Short Communication

TRICHOTHECENE GENOTYPES OF *FUSARIUM GRAMINEARUM* SPECIES COMPLEX AND *F. CEREALIS* ISOLATES FROM MEXICAN CEREALS

M. Osman[1,2], X. He[1], S. Benedettelli[2] and P.K. Singh[1]

[1] International Maize and Wheat Improvement Center (CIMMYT), Apdo. Postal 6-641, 06600 Mexico DF, Mexico
[2] DISPAA – Sezione di Patologia vegetale ed Entomologia, Università di Firenze, Piazzale delle Cascine 28, 50144 Firenze, Italy

SUMMARY

Four of the world's most important crops, wheat, barley, rice and maize, in addition to other small grains are susceptible to *Fusarium graminearum* species complex (FGSC), the most important causal agent of Fusarium head blight (FHB). The major threat from this species complex comes from trichothecene mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV) and their acetylated derivatives, including 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and 4-acetylnivalenol (4-ANIV). Polymerase chain reaction (PCR) assays are being utilized to quickly identify type B trichothecene genotypes in populations of head blight causing Fusaria. In the current study, 388 isolates collected from different locations in 6 Mexican states between 1995 and 2013 were analyzed by chemotype and sub-chemotype specific markers. It was found that the disease has been predominantly caused by FGSC, while *F. cerealis* co-occurred as FHB causal agent in Mexico. Both DON and NIV trichothecene genotypes were identified in isolates belonging to FGSC, with the DON genotype predominating. Furthermore, all DON isolates were shown to be the 15-ADON genotype and no 3-ADON genotype was identified. This was the first detailed study on the trichothecene genotypes of Mexican toxigenic *Fusarium* strains on large scale; wherein we report for the first time the occurrence of FGSC isolates belonging to NIV trichothecene genotype in Mexico.

Keywords: Fusarium head blight, *Fusarium cerealis*, Deoxynivalenol, Nivalenol, PCR.

Wheat, barley, rice and maize, the four most important crops globally along with other small grains crops, are threatened by a number of *Fusarium* species that cause Fusarium head blight (FHB) which is a devastating disease worldwide (McMullen *et al*., 1997; Pritsch *et al*., 2000; McMullen *et al*., 2012). In addition to direct yield losses, FHB infection leads to deterioration in grain quality (Bai and Shaner, 1994; Mardi *et al*., 2006) and contamination with mycotoxins such as nivalenol (NIV), deoxynivalenol (DON), and zearalenone (Schisler *et al*., 2002; Reischer *et al*., 2004; Buerstmayr *et al*., 2012).

Trichothecenes are toxic secondary metabolites, named after the compound 'trichothecin' from the fungus *Trichothecium roseum* (Desjardins *et al*., 1993; Bennett and Klich, 2003). *Fusarium graminearum* and *F. culmorum* isolates are type B trichothecene producers (Ichinoe *et al*., 1983; Miller *et al*., 1991), to which *F. cerealis* also belongs. Based on their type B trichothecene production, there are DON-producing isolates (which are usually further subdivided into 3-ADON and 15-ADON chemotypes) and NIV-producing isolates (Sugiura *et al*., 1994; Desjardins and Plattner, 2003). Both DON and NIV have a carbonyl function at the C-8 position (Brown *et al*., 2003), and are considered to be the principal trichothecenes produced by *F. graminearum*.

The *TRI* genes, like other secondary pathway genes of fungi, are often arranged in clusters within the genome, encoding for enzymes involved in the trichothecene biosynthesis of the fungus (Keller and Hohn, 1997; Pasquali and Migheli, 2014). Since the variation in trichothecene production reflects the allelic polymorphisms of *TRI* genes (Ward *et al*., 2002; Amarasinghe *et al*., 2011), PCR assays were developed in the last two decades to rapidly characterize populations of toxigenic Fusaria in terms of their chemotypes, greatly facilitating studies on the diversity and mycotoxin potential of FHB pathogens worldwide (Lee *et al*., 2001; Chandler *et al*., 2003; Gale *et al*., 2007; Starkey *et al*., 2007; Guo *et al*., 2008; Scoz *et al*., 2009).

Although many studies reported the chemotype-diversity both in North and South America, only few scattered attempts aimed at characterizing Mexican FHB related *Fusarium* isolates. The objectives of this study were to investigate the trichothecene genotype of the predominant cereal FHB causing pathogen in Mexico by PCR based markers targeting several *TRI* genes involved in trichothecene biosynthesis pathway, using recently collected isolates in 2013 and those from previous years to better understand the trichothecene-diversity of FHB related species in Mexico.

Corresponding author: P.K. Singh
Fax: +5258047558
E-mail: pk.singh@cgiar.org

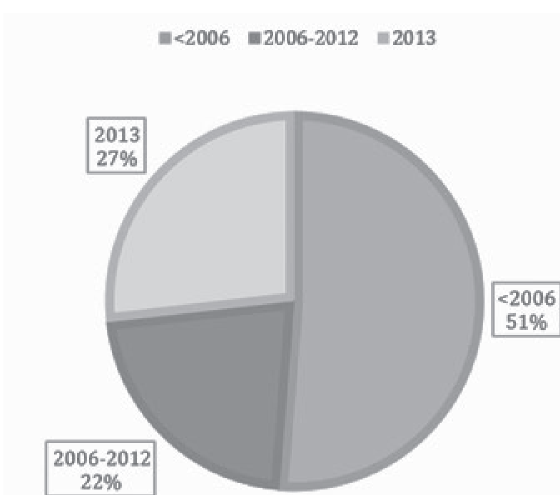

Fig. 1. Percentage of the used isolates based on sampling period: 2013, 2006-2012 and before 2006.

Table 1. The number of studied isolates from each location and their hosts.

	State	City	Host	No. isolates
	State of Mexico	Batan	Wheat	219
		Toluca	Wheat, Corn, Triticale	45
		Boximo	Wheat	5
		Juchitepec	Wheat	1
	Guanajuato	Guanajuato	Wheat	1
	Tlaxcala	Tlaxcala	Barley	2
	Jalisco	Jesús María	Wheat	1
		Tepatitlan	Wheat	24
	Puebla	Agua Fria	Wheat	2
	Michoacan	Patzcuaro	Wheat	48
	Oxaca	Oxaca	Wheat	40
Total	7	11		388

A total of 388 *Fusarium* isolates, belonging to 3 temporal groups, were used in this study (Fig. 1). The first set included a total of 104 isolates collected in 2013, the second set involved 85 isolates collected between 2006 and 2012 and the third one included 199 isolates that were collected in the period between 1995 and 2005. Isolates were collected annually from naturally *Fusarium* infected cereal crops (mostly wheat) about 4 weeks after flowering during August and September from 11 locations in six states of Mexico (Table 1, Fig. 2). Infected kernels were visually inspected to separate out rough, wilted, pink to soft-grey or light-brown in color (tombstone) for fungal isolation (McMullen *et al.*, 1997). Diseased kernels were surface-sterilized for 2 min by vortexing in a 5% sodium hypochlorite solution, rinsed in sterile distilled water for 1 min and dried in a laminar flow cabinet on sterile filter paper for an hour. Then, grains were incubated on Potato Dextrose Agar (PDA) at 25°C for 8 days. Fungal colonies grown from the damaged kernels were examined under microscope or stereoscope and isolates were tentatively identified based on colony characteristics and spore morphology (Nelson *et al.*, 1983; Leslie and Summerel, 2006). The selected isolates were subcultured on water agar and incubated for 18 to 24 h, and mono-spores were identified and transferred to synthetic nutrient agar (SNA).

All of the isolates were grown for 7 days in yeast extract-sucrose broth medium (2 g yeast extract, 6 g sucrose, 1000 ml distilled H_2O) on a rotating shaker at 120 rpm. The resulting mycelial suspension was filtered through Whatman® Grade No. 4 filter paper, freeze dried for 48 h at −80°C, and lyophilized for 48 h. Mycelium was pulverized by vigorous shaking of the blocks for 3 min in a ball mill using stainless steel beads. DNA was extracted and purified according to the CTAB method recommended by the European Community Reference Laboratories for the isolation of maize DNA (European Commission, 2007).

Subsequently the isolates were characterized by a set of PCR-based markers (Table 2). FGSC isolates were confirmed using the *F. graminearum* clade specific marker Fg16NF/R with a PCR product of 280 bp (Nicholson *et al.*, 1998). Similarly, the *F. cerealis* species specific marker CRO-A (Yoder and Christianson, 1998) was employed to confirm the morphology-based results. Then, DON and NIV producers were identified using the chemotype-specific marker ToxP1/P2 with their corresponding PCR products of 300 bp and 360 bp, respectively (Li *et al.*, 2005). The sub-chemotyping of the DON producers was carried out in two concurrent experiments: 1) the multiplex PCR assay using Tri11-CON, Tri11-3ADON, Tri11-15ADON and Tri11-NIV for *Tri11* gene. The *Tri11* based multiplex PCR primers generate a 334 bp fragment from 3-ADON-producing strains, a 279 bp from 15-ADON producers and a 497 bp fragment from NIV producers; 2) a generic PCR assay using a single pair of primers Tri13P1 and Tri13P2 designed from the *Tri13* genes, which was reported to be the determinant for the DON-NIV switching in *Fusarium* (Wang *et al.*, 2008) because it is not functional in DON-producing isolates due to the three deletion sites in this gene (Yörük and Albayrak, 2012). This primer set detects a 583 bp fragment from 15-ADON-genotypes, a 644 bp fragment from 3-ADON- genotypes and an 859 bp fragment from NIV-producing strains. In addition, two primer sets Tri303F/Tri303R (with a 586 bp product amplified from 3-ADON producing strains) and Tri315F/Tri315R (with an 864 bp product amplified from 15-ADON producing strains) were used to further confirm results obtained above, through characterizing the *Tri3* gene which codes for the enzyme C-15 acetylase (Jennings *et al.*, 2004).

All PCR amplifications were performed in a 10 µl reaction volume containing 4.5 µl pre-mixed ReadyMix (Sigma-Aldrich, St. Louis, MO), 250 nM of each primer (except for Tri11 primers where 300 nM of Tri11-CON was used but 100 nM of the rest), and 50 ng template DNA. PCR-grade water was used instead of DNA in one reaction as a negative control. PCR was performed on a Mastercycler® (Eppendorf) following the cycling conditions listed in Table 2.

Fig. 2. Sampling locations of *Fusarium* isolates used in this study.

The amplicons were separated by electrophoresis on a 2% agarose gel, stained with EnviroSafe® DNA/RNA stain and viewed under UV light.

A total of 102 out of 109 isolates collected in 2013 (Group 1) were identified as FGSC members using traditional identification techniques and confirmed by PCR analysis. Two isolates were classified as *F. cerealis* based on morphological characters, whereas the rest of isolates that had different morphology (n = 5) were excluded from further analysis. Of the 284 old isolates (groups 2 and 3), 249 were previously verified to belong to *F. graminearum* clade, and 35 were classified as *F. cerealis.* Afterwards, all sets of isolates were combined for trichothecene genotpe analysis.

Type B trichothecene genotypes were determined for a total of 388 monosporic isolates, of which 351 belonged to FGSC and 37 isolates belonged to *F. cerealis*. The primers ToxP1/2, which were designed from the intergenic sequences between *Tri5* and *Tri6* genes, amplified a 300 bp fragment specific for DON producers from 347 FGSC isolates, but a 360 bp fragment indicative of NIV genotype from 4 of FGSC isolates and all of the 37 *F. cerealis* isolates.

The primers Tri13 P1/P2 for the *Tri13* gene amplified a 583 bp fragment from all the 347 DON producing FGSC isolates, indicating that all of them were of the 15-ADON genotype. An 859 bp fragment for NIV producers was obtained from all the NIV isolates classified by the ToxP1/2 primer set. No 644 bp fragment for the 3-ADON genotype was detected in any of the studied isolates.

To confirm our results and to check the congruity of the different PCR assays, the DON genotypes were tested with the Tri11, Tri315, and Tri303 primer sets. Accordingly, a 279 bp fragment amplified by Tri11 and an 863 bp product by Tri315F/R (all specific to 15-ADON) were produced from all the previously recognized 15-ADON genotypes, whereas no amplicon was obtained with the Tri303 primer set for the 3-ADON genotype. Apparently, the isolates carried chemotype-specific DNA sequences determining their chemotype characters as they were identified by different multiplex PCR assays (Table2), i.e. none of the DON isolates based on Toxp primers showed to belong to NIV genotype in Tri11 or Tri13 based assays and vice versa.

The aim of this study was to identify the trichothecene genotype composition in Mexico for the predominant cereal FHB causing pathogens using PCR based assays. Both morphological and molecular techniques confirmed that FGSC is the major species causing FHB in Mexico which is consistent with a previous study in Mexico (He *et al.*, 2013) and with other studies in North America (Goswami and Kistler, 2004; Ward *et al.*, 2008). Although a few previous reports studied the genetic variation of Mexican FHB related *Fusarium* isolates using chemical analysis (Miller *et al.*, 1991) and molecular markers (Malihipour *et al.*, 2012; He *et al.*, 2013), this study provided the first detailed report on the frequency of type-B trichothecene genotypes of toxigenic FHB related *Fusarium* populations in Mexico. In the present study, more isolates were characterized than in the aforementioned studies, including 347 of FGSC and 37 of *F. cerealis*. Of the 347 FGSC isolates, 99% of the isolates were of the DON genotype based on the amplicon from the intergenic sequences between *Tri5* and *Tri6* genes, while 1% of the FGSC isolates were of the NIV genotype. All of the NIV isolates were recovered from wheat in different states and years. Further characterization of *Tri13*, *Tri11* and *Tri3* genes revealed that all of the DON isolates belonged to the 15-ADON genotype, while the 3-ADON was absent. As expected, all of the *F. cerealis* isolates showed PCR products specific to NIV genotype, as reported previously (Sugiura *et al.*, 1994; Xu and Nicholson, 2009; Gagkaeva, 2010).

According to our results, both NIV and DON genotypes were detected in isolates obtained from different locations and/or years in the states of Mexico, Puebla, Jalisco

Table 2. Primers, their nucleotide sequences and product sizes used in this study.

Primer	Primer sequence (5'–3')	Gene	Amplicon (bp)	Target	PCR program	References
Fg16NF	ACA GAT GAC AAG ATT CAG GCA CA	–	280	*F. graminearum*	95°:2'+(95°:30",63:45",72°:45")$_{\times 35}$ +72:10'	(Nicholson *et al.*, 1998)
Fg16R	TTC TTT GAC ATC TGT TCA ACC CA					
Tri303F	GATGGCCGCAAGTGGA	*TRI 3*	583	3-ADON	94°:2'+(94°:30",58:45",72°:45")$_{\times 35}$ +72:10'	(Jennings *et al.*, 2004)
Tri303R	GCCGGACTGCCCTATTG					
Tri315F	CTCGCTGAAGTTGGACGTAA	*TRI 3*		15-ADON	94°:2'+(94°:30",58:45",72°:45")$_{\times 35}$ +72:10'	(Jennings *et al.*, 2004)
Tri315R	GTCTATGCTCTCAACGGACAAC		863			
ToxP1	GCCGTGGGGRTAAAAGTCAAA	*Tri5-Tri6 intergenic*	360	NIV	95°:5'+(94°:60",55:60",72°:50")$_{\times 30}$ +72:6'	(Li *et al.*, 2005)
ToxP2	TGACAAGTCCGGTCGCACTAGCA		300	DON		
Tri13P1	CTCSACCGCATCGAAGASTCTC	*TRI 13*	859	NIV	94°:4'+(94°:60",58:40",72°:40")$_{\times 35}$ +72:6'	(Wang *et al.*, 2008)
Tri13P2	GAASGTCGCARGACCTTGTTTC		644	3-ADON		
			583	15-ADON		
Tri11-CON	GACTGCTCATGGAGACGCTG	*TRI 11*			94°:4'+(94°:30",58:30",72°:30")$_{\times 25}$ +72:5'	(Wang *et al.*, 2012)
Tri11-3ADON	TCCTCATGCTCG GTGGACTCG		334	3-ADON		
Tri11-15ADON	TGGTCCAGT TGTCCGTATT		279	15-ADON		
Tri11-NIV	GTAGGTTCCATTGC TTGTTC		497	NIV		

and Michoacan, whereas only DON genotype was found in the other locations. Generally, differences in chemotypes distribution could be attributed to hosts distribution, soil type, cultivars, fungicides application and cultural practices in addition to putative climate effects (Jennings *et al.*, 2004; Pasquali and Migheli, 2014), but in our study a clear reason could not be identified. It is noteworthy that although they share very similar chemical structures, DON is more toxic to plants, whereas NIV is more toxic towards animals and human (Ryu *et al.*, 1988). Furthermore NIV producing isolates were found to be less sensitive to tebuconazole than 15-ADON isolates (Umpierrez-Failache *et al.*, 2013), thus high incidence of the NIV genotype should be of considerable concern. Accordingly, the occurrence of the NIV genotypes of FGSC and *F. cerealis* in the states of Mexico, Puebla, Jalisco and Michoacan emphasizes the need for more intensive sampling in different locations across these states and the surrounding regions. Moreover, it is also important to regularly inspect toxin content in cereal products obtained from these states to ensure their suitability for human and animal consumption.

Shifts in trichothecene genotypes within *F. graminearum* have been observed in different reports. For example, Ward *et al.* (2008) detected a shift from 15- to 3-ADON producing isolates in North America, which have the potential to produce higher quantity of this mycotoxin and are reported to be more aggressive. In the Netherlands, a slight increase in NIV frequency, which is more toxic to humans and animals, was detected by Waalwijk *et al.* (2003). In order to investigate any shift in chemotypes of the FHB related species, we tested newly collected 2013 isolates as well as the old collection of *F. graminearum* and *F. cerealis* available in the CIMMYT's wheat pathology lab. Due to the fact that a NIV genotype PCR assay based on only one gene may not be reliable in every case (Chandler *et al.*, 2003; Desjardins *et al.*, 2008) and the failure of Tri13 P1/P2 in discriminating between 15- and 3-ADON genotypes (Pasquali et al., 2011), multiple PCR assays based on different Tri genes were employed to achieve the objective of this study. Although the incidence of *F. cerealis* species, which is notably a NIV producer, was reported previously, this study provides the first report on the occurrence of NIV genotype of FGSC in Mexico, which was not reported previously probably because of the small number of sampled isolates. However, due to the fact that both the *Tri13* and *Tri7* genes, which are required for conversion of DON to NIV and acetylation of NIV, respectively, are nonfunctional in DON producers, it is unlikely that the NIV isolates resulted from DON populations by mutation, and the hypothesis of being introduced into Mexico maybe more acceptable. Hence no substantial change in trichothecene genotypes of Mexican populations of FGSC could be suggested so far, and the 15-ADON always predominated across years and locations.

F. graminearum species complex (FGSC) comprises at least 15 phylogenetically distinct species (Zhang *et al.*, 2012), of which only one (lineage 3 or *F. boothii*) is reported in Mexico (Malihipour *et al.*, 2012; Backhouse, 2014). However the identification of FGSC isolates belonging to the NIV genotype may increase the chance to find a member of the FGSC in Mexico other than *F. boothii*. Thus identification of the fine species or lineages of the *F. graminearum* species complex is needed to obtain more comprehensive results about diversity and stability of the distribution of trichothecene genotypes in Mexico and to detect possible future shifts in chemotypes. In order to achieve this goal, samples from different hosts, new locations especially in north of Mexico and/or different seasons will be helpful.

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