Integrating toxin gene expression, growth and fumonisin B₁ and B₂ production by a strain of *Fusarium verticillioides* under different environmental factors

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1. Introduction

*Fusarium verticillioides* (teleomorph, *Gibberella moniliformis*) is an economically important maize pathogen which colonizes ripening cobs and contaminates the maize with fumonisins (FB; B₁–B₄). These toxins are important as they have toxic effects by inhibition of sphingolipid metabolism which can impact on human and animal health. For example, fumonisins cause leukoencephalomalacia, a brain lesion which can be fatal to horses after consumption of contaminated animal feed. Fumonisins have also been associated epidemiologically with human oesophageal cancer and birth defects, especially neural tube defects in humans [1]. The genome of *F. verticillioides* has been elucidated [2] and the FB biosynthetic genes are clustered together as for other mycotoxigenic fungi (e.g. *Aspergillus flavus*; *Fusarium graminearum*). This may facilitate horizontal transfer and coordinated transcriptional regulation of the genes in the FB biosynthetic cluster which consists of 17 transcriptionally co-regulated genes. Fumonisin polyketide synthase (*FUM1*) is the important gene of the cluster of co-regulated fumonisin biosynthetic genes. The cluster also encodes an amino-transferase (*FUM8*), a C-3 carbonyl reductase (*FUM13*), and cytochrome P450 and other enzymes that catalyse oxygenation at C-5 (*FUM3*), C-10 (*FUM2*) and another undefined site (*FUM6*) [3]. Four other genes (*FUM7*, *FUM10*, *FUM11*, *FUM14*) are also required for tricarballylic acid esterification. At the opposite end of the cluster from *FUM1* there are transporter encoding genes (*FUM19*, *FUM17*...
and FUM18). These have been suggested as being involved in fumonisin self-protection and sphingolipid metabolism.

The biosynthesis of mycotoxins is strongly dependent on growth conditions such as substrate composition [4] or physical factors including pH, water availability, temperature or modified atmospheres [5–8]. Water activity ($a_w$) is a good measure of the amount of freely available water in a substrate for microbial growth and is related to pure water, which has an $a_w$ of 1.00 or 100 per cent equilibrium relative humidity. This $a_w$ is related to the total moisture content of a specific substrate by a moisture sorption curve [9]. Thus, depending on the combination of abiotic factors the biosynthetic pathway for FBs will be activated or completely inhibited. Marin et al. [10] showed that the $a_w \times$ temperature conditions for germination, growth and phenotypic FB production were different with much narrower limits of these two interacting parameters for mycotoxin production. Models of the production of FB in relation to interacting abiotic conditions have been examined [11,12], although such data have not previously been related to the FB gene cluster expression. Studies by Jurado et al. [13] showed that expression of the FUM1 gene was significantly increased under water stress conditions (0.95 $a_w$) which may indicate an increase in FB production by F. verticillioides under such conditions. In contrast, studies with Fusarium proliferatum, which has a much broader host range than the former species, showed that FUM1 gene expression was more stable under water stress, suggesting that FB production may not be an important determinant of pathogen infection of maize by this species [14].

Previous studies using a mycotoxin microarray with sub-arrays for specific mycotoxins, developed by Schmidt-Heydt & Geisen [15] have shown the impact of key environmental factors ($a_w$, temperature) on important mycotoxin genes of the fumonisin gene cluster (A. flavus) and of the trichothecene gene cluster (F. graminearum, Fusarium culmorum) and phenotypic mycotoxin production [16]. Recently, Schmidt-Heydt et al. [17] showed that for F. culmorum and F. graminearum it is possible to integrate such microarray data on relative TRI gene expression under different environmental conditions, growth and deoxynivalenol (DON) production and develop models which can be used to predict DON concentrations. In 2012 Abdel-Hadi et al. [18] demonstrated that data on key genes in the biosynthesis of aflatoxin, including the key regulatory genes (afIR, afIS) could be integrated with growth and aflatoxin production by using a mixed-growth model. This also enabled the relatively accurate prediction of aflatoxin production at increased temperatures and water stress conditions relevant to climate change type scenarios. No such integrated systems approach has been attempted for such datasets for F. verticillioides.

Here, we have used the mycotoxin microarray sub-array described previously [15] for the FB genes as a tool for examining the changes that interacting environmental factors may have on the relative expression of the F. verticillioides gene cluster, as well as effects on growth and phenotypic fumonisin production. The objectives of this study were (i) to examine the effect of $a_w \times$ temperature conditions on growth, FB1 and FB2 production and relative expression of nine genes of the FUM cluster (FUM1, FUM7, FUM10, FUM11, FUM12, FUM13, FUM14, FUM16 and FUM19) in the fumonisin biosynthetic pathway using the mycotoxin gene microarray; (ii) to quantify the amounts of FB1 and FB2 produced under these interacting conditions; (iii) to evaluate the mixed-growth model application to integrate the relationship between the expression of these genes, environmental factors, growth and FB1 and FB2 production; (iv) to validate the model with datasets outside the range of the model and (v) to examine the possible ternary interactions and relationships between FUM1, a key gene, and some other genes in the biosynthetic pathway in relation to $a_w$, temperature and fumonisin FB1 and FB2 production.

### 2. Material and methods

#### 2.1. Fungal strain used

The F. verticillioides strain (MPVP No 294) has been kindly supplied by Prof. P. Battilani, University Sacre Cuore, Piacenza, Italy and is from the Italian Culture Collection and was stored at 4°C or sub-cultured on a 2 per cent maize meal agar when required. It has a known fumonisin production capacity [19].

#### 2.2. Growth studies

These were carried out with a conducive YES medium (20 g yeast extract, 150 g sucrose, 1 g MgSO$_4$·7H$_2$O, 1 l). The agar medium was modified with glycerol to adjust the water availability to 0.995, 0.98, 0.95, 0.93 $a_w$ as described previously [20,21]. The accuracy of the modifications was confirmed using an Aqualab 3TE instrument (Decagon, Pullman, WA, USA) and found to be within ±0.005 of the target $a_w$.

Petri plates (Ø 9 cm) containing media treatments and overlaid with sterile 8.5 cm cellophane discs (P400, Cannings Ltd, Bristol, UK) were inoculated using small agar plugs (Ø 3 mm) taken from 7–8-day-old growing cultures grown at 25°C. Replicates (five per treatment) were incubated at 20, 25 and 30°C for model design and temperature outside the model boundaries: 35°C was used to validate the model. Assessment of growth was made daily during the 10-day incubation period, the optimal time for FUM1 gene expression [13]. Two diameters of the growing colonies were measured at right angles to each other until the whole colony biomass was scraped from the cellophane surface into Eppendorf tubes and frozen at ~80°C for RNA extraction.

#### 2.3. Isolation of RNA from samples

To perform microarray experiments RNA was isolated using the RNAeasy Plant Mini kit (Qiagen, Hilden, Germany). The procedure was made according to the manufacturer’s instructions except for the ground of 1 g of mycelium with a mortar and pestle in liquid nitrogen, of which 250 mg of resulting powder was suspended in 750 μl lysis buffer, mixed with 7.5 μl β-mercaptoethanol and 100 glass beads with a diameter of 1 mm (B. Braun Biotech International GmbH, Melsungen, Germany) in a 2 ml RNase-free micro reaction tube. These extracts were mixed thoroughly and incubated for 15 min at 55°C and 42 kHz in an S10H ultrasonic bath (Elma, Singen, Germany).

#### 2.4. Microarray experiments

cDNA synthesis and labelling were performed using 50 μg of the DNase I treated total RNA and the Micromax Direct Labelling kit (Perkin Elmer Life And Analytical Sciences, Inc., Boston, USA). The cDNA was subsequently purified with the QiaQuick Min Elute kit (Qiagen, Hilden, Germany), dried in a vacuum concentrator (Speed Vac, Savant Instruments, Farmingdale, USA), re-suspended in 60 μl hybridization buffer (Scienion, Berlin, Germany), heated for 2 min at 95°C, placed on ice to prevent strand rearrangement and hybridized for 18 h at 42°C to the microarray by using an automatic hybridization station (Perkin Elmer, Boston, USA). This array was scanned with a confocal laser system (Scanarray lite, Perkin Elmer) at a resolution of 5 μm. The results were normalized using the Lowess algorithm
(locally weighted scatter plot smoothing) together with subtraction of the background signal. The constitutively expressed β-tubulin gene was used as a control.

2.5. Fumonisin B₁ and B₂ analyses

2.5.1. Fumonisins extraction and derivatization

Fumonisins were extracted from agar plugs cut out with the aid of a cork borer (Ø = 5 mm) 10-day-old cultures of *Fusarium*. The plugs were then placed in pre-weighed 2 ml Eppendorff tubes. The tubes and their contents were filtered by means of an on-line degassing device and degassed by means of an on-line degassing device (Agilent). The gradient program was 100 per cent A during 5 min followed by 50/50, v/v) and (B) acetonitrile/water (80/20, v/v). Each mobile phase was filtered by passing through a 0.45 mm hydrophobic membrane filter and degassed by means of an on-line degassing device (Agilent). The mobile phases were (A) methanol/0.05 M sodium dihydrogen phosphate aqueous solution adjusted to pH 5.0 with 2 M NaOH (50/50, v/v) and (B) acetonitrile/water (80/20, v/v).

2.5.2. HPLC-FLD detection and quantification

The derivatized sample (25 μl) was analysed by HPLC (Agilent 1200 series) with a fluorescence detector set at excitation and emission wavelengths of 355 and 440 nm, respectively. Separation was achieved using C18 chromatographic analytical column (Phenomenex Gemini; 150 × 4.6, 3 μm particle size: Phenomenex, CA, USA). The mobile phases were (A) methanol/0.05 M sodium dihydrogen phosphate aqueous solution adjusted to pH 5.0 with 2 M NaOH (50/50, v/v) and (B) acetonitrile/water (80/20, v/v). Each mobile phase was filtered by passing through a 0.45 mm filter and degassed by means of an on-line degassing device (Agilent). The gradient program was 100 per cent A during 5 min followed by a rapid increase to 50 per cent A at 50 per cent B with a final hold of 15 min. The column was kept at 30°C with a flow rate of 1.0 ml min⁻¹. Quantification of fumonisins was achieved through comparison of peak areas of the chromatograms of the samples with those of the fumonisins standard.

2.6. Data analysis and model development

This study has used a mixed-growth-associated product formation model [23], which takes account of both specific growth rate and metabolite accumulation. This includes the fact that product formation is a combination of growth rate and the specific rate of product formation which is given by equation (2.1). This has been previously used for production of compounds such as xanthan gum and for examining *A. flavus* growth and aflatoxins production [18]:

\[ \frac{dp}{dt} = (\alpha + \beta)X \quad \text{(2.1)} \]

where \( \frac{dp}{dt} \) is the total production of fumonisins B₁ or B₂ produced; \( \alpha \) and \( \beta \) are constants of fumonisins production associated with primary and secondary metabolism and \( \mu \) is the specific growth rate. The specific rate of product formation is proportional to the total biomass and the rate of product formation:

\[ q_p = \alpha \mu + \beta \quad \text{(2.2)} \]

The rate of product formation for a growth-associated product is related to the initial biomass \( (X_0) \) and the specific growth rate \( (\mu) \) and the time \( (t) \). Thus, combining equations (2.1) and (2.2) results in

\[ \frac{dp}{dt} = (\alpha + \beta)X \quad \text{(2.3)} \]

and

\[ \frac{dp}{dt} = X_0e^{\mu t} \quad \text{(2.4)} \]

The rate of microbial growth is characterized by the specific growth rate, defined as

\[ \mu = \frac{1}{X} \frac{dx}{dt} \quad \text{(2.5)} \]

After integration form from \( t = 0 \) to \( t \) (days) and \( X(0) = X_0 \)

\[ X = X_0e^{\mu t} \quad X_0 = Xe^{-\mu t} \quad \text{(2.6)} \]

Substituting equation (2.6) and (2.3) we obtain:

\[ \frac{dp}{dt} = (\alpha + \beta)X_0e^{\mu t} \quad \text{(2.7)} \]

A temperature-dependent rate coefficient for growth represented by Arrhenius’s empirical equation is given by

\[ \mu = e^{-E_a/RT} \quad \text{(2.8)} \]

\( E_a \) is the activation energy and \( R \) is the universal constant of the gases \( (8.31 \times 10^{-3} \text{ J mol}^{-1} \text{K}^{-1}) \) and \( T \) is the absolute temperature (K). If we assume that the rate of production is affected directly by fungal growth rate and activation energy, we obtain

\[ \frac{dp}{dt} = (\alpha + \beta)X_0e^{\mu t} \cdot e^{-E_a/RT} \quad \text{(2.9)} \]

Based on previous experiments (data not shown), it was observed that the activation energy could be adjusted as a quadratic function:

\[ E_a = b_1a_n \quad \text{(2.10)} \]

Thus,

\[ \frac{dp}{dt} = (\alpha + \beta)X_0e^{\mu t} e^{-b_{1a_n}/RT} \quad \text{(2.11)} \]

and

\[ \int_0^t \frac{dp}{dt} = (\alpha + \beta)X_0e^{-b_{1a_n}/RT} \int_0^t e^{\mu t} dt \quad \text{(2.12)} \]

After integration:

\[ P = \left( \alpha + \beta \right)X_0e^{-b_{1a_n}/RT} (e^{\mu t} - 1) \quad \text{(2.13)} \]

2.7. Generation of ternary contour surfaces of interactions between gene expression, environmental factors and fumonisins production

In order to plot the ternary contour surfaces of the genetic expression, a standardized signal from the microarray was calculated as follows for \( a_n \) and for temperature to relate the relative abundance of mRNA of the FB cluster genes (e.g. *FUM11*, *FUM13*, *FUM19*) to the regulatory gene (*FUM1*):

\[ f(a_n) = \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \]

\[ f(\text{temperature}) = \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \]

\[ f(\text{temperature}) = \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \]

\[ f(\text{temperature}) = \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \]

\[ f(\text{temperature}) = \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \]

\[ f(\text{temperature}) = \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \left( \begin{array}{c} \text{FUM1} \\ \text{FUM11} \\ \text{FUM13} \end{array} \right) \]
where the standardized value = actual value − minimum value/maximum value−minimum value. For FB1 and FB2 production the following equation was used:

\[
f(f\text{umonisin B1 or B2 (ppm)}) = \frac{FUM1}{(FUM1 + FUM11 + FUM13)} \times \frac{FUM11}{(FUM1 + FUM11 + FUM13)} \times \frac{FUM13}{(FUM1 + FUM11 + FUM13)}.
\]

3. Results

3.1. Effect of environmental factors on growth and fumonisin B1 and B2 production by the strain of *F. verticillioides*

Figure 1 shows the effect of interacting conditions of \(a_w\) and temperature on growth of the *F. verticillioides* strain used in this study. This shows that optimum was at 0.995 \(a_w\) and 20–25°C but this changed to 0.98 \(a_w\) at 30–35°C. At 0.93 \(a_w\) there was some growth but only at 25–30°C and this was less than 20 per cent of that under optimum conditions.

Figure 2 shows the impact of \(a_w\) × temperature effects on the relative expression of the nine genes used in this study. This shows that under some conditions there was an increased expression of certain genes indicative of the effect of drought × temperature stress.

Figures 3 and 4 show the effects of (i) \(a_w\) and (ii) temperature on the production of FB1 and FB2 respectively. The optimal temperature and \(a_w\) for FB1 production were at 20°C and 0.98–0.995, respectively, in contrast to that for growth (30°C and 0.98 \(a_w\)). For FB2 production the optimum \(a_w\) was 0.98, while the optimum temperature for production was broader than that for growth (20–30°C). This suggests marked differences in the growth optimum and in the range of \(a_w\) × temperature conditions when compared with that for FB1 and FB2 production by *F. verticillioides*. These data were used in conjunction with the relative gene expression of nine genes involved in the biosynthesis of fumonisins for modelling their relationship.

3.2. Modelling the relationship between environmental factors, gene expression and fumonisin B1 and B2 production

For assessing the relationship between physiological and thermodynamic conditions, FB1 and FB2 production and the expression of the gene clusters involved in toxin production, the physical model previously described in §2.6 was combined with the gene expression data as a linear combination. The generic cluster was

\[
[g] = a_1FUM1 + a_2FUM7 + a_3FUM10 + a_4FUM11 + a_5FUM12 + a_6FUM13 + a_7FUM14 + a_8FUM16 + a_9FUM19,
\]

where \(a_1\) to \(a_9\) are the parameter estimates from the linear combination of the expression of genes so that \([g]\) represents the sum of the effect of the individual genes expressed under specific conditions. The final model which considers

\[
a_w, \text{ temperature, growth rate and gene expression on the regulation of FB1 and FB2 is given by}
\]

\[
P = [g] \times \left(\alpha + \beta \frac{X_0}{\ln(m_1)} - \frac{1}{RT}\right),
\]

where \(P\) is the FB1 or FB2 production (ppm) and \(b_1, b_2, \alpha\) and \(\beta\) are parameter estimates from the model and \(\mu\) was calculated based on a period of 10 days growth and the assumption that growth occurs in cylindrical fungal hyphal extension with a constant radius simplified as

\[
\mu = \ln\left(\frac{X_t}{X_0} = \ln\left(\frac{m_1}{m_i}\right) = \ln\left(\frac{v_i}{v_i/m_i}\right)\right)
\]

\[
= \ln\left(\frac{\pi \times r_i^2 \times L_i}{\pi \times r_i^2 \times L_i}\right) = \ln\left(\frac{L_i}{L_i}\right).
\]

\(X_t\) and \(X_0\) are final and initial biomass, \(m_t\) and \(m_i\) are initial and final fungal mass, \(v_i\) and \(v_i\) are the initial and final fungal road volume, \(\rho\) is the fungal density, \(r_i\) is the *F. verticillioides* hyphal radius and \(L_i\) and \(L_i\) are the radial growth.

Table 1 shows the actual mean data (\(n = 3\)) for FB1 and FB2 production and that predicted by this model in relation to different combinations of temperature and \(a_w\) and the relative mean growth rate.

Table 2 shows the ANOVA for the fit of the model and the regressed coefficients and the corrected totals for FB1 and FB2. This suggests that the model showed a good fit to the data and was statistically significant. Table 3 shows the overall estimates and the dependence for the main areas that are related to the model. All the parameters are statistically significant at \(p < 0.01\).

Figure 5a,b shows the model fit for the observed versus predicted effects on FB1 and FB2, respectively. The fitted line gave a good correlation between these parameters (FB1, \(r^2 = 0.9736\); FB2, \(r^2 = 0.9530\)).

3.3. Validation of the model

The model was subsequently tested to examine whether it could be used at temperatures of 35°C at different \(a_w\) levels. These conditions were not originally included in the model because of the limited data at these conditions. Table 4 shows the effect on the observed and predicted FB1 and FB2 production under these conditions. At 35°C and 0.98 \(a_w\) the
model predicted much higher FB1 production than was actually observed, whereas the FB2 model-predicted production was slightly lower than observed in the same conditions of \( a_w \) and temperature. In contrast, the FB2 produced was similar to the predicted value at 35°C and 0.995 \( a_w \).

3.4. Ternary relationships between gene expression, environmental factors and FB1 and FB2 production

By using a standardized signal from the microarray dataset, it was possible to examine the relative relationship between the activity of the important FUM1 gene in the biosynthetic pathway and related cluster genes in the pathway (FUM7, FUM10, FUM11, FUM12, FUM13, FUM14, FUM16 and FUM19) at the same time in ternary diagrams in relation to \( a_w \), temperature and FB1 and FB2 production. Only the expression of FUM1, FUM11, FUM13, FUM14 and FUM19 showed a significant effect on the synthesis of both fumonisins, while the other genes exhibited very little response or the effects were variable (data deposited in Cranfield Health share point facility; Applied Mycology).

Figure 6 shows the effect of FUM1, FUM11 and FUM13 relative gene expression on FB1 (i) and FB2 (ii) production, respectively. These were calculated using the model below:

\[
\text{FB1 production (\(\mu g \text{ml}^{-1}\))} = 10.7 \left( \frac{\text{FUM1}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} \right) - 28.28 \left( \frac{\text{FUM11}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} \right) + 32.9 \left( \frac{\text{FUM13}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} \right)
\]

(3.4)

and

\[
\text{FB2 production (\(\mu g \text{ml}^{-1}\))} = -0.67 \left( \frac{\text{FUM1}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} \right) - 11.0 \left( \frac{\text{FUM11}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} \right) + 15.89 \left( \frac{\text{FUM13}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} \right)
\]

(3.5)

The production of FB1 proportionally increased to FUM11 gene transcription but not FUM13. Notably, FUM1 was down-regulated during FB1 overexpression.

For FB2 production, the relative expression of FUM11 and FUM13 was inversely related to this parameter. As the FUM11 and FUM13 expression increased, the production of FB2 was reduced. FUM1 expression was weakly correlated to the production of fumonisin B2.

Similar ternary diagrams for the interaction between FUM1 and the FUM cluster genes FUM19 and FUM14 are presented in figure 7. The relative expression of the three genes in relation to FB1 and FB2 production was calculated based on

\[
\text{FB1 production (\(\mu g \text{ml}^{-1}\))} = 8.7 \left( \frac{\text{FUM1}}{(\text{FUM1} + \text{FUM19} + \text{FUM14})} \right) + 0.8 \left( \frac{\text{FUM19}}{(\text{FUM1} + \text{FUM19} + \text{FUM14})} \right) + 19.7 \left( \frac{\text{FUM14}}{(\text{FUM1} + \text{FUM19} + \text{FUM14})} \right)
\]

(3.6)
**Table 1.** Experimental data and model estimation for FB1 and FB2 at different temperatures, water activity and fungal growth rate of the strain of *Fusarium verticillioides*.

<table>
<thead>
<tr>
<th>temp. (°C)</th>
<th>water activity</th>
<th>$\mu \pm$ s.d. (mm d$^{-1}$)</th>
<th>FB1 $\pm$ s.d. ($\mu$g ml$^{-1}$)</th>
<th>FB2 $\pm$ s.d. ($\mu$g ml$^{-1}$)</th>
<th>model estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FB1 $\pm$ s.d. ($\mu$g ml$^{-1}$)</td>
<td>FB2 $\pm$ s.d. ($\mu$g ml$^{-1}$)</td>
<td>FB1 $\pm$ s.d. ($\mu$g ml$^{-1}$)</td>
</tr>
<tr>
<td>20</td>
<td>0.95</td>
<td>3.52 $\pm$ 0.03</td>
<td>9.27 $\pm$ 0.62</td>
<td>1.67 $\pm$ 0.17</td>
<td>9.32 $\pm$ 0.08</td>
</tr>
<tr>
<td>20</td>
<td>0.98</td>
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<td>13.49 $\pm$ 5.69</td>
<td>4.31 $\pm$ 0.27</td>
<td>13.59 $\pm$ 0.89</td>
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<td>20</td>
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<td>0.00 $\pm$ 0.00</td>
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</tr>
<tr>
<td>25</td>
<td>0.93</td>
<td>1.70 $\pm$ 0.08</td>
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<td>11.65 $\pm$ 2.46</td>
<td>6.96 $\pm$ 1.96</td>
<td>11.58 $\pm$ 0.21</td>
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<tr>
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<td>2.46 $\pm$ 1.83</td>
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</tr>
<tr>
<td>30</td>
<td>0.95</td>
<td>6.21 $\pm$ 0.32</td>
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</tr>
<tr>
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<td>3.56 $\pm$ 0.99</td>
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<td>3.59 $\pm$ 0.09</td>
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</tbody>
</table>

Figure 3. The effect of (a) water activity ($a_w$) and (b) temperature on the fumonisin B$_1$ production by a strain of *F. verticillioides*. Bars indicate standard error of the means.

Figure 4. The effect of (a) water activity ($a_w$) and (b) temperature on the fumonisin B$_2$ production by a strain of *F. verticillioides*. Bars indicate standard error of the means.
increased, the production of FB1 was reduced and that of FUM1, a observed under dried conditions (0.94 related to gene. In contrast, this fumonisin production was inversely increased with higher relative expression of the dized) of the genes (i)

Table 2. Statistical analysis of the ANOVA for the fit of the model and the regressed coefficients and the corrected totals for FB1 and FB2 by F. verticillioides.

<table>
<thead>
<tr>
<th></th>
<th>sum of squares</th>
<th>d.f.</th>
<th>mean squares</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td>ANOVA: FB1</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>regression</td>
<td>1819.656</td>
<td>13</td>
<td>139.9735</td>
<td>120.35</td>
<td>0</td>
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<tr>
<td>residual</td>
<td>23.260</td>
<td>20</td>
<td>1.1630</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>1842.916</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>corrected total</td>
<td>847.539</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>regression versus corrected total</td>
<td>1819.656</td>
<td>13</td>
<td>139.9735</td>
<td>5.2849</td>
<td>0.000061</td>
</tr>
<tr>
<td>ANOVA: FB2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>regression</td>
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<td>13</td>
<td>15.58323</td>
<td>49.97915</td>
<td>0</td>
</tr>
<tr>
<td>residual</td>
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<td>20</td>
<td>0.31179</td>
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</tr>
<tr>
<td>total</td>
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<td></td>
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<td></td>
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<tr>
<td>corrected total</td>
<td>132.6695</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>regression versus corrected total</td>
<td>202.5820</td>
<td>13</td>
<td>15.58323</td>
<td>3.75869</td>
<td>0.001121</td>
</tr>
</tbody>
</table>

and

\[
\text{FB}_2 \text{ production (µg ml}^{-1}
\]

\[
= -2.98 \frac{\text{FUM1}}{(\text{FUM1} + \text{FUM19} + \text{FUM14})} + 1.83 \frac{\text{FUM19}}{(\text{FUM1} + \text{FUM19} + \text{FUM14})} + 5.92 \frac{\text{FUM14}}{(\text{FUM1} + \text{FUM19} + \text{FUM14})}
\]  

(3.7)

The production of both fumonisins FB1 and FB2 was increased with higher relative expression of the FUM19 gene. In contrast, this fumonisin production was inversely related to FUM14 signal. As the FUM1 gene expression was increased, the production of FB1 was reduced and that of FB2 increased.

The effect of \( a_w \) on the relative gene expression (standardized) of the genes (i) FUM1, FUM11 and FUM13 and (ii) FUM1, FUM14 and FUM19 was calculated using the relative fractions \((f)\) of these individual genes (figure 8). For \( a_w \), this was

\[
a_w = 0.99 \frac{\text{FUM1}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} + 1.09 \frac{\text{FUM11}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} + 0.79 \frac{\text{FUM13}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})}
\]  

(3.8)

and

\[
a_w = 0.93 \frac{\text{FUM1}}{(\text{FUM1} + \text{FUM14} + \text{FUM19})} + 1.07 \frac{\text{FUM14}}{(\text{FUM1} + \text{FUM14} + \text{FUM19})} + 0.88 \frac{\text{FUM19}}{(\text{FUM1} + \text{FUM14} + \text{FUM19})}
\]  

(3.9)

As the \( a_w \) increased, the expression of the genes FUM11 and FUM14 was reduced. The higher expression of FUM1 was observed under dried conditions (0.94 \( a_w \)), and FUM13 signal decreased at these \( a_w \) levels. The transcription of FUM19 was less affected by water availability.

Figure 9 shows the relative expression of FUM1 and the biosynthetic genes FUM11, FUM13, and FUM14, FUM19 in relation to temperature. These relationships were calculated as shown below:

\[
T(\text{°C}) = 19.3 \frac{\text{FUM1}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} + 96.07 \frac{\text{FUM11}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})} - 37.7 \frac{\text{FUM13}}{(\text{FUM1} + \text{FUM11} + \text{FUM13})}
\]  

(3.10)

and

\[
T(\text{°C}) = 17.0 \frac{\text{FUM1}}{(\text{FUM1} + \text{FUM14} + \text{FUM19})} + 28.0 \frac{\text{FUM14}}{(\text{FUM1} + \text{FUM14} + \text{FUM19})} + 31.7 \frac{\text{FUM19}}{(\text{FUM1} + \text{FUM14} + \text{FUM19})}
\]  

(3.11)

There was an inverse proportional effect of temperature on the expression of FUM11 and FUM19. Thus, the higher the temperature, the lower FUM11 and FUM19 gene signal. The FUM1 gene transcription was proportionally increased with temperature. The expression of FUM13 and FUM14 was similar under different temperature conditions.

4. Discussion

This study has examined the impact that environmental factors can have on FUM cluster genes and the effects that they have on FB1 and FB2 production by a strain of F. verticillioides. This has shown that temperature and \( a_w \) have a profound effect on both gene expression of key biosynthetic genes as well as significantly affecting growth and the phenotypic production of the toxic secondary metabolites.

The main factors influencing the growth of the strain of F. verticillioides included environmental temperature and water availability of the substrate [10,24]. Previous studies have shown that fungal growth occurs within a wide range of temperatures, with the optimum ranging from 22.5 to 27.5 °C and a minimum water availability of 0.87 [25,26].
Table 3. The estimates and the dependence on the main factors that are related to the model for (a) FB1 and (b) FB2 production by F. verticilloides.

<table>
<thead>
<tr>
<th>parameter</th>
<th>estimate</th>
</tr>
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<tbody>
<tr>
<td>gene cluster for FB1 biosynthesis</td>
<td>6.1055</td>
</tr>
<tr>
<td>a1 (FUM19)</td>
<td>2.5770</td>
</tr>
<tr>
<td>a2 (FUM14)</td>
<td>-4.0061</td>
</tr>
<tr>
<td>a3 (FUM11)</td>
<td>-5.5225</td>
</tr>
<tr>
<td>a4 (FUM13)</td>
<td>-8.1792</td>
</tr>
<tr>
<td>a5 (FUM1)</td>
<td>-6.7091</td>
</tr>
<tr>
<td>a6 (FUM16)</td>
<td>1.5228</td>
</tr>
<tr>
<td>a7 (FUM12)</td>
<td>5.8662</td>
</tr>
<tr>
<td>a8 (FUM10)</td>
<td>6.2729</td>
</tr>
<tr>
<td>a9 (FUM7)</td>
<td>1.4678</td>
</tr>
<tr>
<td>mixed-growth-associated product formation</td>
<td>2.3613</td>
</tr>
<tr>
<td>α</td>
<td>6.8545</td>
</tr>
<tr>
<td>β</td>
<td>4.4922</td>
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<tr>
<td>Arhenius’s coefficient</td>
<td>-8.8764</td>
</tr>
</tbody>
</table>

Our data indicate that 20–25°C and 0.995 a_w were the optimum conditions for growth of the strain used in this study. For FB1 and FB2 production, the optimum conditions were 0.98–0.995 a_w and 20°C or 20–30°C, respectively. The optimum temperature and a_w previously reported for inducing fumonisin production ranged from 20 to 25°C and 0.95–0.99, while no production observed at ≤0.93 a_w and 10°C [10,27]. Similar results were shown by Samapundo et al. [12], although with lowered a_w values and a temperature range of 15–25°C. For a F. verticilloides type strain (NRRL 3248), toxin production was higher when it was grown with 50 per cent water content and 21°C [28]. There are strain differences in terms of both FUM1 gene expression and fumonisin production. Previous studies with different strains of both F. verticilloides and F. proliferatum showed that relative expression on glycerol-modified water stress media resulted in relative similar effects on FUM1 gene expression by different strains. However, with ionic solutes such as NaCl this was not so, perhaps owing to toxicity of the ionic solute at higher concentrations [13,14].

The FB biosynthetic gene cluster consists of 17 transcriptional co-regulated genes, nine of which encode enzymes that catalyse reactions of this pathway [29]. The function of the others is not known yet. Few studies have investigated the relationship between the fumonisin production, FUM gene expression and ecological factors [13,14,30–32]. Here, we developed ternary diagrams to examine the link between the FUM1 key gene and eight other pathway genes (FUM7, FUM10, FUM11, FUM12, FUM13, FUM14 and FUM19) in relation to environmental conditions and fumonisin synthesis. This has shown that the relative expression of FUM1 to that of FUM11, FUM13, FUM14 and FUM19 are related and influenced by a_w and temperature for both FB1 and FB2 production. Jurado et al. [13] described that significant and prolonged increase in water stress (e.g. 0.93 a_w) enhanced FUM1 expression, while a mild water stress (0.98–0.95 a_w) did not significantly affect gene transcripts. According to Marin et al. [14], FUM1 transcription was enhanced with an increase in non-ionic water stress and the peak of expression occurred at 20°C.

Our results also showed the correlation of FUM1, FUM11, FUM13, FUM14 and FUM19 expression with FB1 and FB2 production under different conditions. The synthesis of these fumonisins was reduced at higher expression levels of
while an increased production of both fumonisins. FUM11 modulated positively or negatively the production of FB1 and FB2, respectively. Previously, López-Errasquín et al. [32] 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. Lazzaro et al. [30] identified a
positive correlation between fumonisin synthesis and expression of two FUM genes, FUM2 and FUM21, at different $a_w$ and temperature regimes. In the present study effects on FUM21, considered a regulatory gene of fumonisin biosynthesis [33], was not included because the role of this gene was not elucidated when the microarray was developed. However, effects on the FLU1 gene may provide an insight into the way FUM21 may respond. This variable correlation of gene expression and production of the toxin may not be that unexpected, because usually the expression of only a few genes (key genes) is directly correlated with the toxin biosynthesis. Scherm et al. [34], for example, showed this aspect for aflatoxin biosynthetic genes. Taken together, our findings further support the use of ternary diagrams as a reliable approach to investigate the relationship between different key structural and regulatory genes and their impact on the production of secondary metabolites such as aflatoxins [18,35] and fumonisins (the present study).

Besides water availability, several factors can affect fumonisin production including nitrogen limitation and pH of 5.9 [36]. Kohut et al. [31] reported that FUM1 and FUM8 expression, together with fumonisin production, was induced by nitrogen starvation in F. proliferatum cultures. However, the presence of a mycotoxin gene, for example FUM11, does not completely confirm that the pathogen has the potential to produce fumonisin [37].

It is necessary to also consider this study in the context of what might happen when biotic variables are included. Thus, the interaction with ripening maize kernels, especially where plant physiological and nutritional effects may be important, also needs to be considered [38]. Indeed they suggest that the rate of drying of the maturing maize kernels critically affects the contamination with fumonisins. Thus, at the very early dough stage the moisture content (m.c.) is about 40 per cent $(=0.99 a_w)$ with no water stress effects, this decreases to 30–35% m.c. at the mid-dough stage $(= 0.95 a_w)$ and to 20–25% $(0.90–0.85 a_w)$ at full maturity over a period of about 4–6 weeks [38,39]. This shows that the window of opportunity for colonization by F. verticillioides and fumonisin contamination is limited to more than 0.93 $a_w$. Thus, the range of conditions we have chosen in relation to $a_w \times$ temperature stress interactions is in the relevant range. However, the nutritional quality of maize changes during ripening and this may further influence the capacity for biosynthesis of fumonisins. This may also influence the interaction with other mycobiota including A. flavus, which...
colonizes the ripening maize cobs over a wider aw range than *Fusarium* section *Liseola* during these critical phases of plant development.

Very few attempts have been made to try and integrate molecular expression data under different environmental stresses with phenotypic secondary metabolite data to develop predictive models. Recently, Abdel-Hadi et al. [18] demonstrated that for *A. flavus* and aflatoxin production it was possible to use the mixed growth model for secondary metabolite production and link this to a linear model of the gene expression data of up to 10 key genes for predicting aflatoxin production under different interacting environmental conditions. The present study has used this mixed growth model to try and relate the relative expression of nine biosynthetic genes under different environmental conditions to growth and production of FB1 and FB2 for a strain of *F. verticillioides*. This made it possible to develop a predictive model which provided a good linear regression fit between the predicted and observed fumonisin production. This model is important for developing an integrated systems approach by combining gene expression, ecophysiological influences and growth data to predict fumonisin production. This could further help in developing a more targeted approach to design prevention strategies to control fumonisin biosynthesis in staple food commodities. For example, use of RNAi systems to interfere and reduce the function of the FUM1 or FUM21 gene may be an approach for the minimization of fumonisins in cereals [20]. This approach could be a powerful tool in examining the impact of climate change factors on toxin production [39].

**References**


