Mycotoxin Production, Chemotypes and Diversity of Czech *Fusarium graminearum* Isolates on Wheat

Taťána SUMÍKOVÁ[1], Ludmila GABRIELOVÁ[2], Ladislav KUČERA[3], Martin ŽABKA[2] and Jana CHRPOVÁ[1]

[1]Department of Genetics and Plant Breeding Methods, [2]Department of Mycology and [3]Department of Molecular Biology, Crop Research Institute, Prague, Czech Republic

Abstract

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Fusarium head blight (FHB) is a serious cereal disease in the Czech Republic. The most important pathogen associated with FBH is *Fusarium graminearum*, which can produce trichothecenes, mainly deoxynivalenol (DON) and its derivates. A set of 103 *F. graminearum* isolates were isolated from naturally infected wheat ears collected from 20 localities (25 ears from one locality) within the Czech Republic, in the year 2004. The ears were evaluated for DON content by ELISA. Maximum detected value was 30.7 mg/kg. A group of PCR assays targeting the segments of the *Tri7*, *Tri13* and *Tri3* genes were used to determine the chemotypes of *F. graminearum* isolates. All the isolates belonged to DON producing chemotype. Further discrimination revealed that almost all (99.03%) isolates belonged to 15-ADON chemotype, and only one (0.97%) isolate belonged to 3-ADON chemotype. The genetic variability of the isolates was assessed from their AFLP fingerprints. The populations were highly heterogeneous both within and between locations, and no clear evidence for the association between AFLP profile and geographic origin was found out.

Keywords: food contaminant; electroanalysis; sample preparation; deoxynivalenol; AFLP; *Triticum aestivum* L.

F. graminearum Schwabe (teleomorph: *Giberella zeae* (Schwein.) Petch) is one of the major causative pathogens of Fusarium head blight (FHB) in wheat and barley. Our current understanding of the phylogeny of the *F. graminearum* complex, based on multi-locus sequence data, has identified 15 distinct species (Starkey *et al.* 2007; O'Donnell *et al.* 2008; Yli-Mattila *et al.* 2009, Sarver *et al.* 2011). These classifications are correlated to some extent with the geographical origin, choice of host, pathogenicity towards a range of cereal species, and mycotoxin chemotype. However, the division of *F. graminearum* complex into multiple species is not generally accepted (Leslie & Bowden 2008). The attempts to characterise the genetic variation existing within the populations of *F. graminearum* have taken advantage of a variety of genotyping methods, and an overall review of the population genetics of the species has been given by Miedaner *et al.* (2008).

Natural epidemics of FHB are associated with the yield loss and compromised grain quality, the latter reflecting the presence of the trichothecene mycotoxins. *F. graminearum* strains usually express one of three sets of trichothecenes – nivalenol and its derivates (NIV chemotype), DON and 15-acetyl deoxynivalenol (15-ADON chemotype), and DON and 3-acetyl deoxynivalenol (3-ADON chemotype) (Ward *et al.* 2002). The biological function of these mycotoxins is not precisely understood, although it has been shown that DON and other trichothecenes are linked to the aggressiveness of *F. graminearum* and *F. culmorum* strains towards wheat (Maier *et al.* 2006). However, it is well known that trichothecenes present a serious health hazard for both humans and livestock. The

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synthetic pathway of the trichothecenes involves a number of steps and is controlled by a cluster of *Tri* genes. The biosynthesis of trichothecenes was precisely described by Kimura *et al.* (2007). *Tri3* is necessary for the synthesis of B-trichothecenes (Garvey *et al.* 2009). Gen *Tri13* is responsible for the conversion of DON to NIV, while gen *Tri7* acetylates NIV to produce 4-acetyl-nivalenol (4-ANIV) (Lee *et al.* 2001, 2002). All DON producing *F. graminearum* isolates appear to carry mutations in both these two genes, whereas the NIV producing ones have functional *Tri13* and *Tri7* genes (Lee *et al.* 2001; Chandler *et al.* 2003).

DON content in the grain has been widely studied in central Europe (Polišenská *et al.* 2008; Šudyová *et al.* 2010), nevertheless relatively little is known about the genetic chemotyping and variability of *F. graminearum* strains causing FHB in the Czech Republic. The present study aims to describe the population structure of a set of Czech *F. graminearum* isolates using ALFP genotyping, and to reveal the chemotypes of the isolates tested via a PCR assay targeting the sequences of *Tri7*, *Tri13*, and *Tri3* genes.

MATERIAL AND METHODS

Fusarium isolates and their identification. A random sample of 25 naturally infected mature wheat ears (soft wheat, *Triticum aestivum*) was collected from each of 20 localities within the Czech Republic in the year 2004 (Table 1). The grains from all 25 ears of each locality were threshed to make a pooled sample. 25 visually infected grains of the pooled sample were randomly chosen for mycological analyses. The grains were surface sterilised by immersion in 3–5% sodium hypochlorite for 3 min, and then rinsed three times in sterile water. A total

Table 1. Geographic origins of *F. graminearum* isolates, agricultural production areas, description of the localities, numbers of isolates from each locality and DON contents detected in wheat samples by ELISA

Geographic origin (code in the dendrogram)	Agricultural production area*	Altitude (m)	Average temperature (°C) April–June	Amount of precipitation (mm) April–June	Number of isolates	DON content by ELISA (mg/kg)
Čejkovice (Cej), South Bohemia	potato-growing	395	14.154	304.3	6	27.2
Doubravice (Dou), South Moravia	corn-growing	670	11.938	216.2	1	0
Hlohová (Hlo), South-West Bohemia	potato-growing	362	13.267	244.0	19	30.7
Hospříž (Hos), South Bohemia	potato-growing	525	12.750	142.3	1	under LOQ
Huntířov (Hun), North-West Bohemia	potato-growing	375	13.704	269.9	2	0
Jevíčko (Jev), North-East Bohemia	potato-growing	342	12.891	166.2	3	1
Jevíčko Predmesti (JeP), North-East Bohemia	potato-growing	342	12.891	166.2	3	3.2
Lipov (Lip), South Moravia	beet-growing	176	14.250	227.3	1	12.4
Miletín (Mil), North Bohemia	beet-growing	320	13.521	245.5	1	18
Nové město (NMe), North Bohemia	beet-growing	278	14.338	255.9	1	0.2
Přerov (Pre), North-West Moravia	beet-growing	205	14.321	216.4	3	22.2
Ratiboř (Rat), South Bohemia	potato-growing	615	12.027	251.9	1	0
Sokolov (Sok), South-West Bohemia	potato-growing	603	11.933	287.8	6	9.9
Spytihněv (Spy), East Moravia	corn-growing	235	14.671	206.7	18	5.8
Strížovice (Str), East Moravia	corn-growing	235	14.671	206.7	8	1.8
Tečovice (Tec), East Moravia	corn-growing	235	14.671	206.7	17	12.3
Telč (Tel), South-East Bohemia	potato-growing	569	12.708	264.5	2	0
Ústí nad Labem (UnL), North-West Bohemia	beet-growing	375	13.704	269.9	1	0
Vševily (Vse), Central Bohemia	potato-growing	519	12.863	284.1	6	5.3
Zdislavice (Zdi), Central Bohemia	potato-growing	534	12.488	244.1	3	0.6

*characterisation of agricultural production areas in the Czech Republic – potato-growing: annual average precipitation 550–900 mm, temperature 5–8°C, altitude 350–650 m; corn-growing: annual average precipitation 550–700 mm, temperature: 5–8.5°C, altitude 250–700 m; beet-growing: annual average precipitation 500–650 mm, temperature 8–9°C, altitude 200–350 m

of 103 *F. graminearum* isolates were isolated. A single spore from each isolate was cultured on potato dextrose agar (PDA) plates covered with sterile cellophane. The species identity of the isolates was established by the combination of spore morphology (assessed under a light microscope), growth characteristics when cultured on SNA (Synthetic nutrient-poor agar) and PDA (Samson *et al*. 1996; Leslie & Summerell 2006), and a species-specific PCR assay (Nicholson *et al*. 1998).

Detection of DON content in wheat samples. DON content was evaluated by ELISA with the limit of quantification (LOQ) 0.2 mg/kg. RIDASCREEN® FAST DON kits (R-Biopharm GmbH, Darmstadt, Germany) were used according to the manufacturer's guidelines. The samples were ground and thoroughly mixed. Five grams of the ground sample was shaken for 3 min with 100 ml of distilled water and filtered. 50 µl of the filtrate was used for the test. The analysis was carried out according to manufacturer's guidelines. The absorbancies were measured at 450 nm, using a SUNRISE spectrophotometer. RIDAWINR software (R-Biopharm GmbH, Darmstadt, Germany) was employed for data processing.

DNA extraction and detection of chemotypes by PCR. DNA was extracted from the mycelia of each isolate using a CTAB method and the chemotype of each isolate (DON vs NIV) was determined using PCR assays directed at *Tri7* and *Tri13* genes according to Chandler *et al*. (2003). The published primers for the *Tri3* gene were used for the differentiation of the DON chemotype into either 3 or 15-ADON (Jennings *et al*. 2004) (Table 2). Multiplex PCR assay was developed to allow simultaneous determinations of the chemotypes. The reactions were carried out in 25 µl volume containing 10 × PCR buffer (Mg^{2+} free), 1.5mM $MgCl_2$, 2.5mM dNTPs, 0.4µM each primer, 1U Taq polymerase, and 100 ng DNA. The cycling conditions consisted of the initial denaturation step of 94°C for 1 min, followed by 10 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min and 20 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 5 min with the final extension step of 72°C for 5 minutes.

The amplicons were then separated by electrophoresis using 2% agarose gels and visualised by EtBr staining under UV light.

AFLP. AFLP analysis was performed following the AFLP™ Plant Mapping protocol (Applied Biosystems, Foster City, USA) as described elsewhere (Sumíková *et al*. 2010). Each selective amplification reaction contained MseI and either FAM-, NED- or JOE-labelled EcoRI primers (CAA+AG/AT/TG, CAC+TA/TC/TT, CAT+AC/AG/AT, CTA+AC/AG/AT, CTT+AC/TA/AT). The resulting AFLP fragments were separated by capillary electrophoresis, and the data were processed by GENESCAN and GENOTYPER software (Applied Biosystems, Foster City, USA). The presence/absence of the variable AFLP fragments in the size range 70–500 bp generated a binary 1/0 matrix.

Data analysis. The genotype matrix was used to estimate pair wise genetic similarity between the isolates using DARwin (http://darwin.cirad.fr/darwin) software (Perrier & Jacquemou-Collet 2006). A distance matrix, based on the simple matching Sokal-Michener index, was calculated and used as the basis for constructing an unweighted neighbour-joining tree. The robustness of the tree nodes was assessed from a 1000 bootstrap re-sampling exercise. The analysis of the molecular variance (AMOVA) was performed using Arlequin v 3.1 (Excoffier *et al.* 2005). The degree of the population subdivision between geographical locations was expressed in the form of Wright's fixation index (F_{ST}).

RESULTS AND DISCUSSION

DON content in wheat samples and chemotype identification

DON content was measured in the samples from all localities. Maximum detected value was 30.7 mg/kg, in the sample from the locality Hlohová in South-West Bohemia. DON content below the LOQ was detected in six (30%) samples. Three samples (15%) had DON content between 0.2–1.25 mg/kg and eleven (55%) samples had DON content higher than 1.25 mg/kg, EU limit for unprocessed soft wheat (Table 1). The samples with a low (below 1 mg/kg) or zero DON contents were further analysed for NIV content using HLPC method, however, no NIV was determined (data not shown).

Only two chemotypes, 15-ADON (99.03%) and 3-ADON (0.97%), were detected. All but two of the isolates amplified both a 282 bp *Tri13DON* fragment and a 445 bp *Tri7DON* fragment, failed to amplify any product from either the *Tri13NIV* or the *Tri7NIV* assay. The remaining two isolates failed to amplify any fragment from the *Tri7DON* assay but amplified both a 483 bp *MinusTri7* and a 282 bp *Tri13DON* fragments. The two isolates failed to PCR amplify in the *Tri7DON* assay, but did amplify the expected fragments in both the

MinusTri7 and *Tri13DON* assays; this PCR profile is consistent with the deletion of the entire *Tri7* gene. Such a deletion is present in all isolates of *F. culmorum* tested to date (Chandler *et al*. 2003; Kammoun *et al*. 2010), but it is rather rare in *F. graminearum* (Lee *et al*. 2001; Chandler *et al*. 2003). Our results are consistent with the chemotype screenings from Eastern Europe, where 15-ADON chemotype dominates (Prodi *et al*. 2009; Talas *et al*. 2011), whereas 3-ADON chemotype is more frequent in Northen Europe (Jestoi *et al*. 2008). The present samples included no NIV producing isolates. Talas *et al*. (2011) also detected only 1.2% NIV producing isolates in neighbouring Germany, although low to medium frequencies of this chemotype have been recorded in Europe (Stepien *et al*. 2008; Audenaert *et al*. 2009; Prodi *et al*. 2009). The analysis of a larger collection of Czech *F. graminearum* isolates would probably reveal a frequency of NIV producers that comparable to detected in Germany.

Population diversity

The AMOVA and phylogeny was based on 286 variable AFLP fragments. The resulting tree showed one separated cluster (45 isolates) supported by bootstrap value 58. However, the clusters seemingly formed by the remaining isolates were not particularly well supported, as the bootstrap values associated with most of the nodes were low (Figure 1). None of the isolates shared the same genotype. The genetic relatedness between all pairs of isolates obtained from single geographical sites was low, and there was no clear association between both the genetic relatedness and geographic origin. Nor any other clear correlation was detected between the agronomic characteristic of the localities and strains genetic variability. An especially high level of diversity was present within each location (Table 2). Other surveys of *F. graminearum* have similarly shown that the pathogen is genetically highly heterogeneous (Gale *et al*. 2002; Zeller *et*

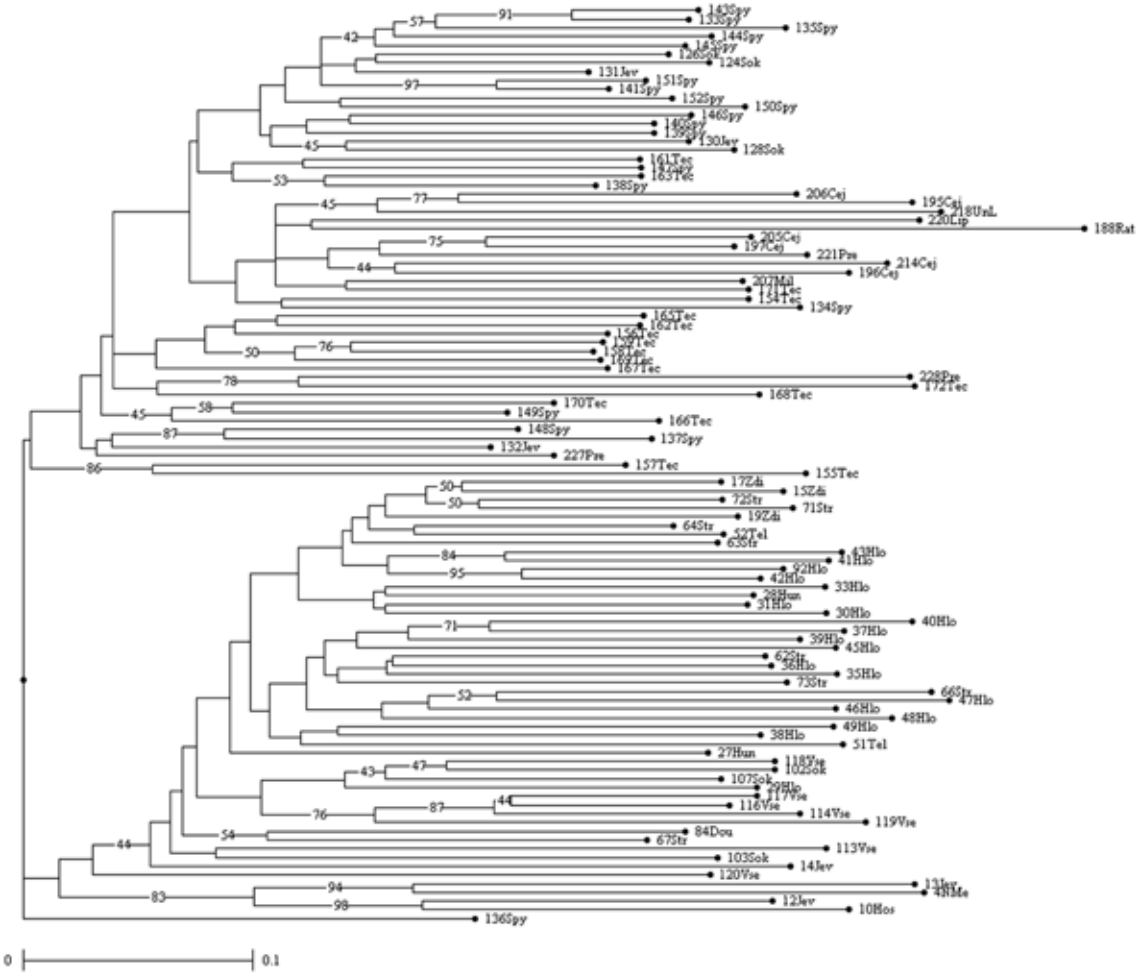

Figure 1. Phylogenetic tree, constructed using the neighbour-joining method, and based on variation at 286 AFLP markers from 103 *F. graminearum* isolates

Table 2. AMOVA of a set of 103 *F. graminearum* isolates, based on AFLP genotype, grouped according to their geographical origins (agricultural production area): potato growing, corn growing, and beet growing agricultural production area

Source of variation	d.f.	Sum of squares	Variance components	*CV*%
Among populations	4	345.813	7.06320 Va	24.78
Within populations	44	943.493	21.44302 Vb	75.22
Total	48	1289.306	28.50622	
Fixation index F_{ST}: 0.24778				
Va and F_{ST}: *P*-value = 0.00000 ± 0.00000				

Va – variation component found among populations; Vb – variation component found within populations; F_{ST} – Wright's fixation index; *CV*% – correlation coefficient

al. 2004; Qu *et al*. 2008). Based on an F_{ST} of 0.248, the isolates originating from different locations differed significantly from one another. The population studies of the pathogen have typically revealed a rather low F_{ST} value, whether the host is wheat (Gale *et al*. 2002; Zeller *et al*. 2004) or some other crop (Naef & Defago 2006). This has been interpreted as implying that *F. graminearum* populations tend to display little hierarchical structure. However, more recent studies have reported higher F_{ST} values that are in agreement with our F_{ST} value, suggesting a higher population substructuring (Gale *et al*. 2011; Talas *et al*. 2011). The genotypic survey of the present collection of *F. graminearum* isolates has confirmed that the pathogen population in any one location can be highly heterogeneous. This level of variability probably reflects the ability of the fungus to reproduce sexually (Bowden & Leslie 1999), the effect of which is a great enhancement of the pathogen capacity to evolve resistance to either fungicides (Gale *et al*. 2002) or potentially to genetic resistance.

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Corresponding author

Mgr. Taťána Sumíková, Ph.D., Výzkumný ústav rostlinné výroby, Odbor genetiky, šlechtění a kvality produkce, Oddělení genetiky a šlechtitelských metod, Drnovská 507, 161 06 Praha, Česká republika; E-mail: sumikova@vurv.cz