The Journal of Animal & Plant Sciences, 24(6): 2014, Page: 1858-1862
ISSN: 1018-7081

CHEMOTYPING OF THE *FUSARIUM GRAMINEARUM* ISOLATES AND VARIATION IN AGGRESSIVENESS AGAINST WHEAT HEADS

F. Mert-Türk[1*], R. Gencer[1], F. and Kahriman[2]

[1]Plant Protection Dept., [2]Crop Science Dept. Agricultural Faculty, Çanakkale Onsekiz Mart University, 17100, Çanakkale, Turkey
[*]Corresponding author: figen.turk@hotmail.com

ABSTRACT

Fusarium head blight (FHB), caused mainly by *Fusarium graminearum*, is a devastating disease of wheat and other small grain cereals. FHB lowers grain yield and quality, and contaminates grain with mycotoxins, predominantly trichotecenes i.e nivalenol (NIV), deoxynivalenol (DON). A survey conducted at three Provinces in Turkey for FHB and 17 isolates were identified as *F. graminearum* using morphological and molecular markers. A PCR assay was carried out to identify the chemotypes of the isolates. Using *Tri13* gene cluster, all 17 isolates that were identified to 15-AcDON type of DON chemotype. None of the isolates displayed 3-ADON or NIV chemotypes. In order to assess variation in aggressiveness among isolates, all isolates were inoculated to a susceptible wheat spikes at field conditions and disease severity and a thousand kernel weight were measured. Aggressiveness (measured as FHB severity or TKW) differed significantly among 17 *F. graminearum* isolates inoculated onto wheat spikes of FHB susceptible cultivar Gönen (P=0,05). Means of FHB severity ranged from 39.75 to 86.33%, averaging 63.29% in total. Reduction in TKW was also reduced significantly by different isolates. Differences in aggressiveness among isolates may due to genetic recombination, mutation or selection in the surveyed area.

Key words: *Fusarium graminearum*, mycotoxins, chemotypes, DON, NIV, pathogenicity, aggressiveness.

INTRODUCTION

The fungal pathogen *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch], is the most common causal agent of Fusarium head blight (FHB) in many parts of the world. This destructive disease, so-called scab, affects wheat, barley and other small grains both in temperate and in semitropical areas and has the capacity to destroy crop within a few weeks of harvest (McMullen *et al*., 1997). Primary inoculum comes from infected plant debris on which the fungus overwinters as saprophytic mycelia. In spring, warm moist weather conditions are favourable for the development and maturation of conidia and perithecia that produce ascospores concurrently with the flowering of cereal crops (Markell and Francl, 2003). The principal mode of fungal spread in wheat from floret to floret inside a spikelet and from spikelet to spikelet is through the vascular bundles in the rachis and rachilla (Ribichich *et al*., 2000).

Infection of wheat heads by *F. graminearum* reduces grain yield by degrading starch granules in the kernels (Jackowiak *et al*., 2005). It also reduces the quality of the grain by contaminating it with harmful mycotoxins such as the trichothecenes, rendering it unsafe for human and livestock consumption. Isolates of *Fusarium graminearum* can be categorised depending on the type B trichothecene they produce. Some isolates produce deoxynivalenol (DON chemotypes) while others produce nivalenol (NIV chemotypes). DON chemotypes can be further divided depending on where DON is acetylated, with 3-acetyl DON (3-AcDON) producers and 15-acetyl DON (15-AcDON) (Miller *et al*., 1991; Jennings *et al*., 2004).

Several studies reported variation in aggressiveness among *F. graminearum* isolates sampled from various parts of the world within a country and even within populations from individual fields (Bai and Shaner, 1996; Miedaner *et al*., 1996; Miedaner *et al*., 2010). Aggressiveness of *F. graminearum* is not geographically structured since isolates with low, medium, and high levels of aggressiveness make up the population in a single location. Most studies revealed a high level of genetic diversity in *F. graminearum* within individual field populations or populations sampled across a definite geographical scale. Knowledge of isolate characteristics in terms of aggressiveness in any location is necessary for predicting the pathogenic potential of FHB pathogens and for the deployment of resistance in a given location.

Lee *at al*. (2002) and Brown et al (2002) reported that *Tri13* from the Fusarium trichothecene biosynthetic cluster is responsible from converting DON to NIV. Differences in the functional and unfunctional *Tri13* genes can be ascertained by PCR assays. The objective of this research is to characterize the chemotypes of *F. graminearum* collected from the blighted ears in North-West of Turkey and to compare aggressiveness of the isolates at field conditions.

MATERIALS AND METHODS

Collection of the *Fusarium graminearum* isolates: Wheat crops were surveyed FHB from May until the beginning of June in the 2009-2010 wheat growing season. Three provinces, Çanakkale, Tekirdağ and Balıkesir, were surveyed covering 4500 ha area. The geographic origins of the isolates collected are given in Table 1.

Infected grain samples were surface sterilized with 1% sodium hypochlorite solution and rinsed three times with sterile distilled water. After drying in a fume hood to eliminate excess moisture, they were placed in potato dextrose agar (PDA) medium containing 100 µg/mL streptomycin sulfate and incubated at 25^0C for 5-7 days. Monoconidial isolates were obtained by streaking spore suspensions onto water agar plates and single conidia were transferred onto new plates.

Identification of the *Fusarium graminearum* isolates: The isolates were examined after 10 days and selected based on pigmentation on PDA. The selected isolates were grown on carnation leaf agar (CLA) and identified as *F. graminearum* by conidial morphology and colony characteristic as described by Leslie and Summerell, 2006.

Fungal DNA Extraction and PCR: The DNA extraction technique was modified from Saitoh *et al* (2006) adding some further cleaning steps. A mycelial mass was picked with a pipette tip and put in a 2 ml Eppendorf tube. The mycelial mass was then homogenised with a micro pestle for a few seconds. One ml of lysis buffer (200 mM Tris-HCl, 50 mM ethylenediaminetetra acetic acid, 200 mM NaCl, 1% *n*-lauroylsarcosine sodium salt, pH 8.0) was added immediately to the mycelia. The mycelial mass was dispersed in the buffer by vortexing for 10 seconds. Two µl of RNAse A (Fermentas, Lithuania) and 4 µl of proteinase K (Fermentas, Lithuania) was added and incubated for about 15 min at 37^0C. The mixtures were centrifuged at 13000 rpm for 5 min. The supernatant was removed to a clean tube containing 1 ml of chloroform. The tubes were inverted briefly before centrifugation for a further 10 min. The supernatant (about 800 µl) was removed into a new tube containing 80 µl sodium acetate (5 M) and 550 µl ice cold isopropanol, then inverted gently. The mixure was centrifuged at 13000 rpm for 10 min following incubation on ice for several minutes. The pellet was washed twice with 70% ice cold ethanol. After drying at room temperature for 30 min, the DNA was dissolved in 100 µl of TE buffer.

PCR amplification was carried out in a 50 µl of reaction mix containing 25 µl of 2X PCR master mix (0.05 units/µl TaqDNA polymerase in reaction buffer, 0.4 mM of each dNTPs, Fermentas, Lithuania), 8 µl (25-50 ng) fungal DNA, 2.5 µl (300 nM) each of primer and 12 µl nuclease free water. A thermal cycler (Bio-RAD, USA) was used for amplification of the specific fragment of DNA.

PCR conditions used for *F. graminearum* detection were: 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 40 s followed by a final extension of 72°C for 5 min. The same conditions were applied for Tri13DON and Tri13NIV with an exception that denaturation temperature was 94°C. The PCR conditions for the generic primer, Tri13P was: 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 60°C for 40 s, 72°C for 40 s, then a final extension of 72°C for 6 min. PCR products were separated by electrophoresis on 2 % agarose gels, stained with ethidium bromide and photographed under UV light in a Bio-Imaging system (Bio-RAD, USA). All assays were repeated at least twice. The primers used to identify *F. graminearum* and its chemotypes are listed in Table 2.

Inoculation of Wheat Head and Disease Assessment: In order to assess variation in aggressiveness among the isolates, a field trial was conducted. The experimental design was a completely randomized block design, comprising four replications for each isolate. The size of each plot was 1 m X 5 m and the spacing between rows was 12.5 cm.

Pathogenicity testing was performed using Gönen wheat variety, one of the most common varieties in the Region. Twenty five-30 individual heads per plot were inoculated with the fungus at mid-anthesis stage. Approximately 10 µl of spore suspension containing $5X10^4$ macroconidia was injected in both sides of the florets at the middle of heads with a hypodermic needle of syringe. Plastic bags were covered over inoculated heads for 2 days for high level of humidity. The percentage of blighted kernels was recorded 14 days after inoculation. The mean percentage of infected kernels per infected head and the percentage of infected heads per plot were calculated for parameters of disease severity. Severity of FHB=blighted kernels% X infected heads% /100 (Snijders and Perkowski, 1990). Relative a thousand kernel weights (TKW) were calculated by dividing a TKW from blighted plot to TKW from respective control plots (Miedaner *et al*., 2003).

RESULTS

Collection and Identification: A total of 17 isolates were described as *F. graminearum* using morphological identification techniques. All isolates were confirmed as *F. graminearum* by species-specific PCR analysis with Fg16 and Fg16N primers developed by Nicholson *et al*. (2004). All samples produced a common band of 280 bp with Fg16N and 450 bp with Fg16 (Figure 1). These data and the morphological examination confirmed that all

studied isolates were belonged to *F. graminearum* clade (Table 2).

Determination of Chemotypes: Chemotyping of collected isolates was done by PCR assays using a set of specific primer of *Tri13* gene sequence including: *Tri13*NIV, *Tri13*DON and *Tri13*P (Table 1). The results obtained from PCR reaction with *Tri13*NIV showed that none of the isolates were NIV chemotypes; however all the isolates gave specific 282 bp fragment with *Tri13*DON assay indicating all population was DON-producer (Figure 2).

The generic PCR detection of 3-AcDON- and 15-AcDON- chemotypes based on Tri13P assay detected only two of acetylated DON chemotypes in a single reaction. All isolates produced a fragment of approximately 583 bp indicating 15-AcDON-chemotypes at the population. The results indicated that although NIV-chemotypes is absent in the surveyed area, only 15-AcDON-chemotype is present among two acetylated DON-producers.

Differences in Aggressiveness: FHB was assessed at 14 days after inoculation. All *F. graminearum* isolates caused visible head blight symptoms in field experiments. No symptoms of disease occurred in the control inoculations. Seventeen isolates of *F. graminearum* tested showed broad genetic variation for aggressiveness, as measured head blight ratings (%). Means of FHB severity ranged from 39.75 % to 86.33 % averaging 63.29 % (Table 3). The results show that although there are some isolates associated with the low level of disease symptoms 14 days after inoculation (T608), some have a high level of aggressiveness such as B114.

TKW was also assessed after harvest and values obtained from each isolate differed greatly (Table 3). Relative TKW from inoculated heads ranged from 0.22 to 0.83 proving high level of variation in yield. The data showed that infection by T608 influenced TKW the least among the isolates, whereas C402, B018 and T118 reduced TKW dramatically.

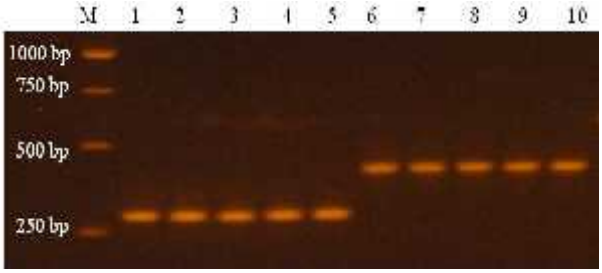

Figure 1. PCR-based identification of *Fusarium graminearum* using Fg16 and Fg16N primer sets. M indicates 1 kb ladder size marker (Fermentas, Lithuania). Lane 1-5 and 6-10 is PCR results of *F. graminearum* using Fg16N and Fg16, respectively. Positive controls were provided kindly by P Nicholson, Norwich, UK.

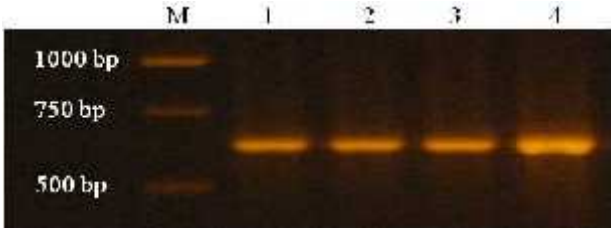

Figure 2. Amplification products using the primer set *Tri13*P. M indicates 1 kb ladder size marker (Fermentas, Lithuania). Lane 1-4 is the PCR results of 4 *F. graminearum* chemotype, 15-AcDON, yielding 583 bp fragments.

Table 1. Code, origin of isolates, amplification through PCR using *F. graminearum* specific primers (Fg16 and Fg16N), and *Tri13*DON, *Tri13*NIV and *Tri13*P assays for NIV and DON and acetylated forms of the isolates.

						Tri13P1		
Code	**Province**	Fg16	Fg16 N	Tri13NIV	Tri13DON	3-AcDON	15-AcDON	NIV
C-601	Çanakkale	+	+	-	+	-	+	-
C-402	Çanakkale	+	+	-	+	-	+	-
C-302a	Çanakkale	+	+	-	+	-	+	-
C-601a	Çanakkale	+	+	-	+	-	+	-
C-606b	Çanakkale	+	+	-	+	-	+	-
B-006	Balıkesir	+	+	-	+	-	+	-
B-018	Balıkesir	+	+	-	+	-	+	-
B-114	Balıkesir	+	+	-	+	-	+	-
B-440	Balıkesir	+	+	-	+	-	+	-
T-604	Tekirdağ	+	+	-	+	-	+	-
T-709	Tekirdağ	+	+	-	+	-	+	-
T-113	Tekirdağ	+	+	-	+	-	+	-
T-507	Tekirdağ	+	+	-	+	-	+	-
T-608	Tekirdağ	+	+	-	+	-	+	-
T-919	Tekirdağ	+	+	-	+	-	+	-
T-118	Tekirdağ	+	+	-	+	-	+	-
T-119	Tekirdağ	+	+	-	+	-	+	-

Table 2. Oligonucleotide primer sequences and sizes of the PCR products.

Primer name	Specificity	Fragment size (bp)
Fg16[1]	*F. graminearum*	450
Fg16N[1]	*F. graminearum*	280
Tri13DON[1]	DON chemotypes	282
Tri13NIV[1]	NIV chemotypes	312
Tri13P[2]	Generic (15-AcDON, 3-AcDON and NIV)	583, 644, 859, respectively

[1] Nicholson *et al*. 2004; Wang *et al*. 2008

Table 3. Mean and significance of isolate variation for percent infected kernels and a thousand seed weight in the Gönen wheat genotype.

Isolates	FHB (%)	TKW
C601	48.06 fg	0.65 de
C402	76.85 bc	0.22 j
C302a	58.84 d	0.62 ef
C601a	46.41 fg	0.78 b
C606b	71.59 c	0.45 h
B006	49.60 ef	0.60 f
B018	77.84 bac	0.23 j
B114	86.33 a	0.44 h
B440	43.18 fg	0.71 c
T604	71.43 c	0.50 g
T709	83.92 ba	0.30 i
T113	41.27 fg	0.67 cd
T507	82.31 ba	0.44 h
T608	39.75 g	0.83 a
T919	60.26 d	0.61 ef
T118	81.42 ba	0.25 j
T119	56.93 de	0.61 ef
Average	63,29	0,52
Tukey MSD	86.767	0.0493
Rep	769.689	0.00046329
Isolates	1098.61347***	0.14687040***
Error	1.130.447	0.00036550

Different letters indicate significant at $P = 0.05$.
*** Significant at $p<0.001$

DISCUSSION

Wheat fields in three provinces in North-West of Turkey were surveyed for FHB symptoms and *F. graminearum* isolates were selected from other Fusarium species collected. *F. graminearum* was first diagnosed morphologically and then by PCR using two sets of species-specific primers. The ability of the isolates to produce trichothecenes was evaluated using chemotype-specific PCR markers (Table 1). Only one chemotype, DON was identified and all displayed the 15-AcDON chemotype (Table 1, Figure 2).

It has been suggested that knowledge of *Fusarium* chemotypes and their distribution could be crucial in forecasting schemes for disease development and mycotoxin contamination within a wheat growing area (Jennings *et al*., 2004). Using a molecular approach in current work, the trichothecene chemotype distribution analysis among the wheat cultivation of these three provinces revealed only one dominant chemotype (DON), furthermore 15-AcDON type was the only sub-chemotype throughout the sampling area.

Carter *et al*. (2002) reported that DON chemotype was dominant comparing NIV in Europe and North America. Previous observations from Luxembourg showed the presence of two populations (Pasquali *et al*., 2009). Strains of *F.graminearum* complex from maize of northwest Argentina were DON and NIV producers (Sampietro *et al*. 2012). Employing 11 *F. graminearum* isolates, the previous report from Turkey by Yörük and Albayrak (2012) showed that although there was a single NIV-producing isolate in the population, the DON-producers were dominant.

In different USA areas, Gale *et al*. (2007) identified 15-ADON as the prevailing chemotype of *F. graminearum sensu stricto*, followed by 3-ADON (5.1% of total), and one to NIV chemotype. In southern Russia 90% of the isolates was 15-ADON (Yli-Mattila *et al*., 2008), Jennings *et al*. (2004) found DON (75%) and NIV (25%) with the predominant 15-ADON chemotype (95%) in England and Wales, the 15-ADON chemotype was the major population (94.3%), the 3-ADON chemotype was not detected and the NIV chemotype was detected sporadically (5.8%) in Luxembourg (Pasquali *et al.*,2010). Yörük and Albayrak (2012) from Turkey reported all DON chemotypes were 15-AcDON-producers.

We previously reported NIV, 3-AcDON and 15-AcDON chemotypes of *F. culmorum* in the same surveyed area (Mert-Türk and Gencer, 2013). However employing 17 *F. graminearaum* isolates, all were described as 15-AcDON-producers in the current study.

In order to analyse the differences in aggressiveness among the isolates, we conducted a field trial. We assessed disease progress as well as a thousand kernel weight. FHB severity was rated visually as the percentage of infected spikelets per plot (0 to 100%). Mean FHB ratings and TKW in wheat showed a wide and significant (P = 0.05) range of variation within population (Table 3). Some isolates produced on average 86% disease severity on the wheat cultivar tested, whereas others were much less virulent on average 22% (Table 3). TKW was also affected variously when inoculated by different isolates.

Differences in aggressiveness among isolates may due to genetic recombination, mutation, or selection.

In *F. graminearum*, there is large genetic variation within a given population, even in samples collected from a small area within a field (McMullen *et al*., 1997). Miedaner *et al*. (210) analyzed the aggressiveness on young winter rye plants of populations of *F. graminearum* and *F. culmorum* collected from natural field epidemics of FHB and rye foot rot. They found high genotypic variance in the two species and a high degree of diversity of aggressiveness within single field populations of either species.

This study demonstrated that *F. graminearum* isolates differ in aggressiveness in *planta* and that the same chemotype- producers could show different degree of virulence in field conditions. The existence of variability in aggressiveness among isolates suggests that wheat breeders should use mixtures of isolates to screen for FHB resistance in wheat lines. In addition, the information can be used by small grain producers to make decisions pertaining to deployment of FHB management strategies.

Acknowledgements: This research was partly supported by TUBITAK, Turkey (Project code: TOVAG-109O830).

REFERENCES

Bai, G.H., and G. Shaner (1996). Variation in *Fusarium graminearum* and cultivar resistance to wheat scab. Plant Dis. 80(9): 975-979.

Carter, J., H. Rezanoor, D. Holden, A.E. Desjardins, R.D. Plattner, and P. Nicholson (2002). Variation in pathogenicity associated with the genetic diversity of *Fusarium graminearum.* Eur. J. Plant Pathol. 108: 573-583.

Gale, L.R., T.J. Ward, V. Balmas, and H.C. Kistler (2007). Population subdivision of *Fusarium graminearum sensu stricto* in the upper Midwestern United States. Phytopathology. 97: 1434-1439.

Jackowiak, H., D. Packa, M. Wiwart, and J. Perkowski (2005). Scanning electron microscopy of Fusarium damaged kernels of spring wheat. International J. Food Microbiol. 98: 113-123.

Jennings, P., M.E. Coates, J.A. Turner, E.A. Chandler, and P.Nicholson (2004). Determination of deoxynivalenol and nivalenol chemotypes of *Fusarium culmorum* isolates from England and Wales by PCR assay. Plant Pathol. 53: 182–190.

Markell, S.G. and L.J. Francl (2003). Fusarium head blight inoculum: species prevalence and *Gibberella zeae* spore type. Plant Dis. 87: 814–820.

McMullen, M., R. Jones, and D. Gallenberg (1997). Scab of wheat and barley: a re-emerging disease of devastating impact. Plant Dis. 81: 1340–1348.

Mert-Türk, F., and R. Gencer (2013). Distribution of the 3-AcDON, 15-AcDON and NIV Chemotypes of *Fusarium culmorum* in the North-West of Turkey. Plant Protect. Sci. 49(1): 57–64.

Miedaner, T., G. Gang, and H.Geiger (1996). Quantitative-genetic basis of aggressiveness of 42 isolates of *Fusarium culmorum* for winter rye head blight. Plant Dis. 80(5): 500-504.

Miedaner, T., A. Schilling, and H. Geiger (2001). Molecular genetic diversity and variation for aggressiveness in populations of *Fusarium graminearum* and *Fusarium culmorum* sampled from wheat fields in different countries. J. Phytopathol. 149(11): 641-648.

Miedaner, T., M. Moldovan, and M. Ittu (2003). Comparison of Spray and Point Inoculation To Assess Resistance to Fusarium Head Blight in a Multienvironment Wheat Trial. Phytopathology. 93(9): 1068-1072.

Miedaner, T., C. Bolduan, and A.Melchinger (2010). Aggressiveness and mycotoxin production of eight isolates each of *Fusarium graminearum* and *Fusarium verticillioides* for ear rot on susceptible and resistant early maize inbred lines. Eur. J. Plant Pathol. 127(1): 113-123.

Miller, J.D., R. Greenhalgh, Y.Z. Wang, and, M. Lu (1991). Trichothecene chemotypes of three *Fusarium* species. Mycologia. 83: 121-130.

Pasquali, M., F. Giraud, C. Brochot, E. Cocco, L. Hoffmann, and T. Bohn (2010). Genetic *Fusarium* chemotyping as a useful tool for predicting nivalenol contamination in winter wheat. Int. J. Food Microbiol. 137: 246-253.

Ribichich, K.F., S.E. Lopez, and A.C. Vegetti (2000). Histopathological spikelet changes produced by *Fusarium graminearum* in susceptible and resistant wheat cultivars. Plant Dis. 84: 794–802.

Sampietro, D.A., M.E. Ficoseco, C.M. Jimenez, M.A. Vattuone, and C.A. Catalán (2012). Trichothecene genotypes and chemotypes in *Fusarium graminearum* complex strains isolated from maize fields of northwest Argentina. Int. J. Food Microbiol. 153(1-2): 229-33

Snijders, C.H.A. and J. Perkowski (1990). Effects of Head Blight Caused by *Fusarium culmorum* on Toxin Content and Weight of Wheat Kernels. Phytopathology. 80: 566-570.

Yli-Mattila, T., K., O'Donnell, T. Ward, and T. Gagkaeva (2008). Trichotecene chemotype composition of *Fusarium graminearum* and related species in Finland and Russia. J. Plant Pathol. 90(3): 60-64.

Yörük, E. and G. Albayrak (2012). Chemotyping of *Fusarium graminearum* and *F. culmorum* isolates from Turkey by PCR Assay. Mycopathologia. 173(1): 53-61.