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Morphological and molecular identification of *Fusarium* species associated with head blight on wheat in East Croatia

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Abstract Fusarium head blight (FHB) is an important fungal disease of wheat. The aim of this research was to determine the diversity of *Fusarium* species infecting winter wheat ears in East Croatia. In 2008 wheat kernels were obtained from three locations in the eastern part of Croatia (Tovarnik, Osijek, Pozega), and in 2009 from two additional locations (Slavonski Brod, Nova Gradiska). In total, 498 visually diseased kernels were selected for morphological identification of *Fusarium* spp. The identity of 226 selected isolates was further investigated by molecular techniques. The predominant species on wheat kernels in East Croatia in 2008 were *F. graminearum*, isolated and confirmed from more than 80% of sampled wheat kernels, followed by *F. avenaceum* (8%) and *F. culmorum* (7%). Incidence of *F. poae* was less than 2%. The most common species identified in 2009 were *F. graminearum* (50%), *F. culmorum* (13%), *F. avenaceum* (12%) and *F. poae* (7%). This is the first report on the identification of *Fusarium* species isolated from naturally infected wheat ears in Croatia.

Keywords *Fusarium* species · Fusarium head blight · *Triticum aestivum* · East Croatia

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Introduction

Wheat (*Triticum aestivum* L.) is one of the major crops grown in Croatia, with average production of approximately 3.87 t ha^{-1} from 1996 to 2006 (Croatian central bureau of statistics in Republic of Croatia, 2007). The main growing areas for wheat production in Croatia are situated in the eastern part of Croatia. Fusarium head blight (FHB), also called ear blight or scab, is economically one of the most important fungal diseases of wheat. In general, the causal agents of FHB in Europe are primarily *F. graminearum* Schwabe (teleomorph *Gibberella zeae* [Schwein.] Petch), *F. culmorum* (W.G.Smith) Sacc. and *F. avenaceum* (Fr.) Sacc. (teleomorph *Gibberella avenacea*) (Lemmens et al. 2004; Stepien et al. 2008). Less frequently represented species are *F. poae* (Peck) Wollenw., *F. cerealis* Burgess, Nelson and Toussoun, *F. equiseti* (Corda) Sacc., *F. sporotrichioides* Sherb., *F. tricinctum* (Corda) Sacc. and, to a lesser extent, *F. acuminatum* Ell. and Ev., *F. subglutinans* (Wollenw. and Reinking) Nelson, Toussoun and Marasas comb. nov., *F. solani* (Martius) Appel and Wollenweber

emend., Snyder and Hansen, *F. oxysporum* Sclecht. Emend. Snyd and Hans., *F. verticillioides* (Sacc.) Nirenberg, *F. semitectum* Berk. and Rav. and *F. proliferatum* (Matsushima) Nirenberg (Bottalico and Perrone 2002). The predominant species causing FHB worldwide is *F. graminearum* (Ban et al. 2008). *F. culmorum* is most aggressive to cereal plants in moderate and colder regions (Bottalico and Perrone 2002). Cosic et al. (2007) reported on species of Fusarium on wheat in Croatia, but no systematic overview has been published on the *Fusarium* species causing the disease in winter wheat in Croatia, nor has polymerase chain reaction (PCR) been used for species confirmation.

Polymerase chain reaction (PCR) can be used for identification of *Fusarium* species, either as an alternative or as a complement to identification based upon morphological characteristics. It has proven to be very useful and more suitable than whole seed plating for the identification of *Fusarium* species (Demeke et al. 2005; Yli-Mattila et al. 2004; Rahjoo et al, 2008). Species-specific primers have been developed and used for PCR detection and screening of *F. graminearum* (Nicholson et al. 1998; Waalwijk et al. 2003, 2004), *F. pseudograminearum* (Aoki and O'Donnell 1999), *F. acuminatum* (Williams et al. 2002), *F. avenaceum* (Schilling et al. 1996; Waalwijk et al. 2003, 2004), *F. crookwellense* (Yoder and Christianson 1998), *F. culmorum* (Nicholson et al. 1998), *F. equiseti* (Mishra et al. 2003) and *F. poae* (Parry and Nicholson 1996). Some authors state that PCR can be used for the routine detection and identification of *Fusarium* species without the need for isolation and morphological investigation of this fungus (Koncz et al. 2008).

Infection of grain with FHB decreases kernel quality, and several causal *Fusarium* species produce mycotoxins that contaminate the grain. FHB mycotoxins can be dangerous to human and animal health if they are ingested as food or in feed mixtures made from cereals (Rohácik and Hudec 2005). The *Fusarium* toxins of primary concern are: deoxynivalenol (DON) produced by *F. graminearum* and *F. culmorum*; diacetoxyscirpenol (DAS) produced by *F. equiseti*; monoacetyl-deoxynivalenol (AcDON) produced by *F. graminearum*; T-2 produced by *F. sporotrichioides*; HT-2 produced by *F. sporotrichioides* and *F. acuminatum*; nivalenol (NIV) produced by *F. graminearum*, *F. culmorum*, *F. poae*, *F. cerealis*; zearalenone (ZEN) produced by *F. graminearum*, *F. culmorum, F. equiseti*, *F. cerealis*; and Fusarenone-X (FUS) produced by *F. graminearum*, *F. poae* and *F. cerealis* (Bottalico and Perrone 2002). Most resistance breeding programmes deal only with the dominant *Fusarium* species, such as *F. graminearum* or *F. culmorum* (Mesterhazy 2005).

The objectives of this study were to morphologically identify *Fusarium* species isolated from naturally infected winter wheat kernels in East Croatia and to confirm the identity of isolates using molecular tools. With this information the type of mycotoxins occurring in wheat grain can be predicted. Furthermore, isolated Croatian *Fusarium* isolates will be used in FHB resistance tests in wheat breeding programs.

Materials and methods

In 2008, wheat heads were obtained from three locations in East Croatia (Tovarnik-near Vukovar, 45°10′N, 19°09′E, Osijek, 45°27′ N, 18°48′E, Pozega, 45°20′, 17°41′E), and in 2009 two new locations were added (Slavonski Brod, 45°10′N, 18°01′E, Nova Gradiska, 45°16′N, 17°22′E) (Fig. 1). The fields were located in the main wheat growing region in Croatia, and represented different types of soil (Tovarnik-black earth, Osijek-eutric cambisol, Nova Gradiska-alluvium, Pozega-pseudogley, Slavonski Brod-amphigley) (Fig. 1). The preceding crop in 2007 was corn (*Zea mays* L.) in Osijek and soybean (*Glycine max* L.) in Tovarnik and Pozega; in 2008, corn in Osijek and Nova Gradiska and soybean in Tovarnik, Pozega and Slavonski Brod. To control seedborne diseases the seed was treated with Vitavax 200 (thiram+carboxin) at a rate of 200 g/100 kg. Fertilization differed during the study at different locations (N:P:K 120–180:80–100:120–150 kg ha^{-1}). The environmental conditions during the two growing seasons were recorded for each location.

To isolate *Fusarium* species, wheat kernels with visible infection symptoms were collected after harvest. The kernels originated from the most popular winter wheat varieties sown in Croatia, such as 'Srpanjka', 'Super Zitarka', 'Zitarka', 'Lucija' and 'Golubica', which all were bred by the Agricultural Institute Osijek.

Fig. 1 Map of Croatia showing the locations where Fusarium infected wheat kernels were collected in 2008 and 2009

Fungal isolates

A total of 498 kernels collected over two seasons were surface-sterilised in 80% ethanol for 2 min, air dried and transferred on to SNA-Synthetic Nutrient Agar (1.0 g KH_2PO_4, 1.0 g KNO_3, 0.5 g $MgSO_4x7H_20$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 1 l double distilled H_2O, 20.0 g agar) with added antibiotics (chlortetracycline, 0.01 g and streptomycinsulphate, 0.05 g) (Nirenberg 1981). After 7–14 days incubation in a controlled environment (temperature 25°C and humidity 80%), a single spore technique was used to obtain pure cultures for identification. Potato dextrose agar (PDA) (Difco, Detroit, MI) medium was used to assess colony characteristics such as pigmentation and growth rate, while SNA medium, supplemented with a piece of filter paper to promote sporulation, was used to examine conidial morphology and detect the presence of chlamydospores. Black UV light (36 W) was used to improve sporulation of isolates. Morphological identification was based on Nelson et al. (1983) and Leslie and Summerell (2006).

DNA extraction

To verify the morphological identification of *Fusarium* spp., 226 selected isolates from the 2008 harvest season were further investigated by molecular techniques at IFA-Tulln, using species-specific PCR assays.

Modified DNA extractions were conducted, as previously described (Abd-Elsalam et al. 2003), from fresh fungal mycelium grown in 25 ml Czapeck-Dox medium (3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4$,

Table 1 Primer pairs used to confirm *Fusarium* species

Species	Sequence forward (5′-3′)	Sequence reverse (5′-3′)
F. graminearum[a]	CTCCGGATATGTTGCGTCAA	GGTAGGTATCCGACATGGCAA
F. avenaceum[a]	GCTAATTCTTAACTTACTAGGGGCC	CTGTAATAGGTTATTTACATGGGCG
F.culmorum[a]	ATGGTGAACTCGTCGTGGC	CCCTTCTTACGCCAATCTCG
F. poae[a]	CAAGCAAACAGGCTCTTCACC	TGTTCCACCTCAGTGACAGGT

[a] Sources of primers: *F. graminearum* and *F. culmorum*—Nicholson et al. (1998), *F. poae*—Parry and Nicholson (1996), *F. avenaceum*—Schilling et al. (1996)

Table 2 Morphologically identified *Fusarium* species in 2 years (2008 and 2009) collected from winter wheat kernels in East Croatia

Fusarium species	Year 2008		Year 2009	
	Number	%	Number	%
Total	276	100	222	100
F. graminearum	186	67	111	50
F. avenaceum	19	7	27	12
F. culmorum	17	6	29	13
F. poae	4	2	16	7
F. cerealis	3	1	2	1
F. solani	2	1	–	–
F. equiseti	–	–	3	1
F. oxysporum	–	–	1	1
Other *Fusarium* species	45	16	33	15

0.5 g 1% $FeSO_4$, 20 g glucose, 2 g yeast extract) in 100 ml Erlenmeyer flasks on a rotary shaker. Subsequently, the mycelium was ground with fine sand (SiO_2; M=60.06 g/mol) and liquid nitrogen. The ground mycelium (200 mg) was transferred into a centrifugation tube and 300 µl sodium dodecyl sulphate (SDS) extraction buffer (250 mM 5 M NaCl, 200 mM 1 M Tris-HCl pH 8, 25 mM 0.5 M EDTA pH 8; 20% SDS 0.50%) added. Homogenization was carried out on a thermoshaker for 20–30 min at 65°C and 750 rpm; 150 µl of 3 M Na-acetate (pH 5.2) was added and then incubated for 10 min at room temperature. Supernatant was transferred to a new centrifugation tube and 600 µl of isopropanol added. The solution was incubated on ice for 20 min, mixed gently and centrifuged at 13,000 rpm for 10 min. The supernatant was removed and the pellet was washed with *Wash I* (76% EtOH, 0.2 M NaOAc, distilled H_20) followed by *Wash II* (76% EtOH, 10 mM NH_4Ac, distilled H_20). The ethanol was left to evaporate, and the pellet was dissolved in 150–200 µl of TE-buffer.

PCR analysis

To confirm the morphological identification of species, conventional PCR was performed by testing isolates with species-specific primers (Table 1).

PCR for single species detection was done in 96-well plates (PCR Primus96plus). The thermocycler program was programmed as follows: for *F. graminearum*—1 min at 95°C, 40 cycles of 30 s at 95°C, 30 s at 62°C, and 40 s at 72°C, and one cycle of 10 min at 72°C; for *F. culmorum*—2 min at 96°C, 40 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C, and one cycle of 10 min at 72°C; for *F. avenaceum* and *F. poae*—2 min at 95°C, 40 cycles of 30 s at 95°C, 30 s at 66°C, and 45 s at 72°C, and one cycle of 5 min at 72°C.

For each reaction, 3 µl (50 ng/µl) of template DNA was mixed with 17 µl of PCR master mix containing 2 µl of each species-specific primer (2.5 µM), 2 µl PCR buffer, 2 µl dNTPs (2 mM) and 0.20 µl Taq DNA Polymerase. Amplification products were separated by electrophoresis (80–90 V, 60 mA, 100 W, 30 min) in 1.5% agarose gels in 1 TAE buffer stained with 2 µl/100 ml ethydium bromide. The gel was scanned with a transluminator (UV BIO-RAD) and analyzed with Quantity one-4.5.2; 1D Analysis Software.

Results

The environmental conditions during the two growing seasons were recorded for each location and differed significantly for the amount of precipitation measured at Osijek (2007/08=623.0 mm and 2008/09=

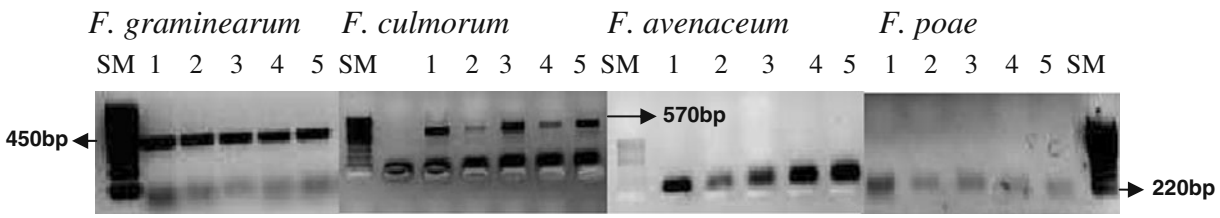

Fig. 2 Confirmation of analyzed strains as *F. graminearum* (450-bp), *F. culmorum* (570-bp), *F. avenaceum* (220-bp) and *F. poae* (220-bp) from winter wheat kernels in East Croatia

367.8 mm) and differed less at Tovarnik (2007/08= 522.5 mm and 2008/09=430.6 mm) and at Pozega (2007/08=685.2 mm and 2008/09=528.1 mm). The amount of precipitation in 2008/09 at Slavonski Brod was 507.9 mm and at Nova Gradiska 492.1 mm. Average temperatures during the growing seasons in Osijek were: 10.2°C and 10.8°C, in 2007/08 and 2008/09, respectively; in Tovarnik 10.8°C and 11.2°C and in Pozega 9.8°C and 10.2°C. In 2008/09 in Slavonski Brod average temperatures were 10.6°C and in Nova Gradiska 10.1°C.

Based on morphological characters, the most common species identified in year 2008 were *F. graminearum* (67%), *F. avenaceum* (7%), *F. culmorum* (6%) and *F. poae* (2%). In the year 2009, the most common species recovered were: *F. graminearum* (50%), *F. culmorum* (13%), *F. avenaceum* (12%) and *F. poae* (7%) (Table 2). Less frequently isolated species in 2008 included *F. cerealis* and *F. solani*, and in 2009 *F. equiseti*, *F. cerealis* and *F. oxysporum* (see Table 2). In total, 226 of 280 *Fusarium* isolates from the year 2008 were selected for molecular identification from which four *Fusarium* species were identified, namely, *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae*. All of the isolates tested on agarose gels produced their respective DNA fragments: *F. graminearum* 450-bp, *F. avenaceum* 220-bp, *F. poae* 220-bp and *F. culmorum* 570-bp (Fig. 2).

Discussion

To reach the goals of this research, visually diseased seeds of winter wheat were collected from three and five locations in 2008 and 2009, respectively. The aim of this research was to identify the *Fusarium* species associated with winter wheat kernels in East Croatia. Morphological identification of *Fusarium* species was based on Nirenberg (1981), Nelson et al. (1983) and Leslie and Summerell (2006). In years 2008 and 2009, *F. graminearum* was the most dominant *Fusarium* species on wheat kernels in East Croatia. These results are in accordance with previous research in other countries in Europe (Birzele et al. 2002; Tomczak et al. 2002; Lemmens et al. 2004; Isebaert et al. 2004; Gromadzka et al. 2008).

In Croatia, Cosic et al. (2007) concluded that the most dominant *Fusarium* species occurring on wheat and barley ears over 10 years, based on morphological identification, was *F. graminearum*. Although the frequency of occurrence of *F. culmorum* was relatively low in our study, this is one of the most frequently isolated species on cereals (Rohácik and Hudec 2005). Unlike *F. graminearum*, *F. culmorum* has no known sexual stage which might put the pathogen as a disadvantage compared to *F. graminearum*. It is likely that *F. culmorum* has always been less prevalent in central Europe compared to northern Europe because of temperature and/or moisture differences.

Despite the difference in precipitation during the two growing seasons at the study locations, the levels of the main species remained relatively constant, albeit with some decline of *F. graminearum* and a rise in *F. avenaceum* and *F. culmorum*.

Conclusion

In this study a PCR assay was used to confirm the morphological identification of *F. graminearum, F. culmorum, F. avenaceum* and *F. poae*. These were the most frequently isolated *Fusarium* species from winter wheat ears in East Croatia in 2008 and 2009. We conclude that the molecular identification of 226 selected isolates was in agreement with the morphological identification. Based on the occurrence of the predominant species, the most important toxins expected to contaminate wheat kernels in Croatia are deoxynivalenol, nivalenol, moniliformin and zearalenone. We recommend the use of isolates of *F. graminearum*, *F. culmorum* and *F. avenaceum* for screening in winter wheat breeding in Croatia.

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