International Journal of Food Microbiology 140 (2010) 84–89

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Trichothecene chemotypes of *Fusarium culmorum* infecting wheat in Tunisia

Lobna Gargouri Kammoun [a,*], Samia Gargouri [a], Christian Barreau [b], Florence Richard-Forget [b], Mohamed Rabeh Hajlaoui [a]

[a] Laboratoire de Protection des végétaux, Institut National de la Recherche Agronomique de Tunis, rue Hédi Karray, 2049, Tunisia
[b] Unité de Mycologie et Sécurité des Aliments, Institut National de la Recherche Agronomique, 71 avenue Edouard Bourleaux, France

ARTICLE INFO

Article history:
Received 25 August 2009
Received in revised form 6 January 2010
Accepted 28 January 2010

Keywords:
Wheat
Mycotoxins
Fusarium culmorum
Deoxynivalenol
Nivalenol
Aggressiveness

ABSTRACT

Fusarium culmorum is a major pathogen associated with Fusarium head blight (FHB) of wheat in Tunisia. It may cause yield loss or produce mycotoxins in the grain. The objectives of the present study were threefold: to evaluate by PCR assays the type of mycotoxins produced by 100 *F. culmorum* isolates recovered from different regions in Northern Tunisia, to determine the amount of mycotoxin production by HPLC analysis, and to analyse for correlations between the amount of mycotoxin produced and the aggressiveness of isolates.

PCR assays of *Tri5*, *Tri7*, *Tri13*, and *Tri3* were used to predict whether these isolates could produce nivalenol, 3-acetyl-deoxynivalenol, or 15-acetyl-deoxynivalenol. Two of the isolates were predicted to produce NIV, whereas the others were predicted to produce 3-AcDON. Trichothecene production was confirmed and quantified by high pressure liquid chromatography (HPLC) in 28 isolates, after growth on wheat grains, and in a liquid Mycotoxin Synthetic medium (MS). All strains produced DON/3-AcDON at detectable levels ranging from 21 µg/g to 11.000 µg/g of dry biomass on MS medium and from 10 µg/g to 610 µg/g on wheat grain. The evaluation of the relationship between 3-AcDON production and aggressiveness of 17 strains revealed a significant difference in aggressiveness among the isolates. Moreover, only a significant correlation was revealed between aggressiveness and the amount of 3-AcDON produced on MS medium ($r = 0.36$). Chemotyping of *F. culmorum* isolates is reported for the first time for isolates from Tunisia, and highlights the important potential of *F. culmorum* to contaminate wheat with 3-AcDON trichothecenes.

1. Introduction

Fusarium head blight (FHB), is a major fungal disease affecting wheat (*Triticum aestivum* L.) worldwide. The disease is associated with several *Fusarium* spp. including *F. graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch), *Fusarium culmorum* (W.G. Smith) Sacc., *F. poae* (Peck) Wollenw., *F. avenaceum* (Fr.) Sacc., and *Microdochium nivale* (Fr.) Samuels & I.C. Hallett (Osborne and Stein, 2007). Globally, *F. graminearum* is the prevalent species, but in several European countries, *F. culmorum* is the main causative agent of FHB (Jennings et al., 2004; Bottalico and Perrone, 2002).

FHB often is accompanied by contamination of the substrate with trichothecenes, a toxin family of considerable concern for human and animal health (Bennett and Klich, 2003). Type B trichothecenes are the principal mycotoxins produced in cereals by *F. culmorum* as well as other species of *Fusarium* (Desjardins, 2006). They include deoxynivalenol (DON) and its acetylated forms 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol (3- and 15-AcDON), and nivalenol (NIV) and its acetylated form 4-acetylnivalenol or fusarenone X (FX). NIV is generally regarded as more toxic to humans and animals than is DON (Ryu et al., 1988), although DON may be more phytotoxic than NIV (Eudes et al., 2000). The production of DON by *F. culmorum* may play a role in pathogenesis (Eudes et al., 2001).

The usual methods for chemotyping *Fusarium* isolates are high performance liquid chromatography (HPLC) or gas chromatography/mass spectroscopy (GS/MS) analysis of extracts from substrates such as wheat, maize or rice artificially inoculated with *Fusarium* (Sugiura et al., 1990; Miller et al., 1991; Muthomi et al., 2000). DNA based methods that rely on the amplification of the genes involved in the biosynthesis of trichothecenes also are available. Specific PCR primers have been developed to the *Tri5* gene which encodes trichodiene synthase, an enzyme that catalyses the first step in the biosynthesis of trichothecenes. Two others genes, *Tri7* and *Tri13*, also have been sequenced and found to be functional in NIV-producing isolates and nonfunctional in DON-producing isolates (Lee et al., 2001, 2002; Chandler et al., 2003). Positive–negative PCR assays based on these two genes have been developed to characterize the DON and NIV genotypes. Primers developed to the *Tri3* gene have enabled the acetyl derivatives of DON (3- or 15-AcDON) to be determined (Chandler et al., 2003; Gale et al., 2007; Jennings et al., 2004; Quarta et al., 2005; Stepiën et al., 2008).

The distribution of each chemotype/genotype varies by geographic region. Thus, strains of *F. culmorum* with DON and NIV chemotype/

* Corresponding author.
E-mail address: lobna_kammoun@yahoo.fr (L.G. Kammoun).

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doi:10.1016/j.ijfoodmicro.2010.01.040

genotype are known from several countries, including UK (Jennings et al., 2004), Germany (Muthomi et al., 2000), the Netherlands and Norway (Langseth et al., 1999), Italy (Gang et al., 1998), France (Bakan et al., 2001) and USA (Mirocha et al., 1994), whereas only the DON chemotype was detected in western Canada (Abramson et al., 2001).

Recent surveys conducted in Tunisia have shown that harvested grains are contaminated with DON and that *F. culmorum* was the dominant *Fusarium* species present (Kammoun et al., 2009; Bensassi et al., 2009). To our knowledge, there are no previous reports on the mycotoxins produced by Tunisian *F. culmorum* isolates. Since wheat represents the major staple food for the people of Tunisia, it is important to assess the mycotoxin production capability of *Fusarium* isolates and to determine the types and amounts of mycotoxins produced to evaluate the risk that might be posed by contaminated food or feed.

Our objectives in this study were to determine the trichothecene genotypes of Tunisian isolates of *F. culmorum*, through PCR analysis of the *Tri5*, *Tri7*, *Tri13* and *Tri3* genes, to quantify mycotoxin production by HPLC analysis, and to determine if there is a correlation between the amount of mycotoxin produced and the aggressiveness of an isolate.

2. Materials and methods

2.1. Isolates

One hundred single-spore isolates of *F. culmorum*, described in detail by Kammoun et al. (2009), were used. These isolates originated from different regions of Northern Tunisia (Table 1). All *Fusarium* isolates were identified based on conidial morphology according to Leslie and Summerell (2006) and then confirmed by polymerase chain reaction (PCR) with specific primers described by Schilling et al. (1996).

2.2. DNA extraction

Isolates of *F. culmorum* were grown on potato dextrose agar medium (PDA) (Sigma Chemical Co., St. Louis, MO) plates for 5–6 days. The mycelia were freeze-dried and ground to a fine powder. DNA was extracted by using a method adapted from Möller et al. (1992).

2.3. Fusarium culmorum species-specific PCR

DNA of 100 isolates identified as *F. culmorum* was amplified with PCR using species-specific primers OPT18F/OPT18R (Schilling et al., 1996). The amplification conditions were: approximately 20 ng of fungal DNA, 1 mM of each dNTP, 1.5 mM $MgCl_2$, 1 unit of GoTaq® DNA polymerase (Promega, USA), 1X PCR polymerase reaction buffer, and 0.25 μM of each forward and reverse primer. DNA amplification was performed in a Thermal Cycler (Biometra, T-1, Göttingen, Germany) as described by Schilling et al. (1996). Amplification products were separated by electrophoresis in 1.5% agarose gels in TBE buffer (0.9 M Tris, 0.9 M boric acid, 2 mM EDTA, pH 8.0). Gels were stained with ethidium bromide (10 mg/μl) and photographed under UV light.

2.4. PCR analysis of trichothecenes genes

PCR assays with primer pair Tox5-1/Tox5-2 (Table 2) developed for the gene *Tri5* were used to determine the potential ability of *F. culmorum* isolates to produce trichothecenes (Niessen and Vogel, 1998). The PCR mixture was as described above and the PCR reactions were: 4 min at 96 °C; 5 cycles of 1 min at 96 °C, 2 min at 68 °C, and 3 min at 75 °C; 30 cycles of 30 s at 96 °C, 30 s at 68 °C, and 1 min at 75 °C followed by a final 10 min incubation at 72 °C.

Primers pairs Tri13NIVF/Tri13R and Tri7F/Tri7NIV were used to identify NIV-producing isolates, and primer pairs Tri13F/Tri13DONR and MinusTri7F/MinusTri7R were used to identify DON-producing isolates (Chandler et al., 2003; Table 2). The cycling protocol for Tri7F/Tri7NIV was 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C followed by a final extension of 5 min at 72 °C. The annealing temperature was 58 °C for the other three primers and the extension time was 45 s for Tri13NIVF/Tri13R and Tri13F/Tri13DONR and 30 s for MinusTri7F/MinusTri7R.

Two primer sets Tri3F971/Tri3R1679 and Tri3F1325/Tri3R1679, were used to distinguish the 15-AcDON and 3-AcDON genotypes (Table 2) (Quarta et al., 2005). The PCR program used was: 94 °C for 3 min (1 cycle only), then 35 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. Negative controls (no DNA template) were included in each set of experiments to test for the presence of DNA contamination in reagents and reaction mixtures. Amplification products were analysed as described above.

2.5. HPLC analysis

Twenty-eight isolates of *F. culmorum* were selected arbitrarily from the collection used for this study. Each isolate was cultured on

Table 1
Isolates of *F. culmorum* examined in this study.

Isolates	Geographic origin	Cultivar of durum wheat
Fcu1, Fcu18, Fcu30, Fcu32, Fcu78.	Cap Bon	Karim
Fcu17, Fcu31.	Cap Bon	Khiar
Fcu 25, Fcu70.	Cap Bon	Razzek
Fcu 9, Fcu38, Fcu39, Fcu44, Fcu47, Fcu43.	Tunis	Karim
Fcu 3, Fcu35, Fcu37, Fcu40, Fcu42, Fcu46.	Tunis	Khiar
Fcu 5, Fcu8, Fcu2, Fcu34, Fcu36, Fcu41.	Tunis	Razzek
Fcu 14, Fcu24, Fcu49, Fcu53, Fcu55, Fcu59, Fcu61, Fcu67, Fcu68.	Jendouba	Karim
Fcu 16, Fcu51, Fcu52, Fcu 56, Fcu58, Fcu60, Fcu64, Fcu66, Fcu88	Jendouba	Khiar
Fcu 48, Fcu50, Fcu54, Fcu57, Fcu62, Fcu63, Fc65, Fcu69	Jendouba	Razzek
Fcu 4, Fcu15, Fcu45, Fcu94, Fcu95, Fcu97.	Mateur	Karim
Fcu 6, Fcu11, Fcu19, Fcu29, Fcu96, Fcu98.	Mateur	Khiar
Fcu 7, Fcu13, Fcu20, Fcu23, Fcu33, Fcu93.	Mateur	Razzek
Fcu 10, Fcu71, Fcu73, Fcu75, Fcu79, Fcu81, Fcu86, Fcu87.	Beja	Karim
Fcu 12, Fcu26, Fcu74, Fcu83, Fcu85.	Beja	Khiar
Fcu 22, Fcu28, Fcu72, Fcu76, Fcu77, Fcu80, Fcu82, Fcu84.	Beja	Razzek
Fcu 21, Fcu90, Fcu100	Bizerte	Karim
Fcu 27, Fcu89, Fcu99	Bizerte	Khiar
Fcu 91, Fcu92.	Bizerte	Razzek

Table 2
Primer sequences used in this study and their references.

Primer	Sequence (5′–3′)	References
Tox5-1	GCTGCTCATCACTTTGCTCAG	Niessen and Vogel (1998)
Tox5-2	CTGATCTGGTCACGCTCATC	Niessen and Vogel (1998)
Tri13NIVF	CCAAATCCGAAAACCGCAG	Chandler et al. (2003)
Tri13R	TTGAAAGCTCCAATGTCGTG	Chandler et al. (2003)
Tri7F	TGCGTGGCAATATCTTCTTCTA	Chandler et al. (2003)
Tri7NIV	GGTTCAAGTAACGTTCGACAATAG	Chandler et al. (2003)
Tri13F	CATCATGAGACTTGTKCRAGTTTGGG	Chandler et al. (2003)
Tri13DONR	GCTAGATCGATTGTTGCATTGAG	Chandler et al. (2003)
MinusTri7F	TGGATGAATGACTTGAGTTGACA	Chandler et al. (2003)
MinusTri7R	AAAGCCTTCATTCACAGCC	Chandler et al. (2003)
Tri3F971	CATCATACTCGCTCTGCTG	Quarta et al. (2005)
Tri3R1679	TT(AG)TAGTTTGCATCATT(AG)TAG	Quarta et al. (2005)
Tri3F1325	GCATTGGCTAACACATGA	Quarta et al. (2005)