World Mycotoxin Journal, 2020; 13 (2): 213-223
SPECIAL ISSUE: *Mycotoxins in China*

https://www.wageningenacademic.com/doi/pdf/10.3920/WMJ2019.2516 - Tuesday, March 28, 2023 3:56:17 AM - Wageningen University and Research Library IP Address:137.224.252.12

Survey for toxigenic *Fusarium* species on maize kernels in China

P.W. Qin[1], J. Xu[2], Y. Jiang[2], L. Hu[2], T. van der Lee[3], C. Waalwijk[3], W.M. Zhang[4] and X.D. Xu[2*]

[1]College of Plant Protection, Shenyang Agricultural University, Shenhe Street, Dongling Road No.120, Shenyang, 110161, China P.R.; [2]Plant Protection Institute, Liaoning Academy of Agricultural Sciences, Shenyang, 110161, China P.R.; [3]Plant Research International, Business Unit Biointeractions, P.O. Box 16, 6700 AA Wageningen, the Netherlands; [4]Plant Protection Station of Liaoning Province, Shenyang 110034, China P.R.; xiudex@163.com

Received: 29 August 2019 / Accepted: 13 December 2019
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RESEARCH ARTICLE

Abstract

Maize is currently the most important crop in China. A major concern in maize production is maize ear rot caused by *Fusarium* spp., which results in yield losses, reduction of seed quality and the accumulation of mycotoxins in the harvested grains. To identify the importance of the different *Fusarium* species in maize infection, we performed a comprehensive survey on 9,000 asymptomatic and randomly collected maize kernels. Seeds were collected from 12 different provinces covering all major maize growing areas in China and included five maize varieties. In total 1,022 *Fusarium* isolates were retrieved that were identified based on morphological characteristics, by species specific diagnostic PCRs and by EF1-α gene sequencing. Eight different species were identified: *Fusarium verticillioides* (75.34%), *Fusarium graminearum* (8.32%), *Fusarium proliferatum* (7.14%), *Fusarium subglutinans* (4.11%), *Fusarium meridionale* (1.57%), *Fusarium oxysporum* (1.37%), *Fusarium semitectum* (1.17%), and *Fusarium asiaticum* (0.98%). The distribution of *Fusarium* species was found to be different in different regions with the largest diversity observed in Hubei province, where all eight *Fusarium* species were isolated. Genetic chemotyping within the *F. graminearum* species complex indicated that all of the 85 *F. graminearum* isolates showed the 15-acetyldeoxynivalenol chemotype, whereas all *F. asiaticum* (n=10) and *F. meridionale* (n=16) isolates had the nivalenol chemotype even when isolated from the same maize field. To our knowledge this is the largest collection of *Fusarium* isolates from maize and further exploitations of this collection are discussed.

Keywords: maize ear rot, *Fusarium* spp., population structure, mycotoxin chemotype

1. Introduction

Maize is one of the principal crops grown for human food, industrial usage and livestock feed in China. Its yield and quality can be affected by many kinds of fungi and it is one of the crops subject to the most critical mycotoxin problems throughout the world. Although many toxic fungal metabolites can be found in maize (Gao *et al.*, 2005), management has focused on a few mycotoxins that occur with greater frequency or are associated with particularly undesirable consequences. These include deoxynivalenol (DON, a trichothecene mycotoxin, produced primarily by *Fusarium graminearum* and *Fusarium culmorum*), zearalenone (ZEN), an estrogenic compound produced primarily by *F. graminearum* and fumonisins (produced primarily by *Fusarium verticillioides* and *Fusarium proliferatum*). Each of these toxins is associated with a specific set of health detriments that have been documented in domestic animals and either documented or suspected for humans (CAST, 2003). *Fusarium* Ear Rot (FER) in maize can be caused by a wide range of fungal pathogens, with *F. verticillioides* and *F. graminearum* being the most important ear-rotting pathogens of maize in many areas of China (Bai, 1997; Xu and Liu, 2009). FER reduces both the yield as well as the grain quality due to the accumulation of mycotoxins produced by these *Fusarium* pathogens. This is of considerable concern to livestock producers and to human health. Besides causing direct and indirect economic losses, these fungi can also affect the health of grain handlers and processors.

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As the incidence and severity of FER is subject to climate and locations as well as agronomic measures and the maize variety, monitoring is important to assess risks and trends. This can guide adequate control and protection measures, that include proper grain drying, storage of grain at low moisture levels, and sanitation of feed preparation and delivery systems. Cultural practices designed to reduce mycotoxin contamination of crops should be based on plant disease epidemiology (reviewed by Munkvold, 2003). In many parts of China, especially in rural areas, farmers produce maize primarily for home consumption and to feed their animals. After harvest, maize is commonly stored in sheds or in containers, such as homemade bins. Grain dries slowly in these bins, consequently the moisture content can remain high enough to allow continued growth of the fungi as well as toxin production by fungi that have infected the kernels preharvest. Insects may continue to feed on maize in the field late in the season, enhancing the ability of fungi to infect the kernels. The aims of this study were: (1) to establish a protocol for surveys in China and to start a collection of viable accessions for future reference; (2) to understand which species of *Fusarium* infect maize kernels and their frequencies in different provinces; and (3) to determine the toxigenic chemotypes of different *Fusarium* spp. The results provide an effective base level on the toxigenic genotype and toxin production capacity of major pathogenic *Fusarium* spp. causing maize kernel rot in China and provide an early warning platform for stakeholders in regional maize production.

2. Materials and methods

Samples collection of maize kernels and *Fusarium* spp. isolation

In 2016, five maize varieties (N28, Qi319, Huang Zaosi, Oh7, Lu Yuan 92) with different resistance levels to *Fusarium* ear rot (Xu *et al*., 2019), were planted in 12 provinces, including Heilongjiang, Jilin, Liaoning, Inner Mongolia, Hebei, Shandong, Shanxi, Hubei, Henan, Gansu, Guizhou and Sichuan Provinces (Table 1). Five cobs per variety were harvested at physiological maturity stage and transported to local research units in the provinces and kernels were removed from the cob after 1-2 weeks on air-drying at room temperature. Maize kernels from each variety were mixed together as one sample and five samples from each location were transported to the Institute of Plant Protection, Liaoning Academy of Agricultural Sciences (IPP-LAAS, Shenyang city). Subsequently, all samples (5×12) were analysed for the percentage of *Fusarium* species contaminating the maize kernels and pathogen chemotypes detection.

For isolation of *Fusarium* spp., a total of 150 maize kernels from each of the 60 samples were taken at random for testing. The kernels were washed with distilled water to remove attached dirt or dust and put on sterilised filter papers to remove extra water. Subsequently, the kernels were surface sterilised in 2% sodium hypochlorite solution for 5 min, rinsed in three changes of sterile distilled water and placed on potato dextrose agar (PDA) medium at a density of ten kernels per 12 cm Petri dish. Plates were incubated at 25 °C for 3-5 days, checked for fungal outgrowth from each kernel. Hyphae from *Fusarium* colonies on PDA were transferred to a fresh poor-nutrient potato dextrose agar (half strength PDA) (potato infusion 100 g, dextrose 20 g, agar 20 g, distilled water 1000 ml) plate and the culture was grown for 5 to 7 days. Single spores were separated with an inoculation needle under stereomicroscope and transferred to individual PDA plates. All *Fusarium* isolates were stored in 30% glycerin, at -80 °C at the IPP-LAAS, Shenyang, China.

Table 1. Maize samples from different province or locations used for isolation of *Fusarium* species.

Province of samples taken	Cities of samples taken	GPS data of collection sites	No. of maize kernels for isolation of fungi
Helongjiang	Harbin	N45°45, E126°41	750
Jilin	Gongzhuling	N43°50', E124°82'	750
Liaoning	Shenyang	N41°50', E123°24'	750
Inner Mogolia	Chifeng	N42°27, E118°92'	750
Hebei	Sijiazhuang	N38°02, E114°30'	750
Shandong	Jinan	N36°40, E117°00'	750
Shanxi	Fenyang	N37°27, E111°75'	750
Henan	Zhengzhou	N34°46, E113°40'	750
Hubei	Nanzhang	N31°78, E111°83'	750
Gansu	Pingliang	N35°55, E106°67'	750
Guizhou	Guiyang	N26°35, E106°42'	750
Sichuan	Luzhou	N28°54, E105°24'	750
Total			9,000

Morphological examination of *Fusarium* spp.

The isolates were identified based on morphological characteristics (Leslie and Summerell, 2006). Strains of *Fusarium* spp. were grown at 25 °C on carnation leaf agar, synthetic nutrient-poor agar (SNA) as well as PDA. Growth rates and colony diameters of cultures incubated for 5 days in the dark were measured on both SNA and PDA. Characteristics, such as size and shape of conidia, the presence of phialides and chlamydospores were recorded from strains grown on CLA or SNA incubated in the dark for 10-14 days. Presence of polyphialides on SNA, CLA and development of sporodochia on PDA was also examined in 4-week old cultures. Polyphialides were identified after staining the fungal structures with cotton blue in lactic

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acid. Macroscopic characters, such as surface texture and colony colours, were described from PDA.

Molecular identification of *Fusarium* spp.

DNA manipulation

Fusarium isolates were grown in shaken cultures (140 rpm) at 26 °C for 4 days in 100 ml flasks containing 50 ml potato-dextrose broth medium. Mycelium was harvested by filtration though cheesecloth and freeze-dried. 10 to 20 mg freeze-dried mycelium was grinded in a 1.5 micro tube with help of glass beads for 10 s at 4,000 rpm, 450 µl cell lysis solution (Gentra Puregene Buccal Cell Kit, Qiagen, Germantown, MD, USA) plus 3 µl proteinase (20 mg/µl) was added and the tube was incubated for 1 h at 55 °C. To remove proteins, 150 µl of protein precipitation solution was added to the cell-lysate and vortexed for 20 s at high speed to mix well. After protein precipitation the samples were centrifugated and the supernatant transferred to a new tube containing 300 µl 100% isopropanol. The tubes were stored at -20 °C for 10 min and DNA was pelleted at 1,400 rpm for 10 min. The pellet was washed with 300 µl 70% ethanol and centrifuged again for 10 min. After removal of the supernatant the pellet was airdried at 65 °C for 10 min and 50 µl distilled water was added to the pellet. RNA was degraded by incubation with RNases (10 mg/ µl) for 30 min at 37 °C. Gel electrophoresis was used to estimate the concentration and integrity of the DNA.

Molecular identification of Fusarium species

Molecular identification of *Fusarium* isolates was done using established PCR protocols described in literature (Mishra *et al.*, 2003; Mulè *et al.*, 2004; Nicholson *et al.*, 1998; O'Donnell *et al.*, 1998) and primers used are listed in Table 2. After amplification PCR products were analysed on agarose gels to confirm species identity.

Sequencing the elongation factor gene

The translation elongation factor gene EF-1α was amplified with the primers EF-1 and EF-2 (Table 2) as previously described (O'Donnell *et al.*, 2004). PCR was performed and each PCR reaction (50 µl) contained 5.0 µl DNA template. Reactions were performed using a GeneAmp PCR System 9700 thermal cycler (Thermo Fisher, Waltham, MA, USA) programmed for 94 °C for 5 min; followed by 35 cycles of (95 °C for 50 s, 53 °C for 50 s, and 72 °C for 60 s) and then a final extension at 72 °C for 10 min. The amplified PCR products were bi-directionally sequenced by Dingguo Biotech (Beijing, China P.R.) and the sequences were compared with DNA sequences in the *Fusarium* ID database at Penn State University (USA).

Molecular identification of toxigenic genes

The detection of the FUM1 gene was conducted using the specific primers: PQF5-F/PQF5-R for *F. verticillioides* isolates, FUM5P2-F/FUM5P2-F for *F. proliferatum* strains, and FUM1-F/FUM1-R for all other *Fusarium* species, respectively (Table 3). The molecular determination of the chemotypes of isolates from the *Fusarium graminearum* species complex (FGSC), including *F. graminearum, Fusarium asiaticum* and *Fusarium meridionale* was conducted using specific primers: Tri13F/DON, Tri13NIV/R and ToxP1/ToxP2 for identification of DON and NIV producers, respectively. To discriminate among DON producers, primer pair Tri303F/R was used for the identification of 3-acetyldeoxynivalenol (3-ADON) producers and primer pair Tri315F/R for 15-acetyldeoxynivalenol (15-ADON) producers. Electrophoretic analysis of the PCR amplified products was performed on a 1% agarose gel.

Table 2. Primers used for identification of *Fusarium* species in this study.

Primer	Primer sequence	*Fusarium* species	Temperature (°C)	Product size (bp)	References
VER1	CTTCCTGCGATGTTTCTCC	*F. verticillioides*	56	578	Mulè *et al.*, 2004
VER2	AATTGGCCATTGGTATTATATATCTA				
PRO1	CTTTCCGCCAAGTTTCTTC	*F. proliferatum*	56	585	Mulè *et al.*, 2004
PRO2	TGTCAGTAACTCGACGTTGTTG				
SUB1	CTGTCGCTAACCTCTTTATCCA	*F. subglutinans*	56	631	Mulè *et al.*, 2004
SUB2	CAGTATGGACGTTGGTATTATATCTAA				
FoF1	ACATACCACTTGTTGCCTCG	*F. oxysporum*	58	340	Mishra *et al.*, 2003
FoR1	CGCCAATCAATTTGAGGAACG				
Fg16NF	ACAGATGACAAGATTCAGGCACA	*F. graminearum*	57	280	Nicholson *et al.*, 1998
Fg16NR	TTCTTTGACATCTGTTCAACCCA				
EF-1	ATGGGTAAGGAGGACAAGAC	*Fusarium* spp.	58	750	O'Donnell *et al.*, 2004
EF-2	GGAAGTACCAGTGATCATGTT				

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Table 3. Primer used for identification of toxigenic genes in this study.

Primer	Primer sequence	PCR conditions	Amplicon (bp)	Mycotoxin chemotype[1]	Reference
Tri13F	CATCATGAGACTTGTKCRAGTTTGGG	58 °C, 45 s	282	DON	Chandler *et al.*, 2003
Tri13RDON	GCTAGATCGATTGTTGCATTGAG				
Tri13R	TTGAAAGCTCCAATGTCGTG	58 °C, 45 s	312	NIV	Chandler *et al.*, 2003
Tri13NIV	CCAAATCCGAAAACCGCAG				
Tri303F	GATGGCCGCAAGTGGA	55 °C, 30 s	586	3-ADON	Jennings *et al.*, 2004
Tri303R	GCCGGACTGCCCTATTG				
Tri315F	CTCGCTGAAGTTGGACGTAA	58 °C, 45 s	864	15-ADON	Jennings *et al.*, 2004
Tri315R	GTCTATGCTCTCAACGGACAAC				
ToxP1	GCCGGGGGRTAAAAGTCAAA	55 °C, 60 s	300	DON	Li *et al.*, 2005
ToxP2	TGACAAGTCCGGTCGCACTAGCA		360	NIV	
PQF5-F	GAGCCGAGTCAGCAAGGATT	60 °C, 60 s	70	FUM (*F. vert*)	López-Errasquín *et al.*, 2007
PQF5-R	AGGGTTCGTGAGCCAAGGA				
FUM5P2-F	CCCCCATCATCCCGAGTAT	60 °C, 60 s	60	FUM (*F. prol*)	López-Errasquín *et al.*, 2007
FUM5P2-R	TGGGTCCGATAGTGATTTGTCA				
FUM1-F	CCATCACAGTGGGACACAGT	56 °C, 50 s	183	FUM1	Bluhm *et al.*, 2004
FUM1-R	CGTATCGTCAGCATGATGTAGC				

[1] DON = deoxynivalenol; NIV = nivalenol; 3-/15-ADON = 3-/15-deoxynivalenol; FUM = fumonisin.

3. Results

Population structure of *Fusarium* species associated with maize ear rot

Based on morphology and molecular assay results, a total of 1,022 *Fusarium* isolates encompassing eight *Fusarium* species were identified, including *F. verticillioides, F. graminearum, F. proliferatum, Fusarium subglutinans, F. meridionale, Fusarium oxysporum, Fusarium semitectum,* and *F. asiaticum,* with isolation frequencies of 75.34% (n=770), 8.32% (85), 7.14% (73), 4.11% (42), 1.57% (16), 1.37% (14), 1.17% (12), and 0.98% (10), respectively (Figure 1). *F. verticillioides* was dominant in all regions ranging from 63.63 to 87.85% of the isolates retrieved. There were differences in the number of isolates and the frequency of *Fusarium* species across different provinces of China. The number of *Fusarium* species from maize kernels in Liaoning, Jilin, Hebei and Hubei provinces were higher than that in other provinces. The distribution of *Fusarium* species is also different among provinces with eight *Fusarium* species in Hubei province. *F. asiaticum* was found in Liaoning, Jilin, Shandong and Hubei province. *F. meridionale* was only found in the south of China, such as Hubei, Sichuan and Guizhou province.

Detection of toxigenic genes and chemotypes

Detection of trichothecene chemotypes of FGSC members associated with maize ear rot in different provinces of China

The total of 111 isolates of the FGSC, including 85 *F. graminearum,* 10 *F. asiaticum* and 16 *F. meridionale* from 12 provinces of China, was tested for trichothecene chemotypes using specific primers (Table 4). All of 85 *F. graminearum* isolates were of the DON chemotype based on the Tri13F/DON, Tri13NIV/R and ToxP1/P2 assays and more specifically all were of the 15-ADON type as determined by the primer pairs Tri303F/R and Tri315F/R. The ten *F. asiaticum* and 16 *F. meridionale* isolates were all of the NIV chemotype as determined by the Tri13F/DON, Tri13NIV/R and ToxP1/P2 assays (Table 4).

Detection of the FUM gene in members of Fusarium fujikuroi species complex associated with maize ear rot in provinces of China

Using the specific primer pairs of PQF5-F/R, FUM5P2-F/R and FUM1F/R, the presence of the fumonisin polyketide synthase gene was detected in 432 out of 770 *F. verticillioides* (56.10%) and 32 out of 73 *F. proliferatum* (43.84%) while none of the 42 *F. subglutinans* isolates tested positive with one or more of these primer combinations (Table 5). The proportion of fumonisin producing *F. verticillioides* strains was different across the 12 provinces ranging from 32.39% (Hebei province) to 70.21% (Shanxi province). Among the

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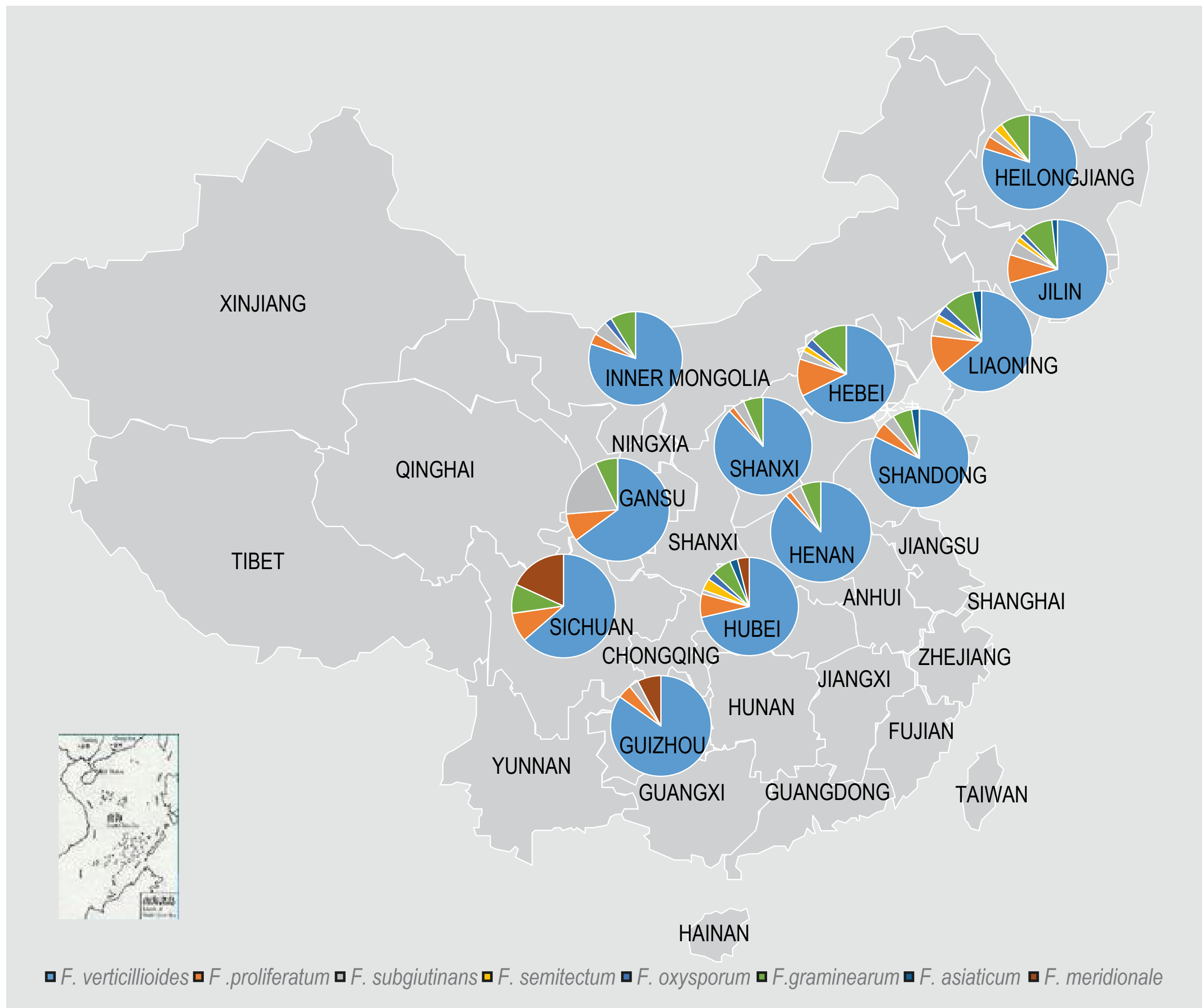

Figure 1. Distribution of *Fusarium* species associated with maize ear rot in different provinces.

73 *F. proliferatum* isolates fumonisin producers ranged from 20.00% in Gansu province to 100% in Inner Mongolia, Shanxi and Guizhou.

4. Discussion

Maize is an important food crop in the world and has become more popular as well in China. *Fusarium* ear rot, FER, is an important disease in most countries were maize is grown. FER not only results in yield losses and the reduction of seed quality, the pathogens involved accumulate mycotoxins in infected tissues that could have dangerous consequences on human and animal health (Bottalico, 1998). Fumonisins, trichothecenes (DON, NIV and derivatives) and ZEN are the most prominent mycotoxins produced by *Fusarium* species that contaminate maize. Members of the *Fusarium fujikuroi* species complex, including *F. verticillioides, F. proliferatum* and *F. subglutinans,* are the most important and prevalent species, causing ear rot world-wide (Bottalico, 1998). In China, studies on mycotoxins in maize focused mainly on detection methods, such as high-performance liquid chromatography (HPLC), rather than on the causal agents, the toxigenic *Fusarium* species. Wang *et al.* (2006) collected 284 maize samples from six provinces of China and detected DON in 67% of the samples ranging from 10 to 3,800 μg/kg using gas chromatography. More recently, Wang *et al.* (2016) collected maize samples in Shandong province for mycotoxin contamination demonstrating that the incidences and average contents of FBs (fumonisin B_1, B_2 and B_3), DON, and ZEN increased from harvest to storage period. Due to the co-occurrence of mycotoxins and their cumulative toxic effects, there is an urgent need for quality control to warrant safe produce.

Species identification

Previous studies on maize ear rot in China focus on symptomatic plant parts (Guo *et al.*, 2014) in which *F. verticillioides* and *F. graminearum* were identified as the predominant species (Qin *et al.*, 2014; Qu *et al.*, 2009).

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Table 4. Detection of mycotoxin chemotypes of *Fusarium graminearum* species complex members associated with maize ear rot in provinces of China.

Isolate codes	*Fusarium* species[1]	Origin	ToxP gene[2]		Tri13 gene[2]		Tri3 gene[2]	
			ToxP1/DON	ToxP2/NIV	Tri13F/DON	Tri13NIV/R	Tri303F/R/3-ADON	Tri315F/R/15-ADON
HLJFG01	FG	Heilonlongjiang	+	-	+	-	-	+
HLJFG02	FG	Heilonlongjiang	+	-	+	-	-	+
HLJFG03	FG	Heilonlongjiang	+	-	+	-	-	+
HLJFG04	FG	Heilonlongjiang	+	-	+	-	-	+
HLJFG05	FG	Heilonlongjiang	+	-	+	-	-	+
HLJFG06	FG	Heilonlongjiang	+	-	+	-	-	+
HLJFG07	FG	Heilonlongjiang	+	-	+	-	-	+
JLFG01	FG	Jilin	+	-	+	-	-	+
JLFG02	FG	Jilin	+	-	+	-	-	+
JLFG03	FG	Jilin	+	-	+		-	+
JLFG04	FG	Jilin	+	-	+	-	-	+
JLFG05	FG	Jilin	+	-	+		-	+
JLFG06	FG	Jilin	+	-	+	-	-	+
JLFG07	FG	Jilin	+	-	+	-	-	+
JLFG08	FA	Jilin	-	+	-	+	-	-
JLFG09	FA	Jilin	-	+	-	+	-	-
JLFG010	FG	Jilin	+	-	+	-	-	+
JLFG011	FG	Jilin	+	-	+	-	-	+
LN01	FG	Liaoning	+	-	+	-	-	+
LN02	FG	Liaoning	+	-	+	-	-	+
LN03	FG	Liaoning	+	-	+	-	-	+
LN04	FG	Liaoning	+	-	+	-	-	+
LN05	FG	Liaoning	+	-	+	-	-	+
LN06	FG	Liaoning	+	-	+	-	-	+
LN07	FG	Liaoning	+	-	+	-	-	+
LN08	FA	Liaoning	-	+	-	+	-	-
LN09	FA	Liaoning	-	+	-	+	-	-
LN10	FG	Liaoning	+	-	+	-	-	+
LN11	FA	Liaoning	-	+	-	+	-	-
LN12	FA	Liaoning	-	+	-	+	-	-
LN13	FG	Liaoning	+	-	+	-	-	+
LN14	FG	Liaoning	+	-	+	-	-	+
LN15	FG	Liaoning	+	-	+	-	-	+
LN16	FG	Liaoning	+	-	+	-	-	+
LN17	FG	Liaoning	+	-	+	-	-	+
LN18	FG	Liaoning	+	-	+	-	-	+
LN19	FA	Liaoning	-	+	-	+	-	-
IM01	FG	Inner Mongolia	+	-	+	-	-	+
IM02	FG	Inner Mongolia	+	-	+	-	-	+
IM03	FG	Inner Mongolia	+	-	+	-	-	+
IM04	FG	Inner Mongolia	+	-	+	-	-	+
IM05	FG	Inner Mongolia	+	-	+	-	-	+
IM06	FG	Inner Mongolia	+	-	+	-	-	+
IM07	FG	Inner Mongolia	+	-	+	-	-	+
HEB01	FG	Hebei	+	-	+	-	-	+
HEB02	FG	Hebei	+	-	+	-	-	+
HEB03	FG	Hebei	+	-	+	-	-	+
HEB04	FG	Hebei	+	-	+	-	-	+

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Table 4. Continued.

Isolate codes	*Fusarium* species[1]	Origin	ToxP gene[2]		Tri13 gene[2]		Tri3 gene[2]	
			ToxP1/DON	ToxP2/NIV	Tri13F/DON	Tri13NIV/R	Tri303F/R/3-ADON	Tri315F/R/15-ADON
HEB05	FG	Hebei	+	-	+	-	-	+
HEB06	FG	Hebei	+	-	+	-	-	+
HEB07	FG	Hebei	+	-	+	-	-	+
HEB08	FG	Hebei	+	-	+	-	-	+
HEB09	FG	Hebei	+	-	+	-	-	+
HEB10	FG	Hebei	+	-	+	-	-	+
HEB11	FG	Hebei	+	-	+	-	-	+
HEB12	FG	Hebei	+	-	+	-	-	+
HEB13	FG	Sandong	+	-	+	-	-	+
SD01	FG	Sandong	+	-	+	-	-	+
SD02	FG	Sandong	+	-	+	-	-	+
SD03	FG	Sandong	+	-	+	-	-	+
SD04	FG	Sandong	+	-	+	-	-	+
SD05	FA	Sandong	-	+	-	+	-	-
SD06	FA	Sandong	-	+	-	+	-	-
SD06	FA	Sandong	-	+	-	+	-	-
SX01	FG	Shanxi	+	-	+	-	-	+
SX02	FG	Shanxi	+	-	+	-	-	+
SX03	FG	Shanxi	+	-	+	-	-	+
SX04	FG	Shanxi	+	-	+	-	-	+
SX05	FG	Shanxi	+	-	+	-	-	+
SX06	FG	Shanxi	+	-	+	-	-	+
SX07	FG	Shanxi	+	-	+	-	-	+
HN01	FG	Henan	+	-	+	-	-	+
HN02	FG	Henan	+	-	+	-	-	+
HN03	FG	Henan	+	-	+	-	-	+
HN04	FM	Henan	-	+	-	+	-	-
HN05	FM	Henan	-	+	-	+	-	-
HN06	FG	Henan	+	-	+	-	-	+
HN07	FG	Henan	+	-	+	-	-	+
HN08	FG	Henan	+	-	+	-	-	+
HN09	FG	Henan	+	-	+	-	-	+
HN10	FG	Henan	+	-	+	-	-	+
HN11	FG	Henan	+	-	+	-	-	+
HUB01	FG	Hubei	+	-	+	-	-	+
HUB02	FM	Hubei	-	+	-	+	-	-
HUB03	FM	Hubei	-	+	-	+	-	-
HUB04	FG	Hubei	+	-	+	-	-	+
HUB05	FG	Hubei	+	-	+	-	-	+
HUB06	FG	Hubei	+	-	+	-	-	+
HUB07	FG	Hubei	+	-	+	-	-	+
HUB08	FG	Hubei	+	-	+	-	-	+
HUB09	FG	Hubei	+	-	+	-	-	+
HUB09	FG	Hubei	+	-	+	-	-	+
HUB10	FM	Hubei	-	+	-	+	-	-
GS01	FG	Gansu	+	-	+	-	-	+
GS02	FG	Gansu	+	-	+	-	-	+
GS03	FG	Gansu	+	-	+	-	-	+
GS04	FG	Gansu	+	-	+	-	-	+

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Table 4. Continued.

Isolate codes	*Fusarium* species[1]	Origin	ToxP gene[2]		Tri13 gene[2]		Tri3 gene[2]	
			ToxP1/DON	ToxP2/NIV	Tri13F/DON	Tri13NIV/R	Tri303F/R/3-ADON	Tri315F/R/15-ADON
GZ01	FM	Guizhou	-	+	-	+	-	-
GZ02	FM	Guizhou	-	+	-	+	-	-
GZ03	FM	Guizhou	-	+	-	+	-	-
GZ04	FM	Guizhou	-	+	-	+	-	-
GZ05	FM	Guizhou	-	+	-	+	-	-
SC01	FG	Sichuan	+	-	+	-	-	+
SC02	FG	Sichuan	+	-	+	-	-	+
SC03	FG	Sichuan	+	-	+	-	-	+
SC04	FM	Sichuan	-	+	-	+	-	-
SC05	FM	Sichuan	-	+	-	+	-	-
SC06	FM	Sichuan	-	+	-	+	-	-
SC07	FM	Sichuan	-	+	-	+	-	-
SC08	FM	Sichuan	-	+	-	+	-	-
SC09	FM	Sichuan	-	+	-	+	-	-

[1] FG = *Fusarium graminearum;* FA = *Fusarium asiaticum*; FM = *Fusarium meridionale*.
[2] +/- indicate the presence/absence of the PCR product; with the ToxP1/P2 primer pair all isolates.

Table 5. Detection of the capacity of fumonisin (FUM) production of in members of *Fusarium fujikuroi* species complex associated with maize ear rot in 12 provinces in China.[1]

Fusarium species	Isolates	Total number	HLJ	JL	LN	IM	HEB	SD	SX	HN	HUB	GS	GZ	SC
F. verticillioides	Number	770	55	77	91	64	71	65	94	84	55	37	56	21
	FUM	432	33	45	56	29	23	45	66	45	28	21	31	10
	Frequency (%)	56.10	60.00	58.44	61.54	45.31	32.39	69.23	70.21	53.57	50.91	56.76	55.36	47.62
F. proliferatum	Number	73	3	10	18	3	13	4	2	3	6	5	3	3
	FUM	32	1	4	5	3	5	3	2	2	4	1	3	1
	Frequency (%)	43.84	33.33	40.00	27.78	100	38.46	75.0	100	66.67	66.67	20.00	100	33.33

[1] HLJ = Heilongjiang; JL = Jilin; LN = Liaoning; IM = Inner Mongolia; HEB = Hubei; SD = Shandong; SX = Shanxi; HN =Henan; HUB = Hubei; GS = Gansu; GZ = Guizhou; SC = Sichuan.

However, sampling of symptomatic plant tissue creates a bias and as symptomatic kernels and cobs may be discarded by maize producers; this may not represent the actual risks. In addition, many samplings in China have been performed on a provincial or regional level. To allow a more comprehensive and less biased sampling, we performed a structured sampling using a fixed set of cultivars, unified harvest time as well as unified grain drying in the laboratory to reduce contamination and/or outgrowth of *Fusarium* spp. after harvest. This evaluation of the *Fusarium* species associated with maize ear rot provides a new instrument to monitor maize throughout China. To collect data on the *Fusarium* species and mycotoxins that are present in maize the establishment of a China database is required. In addition, storing the isolates in viable cultures will provide a reference for future studies. In this study, *Fusarium* species from maize kernel samples in 12 provinces of China were identified by morphological and molecular techniques. The results indicated that *Fusarium* species associated with maize ear rot include *F. verticillioides, F. graminearum, F. proliferatum, F. subglutinans, F. meridionale, F. oxysporum, F. semitectum* and *F. asiaticum,* respectively. *F. verticillioides* and *F. graminearum* were found to be the dominant species on asymptomatic kernels. Since the incidence of *Fusarium*

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spp. in asymptomatic kernels is lower than in symptomatic kernels, this indicates that the pathogen is already present, but somehow triggered to proliferate on the maize cobs.

Toxin capacity

Both *F. verticillioides* and *F. proliferatum* theoretically possess the capacity to synthesise FBs. Several PCR based assays were previously described to detect the genes responsible for FBs production (Bluhm *et al.*, 2004; López-Errasquín *et al.*, 2007). We used these assays to screen our *F. verticillioides, F. proliferatum* and *F. subglutinans* isolates. As expected in none of *F. subglutinans* isolates the primers were able to amplify the FUM amplicon. However, to our surprise also in a large proportion of the *F. verticillioides* and *F. proliferatum* isolates the expected amplicon using the primer pairs PQF5-F/R or FUM5P2-F/R was not obtained. This cannot be caused by quality or quantity of the DNA preparations, since amplicons were generated with the EF primers as well as with the species-specific primers. This lack of amplification might be due to diversity at the primer sites. If this would be true, it would have important implications for the use of these primers to detect fumonisin producing species leading to an underestimation of the problem. Also other researchers have reported this problem. Cheri (2016) applied the PCR assay based on the FUM gene to identify the genetic potential of FUM production of the FFSC strains isolated from ears of wild grasses in Iran and observed that out of 22 isolates only 11 (50%) were determined as fumonisin producers.

Among the trichothecene genotypes, DON was the predominant mycotoxin in maize production areas of China, with all 85 *F. graminearum* isolates showing the 15-ADON chemotype. Remarkably, none of the *F. asiaticum* isolates showed the 3-ADON chemotype that was previously found to be the dominant species on wheat, rice and barley (Zhang *et al.*, 2010, 2012). In addition, a clear association between the different chemotypes and the species was found: all *F. asiaticum* (n=10) and *F. meridionale* (n=16) isolates had the NIV chemotype even when they were isolated from the same maize field.

Population diversity

Our study does not reveal much of the population diversity within the species. The large set of *F. verticillioides* and the different locations from which the isolates originate could be used for such studies. However, a consensus on established methods for diversity studies is currently lacking. Microsatellite distribution in the *F. verticillioides* genome is similar to other ascomycetes, but unlike other previously analysed fungal genomes, the *F. verticillioides* genome has a high frequency of hexanucleotides (Leyva-Madrigal *et al.*, 2014; Reynoso *et al.*, 2009). Consensus on markers, protocols and scoring is required to set up a global genotyping protocol as was previously established for *F. graminearum* (Suga *et al.*, 2008). Harmonising such efforts can be complicated and perhaps in the future such diversity studies can be performed by whole genome sequencing, or mitochondrial sequencing applying next generation sequencing. The collection described in this study and the identification and characterisation of the isolates could be the start of a global database for *Fusarium* isolates including FER isolates as was previously established for *Fusarium* isolates collected from wheat, rice and barley (https://emdelponte.github.io/FGSC/ or https://twitter.com/edelponte/status/898013549758472196). Such a repository would allow more comprehensive studies and monitoring of changes in *Fusarium* populations.

Solutions

The growth of fumonisin-producing *Fusarium* species and the synthesis of mycotoxins in maize are greatly influenced by multiple environmental factors in different geographical areas, including climate, temperature, humidity and rainfall prior to and during pre-harvest and harvest periods, as well as other factors, such as insect infestation and agricultural practices. Processing and storage are key factors to control and reduce toxin contamination (Magan *et al.*, 2003). Our study shows that in China asymptomatic infections with toxigenic *Fusarium* species are very common. It is therefore important to raise awareness and to improve post-harvest practices. Currently, the storage conditions in China for harvested maize are not optimal and may aggravate the contamination of maize grain by fungi and/or increase mycotoxin production by toxigenic fungi. Particularly, the storage facilities and storage conditions should be improved. In addition, development and application of resistant hybrids may prevent both ear rot progress as well as mycotoxin contamination. Although genetic variation for resistance to *Fusarium* ear rot is present among inbred lines and hybrids in field maize, there is no complete resistance to either ear rot or fumonisin accumulation (Presello *et al.*, 2008; Xu *et al.*, 2019). In recent years, more and more attention has been given to the breeding of maize ear rot resistant varieties in China (Li *et al.*, 2018; Zou *et al.*, 2017). As the assessment of resistance is often done visually, immediately after harvest, it is not clear whether asymptomatic kernels of cultivars evaluated as resistant do not pose a mycotoxin risk after storage. A comprehensive monitoring is required to critically evaluate these risks.

Acknowledgements

The research was sponsored by National Key R&D Program of China (2016YFE0112900) and by the European Union's Horizon 2020 research and innovation programme under grant agreement No 678781 (MycoKey).

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Conflict of interest

The authors declare no conflict of interest.

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