

Differential effect of environmental conditions on growth and regulation of the fumonisin biosynthetic gene *FUM1* in the maize pathogens and fumonisin-producers *F. verticillioides* and *F. proliferatum*

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ABSTRACT

The effects of ecophysiological factors, temperature and solute potential, on both growth and regulation of the fumonisin biosynthetic *FUM1* gene were studied and compared in the two closely related fumonisin-producing and maize pathogens *F. verticillioides* and *F. proliferatum*. The effect of solute potential and temperature was examined on *in vitro* mycelia growth and on expression of *FUM1* gene, quantified by species specific real time RT-PCR assays. Although both species showed similar two dimensional profiles of growth, for *F. verticillioides* optimal growth conditions were maintained at higher temperatures and lower solute potential values. *FUM1* gene expression was markedly induced at 20°C in both species, under sub-optimal conditions for growth; however, their expression patterns differed in relation to solute potential. Whereas *FUM1* expression was induced in response to increasing water stress in *F. verticillioides*, *F. proliferatum* showed a stable expression pattern regardless of water potential conditions. These results suggest a differential regulation of fumonisin biosynthesis in these species that might be related to their different host range and play an ecological role. Additionally, environmental conditions leading to water stress (drought) might result in increased risk of fumonisin contamination of maize caused by *F. verticillioides*.

INTRODUCTION

Fusarium verticillioides (*Gibberella moniliformis*, *G. fujikuroi* mating population A) and *F. proliferatum* (*G. intermedia*, *G. fujikuroi* mating population D) are important pathogens of maize. They produce a number of mycotoxins, among which fumonisins are considered the most relevant. Both species show differences in host range. Fumonisin-producing *F. verticillioides* is basically restricted to maize where it causes ear and stalk rot, whereas *F. proliferatum* colonises a wide range of hosts in addition to maize, as diverse as pine trees, asparagus or palm trees, and dietary crops such as wheat and barley. In these crops, it may cause black point symptoms (Conner *et al.*, 1996; Desjardins *et al.*, 2007). Both species have a wide distribution in temperate regions (Desjardins *et al.*, 2000; De Souza & Formento, 2004; Aliakbari *et al.*, 2007; Cavaglieri *et al.*, 2009) and they are particularly relevant in Southern Europe (Logrieco *et al.*, 2002; Soldevilla *et al.*, 2005; Jurado *et al.*, 2006; Medina *et al.*, 2006).

Fumonisin are a family of toxic and carcinogenic mycotoxins which cause serious diseases affecting humans and animals (Marasas *et al.*, 2004). Because of the health risk associated with consumption of contaminated commodities, their occurrence is currently under regulation in many countries including the European Union (Commission Regulation EC No 1126/2007).

Fusarium growth and fumonisin production result from the complex interaction of several factors (biotic and/or abiotic). Water stress and temperature are the most relevant environmental factors which influence fungal growth and mycotoxin production and therefore, they are essential to understand the overall process and to predict and prevent plant diseases and mycotoxin production (Charmley *et al.*, 1994). Recently, several reports point out the impact of diverse stress conditions on mycotoxin production, including osmotic stress, pH and diverse compounds such as fungicides (Schmidt-Heydt *et al.*, 2007; Jurado *et al.*, 2008; Schmidt-Heydt *et al.*, 2008.). These

studies have used real time RT-PCR and microarrays-based approaches, since regulation of mycotoxin biosynthesis has been demonstrated to occur primarily at a transcriptional level in many cases (Proctor *et al.*, 1999; O'Callaghan *et al.*, 2006). Furthermore, high and significant correlation has been observed between the relative amount of *FUM1* transcripts quantified by real time RT-PCR and the fumonisin content in both *F. verticillioides* and *F. proliferatum* (López-Errasquín *et al.*, 2007; Jurado *et al.*, 2010), as well as for *TRI5* gene and desoxinivalenol (DON) in *F. graminearum* (Doohan *et al.*, 1999). This method permits rapid and accurate quantification of the effect of factors and treatments on mycotoxin biosynthesis, reducing incubation times necessary to accumulate the toxin that might be eventually produced at analytically detectable levels. Although stress factors may be only transient, in a situation of changing climatic conditions, they may become more permanent in certain regions (Miraglia *et al.*, 2009) and, therefore, information about the behaviour of the different fungal species regarding growth patterns and toxin production in environmental stress conditions might become critical to improve prediction and control of the mycotoxin risk.

The objectives of this study were (i) to compare the effects of temperature and non-ionic osmotic potential stress on growth and *FUM1* gene expression in *F. verticillioides* and *F. proliferatum* strains and (ii) examine the temporal kinetics of the effect of ionic solute potential on growth and *FUM1* gene expression in *F. proliferatum*.

MATERIALS AND METHODS

Fungal isolate.

F. verticillioides FvA (FvMM7-3) strain and *F. proliferatum* strain FpA (FpMM1-2) were originally isolated from a maize field in Madrid (Spain), in September 2003, and both were reported to produce fumonisins (Jurado *et al.*, 2008; Jurado *et al.*, 2010).

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 University Complutense of Madrid.

Growth in relation to osmotic potential (ionic and non-ionic potential)

The medium used in this study was a fumonisin-inducing solid agar medium previously reported (López-Errasquin *et al.*, 2007), which contained malt extract (0.5 g/L), yeast extract (1 g/L), peptone (1 g/L), KH₂PO₄ (1 g/L), MgSO₄·7H₂O (0.3 g/L), KCl (0.3 g/L), ZnSO₄·7H₂O (0.05 g/L), CuSO₄·5H₂O (0.01 g/L), fructose (20 g/L) and bacteriological agar (15 g/L).

The solute potential (Ψ_s) was modified either with the non-ionic solute glycerol to analyze the effect of water stress and temperature on growth rate and *FUM1* gene expression of both strains, or with the ionic solute sodium chloride (NaCl) to perform a kinetics study with *F. proliferatum* FpA. In the first case, the solute potentials used were -0.7, -2.8, and -7.0 MPa of Ψ_s , corresponding to water activities (a_w) of 0.995, 0.982 and 0.955 respectively, while in the kinetics study the solute potentials were -0.7, -2.8, -7.0, and -9.8 MPa of $\Psi_s = a_w$ of 0.995, 0.982, 0.955 and 0.937 respectively. These solutes were not added to the control medium (-0.7 MPa = a_w of 0.995). All the treatments and replicate agar media were overlayed with sterile cellophane sheets (P400; Cannings, Ltd., Bristol, United Kingdom) before inoculation to facilitate removal of the fungal biomass for RNA extractions.

Inoculation, incubation and growth assessment

A 3-mm-diameter agar disk from the margin of a 7-day-old growing colony of *F. verticillioides* FvA and *F. proliferatum* FpA grown at 25°C was used to centrally inoculate plates for each replicate and treatment. The plates were incubated at 15, 20,

25, 30 and 35 °C for 10 days in the fumonisin-inducing medium modified with glycerol. The experiment consisted of a fully replicated set of treatments with at least three replicates per treatment. Experiments were repeated once.

Assessment 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 as the slope of the line.

An additional time course experiment was carried out in the fumonisin-inducing medium modified with NaCl. This study was carried out at 25°C for 3, 6, 9 and 12 days. Three independent replicates were destructively sampled at each time point and analyzed.

RNA isolation and cDNA synthesis

The biomass was removed from the cellophane at the end of the incubation period, and the total RNA was extracted using the “Total Quick RNA cells and tissues” kit (Talent, Italy), according to the manufacturer’s instructions, and stored at -80°C. DNase I treatment was used to remove genomic DNA contamination from the samples using “DNase I, amplification grade” (Invitrogen; United Kingdom), following the manufacturer’s instructions. 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 µl of oligo(dT)₁₆ (50 µM), 10 µl of 5X RT-PCR buffer, 2 µl of MgCl₂ (25 mM), 2 µl of deoxynucleoside triphosphates (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 of 10 min 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.

Real time RT-PCR and quantitative analysis of the data

Real time RT-PCR assays were used to quantify *FUM1* and *TUB2* expression in both species. The primer pair PQF5-F (5' GAGCCGAGTCAGCAAGGATT 3') and PQF5-R (5' AGGGTTCGTGAGCCAAGGA 3') was used for *F. verticillioides* (López-Errasquin *et al.*, 2007). Primer pair FUM1P2-F (5' CCCCCATCATCCCGAGTAT 3') and FUM1P2-R (5' TGGGTCCGATAGTGATTTGTCA 3') was used for *F. proliferatum* (Jurado *et al.*, 2010). These two species specific primer sets were used to amplify the *FUM1* gene. The pair of primers PQTUB-F2 (5' ACATCCAGACAGCCCTTTGTG 3') and PQTUB-R2 (5' AGTTTCCGATGAAGGTCAAGA 5') was used to amplify a partial region of *TUB2* gene in both species (Jurado *et al.*, 2010). Real time RT-PCR reactions were performed using an ABI PRISM 7700 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 2.6 µl of sterile Milli-Q water, 1.2 µl of each primer (5 µM), and 5 µl of template cDNA, in a final volume of 20 µl. In all 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.

The results were normalized using the *TUB2* cDNA amplifications run on the same plate. The *TUB2* gene is an endogenous control that was used to normalize

quantification of mRNA target for differences in the amount of total cDNA added to each reaction. In real time RT-PCR analysis, quantification is based on the threshold cycle (C_T), which is defined as the first amplification cycle at which the fluorescence signal is greater than the minimal detection level, indicating that PCR products become detectable. Relative quantitation is the analytic method of choice for this study (Ginzinger, 2002). In this method, a comparison within a sample is made with the gene of interest (*FUM1*) to that of the endogenous control gene (*TUB2*). Quantitation is relative to the control gene by subtracting the C_T of the control gene from the C_T of the gene of interest (C_T). In graphic representations (see Figures 2 and 3), we have 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 C_T values. C_T values were transformed to log2 (due to the doubling function of PCR) to generate the relative expression levels.

Statistical analysis of results

The linear regression of increase in radius against time (days) was used to obtain growth rates (mm day^{-1}) as indicated above for each set of treatments. Analysis of variance (ANOVA) was made for the growth rate, *FUM1* gene expression and temporal studies including the data of the three replicates from each experiment. All sets of results were evaluated by using StatsGraphics Centurion XV.II (Statistical Graphics Corp., Herndon, VA). *FUM1* gene expression data were transformed prior to analysis by $y = \log_{10}(\text{FUM1 gene expression})$.

RESULTS

Effects of temperature and non-ionic solute stress on growth

Figure 1 shows the two dimensional profiles of relative growth rates of *F. verticillioides* FvA (Figure1a) and *F. proliferatum* FpA (Figure1b) strains on fumonisin-inducing solid agar medium in response to changes in non-ionic solute potential and temperatures between 15 and 35°C.

Figure 1a shows that the optimal conditions for growth (5-6 mm day⁻¹) of *F. verticillioides* were at between 25 and < 35°C, at both -0.7 and -2.8 MPa Ψ_s . However, $\Psi_s \leq -7.0$ MPa, at all the temperatures, severely reduced growth (1-3 mm day⁻¹). Temperatures below 17°C and above 35°C could be considered marginal for growth. The ANOVA analyses showed that single factors (temperature and water potential) and their interaction were statistically significant (Table 1a). Therefore the most restrictive conditions might be lower and higher temperature and increased solute stress. Differences in growth were significant at all the temperatures, as well as osmotic Ψ_s except at -0.7 and -2.8 MPa of Ψ_s where no significant difference in growth was observed (data not shown).

Optimal conditions for growth (5-6 mm day⁻¹) of *F. proliferatum* were slightly narrower than for *F. verticillioides* (Figure 1b), at between 25 and 30°C and at both -0.7 and -2.8 MPa Ψ_s . Growth was reduced (1-3 mm day⁻¹) at -7.0 MPa Ψ_s or lower at all temperatures. The ANOVA showed statistically significant effects for all the single and interacting factors (Table 1b). Differences in growth were significant at all the temperatures and osmotic potentials (data not shown).

Effects of temperature and non-ionic solute stress on *FUM1* gene expression

Figure 2 shows the relative *FUM1* gene expression of the *F. verticillioides* FvA and *F. proliferatum* FpA strains cultured on fumonisin-inducing agar medium for 10 days in response to both temperature and Ψ_s potential.

FUM1 gene expression profiles by *F. verticillioides* are shown in Figure 2a. The highest fold induction, in all the three Ψ_s potentials, was at 20 °C, and at both -2.8 and -7.0 MPa was statistically different. At -0.7 MPa, *FUM1* gene expression showed induction only at 20°C. Increasing water stress resulted in higher induction of *FUM1* gene in all the temperatures tested. The ANOVA showed statistically significant effects for both single factors (temperature and solute potential) and their interaction for *F. verticillioides* (Table 2a).

Induction of *FUM1* gene in *F. proliferatum* seemed to be predominantly influenced by temperature, and largely restricted to 20°C (Figure 2b). The reduction of *FUM1* gene expression observed at higher temperatures decreases with increasing water stress, suggesting that this condition might have a certain positive effect on gene expression. The ANOVA showed that single factors, temperature and water potential, has a statistically significant effect on *FUM1* gene expression (Table 2a).

The standard error of the mean values of the two amplifications performed for each sample in the same plate, were generally very low (about 0.1%) and never higher than 1%, indicating a high level of reproducibility of the real time RT-PCR assay.

Effects of the ionic solute potential on temporal *FUM1* gene expression

Figure 3 shows the effect of the ionic Ψ_s potential on growth rates, expressed as area of fungal growth, and induction of *FUM1* gene expression by *F. proliferatum* at 25°C after 3, 6, 9 and 12 days. The results indicated that *FUM1* mRNA synthesis was remained stable at all the Ψ_s conditions tested. Figure 3 also shows the results obtained for *F. verticillioides* in a previous study carried out in similar conditions for comparison purposes (Jurado et al., 2008).

DISCUSSION

In this work we have compared the growth rate and relative induction of *FUM1* gene expression profiles of two Spanish isolates of *F. verticillioides* and *F. proliferatum* in response to temperature and Ψ_s potential, the main factors affecting fungal growth and mycotoxin production (Charmley *et al.*, 1994; Magan, 1997). The detailed two dimensional growth maps obtained for both species showed quite similar patterns. Both species had similar optimal growth rates (5-6 mm/day) and they were able to grow over a wide range of conditions, particularly at high temperatures and water potentials. This confirms earlier studies (Marin *et al.*, 1999; Marin *et al.*, 2004; Hope *et al.*, 2005) and they are basically in agreement with epidemiologic studies showing the association of these two species with changes in water stress and high temperatures, close to 30-35°C. However, *F. verticillioides* showed a significantly better performance at higher temperatures and water stress than *F. proliferatum*. This might contribute to its comparatively higher incidence in maize fields from regions affected by increasing temperatures and severe drought (Soldevilla *et al.*, 2005; Jurado *et al.*, 2006).

The analysis of *FUM1* expression in *F. verticillioides* and *F. proliferatum* isolates performed in this study revealed an effect of temperature and water potential at a transcriptional level, similar to other studies reported for diverse toxigenic fungal species (Feng & Leonhard, 1998; O'Callaghan *et al.*, 2006; Jurado *et al.*, 2008; Schmidt-Heydt *et al.*, 2008). The relative highest *FUM1* induction was observed at 20°C for both species, close to but not coincident with their optimal temperature for growth (25°C), excluding the Ψ_s potential factor, suggesting a preference for certain temperatures for maximum *FUM1* gene expression. Interestingly, a similar situation was reported for other mycotoxigenic species (Schmidt-Heydt *et al.*, 2008), although there is no conclusive explanation for this so far. However, the response of both species to water stress was markedly different. In the case of *F. verticillioides*, increasing Ψ_s potential values resulted in an induction of *FUM1* expression, particularly

at the highest water stress conditions tested, confirming the pattern observed previously (Jurado *et al.*, 2008). In the case of *F. proliferatum*, a similar trend could be detected although at a much lower intensity. Similarly, induction of toxin biosynthetic gene expression was reported for the trichothecene-producing *F. culmorum*, the aflatoxin-producing *A. parasiticus* and the OTA-producing *Penicillium verrucosum* (Schmidt-Heydt *et al.*, 2008) in response to Ψ_s potential stress, as well as to pH and temperature, suggesting that environmental stress and toxin biosynthesis by fungi are causally related and it might be a more general phenomenon (Magan, 2007). Furthermore, it has been suggested that stress-activated protein kinases might play a role in the regulation of mycotoxins (Kohut *et al.*, 2009).

Based on the work of Jurado *et al.* (2008), the contribution of Ψ_s potential stress on induction of fumonisin biosynthesis in *F. verticillioides* showed a clear stimulation under water stress conditions of the *FUM1* gene in the temporal study. However, the results obtained for *F. proliferatum* showed a stable pattern of *FUM1* expression in relation to water stress. These results suggest that the relative contribution of these two species to fumonisin production on maize might differ during the course of colonization. Fumonisin production would be expected to be higher, in the case of *F. proliferatum*, at earlier stages (when temperature is still mild), whereas the relative contribution to fumonisin synthesis by *F. verticillioides* would increase at later stages, when water stress progressively increases.

The actual biological role of mycotoxins is far from being elucidated, although in certain cases their role in pathogenesis has been demonstrated. This seems to be the case of DON-producing *F. graminearum* strains in wheat (Desjardins *et al.*, 1996). The role of fumonisins in maize pathogenesis is more controversial. A recent report, however, suggests that fumonisin production by *F. verticillioides* is necessary for development of disease symptoms in maize (Glenn *et al.*, 2008). The diverse mycoflora

present on plants and substrates during colonization suggests a situation where complex interactions among species competing for nutrients and space exist. In this scenario, strategies involving the development of differential nutrient abilities (Wilson & Lindow, 1994) and/or production of secondary metabolites (mycotoxins) might confer advantages to occupy or retain a certain niche. In this context, mycotoxins could be regarded as competitive factors (chemical antagonists). Although conclusive evidence supporting this view is still lacking, experiments on competition between *F. verticillioides*, *F. proliferatum*, *F. graminearum*, *A. flavus* and *A. parasiticus*, all associated with maize, suggest a role of toxins, and fumonisins in particular, in their interactions (see the excellent review by Marin *et al.*, 2004). Some reports also describe a prevalence of *F. verticillioides* with respect to *F. graminearum* or *A. flavus*, particularly in certain environmental conditions, and an effect of their interaction on toxin production (Zimo & Scott, 1992; Velluti *et al.*, 2000). However, accurate quantification of toxin (fumonisin) content variation at the concentrations which might be of biological significance in the course of the interactions is technically difficult to achieve. The use of the sensitive and specific real time RT-PCR protocols may be an alternative approach to evaluate the role of mycotoxins in this ecological context. The importance of these conditions (temperature and water potential) was also demonstrated in a recent study on their effect on carbon nutritional patterns and niche overlap between *A. flavus* and *F. verticillioides* (Giorni *et al.*, 2009). This study showed that these two species assimilate different carbon sources resulting in them occupying different niches in maize under specific ranges of environmental conditions, and that only under extreme conditions was dominance of one over the other species. Nutritional dominance of *F. verticillioides* occurred at 20°C, mainly at 0.955 a_w (= -7.0 MPa water potential). These conditions parallel the highest induction of *FUM1* gene, particularly in *F. verticillioides*. The pattern of *FUM1* induction by this species in

response to water stress, close to its limit for growth, and which are conducive for *Aspergilli*, could be envisaged as advantageous for *F. verticillioides* to retain its niche occupation and preventing dominance by *A. flavus*. A change in regulation of fumonisin biosynthesis might have improved adaptation of *F. verticillioides* to maize, at the expenses of reducing the host range. *F. proliferatum*, in contrast, showed a less specific regulation pattern with a relatively stable and constant *FUM1* gene expression in relation to growth rate, more compatible with a less specific colonization pattern, in agreement with the broad range of host plants which this species is able to colonize.

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(a)

Source of variation	df	Mean square	F ^a
Temperature	4	146,798	886,07*
Ψ_s	2	89,0457	537,48*
Temperature vs Ψ_s	8	4,35065	26,26*

(b)

Source of variation	df	Mean square	F ^a
Temperature	4	92,1769	929,55*
Ψ_s	2	99,5896	1004,30*
Temperature vs Ψ_s	8	5,6849	57,33*

Table 1: ANOVA of the effects of temperature (15, 20, 25, 30 and 35°C) and non-ionic (glycerol) solute potential (-1.4, -2.8 and -7.0 MPa) and their interaction on growth rate in *F. verticilloides* (a) and *F. proliferatum* (b).

^a Snedecor's F Test. *, significant at P<0.05

(a)

Source of variation	df	Mean square	F ^a
Temperature	4	3,78524	27,26*
Ψ_s	2	4,16745	30,01*
Temperature vs Ψ_s	8	1,65083	11,89*

(b)

Source of variation	df	Mean square	F ^a
Temperature	4	10,743	22,20*
Ψ_s	2	5,99196	12,38*
Temperature vs Ψ_s	8	1,18482	2,45*

Table 2: ANOVA of the effects of temperature (15, 20, 25, 30 and 35°C) and non-ionic (glycerol) solute potential (-1.4, -2.8 and -7.0 MPa) and their interaction on *FUM1* gene expression on *F.verticilloides* (a) and *F. proliferatum* (b).

^a Snedecor's F Test. *, significant at P<0.05.

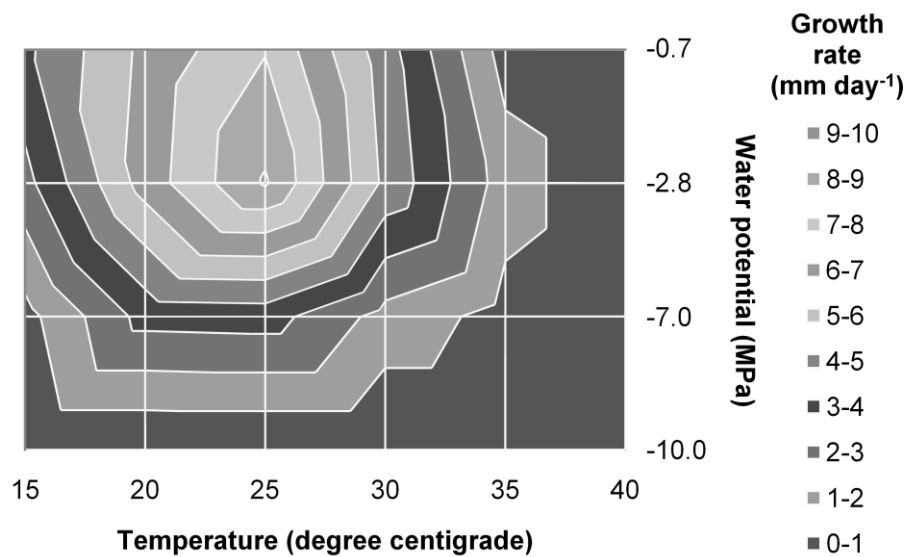


Figure 1: Influence of temperature and non-ionic solute potential growth rate of *F. graminearum* (mean of the three replicates) growing on glycerol modified GYEP solid medium in 10 days of incubation.

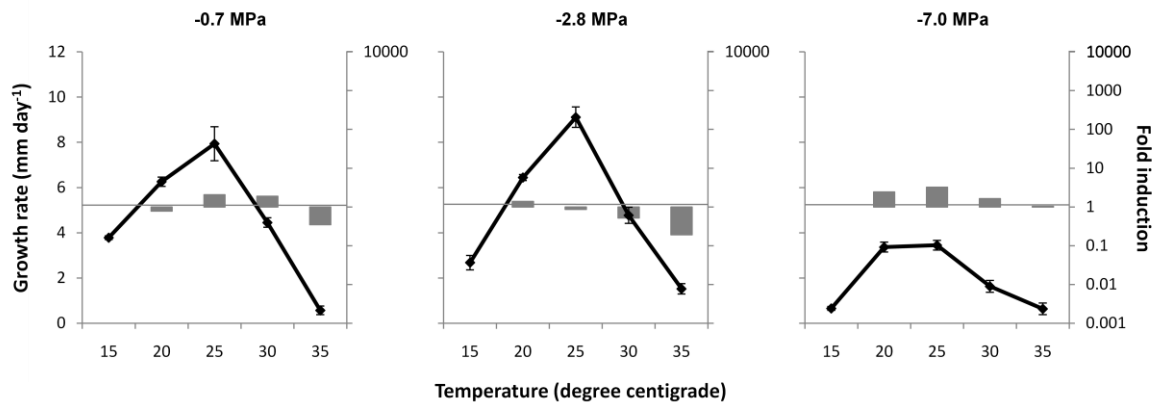


Figure 2: Comparison of induction of growth rate (♦) and *TRI5* gene expression (■) in response to non-ionic (glycerol) osmotic potential in *F. graminearum* for 10 days at three different solute potentials (-1.4, -2.8 and -7.0 MPa) and five temperatures (15, 20, 25, 30 and 35°C). The measured quantity of the cDNA in each experiment was normalized using C_T values obtained for *TUB2* cDNA amplifications run in the same plate. The values represent the number of times *TRI5* is expressed in each experiment compared to its respective culture incubated at 15°C (set at 1.00). The results are averages of the three independent repetitions.

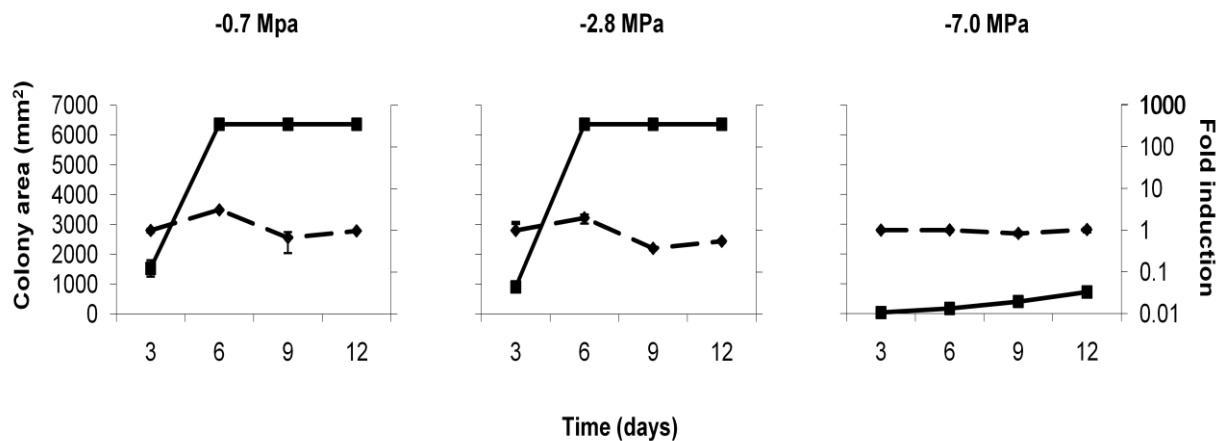


Figure 3: Temporal kinetics study of the effect of ionic (NaCl) solute potential on relative colony size (■) and induction of *TRI5* gene expression (♦) of *F. graminearum* at 25°C at three different solute potentials (-1.4, -2.8 and -7.0 MPa). The measured quantity of the cDNA in each of the experiments was normalized using the C_T values obtained for the *TUB2* cDNA amplifications sun on the same plate. The values represent the number of times *TRI5* is expressed in each experiment compared to its respective 3-day-old culture of the *F. graminearum* strain (set at 1.00). The results are average of the three independent repetitions.



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Differential effect of environmental conditions on the growth and regulation of the fumonisin biosynthetic gene FUM1 in the maize pathogens and fumonisin producers *Fusarium verticillioides* and *Fusarium proliferatum*

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