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Isolation, identification and aggressiveness characterization of *Fusarium* spp. associated with crown rot and Fusarium head blight in Algerian wheat

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Abstract

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Fusarium crown rot (FCR) and fusarium head blight (FHB) are two destructive fungal diseases of wheat in the world. This research was conducted to study the most important species associated with these diseases in Algeria. One hundred and seventeen samples were collected. Sixty-three from the crown and fifty-four from wheat grains in several regions of northeastern Algeria. *Fusarium* spp. have been identified by sequencing internal transcribed spacer regions (ITS1-2) of the rDNA, as well as their life lines (growth and sporulation rates). FCR pathogenicity tests were performed by two methods in vitro (Tube test) and in vivo (Pot test). A total of 34 isolates representing 10 *Fusarium* species was obtained. These results showed five species isolated from crown; *Fusarium culmorum* (16), *F. cerealis* (3), *F. acuminatum* (2), *F. graminearum* (1) and *F. oxysporum* (1). And seven species from grains; *F. culmorum* (3), *F. incarnatum* (3), *F. graminearum* (1), *F. equiseti* (1), *F. asiaticum* (1), *F. fujikuroi* (1) and *F. brachygibbosum* (1). *F. culmorum* strains were dominant and more aggressive. Moreover, *F. graminearum* and *F. cerealis* were aggressive in in-vitro test. However, the rest of species were more saprophytic than aggressive. The dendrogam showed a *Fusarium* rich diversity, in particular *F. culmorum* related to wheat FCR and FHB. A significant correlation was recorded between growth and sporulation rates ($r = 0.35$ $P = 0.012 < 0.05$). Furthermore, a strong correlation was recorded between tube and pot tests ($r = 0.62$, $P = 0.007 < 0.01$). This result provides a simple in vitro test to predict *F. culmorum* FCR aggressiveness on wheat. In addition, this is the first report concerning *F. incarnatum*, *F. asiaticum*, *F. acuminatum* and *F. graminearum* identifications from Algerian wheat.

Keywords: Algeria; biodiversity; fungus; pathogenicity; phytopathology

Abbreviations: FCR: Fusarium Crown Rot; FHB: Fusarium Head Blight; ITS: internal transcribed spacer regions; %GI the Germination Inhibition percentage; AUDPC: the Area Under the Disease Progress Curve

Introduction

Wheat (*Triticum* spp.) being a staple food for about 40% of the world's population plays a major role in global food security (Bockus et al., 2010). Unfortunately, it can be infected by many diseases caused by microorganisms that reduce the harvest quality and quantity. Among these diseases, fusarium crown rot (FCR) and fusarium head blight (FHB). These are two of the most destructive and damaging fungal diseases, transmitted by soils and residues at the worldwide level (Tunali et al., 2008; Shikur Gebremariam et al., 2017).

The major FCR symptoms are; seedling blight, lower stems honey-brown discoloration with nodes occasional pink discoloration or stems under leaf sheaths, and may colonize the entire stem's base up to the third node and white spots at maturity (Dyer et al., 2009; Hollaway & Exell, 2010; Beccari et al., 2011). Damage, caused by FCR pathogens, goes unnoticed until scattered white spots are observed near crop maturity (Paulitz et al., 2002). FHB symptoms include necrosis, head bleaching, shrunken kernels; while flowering is the most favorable stage for wheat infection (György et al., 2020). In addition to yield loss, a powerful mycotoxins variety that accumulate in grains during infection, produced by several *Fusarium* species cause FCR and FHB.

This *Fusarium* species complex varies according to time and geography (Xu & Nicholson, 2009), including, *F. pseudograminearum, F. culmorum* and *F. graminearum* known for their high pathogenicity and cause more severe symptoms and yield losses. They are the most common species in many countries (Smiley et al., 2005; Dyer et al., 2009; Bouanaka et al., 2023a). However, other low pathogenicity species including; *F. acuminatum, F. equiseti, F. oxysporum, F. cerealis, F. asiaticum, F. brachygibbosum, F. incarnatum, F. chlamydosporum* and *F. avenaceum*, are principally isolated from wheat subsoil tissue and are regularly considered secondary colonizers or saprophytes rather than primary pathogens (Bencheikh et al., 2020; Bouanaka et al., 2022, 2023b,c).

Morphological methods are considered as very useful and indispensable for any primary fungi identification, molecular methods have become more than necessary for accurate identification to species level (Abd-Elsalam et al., 2004). The most common sequences used to distinguish *Fusarium* spp. are portions of genomic sequences coding for translation elongation factor -1α (Divakara et al., 2013), β-tubulin (O'Donnell et al., 1998), calmodulin (O'Donnell et al., 2000), intergeneric region (IGS) (Yli-Mattila & Gagkaeva, 2010) and the internal transcribed spacer regions in the ribosomal repeat region (ITS1 and ITS2) (Gaikwad et al., 2013). The use of PCR and DNA sequence analysis of internal transcribed spacer (ITS) regions has become a diagnostic routine for the detection, identification, classification and phylogenetic analysis of many fungi at the species level (Hafizi et al., 2013; Zhao et al., 2014). ITS regions sequences are highly variable in *Fusarium*, and taxon-selective ITS amplification has been used to detect fungal pathogens such as *Fusarium* (White et al., 1990; Abd-Elsalam et al., 2004).

Fusarium include several species that have different characteristics and life lines that can potentially influence their development or toxins production during an epidemic (Picot et al., 2011). The life lines quantification of *Fusarium* spp. and the associated FCR severity variables is an essential step in order to define the links between strain characteristics and their ability to induce FCR.

In the past, only one survey has been conducted to identify associated FCR and FHB species in Algerian wheat (Abdallah-Nekache et al., 2019), whereas other studies have focused on *F. culmorum,* the main species causing wheat FCR and FHB (Bouanaka, 2023; Bouanaka et al., 2021b) and barley (Yekkour et al., 2015). To date, no study has detailed the other species associated with wheat FCR and FHB in Algeria, it is therefore necessary to deepen the information on *Fusarium* spp. Pathogenicity, as well as the severity variables, in order to characterize the strains according to their aggressiveness.

The study's objectives were; (i) To assess the current diversity of *Fusarium* species associated with FCR and FHB in Algeria. (ii) To identify and measure (*in vitro*) the main strains characteristics, life lines and the severity variables. (iii) To evaluate their FCR pathogenicity and aggressiveness on a sensitive durum wheat. (iv) And to try to highlight links between these life lines, easily and rapidly measurable in vitro, and *Fusarium* spp. *in vivo* severity measures.

Material and Methods

Sampling, isolation, purification and conservation of Fusarium spp.

Sampling was carried out in six of the most wheat-producing provinces (Constantine, Mila, Guelma, Oum elbouaghi, Sétif and Bordj Bou Arreridj), located in north-eastern Algeria (Figure 1) (Table 1). Plants symptomatic of FCR, or those suspected of being FHB symptomatic, were collected from different fields in several provinces of the regions surveyed. The samples were kept in sterile plastic bags and stored at 4°C until further use. The infected wheat crowns were cut into fragments of about 10 mm, while seeds were peeled from their glumes. The samples were disinfected in 2% sodium hypochlorite

(NaClO) for 5 min, then rinsed in 3 successive baths of sterile distilled water. Placed to dry on sterilized filter paper for 20 to 30 min under aseptic condition, then deposited on potato sucrose agar (PSA) medium in sterile Petri dishes (5 fragments per dish), and incubated at 28°C for 7 days. The fungal colonies suspected to be *Fusarium* genus were transplanted on the same medium. All isolates were purified by the single-spore technique Noman et al. (2018) and stored in Eppendorf tubes in 20% glycerol at -80°C.

Molecular identification and Phylogenetic analysis

The identification was carried out on the basis of macroscopic data and microscopic characteristics on three culture media; Carnation Leaf-Piece Agar (CLA), Spezieller Nährstoffarmer Agar (SNA) and PSA. These media were prepared according to Leslie & Summerell (2006).

To confirm the isolated *Fusarium* spp. identity, molecular identification was performed by the BIOfidal laboratory (CEDEX-France) as follows:

- *DNA extraction*

The DNA Extraction was carried out using a commercial kit NucleoSpin Plant II (Macherey-Nagel Germany). DNA was extracted from mycelium collected by scraping the surface of Petri dishes cultures of purified isolate. 100 µl lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM EDTA, 3% SDS and 1% 2-mercaptoethanol) was added, and the nucleic acids were isolated according to the microwave mini-prep procedure, described by Goodwin & Lee (1993). The final DNA pellet was supplemented into 100 µl TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and stored at -20°C until used.

- *PCR amplifications and gel electrophoresis*

Internal transcribed spacer (ITS) regions of rDNA were amplified using a fungal-specific primers pair ITS-1 (5' CTT GGT CAT TTA GAG GAA GTA A '3), and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC '3) (Gardes & Bruns, 1993). All amplification reactions were carried out in a 25 µl reaction volume containing 75 mM Tris-HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20, 1.5 µl $MgCl_2$ 0.2 µl dNTP, 1µl of each relevant oligonucleotide primer, 0.2 µl Taq polymerase Promega, 2 µl genomic DNA. The amplification products were revealed after 1.5% agarose gel electrophoresis of a 10 µl deposit of PCR products. Migration is followed by staining in an ethidium bromide bath (0.5 µg/ml). Afterwards, the DNA was visualized and photographed under UV using the Gel doc system of biorad (USA).

PCR temperature condition was as follows: Initial denaturation 95°C for 5 min, followed by 35 cycles, denaturation 95°C for 30 s, hybridization 55°C for 30 s, elongation 72°C for 45 s, final elongation 72°C for 7min, storage before revelation 10°C until use.

- *PCR product Purification*

PCR products were purified by the NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel (Germany), following the protocol described by the supplier. DNA binds in the presence of a chaotropic salt to a silica membrane. The binding mixture was loaded directly onto NucleoSpin® Extract II columns. Contaminations were removed by a washing step with ethanolic NT3 buffer. Finally, the pure DNA was eluted under low ionic strength conditions with a slightly alkaline NE buffer (5 mM Tris-Cl, pH 8.5).

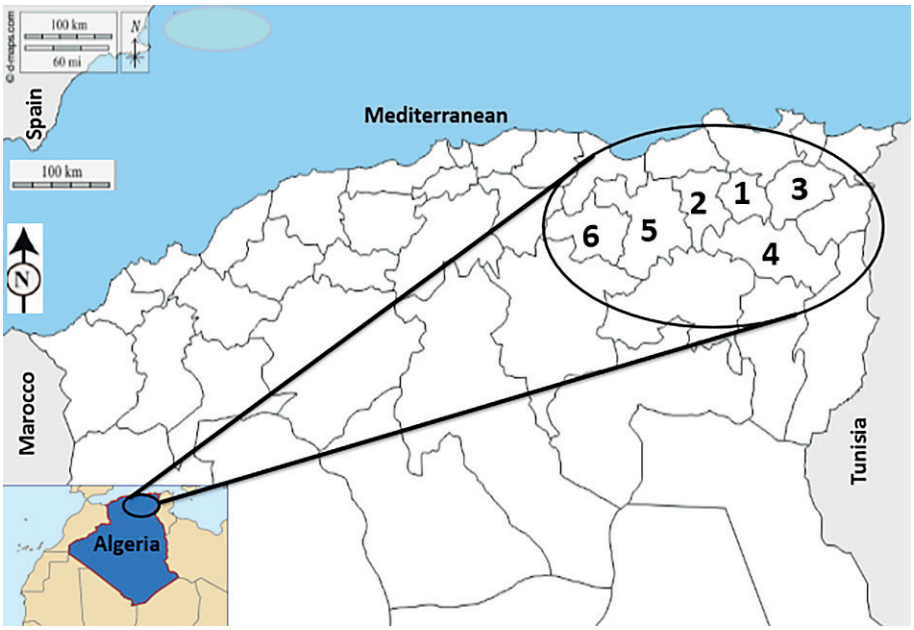

Fig. 1. Map of Northern Algeria showing the sampling provinces
1 – Constantine, 2 – Mila, 3 – Guelma, 4 – OumEl Bouaghi, 5 – Sétif, and 6 – Bordj Bou Arreridj

Table 1. Summary table of regions, provinces, locations, and sampling period

Isolate code	Region	Province	Wheat	Location Coordinates latitude and longitude	Year
FC1	Carrefour Djbel aougueb Oued Athmania	Mila	Durum	36°14′56″N 6°21′08″E	2019
FC2	Messaoud Boudjriou	Constantine	Durum	36°26′03″N 6°29′12″E	2019
FC3	Oued Zenati	Guelma	Durum	36°19′43″N 7°09′31″E	2019
FC4	Bekkouche Ahmed	Guelma	Durum	36°16′52″N 7°07′00″E	2019
FC5	Ain Mlila	Oum El Bouaghi	Durum	36°07′28″N 6°36′07″E	2019
FC6	Guettar El Aich	Constantine	Bread	36°13′35″N 6°36′22″E	2019
FC7	Ain Fakroun	Oum El Bouaghi	Bread	35°49′28″N 7°01′36″E	2019
FC8	Sigus	Oum El Bouaghi	Durum	36°03′14″N 6°49′06″E	2019
FC9	El khroub	Constantine	Bread	36°11′37″N 6°41′55″E	2019
FC10	Oued Arama	Mila	Durum	36°13′59″N 6°26′12″E	2019
FC11	Bordj Lagar	Mila	Durum	36°12′37″N 6°24′15″E	2019
FC12	Oued Seguin	Mila	Durum	36°14′21″N 6°25′05″E	2019
FC13	Oued Seguen	Mila	Durum	36°10′10″N 6°24′53″E	2019
FC14	Zegrour Elarbi Hamma Bouziane	Constantine	Durum	36°14′23″N 6°19′14″E	2019
FC15	Oued Athmania	Mila	Durum	36°21′30″N 6°41′38″E	2019
FC16	El Mridj	Constantine	Durum	36°26′23″N 6°33′17″E	2019
FC17 (INRAA)	Constantine	Constantine	Bread	Nl	2019
FC18 (INRAA)	Constantine	Constantine	Bread	Nl	2019
FC19 (INRAA)	Constantine	Constantine	Durum	Nl	2019
FC20 (INRAA)	Constantine	Constantine	Durum	Nl	2019
FC21 (INRAA)	Constantine	Constantine	Durum	Nl	2016
FC22 (INRAA)	Constantine	Constantine	Durum	Nl	2017
FC23 (INRAA)	Constantine	Constantine	Durum	Nl	2017
FE1 (INRAA)	Constantine/Nord	Constantine	Durum	Nl	2016
FE2 (INRAA)	Constantine/Sud	Constantine	Bread	Nl	2016
FE3 (INRAA)	Constantine/Sud	Constantine	Bread	Nl	2016
FE4 (INRAA)	Constantine/Nord	Constantine	Durum	Nl	2016
FE5 (INRAA)	Constantine/Sud	Constantine	Durum	Nl	2017
FE6 (INRAA)	Constantine/ Nord	Constantine	Bread	Nl	2017
FE7 (INRAA)	Constantine	Constantine	Durum	Nl	2017
FE8 (INRAA)	Constantine	Constantine	Durum	Nl	2017
FE9 (INRAA)	Constantine	Constantine	Bread	Nl	2017
FE10	El Mridj	Constantine	Durum	36°21′10″N 6°45′44″E	2019
FE11	Ain Smara	Constantine	Bread	36°15′31″N 6°28′21″E	2019

Nl: Not located, FC: isolated from wheat crown, FE: isolated from wheat grain, INRAA: Isolate offered by National Institute of Agronomic Research of Algeria

DNA sequencing, alignment and phylogenetic analysis

The amplified and purified PCR products were sequenced using Sanger technique (Sanger et al., 1977) using the Applied Biosystems BigDye v3.1 kit and PCR primers used for the interest fragments amplification. The obtained sequences are analyzed and cleaned by the use of software SnapGene® Viewer 5.2. The final sequences are then compared with those in the GeneBank database by using the program BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi Blast) of NCBI for the identification of the studied *Fusarium* spp. Isolates, based on % homology with the reference strains. The ITS region sequences of the resulting isolates were used for multiple sequence alignment in MEGA format using the Clustal W option of the MEGA-X software. The phylogenetic tree and distance matrix were also constructed using MEGA software, which implemented the Neighbour-Joining (NJ) dendrogram from Saitou & Nei (1987). The phylogenetic distance was based on the Kimura 2-parameter (K2P) model (Kimura, 1980).

Strains characterization and their life lines measurement

Growth rate

From the mother strains in conservation, transplantation was carried out on PSA medium on Petri dishes (9 cm diameter). After 6 days of growth, a second transplantation of 6 mm diameter of the culture was performed on the same medium. The resulting Petri dishes were incubated at 28°C in the dark for 5 days. Then the radial growth diameters were measured to determine the each strain growth rate (mm per day). Three replicates were made for each strain.

Sporulation rate

The conserved *Fusarium* strains were previously transplanted on a Petri dish containing PSA medium and incubated for 30 days at 28°C. After microscopic confirmation that all isolates have been sporulated, an 18 mm mycelium plug from the center of each dish was removed and transferred to a 50 ml Erlenmeyer flask containing 10 ml of sterile distilled water. After 20 min of rotary agitation at a speed of 200 rpm, the suspensions obtained were filtered separately using fine cheesecloth. Then the absorbencies were measured by a spectrophotometer at 530 nm. The sporal concentration was determined by the following linear formula (Concentration = 20090760 × Abs – 100439) (Caligiore-Gei & Valdez, 2015). Spore concentrations were also confirmed by counting at the Malassez cell. Three counts were made for the same sporal suspension as well as 2 repetitions for each strain.

FCR pathogenicity tests

FCR pathogenicity and aggressiveness of *Fusarium* isolates were evaluated by two methods *in vitro* and *in vivo.* The cultivar of Italian origin Simeto, known for its sensitivity to wheat *Fusarium*, was chosen as the plant material for both tests (Purahong et al., 2014).

In vitro test in growth chamber

This test was performed according to Bouanaka et al. (2021a). The bottoms of 105 essay tubes (14 cm × 3 cm) were filled with 4 cm of cotton. Twenty milliliters of distilled water were added to each tube and then covered with aluminum foil. Afterwards, the tubes were sterilized by autoclaving at 180°C for 2 h twice with an interval of 24 h. The wheat (Simeto) seeds were surface disinfected with 2% NaClO for five minutes and rinsed three times in succession with sterile distilled water. After drying, the seeds were carefully placed on moist cotton in each essay tube (5 seeds/ tube).

An 8 mm disc of 7-days old fungal isolate was placed adjacent to the seeds (3 discs in 3 tubes for each isolate). The tubes were closed with transparent caps and incubated at 28°C for 4 days, then transferred to the growth chamber (25/19°C day/night temperature, 16/8 h light/dark cycle). The tubes were arranged according to a complete randomization design. After two weeks, the disease severity (DSt) and the coleoptiles length percentage (%CL) were measured on a scale of 0 to 4 (Figure 2a), FCR disease severity classes were determined in the laboratory, based on symptoms including brown and necrotic discoloration of crown tissue in which: Class 0 = healthy crown; 1 = light browning of the crown; 2 = half crown browning; 3 = complete browning of the crown; and 4 = death of the seedlings (Figure 2a).

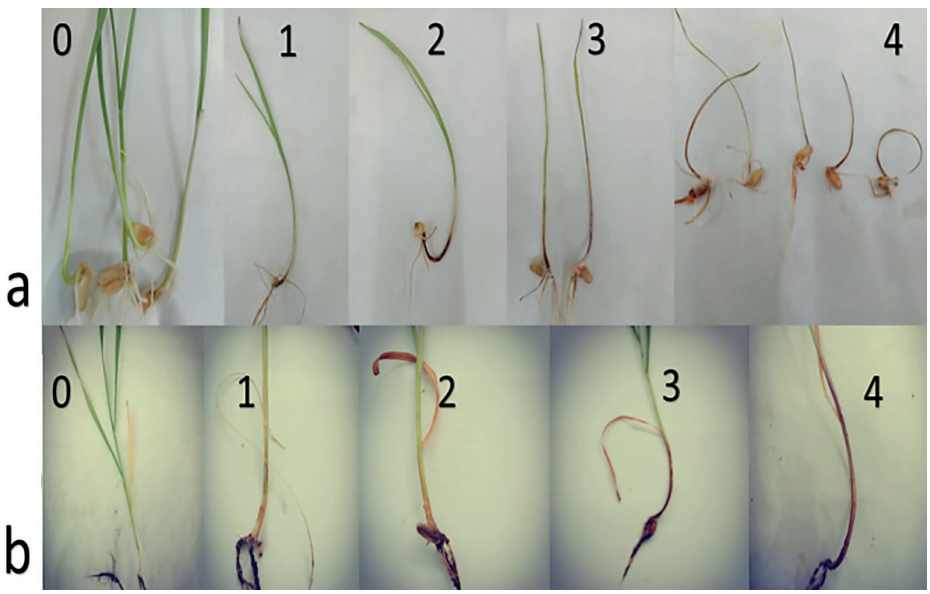

Fig.2. Photos illustrate FCR disease severity classes for the two tests
a: *in vitro* and b: *in vivo*. Class 0 = healthy crown (control). 1 = light browning of the crown; 2 = browning of half of the crown; 3 = complete browning of the crown; and 4 = death of the seedlings

The DSt of each treatment was calculated using McKinney's (1923) index, which expresses the percentage of maximum disease severity (i.e., 100) according to the formulas:

$$DSt = \{\sum(c \times f)/n \times N\} \times 100,$$

where c = disease class, f = frequency, n = number of observations, and N = the highest value of the empirical scale adopted (class 4).

$$\%CL = (CLt \times 100)/CLc,$$

where $\%CL$ = the percentage of coleoptiles length, CLt = the average coleoptiles length, CLc = the average coleoptiles length of the control.

In vivo test in greenhouse

This test was performed according to Bouanaka et al. (2021b). Only selected *Fusarium* spp. isolates among those that showed significant pathogenicity in *in vitro* test were studied for this *in vivo* test. Five seeds of durum wheat (Simeto) were surface-sterilized with 2% NaClO for 8 min and then rinsed six times with sterile distilled water. They were sown in a pot of (8 cm × 12 cm height) containing a mixture of soil and compost (1/2). This mixture was previously sterilized at 180° for 2 h (2 repetitions with 24 h interval). For each isolate, two replicates and two non-inoculated controls (5 pots/ isolate) were used. The pots were placed in growth chamber (25/19°C day/night temperature, 16/8 h light/dark cycle). After 3 weeks, when the seedlings have reached the two-leaf stage (Zadoks' GS 12: Zadoks et al., 1974), the soil around the stems was removed and a PSA disc (13 mm diameter) of a 7-days-old culture of the tested isolate was placed around the stems, 2 cm below the soil, to allow the mycelium to reach the stems base. The soil was then put back in the place around the seedlings. The pots were organized according to a completely random plan. They were watered every 3 days throughout the test. Three weeks after inoculation (Zadoks' GS 20), each plant was carefully removed from the soil and washed with tap water.

Severity classes were assigned to the laboratory on a scale of 0 to 4 (Figure 2b). As well as the disease severity (DSp) of each treatment were calculated using the McKinney's (1923) index as described above (*in vitro* test). The DSp value ranged from 0% (non-pathogenic, absence of infected crown) to 100% (highly pathogenic, all seedlings dead). Koch's postulates were satisfied by re-isolation of *Fusarium* sp. from small or large brown spots on the crowns for both tests.

Statistical analysis

Data analyses, graphs, tables, and descriptive statistics (mean standard deviation and variance), were performed using SPSS software (IBM SPSS Statistics version 25). Correlations between the different parameters were determined by Pearson correlation, the correlation coefficients were considered significant at the 5% level ($P \leq 0.05$) and highly significant at the 1% level ($P \leq 0.01$). The means for the different treatments were compared using Tukey's honestly significant difference test.

Results

Fusarium spp. isolation

Out of 117 samples collected (crown + grain), sixty-three samples from wheat crown showing FCR symptoms and fifty-four samples from wheat grains suspected of being affected by FHB. A total of 34 *Fusarium* spp. isolates were the subject of this study (Table 1). Eighteen isolates of *Fusarium* spp. were obtained from this sampling and sixteen others were provided by the national institute of agronomic research of Algeria (INRAA), Constantine unit (Table 1).

Among the 34 isolates obtained, a high diversity of *Fusarium* spp. was observed, 23 isolates from wheat crown and 11 from wheat grain. The majority of isolates were obtained in the 2019 crop year. And few others were obtained exclusively from wheat grain from the previous seasons 2016 and 2017 (Table 1).

Molecular identification and Phylogenetic analysis

Identification was performed on the basis of macroscopic data and microscopic characteristics (Leslie & Summerell, 2006; Hoshino et al., 2009) and confirmed by molecular analysis. In the total of 34 isolates, ten *Fusarium* species were identified: *F. culmorum* (19), *F. cerealis* (3), *F. incarnatum* (3), *F. graminearum* (2), *F. acuminatum* (2), *F. oxysporum* (1), *F. equiseti* (1), *F. asiaticum* (1), *F. brachygibbosum* (1) and *F. fujikuroi* (1) (Table 2). *F. culmorum* is the predominant species for wheat crown and a remarkable variety of species for wheat grain.

The sequence of each isolate was compared to the reference sequence collected from GenBank. Phylogenetic analysis of the ITS region showed that the strains are form several groups (Figure 3). The phylogenetic analysis groups isolates into 6 distinct clades; the first group includes (FC6, FC16, FC2, FC20, FC11, FC17 and FC4), the second clade includes strains (FC12, FE9, FE1, FC14, FC9 and FE3), the third clade includes strains (FC8, FE7, FC3, FC22, FC5, FC18, FE6 and FC13), this clade is divided into two subclades, the fourth clade (FE2, FC1, FE8, FC15 and FE4), the fifth clade groups FC23 and FC7, the sixth clade groups the rest of the strains. The out-group fungi other than *Fusarium, Aspergillus* and *Trichoderma* appeared side by side

quite distinct. Moreover, the sequence of *Rhizopus* seems far and incomparable with any other strains in this dendrogram.

Growth and sporulation rates

A huge variety between isolates for both life lines was noted (Figure 4). Also a great diversity for growth rates was noticed. *F. culmorum* strains (FC20, FC5, FC11 and FC10) recorded the fastest growth rates. While isolates FC21, FE10, FE2, FC7, FE9 and FE6 ranked in ascending order recorded low growth rates below 10 mm/d (Table 2). The latter isolates belong to the single species isolated *F. acuminatum, F. brachygibbosum, F. equiseti, F. oxysporum, F. fujikuroi, F. asiaticum.*

The highest sporulation rates were recorded by isolates FC20, FE3, FE4, FC10, FC7, FC14 and FC8 with values > 4000000 sp/ml. The isolates with the lowest rates were FC19 < FE7 < FC6 < FC2< FC23 < FC13 < FE6 with values below 740000 sp/ml (Table 2).

Table 2. Molecular identification of *Fusarium* isolates with their life lines (sporulation rate and growth rate) and pathogenicity in vitro and in vivo on FCR

Isolate code	*Fusarium* species	GenBank accession number	Sporulation Rate (sp/ml)		Growth Rate (mm/d)		Disease Severity Tube test (DSt)	the average coleoptiles length (CLt) mm ± Std. Deviation	the coleoptile length (%CL)	Disease Severity Pot test (DSp)
			Mean	Std. Deviation	Mean	Std. Deviation				
FC1	*F. culmorum*	MW366637	776420.33^{ab}	449168.89	13.40^{fghij}	0.87	75.00	66.73±34.80^{abcdefg}	59.58	68.33
FC2	*F. culmorum*	MW165423	555532.00^{a}	407759.70	13.46^{fghij}	0.46	83.33	46.53±40.84^{abcde}	41.54	68.33
FC3	*F. culmorum*	MW363060	2469897.67^{abcdef}	371900.84	14.60^{hij}	1.03	76.66	58.73±50.02^{abcdefg}	52.44	56.66
FC4	*F. culmorum*	MW165434	977228.00^{abc}	771826.13	14.66^{hij}	1.10	81.66	43.60±40.80^{abc}	38.92	48.33
FC5	*F. culmorum*	MW349658	3721598.67^{bcdef}	1628193.45	15.73^{ij}	1.33	81.66	60.20±48.40^{abcdefg}	53.75	60.00
FC6	*F. culmorum*	MW358285	475209.33^{a}	220279.00	14.00^{ghij}	1.74	36.66	41.33±59.39^{ab}	36.90	NA
FC7	F. oxysporum	MW353146	4558296.67^{defg}	387303.58	8.93^{cd}	0.41	6.66	66.60±51.84^{abcdefg}	59.46	NA
FC8	*F. graminearum*	MW349656	4404344.00^{defg}	573035.18	13.80^{ghij}	0.20	78.33	37.13±28.47^{ab}	33.15	58.33
FC9	*F. culmorum*	MW353156	2771109.33^{abcdef}	362011.21	13.60^{fghij}	0.52	60.00	43.67±45.79^{abc}	38.98	55.00
FC10	*F. culmorum*	MW353149	4765798.00^{efg}	801806.47	15.00^{hij}	0.87	76.66	44.20±37.42^{abc}	39.46	56.66
FC11	*F. culmorum*	MW151664	3761760.00^{cdef}	1164742.20	15.33^{ij}	0.70	85.00	30.80±32.27^{a}	27.50	61.66
FC12	*F. culmorum*	MW349657	916985.67^{abc}	258982.17	14.80^{hij}	0.20	70.00	26.53±37.70^{a}	23.69	56.66
FC13	*F. culmorum*	MW353157	676016.67^{a}	548588.23	14.66^{hij}	0.11	56.66	39.93±46.07^{ab}	35.65	NA
FC14	*F. cerealis*	MW358286	4437812.33^{defg}	654707.01	13.40^{fghij}	0.72	25.00	65.86±55.76^{abcdefg}	58.80	NA
FC15	*F. culmorum*	MW447383	883518.00^{abc}	456147.35	14.80^{hij}	0.52	63.33	52.33±46.56^{abcdef}	46.72	50.00
FC16	*F. culmorum*	MW450585	896905.00^{abc}	206092.87	14.73^{hij}	1.50	66.66	59.27±54.47^{abcdefg}	52.91	51.66
FC17	*F. cerealis*	MW447299	214159.00^{a}	101070.62	12.26^{efgh}	0.70	56.66	69.20±42.51^{abcdefg}	61.78	NA
FC18	*F. culmorum*	MW353140	3768453.33^{cdef}	707496.50	14.00^{ghij}	1.21	76.66	50.40±47.68^{abcdef}	45.00	60.00
FC19	*F. acuminatum*	MW353145	200772.00^{a}	60242.00	10.66^{def}	0.57	8.33	110.13±44.76^{defg}	98.33	NA
FC20	*F. culmorum*	MW165435	6767180.33^{g}	4052086.57	15.80^{j}	0.20	88.33	50.00±36.60^{abcdef}	44.64	71.66
FC21	*F. acuminatum*	MW447502	2443123.33^{abcdef}	655732.68	3.20^{a}	0.52	13.33	112.67±32.14^{fg}	100.59	NA
FC22	*F. culmorum*	MW353148	2777803.00^{abcdef}	815519.40	13.66^{ghij}	0.83	75.00	45.40±38.30^{abcd}	40.53	65.00
FC23	*F. cerealis*	MW447774	649242.33^{a}	239570.21	12.80^{fghi}	1.74	60.00	40.93±41.37^{ab}	36.54	NA
FE1	*F. culmorum*	MW353147	1144567.33^{abc}	278970.96	13.00^{fghij}	0.34	65.00	41.27±49.07^{ab}	36.84	40.00
FE2	*F. equiseti*	MW448184	1104406.33^{abc}	313671.47	6.93^{bc}	0.50	10.00	110.93±39.10^{efg}	99.04	NA
FE3	*F. culmorum*	MW353158	5247736.00^{fg}	425661.64	12.13^{efgh}	1.13	61.66	52.80±55.01^{abcdef}	47.14	NA
FE4	F. culmorum	MW450591	4866201.67^{efg}	645505.90	14.40^{hij}	2.11	73.33	54.20±44.38^{abcdefg}	48.39	56.66
FE5	*F. graminearum*	MW358916	3801921.67^{cdef}	162310.81	14.13^{hij}	0.11	65.00	53.20±56.38^{abcdef}	47.50	NA
FE6	*F. asiaticum*	MW366557	736259.00^{a}	209007.59	9.60^{cde}	0.34	11.66	98.07±54.48^{bcdefg}	87.55	NA
FE7	*F. incarnatum*	MW450593	388192.33^{a}	258202.32	12.93^{fghij}	0.30	5.00	82.00±67.51^{abcdefg}	73.21	NA
FE8	*F. incarnatum*	MW448396	1713522.33^{abcd}	960173.70	14.33^{hij}	0.98	41.66	86.00±61.36^{abcdefg}	76.78	NA
FE9	*F. fujikuroi*	MW450594	1258358.33^{abc}	421855.34	9.06^{cd}	1.00	10.00	96.40±41.80^{bcdefg}	86.07	NA
FE10	*F. brachygibbosum*	MW450596	950453.67^{abc}	543418.61	4.93^{ab}	0.30	20.00	106.67±51.36^{cdefg}	95.23	NA
FE11	*F. incarnatum*	MW450597	2068282.67^{abcde}	348388.17	11.06^{defg}	1.00	30.00	118.27±35.92^{g}	105.59	NA
Control								112.00±33.47^{fg}	100	

For each column, the values (mean±SD) with different letters denote the statistical significance determined by ANOVA followed by Tukey test ($P < 0.05$).
NA : not available

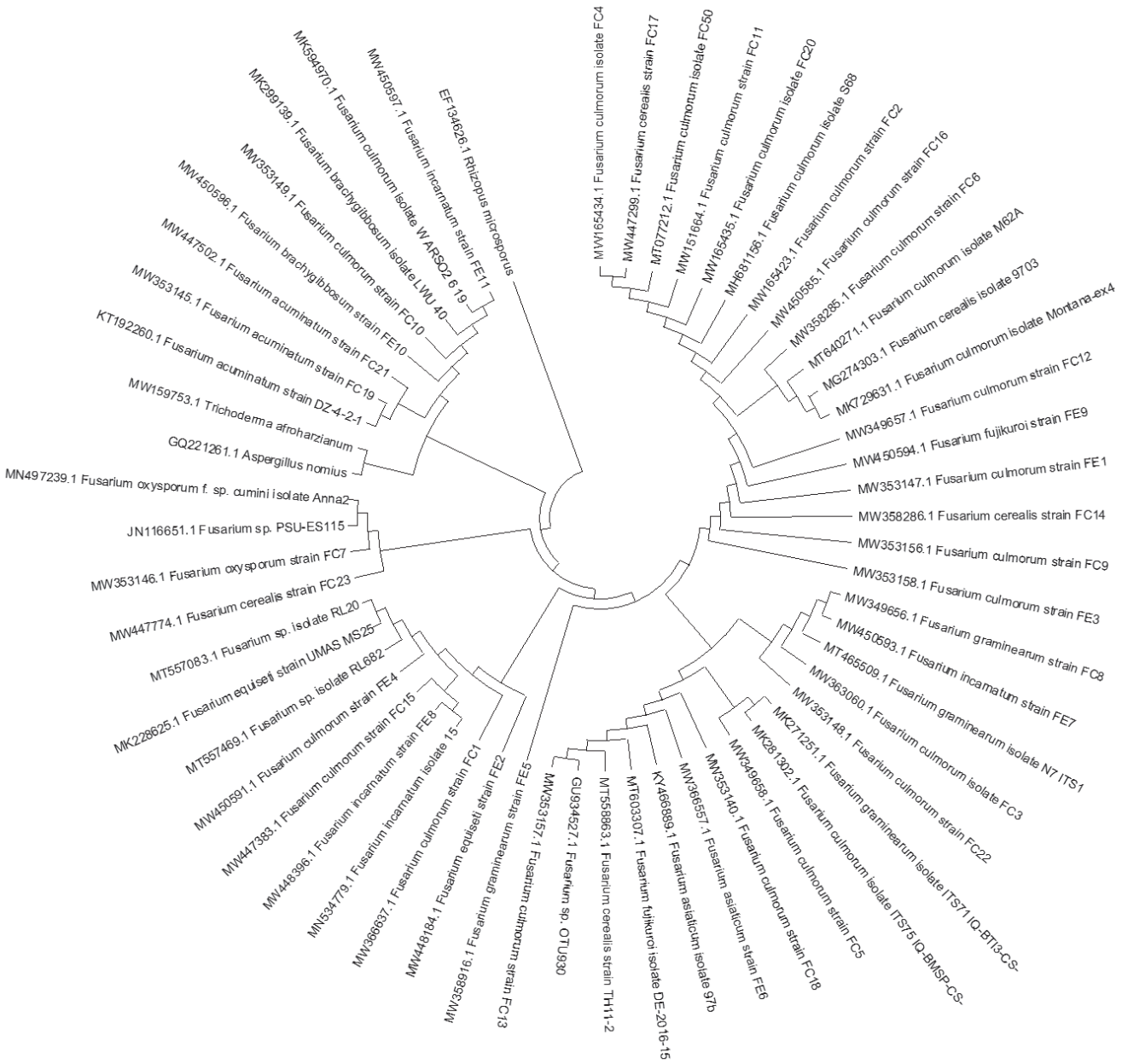

Fig. 3. Phylogenetic relationships of 34 *Fusarium* isolates inferred from ITS sequences analysis by MEGA software, with 21 type strains from the GeneBank database. In addition to out-group *Fusarium* fungi (*Trichoderma*, *Aspergillus* and *Rhizopus*)

FCR pathogenicity tests in vitro and in vivo

Pathogenicity test was performed on the susceptible variety Simeto to determine the isolates impact on wheat basal part (coleoptile). The *in vitro* test showed that all isolates were pathogenic and caused FCR symptoms. None of the strains had a zero DSt, their DSt values varied between 88.33% for FC20 and 5% for FE7 (Table 2). It was also clearly noticed that the DSt value is in tight significant relation with the species.

It was also clearly noticed that the DSt value is in tight significant relation with the species. *F. culmorum* with its 19 strains caused FCR with a wide range of DSt, but also the

highest among all other species. *F. culmorum* strains FC20, FC11 and FC2 recorded the highest aggressiveness with DSt = 88.33, 85 and 83.33%, respectively. While FC6, FC13 and FC9 recorded the lowest rates with DSt = 36.66, 56.66 and 60%), respectively (Table 2).

The 17 *Fusarium* spp. tested for their FCR aggressiveness in greenhouse were only those that showed the most severe pathogenicity in the tube test with a DSt > 60%. It was *F. culmorum* with 16 strains and one strain of *F. graminearum* (Table 2). The results of the pot test aggressiveness revealed significant differences in DSp values among *F. culmorum* strains; FC20, FC22, FC11, FC18 and FC5 recorded DSp = 71.66%, 65%, 61.66%, 60% and 60%, respectively. FC1 and FC2 with the same value 68.33%. While the others were less aggressive, such as FE1 and FC4 with a DSp of 40% and 48.33%, respectively (Table 2).

F. culmorum strain FC12 severely influenced coleoptile development with only a %CL = 23.69, followed by the rest of the same species strains. The strains FE11 (*F. incarnatum*) and FC21 (*F. acuminatum*) did not influence at all on the %CL with values higher than the control 105.59 and 100.59% (Table 2).

Correlation between the different parameters

After measuring the 34 strains life lines and pathogenicity (Table 2), the correlation study was more than necessary to figure out the links between the different standards and parameters. Results showed a strong correlation between the tube test and the pot test ($r = 0.62$ $P = 0.007 < 0.01$) (Figure 5a**)**. Moreover another correlation between growth rate and sporulation rate ($r = 0.35$ $P = 0.012 < 0.05$) (Figure 5b) (Table 3). While for the rest of parameters, there was no significant correlation between disease severity in both tests with the reduction in coleoptiles length ($r = -0.03$, $p = 0.90$ for DSt, and $r = 0.17$, $p = 0.50$ for DSp) (Table 3). No correlation between the two tests *in vitro* and *in vivo* with growth rate or sporulation rate (Table 3).

Discussion

This study was conducted to isolate and identify *Fusarium* spp., causing wheat FCR and FHB in the north-eastern region of Algeria (Figure 1) (Table 1), and to characterize their life lines and their FCR aggressiveness (*in vitro* and *in vivo*). We also attempted to study pathogen diversity and compare our results with those published on FCR species.

Such a study has never been performed in Algeria, with the exception of Abdallah-Nekache et al. (2019), where molecular identification of fungal species was based on conventional PCR by testing isolates with species-specific primers. Whereas in our study, molecular identification was done with ITS1-2 internal transcribed spacer sequencing of the rDNA region (contains internal transcribed spacer 1, 5.8S ribosomal RNA and internal transcribed spacer 2 sequences). ITS sequencing has been used recently by many scientists in their searches for *Fusarium* spp. identification (AL-Taae et al., 2019; Dehghanpour et al., 2019; Irom et al., 2016).

Thirty-four isolates of *Fusarium* spp. were obtained (Table 2), the results after identification showed a diversity of species and the presence of new species never reported in Algeria. Five *Fusarium* species of the crown have been distinguished; *F. culmorum* (16), *F. oxysporum* (1), *F. graminearum* (1), *F. cerealis* (3) and *F. acuminatum* (2) and seven species from grains distributed as follows; *F. culmorum* (3), *F. incarnatum* (3), *F. graminearum* (1), *F. asiaticum* (1), *F. fujikuroi* (1), *F. brachygibbosum* (1), and *F. equiseti* (1) (Table 2). This high species diversity isolated from wheat grains appears to be quantitatively similar to that obtained by Nielsen et al. (2011), where they found up to seven different species of *Fusarium* in the grain samples, confirming the exis-

Table 3. The different correlations between the different parameters measured

different parameters compared	Pearson Correlation (r)	P-value	P > F
DSt X DSp	0.627**	0.007	< 0.01
DSt X Sporulation Rate	0.315	0.218	> 0.05
DSt X Growth Rate	0.314	0.219	> 0.05
DSt X Coleoptile length	-0.030	0.908	> 0.05
DSp X Sporulation Rate	0.236	0.362	> 0.05
DSp X Growth Rate	0.043	0.871	> 0.05
DSp X Coleoptile length	0.173	0.508	> 0.05
Sporulation Rate X Growth Rate	0.355*	0.012	< 0.05
Sporulation Rate X Coleoptile length	0.078	0.766	> 0.05
Growth Rate X Coleoptile length	0.276	0.284	> 0.05

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

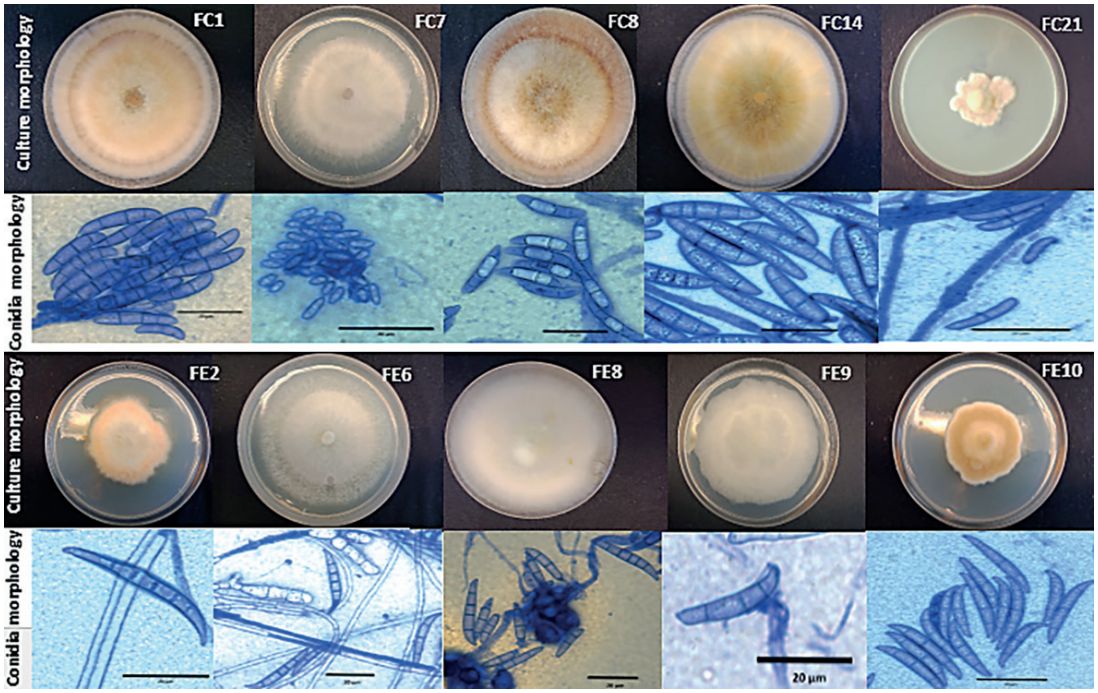

Fig. 4. Photos illustrate culture morphology (10 days on PSA medium), and conidia morphology of 10 *Fusarium* isolates representing the ten species isolated (FC1; *F. culmorum*, FC7; *F. oxysporum*, FC8; *F. graminearum,* FC14; *F. cerealis* FC21; *F. acuminatum*, FE2; *F. equiseti*, FE6; *F. asiaticum*, FE8; *F. incarnatum*, FE9; *F. fujikuroi*, FE10; *F. brachygibbosum*). Scale bar 20 µm

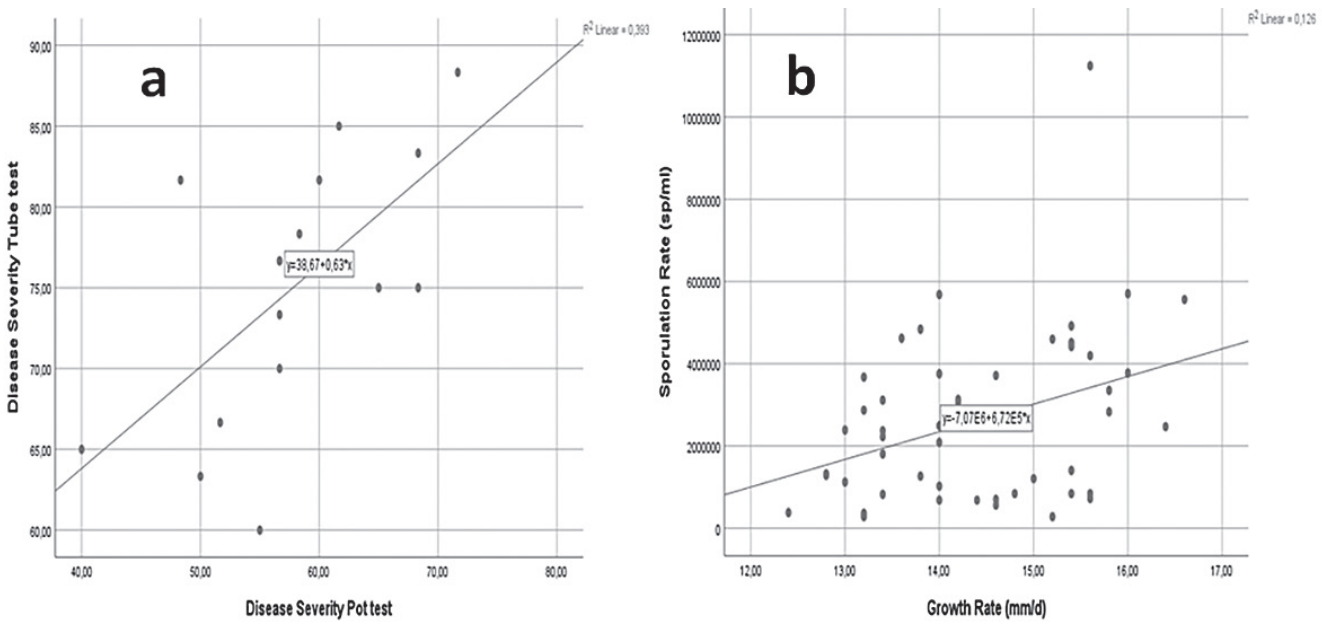

Fig. 5. Simple Scatter with fit line of Correlations between (a) DSt by DSp test (r = 0.627, P = 0.007 < 0.01), (b) Sporulation rate (mm/d) by Growth Rate (sp/ml) r = 0.355, P = 0.012 < 0.05 determinate by Pearson correlation coefficient

tence of a complex of diverse *Fusarium* species at the origin of FHB epidemics. Thus; Audenaert et al. (2009) recorded up to nine *Fusarium* spp. in a sample of cereals and found clear associations and direct synergy between some species, although FHB species frequently compete in the same host.

Phylogenetic analysis of the ITS region groups our isolates into 6 distinct clades, the first clade includes all *F. culmorum* species except FC17 (*F. cerealis*), the second clade include the single strain *F. fujikuroi,* the third clade was classified into two subclades, where *F. graminearum* and *F. incarnatum* (FC8 and FE7) are side by side in the first subclade, the single strain of *F. asiaticum* appeared in the second subclade with only strains of *F. culmorum* (Figure 3). The fourth clade includes the unique strain of *F. equiseti* (FE2), the fifth clade encompasses only two strains that are close in sequence to strains FC23 and FC7, although they belong to two morphologically different species *F. cerealis* and *F. oxysporum*, respectively (Figure 3).

The sixth clade includes the two *F. acuminatum* strains (FC19 and FC21) side by side, as well as the single strain of *F. brachygibbosum* (FE10). The out-group *Fusarium* strains used in the dendrogram are quite distinct from the other strains. *Trichoderma afroharzianum* and *Aspergillus nomius* appeared side by side between the fifth and sixth clades, while *Rhizopus microsporus* alone is far from comparable with the other sequences (Figure 3).

Although *F. culmorum* strains FCR pathogenicity is high overall, there is still potential for low differentiation of aggressiveness due to variation among strains. This illustrates the variability between isolates of the same species, so it is necessary to continue to monitor trends in this pathogenic species in the future. This complex variation between pathogenic isolates associated with FCR has been previously studied using various evaluation standards and inoculation techniques by Yang et al. (2010), and Poole et al. (2012). Most of these studies showed that *F. pseudograminearum* and *F. culmorum* were the most pathogenic species, and *F. avenaceum*, *F. equiseti* and *F. poae* were the least pathogenic species. While *F. pseudograminearum* and *F. graminearum* cause greater crown rot, and *F. culmorum* causes the greatest seedling blight.

Pathogenicity results in the tube assay revealed significant differences in aggressiveness among *Fusarium* isolates; FC20, FC11, FC2, FC4 and FC5, caused a DSt > 80%. While others were much less aggressive with a DSt ≤ 10% (Table 2).

The single species of *F. oxysporum, F. equiseti, F. asiaticum, F. fujikuroi* and *F. brachygibbosum* recorded low to negligible pathogenicity values (Table 2). These species have not been competitive against *F. culmorum* strains. Our results seem to be compatible with the works of Eslahi (2012), Shikur Gebremariam et al. (2017) and Dehghanpour et al. (2019), where they found that *F. proliferatum, F. acuminatum, F. oxysporum* and *F. flocciferum* species had medium to low levels of pathogenicity on wheat, that are rather saprophytic.

According to the study of Smiley & Patterson (1996), *F. acuminatum* includes a few isolates capable of causing brown discoloration of the wheat crown and some strains capable of even killing wheat plants in greenhouse. In contrast to our results, the two strains of *F. acuminatum* were without influence on coleoptiles growth and with very low DSt (Table 2).

In this study, *F. oxysporum* and *F. brachygibbosum* were among the non-pathogenic species. However, Demirci & Dane (2003) also reported that *F. oxysporum* is weakly aggressive. Moreover, Shikur Gebremariam et al. (2017) in their work reported that *F. oxysporum, F. equiseti, F. incarnatum, F. brachygibbosum* and *F. acuminatum* were non-pathogenic on winter wheat.

Pathogenicity was also manifested by a large coleoptile length reduction (%CL) (Table 2). On the other hand, other isolates did not influence %CL at all, with values even higher than those of the control. This was the case for FE11 and FC21, which represent two strains of *F. incarnatum* and *F. acuminatum,* respectively. Three other isolates recorded almost negligible reduction in coleoptiles length, FE2, FC19 and FE10 represent *F. equiseti, F. acuminatum* and *F. brachygibbosum*, respectively (Table 2). In contrast to our single strain of *F. equiseti*, Bencheikh et al. (2020) found that *F. equiseti* had little negative effect on certain growth parameters such as length and fresh weight of the root and vegetative system.

F. culmorum was the most common species among the main pathogenic *Fusarium* species identified in this study. This result is consistent with those obtained in other countries such as Turkey (Tunali et al., 2008), Italy (Scherm et al., 2012), Morocco (El Yacoubi et al., 2012), Tunisia (Rebib et al., 2014), and Iran (Eslahi, 2012), where *F. culmorum* was the dominant species as the FCR causative agent. The other species isolated from the crown were *F. incarnatum, F. oxysporum, F. graminearum, F. acuminatum* and *F. brachygibbosum*. Species isolated from wheat grains *F. equiseti, F. brachygibbosum, F. asiaticum, F. graminearum* and *F. culmorum* (Table 2). Nevertheless, our results appear to be slightly different from a survey conducted in five provinces of China, where *F. asiaticum* was considered the dominant species (Zhang et al., 2015).

The two isolates FE10 and FE11 belonging to *F. brachygibbosum* and *F. incarnatum* species, respectively, were characterized by very low aggressiveness (Table 2). Overall,

F. cerealis isolates were more aggressive than those of *F. incarnatum*, resulting in greater DSt and a greater reduction in coleoptiles length, particularly FC23. On the other hand, *F. acuminatum* isolates were less aggressive than *F. incarnatum* isolates, and so was the reduction in %CL, where it is negligible for *F. acuminatum* (Table 2). Inoculations in the tube test by the single strains *F. equiseti, F. brachygibbosum, F. asiaticum* and *F. fujikuroi* had no effect on the coleoptile length. While *F. oxysporum* significantly reduced %CL, and had very low FCR aggressiveness with a DSt = 6.66% (Table 2).

The pot test results showed that FC20, FC1and FC2, recorded the highest aggressiveness, while FE1, FC4 and FC15 recorded the lowest (Table 2). *F. graminearum* is the species globally associated with FHB worldwide (Nielson et al., 2013; Beccari et al., 2018). The only strain of *F. graminearum* (FC8) tested *in vivo* showed no significant pathogenicity difference from those of *F. culmorum*. Our study revealed the presence of this species for the first time in Algeria, which requires vigilant monitoring of the prevalence of this species and its geographical distribution in the future.

A strong correlation was found between the tube test and the pot test (Figure 5a) (Table 3). This result will allow us to predict *F. culmorum* strains aggressiveness towards wheat FCR by a simple *in vitro* tube test. Furthermore, another correlation between growth rate and sporulation rate was observed ($r = 0.35$ $P = 0.012 < 0.05$) (Figure 5b) (Table 3).

Total lack of correlation between DS in both tests with the reduction in coleoptiles length was noticed ($r = -0.03$, $p = 0.90$ for DSt, and $r = 0.17$, $p = 0.50$ for DSp). This can be explained by the fact that some species did not report FCR symptoms, but they did significantly reduce coleoptiles length, the case of *F. oxysporum* and some strains of *F. incarnatum* (FE7) and *F. cerealis* (FC14) (Table 2). Any correlation between the two tests in vitro and in vivo with growth rate, or sporulation rate was observed, so these two life lines remain far from being parameters for measuring, or predicting the *Fusarium* strains pathogenicity (Table 3). Such pathogenicity, phylogeny and genetic diversity will be useful for monitoring trends in pathogen distribution in different regions and for wheat FCR control strategies adoption in Algeria.

Conclusion

This study reveals a wide range of *Fusarium* species associated with wheat FCR in Algeria. The main finding was that the different *Fusarium* spp. do not all have the same FCR aggressiveness even between strains of *F. culmorum*. This confirms the need for a vigilant surveillance strategy for infected material. The predominant species isolated was *F. culmorum*, followed by *F. cerealis* and *F. incarnatum*. Here, we report the appearance of *F. acuminatum, F. asiaticum, F. incarnatum* and *F. graminearum* for the first time from wheat in Algeria. These strains collected in this research constitute a characterized fungal source for many studies in the future.

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