EVALUATION OF SLOVAK WHEAT CULTIVARS FOR *FUSARIUM CULMORUM* INFECTION BY REAL-TIME PCR AND BY CONVENTIONAL ASSAYS

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Slovak winter wheat cultivars were evaluated for a level of spike and kernel infection, the content of mycotoxin deoxynivalenol (DON) and the amount of *Fusarium culmorum* DNA in kernels after artificial inoculation with the fungus *F. culmorum* (W. G. Sm.) Sacc. The tests included 14 old and 12 modern wheat Slovak cultivars. The cultivars were sown in October 2008 in field conditions of Piešťany and inoculated with pathogen in May 2009. At anthesis, twenty-five spikes from each cultivar were sprayed (block 1) with *F. culmorum* and spikes were covered for 24 hours with a plastic bag. Old Slovak cultivars had lower area under the disease progress curve (AUDPC), *Fusarium* damaged kernels, DON content and lower amount of *F. culmorum* DNA than modern Slovak culti-

vars. An average kernel contamination with DON in the tested cultivars was 24.9 mg/kg and an average amount of *F. culmorum* DNA was 10,528 ng/g. The old cultivars accumulated 35.9% less DON and 51% less *F. culmorum* DNA than modern cultivars. The positive correlation coefficients were significant with AUDPC and DON content, and with the amount of *F. culmorum* DNA and DON content and AUDPC ($P < 0.01$). Correlation coefficients were higher when we used quantification of *F. culmorum* DNA expressed in infection per cent. Our study has confirmed that real-time polymerase chain reaction is very suitable method for evaluation of wheat cultivars for *F. culmorum* infection and presents less time-consuming, more sensitive and more specific assay than conventional assays.

Key words: *Fusarium culmorum* Sacc., wheat, deoxynivalenol, AUDPC, real-time PCR

Fungal pathogen *Fusarium culmorum* (W. G. Sm.) Sacc. as a causal agent for head blight disease leads to reduced yield and quality of grain and is also harmful to consumers due to the production of mycotoxins in the grain, especially deoxynivalenol (DON). Hence, the evaluation of the infection level and studies of host resistance are very important and more methods can be used for it and compared (Ozakman & Schaad 2003). Šíp *et al.* (2002, 2004) showed good correlation between DON content detected by enzyme-linked immunosorbent assay (ELISA) and the spike damage caused by *Fusarium* head blight. Nowadays, molecular diagnostics based on polymerase chain reaction (PCR) are often used for highly specific, sensitive and fast detection and quantification of plant pathogenic fungi in host plant (McCartney *et al.* 2003). SYBR-Green

real-time PCR was successfully used for the specific detection of trichothedene-producing *Fusarium* spp. (Schnerr *et al.* 2001; Moradi *et al.* 2010) and also more specific Taq-Man real-time PCR was used to quantify main *Fusarium* species (Reischer *et al.* 2004; Waalwijk *et al.* 2004; Leišová *et al.* 2006; Yli-Mattila *et al.* 2008). Leišová *et al.* (2006) found a significant correlation between the amounts of *F. culmorum* DNA (expressed by transformed threshold cycle (Ct) values) detected by Taq-Man real-time PCR and the content of DON detected by ELISA in barley, while in wheat the relationship was rather complicated and influenced by environmental conditions.

The goal of our study was to evaluate *F. culmorum* infection in Slovak winter wheat cultivars by the areas under disease progress curves (AUDPC), the percent-

age of visually *Fusarium* damaged kernels (FDK), the amount of DON detected by ELISA and the amount of *F. culmorum* DNA detected by Taq-Man real-time PCR. The aim was to compare the infection of old and modern Slovak cultivars with *F. culmorum* by different methods. The old Slovak genotypes not derived from the known resistance sources could be used for broadening the genetic variability in wheat breeding.

MATERIAL AND METHODS

The examined set included the old wheat cultivars: Šamorínska, Bučianska červenoklasá, Bučianska 316, Košútská, Radošinská Dorada, Radošinská Karola, Radošinská Norma, Radošinská poloranná 562, Radošinská ranná 594, Slovenská 2, Slovenská 777, Trebišovská 76, Víglašská červenoklasá, Vrakúnská and modern wheat cultivars: Ilona, Ignis, Viador, Pavlína, Veldava, Markola, Stanislava, Venistar, Verita, Vanda, Alacris and Genoveva. Single-spore isolate of *F. culmorum* (W. G. Sm.) Sacc. (RA/02) from Radošina (SK), which is held in the microorganism collection at

the Research Institute of Plant Production Piešťany, was used. Culture to prepare inoculum for the trials was grown in laboratory conditions, on potato-dextrose-broth (Sigma, St. Louis, USA), 20 g/L, at 25°C, and a 12-hour light period.

In October 2008, plot experiments with two blocks (inoculated (I) and non-inoculated (N)) were established under natural conditions in Piešťany. This locality has microclimatic conditions similar to the locality Bučany with very low incidence of fungi *F. culmorum* (Roháčik & Hudec 2005). There were five rows per plot, with 1 m length and 150 mm row spacing. At anthesis, 25 heads were inoculated under pressure sprayer to test for reaction to initial infection (resistance type I) with 25 ml of inoculum (inoculum contained 50,000 spores per ml). After inoculation, the spikes were covered with plastic bags for 24 hours. The spikes were visually evaluated on a 0–100% scale at 10, 15 and 20 days after inoculation. The AUDPC was computed. In the stage of maturity 25 ears were collected from each cultivar in two replications, namely from two variants with infection and the same extent also from control variant without infection. The threshed grains were

Figure 1. Correlation between the areas under disease progress curves (AUDPC) and deoxynivalenol (DON) content (mg/kg) in grain of wheat cultivars after artificial inoculation with *F. culmorum* in year 2009, ($r=0.538$)

manually cleaned and the percentage of visually FDK was evaluated. 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).

Mycelia of the isolate *F. culmorum* (RA/02) from Radošina (SR) were harvested from petri dishes, ground to a fine powder in a cooled mortar using liquid nitrogen, homogenised, and pure total genomic DNA was extracted using the DNeasy Plant Maxi

Kit (Qiagen, Germany). Wheat seeds were ground by ultracentrifuge mill ZM 100 (Retschn GmbH & Co.KG., Haan, Germany) and 1 g of powder was used for isolation of the whole DNA by use of the same DNA isolation kit (Qiagen). Concentration and quality of isolated DNA were checked spectrophotometrically using NanoDrop1000 Spectrophotometer (Thermo scientific). All samples of the seed DNA were diluted to a concentration 25 ng/μl.

Quantification of pathogen DNA in wheat seeds

T a b l e 1

The mean values of area under the disease progress curve (AUDPC), *Fusarium* damaged kernels (FDK), deoxynivalenol (DON), amount of *F. culmorum* DNA and DNA infection percent in 26 wheat cultivars (2009)

Cultivar	AUDPC	FDK [arcsin°]	DON [mg/kg]	<i>F.culmorum</i> DNA [ng/g]	DNA infection per cent
Šamorínska	200.0	32.5	9.7	5,849.5	4.46
Bučianska červenoklasá	100.0	29.9	2.1	2,186.2	2.52
Bučianska 316	237.5	44.2	21.4	6,442.2	7.52
Košútská	200.0	65.6	15.9	5,700.9	4.08
Radošínská Dorada	75.0	39.3	25.3	10,994.6	8.57
Radošínská Karola	137.5	37.1	20.6	6,827.1	6.76
Radošínska Norma	162.5	42.5	26.4	9,053.8	7.42
Radošínská poloranná 562	37.5	35.6	13.9	2,546.3	2.40
Radošínska ranná 594	200.0	48.4	22.3	2,498.4	1.38
Slovenská 2	187.5	44.9	23.9	10,003.8	2.15
Slovenská 777	62.5	40.5	16.3	8,604.8	1.92
Trebišovská 76	100.0	53.2	20.7	8,794.5	2.02
Vígfašská červenoklasá	137.5	54.3	22.3	4,032.6	2.81
Vrakúnská	175.0	52.2	35.9	16,411.5	6.09
Mean old cultivars	143.8	44.3	19.8	7,139.0	4.29
Ilona	512.5	51.9	66.7	17,826.9	18.12
Ignis	387.5	36.3	35.6	4,391.5	12.92
Viador	187.5	61.8	42.6	5,948.1	8.83
Pavčina	87.5	46.2	14.8	1,631.5	4.57
Veldava	512.5	59.0	36.7	29,533.9	21.28
Markola	375.0	51.3	20.5	11,491.6	14.19
Stanislava	312.5	52.4	18.4	9,211.7	6.86
Venistar	237.5	58.4	15.8	9,704.9	8.66
Verita	200.0	58.0	17.1	8,162.6	7.24
Vanda	587.5	54.7	34.5	35,645.5	18.00
Alacris	575.0	41.9	24.0	13,203.5	10.11
Genoveva	500.0	53.9	44.6	27,030.1	16.93
Mean modern cultivars	372.9	52.2	30.9	14,481.8	12.31
Mean all cultivars	249.5	47.9	24.9	10,528.0	7.99

was carried out by real time-PCR method using ABI PRISM® 7000 machine (Applied Biosystems, Foster City, USA) in MicroAmp optical 96-well plates (Applied Biosystems). We used TaqMan™ probe FC92s1 5'FAM 3'MGB and primer pair Fc92s1 specific for *F. culmorum* according to Leišová *et al.* (2006). Reaction was carried out in 25 µl reaction volume consisting of 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM each primers, 200 nM TaqMan MGB probe (labelled with FAM fluorescent dye) and 25 ng of DNA in 1 µl. Conditions of PCR were as follows: 95°C for 10 min., 40 cycles: 95°C for 15 s, 60°C for 1 min.

As standards for standard curves we used five dilutions of pure *F. culmorum* DNA (1; 10; 100; 1,000 and 10,000 pg/µl) in triplicate in every run. For evaluation of results we used ABI PRISM® 7000 software (Applied Biosystems), in which unknown samples are quantified from measured Ct values by interpolation using the regression equation derived from standard curves.

The descriptive statistics and graphics were performed using SPSS software for Windows version 8.0.1 (SPSS, Chicago, Illinois, USA) and Microsoft Excel 97 SR-2 software (Microsoft Corporation, Redmond, Washington, USA). For the assessment of the relationship between the traits, Pearson's correlation analysis was applied using SPSS software.

RESULTS AND DISCUSSION

AUDPC, FDK and DON values were high for modern cultivars (Table 1). Average AUDPC in the tested cultivars was 249.5, which is similar to the results of Brunner *et al.* (2009) and was higher in modern cultivars (49.5%) than in old cultivars (Table 1). Between the tested groups there were differences found in FDK. Infection of grains in old cultivars was 7.5% lower than the overall average FDK, and in modern cultivars 8.9% higher. The grains of modern cultivars were infected

Figure 2. Correlation between the areas under disease progress curves (AUDPC) and infection percent (based on amount of *F. culmorum* DNA – % DNA) in grain of wheat cultivars after artificial inoculation with *F. culmorum* in year 2009, ($r=0.852$)

17.8% more than the grains of old cultivars. The average contamination of grains in the tested group with the mycotoxin DON was 24.9 mg/kg, what is similar to DON content in wheat samples without a fungicide treatment found by Leišová *et al.* (2006). The results show that old cultivars had average DON content in grain 35.9% lower than modern cultivars. Accumulation of DON in samples of old cultivars ranged between 2.1 mg/kg and 35.9 mg/kg. Similar range was also found by Brunner *et al.* (2009) in highly *Fusarium* resistant wheat lines. In the case of modern cultivars, the values ranged from 14.8 mg/kg to 66.7 mg/kg, which are similar to the results of these authors in *Fusarium* susceptible wheat lines.

For the quantification of *F. culmorum* DNA we successfully used the real-time PCR assay with Taq-Man probe developed by Leišová *et al.* (2006). The standard curve constructed using dilution series of pure *F. culmorum* DNA showed very high correlation between Ct values and the logarithm of the amount of input DNA ($r^2 = 0.998$) comparable with the results of Leišová *et al.* (2006) and Moradi *et al.* (2010). The contents of *F.*

culmorum DNA in analysed samples expressed in nanograms of pathogen DNA per 1 g of wheat seeds and the infection per cent calculated as (Fusarium DNA/total DNA) $\times 100$ as given in Table 1. These data were compared with results of AUDPC, FDK and with DON contents detected by ELISA. Among the old Slovak wheat cultivars the lowest content of pathogen DNA and DON content and the lowest FDK was found by cultivar Bučianska červenoklasá, among modern cultivars the lowest content of pathogen DNA and DON and the lowest AUDPC was found by cultivar Pavlína. On an average, old Slovak cultivars had lower AUDPC, DON, FDK and lower amount of *F. culmorum* DNA than modern Slovak cultivars (averages given in Table 1). The old cultivars accumulated less DON and 51% less *F. culmorum* DNA than modern cultivars. In all tested cultivars an average DON content was similar to results of Brunner *et al.* (2009). However, an average amount of *F. culmorum* DNA in our samples was lower, an average of our samples was only 10,528 ng/g comparing with an average of their samples 101,000 ng/g. The reason could be also that the total DNA yields

Figure 3. Correlation between deoxynivalenol (DON) content (mg/kg) and infection percent (based on amount of *F. culmorum* DNA – % DNA) in grain of wheat cultivars after artificial inoculation with *F. culmorum* in year 2009, ($r=0.697$)

during our extractions were about 4–5 times lower (an average 163 ng/mg) than total DNA yields obtained by C extraction method of Brunner *et al.* (2009) (an average 774 ng/g). Their isolation method C resulted in the high amounts of total DNA, but also in low co-isolation of PCR inhibitors combined with almost no fragmentation of the isolated DNA. To overcome drawbacks of unequal DNA yield and fragmentation, in each sample they analysed also quantity of wheat DNA by real-time PCR for wheat-specific gene. This reference-gene-based approach could be useful in our future studies.

The positive correlation coefficients were significant between AUDPC and amount of DON ($r=0.538$) (Figure 1); AUDPC and amount of DNA ($r=0.748$); amount of DON and amount of DNA ($r=0.574$). The correlation coefficients were higher between AUDPC and the infection percent ($r=0.852$) (Figure 2) and between the amount of DON and the infection per cent ($r=0.697$) (Figure 3), because we used this infection per cent as compensation for different total DNA yields after extraction. These results are similar to the results of Brunner *et al.* (2009); however, their infection per cent calculated as $(Fusarium\ DNA / (Fusarium\ DNA + wheat\ DNA)) \times 100$ was higher probably also due to their precise quantification of wheat DNA by real-time PCR while we determined total DNA only photometrically. Leišová *et al.* (2006) found higher correlation coefficient ($r=0.89$) between content of *F. culmorum* DNA expressed by transformed Ct values and DON content in wheat samples; however, they found that the relationship was influenced by environmental conditions especially temperature. For an assessment of such factors, we plan to continue in our experiments for more years.

CONCLUSION

The obtained results indicated that quantification of *Fusarium* in kernels refined the characterisation of genotypes for *F. culmorum* resistance and can be helpful in evaluating wheat genotypes for *Fusarium* infection. The evaluation of genotypes for FHB (*Fusarium* head blight) resistance by the quantification of pathogen is more responsible than only using the AUDPC, FDK or DON content. The quantification of *Fusarium* and AUDPC is a practical alternative to AUDPC, FDK

and DON content. The real-time PCR assay can be useful in phytopathological studies and breeding programmes as it is more fast, more sensitive (it detects very low disease levels) and more specific assays than conventional assays.

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