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Full Length Research Paper

Identification and pathogenicity assessment of *Fusarium* spp. sampled from durum wheat fields in Tunisia

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Durum wheat is the major cereal crop cultivated in Tunisia; covering over 40% of the cereal growing areas. Durum wheat production remains below expectation due to its low productivity that is attributed to the chronically abiotic and biotic stresses. Fusarium head blight (FHB) caused by *Fusarium* spp. has become an important disease on durum wheat (*Triticum turgidum* L var. *durum*) in Tunisia, particularly during humid season. To identity the *Fusarium* species associated with FHB, samples were collected from five and six durum wheat fields in Northern Tunisia during 2004 and 2007 crop seasons, respectively. A total of 241 single spore cultures were isolated and seven different *Fusarium* spp. were identified using morphological traits and species-specific PCR assays. Pathogenicity of FHB causal agents was assessed on predominant durum wheat varieties. Aggressiveness of selected *Fusarium* isolates was investigated. Three durum wheat cultivars, characterized by different level of susceptibility to FHB, were artificially inoculated. Symptoms of FHB were rated as percentage of infected spikelets (PIS) at 7, 14 and 21 days after inoculation (DAI) and area under disease progress curve (AUDPC) was analysed. *Fusarium culmorum* was the dominant species representing 36.3% of all sampled isolates. All *Fusarium* species tested caused visible infections to the durum wheat cultivars with significant difference in aggressiveness among species. *F. culmorum* was considered highly pathogenic with an AUDPC=243.7, followed by *Fusarium pseudograminearum* (AUDPC=216.7). The remaining species, which had <150 AUDPC, were moderately and weakly pathogenic. There were significant differences ($P < 0.05$) in aggressiveness among isolates within species suggesting that screening for resistance to FHB requires a mixture of several isolates within and among *Fusarium* species.

Key words: *Fusarium* head blight (FHB), *Fusarium* species, pathogenicity, durum wheat.

INTRODUCTION

Fusarium head blight (FHB) is a widespread disease that affects cereals under warm and humid conditions. The disease has become a serious problem causing signify-cant reduction of grain yield and quality in most of durum wheat production areas (Olivier et al., 2008). Conservation tillage (Wilcoxon et al., 1988; Bai and Shaner, 1994), a common cultural practices and lack of effective fungicide control (McMullen et al., 1997) are considered among the principal conducive factors for the disease development and spread. Head blight results from the development of a complex of two genera of pathogenic fungi: *Microdochium* and *Fusarium* (Simpson et al., 2001). *Microdochium nivale* (*Monographella nivalis*) is one of the predominant causal organisms associated with FHB. *M. nivale* isolates have been classified as two subspecies: *M. nivale* var *nivale* and *Microdochium nivale* var *majus* (Brennan et al., 2005). *Fusarium* species including: *Fusarium graminearum* Schwabe, *Fusarium culmorum* (Wm.G. Sm.) Sacc., *Fusarium acuminatum* Ellis and Everhart, *Fusarium*

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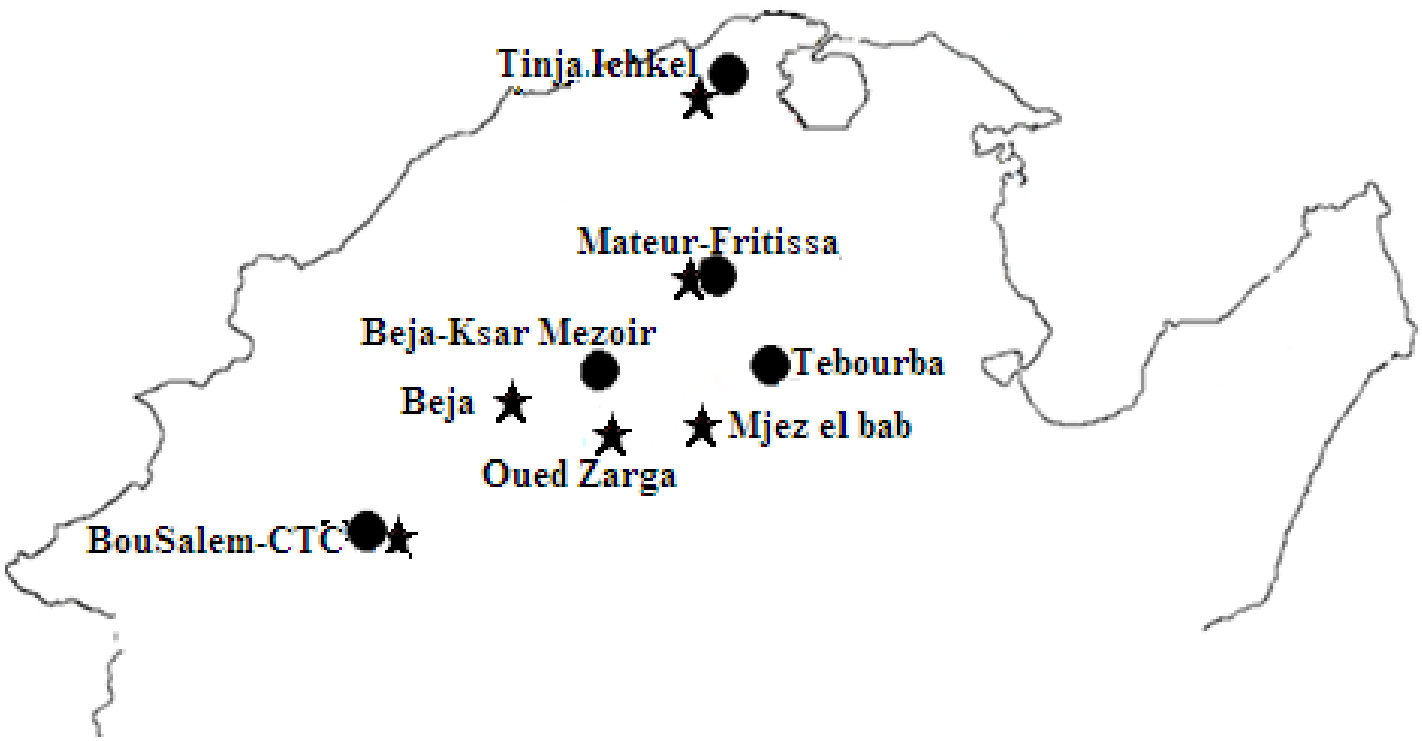

Figure 1. The surveyed fields localisation in north cereals area in Tunisia during 2004 (●) and 2007 (★) cropping seasons.

avenaceum (Corda:Fr.) Sacc., *Fusarium crookwellense* Burgess, (Nelson et al., 1983), *Fusarium equiseti* (Corda) Sacc., *Fusarium poae* (Peck) Wollenw. and *Fusarium sporotrichioides* Sherb., have been isolated from FHB-infected kernels (Fernandez et al., 2001; Xue et al., 2002). *Fusarium pseudograminearum* (teleomorph *Gibberella coronicola*) caused severe FHB epidemics in Australia (Akinsanmi et al., 2004). This specie was recently isolated from infected kernel in Tunisia (Kammoun et al., 2009). The geographical distribution of these *Fusarium* species is related to their temperature requirements (Parry et al., 1995). In warmer regions including the USA, Canada, Australia and central Europe, *F. graminearum* (teleomorph *Gibberella zeae*) is generally regarded as the most important species for FHB, whereas in the cooler regions of north-western Europe, *F. culmorum* predominates (Parry et al., 1995).

Several investigations showed that, *F. graminearum* and *F. culmorum* were the most aggressive among these species (Fernandez et al., 2001; Gilbert et al., 2000; Xue et al., 2002; Gale, 2003; Golinski et al., 2002). Furthermore, significant quantitative variation for aggressiveness has been also observed within individual field populations of theses species (Miedaner et al., 2001). *F. avenaceum* was characterized by intermediate levels of pathogenicity, although, this specie was also reported to be as pathogenic as *F. culmorum* and *F. graminearum* (Golinski et al., 2002). *F. sporotrichioides* was associated with an intermediate pathogenicity (Stack and McMullen, 1985; Wong et al., 1995). Other *Fusarium* species such as *F. equiseti* and *F. poae* were considered the least pathogenic species (Fernandez and Chen, 2005).

The objective of this study was to identity *Fusarium* spp. causing FHB on durum wheat in Tunisia and to assess the ability of the sampled *Fusarium* isolates causing FHB disease on three different resistance durum wheat cultivars.

MATERIALS AND METHODS

Collection of field samples and isolation of *Fusarium* spp.

During 2004 and 2007, five and seven commercially grown durum wheat varieties in Northern Tunisia were surveyed for FHB at the grain filling stage between April and May, respectively. The sampling sites (Figure 1) represented the major durum wheat areas in the country where the annual rainfall was high (> 500 mm) in the sub-humid areas to low (<500 mm) in the semi-arid areas. Sampling was also carried out from irrigated areas. Durum wheat variety “Karim”, the most commonly grown cultivar in this region, covers over 60% of the wheat area and “Khiar”, the recommended durum wheat variety in irrigated area covering less than 20%, are ranked as susceptible to FHB.

Disease incidence and severity were estimated using a hierarchical method. Three transacts of approximately 25 m apart and at 25 m intervals along each transact, were used. At each of

the nine sampling points, disease incidence (DI) and percentage of infected spike (PIS) were estimated from an area of about 0.25 m^2. Disease incidence (DI) was estimated as the proportion of diseased spikes (number of spikes with non zero symptoms divided by the total number of spikes sampled). Percentage of infected spike (PIS) was recorded as the average proportion of diseased spikelets per spike (sum of the proportion of diseased spikelets per spike divided by the total number of spikes sampled with no zero severity). The mass disease index of FHB (MDI) can be calculated following the method of Ding et al. (1993): MDI= (DI x PIS)/ 100. Three to four heads with typical FHB symptoms were collected, at each of the nine sampling points, by turning around once in a circle and sampling from an area of about 1 m^2.

Areas where spikes were collected were BouSalem, Beja, Tinja, Mateur and Tebourba. Precipitations in these areas during March, April and May were recorded every tenth day for 2004 and 2007 crop seasons. Data was collected by Tunisian agriculture climatic services.

Identification of *Fusarium* spp.

From each infected head, 4 to 6 seeds with signs of FHB infection were surface disinfected in a 75% ethanol solution for 30 s and then in 10% sodium hypochlorite NaOCl solution for 1 min then rinsed twice in sterile distilled water, then plated on potato dextrose agar (PDA) and incubated at 25°C for 3 to 5 days. All *Fusarium* colonies growing from kernels were purified by sub culturing onto PDA and incubated at 25°C for 5 to 7 days. Single spore isolates were obtained by streaking a spore suspension onto water agar plates and picking single pre-germinate macro-conidia after 24 h with a sterile needle and transferring to a new PDA culture plate. Single spore cultures were grown on PDA and carnation leaf agar (CLA) to record morphological characters used in identification kits. All isolation plates were preliminarily assigned to species and were selected and grouped based on pigmentation and the arrangement of conidia and conidiophores and also on the color of the colonies, according to the descriptions provided by Burgess et al. (1994). The colony morphology, density, pigmentation of aerial mycelium and of the media and the extent of mycelia growth were recorded on PDA. The shape and size of macro-conidia, presence/absence of micro-conidia, chlamydospores and perithecia were recorded on CLA. When present, the shape, size, septation and the formation of micro-conidia were also recorded.

The selected mono-conidial isolates were subjected to species-specific PCR assay to confirm the morphological identification. For DNA preparations, mycelium from 7 day old colonies grown on PDA was used to inoculate aseptically 75 ml of potato dextrose broth (PDB). Cultures were incubated on an orbital shaker (100 rpm) at room temperature for 10 days. Mycelium was harvested from liquid cultures by filtration onto Whatman no.1 filter paper disks. After rinsing with distillated water, mycelium was lyophilized. Freeze-dried mycelium was ground in liquid nitrogen to a fine powder in a sterile mortar and placed in freezer at -25°C. DNA preparation was carried out in 2-ml Eppendrof tube fill in 2/3 with a quantity of powder dried mycelium. DNA was extracted in 800 µl 2% CTAB (Cetyl trimethylammonium bromide) and incubated at 65°C for 60 min and every 15 min the mixture was homogenized. An equal volume of chlorophorm: isoamylalcohol (24: 1) was added and centrifuged at 12000 rpm to eliminate proteins. The aqueous phase was subsequently removed to a fresh tube and an equal volume of cold isopropanol plus 1/10 volume of ammonium acetate (3 M, pH 8.0) were added followed by centrifugation (10 000 rpm for 5 min) as earlier stated, to precipitate the DNA. The resulting pellet was washed in 2 rinses of cold 70% ethanol, dried, dissolved in 100 µl TE buffer (10 mM tris–HCl (pH 8.0), 0.1 mM EDTA). The DNA was quantified by UV spectrophotometer at 260 ηm and following gel electrophoresis, by comparison with DNA standards. PCR amplification was carried out in a 25-µl reaction mix containing 25 ηg fungal DNA, 100 µM each of dNTPs (dATP, dCTP, dGTP, dTTP), 25 pmoles of forwards and reverse primer for a given species, 0.8 units *Taq* polymerase (Biotech Int.) in 10 X PCR polymerase reaction buffer (tris-HCl, 10 mM pH 8.3, KCl, 50 mM, $MgCl_2$, 2 mM et 0.001% of gelatine). The primers used are indicated in Table 1. A thermocycler was programmed for initial denaturation at 94°C for 3 min, followed by 40 cycles with specific programs for each primer pair (Table 1), followed by a final extension at 72°C for 7 min and 4°C hold. PCR products were separated by electrophoresis on 1.5% agarose gels in TBE buffer (0.9 M tris, 900 mM boric acid, 2 mM EDTA, pH 8.0). Gels were stained with ethidium bromide (10 mg/µl) and photographed under UV light.

FHB pathogenecity assessment of *Fusarium* spp.

Head pathogenicity tests of 222 identified mono-conidial isolates were conducted on three durum wheat cultivars 'Divide', 'Karim' and 'Khiar'. Divide selected from the cross 'Ben' (PI 596557)/D901282//'Belzer' (PI 603286) made in 1993 (Elias and Manthey, 2007) is moderately tolerant (Elias, unpublished data). Karim: *D21563/AA"S"//Fg"S"* (Deghaïs et al., 2007) and Khiar: *Chen"S"/Altar 84* (Deghaïs et al., 2007) were susceptible and highly susceptible to FHB, respectively, based on the field evaluation at BouSalem-CTC in 2007 (Anonymous, 2008). Plants were grown in 8-cm diameter and 20-cm height plastic pots filled with a mixture of clay loam soil and peat moss. Plants were grown in the greenhouse and fertilized at tillering and booting stage using 5 g nitrogen in each pot. A completely random design was used with (222 mono-conidial isolates + check: sterile distilled water) x 3 cultivars × 3 plants per cultivar.

Individual wheat heads were inoculated in the greenhouse at mid-anthesis. Inoculation was performed following the methods described by Stack et al. (2002). The inoculum was prepared by flooding the culture with sterile distilled water and straining the resulting suspension through sterile cheesecloth. The final conidia suspension was adjusted to a concentration of 5.10^4 spores ml^{-1}. A droplet of conidia (about 1000 spores) was injected into a central floret of selected spikes with a hypodermic syringe. Control plants were injected in a similar manner with sterile distilled water. Disease development was recorded on each inoculated spike. In order to enhance disease development and increase the accuracy of the evaluation, humidity was maintained for 72 h post-inoculation period by covering each spike with a plastic bag and misting at least once daily. In the greenhouse, the inoculated plants were misted with tap water from a mist irrigation system. The average temperature was 25°C during the day with a range of 18 to 32°C and 17°C at night with a range of 15 to 19°C. Symptoms on wheat spikes varied from light brown, water-soaked spots on the glumes to bleached spikelets. Each inoculated spike was examined visually. The Percentage of infected spike (PIS) was recorded visually as the average proportion of diseased spikelets per spike at 7, 14 and 21 days after inoculation (DAI), PIS-7, PIS-14 and PIS-21, respectively. Note that, the actual number of spikelets was not counted; rather a single estimate of the proportion of spikelets with symptoms was made for each spike. Area under disease progress curve (AUDPC) was calculated based on the PIS data using a modified formula of Shaner and Finney (1977).

Statistical analysis

The percentage of infected spike and AUDPC average values were calculated for each pot. The effects of durum wheat cultivar, fungal species and interaction cultivar fungal species on percent FHB and AUDPC were tested using Proc GLM of SAS (2007). Within each

Table 1. Species-specific primers used to identify *Fusarium* and *Michrodochium* species.

Species	Primer	Sequence 5' → 3'	Amplification size (bp)	Reference
F. graminearum	Fg 16 NF Fg 16 NR	ACA GAT GAC AAG ATT CAG GCA CA TTC TTT GAC ATC TGT TCA ACC CA	280	Nicholson et al. (1998)
F. graminearum	Fg 16 F Fg 16 R	CTC CGG ATA TGT TGC GTC AA ACT GTG CAC TGT CGC AAG TG	450	Nicholson et al. (1998)
F. graminearum	GaoA-V2 GaoA-R2	AGG GAC AAT AAG TGC AGA ACT GTG CAC TGT CGC AAG TG	898	Nissen and Vogel (1997)
F. pseudograminearum	Fp 1-1 Fp 1-2	CGG GGT AGT TTC ACA TTT CYG GAG AAT GTG ATG ASG ACA ATA	520	Aoki and O'Donnell (1999)
F. avenaceum	AF AR	CAA GCA TTG TCG CCA CTC TC GTT TGG CTC TAC CGG GAC TG	920	Lees (1995)
F. acuminatum	FAC-F FAC-R	GGG ATA TCG GGC CTC A GGG ATA TCG GCA AGA TCG	600	Williams et al. (2002)
F. equiseti	FEF1 FER1	CAT ACC TAT ACG TTG CCT CG TTA CCA GTA ACG AGG TGT ATG	400	Mishra et al. (2003)
F. culmurum	OPT18F OPT18R	GAT GCC AGA CCA AGA CGA AG GAT GCC AGA CGC ACT AAG AT	470	Schilling et al. (1996)
M. nivale var. Nivale	Y13NF Y13NR	ACC AGC CGA TTT GTG GTT ATG GGT CAC GAG GCA-GAG TTC G	310	Nicholson et al. (1996)
M .nivale var. Majus	Y13 MF Y13 MR	CTT GAG GCG GAA GAT CGC ATC CCT TTT CCG GGG TTG	220	Nicholson et al. (1996)
F. sporotrichoides	AFCF AFCR	AAA AGC CCA AAT TGC TGA TG TGG CAT GTT CAT TGT CAC CT	330	Demeke et al. (2005)

Ns, non significant; * and ** , significant at $P < 0.05$ and $P < 0.01$, respectively

species, fungal isolates, durum wheat cultivars and interaction fungal isolates by durum what cultivars were examined for aggressiveness using proc ANOVA of SAS

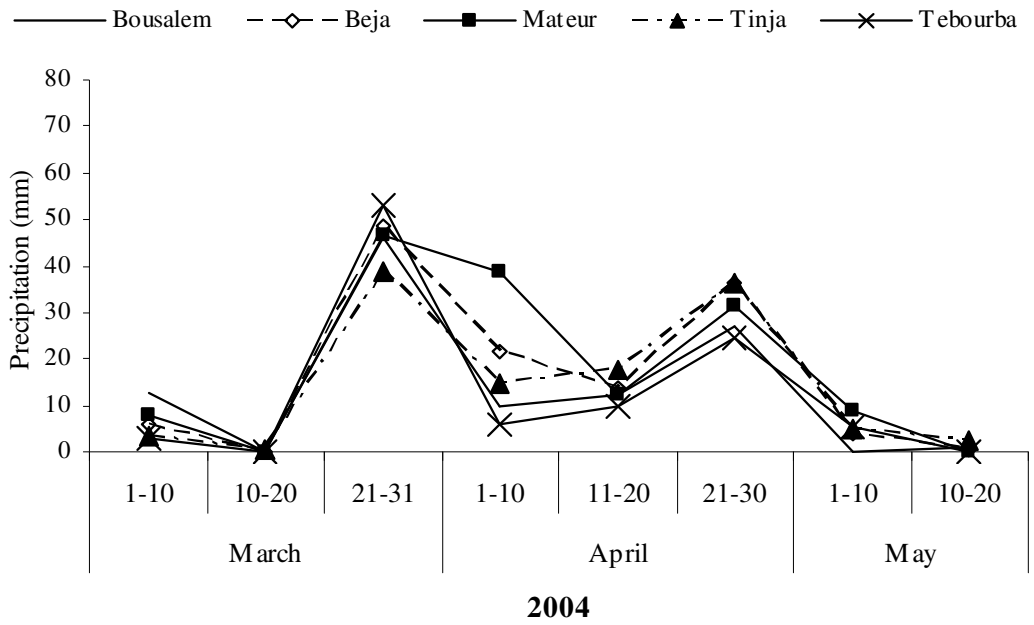

Figure 2. Rainfall during March, April and May 2004 registered in BouSalem, Beja, Mateur, Tinja and Tebourba station nearest prospected fields.

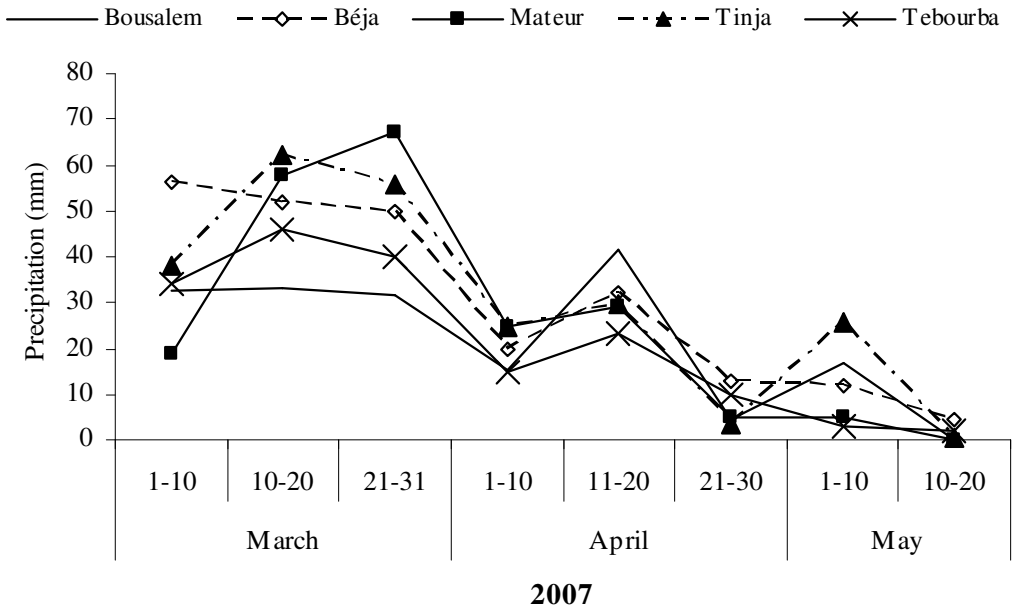

Figure 3. Rainfall during March, April and May 2007 registered in BouSalem, Beja, Mateur, Tinja and Tebourba station nearest prospected fields.

(2007).

RESULTS

Weather conditions

Optimal heading period was from late March to early April for durum wheat grown in the northern area of Tunisia. Precipitation levels in March were higher in 2007 by over 100 mm and were well distributed. Over 30 mm precipitation was recorded almost every 10 days (Figure 3). However, the highest precipitation levels recorded in March 2004 were observed during the last ten days with lower precipitation of less than 10 mm, in the first ten days and dry climatic conditions followed the second ten days of the month (Figure 2). Both precipitation levels in

Table 2. FHB disease incidence (DI), the percentage of infected spike (PIS), the mass disease index (MDI) (%), the number of infected spikes collected and the number of *Fusarium* cultures isolated assessed during 2004 and 2007 cropping seasons from 12 durum wheat fields.

Sampling site	Previous crop	Cultivar	FHB DI (%)	FHB PIS (%)	MDI (%)	Number of sample	Number of single spore isolated
2004							
Tinja-Ichkel	Wheat	Karim	24.4±8.8	17.8±6.7	4.7±2.8	30	20
Mateur-Fritissa	Legume	Karim	17.8±6.7	12.2±4.4	2.1±0.9	34	22
Beja-Ksar Mezoir	sunflower	Karim	15.0±6.1	10.0±2.5	1.4±0.6	34	17
BouSalem-CTC *	Durum wheat	Khiar	56.9±19.1	26.6±11.3	17.0±11.0	35	18
Tebourba	Durum wheat	Karim	21.1±7.8	11.7±3.5	2.4±1.0	35	16
2007							
Tinja-Ichkel	Durum wheat	Karim	37.5±10.0	19.9±6.2	7.9±4.5	33	21
Mateur-Fritissa	Legume	Khiar	86.3±6.5	36.7±10.6	32.2±11.6	36	22
Beja	Legume	Karim	34.6±9.0	19.1±4.6	6.9±3.2	25	32
BouSalem-CTC	Legume	Khiar	70.5±6.7	35.7±7.8	25.5±8.0	33	26
Oued Zarga	Oats	Karim	35.0±7.7	26.0±6.7	9.3±4.1	29	21
Mjez El bab	Durum wheat	Karim	33.2±4.8	22.8±6.6	7.7±3.1	33	25

*Supplemental irrigation was applied in March.

April 2004 and 2007 were equal, although, precipitations were recorded during the third ten days in 2004 and the second ten days in 2007. Precipitation levels during the first ten days in May were higher in 2007 and exceed 12 mm in surveyed fields (Figure 3).

Identification of *Fusarium* spp.

During 2004, disease incidence ranged from 17 to 56.9% with percentage of infected spike ranging from 10 to 26.6%. The highest disease level was observed at BouSalem-CTC experimental site (Table 2). From a total of 168 infected spikes collected in 2004, 93 single spore cultures were isolated: 20 from Tinja-Ichkel, 22 from Mateur-Fritissa, 16 from Tebourba, 17 from Beja-KsarMezoir and 18 from BouSalem-CTC. During 2007 crop season, disease incidence (DI) and percentage of infected spike (PIS) ranged from 33.2 to 86.3% and from 19.1 to 36.7%, respectively. From the infected kernels collected from 189 affected spikes in 2007, 147 *Fusarium* single spore cultures were developed: 22 from Mateur-Fritissa, 21 from Ichkel-Tinja, 26 from BouSalem-CTC, 21 from Oued Zarga, 25 from Mjez bab and 32 from Beja. Seven *Fusarium* species: *F. culmorum, F. avenaceum, F. equiseti, F. pseudograminearum, M. nivale var nivale, F. acuminatum and F. sporotrichoides,* were identified using micro- and macro- morphology and confirmed with species-specific PCR assays. Primer pairs for *F. culmorum* (OPT 18F/OPT18R), *F. avenaceum* (AF/AR), *F. equiseti* (FEF1/FER1), *F. pseudograminearum* (Fp1-1/Fp1-2), *M. nivale* var. *nivale* (Y13NF/Y13NR), *F. acuminatum* (FAC-F/FAC-R) and *F. sporotrichoides* (AFCF/AFCR) gave specific fragments of 470, 920, 400, 520, 310, 600 and 330 bp, respectively. No specific amplification was observed for (Y13M F/Y13M R) specific for *M. nivalea* var. *majus* and no specific amplification was observed for (Fg16NF/Fg16NR), (Fg16F/ Fg16R) and (GaoAF/GaoAR) specific for *F. graminearum*.

From all infected kernels collected in 2004 and 2007, *F. culmorum* was the most common species developed (36.3%). In addition, *F. avenaceaum, F. equiseti, F. pseudograminearaum, M. nivale* var. *nivale* were isolated with intermediate frequencies 17.5, 17.1, 9.2 and 8.3%, respectively. Differences were detected between locations and crop seasons. In 2004, *F. culmorum* was more prevalent, comprising 50.7% of all isolates.

Table 3. Percentage (%) of *F. culmorum (F.cl), F. avenaceaum (F. av), F. equiseti (F. eq), F. pseudograminearaum (F. psdgr), M. nivale* var. *nivale (M. niv* var *niv), F. acuminatum (F. ac), F. sporotrichoides* (*F. spr*) and others *Fusarium* spp. (*F.* spp) isolated from infected kernels collected from different durum wheat fields during 2004 and 2007 cropping season.

Parameter	*F.cl*	*F. av*	*F. eq*	*F. psdgr*	*M. niv var niv*	*F.ac*	*F. spr*	*F.* spp
2004								
BouSalem-CTC	77.8	5.6	5.6	5.6	5.6	0.0	0.0	0.0
Tinja-Ichkel	55.0	15.0	5.0	0.0	10.0	5.0	0.0	10.0
Mateur-Fritissa	36.4	22.7	18.2	0.0	4.5	13.6	0.0	4.5
Beja-Ksar Mezoir	47.1	11.8	23.5	5.9	5.9	0.0	0.0	5.9
Tebouba	37.5	25.0	6.3	12.5	6.3	6.3	0.0	6.3
Mean	50.7	16.0	11.7	4.8	6.4	5.0	0.0	5.3
2007								
BouSalem-CTC	15.4	19.2	11.5	15.4	15.4	0.0	7.7	15.4
Tinja-Ichkel	14.3	52.4	19.0	4.8	9.5	0.0	0.0	0.0
Mateur-Fritissa	13.6	4.5	45.5	4.5	9.1	0.0	4.5	18.2
Beja	9.4	3.1	34.4	25.0	12.5	0.0	3.1	12.5
Mjez bab	76.0	12.0	0.0	8.0	0.0	0.0	0.0	4.0
Oued Zarga	38.1	28.6	9.5	9.5	9.5	4.8	0.0	0.0
Mean	27.8	20.0	20.0	11.2	9.3	0.8	2.6	8.3

Ns, non significant; * and ** , significant at P < 0.05 and P < 0.01, respectively.

F. avenaceaum was moderately frequent (16%) and was the most frequent species isolated from infected kernels from Mateur-Fritissa (22.7%) and Tebourba (25%). *F. equiseti* was detected at lower frequencies (11.7%) with large infection of samples collected from Beja-Ksar Mezoir (23.5%). The other species *F. pseudograminearaum, M. nivale* var. *nivale, F. acuminatum* and *F. sporotrichoides* were isolated in trace amounts (Table 3). In 2007, *F. culmorum* was the most frequently species isolated from infected kernels collected (27.8%). This species was highly recovered from infected kernels sampled from Mjez bab (76%) and Oued Zarga (38.1%). From the infected kernel collected in 2007, *F. avenaceum* was the most prevalent species in infected kernels collected from Tinja-Ichkel (52.4%) and *F. equiseti* was the most dominant species isolated in collected spikes from Mateur-Fritissa and Beja (Table 3). Scabbed kernels collected from BouSalem-CTC during 2007 were infected at least by five causal species with equal frequency (Table 3). *F. pseudograminearum* was mostly isolated from the infected kernels sampling from Beja in 2007 (Table 3).

Assessment of FHB severity

Eighty seven (87) isolates of *F. culmorum,* 42 isolates of *F. avenaceum,* 41 isolates of *F. equiseti,* 22 isolates of *F. pseudograminearum,* 20 isolates of *M. nivale* var. *nivale,* 6 isolates of *F. acuminatum* and 4 isolates of *F. sporotrichoides*, were tested in an FHB bioassay. All species tested produced similar FHB symptoms in appearance. Significant differences in the percentage infected spike (PIS) 7, 14 and 21 days after inoculation (DAI) and AUDPC among species, response of durum wheat cultivars and the species × cultivar interaction were observed. The rate of FHB symptom development of the *Fusarium* species differed according to durum wheat cultivar tested (Table 4). Symptoms mostly started, on susceptible cultivars Khiar, 7 DAI with isolates of *F. culmorum, F. pseudograminearum, F. sporotrichoides* and *F. avenaceum*. However, disease establishment was noted later 7 DAI for other species (*M. nivale* var *nivale, F. equiseti* and *F. acuminatum*) and traces of disease symptoms were observed in the control inoculation.

Percentage infected spike (PIS) after 7, 14 and 21 DAI, appeared to be under the control of both *Fusarium* species and cultivar. Although, *F. culmorum* and *F. pseudograminearum* were the most aggressive on Khiar and Karim, significant differences in AUDPC were noted between theses species in the moderately tolerant cultivar Divide, where only *F. culmorum* was the most severe (Table 4). *F. sporotrichoides* was very aggressive on Khiar (AUDPC= 230.4), while limited disease development was noted on Divide. Significant differences ($P < 0.001$) were observed in AUDPC among seven *Fusarium* species. *F. culmorum* had the greatest AUDPC (243.7) followed by *F. pseudograminearum* (216.7). These species were considered highly pathogenic on the durum cultivars tested. *F. sporotrichoides* and *F. avenaceaum* had AUDPC 136.1 and 123.6, respectively and were considered moderately pathogenic. The remaining species characterized by AUDPC around 80 were considered as weakly pathogenic.

Table 4. Quantitative differences in area under disease progress curve (AUDPC) of seven *Fusarium* species noted on Khiar, Karim and Divide cultivar and the mean of three cultivars (mean AUDPC) with minimum mean value (min. AUDPC) and maximum mean value (max. AUDPC).

Species	M. AUDPC			AUDPC Khiar	AUDPC Karim	AUDPC Divide
	Mean	Min	Max			
F. culmorum	243.7[a]	83.7	505.6	332.1[a]	290.7[a]	108.3[a]
F. pseudograminearum	216.7[b]	42.8	392.8	318.2[b]	255.1[a]	76.9[a]
F. sporotrichoides	136.1[c]	70.0	226.7	230.4[c]	154.6[b]	23.3[b]
F. avenaceum	123.6[c]	23.3	396.7	180.6[c]	147.2[b]	42.9[b]
F. acuminatum	82.3[d]	31.1	126.4	128.3[c]	101.1[b c]	17.5[c]
F. equiseti	79.1[d]	15.6	361.7	115.2[c]	94.5[c]	27.4[c]
M. nivale var nivale	83.8[d]	46.7	132.2	134.2[c]	90.4[c]	26.8[c]
Water	7.8	0.0	35.0	11.7	7.8	3.9
Mean				205.6	161.9	46.2

Means followed by the same letter within a column are not significantly different (P > 0.05).

Table 5. Mean squares from the analysis of variance for percentage of area under disease progress curve (AUDPC) of three cultivars inoculated with 87 isolates of *F. culmorum (F.cl),* 42 isolates of *F. avenaceaum (F. av),* 41 isolates of *F. equiseti (F. eq),* 22 isolates of *F. pseudograminearaum (F. psdgr),* 20 isolates of *M. nivale* var. *nivale (M. nv),* 6 isolates of *F. acuminatum (F. ac)* and 4 isolates of *F. sporotrichoides (F.spr).*

Source of variation	*F. cl*	*F. av*	*F. eq*	*F. psdgr*	*M. nv*	*F. ac*	*F. spr*
Replication	8618.6**	2630.4 [ns]	1300.2 [ns]	3991.7 [ns]	2022.8 [ns]	2252.6*	2177.7 [ns]
Isolate (I)	95472.6**	52865.4**	76614.8**	54440.5**	4777.2**	9817.4**	61250.0**
Cultivar (C)	3700385.2**	649599.4**	258853.4**	1033041.3**	174716.5**	59982.9**	131721.5**
I x C	15620.0**	5348.9**	6094.4**	10328.2*	931.1 [ns]	8745.6**	6907.6 [ns]
Error	5447.9**	2584.5	1203.5	216.7	2206.6	931.4	3862.1

ns, not significant.

Durum wheat cultivar responses were different for all *Fusarium* spp. Divide was considered the most tolerant and did not develop any symptoms after 7 DAI; this cultivar showed the lowest levels of PIS observed at 14 and 21 DAI with limited values of AUDPC. Khiar was the most susceptible characterized by the highest levels of PIS and AUDPC. No difference was detected in early infections between Karim and Khiar, but significant differences were noticed for PIS 14 and 21 DAI and AUDPC. Significant differences in infected spike after 7, 14 and 21 DAI and AUDPC among isolates within *Fusarium* species and durum wheat cultivars were depicted. Isolate x cultivar interactions for AUDPC were observed for all species except for *M. nivale* var. *nivale* and *F. sporotrichoides* (Table 5). Significant differences in pathogenicity were found among isolates within a single field and within single sampling plot. The effects of isolate × cultivar interaction, although, significantly contributed less than 20% of the total variation for the highly pathogenic species and were too low to differentiate any possible races among the isolates tested. Durum wheat cultivar responses to isolates within species were different. Disease progress on tolerant cultivar Divide was more important for the aggressive isolates, whereas less disease levels were noted for the less aggressive isolates. Highly pathogenic isolates were those characterized by AUDPC greater than 250. About 84% of highly pathogenic isolates belong to *F. culmorum*. In addition, 4 isolates of *F. pseudograminearum,* 2 isolates of *F. equiseti* and one isolates of *F. avenaceaum* were highly aggressive. The aggressive isolates of *F. culmorum* were collected from ten locations during both years, while the aggressive isolates of the remained species were collected only during 2007 crop season.

DISCUSSION

During the last decade, FHB was observed on several Tunisian durum wheat varieties in major wheat areas, particularly in 2004 and 2007 when precipitation levels and distribution at heading were abundant and well distributed. The surveys of the FHB during 2004 (Kammoun et al., 2009) and 2007 (Bensassi et al., 2010) suggested that the relative humidity prevailing during heading time could be a major component of FHB development in the sub-humid and higher semi-arid areas in Tunisia. This implies that both timing and amount of moisture are

important for the development of FHB. The level of infected kernels was associated with wheat growing areas and conductive climatic conditions during the cropping season particularly at flowering stage as suggested by Shaner (2003). The FHB disease development was more severe in 2007 season. Climatic conditions, especially precipitations at heading and anthesis were more conductive to disease establishment and spread. Precipitations were well distributed during the second and the third ten days of March and the first ten days of April that coincided with heading and flowering. Among prospected fields, disease development was more severe at BouSalem-CTC during both seasons and at Mateur-Fritissa during 2007. The occurrence of high disease levels in theses sites may have been favored by the growing of the high susceptible durum wheat cultivar "Khiar". Furthermore, spray irrigation applied at BouSalem-CTC field at early heading, mid March 2004, enhanced the disease establishment and increased its severity. These results would support that both timing and amount of moisture are important for the disease development.

The *Fusarium* species predominantly found in the sampling fields were *F. culmorum*, *F. avenaceum* and *F. equiseti. F. culmorum* was the most frequently isolated species from infected kernels of blighted heads collected in 2004 and 2007. This specie was the most prevalent at all prospected sites during 2004 and the most frequently encountered during 2007 at Mjez bab and Oued Zarga. This higher frequency of *F. culmorum* was expected, because this specie was the most causal agent of foot rot disease in Tunisia (Gargouri et al., 2001). Moreover, FHB pathogenicity assessment of this specie indicates no major differences between isolates from infected root rot or wheat spikes (Akinsammi et al., 2004). This result would support that, *F. culmorum* is omnipresent in rotted root or in infected spikes. In Italy, the most predominant species are *F. graminearum* followed by *F. culmorum, F. avenaceum* and *F. poae* (Pancaldi et al., 2004). Our survey shows that, *F. graminearum* (teleomorph *Gibberella zeae*), generally regarded as the most important specie for FHB (Bai and Shaner, 2004) was not found in Tunisia. The absence of this specie from kernels collected during 2004 in Tunisia was also reported (Kammoun et al., 2009). The epidemics of this species occurs through warm temperatures with high humidity (Mesterházy, 2003), which were often not likely to occur during the wheat growing season in Tunisia. In Italy, Shah et al. (2005) observed that the incidence of *F. graminearum* infection tended to decrease following the pattern of high rainfall in the north to limited rainfall in the south during the flowering period. Moreover, this specie is pathogenic specie on maize crop, while in Tunisia only limited areas were grown to maize explaining the absence of *F. graminearum*. Kammoun et al. (2009) showed that the most common species isolated from diseased wheat spikes, collected in several fields in Northern Tunisia during 2004, was *M. nivale var nivale* followed by *F. culmorum, F. pseudograminearum* and *F. avenaceum.* The lower relative frequency of *M. nivale* noted in this investigation could be attributed to the main sampling of field's assessment. The foliar fungicide treatment applied at booting-heading stage for all sampling sites, could contribute to the limitation of the *M. nivale* frequency. In fact, the epidemiology of *M. nivale* is quite different from the *Fusarium* species (Windels, 2000). Its incidence may be greatly influenced by the climatic conditions during the whole cereal growing season, as it may also behave as a leaf parasite before reaching the flowering spikes. The application of foliar fungicides reduced the incidence of *M. nivale* (Ioos et al., 2004). Moreover, the relevance of selected infected kernels in our investigation would explain the difference in frequency of *M. nivale* reported by Kammoun et al. (2009) who selected random kernel samples. Variations of *Fusarium* species frequency from year to year within regions as well as between regions have been well documented (Van Eeuwijiki et al., 1995). Shah et al. (2005) suggest that, species composition and relative frequencies of the pathogens within the FHB complex fluctuate in response to seasonal variation and permanent shifts in cultural practices and cropping systems. Several factors, such as abundance of inoculum and weather conditions at heading, contribute to changes in the spread of *Fusarium* species. During favourable growing conditions, it was apparent that several *Fusarium* species, such as *F. avenaceaum* and *F. equiseti,* were more frequent than the dominant species *F. culmorum* in some fields surveyed in 2007. Similarly, in the same field BouSalem-CTC survived during both seasons, the incidence of several *Fusarium* species other than *F. culmorum* was found to increase from 2004 to 2007. These results converge with that reported by Dill-Mackey and Jones (2000) who suggested that the frequency of isolation of *Fusarium* species between years appeared to be greater than differences between sites within a year. These results might be attributed to particular responsiveness of *Fusarium* species to the humid conditions prevailing prior and after heading. The moist conditions at heading and flowering during 2007 induced severe disease development and to large diversity of *Fusarium* species. These suggestions are in agreement with Ioos et al. (2004) who found that, less pathogenic *Fusarium* species development was associated with high post anthesis moisture caused by frequent rainfalls. Dry conditions prior to heading in 2004, during the second ten days in March, may have negatively affected inoculum development of some *Fusarium* species. Hooker et al. (2002) showed that, warm and moist conditions seven days before heading enhance the formation and maturation of inoculum of many *Fusarium* species to ensure the establishment of FHB.

The seven *Fusarium* spp. identified differ in the rate of FHB symptom development on the three tested durum

wheat cultivars. Our results show that, *F. culmorum* is a highly pathogenic specie causing most rapid and severe disease development on the three durum wheat cultivars. In our study, we observed greater pathogenicity of *F. culmorum* compared with other fungal species identified. These results are in agreement with previous reports (Walker et al., 2001; Xue et al., 2004). The large variability in pathogenicity among isolates of *F. culmorum* implies an important variability within population of this fungus. This FHB aggressiveness variability was also found among isolates sampled from the same field. Similarly, Miedaner and Schilling (1996) found a high degree of genetic variability for aggressiveness within single field populations of *F. culmorum.* Gargouri et al. (2003) have detected a large genetic variability within Tunisian population of *F. culmorum. F. pseudograminearum* caused severe disease development, although, no symptoms appeared during early infection on the tolerant cultivar Divide. High pathogenicity of *F. pseudograminearum* found in our study does not converge with that reported by Liddel (2003) who found that this specie was not able to cause FHB. Nevertheless, Akinsami et al. (2004) have reported that *F. pseudograminearum* caused severe FHB with no significant differences in aggressiveness between this specie and *F. graminearum*. Other species tested, *M. nivale* var. *nivale, F. acuminatum* and *F. equiseti* were associated with lower pathogenicity to FHB disease. *F. equiseti* was never associated with a high level of grain infection (Ioos et al., 2004; Fernandez and Chen, 2005), even though, number of isolates collected during 2007 were aggressive. These differences in pathogenecity among isolates were noted by Nicholson et al. (2003) especially in particular situation. The presence of several *Fusarium* spp. with different levels of FHB aggressiveness and large variability among isolates within species and within a field imply that resistant varieties need to be effective against several species and aggressiveness groups. Aggressive isolates should be used in the improvement of host plant resistance and the need to include representative isolates from dominant *Fusarium* spp.; low aggressiveness may not allow good discrimination among lines and cultivars of different levels of resistance. Moreover, small interactions between isolates and varieties occur, but do not affect ranking of varieties responses to isolates or *Fusarium* species infections (Eeuwijk et al., 1995; Snijders, 2004). The *Fusarium* head blight resistance in wheat can be described as horizontal and not specific for isolates or *Fusarium* species. In theory, any reasonably aggressive isolate should be satisfactory for screening purposes (Tóth et al., 2008). Some breeding programs use a single aggressive isolate in their screening programs, but since isolate aggressiveness has been shown to be affected by environment, most programs use a mixture of isolates (Rudd et al., 2001). Our results stressed the need to use a combination of pathogen isolates to evaluate host genotypes (Mesterharzy, 1987; Akinsami et al., 2004). Similar effects of the highly pathogenic isolates for different species on the three durum wheat cultivars indicate that durum wheat may share common genes for the resistance to these species and suggest that breeding for resistance to *F. culmorum* may also give enhanced resistance to other aggressive species. Similarly, van Eeuwijk et al. (1995) demonstrated that, wheat cultivars showed similar reactions to *F. graminearum* and *F. culmorum.* In addition, Xue et al. (2002) showed that, breeding for these species enhanced resistance to other aggressive species such as *Fusarium crookwellense*.

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