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ORIGINAL ARTICLE

Geographic distribution of *Fusarium culmorum* chemotypes associated with wheat crown rot in Iraq

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Abstract

Fusarium crown rot (FCR) is an important disease of wheat and other grains that has had a significant impact on cereal crop production worldwide. *Fusarium* species associated with FCR can also produce powerful trichothecenes mycotoxins that pose a considerable health risk to humans and animals that consume infected grains. In this study we examined *Fusarium* species of wheat from different regions of Iraq that showed FCR symptoms. Twenty-nine isolates were collected overall, and the marker gene translation elongation factor 1 alpha (TEF-1α) was sequenced in order to determine their taxonomic identities. All isolates were determined to be *F. culmorum*, and primers targeting tri-cluster genes were used in order to further characterize isolates into specific trichothecene chemotype strains. Five of the 29 isolates were determined to be the nivalenol (NIV) chemotype, while the rest of the isolates recovered were the deoxynivalenol (DON) chemotype. All DON--type isolates produced 3Ac-DON, while the 15Ac-DON-type was not detected. The majority of the NIV-type isolates originated from wheat growing regions in the mid-latitudes of Iraq, while the DON-type isolates were recovered from areas distributed broadly across the country. To the best of our knowledge, this study is the first to report on the distribution of specific *F. culmorum* chemotypes from FCR diseased wheat in Iraq.

Key words: *Fusarium culmorum,* PCR, B-Trichothecene, *Triticum aestivum*

Introduction

Crown rot disease has been reported around the world and it is known to significantly impact cereal production, resulting in considerable economic loss (Matny 2015). For example, in Australia, where the disease is a persistent and prevalent agricultural problem, losses in wheat and barley production alone have approached one billion Australian dollars annually (Murray and Brennan 2009, 2010). A number of soil-borne fungal species are associated with crown rot disease (Parry *et al.* 1994; Smiley *et al.* 2005; Moya-Elizondo *et al.* 2011); however, *Fusarium graminearum* and *Fusarium pseudograminearum* are among the more commonly cited causative agents (Summerell *et al.* 2010; Liu and Ogbonnaya 2015). These species appear to exhibit some geographic preferences, for example, with *F. pseudograminearum* being a dominant *Fusarium* crown rot (FCR) disease pathogen in Australia and *F. graminearum* being more common across the northern United States (Summerell *et al.* 2010). *Fusarium culmorum* is another important FCR disease causing species (Backhouse and Burgess 2002; Moya-Elizondo *et al.* 2011) that is commonly isolated within particular regions, such as the Middle East (Motallebi *et al.* 2015) or in specific areas within a region, such as higher elevations of the Pacific Northwest and United State (Poole *et al.* 2013). Distinct biogeographic patterns have also been observed among other *Fusarium* taxa, including for specific chemotypes (O'Donnell *et al.* 2000; Summerell *et al.* 2010; Wang *et al.* 2011; van der Lee *et al.* 2015; Pasquali *et al.* 2016).

For *F. culmorum*, chemotypes have been recognized within the type B trichothecene mycotoxins, which are common contaminants of cereals (Pasquali *et al.* 2016). These trichothecenes disrupt eukaryotic protein synthesis, which within infected grains are highly toxic to humans and animals that consume them, and thus are a major concern for cereal production (Cundliffe *et al.* 1974). The B type trichothecenes include nivalenol (NIV), deoxynivalenol (DON), and acetylated DON derivatives such as 3-acetyldeoxynivalenol (3Ac-DON). The tri-cluster genes (e.g., Tri3 and Tri5) have been used as a marker for distinguishing isolates that produce specific types of trichothecenes (Chandler *et al.* 2003; Niessen 2007). These compounds are powerful phytotoxins that likely play a role in pathogenicity (Eudes *et al.* 2000), and distinct chemotypes are recognized according to their production of DON and related derivatives or NIV (Scherm *et al.* 2013).

The Middle East is an important center of wheat production, with Turkey alone ranking among the top 10 largest wheat producers worldwide (Kan *et al.* 2015). Although FCR disease has been consistently reported from the Middle East (Saremi *et al.* 2007; Tunali *et al.* 2008; Matny *et al.* 2012), there is a paucity of studies focusing on *Fusarium* chemotypes associated with FCR from the region. In this study we determined trichothecenes produced in twenty-nine *Fusarium* isolates collected broadly across Iraq, using PCR analysis of *Tri3*, *Tri5*, and *Tri7* genes to characterize specific *F. culmorum* chemotypes. To the best of our knowledge, this study is the first report demonstrating the presence and geographic distribution of differing *F. culmorum* chemotypes from FCR diseased wheat in Iraq.

Materials and Methods

Sampling and fungal isolation

Wheat plants that showed crown rot disease symptoms were collected from fields within seven provinces of Iraq (Fig. 1; Table 1). All samples were collected in paper bags and given a sample number. For each sample metadata were gathered, including the locality, date of collection, and cultivar type. Samples were then brought to the laboratory and air dried. Parts of wheat plants that exhibited crown rot disease were surface sterilized with 10% sodium hypochlorite (bleach) for 2 min, followed by a sterile water wash. They were then dried on filter paper and cut into 0.5–1.0 cm segments. Each sample segment was placed in a 9 cm Petri dish containing Potato Dextrose Agar (PDA) prepared by dissolving 39 g of PDA powder in 1 l of deionized water and sterilized in an autoclave for 20 min at 121°C under 1.5 kg · cm^{-1} pressure. To suppress bacterial growth, 50 mg of the antibiotic Agromycin was then

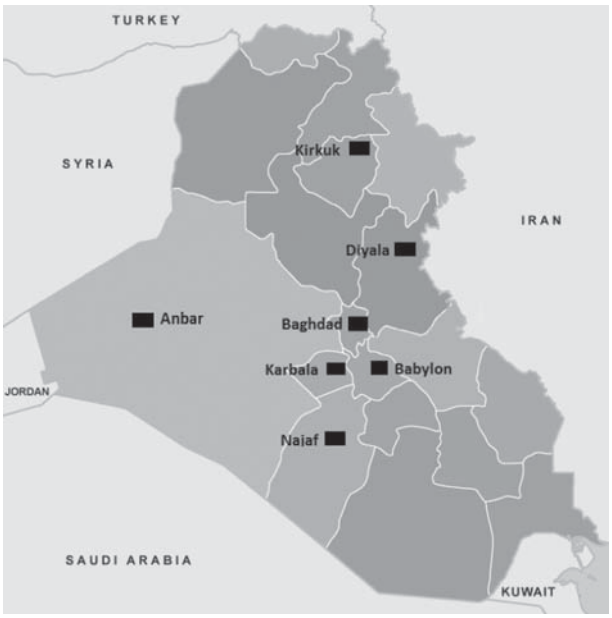

Fig. 1. Map of Iraq showing loci of the sampling sites in this study

Table 1. The *Fusarium* spp. cultures obtained from wheat showing crown rot symptoms and loci of samplings targeted in this study

Culture number	Location
IF 0003	Karbala
IF 0004	Karbala
IF 0005	Diyala
IF 0006	Diyala
IF 0007	Diyala
IF 0008	Diyala
IF 0009	Kirkuk
IF 0013	Anbar
IF 0014	Anbar
IF 0015	Anbar
IF 0017	Najaf
IF 0021	Baghdad
IF 0022	Baghdad
IF 0024	Diyala
IF 0026	Anbar
IF 0028	Baghdad
IF 0029	Baghdad
IF 0030	Kirkuk
IF 0031	Kirkuk
IF 0032	Kirkuk
IF 0033	Kirkuk
IF 0040	Babylon
IF 0041	Babylon
IF 0042	Babylon
IF 0044	Diyala
IF 0045	Baghdad
IF 0046	Baghdad
IF 0047	Baghdad
IF 0052	Anbar

added to the sterilized PDA medium as it cooled. Petri dishes with the crown rot sample segments were incubated at 25°C for 5 days and monitored for growth. A single spore was then removed from each fungal colony observed, and then used to grow a new axenic mycelium on PDA.

Extraction and amplification of fungal DNA

Single spore colonies prepared from the original crown rot sample segment isolates were used in DNA extraction following a modified protocol for the REDExtract-N-Amp Plant Tissue Kits (Sigma-Aldrich, USA). Briefly, a sterilized needle was used to remove a small segment of fungal mycelium, which was then placed into a 0.2 ml vial containing 50 µl of the kit extraction buffer. Each vial containing the mycelial sample and extraction buffer was then incubated in a thermocycler at 65°C for 10 min, followed by an additional 10 min at 95°C. Extracted DNAs were then quantified and checked for quality using a NanoDrop 2000 (Thermo Fisher Scientific, USA).

To aid in the identification of the fungal isolates obtained, sequences of the marker gene translation elongation factor 1 alpha (*TEF-1α*) gene were obtained using the primers EF1 and EF2 (Table 2). Polymerase chain reactions (PCR) were prepared to a total volume of 20 µl, with each reaction containing 10 µl of GoTaq Master Mix (Promega, USA), 0.5 µl (10 nM) of each primer, 5 µl of DNA-free water, and 4 µl DNA template (~5–10 ng). Thermocycling conditions for *TEF-1α* included: denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 50 sec (denaturation), 53°C for 50 sec (annealing), and then 72°C for 1 min (extension), with a final extension at 72°C for 7 min. To characterize the *Fusarium* trichothecene chemotypes, tri-cluster genes (*Tri3*, *Tri5* and *Tri7* regions) were amplified using specific primers developed in previous studies (Table 2). Thermocycling conditions for tri-cluster gene amplification included: denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 sec, 53°C for 30 sec, 72°C for 1 min, with a final extension at 72°C for 5 min. All amplified products were visualized on 1% agarose gels stained with SYBR safe DNA gel stain (Invitrogen, USA) in 1X TAE.

DNA sequencing

Amplified products were prepared for sequencing by using the QlAquick PCR purification kit (Qiagen, USA) following the standard protocol, and then quantified and checked for quality using a NanoDrop 2000. Sequencing was carried out commercially (ACGT, Inc., Chicago, USA). Recovered sequences were read, checked for quality, and contigs were assembled using MEGA6 software (Kumar *et al.* 2016). Sequences were then compared with others in GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST (Altschul *et al.* 1990).

Results and Discussions

The *TEF-1α* gene was sequenced for each of the 29 FCR isolates, and BLAST searches suggested that all isolates were closely related to other *F. culmorum* strains (identities > 97% sequence similarity). A morphological study was subsequently carried out on each of the isolates, which were all verified to be *F. culmorum*. Among the *Fusarium* species known to cause FCR, *F. culmorum* is recognized as a common causative agent, and

Table 2. Primers design used for speciation of *Fusarium* spp and detection of the chemotypes in this study

Primer	Target gene	Nucleotide sequence (5´to 3´)	Product band [bp]	Annealing temperature [°C]	References
EF1 EF2	*TEF-1α*	ATGGGTAAGGA(A/G)GACAAGAC GGA(G/A)GTAC CAGT(G/C)ATCATG	700	53	O'Donnell *et al.* (2000)
N1-2 N1-2R	*Tri5*	CTTGTTAAGCTAAGCGTTTT AACCCCTTTCCTATGTGTTA	200	55	Bakan *et al.* (2002)
*Tri*7F340 *Tri*7R965	*Tri7*	ATCGTG TACAAGGTTTACG TTCAAGTAACGTTCGACAAT	625	50	Quarta *et al.* (2005)
*Tri*3F971 *Tri*3R1679	*Tri3*	CATCATACTCGCTCTGCTG TT(AG)TAGTTTGCATCATT(AG)TAG	708	53	Quarta *et al.* (2005)
*Tri*3F1325 *Tri*3R1679	*Tri3*	GCATTGGCTAACACATGA TT(AG)TAGTTTGCATCATT(AG)TAG	354	53	Quarta *et al.* (2005)

it has been shown to be a dominant and aggressive FCR strain in the Middle East where it poses a threat to wheat production (Matny *et al.* 2012; Motallebi *et al.* 2015). FCR causing species, including *F. culmorum*, are known to produce particular chemical types of trichothecene mycotoxins, and many *Fusarium* species and chemotypes have been shown to exhibit distinct geographical distributions across the globe and within particular regions (Starkey *et al.* 2007; Summerell *et al.* 2010; Backhouse 2014; van der Wall *et al.* 2015).

In order to further chemically characterize our Iraqi *F. culmorum* isolates, we sequenced tri-cluster genes to determine specific trichothecene chemotypes for each of our strains. PCR amplification of the *Tri7* gene cluster was used to assay for the NIV chemotype, while the *Tri3* and *Tri5* gene clusters were amplified to identify the DON chemotype and its sub-chemotypes. Both NIV and DON chemotypes were presented among our 29 *F. culmorum* isolates (Table 3; Fig 2). Of the 29 isolates, 24 (~83%) were DON chemotypes, while five (~17%) of the isolates were NIV-type. Further sub-chemotype characterization showed that all of the 24 DON-type isolates were 3Ac-DON producers, while none of the isolates were 15Ac-DON sub-chemotypes. All of the NIV-type isolates originated from areas around the mid-latitude of Iraq, three from the central region (Baghdad and Karbala) and two from the west-central region (Anbar). The 3Ac-DON-type isolates were found more broadly across the country, Anbar and Baghdad included.

Fusarium culmorum has been shown to be an important FCR causing species within some parts of prominent wheat growing regions of the world; however, other species, such as *F. pseudograminearum*, are

Table 3. Chemotypes of *Fusarium culmorum* isolates shown according to PCR results in this study

Sample number	Culture number	Sequencing similarity	PCR chemotype identification			
			DON	3A DON	15A DON	NIV
1	IF 0003	99	–	–	–	+
2	IF 0004	99	–	–	–	+
3	IF 0005	99	+	+	–	–
4	IF 0006	99	+	+	–	–
5	IF 0007	99	+	+	–	–
6	IF 0008	99	+	+	–	–
7	IF 0009	98	+	+	–	–
8	IF 0013	99	–	–	–	+
9	IF 0014	99	+	+	–	–
10	IF 0015	99	+	+	–	–
11	IF 0017	99	+	+	–	–
12	IF 0021	99	+	+	–	–
13	IF 0022	99	+	+	–	–
14	IF 0024	99	+	+	–	–
15	IF 0026	99	+	+	–	–
16	IF 0028	99	+	+	–	–
17	IF 0029	99	+	+	–	–
18	IF 0030	99	+	+	–	–
19	IF 0031	99	+	+	–	–
20	IF 0032	99	+	+	–	–
21	IF 0033	99	+	+	–	–
22	IF 0040	99	+	+	–	–
23	IF 0041	99	+	+	–	–
24	IF 0042	98	+	+	–	–
25	IF 0044	99	+	+	–	–
26	IF 0045	99	+	+	–	–
27	IF 0046	99	+	+	–	–
28	IF 0047	99	–	–	–	+
29	IF 0052	99	–	–	–	+

DON = deoxynivalenol; 3A DON = 3-acetyldeoxynivalenol; 15A DON = 15A acetyldeoxynivalenol; NIV = nivalenol

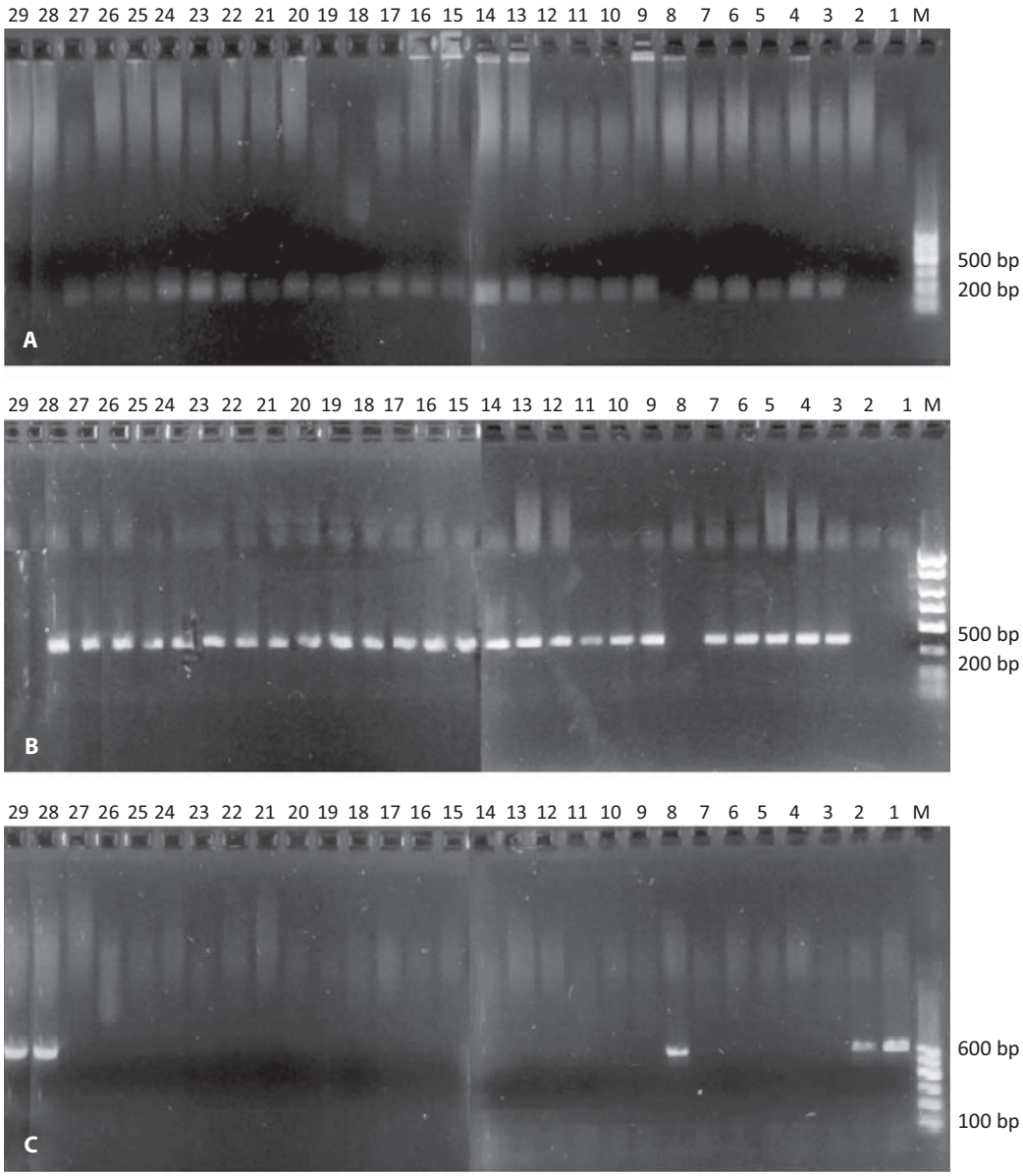

Fig. 2. Amplification of *F. culmorum* DNA associated with crown rot disease to determined chemotypes strains. (A) 1-2, N1-2R primer to deoxynivalenol (DON) producer isolates 200 bp, (B) primer *Tri3* 1325-1679 for 3A DON producer isolates 354 bp, (C) primer *Tri7* F340-R965 for nivalenol (NIV) producer isolates 625 bp

also dominant within the same region. Dominance of *F. culmorum* over other species in a particular area is thought to be influenced by climatic conditions, such as temperature (Poole *et al.* 2013; Backhouse 2014) and precipitation (Backhouse *et al.* 2004). Studies from the Middle East have shown that *F. culmorum* is common in this region (Motallebi *et al.* 2015), although many other FCR causing species have been detected there as well (e.g., Seif El-Nasr and Leath 1983; Saremi *et al.* 2007; Hajieghrari 2009). Few studies have examined the presence or distribution of specific chemotypes for Middle Eastern FCR causing species.

While a number of species have been shown to be associated with wheat FCR in Iraq (Hameed *et al.* 2012; Matny *et al.* 2012), Matny *et al.* (2012) found *F. culmorum* to be the most severe FCR causing species among the Iraqi isolates examined. In that study, all of the Iraqi isolates collected were found to be of the DON-type, with *F. culmorum* isolates producing the highest DON levels (Matny *et al.* 2012), which potentially contributed to their aggressiveness (Eudes *et al.* 2000). Motallebi *et al.* (2015) also reported on the presence of DON chemotypes (3Ac-DON) of *F. culmorum* on FCR diseased wheat from Iran and Syria, and additionally referenced the NIV chemotype in the region that was previously reported from Syria (Alkadri *et al.* 2013). Our results are consistent with these and other studies (Yörük and Albayrak 2012; Alkadri *et al.* 2013; Mert-Turk and Gencer 2013; Motallebi *et al.* 2015) in further demonstrating the dominance of the *F. culmorum* 3Ac-DON chemotype associated with FCR throughout the entire Middle East and under different

climatic conditions. Furthermore, our NIV-type isolates and those of Alkadri *et al.* (2013) appear to be restricted to areas that share very similar climatic conditions in the region (see e.g., USDA Crop Explorer data for Middle East and Turkey growing seasons, http://www.pecad.fas.usda.gov/cropexplorer). While we did not detect the presence of 15Ac-DON chemotypes of *F. culmorum* in Iraq, they have been reported on wheat in the Middle East from Turkey (Mert-Türk and Gencer 2013); however, those isolates were collected from areas very close to the Mediterranean and Black Seas, which experience very different climatic conditions from our collection sites in Iraq.

Overall, our results and those of previous studies suggest that *F. culmorum* is an important and potentially aggressive FCR disease of wheat in the Middle East, and that the 3Ac-DON-type of *F. culmorum* is a dominant and widely distributed DON sub-chemotype within the region. The NIV chemotype of FCR associated *F. culmorum*, however, appears to be restricted to the mid-latitudes of the Middle East, within areas that share common climatic conditions as well as agricultural practices (e.g., reliance on crop irrigation). While other *F. culmorum* chemotypes, such as the 15Ac-DON-type, do not appear to be widely distributed across the Middle East, broader survey efforts in the region will likely find them as well as additional chemotypes and their associated distributions. A more complete knowledge of the FCR causing species and their trichothecene chemotypes, their biogeographical patterns across the Middle East, as well as the factors that influence these patterns will be necessary for improving wheat crop management in the region.

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