Phytopathologia Mediterranea

The international journal of the Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Genetic variability, chemotype distribution, and aggressiveness of *Fusarium culmorum* on durum wheat in Tunisia

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Abstract. *Fusarium culmorum* is the most commonly reported root rot pathogen in Tunisian durum wheat. Isolates of the pathogen from four durum wheat growing areas in the north of Tunisia were analyzed for their chemotypes. Two chemotypes were detected at unequal abundance (96% of 3-ADON and 4% of NIV). Distribution of a SNP mutation located at the position 34 bp after the first exon of the EF-1α partial sequence was analysed, to verify whether the haplotype was specifically associated to Fusarium root rot. A and T haplotypes were homogeneously distributed in three different Tunisian regions (Mateur, Beja and Bousalem) but not for the region of Bizerte, from which greatest number of A haplotype strains were detected. The isolates were tested for their virulence under glasshouse conditions, and a mean of 91% of crown and root infection was observed. Chemotype influenced virulence, but there was no significant influence of the geographical origin or haplotype on virulence. The distribution of three inter simple sequence repeats (ISSR) was examined, to better understand the structure of *F. culmorum* populations in Tunisia. A total of 27 fragments were obtained with eight polymorphic bands. Cluster analysis showed a high level of similarity between isolates. Analysis of molecular variance confirmed that there was little genetic differentiation among *F. culmorum* strains from different locations.

Keywords. *Fusarium* crown and root rots, population structure, trichothecenes.

OPEN ACCESS

Citation: Oufensou S., Balmas V., Scherm B., Rau D., Camiolo S., ProtaV.A., Ben-Attia M., Gargouri S., Pasquali M., El-Bok S., Migheli Q. (2019) Genetic variability, chemotype distribution, and aggressiveness of *Fusarium culmorum* on durum wheat in Tunisia. *Phytopathologia Mediterranea* 58(1): 103-113. doi: 10.13128/Phytopathol_Mediterr-23907

Accepted: November 13, 2018

Published: May 15, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Antonio Moretti, Consiglio Nazionale delle Ricerche, Bari, Italy.

INTRODUCTION

Durum wheat is extensively grown in Tunisia, covering over 40% of the cereal-producing areas (Fakhfakh *et al.*, 2011). *Fusarium culmorum* is reported to be among the most prevalent pathogens responsible of foot and root rot (FRR) of durum wheat and other small grain cereal crops (Burgess *et al.*, 2001; Smiley *et al.*, 2005; Wagacha and Muthomi, 2007; Scherm *et al.*, 2013). FRR is particularly severe in areas affected by water stress, that are usually found in Tunisia (Gargouri *et al.*, 2001), in Southern Italy (Balmas *et al.*, 2006) and in Turkey (Tunali *et al.*, 2006), Iran (Eslahi, 2012) and Syria (El-Khalifeh *et al.*, 2009). *Fusarium culmorum* infects host plants at the initial growing stages, causing rotting of root and crown tissues. In some cases, lesions and browning of the coleoptiles or seedling death may occur. When *F. culmorum* infects wheat plants at later growing stages, brown spots on basal internodes can be observed. Under high humidity conditions, reddish-pink discolourations of the nodes appear due to the development of sporulating pathogen mycelium (Scherm *et al.*, 2013; Balmas *et al.*, 2015).

Fusarium culmorum causes serious problems since it causes yield reductions and mycotoxin accumulation. The pathogen produces type B trichothecenes (Smiley *et al.*, 2005; Miedaner *et al.*, 2008; Obanor *et al.*, 2010; Pasquali *et al.*, 2016). The compounds are harmful to humans and animals, resulting in cancer development and other generally irreversible effects (Bennett and Klich, 2003; Pestka and Smolinski, 2005). Type B trichothecenes are also considered as virulence factors through inhibition of host plant defence mechanisms (Wagacha and Muthomi, 2007; Scherm *et al.*, 2013). Increased virulence of *F. culmorum* strains is associated to their ability to contaminate plant tissues with high doses of trichothecenes, although this correlation has not always been confirmed (Gang *et al.*, 1998; Scherm *et al.*, 2011), or these compounds may have minor effects ith they are translocated from the basal portions of host plants (Winter *et al.*, 2013).

For the most important mycotoxins, the maximum permitted levels have been set, for grains and cereal-derived products used for human or livestock consumption (EC, 2006). However, in some cases, high levels of toxin and the possible interaction between concomitantly occurring mycotoxins, represent toxicological risks, and food safety is seriously hampered (Balmas *et al.*, 2015).

For *F. culmorum*, chemotypes have been recognized within the type B trichothecene mycotoxins (Pasquali *et al.*, 2016). Distinct chemotypes are recognized according to their production of deoxynivalenol (DON) and related derivatives or nivalenol (NIV) (Scherm *et al.*, 2013). Discrimination of DON and NIV may provide insight into the toxigenic potential of *F. culmorum* strains. To our knowledge, all the *F. culmorum* strains from wheat in Italy belong to 3-ADON (Quarta *et al.*, 2005; Covarelli *et al.*, 2014) apart from two NIV strains found in two Italian regions, Tuscany and Emilia-Romagna (Prodi *et al.*, 2010), and one isolate that was characterized as NIV in Sardinia (Balmas *et al.*, 2015). Search for chemotypes associated with FFR throughout the Middle East demonstrated that 100% of *F. culmorum* strains in Turkey belong to the 3-ADON chemotype (Yörük and Albayrak, 2012), while in Syria 55% of the strains were 3-ADON and 45% were NIV chemotypes (Alkadri *et al.*, 2013). In other surveys (Yörük and Albayrak 2012; Alkadri *et al.*, 2013; Mert-Türk and Gencer, 2013; Motallebi *et al.*, 2015) dominance of the *F. culmorum* 3-ADON chemotype was further highlighted.

The DON chemotype is most widely found (Scherm *et al.*, 2013), whereas NIV producers are less frequent in many European countries (Bakan *et al.*, 2001; Jennings *et al.*, 2004), and in Tunisia (Kammoun *et al.*, 2010; Rebib *et al.*, 2014), Turkey (Yörük and Albayrak, 2012) and the United States of America (Mirocha *et al.*, 1994).

The nucleotide sequence of the translation elongation factor 1-α (EF1-α) gene, encoding a part of a highly conserved ubiquitous protein involved in translation, was first used in fungi in *Fusarium* (O'Donnell *et al.*, 1998). As a single-locus identification tool, EF1-α shows a high level of sequence polymorphism among related species, hence it was considered a useful genetic region for phylogenetic and taxonomic studies, allowing reliable identification as an alternative to rDNA or β-tubulin (O'Donnell, 2000; Roger *et al.*, 1999).

Knowledge of chemotype distribution within *F. culmorum* populations originating from several agro-ecological areas in Tunisia would provide useful information on strain fitness in the field, representing a reliable resource for the development of effective disease control strategies (Strange and Scott, 2005).

The goals of the present study were: 1) to characterize a representative *F. culmorum* population isolated from FRR-affected durum wheat plants grown in different agro-ecological areas of Tunisia; 2) to assess genetic variability and population structure of Tunisian *F. culmorum* strains, based on EF1-α sequence polymorphism and ISSR markers; 3) to assess the virulence of *F. culmorum* strains on wheat plants to verify the hypothesis that a specific haplotype is associated with FRR; and 4) to examine the distribution of genetic chemotypes and gather information about the potential toxigenicity of

the fungal population that might contaminate durum wheat in Tunisia.

MATERIALS AND METHODS

Sampling, fungal isolation and isolate storage

FRR distribution on durum wheat was monitored during the 2015 growing season. A total of 88 fields were investigated in two climatic regions (sub humid and higher semi-arid) in Northern Tunisia (Bortoli *et al.*, 1969). These regions include more than 90% of the Tunisian wheat production areas (Figure 1). Sixty-eight fields were from the sub-humid region (Bizerte, Mateur and Beja), and 20 fields were from the higher semi-arid region (Bousalem). The fields, separated by approx. 10 km, were randomly selected. For each field in which FRR symptoms were observed, 20 plants were randomly collected along diagonal transects in different field zones. Plant samples were transferred in paper bags to the laboratory and stored at 4°C until analysed.

All fungal strains were obtained from the basal stems of diseased durum wheat plants. Fungal isolation was carried out according to Balmas *et al.* (2015), and monospore cultures were prepared as described by Burgess *et al.* (1994). All monospore strains collected were identified based on morphological traits, as described by Burgess *et al.* (1994).

For further analyses, all strains were stored at -80°C in 15% glycerol in the *Fusarium* collection of the Dipartimento di Agraria, University of Sassari, Italy.

Genomic DNA extraction and molecular characterization

Mycelia of fungal strains were each collected with a sterile spatula from PDA plates, after 5 d of incubation at 25°C in darkness. Genomic DNA was extracted from each isolate according to Aljanabi and Martinez (1997), and stored at 4°C. For each isolate, a partial sequence of the translation elongation factor 1α (EF1-α) was amplified (Balmas *et al.*, 2015), in a total of 50 µL reaction mixture containing: 10-25 µg of DNA template, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each of the primers TEF1 ATGGGTAAGGA(A/G)GACAAGAC and TEF2 GGA(G/A)GTACCAGT(G/C)ATCATGTT, 2 units *Taq* polymerase (Invitrogen). The PCR programme included one cycle at 98°C for 2 min, 35 cycles at 98°C for 15 s, 60°C for 15 s and 72°C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were purified (PureLink™ Quick PCR Purification Kit, Invitrogen) following the manufacturer's instructions, and amplicon concentration was estimated with a fluorometer (Qubit™, Invitrogen), for sequencing optimisation. Sequencing was performed with 3500 Genetic Analyser (Life Technologies). For each isolate, both forward and reverse strands were sequenced and aligned using the multiple alignment program ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/). To confirm morphological identification, EF1-α partial sequences were compared with consensus sequences available in *Fusarium*-ID and GenBank databases (O'Donnell *et al.* 2012). Based on the polymorphic nucleotide (T–A) located at position 34 after the first exon of the EF1-α partial sequence gene (Balmas *et al.* 2010, 2015), a Single Nucleotide Polymorphism (SNP) was targeted. All the sequences were then deposited in GenBank (Table 1).

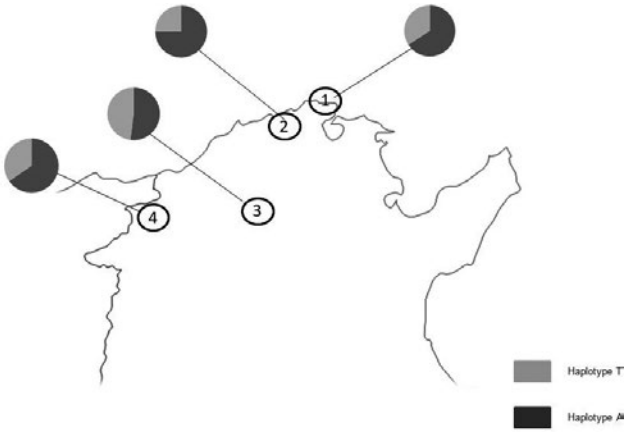

Figure 1. Map indicating sample sites in Tunisia where Fusarium isolates associated with root rot of wheat were obtained. Proportions (%) of haplotypes are indicated. Localities of Bizerte (1), Mateur (2), Beja (3) and Bousalem (4) are also indicated.

To determine the genetic chemotype of each isolate, a *Tri12* multiplex PCR was carried out according to Ward *et al.* (2002) using two different primer sets, 12CON/12NF CATGAGCATGGTGATGTC/TCTCCTCGTTGTATCTGG for NIV discrimination and 12CON/12-3F CATGAGCATGGTGATGTC/CTTTGGCAAGCCCGTGCA for DON discrimination. PCR conditions were performed at 98°C (2 min) for one cycle; 98°C (10 s), 59°C (10 s), and 72°C (20 s) for 30 cycles; and a final cycle of 72°C for 5 min.

Genetic variability among the *F. culmorum* isolates was evaluated by an ISSR-PCR assay, using three different ISSR primers (Table 3). PCR conditions and annealing temperature were modified according to the primer sequence: 48°C for ISSR4 [$(GAGG)_3GG$; Albayrak *et al.* 2016]; and 55°C for both ISSR5 [$(AG)_9G$)] and ISSR6 [$(AC)_8YG$; Mishra *et al.* 2003]. PCR products were visualized on agarose gel (1.5% agarose) after electrophoretic

Table 1. Locations, chemotypes, Single nucleotide polymorphisims (SNP), mean aggressiveness scores and GenBank accession numbers for *Fusarium* isolates associated with Fusarium root rot of wheat in Tunisia.

Isolate	Location	Coordinates	Chemotype[a]	SNP[b]	Aggressiveness[c] Mean ± SD	EF-1α GenBank Accession No.
Fu-BI87	Bizerte	N37 12.423 E940.384	3-ADON	A	90.00 ± 31.6 [abc]	MF510992
Fu-BI84	Bizerte	N37 12.423 E940.384	3-ADON	T	77.50 ± 41.6 [abc]	MF510990
Fu-BI151	Bizerte	N37 13.964 E9 42.388	3-ADON	A	95.00 ± 15.81 [ab]	MF511031
Fu-BI169	Bizerte	N37 13.964 E9 42.388	3-ADON	T	50.00 ± 44.1 [cde]	MF511041
Fu-BI3	Bizerte	N36 36.677 E8 40.158	3-ADON	A	95.00 ± 15.81 [ab]	MF510936
Fu-BI36	Bizerte	N36 36.677 E8 40.158	3-ADON	A	100.0 ± 0.0 [a]	MF510958
Fu-BI135	Bizerte	N36 36.677 E8 40.158	3-ADON	A	77.50 ± 36.2 [abc]	MF511027
Fu-BI33	Bizerte	N37 14.416 E9 43.842	3-ADON	T	100.0 ± 0.0 [a]	MF510955
Fu-BI42	Bizerte	N37 14.416 E9 43.842	3-ADON	A	100.0 ± 0.0 [a]	MF510963
Fu-BI50	Bizerte	N37 14.416 E9 43.842	3-ADON	T	82.50 ± 33.4 [abc]	MF510969
Fu-BI54	Bizerte	N37 14.416 E9 43.842	3-ADON	T	85.00 ± 31.6 [abc]	MF510971
Fu-BI58	Bizerte	N37 14.416 E9 43.842	3-ADON	A	62.50 ± 37.7 [abcd]	MF510975
Fu-BI105	Bizerte	N37 14.416 E9 43.842	3-ADON	T	100.0 ± 0.0 [a]	MF511008
Fu-BI120	Bizerte	N37 14.416 E9 43.842	3-ADON	T	85.00 ± 31.6 [abc]	MF511017
Fu-BI123	Bizerte	N37 14.416 E9 43.842	3-ADON	A	100.0 ± 0.0 [a]	MF511019
Fu-BI13	Bizerte	N37 08.734 E9 46.500	3-ADON	A	55.00 ± 42.2 [bcde]	MF510943
Fu-BI15	Bizerte	N37 08.734 E9 46.500	NIV	A	65.00 ± 39.4 [abcd]	MF510944
Fu-BI4	Bizerte	N37 13.964 E9 42.390	3-ADON	A	92.50 ± 23.72 [ab]	MF510937
Fu-BI118	Bizerte	N37 13.964 E9 42.390	3-ADON	A	90.00 ± 24.15 [abc]	MF511015
Fu-BI67	Bizerte	N37 14.416 E9 43.842	3-ADON	A	87.50 ± 24.30 [abc]	MF510980
Fu-BI150	Bizerte	N37 14.416 E9 43.842	3-ADON	A	90.00 ± 31.6 [abc]	MF511030
Fu-BI170	Bizerte	N37 14.416 E9 43.842	3-ADON	A	100.0 ± 0.0 [a]	MF511042
Fu-BI183	Bizerte	N37 14.416 E9 43.842	3-ADON	A	100.0 ± 0.0 [a]	MF511046
Fu-BI6	Bizerte	N37 06.858 E9 46.944	NIV	T	77.50 ± 41.6 [abc]	MF431609
Fu-BI8	Bizerte	N37 06.858 E9 46.944	NIV	T	72.50 ± 39.9 [abc]	MF510939
Fu-BI28	Bizerte	N37 06.858 E9 46.944	NIV	T	75.00 ± 40.8 [abc]	MF510951
Fu-BI161	Bizerte	N37 07.868 E9 45.396	3-ADON	T	75.00 ± 28.87 [abc]	MF511039
Fu-BI159	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF511037
Fu-BI152	Bizerte	N37 07.868 E9 45.396	3-ADON	A	90.00 ± 31.6 [abc]	MF511032
Fu-BI149	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF511029
Fu-BI128	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF511023
Fu-BI103	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF511006
Fu-BI35	Bizerte	N37 07.868 E9 45.396	3-ADON	A	100.0 ± 0.0 [a]	MF510957
Fu-BI9	Bizerte	N37 10.539 E9 44.507	3-ADON	A	85.00 ± 33.7 [abc]	MF510940
Fu-BI10	Bizerte	N37 10.539 E9 44.507	3-ADON	T	100.0 ± 0.0 [a]	MF510941
Fu-BI19	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF510946
Fu-BI22	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF510948
Fu-BI25	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF510949
Fu-BI27	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF510950
Fu-BI30	Bizerte	N37 10.539 E9 44.507	3-ADON	A	87.50 ±27.00 [abc]	MF510952
Fu-BI76	Bizerte	N37 10.539 E9 44.507	3-ADON	T	100.0 ± 0.0 [a]	MF510984
Fu-BI90	Bizerte	N37 10.539 E9 44.507	3-ADON	T	95.00 ± 15.81 [ab]	MF510994
Fu-BI140	Bizerte	N37 10.539 E9 44.507	3-ADON	A	100.0 ± 0.0 [a]	MF511028
Fu-BI156	Bizerte	N37 10.539 E9 44.507	3-ADON	T	100.0 ± 0.0 [a]	MF511036
Fu-BI98	Mateur	N37 08.774 E9 57.402	3-ADON	A	100.0 ± 0.0 [a]	MF511001
Fu-BI117	Mateur	N37 08.774 E9 57.402	3-ADON	A	100.0 ± 0.0 [a]	MF511014
Fu-BI127	Mateur	N37 08.774 E9 57.402	3-ADON	A	100.0 ± 0.0 [a]	MF511022

(Continued)

Table 1. (Continued).

Isolate	Location	Coordinates	Chemotype[a]	SNP[b]	Aggressiveness[c] Mean ± SD	EF-1α GenBank Accession No.
Fu-MA1	Mateur	N37 08.862 E9 33.401	3-ADON	A	100.0 ± 0.0 [a]	MF431610
Fu-MA12	Mateur	N37 08.862 E9 33.401	3-ADON	T	100.0 ± 0.0 [a]	MF510942
Fu-BI21	Mateur	N37 08.862 E9 33.401	3-ADON	A	16.00 ±16.80 [e]	MF510947
Fu-BI97	Mateur	N37 08.862 E9 33.401	3-ADON	A	92.50 ± 23.72 [ab]	MF511000
Fu-BI100	Mateur	N37 08.862 E9 33.401	3-ADON	T	97.50 ± 7.91 [a]	MF511003
Fu-BI112	Mateur	N37 08.862 E9 33.401	3-ADON	A	77.50 ± 36.2 [abc]	MF511013
Fu-MA95	Mateur	N37 08.862 E9 33.400	3-ADON	A	95.00 ±15.81 [ab]	MF510998
Fu-MA101	Mateur	N37 08.862 E9 33.400	3-ADON	A	100.0 ± 0.0 [a]	MF511004
Fu-BI107	Mateur	N37 10.729 E9 45.649	3-ADON	T	100.0 ± 0.0 [a]	MF511010
Fu-BE31	Beja	N36 42.475 E9 10.687	3-ADON	A	95.00 ± 15.81 [ab]	MF510953
Fu-BE32	Beja	N36 42.475 E9 10.687	3-ADON	T	95.00 ± 15.81 [ab]	MF510954
Fu-BE48	Beja	N36 42.475 E9 10.687	3-ADON	A	80.00 ± 42.2 [abc]	MF510967
Fu-BE55	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF510972
Fu-BE69	Beja	N36 42.475 E9 10.687	3-ADON	T	82.50 ± 37.4 [abc]	MF510981
Fu-BE78	Beja	N36 42.475 E9 10.687	3-ADON	T	95.00 ± 15.81 [ab]	MF510985
Fu-BE106	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF511009
Fu-BE129	Beja	N36 42.475 E9 10.687	3-ADON	A	92.50 ± 16.87 [ab]	MF511024
Fu-BE131	Beja	N36 42.475 E9 10.687	3-ADON	T	67.50 ± 44.2 [abcd]	MF511025
Fu-BE132	Beja	N36 42.475 E9 10.687	3-ADON	T	100.0 ± 0.0 [a]	MF511026
Fu-BE154	Beja	N36 42.475 E9 10.687	3-ADON	T	95.00 ± 15.81 [ab]	MF511034
Fu-BE162	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF511040
Fu-BE171	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF511043
Fu-BE180	Beja	N36 42.475 E9 10.687	3-ADON	A	100.0 ± 0.0 [a]	MF511044
Fu-BE182	Beja	N36 42.475 E9 10.687	3-ADON	T	100.0 ± 0.0 [a]	MF511045
Fu-BE37	Beja	N36 38.159 E9 06.458	3-ADON	A	9750 ± 7.91 [a]	MF510959
Fu-BE38	Beja	N36 38.159 E9 06.458	3-ADON	A	100.0 ± 0.0 [a]	MF510960
Fu-BE41	Beja	N36 38.159 E9 06.458	3-ADON	A	100.0 ± 0.0 [a]	MF510962
Fu-BE52	Beja	N36 38.159 E9 06.458	3-ADON	A	80.00 ± 36.9 [abc]	MF510970
Fu-BE60	Beja	N36 38.159 E9 06.458	3-ADON	A	92.50 ± 23.72 [ab]	MF510977
Fu-BE63	Beja	N36 38.159 E9 06.458	3-ADON	T	92.50 ± 12.08 [ab]	MF510979
Fu-BE72	Beja	N36 38.159 E9 06.458	3-ADON	A	90.00 ± 31.6 [abc]	MF510982
Fu-BE75	Beja	N36 38.159 E9 06.458	3-ADON	T	97.50 ± 7.91 [a]	MF510983
Fu-BE83	Beja	N36 38.159 E9 06.458	3-ADON	T	100.0 ± 0.0 [a]	MF510989
Fu-BE104	Beja	N36 38.159 E9 06.458	3-ADON	T	92.50 ± 23.72 [ab]	MF511007
Fu-BE126	Beja	N36 38.159 E9 06.458	3-ADON	T	92.50 ± 16.87 [ab]	MF511021
Fu-BO44	Bousalem	N36 34.444 E8 54.849	3-ADON	T	100.0 ± 0.0 [a]	MF510964
Fu-BO46	Bousalem	N36 34.444 E8 54.849	3-ADON	A	90.00 ± 31.6 [abc]	MF510965
Fu-BO47	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF510966
Fu-BO56	Bousalem	N36 34.444 E8 54.849	3-ADON	A	75.00 ± 31.18 [abc]	MF510973
Fu-BO57	Bousalem	N36 34.444 E8 54.849	3-ADON	A	85.00 ± 31.6 [abc]	MF510974
Fu-BO59	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF510976
Fu-BO79	Bousalem	N36 34.444 E8 54.849	3-ADON	T	77.50 ± 27.51 [abc]	MF510986
Fu-BO80	Bousalem	N36 34.444 E8 54.849	3-ADON	T	100.0 ± 0.0 [a]	MF510987
Fu-BO82	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF510988
Fu-BO85	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF510991
Fu-BO91	Bousalem	N36 34.444 E8 54.849	3-ADON	A	85.00 ± 33.7 [abc]	MF510995
Fu-BO102	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF511005
Fu-BO110	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF511011

(Continued)

Table 1. (Continued).

Isolate	Location	Coordinates	Chemotype[a]	SNP[b]	Aggressiveness[c] Mean ± SD	EF-1α GenBank Accession No.
Fu-BO111	Bousalem	N36 34.444 E8 54.849	3-ADON	A	97.50 ± 7.91 [a]	MF511012
Fu-BO119	Bousalem	N36 34.444 E8 54.849	3-ADON	A	100.0 ± 0.0 [a]	MF511016
Fu-BO153	Bousalem	N36 34.444 E8 54.849	3-ADON	T	90.00 ± 31.6 [a]	MF511033
Fu-BO155	Bousalem	N36 34.444 E8 54.849	3-ADON	A	95.00 ± 15.81 [a]	MF511035
Fu-BO160	Bousalem	N36 34.444 E8 54.849	3-ADON	A	92.50 ± 23.72 [ab]	MF511038
Fu-BO2	Bousalem	N36 34.444 E8 54.900	3-ADON	T	90.00 ± 31.6 [abc]	MF510935
Fu-BO7	Bousalem	N36 34.444 E8 54.900	3-ADON	T	100.0 ± 0.0 [a]	MF510938
Fu-BO16	Bousalem	N36 34.444 E8 54.900	3-ADON	A	92.50 ± 23.72 [ab]	MF510945
Fu-BO34	Bousalem	N36 30.263 E8 47.347	3-ADON	T	95.00 ± 15.81 [ab]	MF510956
Fu-BO92	Bousalem	N36 37.668 E8 54.874	3-ADON	T	100.0 ± 0.0 [a]	MF510996

[a] 3-acetyldeoxynivalenol, 3-ADON; nivalenol, NIV.
[b] Single nucleotide polymorphism (SNP) at position 34 after first exon of EF-1α (Balmas *et al.* 2010).
[c] Data followed by the same letters are not significantly different from each other (Tukey test).

Table 2. Statistical significance of impacts of location, chemotype or haplotype on pathogenicity of *Fusarium* isolates associated with root rot of wheat.

Variable	Number	Degrees of freedom	P-value
Location		3	0.32
Bizerte	440		
Mateur	120		
Beja	250		
Bousalem	230		
Chemotype		1	0.00
3-ADON	1000		
NIV	40		
Haplotype		1	0.87
A	660		
T	380		

Table 3. ISSR primers used in this study.

Primer	Sequence	(P/M)[a]	Reference
ISS4	$(GAGG)_3GG$	(4/12)	Albayrak *et al.* (2016)
ISSR5	$(AG)_9G$	(0/5)	Mishra *et al.* (2003)
ISSR6	$(AC)_8YG$	(4/10)	Mishra *et al.* (2003)

[a] *P* number of polymorphic markers with polymorphism level above 2%; M monomorphic markers.

separation in 1 × TAE buffer of 1 h for ISSR5 and ISSR6, or 2 h for ISSR4. Fragment size was estimated by comparison with a 1 Kb Plus DNA Ladder (Invitrogen). Gel images were analysed using a Gel-Doc XR+ System (Bio-Rad). ISSR markers were visually scored as presence (1) or absence (0) of each band.

Pathogenicity experiments

For each *F. culmorum* isolate, agar plugs (8 mm diam.) covered with mycelium were cut from actively growing colony margins of 5-d-old cultures grown on PDA, and were used to inoculate durum wheat seeds. A single durum wheat seed (cv. Saragolla) was deposited on each mycelial plug and was planted in the centre of a plastic pot containing sterilized potting mix composed of equal proportions peat soil, sand and redwood soil. The pots were incubated on a bench in a glasshouse for 20 d, with day and night temperatures of 25-30 °C and 18-25 °C, and with daily irrigation. For each isolate, ten replicates were established. According to, After 3 weeks, disease severity index was assessed using an empirical scale of five disease severity classes of disease severity (Balmas *et al.*, 2006), where 0 = no browning; 1 = 1 to 25%; 2 = 50%; 3 = 75%; and 4 = no plant emergence). For each plant, stem browning were also assessed.

Statistical analyses

Disease severity data were analysed using Minitab version 17.1.0 software. *Post hoc* analyses (Tukey's HSD test of multiple comparisons) were subsequently performed considering 95% confidence level.

A phylogenetic tree was constructed from pairwise distance matrix by UPGMA, applying MEGA version 7.0 software. Bootstraps analysis with 1,000 replicates was carried out to estimate the statistical support for differ-

ent tree branches, and the number on each branch represented the bootstrap value.

Analysis of molecular variance (AMOVA), provided by the Arlequin version 3.5.1.2 software, using 1,023 permutations, was used to calculate the variance within the *F. culmorum* collection, based on ISSR molecular markers.

RESULTS

Distribution of Fusarium culmorum isolates in Tunisia, and their molecular chemotyping

A total of 104 *F. culmorum* isolates were obtained from the basal stems of symptomatic durum wheat plants. The greatest number of isolates (44) was collected from the the sub-humid region of Bizerte.

Among the 104 *F. culmorum* isolates 100 (96%) were of the 3-ADON chemotype, and were obtained from all the Tunisian regions considered. Only four isolates were ascribed to the NIV chemotype, and all NIV chemotype isolates were collected from two fields of the sub-humid region of Bizerte

EF1-α haplotype distribution

The identification of both SNPs (A or T) of the EF1-α partial sequence of the *F. culmorum* isolates confirmed the presence of the two haplotypes within the *F. culmorum* population. Both haplotypes were homogenously and equally distributed in the region of Beja, whereas the A haplotype was dominant in the other three tested regions (Figure 1).

ISSR marker analysis

Based on three ISSR primers (ISSR4, ISSR5, and ISSR6), 27 bands were scored, and ranged from 0.39 to 2.6 Kb. The most informative primer was ISSR4 (12 amplicons). Bootstrapping gave values less than 50%. The cluster analysis with the UPGMA using pairwise genetic distances indicated high similarity between the *F. culmorum* isolates (Figure 2). Results from AMOVA provided an estimated pattern of population differentiation. All ISSR variation was distributed among isolates within populations (98.3% of the total variance). A Small proportion (1.7%) of the variability was explained by differences between populations and was not statistically significant ($P = 0.12$; Table 4). In addition, no statistically significant correlations were detected between cluster-

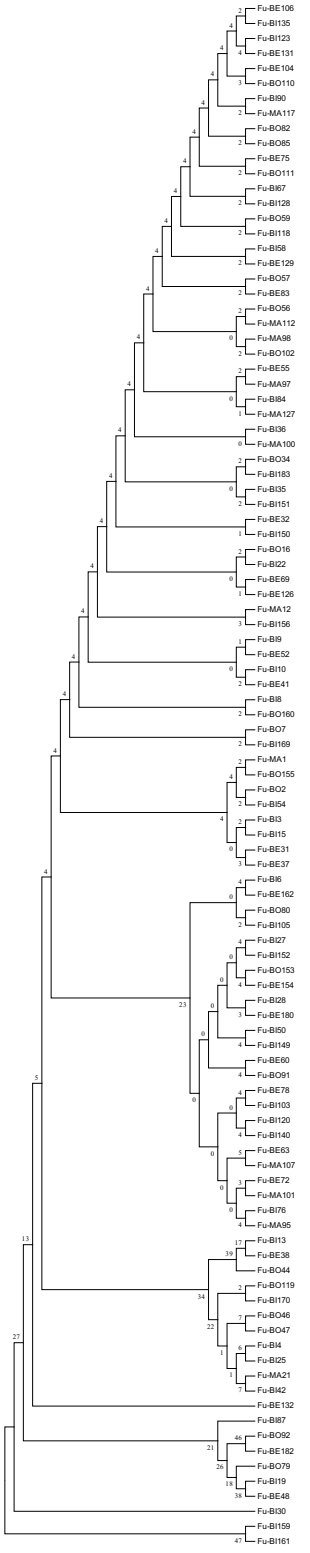

Figure 2 UPGMA analysis of *Fusarium culmorum* populations, based on combined ISSR data analyzed using using pairwise distances matrix. The numbers on the branches represent bootstrap support values.

Table 4 Analysis of molecular variance of ISSR for 104 isolates of *Fusarium culmorum* from four Tunisian populations.

Source of variation	*d.f*[a]	*SSD*[b]	Variance components	Percentage of variation
Among population	*3*	3.142	0.01282 Va	1.71
Within population	99	73.052	0.73790 Vb	98.29
Total	102	76.194	0.75072	
Va and FST : *P* (rand. value > obs. value) = 0.12121 *P* (rand. value = obs. value) = 0.00000 *P*-value = 0.12121±0.01052				

[a] *df* degrees of freedom;
[b] *SSD* sums of squared deviations;
[c] *P* probability of obtaining equal or large value determined by1023 randomizations of the treatments.

ing analyses, virulence, chemotype and haplotype of the isolates.

Aggressiveness of Fusarium culmorum *isolates*

In the pathogenicity test, root symptoms caused by *F. culmorum* isolates were detected in almost all cases (91%), with severity values ranging from 16 to 100%. A highly significant effect of chemotype on isolate aggressiveness was observed, with the 3-ADON isolates being more aggressive than the NIV isolates ($P = 0.007$; Table 1). However, few NIV chemotype strains were analyzed, which did not allow robust statistical analysis. In contrast, no statistically significant effects were detected for geographical origin ($P = 0.324$) or haplotype ($P = 0.877$) on isolate aggressiveness.

DISCUSSION

Fusarium culmorum is reported as the predominant cause of FRR disease in Tunisia, since incidence of the pathogen is promoted by dry springs and irregular rainfall (Gargouri *et al*., 2001). Pedo-climatic conditions in wheat-producing Tunisian regions are very similar to those in Sardinia (Balmas *et al*., 2015), and in Turkey (Tunali *et al*., 2008), where *F. culmorum* is the most common species causing FRR.

The present research aimed to investigate the genetic variability and the structure of *F. culmorum* populations associated with FRR on durum wheat in Tunisia. *F. culmorum* strains were collected from different wheat production areas, showing different climatic conditions. Two distinct chemotypes (3-ADON and NIV) were distinguished in the Tunisian populations of *F. culmorum*, the 3-ADON chemotype was the most common. This confirms previous reports by Rebib *et al*. (2014) on two fields showing FRR symptoms. The NIV chemotype was detected for the first time as associated to FRR in Tunisia, although this chemotype was found only in two fields in the Bizerte region.

Based on chemotype characterization of Italian *Fusarium* species, Covarelli *et al*. (2015) suggested that climatic conditions may strongly affect the occurrence of 3-ADON and 15-ADON, whereas NIV contamination may occur regardless of climatic conditions. Kammoun *et al*. (2010) reported that most isolates (98%) causing Fusarium head blight were 3-ADON producers, while 2% were NIV producers and originated from Bizerte, results which agree with those reported here. However, further investigations are required to better understand if specific agronomic or environmental conditions favour the presence of NIV-chemotype strains (Beyer *et al*., 2014).

While previous reports from Tunisia have focused on FHB chemotyping (Bensassi *et al*., 2009; Kammoun *et al*., 2010), the present study represents the first geographic survey in different areas of Tunisia, and of the chemotypes of *F. culmorum* causing FRR on durum wheat. These results are in agreement with previous studies carried out in northern Mediterranean countries, including Italy, France, Portugal and Yugoslavia (Logrieco *et al*., 2003; Pasquali and Migheli, 2014), as well as Germany and the United Kingdom (Tóth *et al*., 2004; Jennings *et al*., 2004). These reports confirm that the DON chemotype predominates among *F. culmorum* strains recovered from cereal grains.

Genetic chemotyping is an essential tool for characterizing *F. culmorum* populations causing root rot on wheat, but the presence of an amplification product reflects the possibility of a particular toxin being produced, whereas biosynthesis of the toxin remains to be confirmed by chemical analysis.

In our previous study of *F. culmorum* strains collected in the Sardinian region, association of haplotype A (EF1-α polymorphism) with FRR was highlighted (Balmas *et al*., 2015). To verify this association, an *ad hoc* survey on FRR-causing isolates from Tunisia was carried out. In this study, both A and T haplotypes were observed within the Tunisian *F. culmorum* population. No significant association was found between A-haplotype and FRR. It is therefore evident that, at least in Tunisia, the presence of the two haplotypes is not associated with specific fitness or virulence advantage in FRR pathogens on wheat.

ISSR markers were used to assess the genetic variability of *F. culmorum* populations in Tunisia, aiming

to verify the findings of Rebib *et al.* (2014) over a large geographic area. They suggested a high level of similarity among populations comparing two Tunisian fields. The present data confirmed that no clear trends were apparent in the distribution of the genetic variability with regard to the geographic origin within Tunisia. Our observations are in agreement with those reported by Albayrak *et al.* (2016), who showed similarity coefficients of 65.7-94.3% among *F. culmorum* isolates using ISSR4 and 41 other primers. The present report also confirms a previous study on the distribution of RAPD markers, suggesting that the low level of genetic differentiation among Tunisian populations of *F. culmorum* is mostly interpreted as the outcome of asexual reproduction in this pathogen (Gargouri *et al.* 2003). Similarly, Gargouri *et al.* (2003) indicated that no structuring had been observed at small or large geographic scales in this fungal species. Based on this assumption, the lack of a geographic structure in Tunisian populations of *F. culmorum* also suggests that spore dispersal probably occurs over a wide geographic area. These factors probably influence the level of genetic diversity within populations.

Using three markers, two of which (ISSR5 and ISSR6) were the same used in the present work, Mishra *et al.* (2003) found that 81% of the ISSR bands were polymorphic among *F. culmorum* populations: ISSR5 generated 28 ISSR distinct genotypes and ISSR6 produced 22 genotypes among 75 examined isolates. Moreover, the pattern of genetic diversity was largely associated to the geographical origin of the isolates (Mishra *et al.* 2003). In our study, no clear spatial clustering or relationships between variability and geographical regions were observed. Other genotyping assays have failed to show clear correlation between genetic variability and the geographic origin, mostly because the tested populations were from limited agro-ecological areas (Gargouri *et al.* 2003; Mishra *et al.* 2003).

The current survey provides a first insight into the genetic diversity of the *F. culmorum* population causing FRR in the main durum wheat growing regions of Tunisia. Further analyses with greater numbers of *F. culmorum* isolates from throughout Tunisia are warranted. Nonetheless, these preliminary data provide knowledge at the country scale on chemotyping and haplotyping, as an aid to ensure food safety monitoring, and for development of effective disease prevention and control strategies.

ACKNOWLEDGEMENTS

This research was supported by Fondazione di Sardegna and by Regione Sardegna ["Selection of sustainable phenolic inhibitors of trichothecene biosynthesis: in vitro studies and computational insights into the structure-activity relationship (SSPIT)"]; by Ministère Tunisien de l'Enseignement Supérieur et de la Recherche Scientifique [Faculté des Sciences de Bizerte, Laboratoire de Bio-surveillance de l'environnement (LR01/ES14)]. S.O. acknowledges financial support by SIPCAM Italia SpA.

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