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Phylogenetic analysis and growth profiles of Fusarium incarnatum-equiseti species complex strains isolated from Tunisian cereals

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Abstract:	The Fusarium incarnatum-equiseti species complex (FIESC) is a phylogenetically rich complex. It includes more than 30 cryptic phylogenetic species, making morphological identification problematic. FIESC has previously been detected in Tunisian cereals, but knowledge on the phylogeny and the ecophysiology of their species is lacking. In this work a phylogenetic analysis was performed using partial sequences of the translation elongation factor 1a gene (EF1a) of three FIESC strains isolated from barley and wheat from Tunisia, situated south in the Mediterranean basin, and additional strains from other countries. The results indicated that all Tunisian strains clustered with FIESC 5 group (F. clavum) together with other Spanish FIESC 5 strains also isolated from cereals. Growth rate profiles of the Tunisian strains were also determined on wheat and sorghum based media at a range of temperatures (15, 20, 25, 30, 35 and 40 °C) and water potential values (-0.7, -2.8, -7.0, and -9.8MPa, corresponding to 0.995, 0.98, 0.95 and 0.93 aw values). Optimal growth was observed at 20-30 °C and between -0.7 and -7.0 MPa on both substrates (wheat and sorghum). The highest growth rate for the three strains was seen at 25 °C combined with -2.8 MPa. The comparison between the growth profiles of Tunisian and Spanish FIESC 5 strains showed similar trends with some interesting diferences regarding temperature and water potential factors. Tunisian strains seem to perform better between 15-30 ºC and, notably, at even lower water potentials included -9.8 Mpa. This might suggest that tolerance to low water potentials might be for Tunisian strains a more important selective clue than to higher temperatures. These results appeared to be consistent with a population well adapted to the present climatic conditions and predicted scenarios for North Africa.

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Abstract

The *Fusarium incarnatum-equiseti* species complex (FIESC) is a phylogenetically rich complex. It includes more than 30 cryptic phylogenetic species, making morphological identification problematic. FIESC has previously been detected in Tunisian cereals, but knowledge on the phylogeny and the ecophysiology of their species is lacking. In this work a phylogenetic analysis was performed using partial sequences of the translation elongation factor 1a gene (*EF1a*) of three FIESC strains isolated from barley and wheat from Tunisia, situated south in the Mediterranean basin, and additional strains from other countries. The results indicated that all Tunisian strains clustered with FIESC 5 group (*F. clavum*) together with other Spanish FIESC 5 strains also isolated from cereals. Growth rate profiles of the Tunisian strains were also determined on wheat and sorghum based media at a range of temperatures (15, 20, 25, 30, 35 and 40 °C) and water potential values (-0.7, -2.8, -7.0, and -9.8MPa, corresponding to 0.995, 0.98, 0.95 and 0.93 aw values). Optimal growth was observed at 20-30 °C and between -0.7 and -7.0 MPa on both substrates (wheat and sorghum). The highest growth rate for the three strains was seen at 25 °C combined with -2.8 MPa. The comparison between the growth profiles of Tunisian and Spanish FIESC 5 strains showed similar trends with some interesting diferences regarding temperature and water potential factors. Tunisian strains seem to perform better between 15-30 ºC and, notably, at even lower water potentials included -9.8 Mpa. This might suggest that tolerance to low water potentials might be

for Tunisian strains a more important selective clue than to higher temperatures. These results appeared to be consistent with a population well adapted to the present climatic conditions and predicted scenarios for North Africa.

Keywords: FIESC; Phylogenetics; *EF1a*; Growth rate; Environmental factors; Cereal based media

1. Introduction

Fusarium is one of the most diverse fungal genera that has been given much attention by mycologists and plant pathologists (Aoki et al. 2014, 2018; Maryani et al. 2019). The identification of *Fusarium* species is traditionally based on the detection of morphological and physiological features. However, such methods are not able to discriminate among similar species, nor to detect intraspecific variability. Fortunately, phylogenetic analyses using DNA sequence data have led to a better understanding of the *Fusarium* systematics, which is an essential element for establishing inter and intra-specific relationships (Jurado et al. 2006; Kristensen et al. 2005; Mirete et al. 2004; O'Donnell et al. 2009). Several genomic sequences have been used to analyse the intra-specific variability of *Fusarium,* including the *EF 1a* gene that has been used as a single-locus identification tool and is a suitable genetic marker for discriminating between *Fusarium* species (Geiser et al. 2004).

The *F. incarnatum-F. equiseti* species complex (FIESC) is a highly diverse group which currently includes 38 recognised phylospecies (FIESC 1–38)), across a wide range of habitats/hosts around the world, the majority of which have recently been linked to Latin binomials (Avila et al., 2019; Hartman et al., 2019; Lima et al 2021; Maryani et al., 2019; O'Donnell et al. 2009, 2010, 2012, 2018; Santos et al.,

2019; Short et al., 2011; Villani et al., 2016, 2019; Wang et al., 2019; Xia et al., 2019).

The classification within this group is constantly evolving. Several reports had previously indicated that FIESC 14 and FIESC 5 were both associated with *F. equiseti* clade (Villani et al. 2016), noting the existence of intraspecific diversity therein, particularly between Northern and Southern European strains which were separated into two distinct clusters FIESC 14 (*F. equiseti* type I) and FIESC 5 (*F. equiseti* type II), respectively (Castellá and Cabañes 2014; Jurado et al. 2006; Kosiak et al. 2005; Kristensen et al. 2005; Marín et al. 2012). Currently, *F. equiseti* represents the phylospecies FIESC 14 as reported in O'Donnell et al. 2009, while FIESC 5 was recently named *F. clavum* (Xia et al. 2019).

FIESC species have been found in grains of wheat, barley, rice, oats and maize (Amatulli et al., 2010; Avila et al 2019; Castellá and Cabañes, 2014; Marín et al., 2012; O'Donnell et al., 2018; Piacentini et al., 2019; Villani et al., 2016). Strains of FIESC have been reported to produce a range of different mycotoxins or a combination of them, including type A and B trichothecenes, and other mycotoxins such as zearalenone (ZEA) and fusarochromanone (FUSCHR) (Avila et al., 2019; Bennett and Klich 2003; Bottalico and Perrone 2002; Kosiak et al. 2005; Marín et al. 2012; O'Donnell et al., 2018). In fact, isolated strains in Southern Europe, particularly in Spain (Marín et al. 2012), showed a different toxin profile comparing to that previously described for strains in Northern Europe (Norway) (Kosiak et al. 2005). Indeed, Norwegian *F. equiseti* strains (FIESC 14) were found able to produce high levels of type A trichothecene (diacetoxy scirpenol (DAS), 15-monoacetoxy-scirpentriol (MAS), neosolaniol (NEO) but no T-2 or HT-2 toxins). However, for type B trichothecenes no detectable levels of DON or DON derivatives have been found, but

significant amounts of NIV and 4-acetylnivalenol (FUS-X) have been reported. On the other hand, Spanish FIESC 5 strains were found able to produce trichothecenes type B (DON, DON derivatives, and NIV and FU-X) and trichothecenes type A (NEO and DAS, but no T-2 nor H-2) (Kosiak et al. 2005; Marín et al. 2012).

Fungal growth and mycotoxin production are influenced by several variables, such as temperature, water potential, pH, substrate, interaction between species and time. In general, temperature and water potential are considered to be the most critical factors (Magan and Aldred 2007). However, there is scarce information about it regarding FIESC phylogenetic species. Thus, although ecophysiological differences between FIESC 14 and FIESC 5 could be envisaged according to their prevalence in two regions which notably differ in their climate, to our knowledge, no reports on the ecophysiological profile of FIESC 14 nor for any other FIESC population have been published apart from a previous work (Marín et al. 2015) focused on FIESC 5.

The effect of these factors on trichothecene production, and pathogenicity of FIESC strains has been previously reported (Kosiak et al. 2005; Marín et al. 2012, 2015; Palmero et al. 2011). Additionally, there is also information on their effect on the *TRI5* gene expression, a key gene for trichothecene biosynthesis (Marín et al. 2015).

In Tunisia, species within FIESC mostly designated as *F. equiseti* has been identified as one of the most pathogenic species infecting Tunisian durum wheat during 2004 and 2007 crop seasons, and causing *Fusarium* Head Blight (FHB) (Fakhfakh et al. 2011). *F. equiseti* was previously detected also in Moroccan wheat grains (Hajjaji et al. 2006). More recently, this species has been reported to be contaminant of Tunisian wheat and barley (Jedidi et al. 2018, 2021). In addition, members within FIESC was found to be the predominant *Fusarium* species (37/59 isolates) contaminating Tunisian and Egyptian sorghum seeds marketed in Tunisia

(Lahouar et al. 2015). The occurrence of DON and ZEA, which may be produced by various species, including members of FIESC, was previously reported in Tunisian cereals, particularly wheat and sorghum (Bensassi et al. 2010; Ghali et al. 2008; Jedidi et al. 2021; Zaied et al. 2012). To date, only few data exist on the mycotoxigenic ability of FIESC strains isolated in Tunisia except for those reported for *F. incarnatum* by Lahouar et al. (2015), showing its ability to produce ZEA. The influence of abiotic factors (temperature and water availability) on mycelial growth and ZEA accumulation by these strains has been studied *in vitro* on a sorghum grain medium (Lahouar et al. 2017); this study has shown that the optimal conditions for *F. incarnatum* proliferation are 25 °C and 0.99 aw, whereas those for ZEA production were not well defined, showing variability from one strain to another. However, there are no available DNA sequences from those strains which might permit their assignment to a particular FIESC species nor from any other studies carried out in the North African region to our knowledge. That is why in this work, we focus on describing the phylogenetic analysis of FIESC strains isolated from cereals grown in Tunisia and the evaluation of their growth under a range of ecophysiological conditions related to possible climatic scenarios in order to obtain useful information to improve prevention and control of mycotoxin risk strategies in cereals in Tunisia.

The aims of this work were: (1) to examine, using partial sequences of the *EF-1a* gene, the phylogenetics of three FIESC strains isolated from Tunisian wheat and sorghum and (2) to evaluate the effects on their growth of the interacting conditions of temperature and water potential on wheat and sorghum based substrates. The results are compared and discussed in relation to other FIESC strains isolated from cereals grown in Spain.

2. Material and methods

2.1. Fusarium strains

Three Tunisian *Fusarium* strains identified as FIESC, using the PCR protocol described by Jurado et al. (2005) which amplified strains from FIESC 14 and FIESC 5 (both belonging to the Equiseti clade), were used in this work: EQUF6, isolated from wheat cultivated in Kairouan (Center of Tunisia); and EQUF56 and EQUF85 strains, isolated from barley samples grown in Sousse (East of Tunisia) and Kairouan (Center of Tunisia), respectively (Table 1). Fungal cultures were maintained on potato dextrose agar medium (PDA) (CONDA, Pronadisa, Madrid, Spain) at 4 °C and stored as spore suspensions in 15% glycerol at -80 °C. Given their different cities of origin (Kairouan and Sousse) as well as their different matrices (wheat and barley), the three FIESC strains, subject of this study, may be considered good representatives of Tunisian FIESC isolates in general.

2.2. Phylogenetic analysis using partial sequences of EF1a

Extraction of genomic DNA from fungal cultures was basically performed according to Querol et al. (1992) using three mycelial disks which were excised from the margin of a seven-day-old PDA plates and crushed against the wall of a 2 mL microcentrifuge tube, using a sterile pipette tip. The DNA concentration was estimated using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA).

The partial sequences of the *EF1a* gene were obtained by PCR using the primers and the amplification program described elsewhere (O'Donnell et al. 1998). The PCR-amplified fragments were purified using the UltraClean™ PCR Clean-Up™ kit (MoBio Laboratories Inc., Carlsbad, CA, USA), and sequenced using the ABI 3730

176 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) according to the
177 manufacturer's instructions in the Genomic and Proteomic Unit of the Complutense
178 University of Madrid (Spain). Sequences were corrected using Chromas v 1.43
179 software (Brisbane, Australia) and analysed and edited using Bioedit Sequence
180 Alignment Editor v 7.0.9.0 software (Hall 1999).

181 Using PAUP v 4.0 b10 software (Marín et al. 2012; Swofford 2003), individual
182 maximum-parsimony (MP) phylogenetic analyses were performed for the Tunisian
183 FIESC strains using the partial sequences of the *EF1a* gene obtained. Additional
184 FIESC sequences obtained previously and other retrieved from databases were also
185 included. A *F. graminearum* strain was used as an outgroup in the analyses. A total
186 of 60 strains were used in the phylogenetic analyses (Table 1). Gaps were coded as
187 missing data and were excluded from the analyses. Unweighted parsimony analyses
188 were performed on the individual data sets using the heuristic search option with
189 1000 random additional sequences with tree bisection-reconnection (TBR) branch
190 swapping. Clade stability was assessed via 1000 bootstrap replications (Hillis and
191 Bull 1993).

192

193 *2.3. Growth profiles, in relation to temperature and water potential, on sorghum*
194 *and wheat based media*

195 The medium used in this study was a 6% (w/v) either sorghum or wheat
196 extract agar. Both cereal extract agar media were made by boiling 60 g of milled
197 wheat or sorghum grain in 1 L of distilled water for 30 min. The resulting mixture was
198 filtered through a double layer of muslin and the volume was made up to 1 L.
199 Subsequently, 20 g of bacteriological agar (CONDA, Pronadisa, Madrid, Spain) were
200 added to the mixture. Each cereal medium was modified with the non-ionic solute

glycerol to obtain the water potentials (Ψw) -2.8, -7.0 and -9.8 MPa corresponding to water activities (aw) of 0.98, 0.95 and 0.93 respectively. The control medium had a water potential of -0.7 MPa (=0.995 aw). All agar media were flowed in 9 cm Petri plates.

A 5-mm-diameter agar disk from the margin of 7-day-old growing colony of each of the three FIESC strains grown at 25 °C was used to centrally inoculate each replicate and treatment. The plates were incubated at 15, 20, 25, 30, 35 and 40 °C for 10 days. The experiment consisted of three replicates per treatment.

Assessments of growth were made daily during the 10-day incubation period. Two diameters of the growing colonies, at right angles to each other, were measured until the colony reached the edge of the plate. The radii of the colonies were plotted against time and a linear regression was applied to obtain the growth rate (mm/day) as the slope of the line, for all replicates and treatments. Two dimensional growth rate profiles were obtained for each strain in relation to temperature × water potential treatments with both sorghum and wheat extracts agar media.

Multifactor ANOVA of all the 4 factors (strain/substrate/temperature/ Ψw) and three ways ANOVA of factors (strain/temperature/Ψw) for each substrate were performed for growth rate of FIESC isolates, including all the replicates per treatment. Subsequent *post hoc* analyses (Tukey's HSD test of multiple comparisons) were carried out at a 95% confidence level ($P<0.05$). These statistical analyses were performed by using STATGRAPHICS CENTURION XV.II (Statistical Graphics Corp., Herndon, VA).

3. Results

3.1. Phylogenetic analyses

226 The total number of nucleotides (nt) of the partial sequence of the *EF1a* gene
227 analysed, excluding indels, was 631. Of these, 466 nt were constant, 95 nt were
228 parsimony-uninformative characters and 70 nt were parsimony-informative
229 characters. Fig. 1 shows the bootstrap 50% majority consensus tree based on MP
230 analysis of FIESC isolates and the consistency (CI), retention (RI) and rescaled
231 consistency (CR) indexes. The phylogenetic analysis revealed three distinct clusters
232 of isolates corresponding to FIESC 14 (*F. equiseti*), we named in previous works *F.*
233 *equiseti* type I, FIESC 5 (*F. clavum*), similarly named *F. equiseti* type II, and FIESC 9
234 (*F. scirpi*). The Northern European and Southern European FIESC strains in this
235 analysis were separated into the FIESC 14 and the FIESC 5 phylogenetic clusters,
236 respectively. In fact, 12 out of the 13 Spanish strains described in previous studies
237 (Jurado et al. 2006; Maciá-Vicente et al. 2008; Marín et al. 2012) fell into FIESC 5
238 cluster, while the other fell into the *F. equiseti* cluster. Otherwise, 17 out of the 18
239 Northern European strains (Kristensen et al. 2005; Nitschke et al. 2009; O'Donnell et
240 al. 2009) were clustered in *F. equiseti* cluster, while the other was clustered into *F.*
241 *scirpi* cluster. All Tunisian FIESC strains were clustered within *F. clavum* cluster.
242 Strains from other locations (Table 1) fell into either the *F. clavum*, *F. equiseti* or *F.*
243 *scirpi* clusters.
244
245 *3.2. Growth profiles of Tunisian FIESC strains*
246 Fig. 2 shows the two-dimensional maps of relative growth rate of the 3 FIESC
247 5 strains on wheat and sorghum based media in response to water potentials
248 (between -0.7 and -9.8 MPa) and temperatures (between 15 and 40 °C). The results
249 of our study indicate that the strains showed a wide range of permissive conditions
250 and, notably, showed that growth could be even sustained at water potential so low

as -9.8 MPa (between 15-35 ºC), Optimal growth was observed at 20-30 °C and between -0.7 and -7.0 MPa in both wheat and sorghum for all tested strains. The maximum growth rates were obtained at 25 °C combined with -2.8 MPa: mean values (of the 3 replicates) for EQUF6, EQUF56 and EQUF85 were 6.53±0.06 mm/day, 6.84±0.13 mm/day and 7.29±0.06 mm/day, respectively in wheat based medium, being slightly higher in sorghum based medium (6.87±0.04 mm/day, 7.07±0.15 mm/day and 7.25±0.04 mm/day, respectively). The effects on growth rate of single factors (strain, substrate, temperature and Ψw), as well as the effects of all their interactions were significant, except that of substrate × strain (Table 2). In general, growth rate appeared to be better on sorghum than in wheat, although the interactions substrate x water potential x strains are complex, as it was observed in a previous study performed on wheat and barley based substrates (Marín et al. 2015). Indeed, subsequent separate analyses for each substrate revealed significant effects of strain, with significant differences among them in response to different temperatures, water potentials and their interactions in the Tukey tests (Table 3) and this might reflect the existence of genetic variability among them.

3.3. Comparison of the growth profiles of Tunisian and Spanish FIESC 5 strains

Table 4 shows the growth rate average values, obtained on wheat based medium, of the 3 Tunisian FIESC 5 strains and those obtained for 4 FIESC 5 Spanish strains (EQU 5, EQU 7, EQU 9 and EQU 10), previously reported by Marín et al. (2015) and included in the phylogenetic study carried in this work. Based on statistical analyses, the comparison between growth patterns of these two populations indicates significant differences (data not shown). Although both groups showed a wide range of growth in relation to the temperature (15-35 ºC), being

optimal at 25 °C and lower at 15 and 35 °C, However, the optimal growth for Spanish strains was obtained at -0.7MPa and -2.8MPa and was notably reduced by high water stress conditions (especially at -9.8MPa), whereas Tunisian strains showed their optimal growth at -2.8MPa and a lower reduction at -7.0 and -9.8MPa.

4. Discussion

The geographical position of Tunisia and its climate are critical factors influencing the infestation by various species of *Fusarium* and the accumulation of their mycotoxins in cereals. Situated south in the Mediterranean basin, Tunisia shares a large and ancient tradition of growing cereals with other North African, middle-east and south European countries surrounding the Mediterranean sea. To prevent and control such risk, some measures and programs for food surveillance should be taken. Therefore, the study of the physiology of potentially mycotoxigenic species help to understand their performance facing climatic factors and to set environmental conditions limiting their growth and their mycotoxin production and are useful for such prevention and control of mycotoxigenic risk. Conventional methods for identifying *Fusarium* species may overlook the intraspecific diversity and phylogenetic analyses may be fortunately an alternative tool for revealing such variability; they can efficiently help in the identification of closely related fungal strains, and they permitted to associate growth patterns and toxigenic profiles with particular populations or species. Additionally, the use of phylogenetics with significant or diagnostic sequences (such as partial sequences of *EF1a*) helps to identify the FIESC isolates, situate new FIESC groups and delimitate species and populations, as well as to know their population structure and variability. All this information is crucial for the evaluation of their ecophysiological and toxic profiles to

be used for more efficient prediction and control strategies aimed to reduce the risk of toxigenic and pathogenic fungi for safety and security of food and feed.

4.1. Phylogenetic analysis of Tunisian FIESC strains

In the present work, we examined three FIESC strains, isolated from cereals cultivated in Tunisia and not characterised previously. We aimed to situate these Tunisian strains within a wider geographical context including *EF1a* sequence data of FIESC strains obtained in our previous studies, as well as those available from data bases.

This phylogenetic analysis revealed FIESC strains from cereals basically grouped in two different species FIESC 14 (*F. equiseti*) and FIESC 5 (*F. clavum*), prevalent in north and south Europe regions, respectively, showing distinct climatic characteristics. These results are consistent with previous studies reported by Jurado et al. (2006) and Marín et al. (2012), indicating that the two main clusters I and II reported therein would correspond to FIESC 14 and FIESC 5 phylospecies, respectively.

The 3 Tunisian FIESC strains appeared included in FIESC 5 cluster according the phylogenetic analysis described in this work. Additionally, they were compared for identity with reference sequences named according to FIESC (and binomial) denomination. The Tunisian strains EQUF6 and EQU85 showed 100% identity to *Fusarium* sp. FIESC_5c clone wxwh12 (accession number MG826864.1) and *Fusarium* sp. FIESC_5c clone wxwh06 (accession number MG826858.1) (Funnell-Harris et al. 2019) and EQUF56 showed 100% identity with *Fusarium* sp. FIESC 5 ITEM10393 (accession number LN901566.1) (Villani et al. 2016). These results provide important information to predict the risk that may pose FIESC 5 as a

pathogen and a mycotoxin producer. Indeed, the toxin profile reported for strains grouped in the FIESC 5 indicate production of DON, DON derivatives, NIV, FUS-X, NEO and DAS, though no production of type A thricothecenes T2 and HT-2 was detected (zearalenone was not analysed) (Marín et al., 2015). In this study, over 80% of the strains tested produced at least DON and 25% produced NIV as well and less than 20% were negative for the toxins analysed. Additionally, it was observed diversity among individuals regarding the set of toxins produced and the relative quantities manufactured.

4.2. Growth profiles of Tunisian FIESC 5/ F. clavum strains

In this work, the influence of climatic factors, temperature and water potential, on the fungal growth of the 3 Tunisian FIESC 5 strains was examined on two different substrates (wheat and sorghum) (Figure 2, Tables 2 and 3). The climatic conditions tested included high temperatures (up to 40 °C) and low water potentials (up to -9.8 MPa) to account for the most extreme scenarios among those predicted for Tunisia. The choice of wheat and sorghum as substrates comes from the importance of these two cereals in Tunisian population diet. The consumption of wheat in form of pasta, "Couscous", traditional bread, "Frik", and "Bsissa" is a cultural tradition. Sorghum can also be counted as one of the most important cereals in Tunisia, given its intense use in animal feed, and especially during Ramadan month, where its human consumption increases in the form of "Bouza". In addition, contamination of these cereals by FIESC has already been reported in Tunisia (Jedidi et al. 2018, 2021; Lahouar et al. 2015).

The results of our study indicate that the strains analysed a wide range of permissive conditions and, notably, showed that growth could be even sustained at

351 water potential so low as -9.8 MPa (between 15-35 ºC), hardly possible for other
352 *Fusarium* species common in cereals such as *F. verticillioides*, *F. proliferatum* and
353 even more for *F. graminearum* (Marín et al. 2010). These results are basically in
354 agreement with the incidence and diversity of the *Fusarium* species in cereals
355 (maize, wheat, barley and sorghum) reported in Tunisia and neighbouring countries.
356 "*F. equiseti*" species, occasionally and particularly in certain regions related with
357 *Fusarium* Head Blight, are commonly found, with variable relative incidence in wheat,
358 barley and sorghum, probably due to differences in climatic and environmental
359 conditions in fields among locations and years (Fakhfakh et al. 2011 ; Hajjaji et al.
360 2006; Jedidi et al. 2018, 2021; Lahouar et al. 2015). It cannot be assessed if those
361 "*F. equiseti*" strains were FIESC 5, though it might be possible in the case of the last
362 two Jedidi et al. reports since they used the same PCR assay we did in the present
363 work. *F. graminearum* had shown optimal growth rate at 25 ºC and -2.8 MPa with
364 much higher values (9-10 mm/day) (Marín et al. 2010) than any of the FIESC 5
365 strains analysed but substantially decreases at temperatures higher than 30-35 ºC
366 and water potential lower than -2.8 MPa. Under these conditions, FIESC 5 strains
367 might become more competitive. Similarly, *F. verticillioides* appeared to be more
368 prevalent in maize than *F. graminearum* in the studies above mentioned. These
369 results are in agreement with the higher growth reduction of *F. graminearum* in
370 comparison with *F. verticilliodes* at temperatures of 30 ºC and 35 ºC in combination
371 with lower values of water potentials rates (Marín et al. 2010). Furthermore, some of
372 the reports above mentioned on the occurrence of *Fusarium* species on cereals in
373 Tunisia and neighbouring countries also analysed a number of mycotoxins in their
374 samples reporting frequently DON and NIV, which can be produced by either *F.*
375 *graminearum* or FIESC 5 (Hajjaji et al. 2006; Jedidi et al. 2021). On the other hand,

376 the highest induction of *TRI5* gene expression in FIESC 5 strains has been reported
377 between 25-35 ºC at water potential between -0.07 and - 2.8 MPa in barley and
378 wheat based media (Marín et al. 2015). These ranges of conditions include the
379 optimal conditions for growth of the FIESC 5 strains analysed in present work
380 suggesting that trichothecene biosynthesis will be within the range of the most
381 favourable conditions for host colonisation, increasing the potential risk for
382 trichothecene contamination. These data provide useful information about the effect
383 on the fate of FIESC 5 population, that could have future conditions of higher
384 temperatures and long drought periods predicted by climatic change scenarios for
385 Tunisia and other Mediterranean countries, and they can direct the concerned
386 authorities and organisations to apply preventive strategies to reduce growth of this
387 fungal species in cereal grains

388 The influence of the substrate on colonisation by FIESC strains has been
389 recognised by several authors (Llorens et al. 2004; Marín et al. 2004; Ramírez et al.
390 2006). Higher levels of fungal contamination of sorghum than of wheat by these
391 species have been reported in Tunisia (Jedidi et al. 2018; Lahouar et al. 2015). This
392 agreed with our results in the present work showing significant differences pointing
393 out that sorghum may be more favourable than wheat for proliferation of Tunisian
394 strains.

395

396 *4.3. Comparison between growth patterns of Tunisian and Spanish FIESC 5*
397 *strains*

398 The comparison between the growth profiles of Tunisian and Spanish FIESC 5
399 strains showed similar trends with some interesting differences regarding
400 temperature and water potential factors. Tunisian strains seem to perform better

between 15-30 ºC and, notably, at even lower water potentials including -9.8 Mpa. This might suggest that tolerance to low water potentials might be for Tunisian strains a more important selective clue than to higher temperatures. That might be advantageous for them to occur for instance in saline soils and that might be the case of the strain D3 (MK361175.1) reported as *F. equiseti* with an identity of 100% to FIESC 5 (included in a study of halotolerant and halophilic fungi from Algeria, Chamekh et al. 2019); these are demanding environments where tolerance to low water potentials is necessary.

In conclusion, the present work reports the occurrence of FIESC 5 in Tunisia, showing growth profiles similar to Spanish FIESC 5 population although with higher growth values at lower water potentials (-9.8 MPa, between 15-35 ºC), hardly possible for other *Fusarium* species common in cereals such as *F. verticillioides*, *F. proliferatum* and, particularly for *F. graminearum*. Additionally, they showed slightly higher growth rates on sorghum than on wheat based medium in agreement with their higher incidence in sorghum than in wheat fields. Finally, the occurrence of FIESC 5 population in Tunisia suggest that its members might pose a veritable risk of cereal contamination in a large geographical and climatic area where it was not reported previously and which deserves further investigation given the importance of cereals for the North African region.

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Table 1

Fusarium strains used in this study, indicating host, origin and accession number of the *EF1a* partial sequences.

F. incarnatum-equiseti species complex						
Isolate name	**Strain**	**Phylogenetic species/Species complex**	**Host**	**Origin**	**Accession number**	**References**
EQU1	H3SA.042	FIESC 5	Barley	Spain	JF496568	Marín et al. 2012
EQU2	C1SA.060	FIESC 5	Barley	Spain	JF496569	Marín et al. 2012
EQU3	C1SA.063	FIESC 5	Barley	Spain	JF496570	Marín et al. 2012
EQU4	C3RA.065	FIESC 5	Barley	Spain	JF496571	Marín et al. 2012
EQU5	C1SA.073	FIESC 5	Barley	Spain	JF496568-1	Marín et al. 2012
EQU6	D24SZ.090	FIESC 5	Barley	Spain	JF496572	Marín et al. 2012
EQU7	C1SA.102	FIESC 5	Barley	Spain	JF496573	Marín et al. 2012
EQU8	C3SH.103	FIESC 14	Barley	Spain	JF496574	Marín et al. 2012
EQU9	H2-2-5B	FIESC 5	Durum wheat	Spain	JF496575	Jurado et al. 2006
EQU10	L1-2-2	FIESC 5	Durum wheat	Spain	JF496575-1	Jurado et al. 2006
EQU11	L3-1-2J	FIESC 5	Durum wheat	Spain	JF496576	Jurado et al. 2006
EQU12	U6-1-1	FIESC 5	Durum wheat	Spain	JF496577	Jurado et al. 2006
EQU13	VI01066	*F. scirpi*	Soil	Malta	AJ543571	Kristensen et al. 2005
EQU14	VI01067	FIESC 14	Beet	Denmark	AJ543558	Kristensen et al. 2005
EQU15	VI01068	FIESC 14	Barley	Sweden	AJ543557	Kristensen et al. 2005
EQU16	VI01069	FIESC 14	Onion	Denmark	AJ543561	Kristensen et al. 2005
EQU18	VI01071	FIESC 14	Wheat	Denmark	AJ543563	Kristensen et al. 2005
EQU19	VI01072	FIESC 14	Barley	Denmark	AJ543559	Kristensen et al. 2005
EQU20	VI01079	FIESC 14	Wheat	Norway	AJ543564	Kristensen et al. 2005
EQU22	VI01087	*F. scirpi*	Wheat	Norway	AJ543570	Kristensen et al. 2005
EQU23	VI01093	FIESC 14	Barley	Norway	AJ543566	Kristensen et al. 2005
EQU24	VI01095	FIESC 14	Barley	Norway	AJ543560	Kristensen et al. 2005
EQU25	VI01096	FIESC 14	Barley	Norway	AJ543567	Kristensen et al. 2005
EQU26	VI01104	FIESC 14	Wheat	Norway	AJ543568	Kristensen et al. 2005
EQU27	VI01105	FIESC 14	Oats	Norway	AJ543569	Kristensen et al. 2005
EQU30	11_ZP_2	FIESC 14	Soil	Canada	DQ842055	_

EQU31	34/2.1.1	FIESC 5	Esparto grass	Spain	DQ854854	Maciá-Vicente et al. 2008
EQU33	DAOM194187	FIESC 5	Wheat	Canada	DQ842084	_
EQU35	DAOM232362	FIESC 5	Barley	Canada	DQ842096	_
EQU36	DAOM236361	FIESC 5	Wheat	Canada	DQ842099	_
EQU38	G4_2_QC_ND_3_2_1_2	*F. scirpi*	Soybean	Canada	DQ842101	_
EQU43	11_ZP_1	FIESC 14	Ginseng soil	Canada	DQ842054	_
EQU44	16_ZP_2	FIESC 14	Wheat	Canada	DQ842058	_
EQU46	22_ZP_2	FIESC 14	Straw	Canada	DQ842061	_
EQU48	2_ZP_2	FIESC 14	Straw	Canada	DQ855945	_
EQU49	7_ZP_1	FIESC 14	Ginseng root	Canada	DQ842078	_
EQU50	60	FIESC 5	Sugar beet	France	FJ939674	Nitschke et al. 2009
EQU52	113	*F. scirpi*	Sugar beet	US	FJ939678	Nitschke et al. 2009
EQU53	90	FIESC 14	Sugar beet	Sweden	FJ939675	Nitschke et al. 2009
EQU58	157	FIESC 14	Sugar beet	Germany	FJ939684	Nitschke et al. 2009
EQU60	149	FIESC 14	Sugar beet	Germany	FJ939680	Nitschke et al. 2009
EQU61	174	FIESC 5	Sugar beet	Italy	FJ939686	Nitschke et al. 2009
EQU62	DAOM194188	FIESC 5	Wheat	Canada	DQ842085	_
EQU64	DAOM215463	*F. scirpi*	Corn	Canada	DQ842094	_
EQU65	DAOM232364	*F. scirpi*	Wheat	Canada	DQ842098	_
EQU66	GLS2	FIESC 5	Rice	Italy	GQ848542	Amatulli et al. 2010
EQU68	NRRL20697	FIESC 5	Beet	Chile	GQ505594	O'Donnell et al. 2009
EQU69	NRRL26419	FIESC 5	Soil	Germany	GQ505599	O'Donnell et al. 2009
EQU70	NRRL36136	FIESC 5	_	_	GQ505644	O'Donnell et al. 2009
EQU71	NRRL36321	FIESC 5	Soil	Netherlands	GQ505647	O'Donnell et al. 2009
EQU72	NRRL36466	FIESC 5	Potato peel	Denmark	GQ505356	O'Donnell et al. 2009
EQU73	NRRL43636	FIESC 14	Dog	US	GQ505663	O'Donnell et al. 2009
SCI1	NRRL36478	*F. scirpi*	Pasture soil	Australia	GQ505654	O'Donnell et al. 2009
SCI2	NRRL29134	*F. scirpi*	Pasture soil	Australia	GQ505605	O'Donnell et al. 2009
SCI3	NRRL26922	*F. scirpi*	Soil	France	GQ505601	O'Donnell et al. 2009
SCI4	NRRL13402	*F. scirpi*	Pine soil	Australia	GQ505592	O'Donnell et al. 2009
EQUF6	W3Hp2g10B1	FIESC 5	Wheat	Tunisia	KP881270	Present work

EQUF85	B9Hp1g4B1	FIESC 5	Barley	Tunisia	KP881272	Present work
EQUF56	B4Hp1g1B2	FIESC 5	Barley	Tunisia	KP881271	Present work
			F. graminearum			
GRA1	GRA1	NRRL29169	Wheat	US	AF212461	O'Donnell et al. 2000

_ unpublished

Table 2

Multifactorial ANOVA (strain/substrate/temperature/Ψw) of growth rate of the three FIESC 5 strains (EQUF6, EQUF56 and EQUF85) incubated with different substrates (wheat and sorghum) for 10 days at different temperatures (15, 20, 25, 30 and 35 ºC) and water potentials (-0.7, -2.8, -7.0 and -9.8 MPa) and their interactions. Since no growth was observed at 40 ºC, this temperature value is not considered for ANOVA.

Source of variation	**d.f.**	**Mean square**	**F-Snedecor**
Substrate	1	7.825	49.567*
Strain	2	1.772	12.231*
Temperature	4	512.344	3536.179*
Ψ_w	3	829.723	5726.716*
Substrate × Strain	2	0.101	0.695
Substrate × Temperature	4	3.447	23.788*
Substrate × Ψ_w	3	1.325	9.145*
Strain × Temperature	8	3.515	24.258*
Strain × Ψ_w	6	4.154	28.671*
Temperature × Ψ_w	12	21.938	151.414*
Substrate × Strain × Temperature	8	0.896	6.186*
Substrate × Strain × Ψ_w	6	0.751	5.180*
Substrate × Temperature × Ψ_w	12	0.815	5.622*
Strain × Temperature × Ψ_w	24	1.109	7.654*
Substrate × Strain × Temperature × Ψ_w	24	0.431	2.972*

* Significant at P<0.001.

Table 3

Three ways ANOVA of factors (strain/temperature/Ψw) per substrate (wheat and sorghum) of growth rate for FIESC 5 strains (EQUF6, EQUF56 and EQUF85). Tukey's HSD tests for each strain, temperature and water potential were separately performed. Different letters indicate significant differences at P<0.05. Since no growth was observed at 40 ºC, this temperature value is not considered for ANOVA.

Source of variation: wheat	**d.f.**	**Mean square**	**F-Snedecor**
Strain	2	1.335	7.955**
Temperature	4	246.895	1470.785**
Ψ_w	3	422.503	2516.904**
Strain × Temperature	8	3.531	21.032**
Strain × Ψ_w	6	2.348	13.984**
Temperature × Ψ_w	12	10.391	61.900**
Strain × Temperature × Ψ_w	24	0.848	5.045**

Tukey's HDS test

Ψ_w (MPa)						**Temperature (ºC)**			
	15 ºC	**20 ºC**	**25 ºC**	**30 ºC**	**35 ºC**	**-0.7MPa**	**-2.8MPa**	**-7.0MPa**	**-9.8MPa**
EQUF6	a/b/c/d	a/b/c/d	a/a/b/c	a/a/b/c	a/b/c/d	a/b/c/b/d	a/ab/b/a/c	a/b/b/a/c	a/a/b/a/c
EQUF56	a/b/c/d	a/b/c/d	a/b/c/d	a/b/c/d	a/b/a/c	a/ab/b/b/c	a/a/b/a/c	a/b/c/b/d	a/b/c/b/d
EQUF85	a/b/c/d	ab/a/b/c	a/b/c/d	a/b/c/d	a/b/a/c	a/b/c/bc/d	a/a/b/c/d	a/b/b/c/d	a/b/c/d/e

Strain

	15 ºC	**20 ºC**	**25 ºC**	**30 ºC**	**35 ºC**
-0.7MPa	a/a/b	a/b/b	a/b/a	a/a/a	a/a/a
-2.8MPa	a/a/b	a/b/b	a/a/b	a/a/b	a/b/a

-7.0MPa	a/a/b	a/b/a	a/a/a	a/b/b	a/a/b
-9.8MPa	a/a/a	a/a/b	a/a/b	a/a/a	a/a/a

Source of variation: sorghum	**d.f.**	**Mean square**	**F-Snedecor**
Strain	2	0.538	4.409*
Temperature	4	268.895	2205.753**
Ψ_w	3	408.544	3351.298**
Strain × Temperature	8	0.880	7.221**
Strain × Ψ_w	6	2.557	20.976**
Temperature × Ψ_w	12	12.362	101.402**
Strain × Temperature × Ψ_w	24	0.691	5.669**

Tukey's HDS test

Ψ_w (MPa)						**Temperature (ºC)**			
	15 ºC	**20 ºC**	**25 ºC**	**30 ºC**	**35 ºC**	**-0.7MPa**	**-2.8MPa**	**-7.0MPa**	**-9.8MPa**
EQUF6	a/b/c/d	a/b/c/d	a/b/c/d	a/b/c/d	a/b/a/c	a/a/b/c/d	a/b/c/a/d	a/b/c/a/d	a/b/c/d/e
EQUF56	a/b/c/d	a/b/a/c	a/b/c/d	a/b/c/d	a/b/a/c	a/a/b/b/c	a/a/b/c/d	a/b/c/d/e	a/b/b/a/c
EQUF85	a/b/a/c	a/b/a/c	a/b/c/d	a/b/c/d	a/b/a/c	a/a/b/b/c	a/b/c/a/d	a/b/c/a/d	a/bc/c/ab/d

Strain

	15 ºC	**20 ºC**	**25 ºC**	**30 ºC**	**35 ºC**
-0.7MPa	a/a/b	a/b/b	a/a/a	a/a/b	a/a/a
-2.8MPa	a/a/b	a/a/a	a/a/a	a/a/a	a/b/a
-7.0MPa	a/a/a	a/a/a	a/a/b	a/b/b	a/a/a
-9.8MPa	a/a/a	a/a/a	a/a/a	a/a/a	a/a/a

* Significant at P<0.01 and **Significant at P<0.001.

Table 4

Growth rate* (mm/day) of Tunisian and Spanish FIESC 5 strains on wheat based medium, depending on temperature and Ψw (mm/day)

	Tunisian strains[1]				Spanish strains[2]			
Temperature (°C)	**Water potential (MPa)**							
	-0.7	**-2.8**	**-7.0**	**-9.8**	**-0.7**	**-2.8**	**-7.0**	**-9.8**
15	4.24±0.69	5.51±0.50	2.57±0.16	1.60±0.08	3.77±1.37	3.78±0.69	1.67±0.15	0.00±0.00
20	5.08±0.49	5.70±0.58	3.91±0.44	2.23±0.24	5.06±0.37	4.56±0.46	1.96±0.28	0.11±0.21
25	6.01±0.38	6.89±0.38	4.15±0.08	2.94±0.56	7.45±0.26	5.90±0.82	3.24±0.52	1.23±0.17
30	5.45±0.14	6.04±0.21	3.17±0.37	2.05±0.13	6.21±1.73	5.54±0.55	2.35±0.61	0.67±0.43
35	1.34±0.05	2.72±0.51	1.61±0.20	0.55±0.07	2.65±0.81	3.35±0.50	1.23±0.39	0.06±0.11
40	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

*mean ± standard deviation

[1]strains analysed in this work

[2]4 strains. Data provided by the authors of the article Marín et al. 2015.

Figure 1

Click here to access/download;Figure;Figure 1.tif

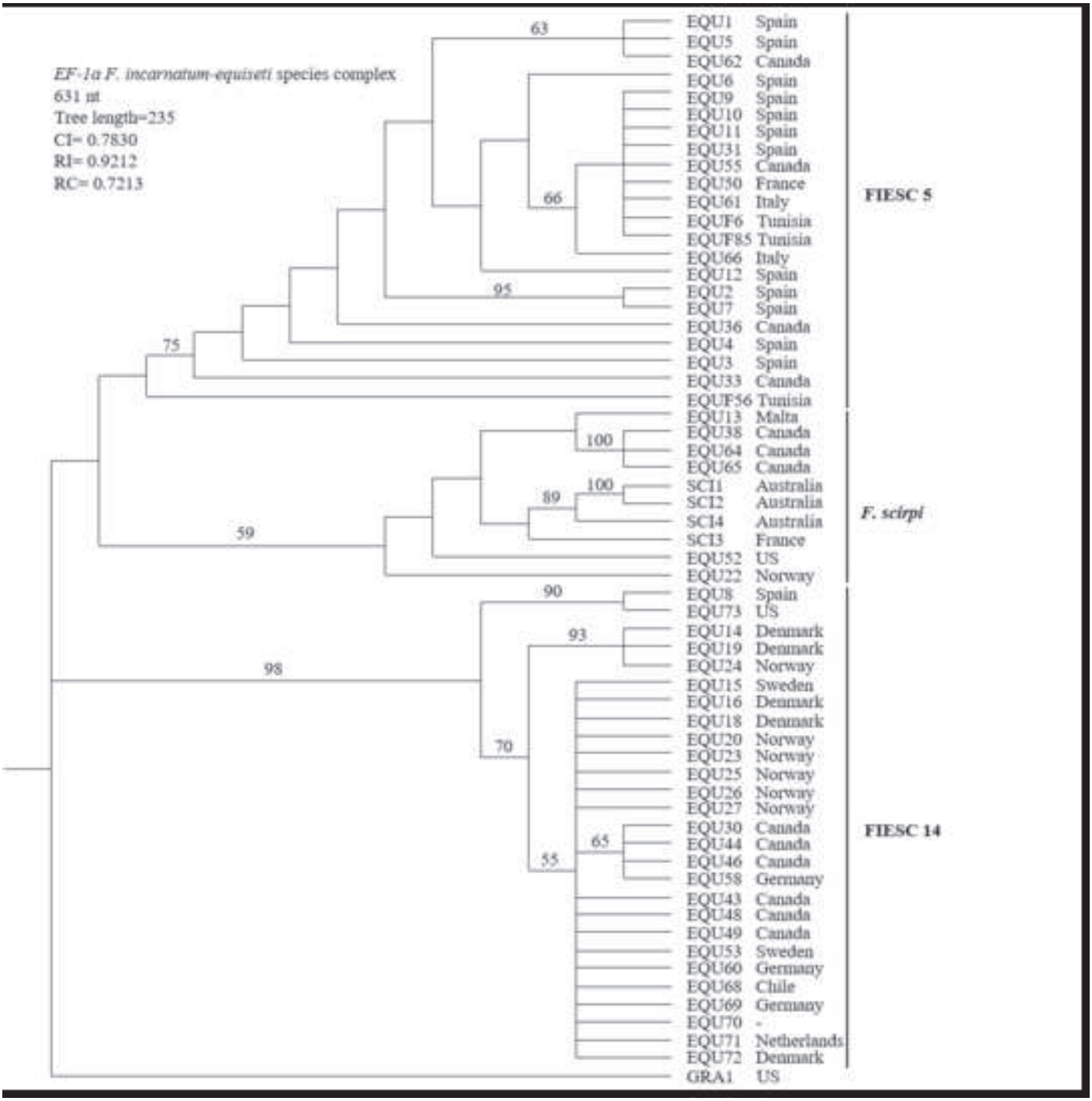

Figure 2

Click here to access/download;Figure;Figure 2.tif

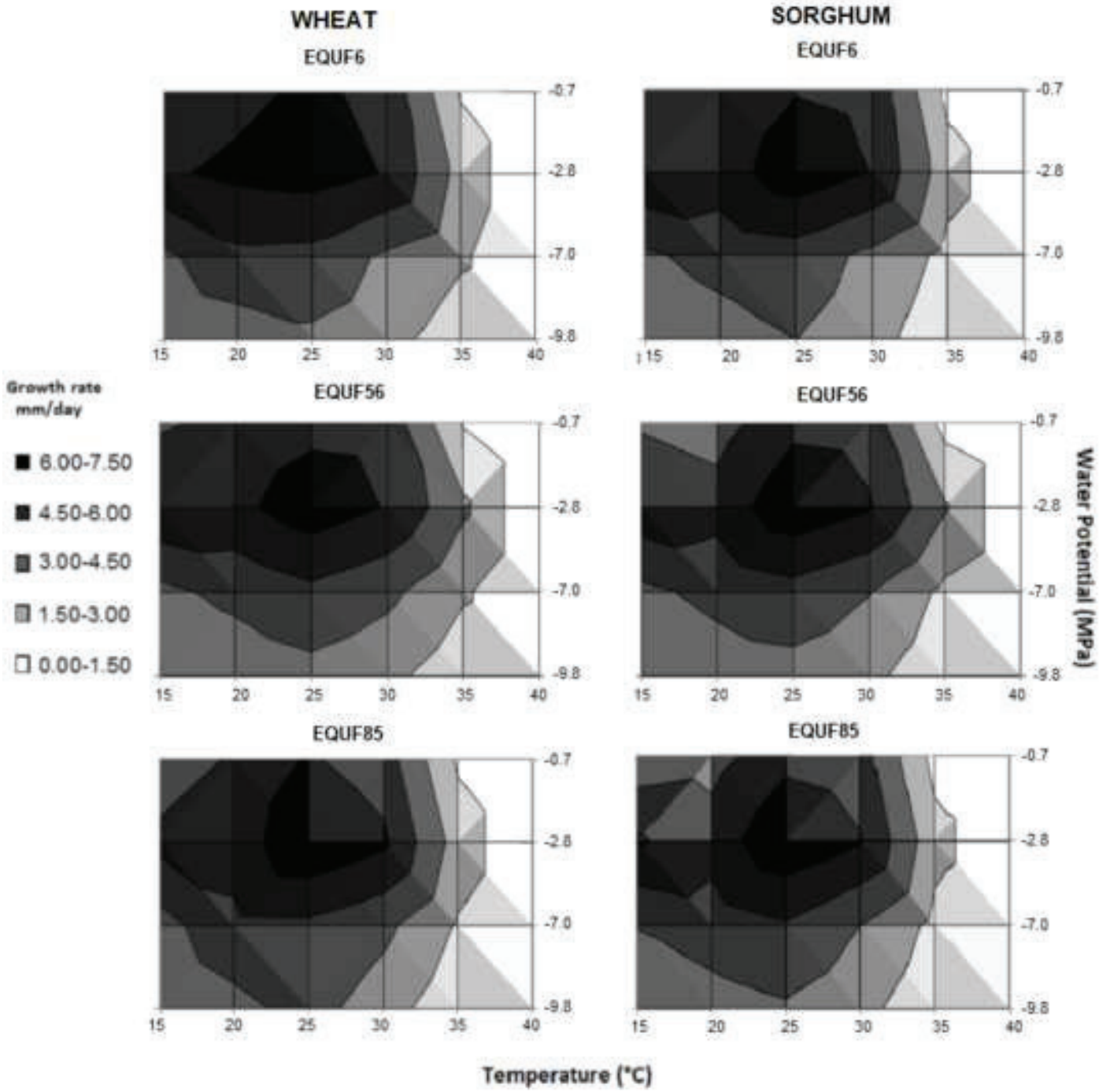

Figure captions

Fig. 1. Bootstrap 50% majority-rule consensus tree based on MP analysis of the FIESC isolates. CI: Consistency index, RI: Retention index, RC: Rescaled consistency index.

Fig. 2. Two-dimensional maps of the relative growth rate of the 3 Tunisian FIESC 5 (*F. clavum*) strains on wheat and sorghum substrates in response to water potentials and temperatures.