FUM and BIK gene expression contribute to describe fumonisin and bikaverin synthesis in Fusarium verticillioides

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**FUM and BIK gene expression contribute to describe fumonisin and bikaverin synthesis in *Fusarium verticillioides***

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**Abstract**

*Fusarium verticillioides* is a maize pathogen that produces toxic secondary metabolites, including fumonisins and bikaverin. The regulation of biosynthetic gene expression and the production of these metabolites are not fully understood and in this study we investigated the influence of water activity (0.955 and 0.990) on the expression of 5 genes (*FUM3–FUM6–FUM13–FUM14 and BIK1*) in *F. verticillioides* strains after 14 and 21 days incubation. Fumonisin production and biosynthetic gene expression were greatest at aw = 0.990, and the same trend was observed for bikaverin production, and BIK1 expression. *FUM3* and *FUM14* were the most highly expressed genes and were positively correlated with the production of FB1, FB2 and FB3. When *FUM14* is more highly expressed than *FUM3* the amount of FB1 quantified is higher with respect to FB3; this could be explained by the role of *FUM3* in the hydroxylation of FB3 to FB1.

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1. Introduction

*Fusarium verticillioides* (Sacc.) Nirenberg (teleomorph Gibberella moniliformis Wineland) is a predominant species isolated from maize grown in Italy (Logrieco et al., 2003). It is known to cause pink ear rot and produce toxic secondary metabolites, mainly fumonisins and bikaverin. Fumonisins are mycotoxins harmful to animal and human health (Marasas, 2001); in particular, fumonisin B1 (FB1) is classified as a potential carcinogen to humans (Group 2B) (IARC, 1993). Bikaverin is a reddish pigment with antibiotic activity against fungi (Son et al., 2008) and antitumor action (Zhan et al., 2007).

During the last decade, the biosynthetic pathway leading to formation of fumonisin has been described and a cluster of biosynthetic genes (*FUMs*) has been characterized in *F. verticillioides*. The first step in the pathway is catalyzed by the *FUM1*-encoded polyketide synthase and results in the formation of a linear polyketide with two methyl groups and a terminal carboxyl group (Proctor et al., 1999). The *FUM8*-encoded α-oxoamine synthase then catalyzes condensation of the polyketide and alanine to form the 20-carbon-long fumonisin backbone (Proctor et al., 2008). The *FUM6*-encoded cytochrome P450 monooxygenase is postulated to catalyze hydroxylation at carbon atoms 14 (C-14) and C-15 (Seo et al., 2001). The *FUM13*-encoded dehydrogenase catalyzes the reduction of the C-3 carbonyl to a hydroxyl (Butchko et al., 2003b). The *FUM2*-encoded cytochrome P450 monooxygenase then catalyzes the hydroxylation of C-10 (Proctor et al., 2006). The *FUM14*-encoded protein then catalyzes the esterification of trycarballylic acids to the C-14 and C-15 hydroxyls (Zaleta-Rivera et al., 2006). Lastly the *FUM3*-encoded dioxygenase catalyzes the hydroxylation of C-5, leading to fumonisin FB1 and FB2 production (Butchko et al., 2003a).

Fumonisins are produced optimally between 20 and 30 °C and water activity (aw) between 0.955 and 0.990 and ceases below 10 °C and at aw lower than 0.930 (Marin et al., 1999). *FUM1* and *FUM19* transcripts were observed from *F. verticillioides* cultures incubated at 20 °C for 14 days and found to be linearly correlated to fumonisin production (López-Erasquín et al., 2007). These findings have been confirmed by de Oliveira Rocha et al. (2011) in *F. verticillioides* 7 day-old cultures grown at 25 °C.

*FUM1* expression was also used to assay the effects of osmotic and matrix potential in *F. verticillioides*. *FUM1* expression was highest at 12 days during water stress (i.e. aw = 0.930) whereas no significant variations were observed under mild water stress (aw = 0.950–0.980) (Jurado et al., 2008). Marin et al. (2010) confirmed that *FUM1* transcription was induced under non-ionic water stress at temperatures between 15 and 35 °C in 10 day-old *F. verticillioides* and *Fusarium proliferatum* cultures. The effect of aw on *FUM2* and *FUM21* expression in *F. verticillioides* 7, 14, and 21 day-old cultures was investigated, showing that gene expression increases at lower aw and that fumonisin production is significantly correlated (Lazzaro et al., 2012).

The *F. verticillioides* bikaverin biosynthetic gene cluster consists of six genes, *BIK1* through *BIK6* (Brown et al., 2008). *BIK1* is the main biosynthetic gene and encodes a polyketide synthase that catalyzes...
the condensation of acetyl- and malyonyl-CoA units into the bikaverin precursor (Linennmannstöns et al., 2002; Wiemann et al., 2009). Giordano et al. (1999) demonstrated that high nitrogen concentrations and pH > 5 can inhibit bikaverin production while a low carbon to nitrogen ratio and pH < 5 induces bikaverin production. In Fusarium fujikuroi, BIK1 expression is repressed at pH ≥ 5 and by high nitrogen concentrations, as well as all other BIK genes (Linennmannstöns et al., 2002; Wiemann et al., 2009). In F. verticillioides, the same conditions are conducive for BIK gene cluster expression and bikaverin production (Brown et al., 2008).

The role of environmental conditions on fumonisins and bikaverin production is complex and a better understanding of the mechanisms that regulate gene expression and secondary metabolite production could help clarify the roles of these compounds in nature. Therefore, the aim of this study was to investigate the influence of aw over time on the expression of four FUM genes (FUM3, FUM8, FUM13 and FUM14) as well as one BIK gene (BIK1) in two different F. verticillioides strains.

2. Materials and methods

2.1. Fungal isolates, media and inoculum preparation

Fungal strains used in this study are two fumonisin-producing F. verticillioides, ITEM 10027 (MPVF 294) and ITEM 1744, isolated from maize in Tuscany and Sardinia (Italy), respectively. Strains are preserved in 18% glycerol at −80 °C in the fungal collection of ISPA-CNR, Bari (code ITEM) and at the Institute of Entomology and Plant Pathology-UCSC, Piacenza (code MPVP); isolates were cultured on V8 juice (Campbell Soup Company, Camden, NJ) agar at 25 °C for 7 days under UVA light (12 h light/12 h dark). Conidia were harvested from plates in 5 ml of sterile water and 1 ml was used to inoculate liquid media to a final concentration of 106 conidia/ml. Fumonisin-inducing Malt Extract (ME) and fumonisin-inhibiting Czapek Yeast Autolysate (CYA) were utilized as liquid media (100 ml) for F. verticillioides cultures (Frisvad et al., 2007). The aw of ME and CYA were adjusted to 0.955 and 0.990 by adding appropriate amounts of glycerol (Giorni et al., 2011). Cultures were incubated in the dark at 25 °C for 14 and 21 days in static conditions. Three biological replicates were done for each condition (strain, liquid medium, aw, incubation time) studied. The mycelium was weighed and used for RNA isolation and gene expression analysis, while the liquid medium from culture filtration was used for metabolite analysis.

2.2. RNA isolation and gene expression data analysis

The mycelium was collected by filtration over vacuum on Miracloth (Calbiochem, Germany), frozen in liquid nitrogen, lyophilized overnight and stored at −80 °C. Total RNA was purified from lyophilized mycelium with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufactures protocol with the addition of the DNase digestion step and stored at −80 °C. The amount and quality of total RNA was estimated by Nanodrop (Thermo Scientific, Wilmington, DE) and via Bioanalyzer (Agilent, Santa Clara, CA). Total RNA quality control was performed using the RIN (RNA integrity number) (Schroeder et al., 2006).

First strand cDNA was synthesized using 1 μg of total RNA, following the iScript cDNA Synthesis kit protocol (Bio-Rad, Hercules, CA) and was stored at −20 °C. Primer pairs specific to target genes were designed using Primer3 Plus (Undergasser et al., 2007) and DNAMAN 7.0 (Lynnorn Corporation, Quebec), on exon–exon junctions in the target mRNA to prevent amplification of genonic DNA template. Primer pairs for FUM3, FUM8, FUM13, FUM14, and BIK1 and for the house-keeping gene β-tubulin (BT) are listed in Table 1. Primer-gene specificity was tested with F. verticillioides cDNA by

PCR using an amplification reaction of 20 μl total volume: 10 μl Go Taq Master Mix (Promega, Madison, WI), 1 μl cDNA (<250 ng), 2 μl each primer (10 μM) and 5 μl nuclease free water.

Gene expression profiles were determined through quantitative real-time PCR using an MJ Research PTC-200 fitted with a Chromo4 Real-Time PCR detector (Bio-Rad, Hercules, CA). Single strand cDNA (20 ng) was amplified using the following protocol: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Melting curve analysis (60 °C to 95 °C) confirmed primer pairs specificity. Each sample (biological replicate) was analyzed in triplicate (technical replicate) together with a template-free negative control in each analysis of both target and house-keeping genes. The amplification reaction contained 12.5 μl 2X iQ STBR Green Supermix (Bio-Rad, Hercules, CA), 3 μl of each primer (10 μM) and 40 ng of template cDNA and RNase free water to a final volume of 25 μl.

Data were analyzed using Option Monitor Version 3.1.34 software (Bio-Rad, Hercules, CA). Relative quantification of gene expression was calculated using the 2−ΔΔCT method (Applied Biosystems User Bulletin #2 ABI PRISM 7700 Sequence Detection System). The method assumes that when comparing multiple samples using relative quantification, the untreated/control sample is chosen as calibrator (Fusarium cultures on CYA in this study), and the corresponding treated sample is chosen as test (Fusarium cultures on ME in this study). The expression of the target gene correspond to the fold increase (or decrease) of the gene in the test sample compared to the calibrator sample. All data were normalized to β-tubulin as internal control (Real-Time PCR Application Guide, Bio-Rad, Hercules CA). ANOVA was performed on all gene expression values and fumonisin and bikaverin toxin levels, transforming values by y = ln before analysis by PASW statistic 18 (IBM, Armonk, NY).

2.3. Fumonisin and bikaverin analysis

Liquid medium from fungal cultures was filtered through 0.2 μm acetate filters (Thermo Fisher Scientific, Rochester, NY) and stored at 4 °C. All LC-MS and LC-MS/MS experiments were conducted utilizing a ThermoFinnigan LCQ-DECA (Thermo Scientific, San Jose, CA) ion trap mass spectrometer equipped with an electrospary ionization source, coupled to a ThermoSpectraPhysics high performance liquid chromatography system consisting of an AS4000 autosampler coupled to a P2000 gradient pump. Operation of the chromatography and mass spectrometry instrument and quantitation of the eluting fumonisins and bikaverin were done utilizing ThermoFinnigan Xcalibur software. Elution of analytes was achieved with a gradient

Table 1

<table>
<thead>
<tr>
<th>Primers pair</th>
<th>Gene</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
<th>GenBank ID</th>
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<tr>
<td>TUB2 F</td>
<td>β-tubulin</td>
<td>5'-GGTTCAGTGGCCCGAATACAAAT-3'</td>
<td>107</td>
<td>AF155771.5</td>
</tr>
<tr>
<td>TUB2 R</td>
<td>β-tubulin</td>
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<td>137</td>
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<td>115</td>
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</tr>
<tr>
<td>FUM3 R</td>
<td>FUM3</td>
<td>5'-GGCTATTTGGTCCCCTCAGA-3'</td>
<td>116</td>
<td>AF155771.5</td>
</tr>
<tr>
<td>FUM8 F</td>
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<tr>
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<td>FUM13</td>
<td>5'-CCCTGCAAGACTGTCAGGACCA-3'</td>
<td>116</td>
<td>AF155771.5</td>
</tr>
<tr>
<td>FUM14 F</td>
<td>FUM14</td>
<td>5'-GGTTCAGTGGCCCGAATACAAAT-3'</td>
<td>107</td>
<td>AF155771.5</td>
</tr>
<tr>
<td>FUM14 R</td>
<td>FUM14</td>
<td>5'-GACGACCATTCTTCCTCAGA-3'</td>
<td>137</td>
<td>AF155771.5</td>
</tr>
<tr>
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<td>114</td>
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</tr>
<tr>
<td>BIK1 R</td>
<td>BIK1</td>
<td>5'-GACGACCATTCTTCCTCAGA-3'</td>
<td>114</td>
<td>AF155771.5</td>
</tr>
</tbody>
</table>

F = forward, R = reverse.
flow of methanol and water (0.3% acetic acid was added to the mobile phase). The solvent program used a 35–95% methanol gradient over 25 min. The entire chromatography column flow was directed to the ion source of the mass spectrometer. Operation of the mass spectrometer was in the positive mode. Fumonisin determinations were made in LC-MS mode, monitoring the detection of m/z 722 [M + H]+ ions for FB1 and m/z 706 [M + H]+ ions for FB2 and FB3 (Plattner et al., 1996). Multiple reaction monitoring MS/MS experiments were used for the detection of bikaverin. To achieve effective fragmentation of the bikaverin [M + H]+ ions (m/z 383), a normalized collision energy of 50% was used. The monitoring of the 355 m/z fragment ion was used as the basis for quantitation of bikaverin while integrations of LC-MS/MS response were recorded for the m/z 337, 340, 355 and 365 distinctive fragment ions. Quantitations were made by comparison to calibration standards of the fumonisins (Plattner et al., 1996) and bikaverin (Vesonder et al., 1989).

3. Results

3.1. RNA isolation

All RNA samples had a very good quality, with a RIN ranging from 8 to 10 (RIN values range from 10 = intact to 1 = totally degraded).

3.2. Effect of water activity on metabolite production and gene expression

Fumonisins were produced by both F. verticillioides strains in all conditions in ME media, while it was absent in CYA cultures. ITEM 10027 produced four times as much total fumonisins as ITEM 1744 (43.83 versus 10.17 mg/kg; average total FBs respectively; Fig. 1). FB1 was the predominant fumonisin in both strains. Fumonisin ratios and 0.13 (FB2/FB1) and 0.09 (FB3/FB1) in cultures of ITEM 1744. Water activity of 0.955 reduced significantly fumonisin production (around 50%) compared to aw = 0.990. Fumonisin production was higher at the later time point when ITEM 10027 was cultured at aw = 0.955. In contrast, fumonisin accumulation was higher at the early time point when ITEM 10027 was cultured at aw = 0.990. Interestingly, ITEM 1744 was affected differently with regard to fumonisin production at different water activities: it accumulated more fumonisins at 14 days versus 21 days when cultured at aw = 0.955, but accumulated more fumonisins at 21 days versus 14 days when cultured at aw = 0.990 (Fig. 1), as pointed out by the significant interaction strain × aw × time. Bikaverin production was significantly (P < 0.01) higher in ITEM 10027 than in ITEM 1744 (5.11 versus 1.75 mg/kg; average bikaverin respectively). Bikaverin was significantly (P < 0.01) reduced (around 66%) at lower aw respect to aw = 0.990 (Table 2). Bikaverin production by ITEM 10027 and ITEM 1744 reflected the trend of fumonisin production.

The production of fumonisin appeared to be correlated with the mycelial growth, and tended to increase with mycelial production, especially with aw = 0.990. Also bikaverin production was positively correlated with mycelial growth, and showed in both strains an exponential increase with the increment of fungal biomass (R² = 0.854).

FUM genes and BIK1 were significantly (P < 0.01 and P < 0.05 respectively) more highly expressed in ITEM 1744 (Table 2). Fumonisin and bikaverin production were significantly (P < 0.01) higher, however, in ITEM 10027. FUM3 and FUM14 were the most highly expressed genes at all conditions considered and the ones most affected by aw variations. Their expression was lower at aw = 0.955 than at aw = 0.990 (Fig. 2). BIK1 expression was significantly reduced with low aw values compared to aw = 0.990, as FUM3 and FUM14 (Table 2). The combined effect of strain × aw × incubation time was significant for all FUM gene expression and FBs production (Table 2).

4. Discussion

Environmental conditions could affect secondary metabolite gene expression and production. In order to observe the role of aw on fumonisin metabolism in a time-course during which F. verticillioides could produce fumonisin on maize ears, we studied the effects of high (aw = 0.990) and low (aw = 0.955, commonly measured in ripe maize (Battilani et al., 2011)) water activity on the expression of BIK and FUM genes and on production of bikaverin and fumonisins, in 14 and 21 day-old cultures. F. verticillioides strains used in this study were recovered from maize and were confirmed to be fumonisin-producers (Etcheverry et al., 2009; Moretti et al., 1995). They showed different patterns of fumonisin production: ITEM 1744 had a classical pattern of FBs produced (FB1 > FB2 > FB3; de Oliveira Rocha et al., 2011; López-Erasquín et al., 2007) while ITEM 10027 presented one of the minor chemotypes: FB1 > FB2 > FB3 (Szeczki et al., 2010), in accord with our previous data (ITEM1744: 2800 FB1 > 521 FB2 > 480 FB3 μg/kg; ITEM 10027: 7720 FB1 > 2180 FB2 > 2070 FB3 μg/kg) (Lazzaro et al., 2012). Water activity significantly affected fumonisin production with aw = 0.990 supporting more production than aw = 0.955. This is in accord with Mogensen et al. (2009) who reported decreasing total fumonisin production at aw between 0.990 and 0.955, and with Marín Fig. 1. Toxin analysis. Fumonisin B1 (FB1) and bikaverin (BIK) values from strains ITEM 10027 and ITEM 1744 cultured for 14 and 21 days in triplicate at two water activities (0.955 and 0.990).
was higher at \( aw = 0.990 \) compared to 0.955. Likewise, fumonisin production in this study we found that the expression of all genes observed at different temperatures (Marín et al., 2010). In cultures grown with \( aw = 0.990 \) compared to cultures from \( aw = 0.955 \; at\ aw = 0.990, \) an increase in osmotic pressure; therefore, the High Osmolarity trend (Proctor et al., 2003).

expression and secondary metabolite production followed the same differences observed at different temperatures (Marín et al., 2010). In this study we found that the expression of all FUM genes analyzed was higher at \( aw = 0.990 \) compared to 0.955. Likewise, fumonisin production was higher at \( aw = 0.990 \) than at 0.955, underlining that gene expression and secondary metabolite production followed the same trend (Proctor et al., 2003).

FUM14 is required for FB1 and FB2 formation, while FUM3 catalyzes the hydroxylation of FB1 and FB2 to FB1 and FB2 respectively (Butchko et al., 2003a). Interestingly, the FB2/FB1 ratio was higher in cultures grown with \( aw = 0.990 \) compared to cultures from \( aw = 0.955; \) at \( aw = 0.990, FUM14 \) is more highly expressed than FUM3 and we hypothesize that the higher amount of FB2, quantified with respect to FB1, is a consequence of this difference. The last step of fumonisin biosynthesis, FUM3 mediated hydroxylation of FB3 to FB1 is less enhanced due to the lower FUM3 expression, which results in potentially less FUM3 enzyme to catalyze C-5 hydroxylation.

The addition of glycerol into the growth medium modify \( aw \) by caus- ing an increase in osmotic pressure; therefore, the High Osmolarity Glycerol (HOG) pathway should be taken into consideration. Kohut et al. (2009) found that the HOG-type MAP kinase (MAPK) influenced both the expression of FUM1 and FUM8 and FB1 production during N-starvation; moreover Adám et al. (2008) observed that Fphog1, a HOG-type MAPK gene cloned from F. proliferatum, was not involved in transcriptional regulation under salt and hyperosmolar stress conditions. A similar role of HOG-type MAPK in F. verticillioides could be hypothesized and it would be interesting to study the HOG response in F. verticillioides cultures.

We investigated the expression of BIK1 observing that it was enhanced by the same conditions useful for the FUM genes, with the expression higher at \( aw = 0.990 \) compared to 0.955, in agreement with Brown et al. (2008). Furthermore, bikaverin production was higher at \( aw = 0.990 \) compared to 0.955.

To our knowledge, this is the first report of the effects of \( aw \) on FUM3, FUM8, FUM13, FUM14 and BIK1 gene expression (2 \(-\Delta\Delta \text{Ct}\)) in 2 F. verticillioides strains grown on ME adjusted at 2 \( aw \) (0.955–0.990) and incubated for 14–21 days.

et al. (1995) that indicated an \( aw \) between 0.956 and 0.968 as supporting the production at both 25 and 30 °C. Previous studies on FUM gene expression were limited to FUM1. Jurado et al. (2008) found that FUM1 transcription was higher at \( aw = 0.98 \) compared to 0.93 at 25 °C when \( aw \) was modified with glycerol, while with an ionic stress, FUM1 expression was highest at \( aw \) between 0.93 and 0.95. More recently, it has been reported that FUM1 expression is greater at \( aw = 0.955 \) compared to 0.982, with differences observed at different temperatures (Marín et al., 2010). In this study we found that the expression of all FUM genes analyzed was higher at \( aw = 0.990 \) compared to 0.955. Likewise, fumonisin production was higher at \( aw = 0.990 \) than at 0.955, underlining that gene expression and secondary metabolite production followed the same trend (Proctor et al., 2003).

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We investigated the expression of BIK1 observing that it was enhanced by the same conditions useful for the FUM genes, with the expression higher at \( aw = 0.990 \) compared to 0.955, in agreement with Brown et al. (2008). Furthermore, bikaverin production was higher at \( aw = 0.990 \) compared to 0.955.

To our knowledge, this is the first report of the effects of \( aw \) on FUM3, FUM8, FUM13, FUM14 and BIK1 expression in F. verticillioides. We have also demonstrated the effects of \( aw \) on bikaverin production and the expression of BIK1 and also confirmed the production of bikaverin under fumonisin inducing conditions. Furthermore, we identified a correlation between FUM gene and BIK gene expression.

The similar effects of \( aw \) on FUM and BIK gene expression and production of the corresponding metabolites suggest that fumonisin and bikaverin synthesis could be connected by a genetic regulatory systems that relays environmental signals, in this case \( aw \), to the nucleus in order to suppress or activate FUM and BIK gene expression. It was reported that nitrogen starvation resulted in induced FUM1 and FUM8 expression

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**Table 2**

ANOVA of fumonisin B and bikaverin production and FUM3, FUM8, FUM13, FUM14 and BIK1 gene expression (2 \(-\Delta\Delta \text{Ct}\)) in 2 F. verticillioides strains grown on ME adjusted at 2 \( aw \) (0.955–0.990) and incubated for 14–21 days.

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<thead>
<tr>
<th>Strain</th>
<th>FB1</th>
<th>FB8</th>
<th>BIK</th>
<th>FUM3</th>
<th>FUM8</th>
<th>FUM13</th>
<th>FUM14</th>
<th>BIK1</th>
<th>FB2/FB1</th>
<th>FB3/FB1</th>
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<tr>
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<td>43.83</td>
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<td>0.15</td>
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<tr>
<td>1744</td>
<td>8.3</td>
<td>10.17</td>
<td>1.75</td>
<td>1.52</td>
<td>1.02</td>
<td>1.17</td>
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<td>aw</td>
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</tbody>
</table>

\[ \text{**} \text{ indicates significant at } P \leq 0.01; \text{*} \text{ indicates significant at } P \leq 0.05; \text{ and } \text{–} \text{ indicates not significant.} \]

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**Fig. 2.** Gene expression analysis. Expression of four FUM genes and the bikaverin PKS gene (BIK1) were assayed by quantitative real-time PCR from cultures of ITEM 10027 and ITEM 1744 grown for 14 and 21 days at two water activities (0.955 and 0.990).
and increased fumonisin production in both F. proliferatum (Kohut et al., 2009) and F. verticillioides, suggesting that fumonisin biosynthesis is regulated by the global nitrogen regulatory gene AREA (Kim and Woloshuk, 2008). AREA is postulated to regulate BIK gene expression in F. fujikuroi as well (reviewed in Limón et al., 2010). Even though there are correlations between the expression of the FUM and BIK gene clusters and production of the corresponding secondary metabolites, strain specific differences are apparent, probably due to different genetic backgrounds.

The regulation of secondary metabolite gene clusters and the production of metabolites are of interest both with regard to mechanisms of transcriptional regulation as well as for food safety. As bikaverin is known to have an antitumor activity against mammalian cell lines (Zhan et al., 2007), the toxicity of fumonisin could be hypothesized to be reduced when the compounds co-occur in contaminated products. Studying relationships between environmental factors and secondary metabolism production could also provide insights into the role of secondary metabolites in the ecology of the organisms that produce them and into the possible co-occurrence of different metabolites and their interaction in causing toxic effects in animals and humans.

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References