the cycler ABI PRISM 7700 in MicroAmp optical 96-well plates (Applied Biosystems, Foster City, USA). The 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. The Sequence Detection Software (Applied Biosystems, Foster City, USA) collected data for reported dye from each well, generating a fluorescence profile for each sample. 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.

A dilution series of *Fusarium culmorum* isolate B DNA (from 0.9 pg to 1000 ng) was included in triplicate as standards in every Taq Man experiment. Standard curves for *F. culmorum* 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 (Leišová *et al.* 2006).

For a statistical analysis the Ct values were transformed in the following way: $Ct_{Fus\ transf} = 10^7 \times 2^{-Ct\ (Fus)}$. Transformed Ct values have positive relationships with DNA amounts of *F. culmorum* and take into account the kinetics of PCR reactions.

Statistical analysis. The UNISTAT 5.0 package (UNISTAT Ltd., London, UK) was used for statistical analyses of the data and STATISTICA package (StatSoft, Inc., Tulsa, OK) for graphics. The data obtained from non-inoculated plots were not included in statistical analyses (they were used to determine reductions in the examined yield components). The analysis of DON content

Table 2. The sum of average daily temperatures in a 30-day period after inoculation at GS 64, average daily temperature (AVE) and variation (s^2) in daily temperatures in four years (the interval expression reflects differences in cultivar flowering – inoculation – dates); number of days with temperature maximum > 25°C and > 30°C

Year	Sum of 30 days (°C)	AVE (°C)	s^2	> 25°C	> 30°C
2001	508–512	16.93–17.06	5.68–7.04	6–8	0
2002	541–561	18.03–18.70	7.94–7.46	10–13	2–4
2003	605–565	20.18–18.83	7.10–7.04	19–12	7–5
2004	500–496	16.67–16.53	4.98–4.32	6–7	0

in control plots (C and CF) showed only traces of seed contamination. The respective average values for DON content in C and CF plots were 0.46 mg/kg and 0.31 mg/kg. The experiments were not apparently affected by other diseases and pests or abiotic stress factors. The efficacy of fungicide treatment (%) for each examined trait was calculated in the following way: 100 – (IF/I) × 100, where IF is the value obtained after inoculation and fungicide treatment and I is the value obtained from inoculated plots. For correlation analyses complementary data collected on cultivar heading date (expressed as days after the 1st of January), plant height in the respective year, and sums of average daily temperatures (air temperatures at 2 m above the ground) in a 30-day period after inoculation were also employed (Table 2).

RESULTS

Analysis of factors determining infection severity and DON content

Table 3 shows the results of analyses of variance for DON content, pathogen DNA content (Ct), DON/Ct ratio, percentage of Fusarium damaged grains (FDG), symptom scores (VSS) and reductions in grain yield components of thousand grain weight (TGWR) and grain weight per head (GWHR). The block effects were not significant ($P > 0.05$); they are included in error variance. Analyses of variance showed statistically significant effects of genotype and experimental year on all traits. The fungicide treatment significantly affected all traits with the exception of DON content per pathogen unit (DON/Ct ratio). Year effects prevailed over fungicide treatment effects for FDG and Ct values (besides DON/Ct ratio), while in the other traits the fungicide treatment effect was predominant. Cultivars responded differently in all traits to experimental years and, except for DON content, to fungicide treatment. Year × treatment interactions, and three-way interactions were also mostly significant.

Effects of individual years on trait performance are shown in Table 4. Average DON content and also pathogen DNA content, FDG and VSS were high in 2001. This year could be characterized by a long period of disease development (Figure 1). In 2002 an early outbreak of FHB highly affected particularly grain weight per head (high reduction in grain number per head: 38.6%). The colonization of grain by the pathogen was high in that year, but the content of DON was significantly lower than in 2001. In 2003, with quite similar development of FHB symptoms like in 2002, the same average DON content as in 2002 was obtained at half the pathogen content. In 2002 and 2003 disease development was accompanied by temperature extremes (Table 2). A low disease incidence was characteristic of 2004, but not all cultivars exhibited a low accumulation of DON in that year (Figure 2). The losses of average grain weight per head due to infection were similar in 2001, 2003 and 2004, but symptom expression and colonisation of grain by the pathogen were extremely low in 2004.

Table 3. ANOVA mean squares for each source of variation in seven traits

Source of variation	df	DON content	Pathogen DNA content (Ct)	DON/Ct ratio	Fusarium damaged grains	Visual scoring of symptoms	1000-grain weight reduction	Grain wt./head reduction
Cultivar (C)	8	993**	63.03**	88**	1472**	18.21**	627**	1042**
Year (Y)	3	3250**	507.28**	650**	15066**	30.79**	484**	4136**
Treatment (T):I/IF	1	8474**	182.18**	0^{NS}	10702**	74.40**	2624**	9454**
C × Y	24	197**	27.85**	119**	330**	2.04**	146**	358**
C × T	8	79^{NS}	23.89**	59**	245**	1.70**	204**	549**
Y × T	3	206**	24.47**	32*	496**	1.84**	93**	85^{NS}
C × Y × T	24	163**	18.44**	121**	376**	0.80**	186**	542**
Error	140	28	4.22	12	74	0.21	5	63

**$P < 0.01$; *$P < 0.05$; ^{NS}not significant ($P > 0.05$)

Table 4. Cultivar and year means of inoculated plots (I) and total means of I and IF (inoculation and fungicide treatment) for six traits

Cultivar/year/ treatment	DON content (mg/kg)	Pathogen DNA content (Ct)	Fusarium damaged grains (%)	Visual scoring of symptoms (0–9)	1000-grain wt. reduction (%)	Grain wt./head reduction (%)
Arina	16.17^{a}	3.46^{ab}	26.31^{a}	2.50^{a}	10.70^{ab}	26.25^{ab}
Petrus	16.68^{a}	2.66^{a}	31.85^{ab}	2.50^{a}	6.88^{a}	20.34^{a}
Nela	20.58^{ab}	5.14^{abc}	37.59^{ab}	3.92^{bc}	13.99^{ab}	39.39^{cde}
Bona	22.20^{ab}	6.83^{bc}	35.98^{ab}	3.83^{bc}	11.44^{ab}	33.10^{bc}
Saskia	24.50^{bc}	7.15^{bcd}	48.15^{bcd}	5.67^{e}	22.77^{d}	41.01^{cde}
Šárka	27.27^{bcd}	3.73^{ab}	46.60^{bcd}	4.50^{cd}	21.49^{cd}	39.41^{cde}
Ebi	33.25^{cde}	5.05^{abc}	40.29^{abc}	3.17^{ab}	16.13^{bc}	35.05^{bcd}
Sepstra	34.68^{de}	7.86^{cd}	55.83^{cd}	4.25^{c}	27.13^{d}	47.03^{e}
Siria	39.88^{e}	10.65^{d}	59.51^{d}	5.25^{de}	27.68^{d}	45.29^{de}
2001	38.65^{c}	8.27^{c}	53.02^{c}	4.48^{c}	15.30^{ab}	31.00^{a}
2002	26.57^{b}	9.83^{c}	59.09^{c}	4.56^{bc}	21.81^{c}	49.78^{b}
2003	26.31^{b}	4.18^{b}	42.71^{b}	4.00^{b}	20.25^{bc}	32.35^{a}
2004	15.45^{a}	1.61^{a}	17.37^{a}	2.78^{a}	13.88^{a}	33.37^{a}
I	26.41^{b}	5.91^{b}	42.76^{b}	3.96^{b}	17.88^{b}	36.79^{b}
IF	14.20^{a}	3.83^{a}	28.50^{a}	2.80^{a}	10.81^{a}	23.37^{a}

The means in columns followed by the same letter are not significantly different from each other at $P < 0.05$ of LSD test

Cultivar differences in reactions to FHB are shown in Table 4. It is important that moderately resistant cultivars Arina and Petrus were included in the first homogeneous group in all examined traits, including pathogen DNA content, which may indicate relatively higher "complex" resistance. In accordance with our previous findings (Šíp & Stuchlíková 1997; Šíp *et al*. 2002a), the cultivar Bona could be classified from different aspects as having medium resistance and also the cultivar Nela except for grain weight per head reduction. Susceptible reactions in all traits were characteristic of cultivars Sepstra and Siria. The early cultivars (Saskia and Šárka) showed medium DON content, but symptom scores and reductions in grain yield components were high. The late Ebi, considered to have medium resistance to FHB, exhibited susceptibility to DON accumulation.

Striking cultivar differences in the accumulation of DON in single years are evident from Figure 2. Resistance to the accumulation of DON in Arina and Petrus was highly expressed in 2001, 2002 and 2004, but in 2003 these cultivars exhibited a similarly high DON content like the other cultivars. In 2003, the cultivar effect on DON content was not significant ($P > 0.01$). The 2001 season favoured a high DON content in late susceptible cultivars Ebi and Siria, and 2002 in early cultivars Saskia and Šárka. Under conditions of low disease attack (2004), only the cultivars Ebi, Sepstra and Siria showed a significantly higher DON content (35.9–38.7 mg/kg) than the other cultivars (2.8–5.7 mg/kg). To determine cultivar differences, multiyear comparisons were evidently needed.

Effect of fungicide treatment on different traits

Tables 3 and 4 show that the fungicide treatment had a significant effect on a reduction in the average values of all traits, except DON/Ct ratio, and significant interactions of fungicide treatment with year and/or cultivar were often detected. The absence of cultivar × fungicide treatment interaction for DON indicates a significant difference in cultivar reactions to fungicide applica-

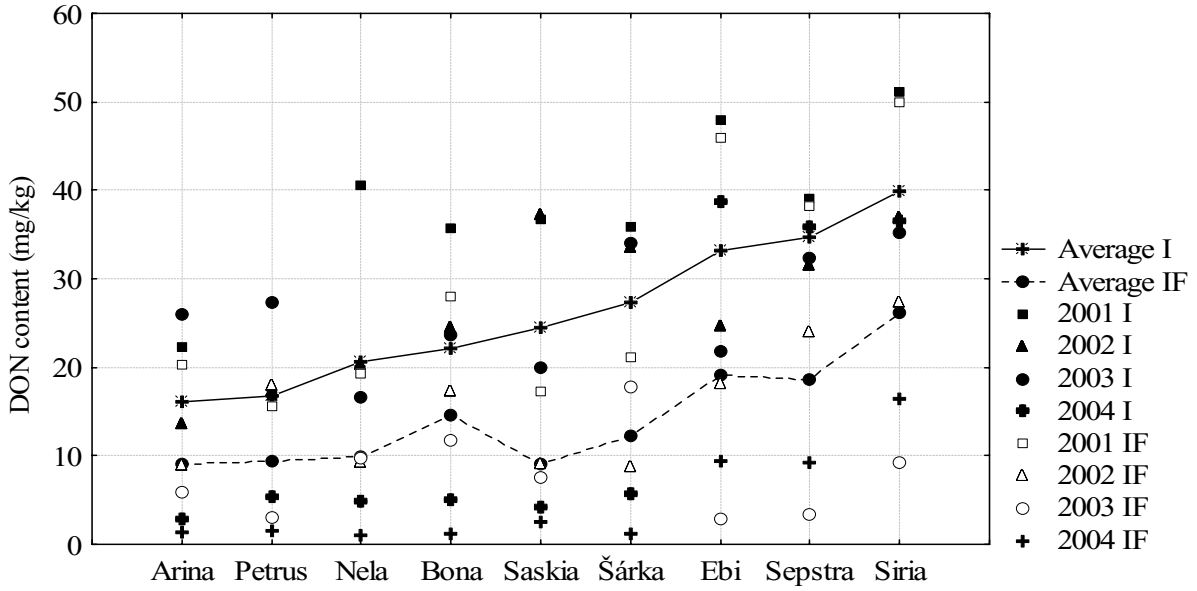

Figure 2. DON content in nine winter wheat cultivars in four years (2001, 2002, 2003 and 2004) after inoculation by *F. culmorum* (I) and fungicide treatment of inoculated plots (IF)

tions. As shown in Figure 2, DON reductions due to fungicide treatment in the cultivars were very variable in the different years, but the application of fungicide caused a reduction in DON in all cultivars. The average efficacy of fungicide treatment reached almost 50% for DON content while these values ranged between 29% and 39% for the other examined traits (Table 5). For individual cultivar traits the efficacies ranged between 7% and 60%, and Šárka and Ebi showed on average a higher pathogen DNA content after fungicide treatment.

Besides cultivars the experimental years also highly influenced the efficacy of fungicide treatment (Table 5). The year 2001 was characterized by a low efficacy for DON content (particularly in late genotypes Arina, Petrus, Ebi and Siria, and medium early Sepstra; Figure 2) and a relatively high reduction in pathogen content and grain weight per head after the application of fungicide. Low efficacy for DON and high efficacy for a reduction in pathogen content led to a significantly higher level of DON per unit of fungal DNA after fungicide treatment (Figure 3). In 2002 and 2003 the efficacy was lower and highly variable for the pathogen DNA content. It was not exceptional in these years that the fungicide treatment led to a higher pathogen content (Figure 4). In 2003, at lower pathogen content and relatively lower effects of FHB on the other traits of disease severity, the efficacy for DON was higher (69%) than in 2002 (38%) although the average DON content in inoculated plots was similar in these years (Tables 4 and 5). In 2004 the effect of fungicide treatment was high both on DON content (FDG) and on pathogen content. The proportionality in fungicide effects on a reduction in DON content and pathogen DNA content led to similar DON/Ct ratios in inoculated (I) and fungicide treated (IF) plots (Figure 3).

Relations between DON content and other examined traits as affected by environment, genotype and fungicide treatment

It follows from Table 6 that in both treatments (I and IF) and in all years DON content was positively related to the pathogen DNA content, and correlation coefficients ranged between 0.42 and 0.92. Both DON content and pathogen content were mostly positively related also to the other traits, but these relationships were highly variable. Among the examined FHB traits especially the percentage of Fusarium damaged grains (FDG) appeared to have practical relevance to the prediction of DON content and grain yield losses. The relations of head symptoms (VSS) to DON and pathogen contents were not often so strong. With

Table 5. Efficacy of fungicide treatment [100 – (IF/I) × 100] for cultivars and years in six traits

Cultivar/year	DON content	Pathogen DNA content (Ct)	Fusarium damaged grains	Visual scoring of symptoms	1000-grain weeght reduction	Grain wt./head reduction
Arina	43.24	42.72	19.40	37.50	28.60	42.75
Petrus	52.80	33.24	43.82	34.72	38.97	27.71
Nela	56.59	55.41	36.74	23.93	52.21	55.87
Bona	44.81	24.56	26.03	7.14	38.64	12.93
Saskia	57.82	31.77	44.56	37.38	44.21	53.02
Šárka	60.19	**	42.74	30.00	52.96	51.05
Ebi	48.39	**	37.24	21.02	13.14	42.17
Sepstra	47.53	53.73	35.82	33.96	50.12	27.84
Siria	38.20	52.48	33.31	30.57	32.37	27.81
2001	22.77	51.62	16.12	24.04	25.00	44.90
2002	37.87	(10.10)*	29.43	21.32	62.58	35.27
2003	68.69	(35.36)*	30.79	40.37	42.00	42.76
2004	67.15	64.00	62.65	28.15	31.25	28.70
Mean	49.87	35.20	35.28	28.47	39.31	37.91

*highly variable (not exceptionally IF > I); **negative value (IF > I)

the exception of 2004 DON content per unit of pathogen (DON/Ct ratio) was negatively correlated with the pathogen DNA content. The inter-annual correlation coefficient was –0.59 ($P < 0.001$) for inoculated plots (I) and –0.46 ($P < 0.001$) when the fungicide was applied (IF). These findings are in accordance with the results of Gosman *et al.* (2005) showing that higher levels of colonisation were strongly associated with reduced levels of DON per unit of fungal pathogen. Relations between DON content and DON/Ct ratio were mostly insignificant.

It was also useful to compare trait relations in different years and treatments I and IF. As shown in Table 6, the relations of DON content and pathogen DNA content with the other traits were more

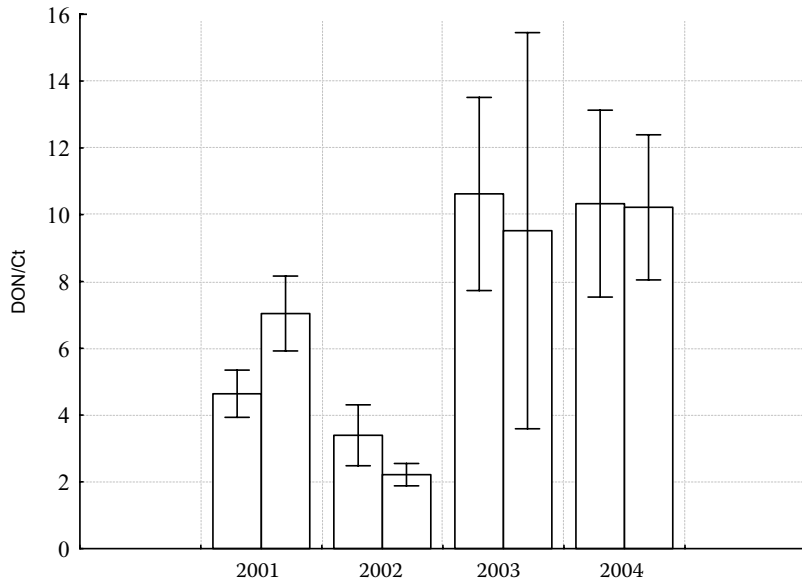

Figure 3. The DON content/fungal DNA (Ct) ratio in four years (2001, 2002, 2003 and 2004) and for □I (inoculation) and ▨ IF (inoculation and fungicide) treatments; the bars are 95% confidence interval

Table 6. Coefficients of correlation between the examined traits in four years (2001, 2002, 2003 and 2004) and for treatments I (inoculation) and IF (inoculation and application of fungicide)

Combination of traits	2001		2002		2003		2004	
	I	IF	I	IF	I	IF	I	IF
DON vs. Ct	0.50**	0.74***	0.61***	0.76***	0.42*	0.57***	0.81***	0.92***
DON vs. FDG	0.41*	0.87***	0.83***	0.35*	0.56**	0.76***	0.97***	0.96***
DON vs. VSS	0.45*	0.48**	0.74***	0.10	0.23	0.72***	0.39*	0.69***
DON vs. TGWR	0.19	0.71***	0.69***	0.46**	0.34*	0.59***	0.88***	0.71***
DON vs. GWHR	0.34	0.79***	0.61***	0.39*	0.29	0.55**	0.46**	0.37*
DON vs. DON/Ct	0.32	–0.08	0.15	0.22	–0.08	–0.27	0.42*	–0.05
Ct vs. FDG	0.05	0.59**	0.60***	0.38*	0.65***	0.20	0.84***	0.89***
Ct vs. VSS	–0.04	0.07	0.65***	0.24	0.25	0.43*	0.68***	0.59***
Ct vs. TGWR	–0.43*	0.42*	0.43*	0.21	0.16	0.67***	0.83***	0.70***
Ct/GWHR	–0.43*	0.52**	0.38*	0.08	0.58***	0.21	0.51**	0.26
Ct vs. DON/Ct	–0.60***	–0.46*	–0.57**	–0.41*	–0.77***	–0.48**	–0.10	–0.26
FDG vs. VSS	0.64***	0.44*	0.78***	0.03	0.39*	0.63***	0.46**	0.62***
FDG vs. TGWR	0.48**	0.82***	0.70***	0.38*	0.53**	0.11	0.91***	0.65***
FDG vs. GWHR	0.43*	0.88***	0.76***	–0.11	0.56**	0.61***	0.45**	0.30
VSS vs. TGWR	0.64***	0.70***	0.73***	–0.51**	0.62***	0.43*	0.53**	0.53**
VSS vs. GWHR	0.54**	0.44*	0.77***	–0.52**	0.80***	0.86***	0.41*	0.51**
TGWR vs. GWHR	0.75***	0.89***	0.71***	0.62***	0.42*	0.26	0.64***	0.60***

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Ct = pathogen DNA content (transformed values); DON = DON content; FDG = percentage of Fusarium damaged grains; VSS = visual symptom scores; TGWR = reduction in thousand grain weight; GWHR = reduction in grain weight per head

often insignificant (or even negative) in 2001 and 2003, which indicates that particularly in these years the inoculation by the pathogen (I) had a variable impact on grain colonization by the pathogen, disease symptoms, DON content and grain yield losses. However, the fungicide treatment in inoculated plots (IF) established significantly positive relations between DON content and the other examined traits. The relations between DON content and other traits in 2002 were closer after I treatment and mostly insignificant after the application of fungicide (IF). The trait relations did not apparently differ between treatments I and IF in 2004, which is another evidence of the proportionality of fungicide effects on different traits in that year.

DON content, pathogen DNA content (Ct), grain weight per head reduction (GWHR) obtained after treatments I and IF, as well as the efficacies of fungicide treatment for these traits, were also examined for interrelations across environments and relations with additional data on heading date, plant height and sums of average daily temperatures (SADT) during the period of disease development. Table 7 shows that the efficacies of fungicide treatment for DON, Ct and GWHR were positively correlated. High efficacy of fungicide treatment was closely connected with low performance in each trait after fungicide treatment (IF), and the insignificance of the correlation between efficacy and performance after inoculation (I) indicates that under certain conditions and in certain cultivars the treatment led to variable reductions in DON, pathogen content and grain yield losses. The efficacy for DON was negatively associated with pathogen content.

In these experiments early heading contributed to lower DON content and higher yield reductions. With early heading the fungicide efficacy for DON tended to be higher. The relations of pathogen content to heading date were not significant, similarly like the effects of plant height on examined traits,

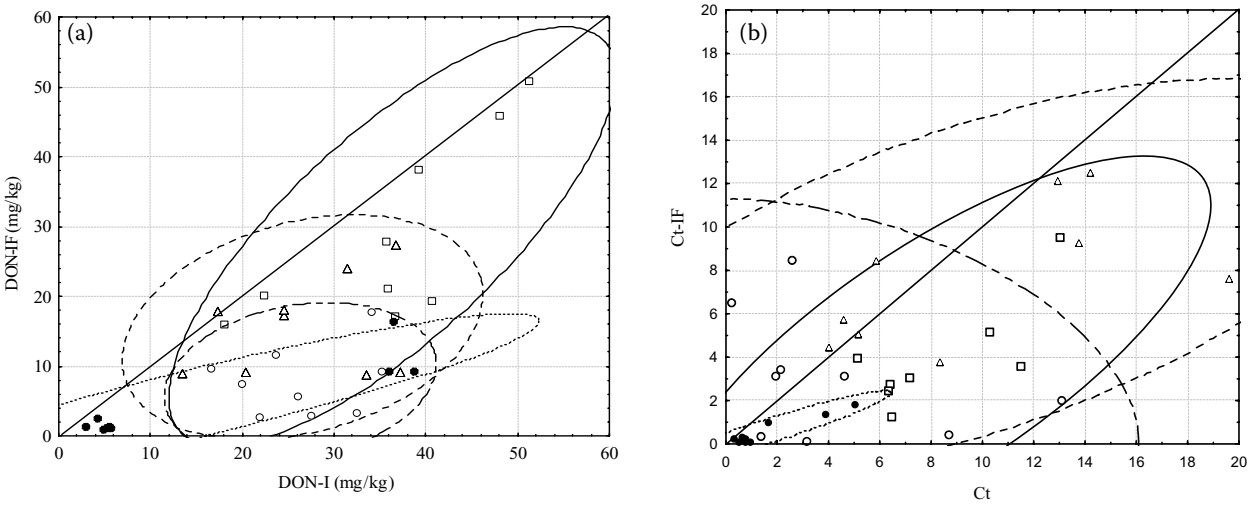

Figure 4. Comparison of cultivar DON content (a) and pathogen DNA content (Ct) (b) for I (inoculation) and IF (inoculation and fungicide) treatments. The ellipses mark the distribution of points (P = 95%) belonging to single experimental years (2001 □ ——, 2002 △ −−−−−2003 ○ — − − —, 2004 ● ·····)

Table 7. Inter-annual coefficients of correlation between DON content, pathogen DNA content (Ct) and grain weight per head reductions (GWHR) for treatments I and IF, efficacies (EF) of fungicide treatment for these characteristics and heading date, plant height and sums of average daily temperatures (SADT) in periods of disease development

	DON-I	DON-IF	EF DON	Ct-I	Ct-IF	EF Ct	GWHR-I	GWHR-IF	EF GWHR
DON-I	–	0.71***	−0.27	0.60***	0.38*	0.02	0.33*	−0.11	0.28
DON-IF	0.71***	–	−0.81***	0.54***	0.54***	−0.19	0.06	0.19	−0.20
EF DON	−0.27	−0.81***	–	−0.37*	−0.54***	0.44**	0.10	−0.40**	0.42**
Ct-I	0.60***	0.54***	−0.37*	–	0.62***	0.03	0.43**	0.05	0.08
Ct-IF	0.38*	0.54***	−0.54***	0.62***	–	−0.51**	0.32*	0.37*	−0.20
EF Ct	0.02	−0.19	0.44**	0.03	−0.51**	–	−0.16	−0.55***	0.33*
GWHR-I	0.33*	0.06	0.10	0.43**	0.32*	−0.16	–	0.24	0.21
GWHR-IF	−0.11	0.19	−0.40**	0.05	0.37*	−0.55***	0.24	–	−0.80***
EF GWHR	0.28	−0.20	0.42**	0.08	−0.20	0.33*	0.21	−0.80***	–
Heading date	0.30*	0.52***	−0.47**	0.11	0.02	0.18	−0.34*	−0.07	−0.10
Plant height	−0.29*	−0.08	−0.18	−0.04	0.16	−0.19	0.06	0.40**	−0.25
SADT 5 days	−0.43**	−0.70***	0.63***	−0.49**	−0.34*	0.00	−0.11	0.01	0.04
SADT 6–10 days	−0.08	0.00	−0.16	0.01	0.41**	−0.63***	−0.04	0.50**	−0.41**
SADT 10 days	−0.29*	−0.40**	0.28	−0.27	0.06	−0.40*	−0.09	0.32*	−0.22
SADT 11–15 days	0.28	0.14	−0.15	0.41**	0.60***	−0.52***	0.31*	0.23	0.04
SADT 15 days	−0.14	−0.26	0.15	−0.06	0.26	−0.49**	0.02	0.32*	−0.17
SADT 16–20 days	0.36*	0,17	0.03	0.51***	0.27	0.13	0.38*	−0.31*	0.37*
SADT 20 days	−0.03	−0.21	0.16	0.10	0.34*	−0.44**	0.14	0.22	−0.06
SADT 21–25 days	0.26	0.15	−0.03	−0.04	−0.06	−0.10	−0.22	−0.32*	0.24
SADT 25 days	0.01	−0.17	0.14	0.08	0.31*	−0.43**	0.10	0.16	−0.02
SADT 30 days	0.01	−0.11	0.04	0.07	0.36*	−0.54***	0.08	0.26	−0.09

***P < 0.001, **P < 0.01, *P < 0.05

with the exception of a positive relation ($P < 0.01$) between plant height and GWHR after fungicide treatment. Relatively lower temperatures in a 5-day period following the inoculation at mid-anthesis (GS 64) (average temperatures in this period ranged between 13.5°C and 22.4°C) contributed to higher DON and pathogen DNA contents in mature grain. The effect of fungicide treatment on a reduction in DON content could be lower under such conditions. In these experiments higher average daily temperatures in the period between day 11 and 20 (temperature range 14.7°C–21.9°C) could favour the colonization of grain by the fungus while grain yield losses tended to be higher. The reduction in pathogen content due to fungicide treatment in connection with higher temperatures in a 30-day period was found lower.

DISCUSSION

A microplot system with the inoculum sprayed onto plots can be considered useful for these types of studies (Mesterházy *et al.* 2003) because it enables to simulate conditions that are likely to occur on a larger scale in agricultural practice. In these experiments the mist irrigation of plots guaranteed progressive development of the disease in all years and, therefore, the variation in disease attack over years could mainly be ascribed to cultivar reactions and their interactions with treatments, as well as to inoculation timing and key environmental factors (temperature, moisture) during disease progression. Error variance in trials with replications was quite low in all examined traits compared to treatment, year and genotype effects (Table 3), which made it possible to properly evaluate the effects of inoculation with the fungus and fungicide treatment. Control variants, used to evaluate the effects of FHB infection and fungicide treatment on yield components, were maintained uncontaminated by DON producing Fusarium pathogens.

Mycotoxin content, grain yield losses and effects of infection on seed quality are undoubtedly the most important characteristics from practical aspects. However, it is widely acknowledged that FHB is a complicated disease and many traits are needed to evaluate damage to the crop by the disease and cultivar resistance. Breeding for FHB resistance and low accumulation of mycotoxins would be greatly facilitated if the mycotoxin (DON) accumulation of a genotype could be predicted from indirect, easily determined FHB traits. Among the included traits, particularly determination of the percentage of Fusarium damaged grains (FDG) appeared to be useful from these aspects. This trait was found positively related to DON content and reductions in the examined yield components in inoculated plots in all years. FDG was considered as a valuable trait by many authors (e.g. Arseniuk *et al.* 1999; Ittu *et al.* 2000; Lemmens *et al.* 2003; Chrpová *et al.* 2004), but due to variable relationships multiple environment examinations for this trait are evidently necessary. The experiments of Mesterházy *et al.* (2005), in which reactions of wheat cultivars to different *Fusarium* spp. were studied, revealed close correlations between FDG and DON contamination, however, this research demonstrated the importance of measuring both FDG and DON in the breeding and selection of resistant germplasm and cultivars. Similarly like in previous experiments, the examination of symptoms was found (Šíp *et al.* 2002a) inferior to the determination of DON by means of traits measuring infection effects on seed size and appearance. In the experiments of Brennan *et al.* (2005) a visual disease assessment clearly reflected yield losses, but no significant relation was detected between symptom scoring and fungal DNA content of grain. Stronger relationships between symptom expression and other FHB traits could be obtained from the examination of AUDPC (Shaner & Finney 1977), but in these types of experiments (when the spraying of inoculum onto plant stands not uniform in heading is used) and in practice (when evaluating natural infections) this examination is not easy to perform and is unlikely to bring more valuable results. The relations between DON content and reductions in the examined yield traits, similarly like the relations between DON and symptom scores, were also found more variable than the relations between DON and FDG.

Contrasting conditions of experimental years highly influenced performance in the examined traits and resulted in mostly significant interactions of years with cultivars and treatments. Surprising high effects caused by the environment were reported by many authors (Mesterházy *et al.* 1999; Miedaner *et al.* 2001; Šíp *et al.* 2003; Wiśniewska *et al.* 2004). Field experiments with artificial infection of winter wheat cultivars with *F. culmorum*, conducted in Prague-Ruzyně since 1992, showed striking differences in the development of FHB between years in these Central European

conditions (Šíp & Stuchlíková 1997; Šíp *et al.* 2002b). In accordance with the results of previous experiments in 1998–2000 (Šíp *et al.* 2002a), it can be suggested also from these experiments that the slower disease development in relatively colder and humid weather conditions may result in a higher accumulation of toxins, while conditions that favour the accelerated and relatively shorter disease development can lead to a higher severity of the disease (higher yield losses), but not necessarily to a high accumulation of DON in grain. At genotype earliness (short vegetation period) the reaction to FHB may be similar like under environmental conditions that promote the shorter disease development. The early cultivars Hana (Šíp *et al.* 2002a) and now Saskia and Šárka showed high yield losses due to infection, but a relatively lower accumulation of DON in grain. Therefore, the growth habit (especially the length of the grain filling period) may contribute to different resistance to the accumulation of DON and FHB in a genotype.

As FHB causes very miscellaneous and often not clear symptoms of infection, it was suggested that the real-time PCR analysis could be particularly helpful for quantifying FHB and explaining differences in FHB development and accumulation of DON under variable environmental conditions. Nicholson *et al.* (2003) reported that competitive and real-time PCR analyses now enable to specify more clearly also fungicide effects on the disease and mycotoxin accumulation. Doohan *et al.* (1999) found that PCR provided a higher resolution than the visual disease assessment for the determination of fungicide efficacy. The real-time PCR assay used in this study was found to be highly specific and sensitive. A close linear relationship was detected between the amount of fungal genomic DNA and transformed Ct values (r^2 = 0.993). Probably the first report on the use of a comparative threshold cycle (Ct) method to quantify the DNA of a plant pathogen relative to its host DNA was published by Gao *et al.* (2004). It was shown by Leišová *et al.* (2006) that transformed Ct values (used also in this study) might well suit the demands because they take into account the amount of target PCR amplicons in the best way. The calibration curve was based on Ct values of diluted pathogen DNA isolated from pure fungal mycelia while DNA isolated from the infected and damaged plant tissue will represent a different matrix even though the DNA isolation procedure is identical.

The relationship between Ct values and DON contents appeared to be rather complicated in wheat. Though our experiments showed significantly positive relationships between Ct values (relative pathogen DNA estimation) and DON content under different conditions, these relationships were not always close. Large differences in DON content per unit of pathogen were detected between the years. It also follows from the results presented by Gosman *et al.* (2005) that similar amounts of fungal biomass may be associated with different mycotoxin contents or *vice-versa*. Reduced levels of DON per unit of pathogen were characteristic of the years 2001 and 2002 with similarly high pathogen contents, but the slower (longer lasting) accumulation of pathogen in plant tissues at relatively lower temperatures probably resulted in a significantly higher DON content in 2001 compared to 2002. The disease saturation is likely to occur at a higher temperature, which may cause less obvious differences in disease progress between cultivars (Brennan *et al.* 2005). Temperature extremes that unequally affected individual cultivars differing in flowering time (inoculation dates) might contribute to the highly variable DON to fungal biomass ratio in cultivars and differential expression of cultivar resistance in 2003. In that year the least genotypic variation was detected for DON content and examined yield traits (data not shown here). Under these conditions the most resistant late cultivars Arina and Petrus did not express high resistance to the accumulation of DON. In 2003, similarly conducted experiments with winter wheat and spring barley genotypes brought about different results: close relations between DON and pathogen content in spring barley (Šíp *et al.* 2004) and relatively the weakest correlation in winter wheat. These differences can partly be explained by the biological specificity of both crops, and a different impact of weather conditions on inoculated plants can be reckoned as the most decisive factor.

It was indicated by these experiments that under conditions of water availability the temperature in a 5-day period following the inoculation could influence both DON and pathogen content in mature grain. Hooker *et al.* (2002) demonstrated that relatively narrow time periods around heading (4–7 days before heading and 3–6 days after heading) were particularly decisive for the prediction of DON content. Weather conditions (temperature, moisture) around anthesis can be

considered as a critical factor in FHB epidemics (Parry *et al.* 1995), and Kang and Buchenauer (2002) documented that the accumulation of DON secondary metabolites in host tissues might start very soon (36 h after inoculation). While temperatures in the subsequent periods (days 6 to 30 after inoculation) did not appear to affect the content of DON strongly, relatively higher temperatures especially between days 11 and 20 contributed to an increase in pathogen content and probably also to an increase in grain yield losses due to infection. Brennan *et al.* (2005) showed that the FHB disease severity was significantly higher in terms of reduced grain weight, visual disease symptoms and amounts of fungal DNA at the higher temperature of 20°C compared with 16°C. Studies of Hope *et al.* (2005) on the colonization of wheat grains by *F. culmorum* and *F. graminearum* in a 40-day period revealed that the growth of both species was highly affected by temperature, moisture conditions and their interactions. *F. culmorum* grew optimally at a high moisture content in grain (0.98 a_w) at 15°C and 25°C. The relationships between temperatures and moisture content in grain were not evaluated in the field experiments under study, but it can be expected that the mist irrigation of plots enabled to escape dry conditions and the temperature could become crucial in determining the infection and mycotoxin production.

Key environmental factors such as water availability and temperature can evidently influence also the efficacy of fungicide treatment (Magan *et al.* 2002). The presented results are without doubt highly attributable to conditions of the examined years and fungicide treatments, and it can be concluded from the correlation analysis that fungicide effects on a reduction in DON and pathogen content were usually higher under conditions that promoted lower performance in these traits. While the efficacy for DON appeared to be higher at a relatively higher temperature in the narrow time period following the inoculation (5 days after inoculation), the fungicide efficacy for pathogen content was higher in "relatively colder" years 2001 and 2004. The results seem to contrast with those of Ramirez *et al.* (2004), who reported the majority of the fungicides to be more effective in controlling the fungal growth at 25°C rather than at 15°C, but it is likely that the temperature extremes in 2003 and 2002 could contribute to high fluctuations of fungicide efficacy and to unsatisfactory results particularly in some cultivars that showed an increase in pathogen content after the application of fungicide.

There is enough evidence that the efficacy of fungicides in controlling FHB is highly variable and often unsatisfactory (Mesterházy *et al.* 2003). Some of the variability is related to the fungicide used, its timing and coverage; other sources of variability are the timing and severity of infection under different weather conditions, virulence of the isolates, and the level of resistance in the cultivars. In general, the active ingredient tebuconazole was reported to be more effective in reducing FHB than the other fungicides, but also metconazole or other fungicides were reported to possess a high activity against both *F. culmorum* and *F. graminearum* (Pirgozliev *et al.* 2002; Magan *et al.* 2002). In these experiments the differences between used fungicides were evidently outweighed by year effects. While in 2002 tebuconazole caused only a 38% reduction in DON, in 2003 this fungicide reached 69% efficacy at a similar average content of DON like in 2002 and at the same fungicide dosage and timing. Large differences between years and cultivars were detected in fungicide effects on a reduction in all examined traits, and the efficacies of fungicide treatment for DON, pathogen content and grain yield loss (GWHR) were positively correlated. Mesterházy *et al.* (2003) detected significant correlations between the fungicide efficacies of the four parameters measured (FHB ratings, percentage of FDG, DON content and relative yield loss). However, it follows from the presented results that the effect on a reduction in one trait was not often proportional to the effect on the other traits. Owing to unproportional reductions in DON and pathogen content, the fungicide treatment in 2001 led to increased production of DON per unit of pathogen, similarly like the application of azoxystrobin in experiments of Simpson *et al.* (2001). The fungicide treatment with either metconazole (2001) or tebuconazole (2002–2004) did not increase the content of DON, though particularly in 2001 the efficacy in late genotypes was close to zero. On the contrary, an increase in pathogen content after the application of fungicide was not exceptional. The $P = 0.95$ ellipses drawn in Figure 4 indicate a high fluctuation of Ct–I/Ct–IF points for cultivars in 2002 and 2003 and a rather negative correlation between Ct–I and Ct–IF in 2003.

In spite of the high variability of fungicide effects in cultivars and years, the examination of

FHB development in different conditions may help to adopt integrated measures to decrease disease damage and to define requirements for fungicides. These experiments also indicated the importance of the relatively narrow time period around anthesis for manifestation of the disease and efficacy of fungicide treatment. Therefore, the proper timing of fungicide application appears to be an important prerequisite of success. The conditions that enable long lasting development of the disease and lead to a high accumulation of both DON and pathogen evidently make the protection highly problematic. The long persistence of fungicide action or other spraying is evidently necessary to combat such an infection type. According to Kászonyi *et al.* (2004) the best fungicides have now about one-month protective action and it should be taken into consideration that in some years (in our case 2001) or regions the one-month protection need not be sufficient. Under conditions of shorter and not so harmful development of the disease (with relatively lower DON and pathogen contents in 2003 and 2004) the application of fungicide with high immediate effect can give satisfactory results. The effect of the treatment with tebuconazole fungicide on a reduction in both DON and pathogen content was low in 2002, which was characterised by high pathogen content and high reductions in yield traits. Mesterházy *et al.* (2004) reported that the most severe infections (in the most sensitive wheat cultivars) could be controlled successfully only with the prothioconazole fungicides.

To increase the effectiveness of disease control under natural conditions the protection should be differentiated with respect to the type of pathogens (Simpson *et al.* 2001) and it should evidently consider the conditions for disease development and cultivar type. Long-term experience with the disease in a certain growing region, respectful of other risk factors, particularly those influencing the number of viable airborne propagules of Fusarium pathogens at flowering stage (Schaafsma *et al.* 2005), and weather forecasts for critical periods may enable to make the prognosis of disease development, decisive for the choice of control measures. Using important weather variables, a weather-based model to predict the concentration of DON in grain at heading was developed by Hooker *et al.* (2002). A quantitative assessment model to predict the risk of FHB and the associated production of mycotoxins (Xu *et al.* 2004) appeared to be useful for making practical disease management decisions involving fungicide applications. Cultivar resistance is another important factor that may significantly contribute to the efficient control of the disease. It also follows from these experiments that in combination with fungicide treatment the cultivars that are more resistant to FHB attack are better protected from disease attack and accumulation of DON in grain under different conditions. From practical aspects the "double protection" (consisting in growing less susceptible cultivars and fungicide treatment) with respect to the factors decisive for FHB development and accumulation of toxins in certain conditions is evidently needed to control the disease in a more efficient way. In these experiments the exploitation of moderate resistance (cultivars Arina and Petrus) resulted in a 68% reduction in DON when combined with fungicide treatment and in the years of high fungicide efficacy (2003 and 2004) even in an 89% DON reduction (96% reduction in pathogen content). Data collected in this study could help to specify the impact of different crucial factors in the examined Central European conditions and to improve protective measures.

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