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Special Report

Fusarium Species Associated with Fusarium Head Blight in Hungarian Wheat Fields

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Abstract

The species composition of the genus *Fusarium* associated with Fusarium head blight (FHB) in wheat fields of Hungary in the year 2019 was assessed. Symptomatic wheat heads were collected at 20 geographical locations representing different ecosystems. A total of 256 *Fusarium* strains were isolated and identified by partial sequences of the translation elongation factor 1-alpha gene and, where required, the second-largest subunit of the DNA-directed RNA polymerase gene. Overall, *Fusarium graminearum* (58.2%) proved to be the dominant species, followed by *F. annulatum* (formerly *F. proliferatum*) (17.2%) and *F. verticillioides* (7.4%). The presence of all other species, including *F. culmorum*, in the population was less than 5%. *F. graminearum* was identified as the main species associated with FHB at 14 sampling sites. Fumonisin-producing *F. annulatum*, primarily known as the pathogen of maize in Hungary, was detected nearly as frequently as *F. graminearum* at three locations and dominated at two other sites. *F. poae* was not found during the survey. *F. vorosii*, a species that is believed to be of Asian origin and was already found in Hungary in 2002, was identified at two locations.

Keywords: fumonisin, Fusarium head blight, *RPB2*, survey, translation elongation factor 1-alpha, *Triticum aestivum*

Fusarium head blight (FHB) is a disease of grain cereals and causes major economic damage worldwide. FHB in wheat reduces flour quality and can result in significant mycotoxin contamination (Spanic et al. 2020, 2021). Several species of the genus *Fusarium* can be associated with FHB. The most important members of them in Europe are *Fusarium graminearum*, *F. culmorum*, and *F. poae* (Bottalico and Perrone 2002) of the *F. sambucinum* species complex (FSASC) (Geiser et al. 2021; O'Donnell et al. 2013). Commonly occurring FHB-associated species are *F. cerealis* (FSASC), *F. sporotrichioides* (FSASC), members of the *F. tricinctum* species complex (FTSC; e.g., *F. avenaceum* and *F. tricinctum*) or the *F. fujikuroi* species complex (FFSC; e.g., *F. annulatum*, formerly *F. proliferatum*), and some species of the *F. incarnatum-equiseti* species complex (FIESC). In addition, other *Fusarium* and *Microdochium* species may become involved in the development of the disease (Bottalico and Perrone 2002; Xu et al. 2005).

Several FHB species composition shifts have been observed in Europe in the last decades. *F. graminearum* has generally become a prevalent species instead of *F. culmorum*, *F. graminearum* has been replaced by *F. poae* in Italy, and new pathogenic species such as *F. vorosii* (FSASC) have been found in Hungary (Starkey et al. 2007; Valverde-Bogantes et al. 2020).

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Until the late 1990s, the identification of *Fusarium* species was primarily based on morphological diagnostic features. Then, phylogenetic analyses focusing on various genetic loci became widespread, and species complexes based on molecular phylogenetics were introduced instead of heterogeneous sections defined morphologically that were placed between the ranks of genus and species (O'Donnell et al. 2013). Among several DNA loci that have been tried to clarify phylogenetic relationships, the translation elongation factor 1-alpha (*TEF-1α*) gene (O'Donnell et al. 1998), largest subunit of RNA polymerase II (*RPB1*), and second-largest subunit of DNA-directed RNA polymerase (*RPB2*) (O'Donnell et al. 2013) proved to have enough variability to resolve at or near the species level within the genus *Fusarium*. Multilocus analyses are now widely used to define old well-known and new species limits phylogenetically within the genus *Fusarium* (Laraba et al. 2021; Lombard et al. 2019; O'Donnell et al. 2022; Yilmaz et al. 2021).

Our aim was to reexamine whether there is any change in the prevalence of the FHB-associated *Fusarium* species in Hungary in light of the state-of-the-art taxonomic system.

Materials and Methods

Isolation of monoconidial fungal cultures

Symptomatic wheat heads were collected in 2019, a year when the high amount and frequency of rain in May, from flowering until the milky stage of seed development, favored the high incidence of FHB. Sampling was implemented during the wheat ripening period (June to July) at 20 different locations representing the main wheat-producing areas of Hungary (Fig. 1 and Table 1). The number of wheat heads gathered (see Table 1) corresponded to the severity of the disease at the investigated locations. Samples were stored at room temperature in separate paper bags until further preparation. The samples were not surface sterilized to allow isolation not only of the internal infestation but also of all *Fusarium* spp. present in the wheat fields. Their presence on the surface of wheat heads can lead to significant contamination of the crop during postharvest storage if

conditions are not suitable. Symptomatic sections of wheat heads containing three to four complete spikelets (chaff, seed, and rachis) were placed on sterilized filter paper moistened with 1 ml of sterile distilled water in glass Petri dishes (d = 9 cm) and incubated at 25°C in the dark for 2 weeks. Petri dishes were checked every second day, and the filter paper was rewetted if necessary. Conidia originated from aerial hyphae or sporodochium, and mycelial fragments developed on the surface of the plant material or the filter paper were transferred to peptone pentachloronitrobenzene (PCNB) agar (15 g of Bacto peptone, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.75 g of PCNB, and 15 g of agar in 1 liter) (Leslie and Summerell 2006). After incubation (at 25°C in the dark for 2 days), colonies that proved to be viable on the surface of peptone PCNB agar were transferred to synthetic low-nutrient agar (SNA; Nirenberg and O'Donnell 1998) overlaid with pieces of sterile filter paper and incubated at 20°C for 5 to 7 days under 12-h dark and 12-h near-UV plus white fluorescent light tubes for sporulation. Monoconidial cultures were obtained using the dilution plating technique described in the manual of Leslie and Summerell (2006), and pure cultures were subcultured on potato dextrose agar (PDA) and SNA with small pieces of sterile filter paper on the agar surface. Colony morphology was characterized on PDA after 8 days of incubation at 25°C in the dark, whereas micromorphological characteristics including shape and size of macroconidia produced in sporodochia or on the aerial mycelium; shape, size, and mode of formation of microconidia; and chlamydospores were examined on SNA plates incubated as described previously. Morphological characteristics served for preliminary identification of *Fusarium* species.

DNA isolation, sequencing, and identification of *Fusarium* species

The simplified method of Pintye et al. (2020) was used for DNA extraction. A loop of mycelium from a culture on PDA was boiled at 97°C for 7 min in 20 µl of Tris-EDTA (TE, pH 8, Lonza, Basel, Switzerland) buffer. The mixture was centrifuged, and the supernatant served as a template solution for the following PCR reaction. Each *Fusarium* isolate was identified by sequencing the *TEF-1α* locus; however, in species complexes where species-level identification remained questionable (FIESC and *F. solani* species complex [FSSC]), the *RPB2* locus was additionally used. *TEF-1α* sequences were amplified and sequenced with the primer pair EF1 (5′-ATGGGTAAGGARGACAAGAC-3′) and EF2

Table 1. Sampling sites and collected diseased wheat heads[a]

Location (codes)	GPS coordinates	DH	FH
Bulgárföld (BG)	47°19′53″N, 18°47′05″E	16	14
Bakonysárkány (BS)	47°26′51″N, 18°04′11″E	1	1
Dobri (DO)	46°31′00″N, 16°35′14″E	11	5
Eszterágpuszta (EP)	45°55′34″N, 18°13′50″E	6	4
Füzesabony (FA)	47°44′05″N, 20°24′13″E	10	3
Galgaguta (GG)	47°51′00″N, 19°24′02″E	19	10
Hantos (HA)	46°58′44″N, 18°41′59″E	5	2
Kocs (KC)	47°37′11″N, 18°12′44″E	13	10
Kompolt (KM)	47°44′22″N, 20°13′50″E	3	3
Kisújszállás (KU)	47°11′26″N, 20°44′58″E	18	11
Lászlópuszta (LP)	47°18′06″N, 18°49′04″E	25	21
Martonvásár (MV)	47°19′04″N, 18°46′30″E	9	9
Sarkad (SA)	46°45′50″N, 21°23′04″E	45	41
Szerencs (SC)	48°04′23″N, 21°04′31″E	11	7
Székkutas (SK)	46°31′02″N, 20°31′25″E	27	15
Szentlőrinc (SL)	46°02′11″N, 18°01′55″E	16	10
Solt (SO)	46°49′34″N, 19°00′27″E	15	15
Tiszaderzs (TI)	47°30′22″N, 20°39′42″E	6	4
Tordas (TO)	47°19′42″N, 18°44′54″E	8	8
Újmohács (UM)	46°00′13″N, 18°43′05″E	15	6

[a] DH = number of diseased wheat heads collected; FH = number of wheat heads from which *Fusarium* isolation was successful.

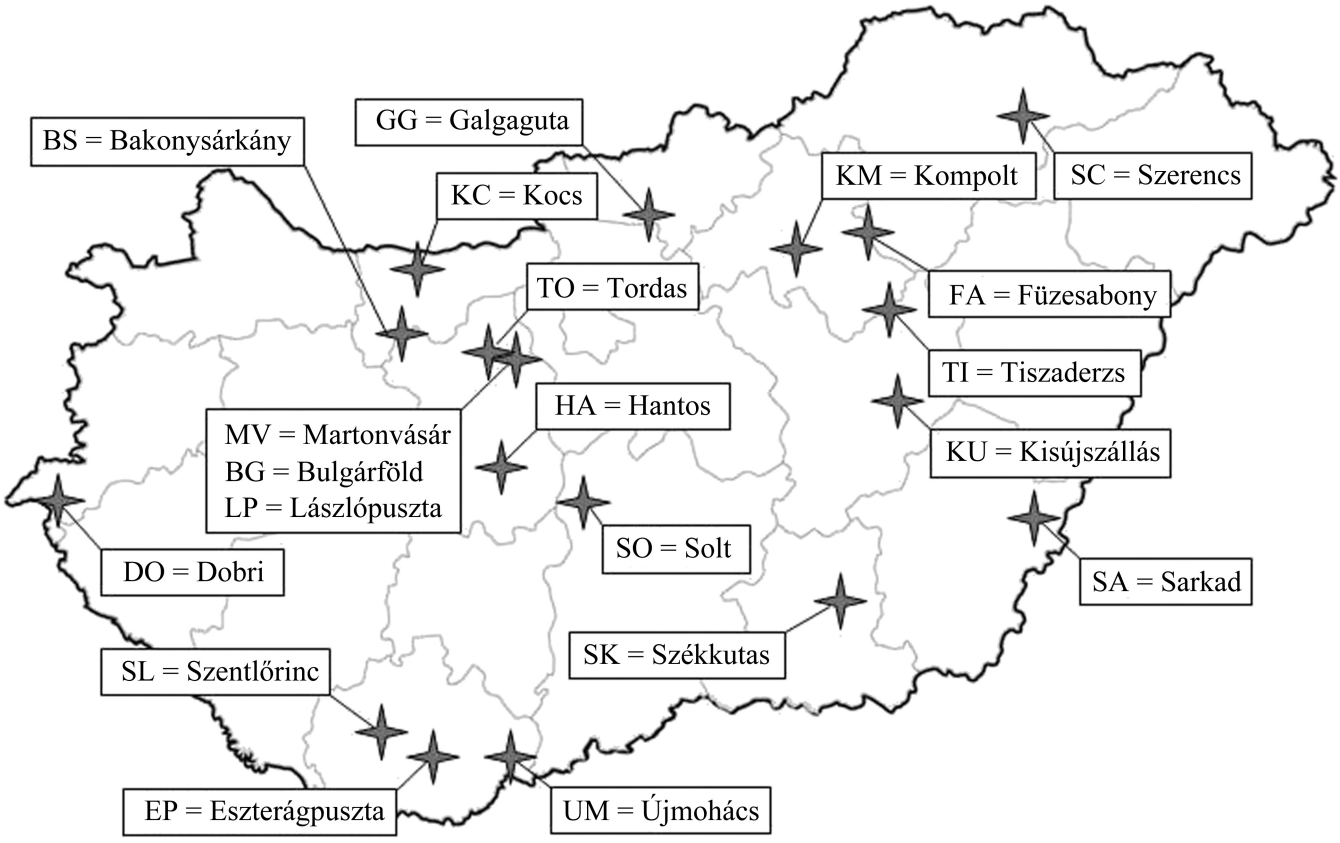

Fig. 1. Sampling sites.

(5′-GGARGTACCAGTSATCATG-3′) (O'Donnell et al. 1998); *RPB2* sequences were amplified and sequenced with the primer pair 5f2 (5′-GGGGWGAYCAGAAGAAGGC-3′) (Reeb et al. 2004) and 7cR (5′-CCCATRGCTTGYTTRCCCAT-3′) (Liu et al. 1999). All PCRs were performed in a 20-μl final volume that included 1 μl of 10 μM forward and reverse primers (Sigma-Aldrich GmbH, Steinheim, Germany), 1 μl of DNA template solution, and the Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). PCR cycling times and temperatures were as follows: 98°C for 2 min, followed by 38 cycles of 5 s at 98°C, 5 s at 60°C, and 30 s at 72°C and a final extension step of 72°C for 5 min. The PCR products were sent to LGC Genomics GmbH (Berlin, Germany) for sequencing. The Staden software package with Pregap4 and Gap4 tools was used to edit the electropherograms (Staden et al. 2000). All resulting sequences were deposited at NCBI GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/).

Based on BLASTn hits in NCBI GenBank, *Fusarium* isolates were tentatively grouped into species complexes. Phylogenetic analyses were conducted to clarify the relationships for species identification. *Fusarium* species belonging to different species complexes were treated separately, as recommended by O'Donnell et al. (2022). In two species complexes, FIESC and FSSC, *RPB2* sequences were analyzed together with *TEF-1α* sequences. Whenever they were available, diagnostic DNA barcode sequences of the type strains (Crous et al. 2021) and the most similar sequences resulting in BLASTn searches in GenBank were analyzed together with our sequences (Supplementary Fig. S1). DNA sequences of *TEF-1α* and *RPB2* were separately aligned with MAFFT 7 (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley 2013), using the G-INS-i method. Alignments were visually inspected and edited; leading and trailing gaps were coded as unknown characters in MEGA 7 (Kumar et al. 2016). The length of the alignments within each species complex was as follows: 678 nucleotides were within FSASC (*TEF-1α*), whereas 663 nucleotides were within FFSC (*TEF-1α*), 647 (*TEF-1α*) and 837 (*RPB2*) within FIESC, 638 within the *F. oxysporum* species complex (FOSC; *TEF-1α*), 644 within FTSC (*TEF-1α*), and 671 (*TEF-1α*) and 860 (*RPB2*) within FSSC. Maximum likelihood (ML) phylogenetic analysis was conducted using raxmlGUI 1.3 (Silvestro and Michalak 2012; Stamatakis 2014). A GTR + G nucleotide substitution model was used with ML estimation of base frequencies. ML bootstrap analysis with 1,000 replicates was used to assess clade support. Phylogenetic trees were visualized and edited in MEGA 7 (Kumar et al. 2016).

Results

A total of 279 diseased wheat heads were collected, and *Fusarium* strains were isolated from 199 of them (Table 1). In total, 256 *Fusarium* strains were isolated and identified at the rank of species level. The numbers of the isolates at the different locations and their classifications are summarized in Table 2. Phylogenetic trees of the investigated species complexes are shown in Supplementary Fig. S1. The *TEF-1α* sequences were found to be diverse enough to allow species-level identification of isolates in most species complexes. O'Donnell et al. (2022) recommended *TEF-1α* sequences as the sole information source as a time- and cost-saving method since this locus contains virtually all phylogenetic signals through its long introns. However, the further recommended locus *RPB2* (O'Donnell et al. 2022) was needed for the identification of species in the FIESC and FSSC. *TEF-1α* (OP205325 to OP205361, OP547875, OQ925410 to OQ925418, OQ925434 to OQ925636, and OR100688 to OR100693) and *RPB2* (OQ925419 to OQ925433) sequences were deposited in NCBI GenBank (Supplementary Fig. S1).

The dominant species complex to which most of the isolates from the FHB symptomatic wheat heads were classified was the FSASC, especially the *F. graminearum* clade within (Laraba et al. 2021). This was followed by the FFSC and FIESC. Species of the FTSC, FOSC, and FSSC were rarely detected.

Overall, the dominant species was *F. graminearum* (58.2%), followed by *F. annulatum* (17.2%) and *F. verticillioides* (7.4%). The proportion of the remaining species including *F. culmorum* in the population was less than 5%.

Two or three different *Fusarium* species were identified from 50 out of the 199 infested wheat heads. Among the 149 cases, only one species was detected from the sample, that is, *F. graminearum* in 70%, *F. annulatum* in 11%, *F. sporotrichioides* in 4%, *F. verticillioides* in 3%, *F. clavus* in 2%, and *F. culmorum* in 2% of the cases. With the exception of *F. subglutinans* and *F. tanahbumbuense*, all other identified species could also be isolated as single species from some samples. *F. graminearum* was isolated as a single species in 77% of its occurrences. For the more common species, this value was

Table 2. Number of isolates of identified species at each site and their proportion in the total sample

Species identified[a]	Sampling sites[b]																					Total (number)	Total (%)
	BG	BS	DO	EP	FA	GG	HA	KC	KM	KU	LP	MV	SA	SC	SK	SL	SO	TI	TO	UM			
Fusarium cerealis (FSASC)	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		1	0.4
F. culmorum (FSASC)	1	–	1	–	–	–	–	–	–	–	–	–	1	–	–	1	–	–	–	–		4	1.6
F. graminearum (FSASC)	11	1	2	–	1	5	1	6	3	7	11	10	43	7	9	6	14	4	7	1		149	58.1
F. sporotrichioides (FSASC)[c]	2	–	–	–	1	3	1	1	–	–	2	–	–	–	–	–	1	–	–	–		11	4.3
F. vorosii (FSASC)	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–		2	0.8
F. annulatum (FFSC)	4	–	1	4	1	3	–	–	–	7	9	–	1	–	–	6	1	–	1	6		44	17.1
F. subglutinans (FFSC)	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–		1	0.4
F. verticillioides (FFSC)	3	–	–	1	1	–	–	3	–	2	5	–	–	–	–	3	1	–	–	–		19	7.4
F. citri (FIESC)	–	–	1	–	–	–	–	–	–	–	–	1	–	–	1	–	–	–	–	–		3	1.2
F. clavus (FIESC)	1	–	–	–	–	–	–	–	–	1	–	–	–	–	5	–	–	–	–	–		7	2.7
F. tanahbumbuense (FIESC)	–	1	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		2	0.8
F. curvatum (FOSC)	–	–	–	–	–	–	–	–	–	–	2	1	–	–	–	–	–	–	–	–		3	1.2
F. inflexum (FOSC)	–	–	–	1	–	1	–	–	–	–	–	–	–	–	1	–	–	–	–	–		3	1.2
F. acuminatum (FTSC)	–	–	–	–	–	2	–	–	–	–	–	–	1	–	–	–	–	–	–	–		3	1.2
F. tricinctum (FTSC)	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–		1	0.4
F. martii (FSSC)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–		1	0.4
F. solani (FSSC)	1	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–		2	0.8

[a] Species complex designations are given in brackets: FSASC = *Fusarium sambucinum* species complex; FFSC = *Fusarium fujikuroi* species complex; FIESC = *Fusarium incarnatum-equiseti* species complex; FOSC = *Fusarium oxysporum* species complex; FTSC = *Fusarium tricinctum* species complex; FSSC = *Fusarium solani* species complex.

[b] Location codes are explained in Table 1.

[c] *F. sporotrichioides* is the only species identified within the FSASC, which does not belong to the *F. graminearum* clade (Laraba et al. 2021).

followed by *F. sporotrichioides* (67%), *F. annulatum* (41%), and *F. verticillioides* (26%).

F. graminearum occurred at all but one sampling site; furthermore, it was the dominant species at 14 out of the 20 locations. *F. annulatum* was found nearly as often as *F. graminearum* at three sites (Kisújszállás, Lászlópuszta, and Szentlőrinc) and was found to be dominated at two additional sites (Eszterágpuszta and Újmohács). *F. poae* was not found during the survey. *F. vorosii*, previously described as an FHB-causing species in Hungary with Asian origin, was detected at two sites (Lászlópuszta and Solt).

Discussion

Population surveys of the FHB pathogens have been regularly conducted in Hungary since 1971, but caution is needed in the comparison because of the different sampling strategies applied, the actual alterations in the fungal taxonomy, and the evolving methodology. This study is a reexamination of *Fusarium* species associated with symptomatic FHB-diseased wheat heads in Hungary using *TEF-1α* or, in the case of FIESC and FSSC, using *TEF-1α* and *RPB2* sequence phylogeny. We do not claim that the *Fusarium* species identified in our study are causative of FHB, as we cannot do so in the absence of pathogenicity studies. Moreover, in the case of multiple infections, it is impossible to determine which fungus infected first and which infected later or whether only its conidia were present on the diseased spikelets. In any case, the *Fusarium* species identified were isolated from the diseased spikelets.

Our result demonstrated that *F. graminearum* is the most frequently isolated pathogen of FHB, which is in agreement with the 50-year-old statement of earlier studies on symptomatic wheat heads (Békésy and Hinfner 1971; László et al. 2011; Mesterházy 1984; Szunics et al. 1978) and the conclusion of Bottalico and Perrone (2002). By contrast, we identified *F. culmorum* only four times (1.6%) at four different sites. Mesterházy (1984) found it as the second most common species (21.9%) associated with diseased wheat head samples during the years 1970 to 1983 in south Hungary. The survey of László et al. (2011) and the results of the present study show that *F. culmorum* has been in decline since Mesterházy's survey in 1984. The reason for the replacement of *F. culmorum* by *F. graminearum* in Hungary may be the global warming and increased maize production, as identified in Europe by Valverde-Bogantes et al. (2020).

F. poae was not present in any of our samples. Studies of randomly selected wheat heads showed that this species was prevalent in the last decades (Tóth 1997; Xu et al. 2005), but monitoring of diseased wheat heads revealed that it occurred less frequently (Békésy and Hinfner 1971; Mesterházy 1984) or not at all (László et al. 2011; Szunics et al. 1978). The absence of *F. poae* on symptomatic wheat heads may be the result of competition between pathogens under weather conditions favorable to FHB. Békésy and Hinfner (1971) stated that there is a significant difference in the species spectrum of apparently diseased and randomly sampled grain.

The occurrence of *F. sporotrichioides* was sporadic in different locations (4.3%; Table 2) among our samples. Most of the earlier Hungarian surveys reveal this species as rare; only Tóth (1997) found it in surface-sterilized wheat grain samples from the central region of Hungary in a higher quantity (13.63%).

The accidental occurrence of *F. cerealis* (FSASC), *F. subglutinans* (FFSC), and some species belonging to the FOSC (*F. curvatum* and *F. inflexum*) (Crous et al. 2021; Lombard et al. 2019), FTSC (*F. acuminatum* and *F. tricinctum*) (Senatore et al. 2021; Torbati et al. 2019), FIESC (*F. clavus*, *F. citri*, and *F. tanahbumbuense*) (Xia et al. 2019), and FSSC (*F. martii* and *F. solani*) (Geiser et al. 2021; O'Donnell et al. 2022) is in accordance with earlier Hungarian survey studies. Newly recorded FHB-associated species in the present study may cause some uncertainty, but we believe that they may have existed in Hungary for a long time as cryptic species. To facilitate future research, the *TEF-1α* and *RPB2* sequences of the present study have been deposited in NCBI GenBank.

F. vorosii was first isolated from blighted wheat in Ipolydamásd, Hungary, in 2002 (Starkey et al. 2007). Based on the phylogenetic analysis of 13 gene sequences, the closest relative of *F. vorosii* in the Hungarian mycobiota is *F. graminearum* (Starkey et al. 2007; Yli-Mattila et al. 2009). Meanwhile, the species was detected in Japan (Starkey et al. 2007), Korea (Lee et al. 2016), and Serbia (Obradović et al. 2022), causing head blight on several small grain cereals. Yli-Mattila et al. (2009) suggested that this species may be of Asian origin and have been introduced to Europe. Seventeen years after the original strain was isolated, this species has been found in two locations, approximately 60 and 114 km from the 2002 isolation site. We can therefore conclude that *F. vorosii* is now a permanent component of the Hungarian mycobiota.

F. verticillioides and *F. annulatum* (formerly known as *F. proliferatum*) were found to be highly prevalent in the wheat samples collected in this study. In older Hungarian studies, *F. verticillioides* represents only a small proportion of the isolates identified (1 to 2%) (Békésy and Hinfner 1971; László et al. 2011; Mesterházy 1974, 1984; Tóth 1997), but in 2019, 7.4% of our isolates were identified as *F. verticillioides*. It has been documented earlier that *F. annulatum* may also be involved in the development of FHB (Amato et al. 2015; Bottalico and Perrone 2002). Under artificially inoculated circumstances, *F. annulatum* can cause remarkable internal kernel infection without any obvious symptoms of FHB on the wheat heads (Puskás et al. 2002), but it has not yet been reported from naturally infected wheat in Hungary. *F. annulatum* was identified in a surprisingly high percentage (17.2%) in the present survey. *F. verticillioides* and *F. annulatum* are well-characterized with conidial chains and are quite tolerant of extreme conditions (Yilmaz et al. 2021). As it is unlikely that these species were hidden in previous morphology-based surveys, we believe that *F. verticillioides* and, in particular, *F. annulatum* are actually spreading pathogenic species. It is in agreement with Molnár (2016), who reported *F. annulatum* as the causal agent of FHB in oats in western Hungary. The spreading of *F. verticillioides* and *F. annulatum* may be caused by the warming of the climate in Hungary, as microconidia of these species germinate optimally at 30°C (Marín et al. 1996). In addition to *F. graminearum*, these two species are the primary pathogens of maize, the other main cereal of Hungary. Environment-friendly tillage trends could contribute to the accumulation of infected plant debris on the soil surface and thus increase pathogen pressure.

Even though they are not considered the primary cause of the typical severe FHB symptoms, the presence of *F. verticillioides* and *F. annulatum* on the grain poses a mycotoxin risk. Both species produce fumonisins that are carcinogenic mycotoxins (Gelderblom et al. 1988; Logrieco et al. 1995). As fumonisins cause toxicoses, particularly from maize-based food and feed contaminated with *F. verticillioides* or *F. annulatum* (Chen et al. 2021; Wild and Gong 2010), the European Commission has issued guidance values for fumonisins in maize and maize products in the European Union (European Commission 2006). In a review, Leslie et al. (2021) cited some works about wheat naturally contaminated with fumonisins but found little evidence that they occur commonly at any significant level in wheat. As a result, there are no such guidance values for fumonisins in wheat and wheat products yet. However, special care should be taken to avoid infection of wheat seeds with *F. verticillioides* or *F. annulatum*, as their presence in the crop provides an opportunity to serve as a source of further mycotoxin contamination, especially in the postharvest environment.

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