International Journal of Food Microbiology 253 (2017) 12–19

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

# Abiotic conditions leading to *FUM* gene expression and fumonisin accumulation by *Fusarium proliferatum* strains grown on a wheat-based substrate

Eugenia Cendoya[a,c], Laetitia Pinson-Gadais[b], María C. Farnochi[a], María L. Ramirez[a,c,*], Sylvain Chéreau[b], Giselè Marcheguay[b], Christine Ducos[b], Christian Barreau[b], Florence Richard-Forget[b]

[a] Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Fco-Qcas y Naturales, Universidad Nacional de Río Cuarto, Ruta 36 Km 601, 5800, Río Cuarto, Córdoba, Argentina
[b] Institut National de la Recherche Agronomique (INRA), UR1264 MycSA, 71 avenue Edouard Bourlaux, cs20032, 33883 Villenave d'Ornon cedex, France
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

ARTICLE INFO

*Keywords:*
Fumonisin
*Fusarium proliferatum*
Wheat
*FUM* gene
Gene expression
Abiotic stress

ABSTRACT

*Fusarium proliferatum* produces fumonisins B not only on maize but also on diverse crops including wheat. Using a wheat-based medium, the effects of abiotic factors, temperature and water activity ($a_W$), on growth, fumonisin biosynthesis, and expression of *FUM* genes were compared for three *F. proliferatum* strains isolated from durum wheat in Argentina. Although all isolates showed similar profiles of growth, the fumonisin production profiles were slightly different. Regarding *FUM* gene transcriptional control, both *FUM8* and *FUM19* expression showed similar behavior in all tested conditions. For both genes, expression at 25 °C correlated with fumonisin production, regardless of the $a_w$ conditions. However, at 15 °C, these two genes were as highly expressed as at 25 °C although the amounts of toxin were very weak, suggesting that the kinetics of fumonisin production was slowed at 15 °C. This study provides useful baseline data on conditions representing a low or a high risk for contamination of wheat kernels with fumonisins.

## 1. Introduction

*Fusarium proliferatum*, which belongs to the *Fusarium fujikuroi* species complex (FFSC) is an important pathogen of maize that produces a number of mycotoxins. In addition to maize, this fungus is able to colonize a wide range of other plants as diverse as pine trees, asparagus, fig, garlic, pea, onion, tomato, pineapple or palm trees, as well as dietary crops such as wheat, barley and rice (Abdalla et al., 2000; Aliakbari et al., 2007; Kenényi et al., 2002; Stankovic et al., 2007; Stępień et al., 2011; von Bargen et al., 2009; Waśkiewicz et al., 2013). This wide spectrum of hosts together with a worldwide geographical distribution provides evidence of the extraordinary adaptation ability of this species to enable it to colonize new environments in diverse climatic conditions.

*F. proliferatum* produces various mycotoxins, including fumonisins B, moniliformin, beauvericin, fusaric acid and fusaproliferin (Desjardins, 2006). Fumonisins B are of high concern as they can interfere with sphingolipid metabolism, leading to adverse effects on human and animal health. Epidemiological studies have associated fumonisins with human esophageal cancer and neural tube defects (Marasas et al., 2004; Missmer et al., 2006). Fumonisin $B_1$ ($FB_1$), the most frequently occurring form, has been classified by the International Agency for Research on Cancer (IARC) in Group 2B as "a possible carcinogenic to humans" (IARC, 2002). Because of the health risk associated with the consumption of contaminated crops, occurrence of fumonisins is currently under regulation in many countries.

Phylogenetic studies of a large number of *F. proliferatum* strains isolated from different hosts has revealed a high genetic variability and showed that the ability to produce fumonisins was widely distributed suggesting that this species can represent a risk for health similar to that associated with *F. verticillioides* (Jurado et al., 2010; Palacios et al., 2015). Moreover, Jurado et al. (2010) concluded that although there is no evidence that fumonisins play a role in pathogenesis, the high proportion of *F. proliferatum* strains able to produce fumonisins indicates that fumonisin production can be considered a widely distributed feature within this species that might play some ecological

* Corresponding author at: Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Fco-Qcas y Naturales, Universidad Nacional de Río Cuarto, Ruta 36 Km 601, 5800, Río Cuarto, Córdoba, Argentina.
*E-mail address:* mramirez@exa.unrc.edu.ar (M.L. Ramirez).

http://dx.doi.org/10.1016/j.ijfoodmicro.2017.04.017
Received 30 November 2016; Received in revised form 20 March 2017; Accepted 24 April 2017
Available online 25 April 2017
0168-1605/ 

role during colonization. Therefore, the occurrence of *F. proliferatum* on commodities may lead to the contamination of grains with fumonisins B as occurs with *F. verticillioides*, this being of particular relevance in crops that have not been considered so far as a potential source of dietary exposure to these mycotoxins. Several papers reported the contamination of wheat by *F. proliferatum* (Amato et al., 2015; Conner et al., 1996; Moretti et al., 1999; Palacios et al., 2011). In addition, some recent studies reported the occurrence of wheat harvests contaminated with fumonisins (Amato et al., 2015; Cendoya et al., 2014b; Mendes et al., 2015; Palacios et al., 2011; Stankovic et al., 2012). As wheat is an important crop in the human diet, its quality and safety is of major concern and presence of the potentially carcinogenic fumonisin must be avoided.

*Fusarium* growth and fumonisin production result from the complex interaction of several biotic and/or abiotic factors where water stress and temperature are the most relevant. Deciphering this complex process is essential to be able to predict and prevent plant diseases and mycotoxin production (Charmley et al., 1994). Thus, depending on the combination of abiotic factors, the biosynthetic pathway for fumonisin B will be activated or inhibited. Extensive work has been carried out on the combined effect of temperature and water stress (*via* the measure of $a_W$, which determines the amount of available water in a substrate) on fumonisin production on maize-based media and maize grains (Marín et al., 2004). In addition, we recently provided the first detailed study on fumonisin production as affected by $a_W$ and temperature on a wheat-based media (Cendoya et al., 2014a).

The fumonisin biosynthetic genes (*FUM*) are organized in clusters as for other toxins produced by filamentous fungi so far described. At least 17 clustered genes encode enzymatic activities responsible for fumonisins. The expression of these genes seems to be co-regulated (Desjardins and Proctor, 2007; Proctor et al., 1999, 2003). Fumonisin production is directly linked to the expression of the *FUM* genes, which in turn is influenced by ecological conditions. *FUM1*, encodes a polyketide synthase required for fumonisin production (Proctor et al., 1999), which catalyzes the initial step in fumonisin biosynthesis (Bojja et al., 2004). Significant positive correlations have been observed between the relative amount of *FUM1* transcripts quantified by real-time RT-PCR and the amount of fumonisins produced by both *F. verticillioides* and *F. proliferatum* (López-Errasquín et al., 2007). *FUM19* is located about 35 kb downstream from the *FUM1* gene and encodes an ABC (ATP-binding cassette) transporter involved in extracellular export of fumonisins (Proctor et al., 2003), and *FUM8* encodes an aminotransferase required to set up the mature, biologically active $FB_1$ molecule (Seo et al., 2001). To date, there have been few attempts to relate key environmental factors to the expression of genes involved in fumonisin production by *F. proliferatum*, and these studies have been mainly restricted to maize as a major crop infected by the pathogen (Fanelli et al., 2012; Kohut et al., 2009; Marín et al., 2010).

The objectives of this study were to compare the effects of temperature and osmotic stress on growth, fumonisin production, and *FUM8* and *FUM19* gene expression in three *F. proliferatum* strains, in order to investigate the relationship between *FUM* gene expression and fumonisin production by this species cultivated in a wheat-based medium.

## 2. Materials and methods

### 2.1. Fusarium strains

Three *F. proliferatum* strains ITEM 15654, ITEM 15661 and ITEM 15664 (ITEM: Agri-Food Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production, CNR, Bari, Italy; http://www.ispa. cnr.it/Collection) previously isolated from wheat grains in Argentina during 2007–2008 harvest season were used. These isolates have been characterized by morphological, biological and molecular criteria (Leslie and Summerell, 2006; Nelson et al., 1983). For the molecular characterization, sequences of elongation factor (EF-1α), calmodulin, and *FUM8* genes indicated that these isolates belong to the *F. fujikuroi* species complex (FFSC) and allowed confirming the identity of the three strains as *F. proliferatum* (Palacios et al., 2015). In order to determine their mating population (MP) inside the FFSC, crossing experiments were performed with standard testers as female parents and the uncharacterized field isolates as male parents (Klittich and Leslie, 1998). In agreement with the previous identification, all these isolates belong to the mating population D. Also, all isolates produce fumonisins (Palacios et al., 2011). The three isolates were used as working strains and maintained in 15% glycerol at − 80 °C. When inocula were required, the strains were grown on PDA slants at 25 °C in the dark for 7 days and spore suspensions were prepared by adding 5 mL of sterile distilled water to the PDA slant with gentle shaking. Spore suspension concentrations were adjusted to $10^6$ spores/mL after counting using a Neubauer chamber.

### 2.2. Medium and culture conditions

Argentinean wheat grains (BioInta 3004) were finely milled. Mixtures of 3% (w/v) of milled wheat in water were prepared and 2% (w/v) agar added. The $a_W$ of the basic medium was adjusted to 0.97, and 0.95 by addition of different amounts of glycerol (Dallyn and Fox, 1980). The control medium was not supplemented with glycerol (control condition, maximum free water availability, $a_W \sim 1$). The media were autoclaved at 120 °C for 20 min. Flasks of molten media were thoroughly shaken, prior to be poured into 9 cm sterile Petri dishes (15 mL/Petri dish). The $a_W$ of two representative samples of media for each treatment was checked. Additional, non-inoculated control plates were prepared and $a_w$ were measured at the end of the experiment in order to detect any significant deviation. Petri plates were covered with Amersham Hybond $N^+$ membranes just before inoculation in order to facilitate the fungal biomass removal for RNA extraction.

### 2.3. Inoculation, incubation and growth assessment

Petri plates were inoculated with 0.1 mL of a fresh spore suspension containing $10^6$ spores/mL that was deposited in the center of the membrane. Fungal cultures of each strain were incubated at 25 °C or 15 °C in the dark for 5 days. A full factorial design was used where the factors were $a_W$, temperature and strain, and the response was growth (total numbers of plates: 3 $a_W$ × 2 temperatures × 3 strains × 3 replicates). Growth assessment was made every day during the incubation period, by measuring two perpendicular diameters of the growing colonies. Growth measurements were plotted against time, and linear regression was applied in order to obtain the growth rate (mm/day) as the slope of the line. At the end of the incubation period, the mycelium was removed and immediately frozen at − 80 °C, then lyophilized, and stored at − 80 °C until RNA extraction. Agar plates were frozen for later fumonisin extraction and quantification.

### 2.4. Fumonisin analysis

For fumonisin extraction, five day-old Petri plates of each strain at different incubation conditions ($a_W$ and temperature) were used. Toxins were extracted with 20 mL of acetonitrile: water (1:1 v/v) by shaking the whole crushed culture media (~15 mL) with the solvent for 30 min on an orbital shaker (200 rpm). After filtration (0.45 μm), the extracts were stored at 4 °C until HPLC-MS-MS analysis.

An aliquot of the extracts (1000 μL) was taken and filtered (0.2 μm) for high performance liquid chromatography (HPLC-MS-MS) analysis. Fumonisin analysis was performed using a HPLC MS-MS system, composed of an Agilent G1311 pump, an Agilent G1313 autosampler, an Agilent G1315 PDA diode array detector interfaced with a Q-Trap 2000 ABSciex with an electrospray ionization (ESI) source operating in

**Table 1**
Parameters used to investigate fumonisins by LC-MS/MS.

| Compound | $[M + H]^+$ | $t_R$ (min) | Product ions ($m/z$) | CV (V) | CE (V) |
|---|---|---|---|---|---|
| $FB_1$ | 722 | 5.4 | 334 | 91 | 57 |
| | | | 352[a] | | 55 |
| $FB_2$ | 706.3 | 17.6 | 318.5 | 96 | 51 |
| | | | 336.3[a] | | 59 |
| $FB_3$ | 706.3 | 9.3 | 318.5 | 96 | 51 |
| | | | 336.3[a] | | 59 |

$[M + H]^+$, precursor ion; $t_R$, retention time; Product ions ($m/z$), daughter ions; CV (V), cone voltage; CE (V), collision energy.
[a] Transition used for quantification.

a positive mode.

For chromatographic separation, a Kinetex C18 2.6 µm column (4.6 × 150 mm) (Phenomenex) with a guard column of the same material was used. A gradient elution was performed with mobile phase A: 0.1% formic acid and methanol:acetonitrile (50:50 v/v), and mobile phase B: 0.1% formic acid. The flow rate was 0.7 mL/min with a split guiding 50% of the flow to the ionization source. The column temperature was maintained at 45 °C and injection volume was 5 µL. Regarding mass spectrometry, multiple-reaction monitoring (MRM) was used for toxin determination. The nitrogen flow was adjusted to 40 and 60 (arbitrary units) for cone and desolvation gases, respectively. The capillary voltage was 4.5 kV. For each compound, two transitions were monitored either for qualification or quantification. MRM parameters, as well as the retention times are collected in Table 1. Trace $m/z$ 722 > 352 was used for the quantification of $FB_1$, while 706 > 336 was used for both $FB_2$ and $FB_3$. For quantification purposes, a calibration curve was obtained injecting different mixed standard solutions (10; 25; 50; 100; 250; 500; 1000 and 5000 ng/mL). Good linearity with a correlation coefficient higher than 0.996 was obtained within calibration range. The calculated instrumental LOD (lowest concentration presenting a signal with S/N = 3) for $FB_1$ and $FB_2$ was 2.5 ng/mL and LOQ (S/N = 10) was 10 ng/mL.

### 2.5. Statistical analysis

The growth rates and mycotoxin concentrations were evaluated by analysis of variance (ANOVA) to determine the effect of $a_W$ and temperature for each *F. proliferatum* strain and two-way interaction.

When the analysis was statistically significant ($p \leq 0.01$), the *post hoc* Tukey's multiple comparison procedure was used for separation of the means ($p > 0.05$). Statistical analysis was done using SigmaStat for Windows Version 2.03 (SPSS Inc.).

### 2.6. Total RNA extraction and cDNA synthesis

Total RNA was extracted from 5-day-old mycelium of the three strains of *F. proliferatum* grown in wheat-based medium with or without glycerol. Three biological replications were prepared for each condition. The biomass was removed from the membrane at the end of the incubation period. Frozen mycelium (50 mg) was lyophilized and ground with a TissueLyser System® (Qiagen, France) for 1 min at 30 Hz. Total RNA was extracted following the RNeasy Mini Kit (Qiagen) manufacturer's instructions. DNase treatment (Ambion®, Life technologie SAS, France) was performed following manufacturer's instructions. A qPCR assay with *β-tubulin* primers (see conditions in Table 2) was performed to check the lack of genomic DNA contamination. Quality of the prepared RNA was assessed by agarose gel electrophoresis (2%), and by measuring absorbance ratios A260/A280 and A260/A230. RNA was quantified by absorbance measurements at 260 nm. RNA (1 µg) was reverse transcribed using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Life technologie SAS, France). Reverse transcription was performed with an iCycler® (Bio-Rad, France) programmed according to the thermal cycling conditions recommended by the manufacturer (Invitrogen, Life technologie SAS, France). cDNA samples were stored at − 20 °C until used for PCR analysis.

**Table 3**
PCR efficiencies obtained for *Fusarium proliferatum* strains.

| Primer | *F. proliferatum* strains | | |
|---|---|---|---|
| | ITEM 15661 | ITEM 15664 | ITEM 15654 |
| *β-tubulin* | 2.012 | 2.011 | 1.930 |
| *histone3* | 2.006 | 2.011 | 1.991 |
| *FUM8* | 2.001 | 2.016 | 2.011 |
| *FUM19* | 2.004 | 2.016 | 2.014 |

### 2.7. Real-time RT-PCR analysis

Real-time PCR was used to amplify *FUM8*, *FUM19*, *β-tubulin* and *histone3* genes using the primers listed in Table 2. *FUM8* primers were based on *FUM8* gene sequence (NCBI GenBank Accession No KF415158.1) and *FUM19* primers were based on *FUM19* gene sequence (NCBI GenBank Accession No KF415190.1). These primers were designed in our laboratory using the Primer3 software (Koressaar and Remm, 2007; Untergasser et al., 2012). Primers were used at a final concentration of 1 mM each. *β-tubulin* and *histone3* expression were used as endogenous references. Real-time RT-PCR analyses were performed with a LightCycler® 1.5 system and LightCycler Software 3.5.3 (Roche, France). Abundance of the transcripts of the genes *FUM8* and *FUM19* was evaluated in 1 µL of each cDNA sample (corresponding to 10 ng of RNA) in a final reaction volume of 10 µL, using the QuantiFast SYBR® Green PCR Kit (Qiagen, France). The expression of *β-tubulin* and *histone3* genes were stable under all tested conditions. Real-time RT-PCR amplifications were performed using 40 cycles according to the thermal cycling conditions recommended by the kit manufacturer (Qiagen, France). Amplifications were performed in triplicate for each of the three biological replications. Negative controls containing no template were performed in the same conditions. The

**Table 2**
Primer sequences and conditions used for amplification of *FUM8*, *FUM19*, *β-tubulin* and *histone3* genes.

| Primer | Sequence | Amplification | Pb | Reference |
|---|---|---|---|---|
| *FUM8* | F: 5′-TCCAGATCAAAGGCGTGACT-3′<br>R: 5′-CCCCAGGGTACTTCTTGGAC-3′ | 95 °C 5 min-40 cycles (10 s-95 °C and 40 s-60 °C) | 149 | [a] |
| *FUM19* | F: 5′-TGGCGTTATTGACCGTTGTG-3′<br>R: 5′-CCTTCTGCACACTCACGATG-3′ | 95 °C 5 min-40 cycles (10 s-95 °C and 40 s-62 °C) | 202 | [a] |
| *β-tubulin* | F: 5′-GGTAACCAAATCGGTGCTGCTTTC-3′<br>R: 5′-GATTGACCGAAACGAAGTTG-3′ | 95 °C 5 min-40 cycles (10 s-95 °C and 40 s-58 °C) | 295 | Glass and Donaldson (1995) (Forward)<br>Pinson-Gadais et al. (2007) (Reverse) |
| *histone3* | F: 5′-ACTAAGCAGACCGCCCGCAGG-3′<br>R: 5′-GCGGGCGAGCTGGATGTCCTT-3′ | 95 °C 5 min-40 cycles (10 s-95 °C and 40 s-62 °C) | 377 | Kohut et al. (2009) |

[a] Primers designed using Primer3.

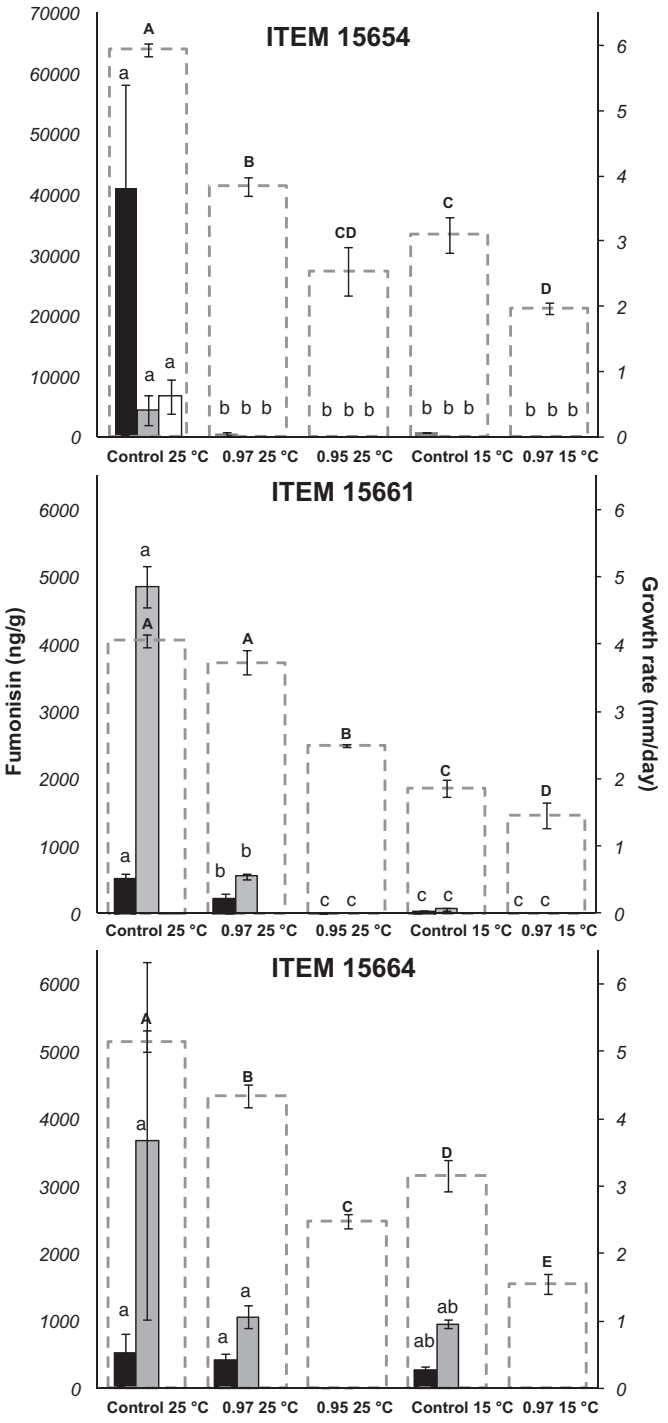


**Fig. 1.** Effect of water activity (1.0, 0.97 and 0.95) and temperature (25 and 15 °C) on the growth rate. (▨) and the production of fumonisins production (■$FB_1$, ▩$FB_2$ and □$FB_3$) by three *Fusarium proliferatum* strains cultivated on wheat-based media. Error bars represent the standard error measured between independent replicates. Means values based on triplicate data with letters in common for each type of fumonisins (lower case letters) or for growth rates (capital letters) are not significantly different according to Tukey HSD Test ($p \geq 0.05$).

absence of non-specific PCR amplification products and primer-dimer formation was checked by running melting curves.

In order to determine PCR efficiencies, a standard cDNA mixture was prepared by mixing a fraction of each cDNA sample. A range of four log dilutions was prepared from the standard mixture. For each gene, PCR efficiency (E) was determined using the serial dilutions as described by Ponts et al. (2007) (Table 3).

### 2.8. Real-time RT-PCR data analysis

Crossing points (Cp) values obtained experimentally for the target *FUM* genes were compared in control and treated conditions and normalized relative to the Cp values obtained for the reference genes *β-tubulin* and *histone3* using the REST[a] software REST-384 (Relative Expression Software Tool). The mathematical model used accounts for differences in efficiencies for the reference gene and the target gene and for the mean crossing point deviation between control and treated conditions (Pfaffl, 2001; Pfaffl et al., 2002).

The expression levels of the target genes, normalized to reference gene expression, were reported as a regulation factor in the treated condition relative to the control, with the ratio = $(E_{target})^{\Delta CP\ target\ (control\text{-}treated)} / (E_{ref})^{\Delta CP\ ref\ (control\text{-}treated)}$.

The expression ratio results were tested for significance by running Pair Wise Reallocation Randomisation Test[a] with a $p$ value of 0.001 using the REST[a] software.

## 3. Results

### 3.1. Effect of $a_W$ and temperature on growth and fumonisin production

The effect of the interaction of $a_W$ and temperature on growth rate and fumonisin production by the three investigated *F. proliferatum* strains cultivated on a wheat-based medium is depicted in Fig. 1. Maximum growth rates were reached at the highest $a_W$ (control condition) and at 25 °C for the three strains with, however some differences between the three isolates. The strain ITEM 15654 exhibited the highest growth rate (6 mm/day), 1.5 times higher than that determined for the strain ITEM 15661. The three *F. proliferatum* strains were able to grow at 15 °C for the highest $a_W$ value. For each strain and each temperature, the growth was significantly reduced with the decrease in $a_W$ in the media. At 25 °C, the decrease of $a_W$ from 1 to 0.95 led to an inhibition of the growth rate by a factor close to 2. Similarly, at 15 °C, change in $a_W$ from 1 to 0.97 had a significant repressing effect on growth; a 2-fold decrease in growth rate was highlighted for the ITEM 15654 and 15664 isolates. This decrease was less pronounced for ITEM 15661, which was also the strain characterized by the lowest growth rate in control conditions (25 °C, $a_W = 1$). It was noticeable that at 15 °C and 0.95 $a_W$ no growth was observed during the incubation period for the tested strains.

With regard to fumonisin, the production profiles of each strain were the same as previously described by Palacios et al. (2011) and Cendoya et al. (2014a). The ITEM 15654 strain produced mainly $FB_1$ and low levels of $FB_2$ and $FB_3$, whereas the ITEM 15661 and ITEM 15664 strains only produced $FB_1$ and $FB_2$, with higher levels of $FB_2$ than $FB_1$. Actually, the two last strains showed levels of $FB_2$ in the same range as the strain ITEM 15654 but produced very low amounts of $FB_1$. For each strain, the maximum amounts of fumonisins were obtained in the control condition ($a_W = 1$) at 25 °C. Only traces of fumonisins were detected for the *F. proliferatum* strains cultivated at 15 °C, except for the ITEM 15664 strain that was shown to produce significant residual levels of both $FB_1$ and $FB_2$. Similarly, decrease in $a_W$ from 1 to 0.97 had a drastic impact on the amounts of quantified toxins, regardless of the temperature of incubation and strain. When considering the ITEM 15654 strain cultivated at 25 °C, the concentration of toxins declined from 40 μg/g in control conditions to < 1 μg/g when the $a_W$ was set at 0.97. Our data also indicated that the production of $FB_1$ and $FB_2$ by the ITEM 15664 strain was less affected by osmotic stress, with a reduction factor lower than 5.

Altogether, our results demonstrated that abiotic stresses ($a_W$ and temperature) and their combination significantly influenced the production of fumonisins and the growth rates of the three *F. proliferatum* strains cultivated in wheat based media during 5 days, with temperature having the strongest impact (Table 4).

**Table 4**
Analysis of variance on the effects of water activity ($a_W$), and temperature (T) and their interaction on growth and fumonisin production of *Fusarium proliferatum* strains on wheat-based media.

ITEM 15654

| S.V[a] | df[b] | $FB_1$ | | $FB_2$ | | $FB_3$ | | Growth rate | |
|---|---|---|---|---|---|---|---|---|---|
| | | MS[c] | F[d] | MS[c] | F[d] | MS[c] | F[d] | MS[c] | F[d] |
| $a_W$ | 2 | $77 \times 10^7$ | 13,1* | $9 \times 10^6$ | 7,2 | $20 \times 10^6$ | 12,6* | 5,51 | 109,1* |
| T | 1 | $12 \times 10^8$ | 21,2* | $15 \times 10^6$ | 12,0* | $34 \times 10^6$ | 20,8* | 16,6 | 329,5* |
| $a_W \times T$ | 1 | $12 \times 10^8$ | 20,2* | $14 \times 10^6$ | 11,6* | $33 \times 10^6$ | 20,4* | 0,7 | 13,6* |

ITEM 15661

| S.V[a] | df[b] | $FB_1$ | | $FB_2$ | | Growth rate | |
|---|---|---|---|---|---|---|---|
| | | MS[c] | F[d] | MS[c] | F[d] | MS[c] | F[d] |
| $a_W$ | 2 | $8 \times 10^4$ | 55,1* | $9,4 \times 10^6$ | 495* | 0,3 | 16,4* |
| T | 1 | $4 \times 10^5$ | 259,4* | $2,1 \times 10^7$ | 1125* | 15 | 815* |
| $a_W \times T$ | 1 | $5 \times 10^4$ | 34,2* | $1,3 \times 10^7$ | 710,1* | 5E-3 | 0,3 |

ITEM 15664

| S.V[a] | df[b] | $FB_1$ | | $FB_2$ | | Growth rate | |
|---|---|---|---|---|---|---|---|
| | | MS[c] | F[d] | MS[c] | F[d] | MS[c] | F[d] |
| $a_W$ | 2 | $2 \times 10^5$ | 9,37* | $7 \times 10^6$ | 5,07* | 3,58 | 155,64* |
| T | 1 | $3 \times 10^5$ | 18,38* | $1 \times 10^7$ | 7,6* | 17,2 | 746,11* |
| $a_W \times T$ | 1 | $2 \times 10^4$ | 1,22 | $2 \times 10^6$ | 1,47 | 0,48 | 21,05* |

[a] Source of variation.
[b] Degrees of freedom.
[c] Mean square.
[d] Snedecor-F test.
* Significant at $p < 0.05$.

### 3.2. Effect of $a_W$ and temperature on expression of *FUM8* and *FUM19* genes

The expression of *FUM8* and *FUM19* of *F. proliferatum* ITEM 15654, ITEM 15664 and ITEM 15661 was evaluated at two temperatures (15 °C and 25 °C) and three $a_W$ (control, 0.97, and 0.95) and compared with the values determined at 25 °C in the control conditions ($a_W = 1$). Expression of *β-tubulin* and *histone3* served as reference genes in all conditions. Results are shown in Fig. 2.

For *F. proliferatum* ITEM 15654 strain cultivated at 25 °C, the decrease in $a_W$ led to a significant reduction of the expressions of *FUM8* and *FUM19* compared to control conditions ($a_W = 1$). This was in agreement with the decrease in fumonisin production reported in Fig. 1. Surprisingly, at 15 °C, the expression of the two considered *FUM* genes was not significantly reduced, compared to the 25 °C condition. This observation was in contradiction with the significant decrease in $FB_1$ accumulation that was previously highlighted (Fig. 1). The expression of *FUM8* and *FUM19* became significantly repressed at 15 °C when the $a_W$ was lowered to 0.97, in agreement with the very low fumonisin production.

When considering the results obtained for *F. proliferatum* ITEM 15661 strain, a significant inhibition of the expression of *FUM8* and *FUM19* was observed at 25 °C, as a result of the decrease of $a_W$ to 0.95. It was also noted that the repression of *FUM19* at $a_W$ 0.97 was not significant whereas a there was major decrease in fumonisin production (Fig. 1). For the previous strain cultivated at 15 °C, either in the control condition ($a_W = 1$) or at $a_W$ 0.97, again although fumonisin production was considerably affected, the expression of *FUM8* and *FUM19* was not significantly affected.

A similar complex situation was observed for *F. proliferatum* ITEM 15664 strain. The expression of *FUM8* and *FUM19* was not reduced by decreasing the $a_W$ to 0.97 or when the incubation temperature was 15 °C instead of 25 °C. This down regulation correlated with the fumonisin production levels that were only partially reduced in comparison with the levels obtained in the control conditions at 25 °C. Although it was only significant for *FUM8*, the expression of *FUM* genes was reduced at 25 °C/$a_W = 0.95$ in agreement with the lower fumonisin production.

As a whole, our data showed that responses to $a_W$ or temperature modifications varied according to the considered strain of *F. proliferatum*. Nonetheless, lowering the temperature from 25 °C to 15 °C led to a significant reduction in fumonisin production. Surprisingly, lowering the temperature did not affect significantly the expression of *FUM* genes although non-significant trends of lesser expression levels could be observed, in particular for the strong $FB_1$-producer strain *F. proliferatum* ITEM 15654. Our observations suggested also that an osmotic stress could down-regulate the expression of *FUM8* and *FUM19* genes.

## 4. Discussion

This study compared for the first time the impact of $a_W$ and temperature on growth, fumonisin production, and *FUM8* and *FUM19* gene expression on a wheat-based medium, for three strains of *F. proliferatum* isolated from wheat in Argentina. As expected, both variables affected growth, and the observed modulation pattern was similar whatever the considered strain. Among tested conditions, the highest growth rates were observed at 25 °C in the absence of osmotic stress for all the evaluated strains. Previous studies related to $a_W$ and temperature requirements for *F. proliferatum* have indicated that this fungus has a minimum $a_W$ of 0.90, and temperature range from 4 to 37 °C for growth in maize extract agar media and that the optimal

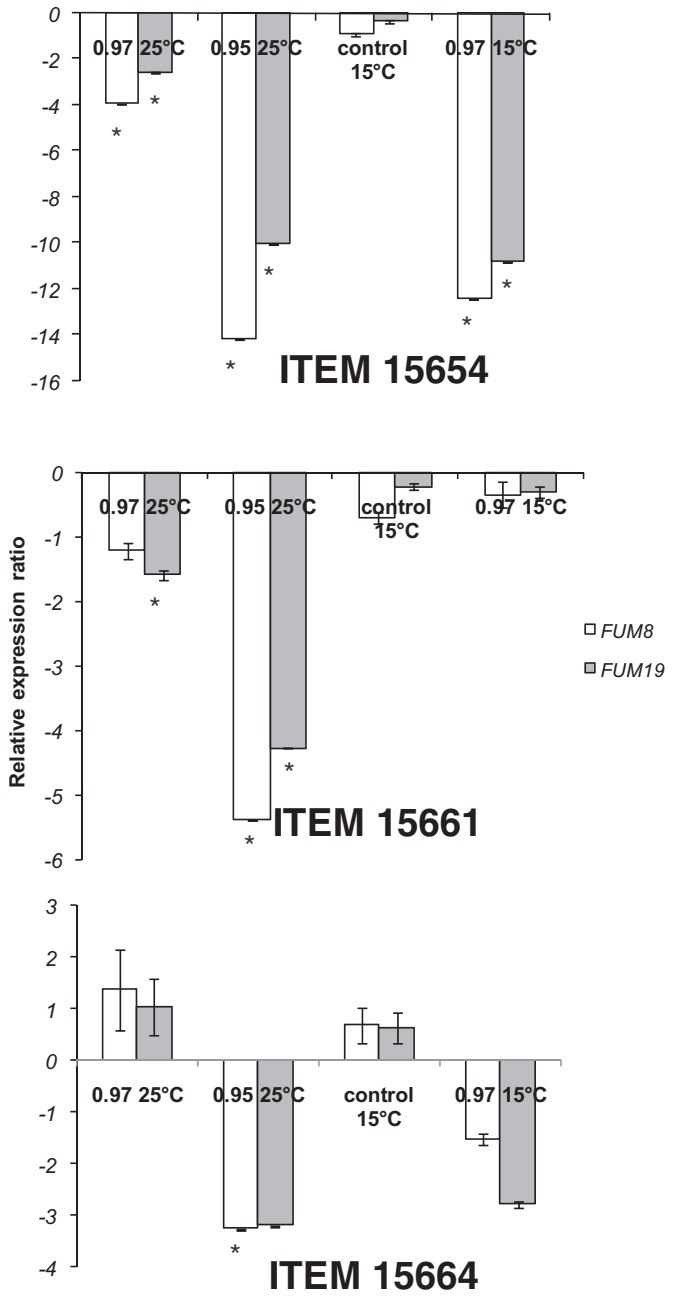


**Fig. 2.** Relative expression of *FUM8* and *FUM19* genes of three *Fusarium proliferatum* strains incubated for 5 days at 15 and 25 °C and three water activities (control, 0.97 and 0.95). The *FUM8* and *FUM19* cDNA amount was normalized in each experiment using the *Cp* values obtained for *β-tub* and *H3* cDNA amplifications. The values represent the regulation factor obtained for the target gene in the different culture conditions relative to the control culture (25 °C, $a_W$ = 1). The data represent the means of three independent repetitions. Error bars represent the standard error measured between independent replicates. Values were calculated using the REST software (Pfaffl, 2001; Pfaffl et al., 2002). * significant $p \leq 0.001$.

condition for growth was at the highest $a_W$ tested (0.994) (Marín et al., 2004). The results obtained in the present work were similar to those reported by Marín et al. (2010) for *F. verticillioides* and *F. proliferatum* strains (5–6 mm/day) and confirmed the values that we have assessed in a previous study (Cendoya et al., 2014a). Nevertheless, in the present study, growth was analyzed during only five days of incubation for each strain. In their environment, *Fusarium* species may be present on a substrate for very long periods during which $a_W$ may change. For this reason, it is highly important to precisely know the optimal $a_W$ range for growth and the range enabling only sub-optimal growth. Under field conditions, temperature fluctuations, changes in relative humidity, and rainfall would strongly influence colonization of the developing grains by *F. proliferatum*.

For all the strains tested in the present study, the highest amounts of fumonisins were produced at 25 °C in the control condition in the absence of osmotic stress (maximum $a_W$). According to previous data (Cendoya et al., 2014a) obtained with the same set of *F. proliferatum* strains but for 28 days of incubation, maximum amounts of total fumonisins were observed at 25 °C and 0.99 $a_W$ for two of the strains, and at 15 °C and 0.98 $a_W$ for the third strain. Different fumonisin production profiles were found among the analyzed strains. At all tested temperatures, two of the three strains of *F. proliferatum* produced almost exclusively $FB_2$ and very low amounts of $FB_1$ on wheat based-media. These results support those previously reported by Palacios et al. (2011) for the same strains and are in agreement with the reported natural fumonisin contamination patterns of some wheat samples harvested in Argentina that were more contaminated with $FB_2$ than with $FB_1$ (Palacios et al., 2011).

In a recent study, Medina et al. (2013) have highlighted the profound effect of temperature and $a_W$ on the growth, production of $FB_1$ and $FB_2$, and expression of nine genes of the biosynthetic *FUM* cluster, using a *F. verticillioides* strain as model strain. The reported data showed the correlation of *FUM1*, *FUM11*, *FUM13*, *FUM14* and *FUM19* expression with $FB_1$ and $FB_2$ production and notably, the high expression of *FUM19* associated with the increased production of both fumonisins. Similarly, López-Errasquín et al. (2007) suggested a linear relationship between *FUM1* and *FUM19* transcripts and fumonisin production for a strain of *F. verticillioides* incubated at 20 °C for 14 days. Our results support the conclusions of the two studies mentioned above: when there were no detectable levels of fumonisins, the expression of the *FUM19* gene was repressed. Regarding *FUM8* gene expression, our results are in agreement with those reported by Kohut et al. (2009) who observed a positive correlation between *FUM8* gene expression and fumonisin production in *F. proliferatum*. Considering the transcriptional control of *FUM* genes, the most surprising finding of the present study was that although no fumonisin was detected after five days of incubation at 15 °C in the absence of osmotic stress, no inhibition of the expression of *FUM8* and *FUM19* genes compared to the conditions at 25 °C ($a_W$ = 1) was highlighted. One possible explanation could be that the biochemical process of fumonisin biosynthesis is slower at this temperature, leading to a delay in the accumulation of fumonisins in wheat–based media and consequently to the lack of detectable levels of the toxin after five days of incubation. Indeed, in previous studies, significant amounts of fumonisins were quantified in wheat-based media inoculated by the same set of *F. proliferatum* strains and incubated for 28 days at 15 °C (Cendoya et al., 2014a). Accordingly, the results of Ryu et al. (1999) indicated that, compared to *F. verticillioides*, *F. proliferatum* was able to produce greater amounts of $FB_1$ at low temperatures, leading to the hypothesis that *F. proliferatum* could show a better adaptation to low temperatures. In addition, Marín et al. (2010) who have studied the effects of temperature and solute potential on growth and transcriptional control of *FUM1* gene in *F. verticillioides* and *F. proliferatum*, have concluded that the relative contribution of these two species to fumonisin production on maize might differ during the course of colonization, depending on the environmental conditions. The contamination of maize with fumonisins could be mainly ascribed to *F. proliferatum*, at the earliest stages of kernel development, when the temperature is still mild, whereas the relative contribution of *F. verticillioides* would increase during the later stages, when water stress progressively increases. Deciphering the effect of low temperatures on fumonisin production by *F. proliferatum* is essential when addressing natural fumonisin contamination of wheat, which is a winter crop and requires for cultivation an optimal temperature range between 10 and 24 °C.

Considering that field conditions are likely to be conducive to *F. proliferatum* growth and toxin production in wheat grains, and that

fumonisin contamination has been reported in wheat and sub-products in several countries (Amato et al., 2015; Busman et al., 2012; Castoria et al., 2005; Cendoya et al., 2014b; Chehri et al., 2010; Jakšić et al., 2012; Kushiro et al., 2009; Mendes et al., 2015; Palacios et al., 2011; Roscoe et al., 2008; Serrano et al., 2012; Wang et al., 2013); studying how this fungus can grow and contaminate this crop, as well as how expression of fumonisin biosynthetic genes are contributing in this process is of concern. Our results provide useful preliminary information for predicting the possible risk factors for fumonisin contamination of wheat.

## Acknowledgements

E. Cendoya is grateful to BECAR for a short stay fellowship held in MycSA, Institut National de la Recherche Agronomique (INRA), Bordeaux, France. This work was supported by (ANPCyT Agencia Nacional de Promoción Científica y Tecnológica) PICT-2012-1436. Cendoya E. is a fellow of CONICET and Ramirez, M.L. is members of the Research Career of CONICET.

## References

Abdalla, M.Y., Al-Rokibah, A., Moretti, A., Mulè, G., 2000. Pathogenicity of toxigenic *Fusarium proliferatum* from date palm in Saudi Arabia. Plant Dis. 84, 321–324.

Aliakbari, F., Mirabolfathy, M., Emami, M., Mazhar, S.F., Karami-Osboo, R.K., 2007. Natural occurrence of *Fusarium* species in maize kernels at Gholestan province in northern Iran. Asian J. Plant Sci. 8, 1276–1281.

Amato, B., Pfohl, K., Tonti, S., Nipoti, P., Dastjerdi, R., Pisi, A., Karlovsky, P., Prodi, A., 2015. *Fusarium proliferatum* and fumonisin $B_1$ co-occur with *Fusarium* species causing *Fusarium* head blight in durum wheat in Italy. J. Appl. Bot. Food Qual. 88, 228–233.

von Bargen, S., Martinez, O., Schadock, I., Eisold, A., Gossmann, M., Buttner, C., 2009. Genetic variability of phytopathogenic *Fusarium proliferatum* associated with crown rot in *Asparagus officinalis*. J. Phytopathol. 157, 446–456.

Bojja, R.S., Cerny, R.L., Proctor, R.H., Du, L., 2004. Determining the biosynthetic sequence in the early steps of the fumonisin pathway by use of three gene disruption mutants of *F. verticillioides*. J. Agric. Food Chem. 52, 2855–2860.

Busman, M., Desjardins, A.E., Proctor, R.H., 2012. Analysis of fumonisin contamination and the presence of *Fusarium* in wheat with kernel black point disease in the United States. Food Addit. Contam. Part A 29, 1092–1100.

Castoria, R., Lima, G., Ferracane, R., Ritieni, A., 2005. Occurrence of mycotoxin in farro samples from southern Italy. J. Food Prot. 68, 416–420.

Cendoya, E., Farnochi, M.C., Chulze, S.N., Ramirez, M.L., 2014a. Two-dimensional environmental profiles of growth and fumonisin production by *Fusarium proliferatum* on a wheat-based substrate. Int. J. Food Microbiol. 182–183, 9–17.

Cendoya, E., Monge, M.P., Palacios, S., Chiacchiera, S., Torres, A., Farnochi, M.C., Ramirez, M.L., 2014b. Fumonisin occurrence in naturally contaminated wheat grain harvested in Argentina. Food Control 37, 56–61.

Charmley, L.L., Rosenber, A., Trenholm, H.L., 1994. Factors responsible for economic losses due to *Fusarium* mycotoxin contamination of grains, foods and feedstuffs. In: Miller, J.D., Trenholm, H.L. (Eds.), Mycotoxins in Grain Compounds Other Than Aflatoxin. Eagan Press, St Paul, MN, pp. 471–486.

Chehri, K., Jahromi, S.T., Reddy, K., Abbasi, S., Salleh, B., 2010. Occurrence of *Fusarium* spp. and fumonisins in stored wheat grains marketed in Iran. Toxins 2, 2816–2823.

Conner, R.L., Hwang, S.F., Stevens, R.R., 1996. *Fusarium proliferatum*: a new causal agent of black point in wheat. Can. J. Plant Pathol. 18, 419–423.

Dallyn, H., Fox, A., 1980. Spoilage of material of reduced water activity by xerophilic fungi. In: Corry, J.E., Gould, G.H. (Eds.), Microbial Growth and Survival in Extreme Environments. Academic Press, London/New York, pp. 129–139.

Desjardins, A., 2006. *Fusarium* Mycotoxins: Chemistry, Genetics and Biology. APS Press, St. Paul, MN, pp. 79–108.

Desjardins, A.E., Proctor, R.H., 2007. Molecular biology of *Fusarium* mycotoxins. Int. J. Food Microbiol. 119, 47–50.

Fanelli, F., Schmidt-Heydt, M., Haidukowski, M., Geisen, R., Logrieco, A., Mule, G., 2012. Influence of light on growth, fumonisin biosynthesis and *FUM1* gene expression by *Fusarium proliferatum*. Int. J. Food Microbiol. 153, 148–153.

Glass, N.L., Donaldson, G., 1995. Development of primer sets designed for use with the PCR to amply conserved genes from filamentous ascomycetes. App. Environm. Microbiol. 61, 1323–1330.

International Agency for Research on Cancer (IARC), 2002. Fumonisin $B_1$. IARC Monograph on the Evaluation of Carcinogenic Risk to Humans, Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene. vol. 82. IARC, Lyon, France, pp. 301–366.

Jakšić, S., Abramović, B., Jajić, I., Baloš, M.Ž., Mihaljev, Ž., Despotović, V., Šojić, D., 2012. Co-occurrence of fumonisins and deoxynivalenol in wheat and maize harvested in Serbia. Bull. Environ. Contam. Toxicol. 89, 615–619.

Jurado, M., Marín, P., Callejas, C., Moretti, A., Vázquez, C., González-Jaén, M.T., 2010. Genetic variability and fumonisin production by *Fusarium proliferatum*. Food Microbiol. 27, 50–57.

Kenényi, Z., Mulé, G., Moretti, A., Waalwijk, C., Hornok, L., 2002. Fertility and mating type assessment within *Fusarium proliferatum* isolates from different host plants. J. Appl. Genet. 43, 55–68.

Klittich, C.J.R., Leslie, J.F., 1998. Nitrate reduction mutants of *Fusarium moniliforme (Gibberella fujikuroi)*. Genetics 118, 417–423.

Kohut, G., Adàm, A.L., Fazekas, B., Hornok, L., 2009. N-starvation stress induced *FUM* gene expression and fumonisin production is mediated via the HOG-type MAPK pathway in *Fusarium proliferatum*. Int. J. Food Microbiol. 130, 65–69.

Koressaar, T., Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23, 1289–1291.

Kushiro, M., Zheng, Y., Nagata, R., Nakagawa, H., Nagashima, H., 2009. Limited surveillance of fumonisins in brown rice and wheat harvested in Japan. J. Food Prot. 72, 1327–1331.

Leslie, J.F., Summerell, B.A., 2006. The *Fusarium* Laboratory Manual. Blackwell Professional, Ames, IA.

López-Errasquín, E., Vázquez, C., Jiménez, M., González-Jaén, M.T., 2007. Real time RT PCR assay to quantify the expression of *FUM1* and *FUM19* genes from the fumonisin-producing *Fusarium verticillioides*. J. Microbiol. Methods 68, 312–317.

Marasas, W.F.O., Riley, R., Hendricks, K., Stevens, W.L., Sadler, T.W., Gelineau van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martinez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M.C., Roman, A.V., Voss, K.A., Wang, E., Merrill Jr., A.H., 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. J. Nutr. 134, 711–716.

Marín, S., Magan, N., Ramos, A., Sanchis, V., 2004. Fumonisin-producing strains of *Fusarium*: a review of their ecophysiology. J. Food Prot. 67, 1792–1805.

Marín, P., Magan, N., Vázquez, C., González-Jaén, M.T., 2010. 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*. FEMS Microbiol. Ecol. 73, 303–311.

Medina, A., Schmidt-Heydt, M., Cárdenas-Chávez, D., Parra, R., Geisen, R., Magan, N., 2013. Integrating toxin gene expression, growth and fumonisin $B_1$ and $B_2$ production by a strain of *Fusarium verticillioides* under different environmental factors. J. R. Soc. Interface 10, 20130320.

Mendes, R.L.G., Reis, A.T., Corrêa, B., Badiale-Furlong, E., 2015. Mycobiota and occurrence of fumonisin $B_1$ in wheat harvested in Southern Brazil. Cienc. Rural. 45, 1050–1057.

Missmer, S., Suaz, L., Felkner, M., Wang, E., Merrill, A., Rothman, K., Hendricks, K., 2006. Exposure to fumonisins and the occurrence of neural tube defects along the Texas–Mexico border. Environ. Health Perspect. 114, 237–241.

Moretti, A., Mulè, G., Perrone, G., Ritieni, A., Bottalico, A., D'Erchia, A.M., Logrieco, A., 1999. *Fusarium proliferatum* from various plants: fertility, toxigenicity, and characterization by RAPDs. Bull. Inst. Compr. Agric. Sci. Kinki Univ. 7, 27–36.

Nelson, P.E., Toussoun, T.A., Marasas, W.F.O., 1983. *Fusarium* Species: An Illustrated Manual for Identification. Pennsylvania State Univ. Press, University Park, PA.

Palacios, S.A., Ramirez, M.L., Cabrera Zalazar, M., Farnochi, M.C., Zappacosta, D., Chiacchiera, S.M., Reynoso, M.M., Chulze, S.N., Torres, A.M., 2011. Occurrence of *Fusarium* spp. and fumonisin in durum wheat grains. J. Agric. Food Chem. 59, 12264–12269.

Palacios, S.A., Susca, A., Haidukowski, M., Stea, G., Cendoya, E., Ramírez, M.L., Chulze, S.N., Farnochi, M.C., Moretti, A., Torres, A.M., 2015. Genetic variability and fumonisin production by *Fusarium proliferatum* isolated from durum wheat grains in Argentina. Int. J. Food Microbiol. 201, 35–41.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 2002–2007.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, 1–10.

Pinson-Gadais, L., Barreau, C., Chaurand, M., Gregoire, S., Monmarson, M., Richard-Forget, F., 2007. Distribution of toxigenic *Fusarium* spp. and mycotoxins production in milling fractions of durum wheat. Food Addit. Contam. 24, 53–62.

Ponts, N., Pinson-Gadais, L., Barreau, C., Richard-Forget, F., Ouellet, T., 2007. Exogenous $H_2O_2$ and catalase treatments interfere with Tri genes expression in liquid cultures of *Fusarium graminearum*. FEBS Lett. 581, 443–447.

Proctor, R.H., Desjardins, A.E., Plattner, R.D., Hohn, T.M., 1999. A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in *Gibberella fujikuroi* mating population A. Fungal Genet. Biol. 27, 100–112.

Proctor, R.H., Brown, D.W., Plattner, R.D., Desjardins, A.E., 2003. Coexpression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genet. Biol. 38, 237–249.

Roscoe, V., Lombaert, G.A., Huel, V., Neumann, G., Melietio, J., Kitchen, D., Kotello, S., Krakalovich, T., Trelka, R., Scott, P.M., 2008. Mycotoxins in breakfast cereals from the Canadian retail market: a 3-year survey. Food Addit. Contam. A. 25, 347–355.

Ryu, D., Munimbazi, C., Bullerman, L.B., 1999. Fumonisin $B_1$ production by *Fusarium moniliforme* and *Fusarium proliferatum* as affected by cycling temperatures. J. Food Prot. 62, 1456–1460.

Seo, J.A., Proctor, R.H., Plattner, R.D., 2001. Characterization of four clustered coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. Fungal Genet. Biol. 34, 155–165.

Serrano, A.B., Font, G., Ruiz, M.J., Ferrer, E., 2012. Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area. Food Chem. 135, 423–429.

Stankovic, Z.S., Levic, J., Krnjaja, V.S., Bocarov-Stancic, A.S., Tancic, S.L., Kovacevic, T.M., 2007. Frequency of toxicogenic *Fusarium* species and fusariotoxins in wheat grain in Serbia. Proc. Nat. Sci. Matica Srpska Novi Sad. 113, 93–102.

Stankovic, S., Levic, J., Ivanovic, D., Krnjaja, V., Stankovic, G., Tanci, S., 2012. Fumonisin $B_1$ and its co-occurrence with other fusariotoxins in naturally-contaminated wheat

grain. Food Control 23, 384–388.
Stępień, L., Koczyk, G., Waśkiewicz, A., 2011. Genetic and phenotypic variation of *Fusarium proliferatum* isolates from different host species. J. Appl. Genet. 52, 487–496.
Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3 — new capabilities and interfaces. Nucleic Acids Res. 40, e115.
Wang, Y.-K., Shi, Y.-B., Zou, Q., Sun, J.-H., Chen, Z.-F., Wang, H., Li, S.-Q., Yan, Y.-X., 2013. Development of a rapid and simultaneous immunochromatographic assay for the determination of zearalenone and fumonisin $B_1$ in corn, wheat and feedstuff samples. Food Control 31, 180–188.
Waśkiewicz, A., Stępień, L., Wilman, K., Kachlicki, P., 2013. Diversity of pea-associated *F. proliferatum* and *F. verticillioides* populations revealed by *FUM1* sequence analysis and fumonisin biosynthesis. Toxins 5, 488–503.