Growth rate and *TRI5* gene expression profiles *of Fusarium equiseti* strains isolated from Spanish cereals cultivated on wheat and barley media at different environmental conditions

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A B S T R A C T

Fusarium equiseti is a toxigenic spedes that often contaminates cereal crops from diverse climatic regions such as Northern and Southern Europe. Previous results suggested the existence of two distinct populations within this species with differences in toxin profile which largely corresponded to North and South Europe (Spain). In this work, growth rate profiles of 4 F *equiseti* strains isolated from different cereals and distinct Spanish regions were determined on wheat and barley based media at a range of temperatures (15,20,25,30,35 and 40 t) and water potential regimens (- 0.7, - 2.8, - 7.0, and - 9.8 MPa, corresponding to 0.99,0.98,0.95 and 0.93 a_w values). Growth was observed at all temperatures except at 40 °*C*, and at all the solute potential values except at — 9.8 MRa when combined with 15 °*C*. Optimal growth was observed at 20-30 "Cand —0.7/—2.8 MPa The effect of these factors on trichothecene biosynthesis was examined on a F *equiseti* strain using a newly developed real time RT-PCR protocol to quantify *TRI5* gene expression at 15,25 and 35 °C and — 0.7, — 2.8, — 7.0 and — 9.8 MPa on wheat and barley based media. Induction of *TRI5* expression was detected between 25 and 35 °C and —0.7 and —2.8 MPa, with maximum values at 35 °C and—2.8 MPa being higher in barley than in wheat medium. These results appeared to be consistent with a population well adapted to the present climatic conditions and predicted scenarios for Southern Europe and suggested some differences depending on the cereal considered. These are also discussed in relation to other *Fusarium* species co-occurring in cereals grown in this region and to their significance for prediction and control strategies of toxigenic risk in future scenarios of climate change for this region.

1. Introduction

Fusarium equiseti (Corda) Saccardo is a cosmopolitan fungus distributed across regions with cool through to hot and arid climates (Leslie and Summerell, 2006). It behaves as a soil saprophyte associated with rotting fruit and other decaying plant material, and as a pathogen of a wide range of crops. These include cereals, wheat, triticale, maize, oat and rice, participating as a minor component of the *Fusarium* head blight (FHB) disease complex (Kosiak et al., 2003; Logrieco et al., 2003; Xu et al., 2008). Other susceptible crops are asparagus, cotton, potato, tomato, cowpea, onion, bean, bush bean, broad bean, chickpea, pea, alfalfa, canola, kohlrabi, melon and ginseng (Palmero et al., 2011; Punja et al., 2008). Besides cultivated plants, it also colonizes wild species, including herbal plants and trees, e.g. *Equisetum* L, *Chenopodium* L and *Pinus* L (Goswami et al., 2008). AlthoughF. *equiseti* is considered as a moderately aggressive pathogen for these plants, it might also become a long-term root endophyte with beneficial effects for other host plants, such as protecting them against other phytopathogenic fungi (Macia-Vicente et al., 2009). Additionally, *F. equiseti* is reported to produce a vast range of toxic secondary metabolites such as A and B trichothecenes, and others such as butenolide, beauvericin (BEA), equisetine (EQJ, fusarochromanone (FUSCHR) and zearalenone (ZEA) (Adejumo et al., 2007; Kosiak et al., 2005; Leslie and Summerell, 2006; Marin et al., 2012). Trichothecenes are potent inhibitors of protein synthesis in eukaryotic cells (Brown et al., 2001). They cause different acute and severe diseases in humans and animals depending on the type of trichothecene ingested (Trenholm et al., 1989). Accordingly, their presence in foods and feeds is under legal regulation.

The phylogenetic status of this species is far from being elucidated. This represents a crucial issue in determining the risk that this species poses both as a pathogen and as a mycotoxin producer. *F. equiseti* is included in the so-called *Fusarium incarnatum-F. equiseti* species complex (FIESC) based on phylogenetic studies using a multilocus DNA sequence approach. This complex is a highly diverse group which may contain several genetically isolated populations or cryptic species (ODonnelletal., 2009,2012). This species frequently contaminates cereals in Northern and Southern Europe in spite of highly differing climatic characteristics ofthese regions (Jurado et al., 2006; Kosiak et al., 2005; Logrieco et al., 2003; Marin, 2010; Soldevilla

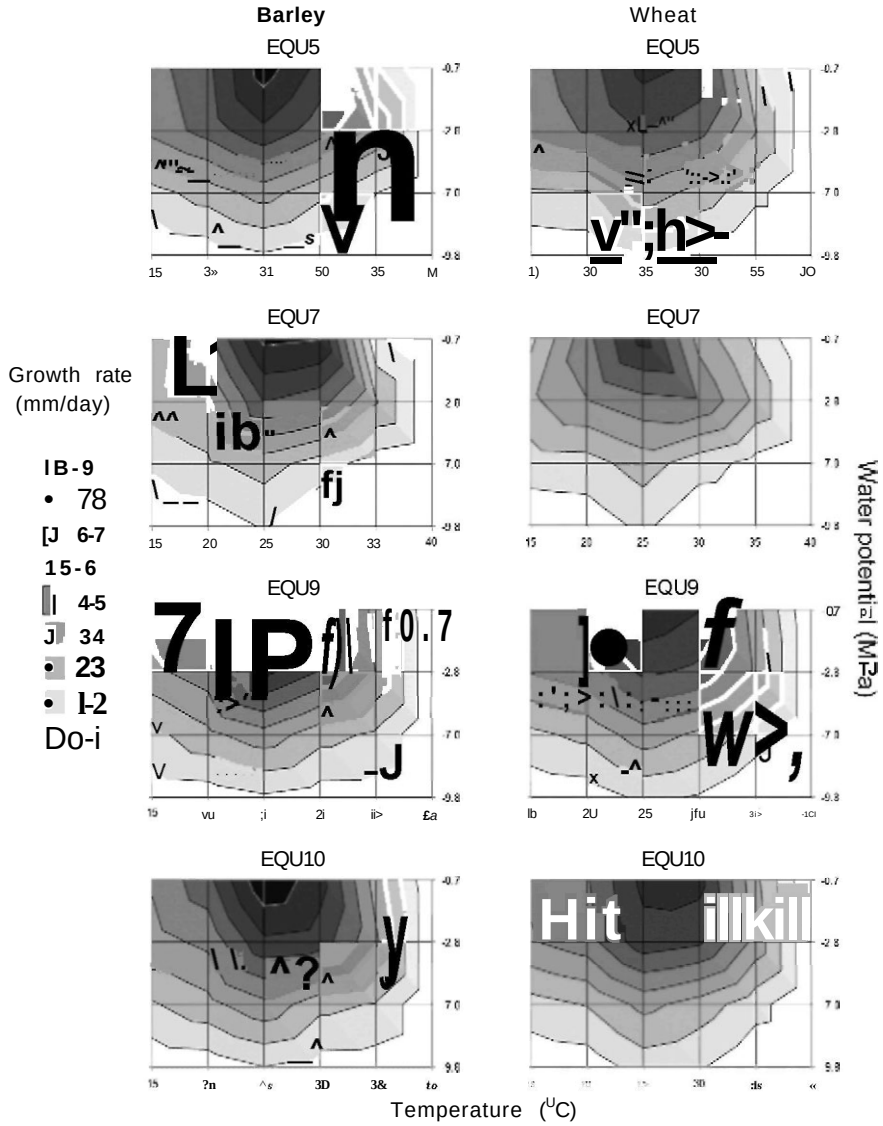

Fig. 1. Bi-dimensional maps of relative growth rate of the four *F. equiseti* strains on barley and wheat in response to water potentials and temperatures.

et al., 2005; Sţepień et al., 2012). Subsequent studies indicated that the Northern and Southern European *F. equiseti* strains are separated largely into two phylogenetically distinct clusters (Jurado et al., 2006; Marin et al., 2012), which basically correspond to FIESC-14 *(F. equiseti sensu stricto)* and F IESC-5c, respectively. *F. equiseti* strains from F IESC-5 group were also reported in Italy (Balmas et al., 2010) and in a recent study carried out in Spain (Castella' and Cabanes, 2014). Moreover, strains isolated from Spain showed a different mycotoxin profile (Marin et al., 2012) to that previously described for strains from Northern Europe (Kosiak et al., 2005). These reports indicating the existence of distinct populations within this species might also indicate the existence of ecophysiological differences between them.

Temperature and water potential are major environmental factors affecting fungal growth and mycotoxin biosynthesis. Previous studies have shown that the patterns of fungal growth and toxin biosynthesis in the pathogenic and mycotoxigenic *Fusarium* verticillioid.es, *Fusarium proliferation and Fusarium graminearum* are consistent with their occurrence within Europe and especially in Spanish cereal fields (Jurado et al., 2008; Marin et al., 2010a, 2010b). The effect of water potential and temperature on fungal growth, trichothecene production and pathogenicity was reported previously (Kosiak et al., 2005; Marin et al., 2012; Palmero et al., 2011). However, there are no studies that link these data at a fundamental level to expression of key regulatory genes such as *TRI5* in the case off. *equiseti*. *TRI5* is the key gene of trichothecene biosynthesis and encodes trichodiene synthase, which catalyzes the first step in the trichothecene biosynthetic pathway, the isomerisation and cyclation of farnesil pyrophosphate to trichodiene (Foroud and Eudes, 2009). Previous studies reported that the expression of key genes of mycotoxin biosynthetic pathways, such as *FUM1* or *TRI5*, measured by real time RT-PCR is positively related to production of fumonisin and trichothecenes in *F. verticillioid.es* or *F. proliferation* and *F. graminearum*, respectively (Doohan et al., 1999; Jurado et al., 2010; Lopez-Errasquin et al., 2007). These and related approaches were useful to unravel the influence of environmental factors on regulation of toxin biosynthesis in a number of species and toxins and to develop models to predict toxin risk (Marin et al., 2010a, 2010b; Schmidt-Heydt et al., 2011).

The objectives of this study were to examine the effects of interacting conditions of temperature, water potential and substrates (wheat and barley) on (i) growth and (ii) expression of the trichothecene biosynthetic *TRB* gene using a newly developed protocol of real time RT-PCR for *F. equiseti*. The results are compared and discussed in relation to other *Fusarium* species co-occurring in cereals grown in Spain and to

Table 1
Multifactorial ANOVA (strain/$*_w$/temperature) of growth rates of the four F *equiseti* strains EQU5, EQU7, EQU9 and EQU10 F *equiseti* strains incubated with two substrates (barley or wheat) for 10 days at different temperatures (15, 20, 25, 30, 35 and 40 °C) and water potentials (- 0.7, - 2.8, - 7.0 and - 9.8 MPa) and multifactorial ANOVA (substrate/$*_w$/temperature) for EQU9 (similar results were obtained for the other 3 strains).

Source of variation	df	Mean square	F*
Barley			
Strain	3	38.879	11.432***
$*_w$	3	1381.043	29.990***
Temperature	4	280.703	5.941**
Strain x $*_w$	9	3.452	1.566
Strain x temperature	12	3.401	1.543
$*_w$ x temperature	12	46.050	20.897***
Strain x $*_w$ x temperature	36	2.204	10.091***
Wheat			
Strain	3	48.280	13.803***
$*_w$	3	1140.615	58.490***
Temperature	4	214.703	10.322***
Strain x $*_w$	9	5.599	2.547*
Strain x temperature	12	3.498	1.591
$*_w$ x temperature	12	19.501	8.872**
Strain x $*_w$ x temperature	36	2.198	10.758***
EQU9			
Substrate	1	1.380	0.848
$*_w$	3	711.631	61.060***
Temperature	4	113.687	9.169***
Substrate x $*_w$	3	0.589	0.667
Substrate x temperature	4	1.627	1.843
$*_w$ x temperature	12	11.655	13.202***
Substrate x $*_w$ x temperature	12	0.883	10.122***

* Significant at $P < 0.05$.
** Significant at $P < 0.01$.
*** Significant at $P < 0.001$.
* Snedecor's F-test

their significance for the predicted scenarios of climatic change in this and similar regions.

2. Material and methods

2.1. Fungal strains

F. equiseti EQU5 and EQU7 strains were isolated from barley fields in Albacete (Spain) (Marin et al., 2012) and *F. equiseti* EQU9 and EQU10 strains were isolated from durum wheat fields in Huelva and Sevilla (Spain), respectively (Jurado et al., 2006). All four strains were able to produce trichothecenes and their toxin profiles are described elsewhere (Marin et al., 2012). Fungal cultures were maintained on potato dextrose agar medium (Scharlau Chemie, Barcelona, Spain) at 4 °C and stored as spore suspensions in 15% glycerol at —80 °C in the Department of Genetics of the Complutense University of Madrid (UCM).

2.2. Primer design and PCR amplification

TRI5 specific primer pairs *for F. equiseti* were designed on the basis of a wide alignment using *TRI5* sequences retrieved for data bases off. *equiseti* (GQ865563; Proctor et al., 2009) and the main trichothecene-producing species of *Fusarium* associated with cereals and related to *F. equiseti*. The software Primer Express® (Applied Biosystems, Foster City, CA) was used to design the suitable primers for real time RT-PCR assays. The alignment was performed by Clustal method using Bioedit Sequence Alignment Editor v 7.0.9.0 (Hall, 1999) and the following *TRI5* sequences: *F. graminearum* (AY102605, AY102603 and AY102599), *F. austroamericanum* (AY102588), *F. meridionale* (AY102586), *F. boothii:* AY102595; *F. mesoamericanum:* AY102598; F *acacia-meamsii:* AY102578; *F. asiaticum:* AY102570; *F. cortaderiae:* AY102601; *F. pseudograminearum:* AY102585 and AY102583; *F. culmorum:* AY102602, AY102571 and AY130291; *F. poae:* AY130294; F *sporotrichioides:* AF364179, AY130293 and AY032745; *F. langsethiae:* JF9662559 and AF449793; *F. scirpi:* GQ915553; F. *semitectum:* GQ915550; F. *camptoceras:* GQ915545). The selected primer pair was: TRI5EQPQ-PF (5' AAGATC CCCAGGTGATGGAAA 3') and TRI5EQPQ-PR (5' TGACGTAGCCGTGCATGAAG 3'). The specificity of these primers was assessed with genomic D NA from a number ofi*F. equiseti* strains and related trichothecene-producing and non-producing *Fusarium* species. Al F. *equiseti* strains amplified the expected 51 bp band. No amplification product was observed with DNA from other *Fusarium* strain tested (data not shown).

The amplification reactions were performed in volumes of 25 µL containing 100 ng of template D NA in 3 µL, 1.25 µL of each primer (20 µM), 0.2 µL of Taq polymerase (5 U/µL), 2.5 µL of lOx PCR buffer, 1 µLofMgCI_2(50 mM) and 0.25 µL of dNTPs (100 mM) (Ecogen, Barcelona, Spain). PCR was performed in a thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany). Amplification products were detected by electrophoresis on 2.5% agarose ethidium bromide gels in 40 mM Tris-acetate and 1.0 mM EDTA lx buffer. The amplification protocol for both pairs of primers was: 1 cycle of 120 s at 94 °C, 35 cycles of 35 s at 94 °C (denaturation), 30 s at 64 °C (annealing), 30 s at 72 °C (extension), and 1 cycle of 10 min at 72 °C

The *TUB2* gene was used as endogenous control gene to normalize the results of real time RT-PCR experiments. The pair of primers used and the amplification protocol was reported previously elsewhere (Marin et al., 2010a) and their sequences were as follows: TUB2PQ-PF (5' CCGAGGCCCAGTCCAACT 3') and TUB2PQ-PR (5' GGCGTCTTGGTA TTGCTGGTA3').

2.3. Growth in relation to water potential on barley and wheat extract media

The medium used in this study was a 3% (w/v) either barley or wheat extract agar (Ramos et al., 1998). Both cereals extract agar media were made by boiling milled wheat or barley grain in 1 L of water for 30 min. The resulting mixture was filtered through a double layer of muslin and the volume was made up to 1 L Subsequently, 20 g/L of bacteriological agar (Pronadisa, Madrid, Spain) was added.

This cereal medium was modified with the non-ionic solute glycerol to obtain the water potentials ($*_w$) of - 2.8, - 7.0 and - 9.8 MPa corresponding to water activities (a_w) of 0.98,0.95 and 0.93 respectively. The control medium had a water potential of —0.7 MPa (= 0.995 a_w). Glycerol modified and un-modified media were prepared with both barley and wheat extracts. Al agar media (in 9 cm Petri plates) were overlaid with sterile cellophane sheets (NatureFlex™ 28 NP, Novocel Flexibles SL ., Barcelona, Spain) before inoculation to facilitate removal of the fungal biomass for RNA. extractions.

2.4. Inoculation, incubation and growth assessment

A 3-mm-diameter agar disk from the margin of 7-day-old growing colony of each of the four F. *equiseti* strain 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 at least four replicates per treatment.

Assessments of growth was made daily during the 10-day incubation period. Two diameters of the growing colonies were measured at right angles to each other 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.

Two dimensional growth rate profiles were obtained for each strain in relation to temperature x water potential treatments with both barley and wheat extracts.

Table 2
Two way ANOVA of temperature x water potential per substrate (barley and wheat) of growth rate for EQU5, EQU7, EQU9 and EOJJ10 *F. equiseti* strains. Tukey's HSD tests of each temperature and water potential were separately performed. Different letters indicate significant differences at P < 0.05.

EQU5

Barley Source of variation	d.f	Mean square	F*
Temperature	4	65,391	337.289***
Ψ_w	3	377.054	1944.864***
Temperature x *„	12	10.118	52.188***.**

Tukey's HDS test

Temperature (°C)					9_w (MPa)			
15	20	25	30	35	-0.7	-2.8	-7. 0	-9.8
aabc	aabc	abed	abed	abed	abede	abeba	abebd	aabed

Wheat Source of variation	d.f	Mean square	F*
Temperature	4	57.651	224.307***
\tr * w	3	320.787	1248.103***
	12	6.704	26.085***. *

Temperature x *„

Tukmpesa HDu^test)					*„ (MPa)			
15	20	25	30	35	-0.7	-2.8	-7. 0	-9.8
aabc	aabc	abed	abed	abed	abeed	aabbc	aabed	aabca

EQU7

Barley Source of variation	d.f	Mean square	F*
Temperature	4	99.246	261.919***
Ψ_w	3	272.793	719.925***
Temperature x *„	12	23.666	62.458***

Tukey's HDS test

Temperature (°C)					*„ (MPa)			
15	20	25	30	35	-0.7	-2.8	-7. 0	-9.8
aabc	abbe	abed	abed	abec	abeca	aabba	abebd	aabca

Wheat Source of variation	d.f	Mean square	F*
Temperature	4	53.066	136.698
\tr * w	3	188.148	484.668***
	12	11.152	28.727***

Temperature x *„

Tuk mperature test)					*„ (MPa)			
15	20	25	30	35	-0.7	-2.8	-7. 0	-9.8
abac	abed	abed	abed	abec	abeda	abbba	aabac	aabaa

EQU9

Barley Source of variation	d.f	Mean square	F*
Temperature	4	61.887	526.018***
\tr * w	3	376.536	3200.433***
	12	8.710	74.032***

Temperature x *„

Tukmpesa HDu^test)					*„ (MPa)			
15	20	25	30	35	-0.7	-2.8	-7. 0	-9.8
abed	aabc	abed	abed	abed	abede	abeda	abeba	aabec

Wheat Source of variation	d.f	Mean square	F*
Temperature	4	53.427	940.845***
Ψ_w	3	335.684	5911.314" ''

(continued on next page)

Table *2 (continued)*

Temperature x *„ Tukey's HDS test			12		3.827		67.399***	
Temperature (°C)					* „ (MPa)			
15 abed	20 abed	25 abed	30 abed	35 abed	-0.7 abede	-2.8 abeda	-7. 0 abeda	-9.8 aabba
EQU10								
Barley Source of variation			d.f		Mean square		F*	
Temperature			4		64.383		351.705***	
Ψw			3		365.015		1993.980***	
Temperature x *„			12		10.166		55.537***	
Tukey's HDS test								
Temperature (°C)					* „ (MPa)			
15 abed	20 abed	25 abed	30 abed	35 abed	-0.7 abeda	-2.8 aabba	-7. 0 abeba	-9.8 abebb
Wheat Source of variation			d.f		Mean square		F*	
Temperature			4		61.052		529.596***	
\tr * w			3		312.794		2713.342***	
			12		4.412		38.269***	
Temperature x *„ Tukey's HDS test)					* „ (MPa)			
15 abed	20 aabc	25 abed	30 abed	35 abed	-0.7 abeed	-2.8 abeca	-7. 0 abeda	-9.8 abede

*** Significant at $P < 0.001$.
* Snedecor's F-test

25. RNA isolation and cDNA synthesis

The biomass of each plate was removed from the cellophane at the end of the incubation period, and the total RNA was extracted using both the "RNeasy® Plant Mini Kit" and "RNeasy® Mini Kit" (Hilden, Germany), according to the manufacturer's instructions, and stored at — 80 °C. First-strand cDNA was synthesized using the "GeneAmp Gold RNA PCR reagent kit" (Applied Biosystems). Each 20 µL reaction mixture contained 500 ng of total RNA 0.5 µLof oligo$(dT)_{16}$ (50 µM), 10 µLof 5X RT-PCRbuffer, 2 µLof $MgCl_2$ (25 mM), 2 µLofdeoxynucleoside triphosphate (dNTP) (10 mM), 2 µL of dithiothreitol (100 mM), 0.5 µL(10 U) of RNase inhibitor (20 U/µL), 0.3 µL (15 U) of MultiScribe reverse transcriptase (50 U/µL), and sterile diethyl pyrocarbonate-treated water up to the final volume. Synthesis of cDNA was performed in a Mastercycler gradient thermal cycler (Eppendorf, Germany) according to the following procedure. After a hybridization step oflOmin at 25 °C, RT was carried out for 12 min at 42 °C. The cDNA samples were kept at — 20 °C. Samples incubated in the absence of reverse transcriptase were used as controls.

2.6. Real time RT-PCR and quantitative analysis of the data

Real time RT-PCR assays were performed to quantify *TRI5* and the constitutive *TUB2* gene expression in *F. equiseti* EQU9 strain using both pairs of primers described above. Real time RT-PCR reactions were performed using an ABI PRISM 7900 HT sequence detection system (Applied Biosystems). The PCR thermal cycling conditions for both genes were as follows: an initial step at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. SYBR green PCR master mix (Applied Biosystems) was used as the reaction mixture with the addition of 13 µL of sterile Mlli-Q water, 0.6 µL of each primer (5 µM) and 2.5 µLof template cDNA in a final volume of 10 µL In all the experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination or carry-over. Each sample was amplified twice in each experiment. Both *TRI5* and *TUB2* cDNA amplifications were run on the same plate.

The PCR efficiencies for both genes were measured by performing a 10 fold serial dilution of positive control template to generate a standard curve, and by plotting the C_T as a function of logio of template. The slope in the standard curve for the amplification of the *TUB2* gene was — 3.24 while the slope in the standard curve for the amplification of *TRI5* was — 3.22. The efficiency of both pairs of primers was 103.57 and 104.61%, respectively and differing less than 10%; thus, within the range of optimal efficiency (90-105 %) and the conditions required to quantify the relative gene expression using the $2^{\sim AACT}$ method (Livak and Schmittgen, 2001). Relative quantification (ACT) was performed by subtracting the CT of the control gene *(TUB2)* from the CT of the gene of interest *(TRI5).* In graphic representations (Fig. 2), we used the average C_T mean value of the three replicates performed in each experiment, and this was subtracted by the calibrator mean value of the three replicates to obtain the corresponding AACT values. These AACT values were transformed to $\log_2$ (due to the doubling function of PCR) to generate the relative expression levels.

2.7. Statistical analysis of results

The linear regression of the increase of radius (mm) against time (days) was used to obtain the growth rates (mm/day) for all replicates and treatments. Multifactor ANOVA of all the three factors (substrate x temperature x water potential) and two way ANOVA for each substrate (temperature x water potential) were performed separately per each *F. equiseti* strain for growth rate and in EQU9 for *TRI5* gene expression results, 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. Consistency of *TUB2* C_T values was evaluated using a one-way ANOVA in all the experiments. The results indicated

that expression of this gene showed a significant stability and consistency in all of the experiments. All sets of results were evaluated using STATGRAPHICS CENTURION XV. I I (Statistical Graphics Corp., Herndon, VA) and SPSS 17.0.0 (Rel, 2008. Chicago: SPSS Inc). The *TRI5* gene expression data used were the ACT values.

3. Results

3.1. Effects of temperature x water potential on growth rate on both barley and wheat extract media

Fig. 1 shows the bi-dimensional maps of relative growth rate of the four *F. equiseti* strains on barley and wheat in response to water potentials (between -0.7 and -9.8 MPa) and to temperatures (15-40 °C). Optimal growth was observed at 20-30 °C and between — 0.7 and — 2.8 MPa on both substrates. The highest growth rate was observed generally at 25 °C at — 0.7 MPa with values of 8-9 mm/day in barley and 7-8 mm/day in wheat. No growth was observed at 40 °C, and at 15 °C if combined with - 9.8 MPa. Tables 1 and 2 show the ANOVA analyses and subsequent Tukey's HSD tests. In both barley and wheat substrates, the effects on growth rate of single factors (strains, water potential and temperature) and their interaction were significant. Two-factor interaction between water potential and temperature was also significant. Subsequent separate analyses on each strain revealed significant effects of water potential and temperature (and the three-factor interaction) but not of substrate or the interaction where this factor was participating and were similar in all four isolates (Table 1 only shows only the results of three-factor ANOVA for EQU9). The subsequent separate two-factor ANOVA and Tukey's HSD tests include the results of all the strains and showed quite similar patterns among strains and for both barley and wheat based substrates (Table 2). A comparison of the effect of high temperatures (30-35 °C) in combination with water potentials on strains of common *Fusarium* species in cereals in similar in vitro experiments is summarized in Table 4.

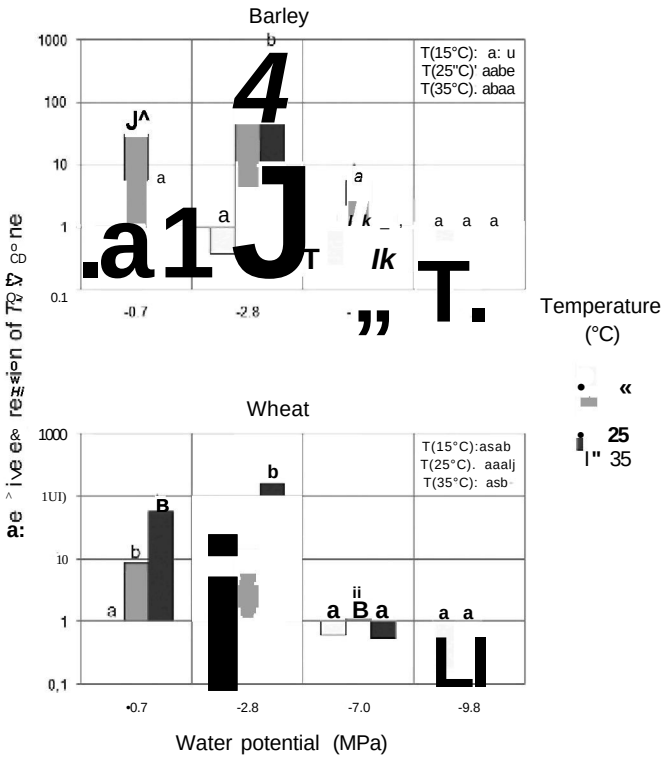

Fig. 2. Relative gene expression pattern of F. *equiseti* strain **EQU9** cultured for 10 days in response to temperature and water potential.

3.2. Effects of temperature x water potential on TR15 gene expression on both barley and wheat extract media

Fig. 2 shows the relative gene expression pattern off. *equiseti* strain EQU9 cultured for 10 days in response to temperature (15,25 and 35 °C*)* and water potential (— 0.7, — 2.8, — 7.0 and — 9.8 MPa) grown on either barley or wheat based medium. Table 3 shows the multifactor ANOVA (substrate/^w/temperature) and subsequent Tukey's HSD tests performed. Two way ANOVA (^w/temperature) were significant ($P < 0.001$) for single factors and their interaction both in wheat and barley (data not shown). The general gene expression pattern looked like quite similar in both media. However, all single factors considered, the interaction temperature-solute potential and the interaction among the three factors, showed significant differences. Induction of *TRI5* expression was detected between 25 and 35 °C and —0.7 and — 2.8 MPa (also at — 7.0 MPa in barley), with maximum values at 35 °C and -2.8 MPa in both media.

The standard error of the mean values of the two amplifications performed for each sample in the same plate was less than 0.1%, indicating that the real time RT-PCR assay showed high reproducibility.

4. Discussion

This work describes the ecophysiological patterns of 4 strains isolated from cereals grown in Spain belonging to a recently identified population within the so-called *F. incamatum-F. equiseti* species complex (ODonnell et al., 2009). The conditions tested considered high temperatures and low water potentials to account the most extreme scenarios among those predicted for Spain. The results obtained in this study indicated that although significant variability among strains was detected, the general growth pattern was similar. This pattern indicated a wide range of permissive conditions of temperature (15-35 °C) and optimal values from 20 to 30 °C. No growth was observed at 40 °C. In the case of water potential, growth was severely decreased at —9.8 MPa when it was combined with 15 or 40 °C and optimal growth was detected between —0.7 and —2.8 MPa. This ecophysiological pattern agreed with the wide distribution throughout different agroclimatic regions within Spain. Although no similar studies have been reported to our knowledge *on F. equiseti* from Northern Europe, it might be reasonable to expect that these strains should perform better at lower temperatures and higher water potentials. Taking together the phylogenetic data, toxin and ecophysiological profiles, we suggest that this population might have been genetically isolated for a long time, enough to become well adapted to the environmental conditions of the agroclimatic regions where it is more frequent (Jurado etal., 2006; Marin, 2010; Marin et al., 2012; Soldevilla et al., 2005). The extent of the region where this

Table 3
Multifactorial ANOVA of the relative **TPJ5** gene expression of **EQU9 F.** *equiseti* strain incubated with two substrates (barley or wheat) for 10 days at different temperatures (15, 25 and 35 °C) and water potentials (— 0.7, —2.8, -7.0 and-9.8 MPa).

EQU9			
Source of variation	d.f	Mean square	F*
Substrate	1	8.804	4.827
^ w	3	113.695	62.331***
Temperature	2	94.138	51.609***
Substrate x *„	3	2.544	1.395**
Substrate x temperature	2	5.114	2.804
IV, x temperature	6	25.363	13.904***
Substrate x *P„ x temperature	5	9.649	5.290***

* Significant at P< 0.05.
** Significant at P< 0.01.
*** Significant at P< 0.001.
* Snedecor's F-test

Table 4
Changes in growth rate by *Fusarium* species under different temperatures and water potentials.

	Maximum growth rate conditions	Percentage of reduction on growth rate compared to maximum							
		30 °C				35 °C			
		-0.7 MPa	-2.8 MPa	-7.0 MPa	-9.8 MPa	-0.7 MPa	-2.8 MPa	-7.0 MPa	-9.8 MPa
F. *verticillioides*	0.995, 25 *X*	7.48	2.23	50.65	100	30.58	17	55.68	100
F. proliferatum	0.98, 25 *X*	16.75	12.45	50.27	100	33.71	16.09	58.19	100
F. graminearum	0.98, 25 *X*	51.15	47.62	82.10	100	93.75	83.33	93.02	100
F. equiseti (wheat)	0.995, 25 *X*	10.09	26.56	64.5	88.38	56.32	50.83	79.82	100
F. *equiseti* (barley)	0.995, 25 *X*	21.95	33.03	72.96	94.34	59.50	55.20	79.52	94.68

population might be prevalent is not yet fully determined but strains from Italy and France are included in this group (Marin et al., 2012).

The influence of the substrate or host on colonization or ability to grow by *F. equiseti* has been acknowledged by several authors (Llorens et al., 2004; Marin et al., 2004; Ramirez et al., 2006). Higher levels of fungal contamination of barley than of wheat have been observed (Gil-Serna et al., 2013; González-Jaen et al., 2008; Medina et al., 2006; Soldevilla et al., 2005). Differences in growth rate off. *equiseti* also have been reported among diverse host plant species (Goswami et al., 2008; Palmero etal., 2011). In our case, although growth profiles were similar on barley and wheat media, there were some interesting differences in certain aspects such as absolute maximal growth rate values or counteracting interactions with temperature and water potential. These might be relevant and they will be considered in future studies.

The wide distribution off. *equiseti* in Spain overlaps to a certain extent with *F. verticillioides* and *F. proliferatum* in some regions and with *F. graminearum* in others (Gonzalez-Jaén et al., 2008; Jurado et al., 2006; Marin, 2010; Marin et al., 2012; Soldevilla et al., 2005). This distribution seems to be consistent with the growth rate patterns of Spanish strains of these species observed in similar in vitro studies (Jurado et al., 2006; Marin et al., 2010a, 2010b, 2012). These data, compared in Table 4, highlight the impact on growth rate that might be expected in future conditions of higher temperatures and more frequent/long drought periods predicted by climatic change scenarios for Spain and other Mediterranean regions. The results suggest that *F. equiseti* would show an intermediate pattern between *F. verticillioides*/ *F. proliferatum* and the less tolerant ,*F. graminearum*, particularly at moderate/low water potential and 35 °C The limit between these two regions might depend on the weather conditions of particular years or cultural practices (for instance, irrigation or not). Interactions of these factors with an increase of 2x and 3x $C0_2$ above existing levels which may be produced by climate change should also be taken into account (Magan etal., 2011).

Environmental conditions also showed a significant effect on *TRI5* mRNA synthesis producing a pattern distinct from that obtained from growth rate analyses. *TRI5* gene expression was highly induced between 25 and 35 °C and — 0.7 and — 2.8 MPa water potential on both barley and wheat media. Lower, but still significant, induction was also observed at —7.0 MPa (25-35 °C) in barley medium. Interestingly this range includes the optimal conditions for growth, particularly at 25 °C and between —0.7 and —2.8 MPa, suggesting that trichothecene biosynthesis may occur within the range of conditions most favorable for successful colonization. Even at 35 °C and — 2.8 MPa, the *F. equiseti* strains were able to grow around 3-4 mm/day average, indicating a high level of constant trichothecene production in conditions considered stressing for most *Fusarium* species. This pattern contrasted with that *TRI5* by *F. graminearum* (Marin et al., 2010a). A similar situation was observed when expression patterns of the *FUMl* gene (key gene of fumonisin biosynthesis) produced by *F. verticillioides and F. proliferatum* were compared (Marin et al., 2010b). These differences of regulation of mycotoxin biosynthesis, even for the same toxin, by fungal species might contribute to improving their differential adaptation to different environments (or hosts) as well as to environmental changes during their life cycle (Marin et al., 2012). *F. equiseti* might represent the highest risk for toxin contamination at moderate/low water potential and 30-35 °C

In conclusion, this species might be expected to continue playing a role in future scenarios and contributing to toxin risk. Additionally, the study suggested that substrate/host/cultivar may influence toxin production by direct regulation of toxin biosynthesis independently of fungal growth. The results and the in vitro approach described in this work could be useful in control and breeding strategies to reduce mycotoxin contamination on cereals.

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